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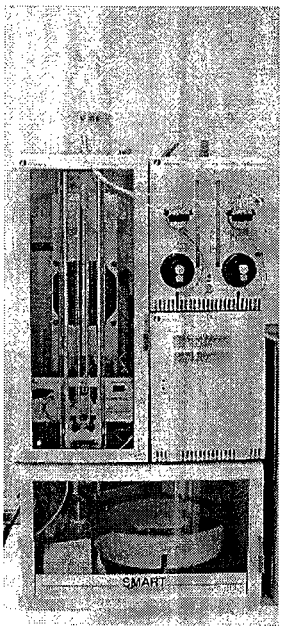
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Smart System/µPeak

Handbook



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1. Introduction

2. Technical description

3. Unpacking and installation

4. Operation

5. SMART® Manager - screens and menus

6. System maintenance

7. Trouble-shooting guide

8. Accessories

9. Technical specifications

10. Method manual

11. Column instructions

12. Application notes

13. Technical notes

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Pharmacia Biotech

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Important user information

Reading this entire manual is recommended for full understanding of the use of this product.



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The lightning symbol within an equilateral triangle is intended to alert the user to the risk of exposure to high voltages.



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1. Introduction

1.1 Introducing SMART® System

SMART® System (Fig. 1.1) is a fully integrated micropurification system for large and medium sized biomolecules. Through the combination of specially developed columns, precision hardware, unique software and a knowledge information system, SMART System enables nanogram to microgram quantities of biologically active material to be securely isolated into individual fractions as small as 5 µl. The versatile hardware and the broad column range allow a wide range of chromatographic techniques to be used, enabling the most refined purification strategies to be developed and optimised. The software, SMART® Manager, controls the system and evaluates the results of the separations. The knowledge information system, SMART Assistant, assists in planning strategies for protein purification.

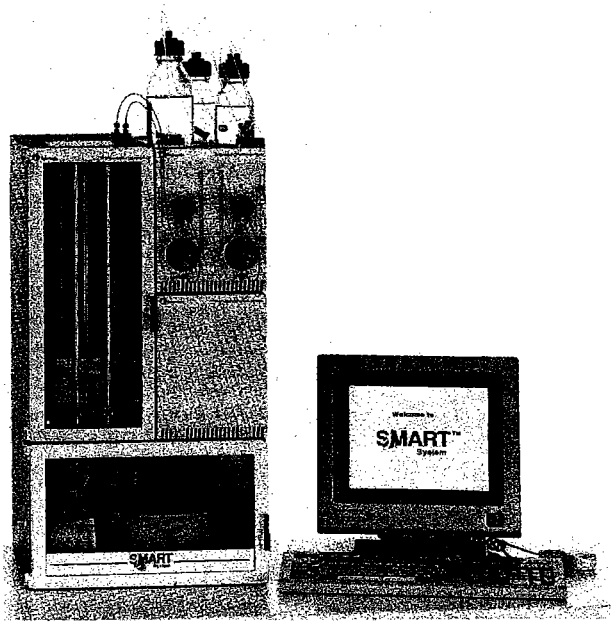


Fig. 1.1. SMART® System

The eluent is delivered to the µSeparation Unit by the µPrecision Pump. This dual cylinder syringe pump is fitted with cylinders and piston heads manufactured in titanium. It provides a high pressure, pulse-free flow and accurate gradients at flow rates as low as 10 µl/min. This feature is critical for precision isocratic systems and for the accurate formation of a gradient.

The µSeparation Unit, which can be cooled, houses the chromatographic, detection and collection components. All parts of the flow path are manufactured from inert materials and particular attention has been paid to the elimination of dead volume.

SMART Manager is the user interface for SMART System. All automatic chromatographic separations are controlled by a Separation Method created by the user. Manual changes can be made before and during the run using SMART Manager. Following the run, the chromatogram can be evaluated and the results presented on screen or on hard copy.

Additional help in method development for the purification of proteins is provided by SMART Assistant, a powerful knowledge

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the aim of the study and sample details, such as molecular weight, isoelectric point and other physical data, SMART Assistant will recommend a purification strategy, a chromatographic technique and a separation medium. The running conditions are provided in the form of a Separation Method. If the separation proves to be unsuccessful, the user can return to SMART Assistant for help in identifying the cause of and remedying the problem.

1.2 How to use SMART System Handbook

In SMART System Handbook all aspects of the system are described, from stepwise instructions for installation to comprehensive details on routine operation, general care and maintenance procedures. The contents of the handbook and how to use each section are described below.

Section 1. Introduction

This gives a brief introduction to SMART System and the System Handbook.

Section 2. Technical description

This fully describes the hardware and the operating principles of each part of the system. Where relevant, the software control by SMART Manager is described.

Section 3. Unpacking and installation

This section includes details of the laboratory site requirements for SMART System and a stepwise guide to installing the hardware and the software. Read this section carefully when installing a new system.

Section 4. Operation

This comprises several sub-sections, all relating to the different operations of SMART System. Examples of how to operate the system manually, how to program a method and how to evaluate the results are given.

Section 5. Screens and menus

This is a user reference section in where the individual parts of SMART Manager and SMART Assistant software are described in detail. It also includes a Short Instruction which shows SMART Manager graphically.

Section 6. System maintenance

This section describes how to carry out the general maintenance procedures, such as remaking tubing connections and replacing worn piston seals.

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Section 7. Trouble-shooting guide

This section helps to identify the possible cause of a problem occurring with SMART System. Symptoms of the fault are listed together with recommended remedies which include a reference to the relevant section of the manual.

Section 8. Accessories

This is a listing of the accessories and commonly used spare parts for SMART System.

Section 9. Technical specifications

A listing of the specifications of SMART System.

Section 10. Method manual

Practical hints on the use of SMART System for individual applications.

Section 11. Column instructions

This is for filing Column instructions for future reference.

Section 12. Application notes

This is for filing Application notes.

Section 13. Technical notes

This is for filing Technical notes.

Section 14. Appendices

Section 15. Index

1.3 Conventions used in this handbook

For simplicity, certain conventions and assumptions have been used in this System Handbook. These are listed below.

1. SMART Manager operates within the Presentation Manager environment of OS/2. In this System Handbook, it is assumed that all users of SMART System already have an understanding of Presentation Manager. Users unfamiliar with this system are advised to read the Learning Guide supplied with the OS/2 software and make themselves completely familiar with the use of the list boxes, option buttons, dialogue boxes etc. which are so much a part of this easy to use graphic environment.
2. SMART Manager can be operated using either the keyboard or a mouse. However, since the graphic environment of Presentation Manager is more compatible with the use of the mouse than the keyboard, this System Handbook assumes the use of a mouse.
3. To make a selection using the mouse, the screen cursor (arrow) is moved onto the item to be selected and the mouse button is pressed. In the System Handbook, this is described as "clicking

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on", with the option to be selected indicated in upper case letters. For example, if the pump flow rate is to be programmed within a Separation Method, this is expressed as "click on PUMP, FLOW", where PUMP is the menu header and FLOW is the menu option.

4. In this handbook, a key enclosed in brackets denotes a specific keyboard key. For example, <ENTER>.

5. For full details of how to use SMART Manager from the keyboard, follow the instructions in the Learning Guide supplied with the OS/2 software. The keyboard command which selects an option within SMART Manager is indicated by an underlined letter on the screen display. To move between different fields within one window, use the tab key.

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2. Technical description

2.1 System overview

SMART System is an integrated micropurification system for the separation of biomolecules present in the sample in small quantities. SMART System (Fig. 2.1) consists of the µPrecision Pump, µSeparation Unit, µPeak Monitor or Monitor UV-M II, SMART Manager software and a wide range of Precision Columns.

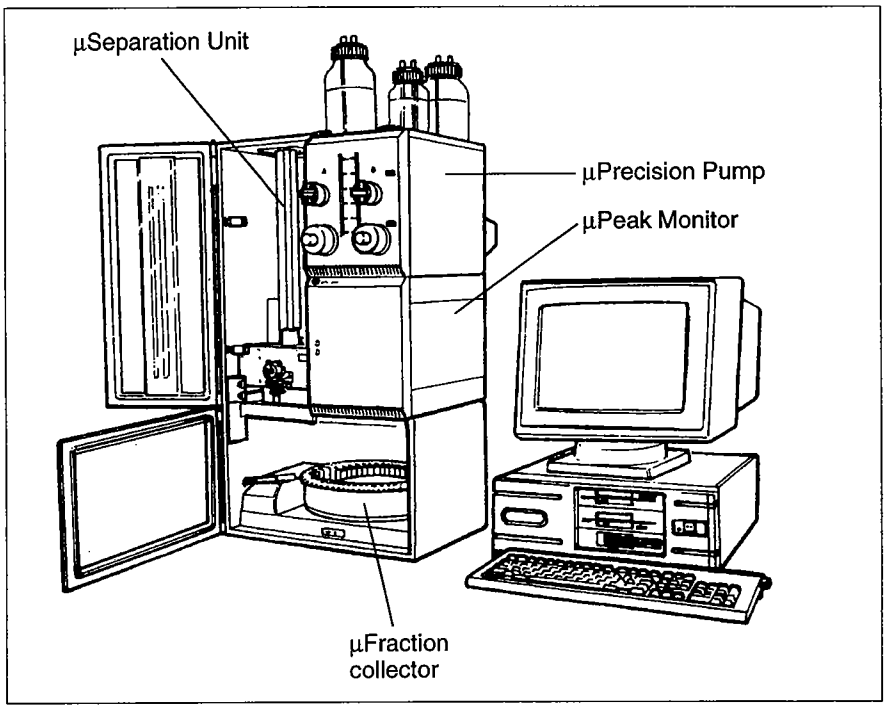


Fig. 2.1. SMART™ System

The µPrecision Pump is designed to deliver pulse-free flow with accurate and reproducible gradient formation in the low flow rate range, 10 µl/min to 2 ml/min, required for high sensitivity, micropurification work. The pump is a syringe pump with two cylinders driven by two separate stepper motors. It can be run in either gradient mode, when the cylinders are pumping different eluents or in continuous mode when the both cylinders alternate to deliver the same eluent.

The µSeparation Unit houses the rest of the systems flow path: the mixer, the injection valve, the automatic column holder, the µPeak Monitor flow cell, the gradient monitor and fraction collector. The compact design minimizes dead volumes and thereby maintains column resolution and monitor sensitivity which is critical for microscale separations. The entire µSeparation Unit can be temperature controlled 4-20 °C to ensure maintained biological activity during the separation. All system components in contact with liquid are manufactured in inert materials.

Each component in the µSeparation Unit will be described in more detail in the following sections.

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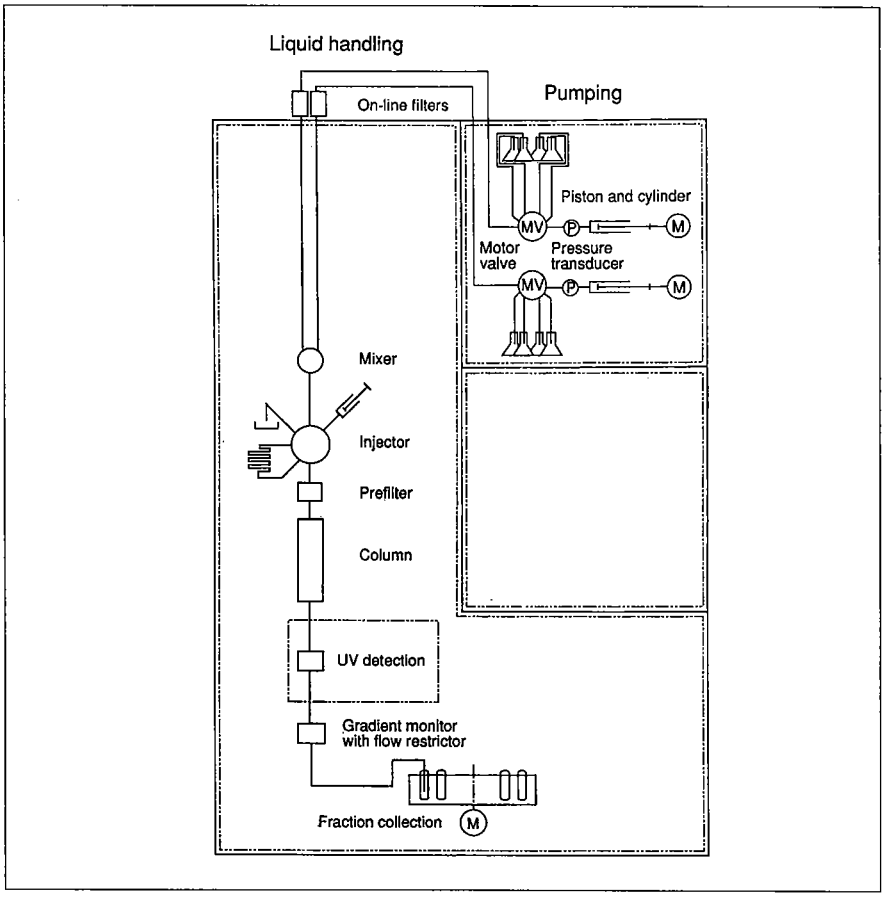


Fig. 2.2. Flow path in SMART™ System

A central System Power Supply distributes 24 V DC power to all units in SMART System via MultiNet power and communication link.

SMART Manager software is designed for easy system control, data evaluation and reporting. It runs in OS/2 operating system with Presentation Manager graphics interface.

All system control is through SMART Manager where automatic separation methods can be programmed or manual settings of parameters be done. Instructions such as flow rates, gradient profile, wavelength settings and fraction collection can be programmed in time or volume basis. A separation method normally runs automatically but manual adjustments can be done at any time during a run.

During a run data is stored for post run evaluation. Evaluation can be done manually or through automatic evaluation procedures. SMART Manager has extensive data manipulation capabilities that are described in detail in Chapter 5.

SMART Manager also incorporates SMART Assistant a knowledge information system. SMART Assistant is designed to assist in the planning of a separation strategy. It gives advice on suitable media to use for specific separation and it also gives a suggested method which can be directly transferred to SMART Manager.

SMART Assistant is described in detail in Chapter 5.

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2.2 µPrecision Pump design and construction

SMART System uses a high precision dual cylinder syringe pump for both gradient and isocratic separations (Fig. 2.3).

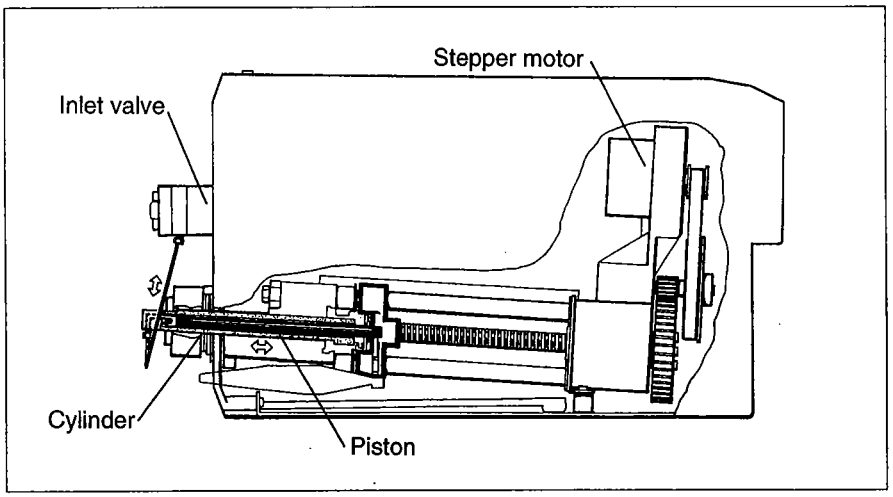


Fig. 2.3. Schematic diagram of the µPrecision Pump

The two titanium cylinders of the syringe pump are identical in design. To ensure that air bubbles are easily expelled, the two cylinders are inclined at an angle within the pump housing.

Each piston has a titanium head fitted with a sealing ring. Each piston is driven by a separate stepper motor giving independent pumping action for both cylinders. With 2 600 steps of the motor per microliter, a pulse-free flow of extremely high accuracy and reproducibility is ensured, even at the lowest flow rates.

Three sizes of cylinder are available. These are 10, 20 and 50 ml, with 10 ml cylinders being fitted on delivery. Instructions for exchanging the cylinders are given in Section 6.4. The cylinder size installed in the pump is programmed in the Method Editor, configuration part of SMART Manager. The µPrecision Pump can recognise the type of cylinder fitted in the pump and give a warning message if any attempt is made to run a Separation Method with incorrect cylinders size. The operator then has the choice of either changing the cylinders or the Separation Method.

There are two indicator lights on the front panel of the µPrecision pump. The green READY light indicates that the power is on and that the pump is ready for a run. When the green light flashes, communication between the pump and the computer has been interrupted. The yellow RUN light is illuminated while the pump is running. If the pump is not delivering eluent as programmed or if the programmed pressure limit has been reached, this yellow light starts to flash and the screen displays an error message.

2.3 µPrecision Pump operating principle

Both pistons have a single stroke action. In gradient mode this limits the gradient volume to the total volume of the two cylinders. In continuous mode the volume is unlimited. Before the start, each cylinder is filled with eluent through the inlet valve from the selected reservoir (Fig. 2.4). Two level indicators on the front panel show the amount of buffer remaining in each cylinder. The volume in the cylinders is checked automatically before the start of the method.

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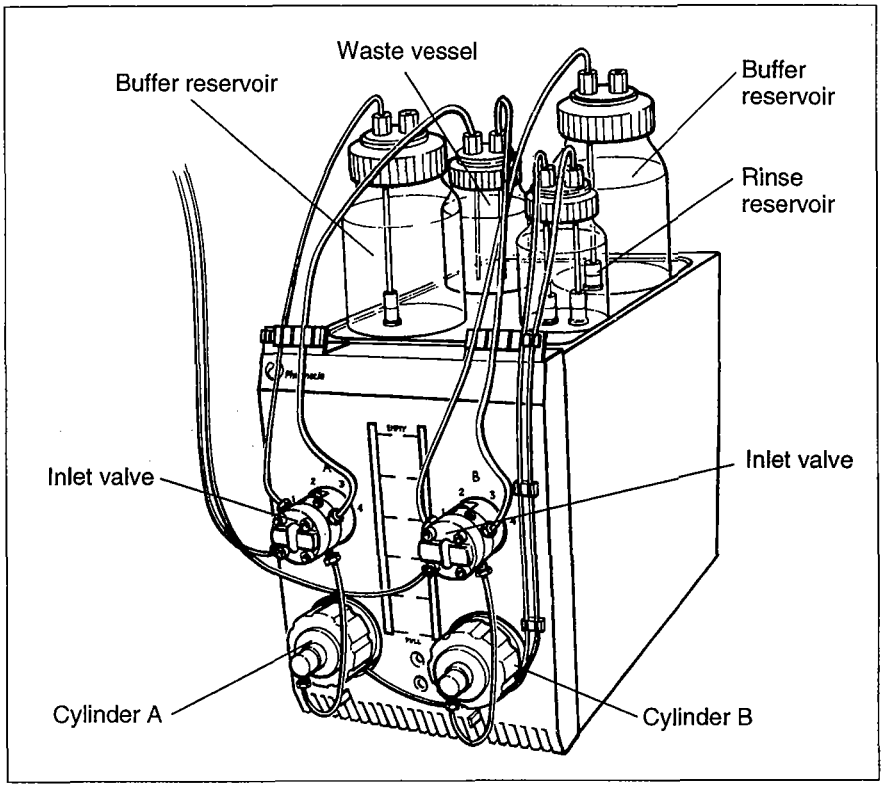


Fig. 2.4. µPrecision Pump

The pumps working mode is programmed in the Separation Method, Configuration. If the pump is running in Gradient Mode any shape of gradient can be programmed by selecting a percentage B at a specified time. Delivery from the two cylinders is simultaneous with the speed of the individual pistons being varied to produce the required composition.

By selecting Continuous Mode the syringe pistons will operate alternately resulting in a continuous flow. In this case, the option of entering the concentration of eluent B is not available.

2.4 Flow rate ranges

The flow rate can be programmed in the Separation Method or set manually in Run/Control. The combined delivery from the two pump cylinders produces the selected rate of flow.

Operating flow rates and pressure limits are as follows:

Cylinder	Flow rate	Pressure limit
10 ml	10-2 000 µl/min	25 MPa
20 ml	20-2 000 µl/min	25 MPa
50 ml	50-2 000 µl/min	12.5 MPa

2.5 Inlet valve

Liquids are drawn from the inlet tubings in the reservoirs into the pump cylinders through a motorised valve which has four inlet ports, numbered 1 to 4. Inside the valve, a rotating channel plate aligns the internal channels so that only one of the four inlet ports can be open to the fifth, pump cylinder port at any time (Fig. 2.5 a, b). The valve positions at filling and emptying of the cylinders are programmed in SMART Manager.

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Eluent is pumped through port 6, marked "OUT" to the system (Fig. 2.5c).

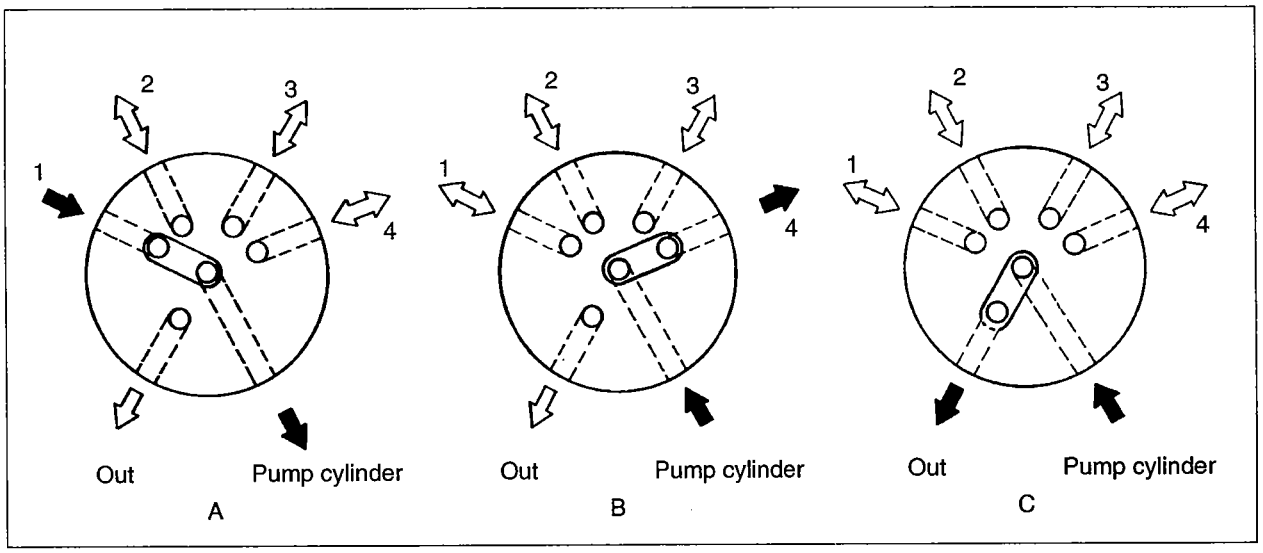


Fig. 2.5a, b, c. Fluidic pathways through inlet valve

Each valve has a closed position between port 1 and OUT which is used during the Leakage Test and when the cylinders are being pressurised after filling.

Inlet tubings to the valve are 1.9 mm i.d. and are manufactured in ETFE. All inlet tubings are fitted with filters to prevent any particulate material being drawn into the system from the buffer reservoirs. The tubing between the inlet valve and pump cylinder is made of titanium.

2.6 Piston rinse

To prevent accumulation of buffer salts, the piston and piston seal are rinsed continuously at the back of the piston assembly. Liquid from the rinse reservoir is automatically drawn in behind the piston as eluent is delivered from the pump. Similarly, rinse liquid flushes out of the rinse chamber as the cylinder is refilled. Since the same volume of rinse liquid is used continuously, the liquid must be changed regularly. To prevent bacterial growth use 20% ethanol.

At the back of the cylinder, the piston is sealed by the rinsing seal. Both the rinsing seal and the piston seal can be replaced when worn. If the rinsing seal becomes worn and a leakage occurs, the rinse liquid collects safely in a tray beneath the pump cylinder and drains to the front of the pump.

2.7 Pump control functions and pressure tests

In addition to the system pressure measurement, several pump functions are performed to enhance the precision of the pump. While most of these functions are automatic and operate without any user intervention, some can be switched on or off by the operator in the Separation Method configuration and are saved together with the method.

2.7.1 System pressure measurement

System pressure is measured automatically by a pressure sensor built into each inlet valve. The pressure signal can be monitored during the

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run and it can be stored together with the chromatogram. The pressure unit is MPa.

The upper pressure limit is either defined by the user or determined automatically by the type of column installed. This is entered in the Separation Method configuration and is saved with the method. If this limit is reached during the run, SMART System will pause automatically.

The system pressure depends of the column. It is proportional to the flow rate, length of column and the viscosity of the eluent. It is inversely proportional to the diameter of the column and the column constant.

2.7.2 Air detection test

After a cylinder refill, the air detection test is performed. This automatic test checks the pressure increase against time. A delayed increase indicates air in the cylinder and the pump is switched off automatically and a warning message is given.

2.7.3 Compressibility compensation

After every pump wash, the compressibility of the new liquid in the cylinder is measured automatically. To do this, the inlet valve is closed and the pump operates at a low flow rate while the pressure increase is measured against time. The program calculates the compressibility factor of the liquid by comparing the resulting curve with a known standard (Fig. 2.6). This measurement is reported in the run log and used later to help maintain a precise and accurate flow rate during gradient formation under conditions when system pressure varies during the run, such as Reversed Phase gradients with viscosity changes.

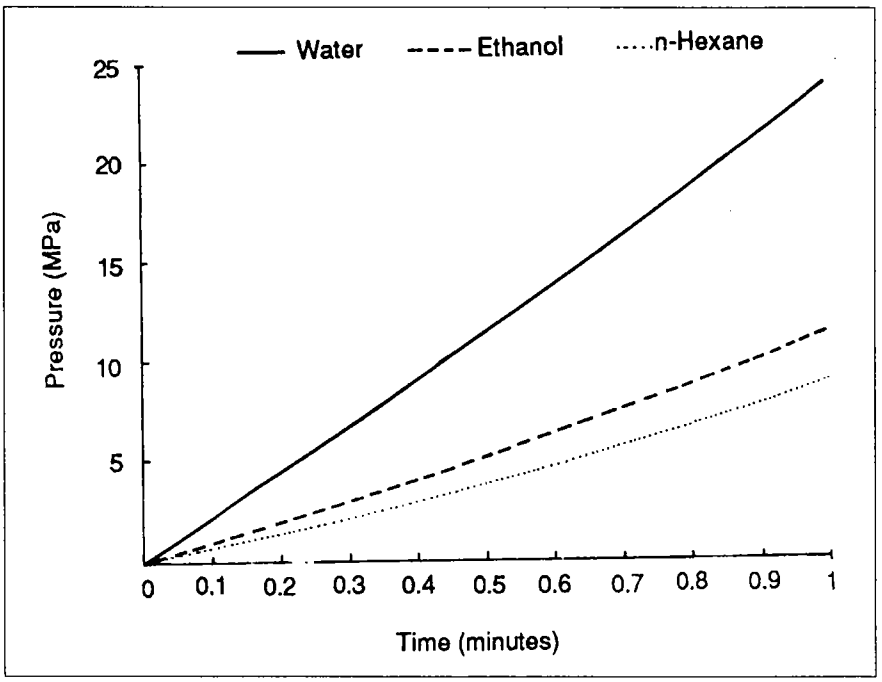


Fig. 2.6. Graph showing the compressibility of eluents

Compensation for the compressibility of a liquid is a function which can be activated by the operator in the Separation Method

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configuration. The user is recommended to activate this function when running RPC gradients where viscosity changes can be expected during the run. It is especially important when working with high backpressure.

2.7.4 Leakage test

A leakage test can be performed on each cylinder to test the pump for leaks. For this test, the inlet valve is closed and the cylinder is pressurised to a pressure defined by the user. If this pressure cannot be maintained, a leakage has been detected.

This test, which is initiated by the user, should be performed on a regular basis. Further details on the operation of the test are given in Section 6.3.

2.7.5 Pressure follow

This function maintains the system pressure in the non working cylinder, when only one cylinder is pumping. This ensures that the gradient composition is accurate at the start of the gradient (Fig. 2.7).

The function is activated by default in the Separation Method configuration and should be used for all gradient applications.

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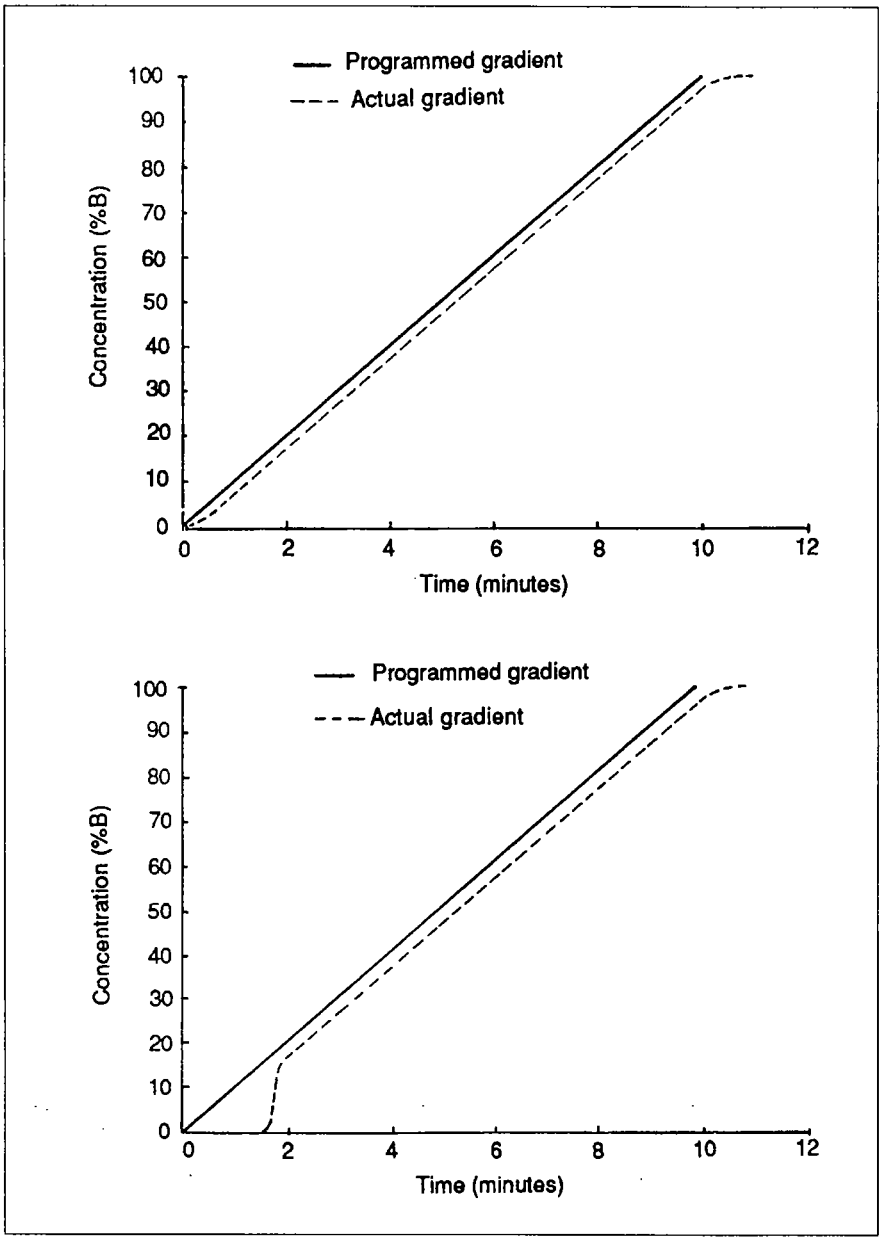


Fig. 2.7. Effect of pressure follow; not activated (lower) and activated (upper)

2.7.6 Fast pressurisation

With this function activated, the column is rapidly pressurised to a stable target pressure. This shortens the time for the system to reach the final run pressure.

The target pressure calculated by the program takes in account the type of column, the selected flow rate and the viscosity of the eluent.

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2.8 On-line Filter

To protect the column, the buffer dispensed from the pump is filtered through a 2 µm titanium filter as it enters the µSeparation Unit (Fig. 2.8).

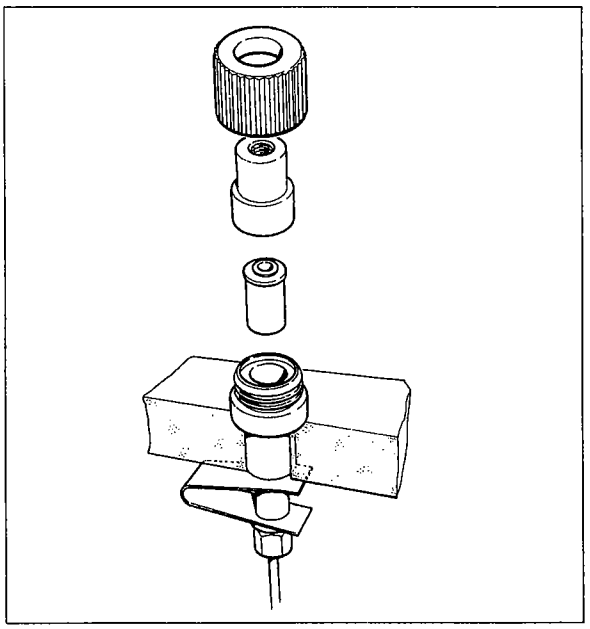


Fig. 2.8. On-line Filter

If the back pressure in the system increases the filters might be blocked and needs cleaning. Instructions for this are given in Section 6.9.

2.9 µMixer

From the filter, PEEK tubing of 0.25 mm i.d. carries the two eluents into the mixer where they are thoroughly combined. The mixer has two chambers, connected in series, one above the other (Fig. 2.9). The two eluents enter the lower chamber of the mixer first and then pass into the upper chamber. The chambers are interchangeable.

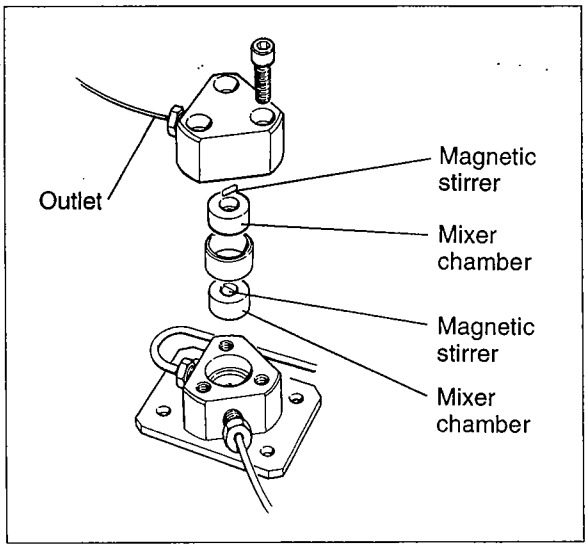


Fig. 2.9. µMixer

Each chamber contains a small PTFE coated magnetic stirrer operating at 600 rpm. The stirrer drive motor, which is located under the mixer, can be viewed through the window in the automatic column holder.

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As standard, the system is fitted with two 30 µl chambers. Three alternative chamber sizes are available in volumes of 12 µl, 75 µl, 150 µl as well as a zero volume chamber bypass. The five chambers can be combined in almost any configuration. However it is not recommended to use 2 x 12 µl and when a 12 µl chamber is used it should be placed in the lower position. To change to another mixer volume, refer to Section 6.10.

The mixing of the two eluents can be optimised by using different chamber volumes for different flow rates. The table below may be used as a guide for selecting the chamber volume for different flow rate ranges.

Flow rate µl/min	Chamber volume µl
10 - 50	0 + 12
30 - 100	0 + 30
50 - 600	30 + 30
300 - 2000	150 + 150

When working with eluents of low miscibility, or at high UV sensitivity, use the larger of the recommended mixer volumes. The optimum mixer volume is also dependant on the gradient time; for shorter gradient times, smaller mixer volumes should be used.

To ensure that the tubing from the pump to the mixer and the mixer itself are filled with eluent at the required composition for the start of the run, the Mixer Wash function can be selected when creating the Separation Method. If activated for a gradient starting with a concentration of 0 %B, this function uses eluent A to wash the mixer before the run starts. During this time, the injection valve is automatically switched to the waste position.

This Mixer Wash function should be used after changing the eluent, after filling, before the first run of the day and when the column is not regenerated with the starting eluent.

2.10 µInjection valve

Titanium tubing, 0.2 mm i.d., carries the eluent from the upper chamber of the mixer to the entry port of the sample injection valve (Fig. 2.10).

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New injection valve in SMART™ System

INSTRUCTIONS

The injection valve in SMART System has been redesigned. Please disregard the information in the System Handbook regarding Technical description Section, 2.10 and Maintenance, Section 6.13.

Please insert this instruction in your System Handbook for future reference.

Technical description

The injection valve is a five port valve for injection of sample via fixed volume sample loops connected to the valve. A switching plate contains the internal flow channels through the valve. This switching plate rotates to align the internal pathways between the five ports.

The valve has three different positions, LOAD, INJECT and WASTE. The valve can be programmed to switch between these positions at a specific time or volume.

Functions of the three valve positions:

LOAD

The sample can be loaded into the sample loop. The eluent from the pump passes through the column.

INJECT

Eluent from the pump passes through the loop taking the sample onto the column.

WASTE

Eluent is pumped directly to waste.

Note: This new valve does not have an internal loop.

Maintenance

Replacement of injection valve switching plate

Wear on the internal switching plate is indicated by leakage around the injection valve. To confirm the fault, set the injection valve in Load position, disconnect the sample loop and start the flow. If eluent is coming out through the sample loop ports, the switching plate is damaged. If this is not the case, replace the sample loop and run eluent at high flow rate through the valve, switching it between inject and load position. If you see a difference in back pressure for the two valve positions, a damaged switching plate can also be suspected.

Warning: The channel plate is coated in a fluoro plastic grease to ensure smooth movement. This substance is toxic and the operator is advised to wear protective gloves when handling the channel plates during this procedure. Wash your hands after accidental contact with the channel plate.

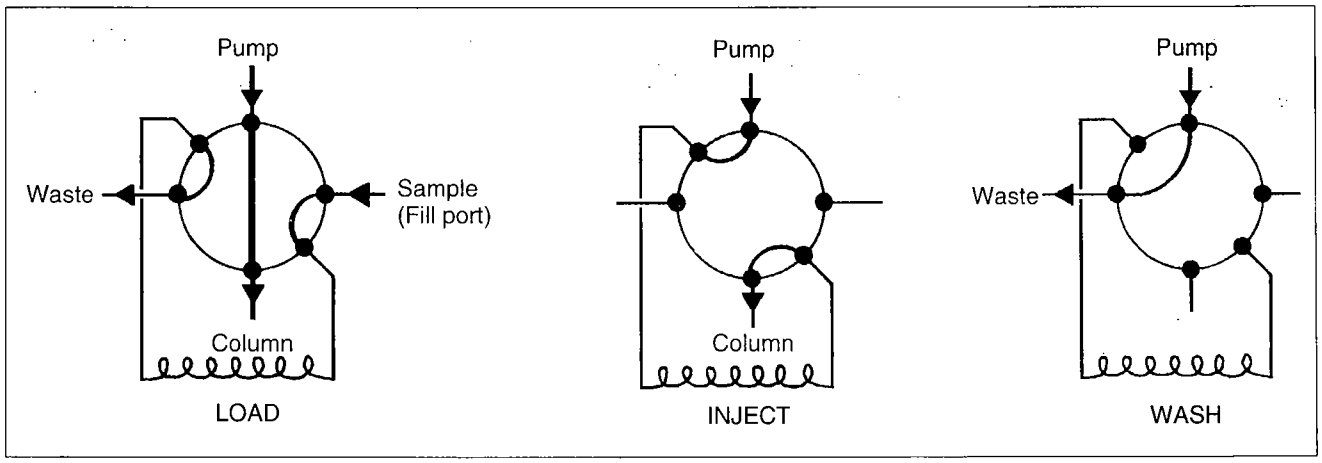


Fig. 1. Valve positions

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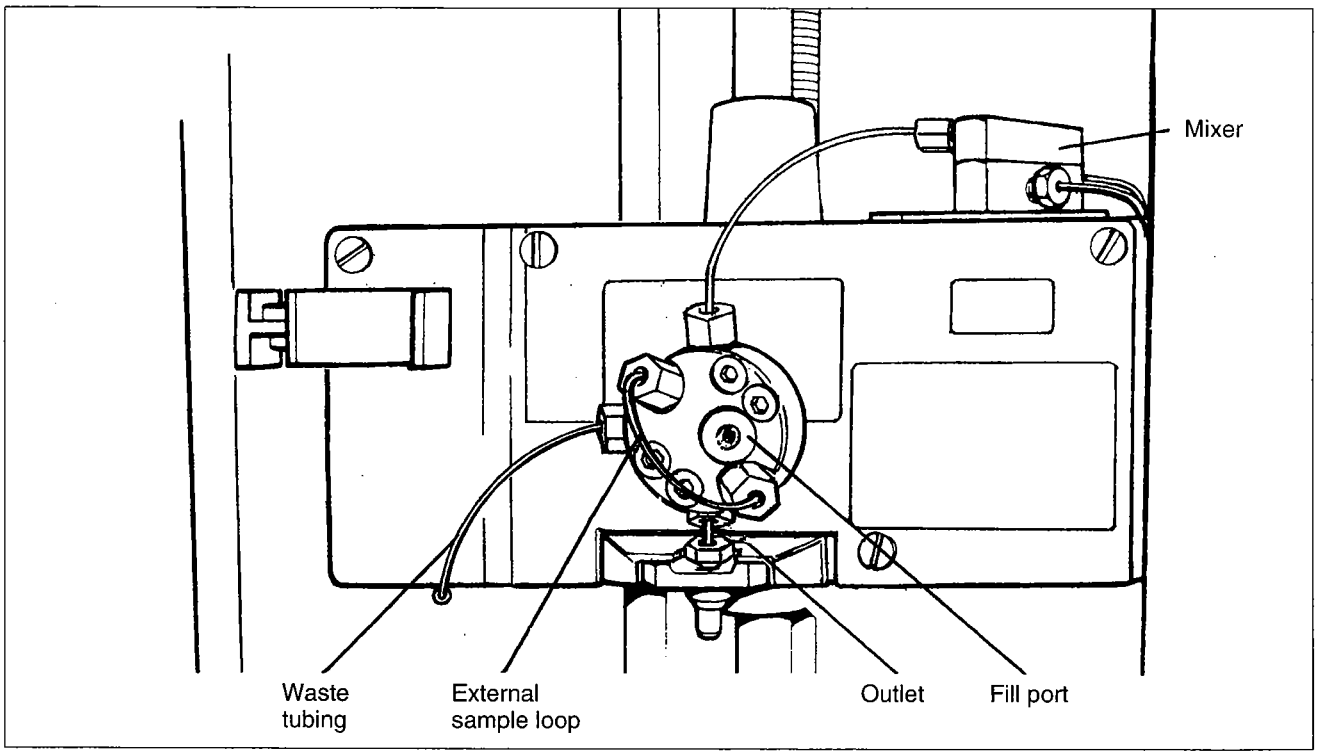


Fig. 2. Injection valve

Before starting to disassemble the injection valve, ensure that you have the Switching Plate Code No. 18-1037-31 available as a spare part. In case of damage occurring during this procedure, it is a wise precaution to have Tubing, valve to column, (Code No. 18-1037-34) also available.

1. Disconnect the waste channel tubing and the tubing from the mixer. Remove the fill port.
2. Use a 3 mm Allen key to loosen the four Allen locking screws in turn slightly. Hold the valve housing while loosening the locking screws.
3. Carefully bend down the outer part of the valve. Care must be taken with the titanium tubing between the valve and the column.

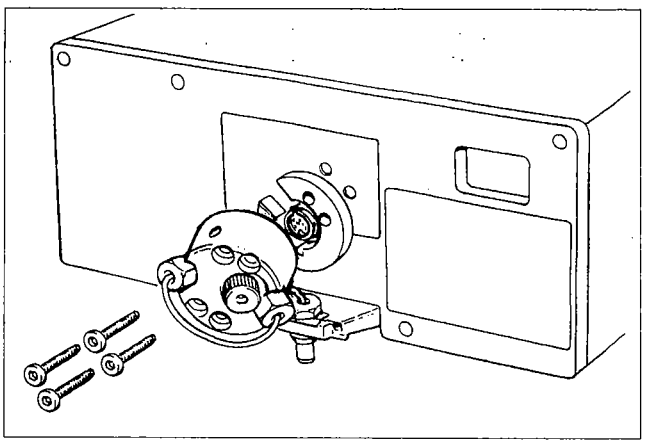


Fig. 3. Removing the outer part of the valve.

4. Remove the switching plate assembly from the valve housing by levering it out gently with a screwdriver.
5. Remove the switching plate (the outer plastic part) with a screwdriver. Replace it with the new switching plate.

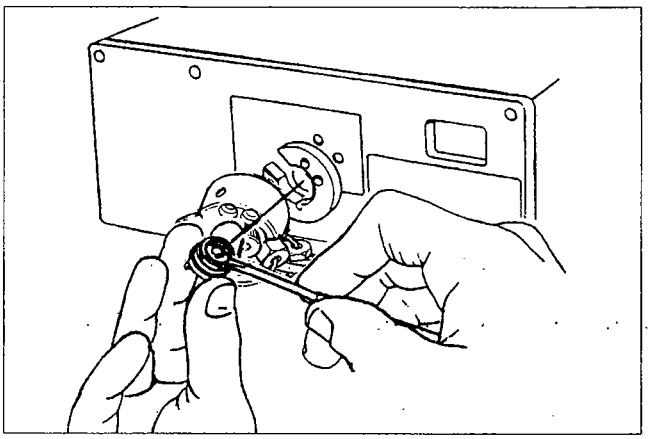


Fig. 4. Removing the switching plate

6. Fit the switching plate in the same alignment as the old plate in the valve housing. Push it in evenly until it is seated correctly in position.
7. Carefully replace the outer part of the valve. Replace the four Allen screws and tighten each in turn slightly.
8. Replace the fill port, waste tubing and tubing from the mixer. Ensure that all connections are tight.

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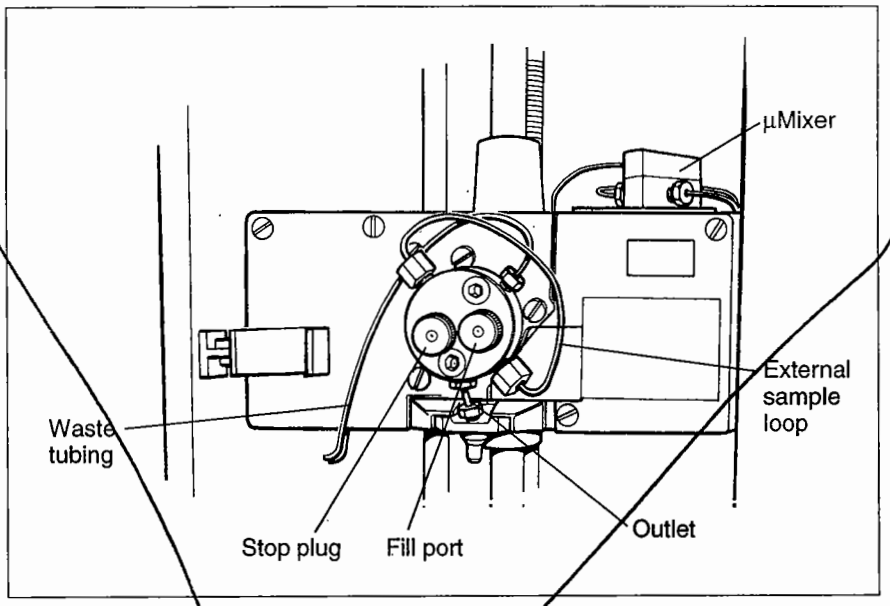


Fig. 2.10. µInjection valve

The injection valve is a three-position six-port valve connecting the mixer and the column with sample loop and the sample fill port. A channel plate contains the internal flow channels through the valve. This channel plate rotates to align the internal pathways between the six ports (Fig. 2.11). The plate can be replaced when worn.

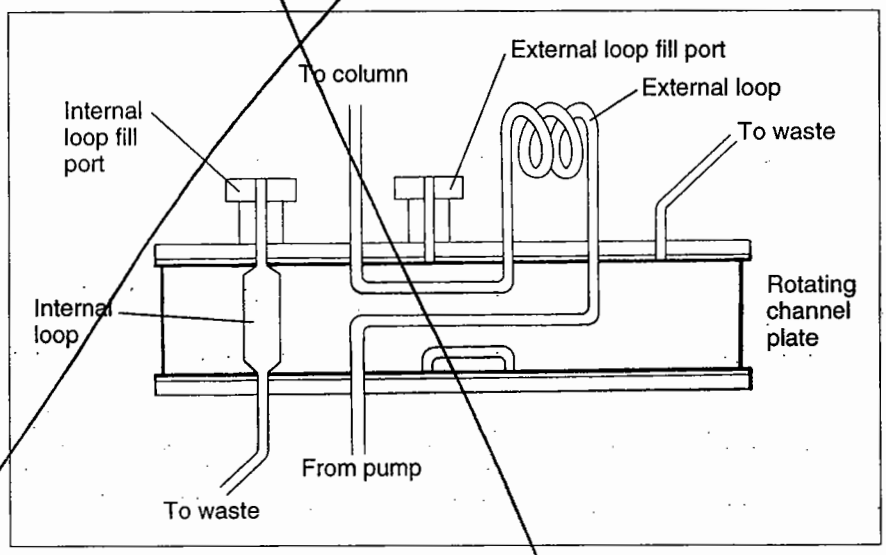


Fig. 2.11. µInjection valve in position for injecting sample from the external loop

2.10.1 Sample loops

A range of sample loops is available. The fixed internal sample loop has a volume of 0.75 µl. Loops of 10 µl, 20 µl, 50 µl and 100 µl are supplied, with the 50 µl loop connected on delivery. Loops of 5 µl, 200 µl, 500 µl, 1 000 µl, 2 000 µl volumes are available as accessories. For even larger injection volumes, Superloop 10 ml or 50 ml can be connected to the valve. These can be fitted in the two hooks on the left wall in the µSeparation Unit.

The material used for the loops varies with loop volume.

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Loops, 5 µl - 10 µl	titanium
Loops, 20 µl - 500 µl	PEEK
Loops, 1 000 µl - 2 000 µl	ETFE

The internal and external loops have separate sample fill ports which are marked with E for external (right) and I for internal (left). When delivered, the system has a fill port fitted to the external side and a plug in place of the internal fill port. The plug must always be in position in the fill port which is not in use. The fill ports can be replaced when worn.

Sample loading into the fill ports requires a syringe with a flat-tipped needle of 0.7 mm o.d. A 100 µl syringe is supplied with the instrument and a similar 10 µl syringe is available as an accessory. The syringe needle can be inserted into either of the two injection ports through the film covered slit in the upper door of the µSeparation Unit. To support the syringe during loading, the syringe support can be positioned at the height of the injection port.

2.10.2 Valve positions

The channel plate inside the injection valve can switch to three different positions, LOAD, INJECT and WASTE (Fig. 2.12a,b). The valve can be programmed to switch at a specific time or volume, or the positions can be changed manually in the Run/Control window.

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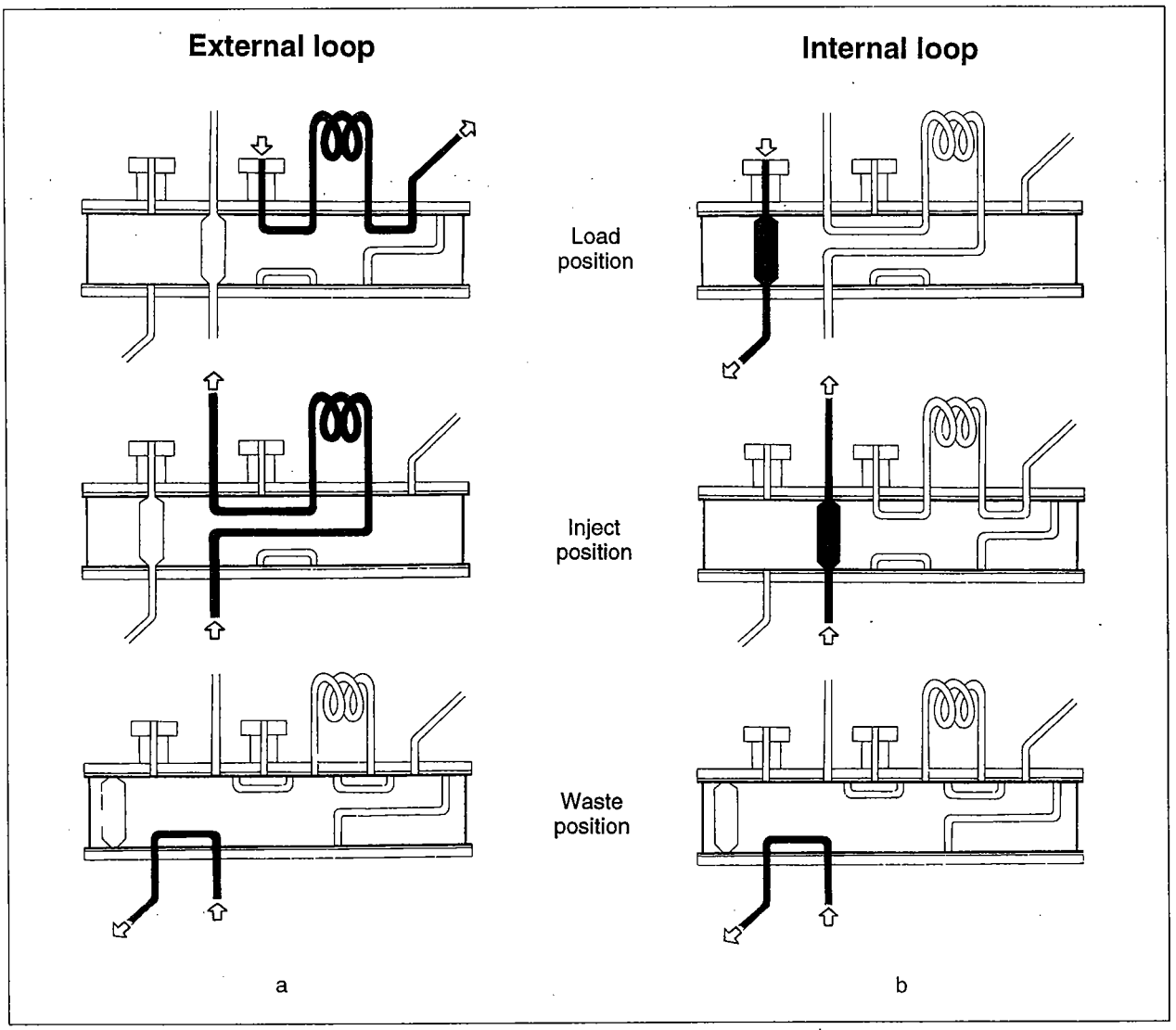


Fig. 2.12a, b. Valve positions for external loop and internal loop

The functions of these positions are described below.

LOAD

The sample can be loaded into the sample loop. The eluent from the pump passes through the column.

INJECT

Eluent from the pump passes through the loop taking the sample onto the column.

WASTE

Eluent is pumped directly to waste.

2.11 Prefilter

A short length of titanium tubing of 0.2 mm i.d. carries the eluent and sample from the injection valve to the titanium fitting which connects into all types of Precision Columns. The prefilter, a replaceable 0.5 µm titanium frit held within a collar, clips directly onto this titanium fitting (Fig. 2.13).

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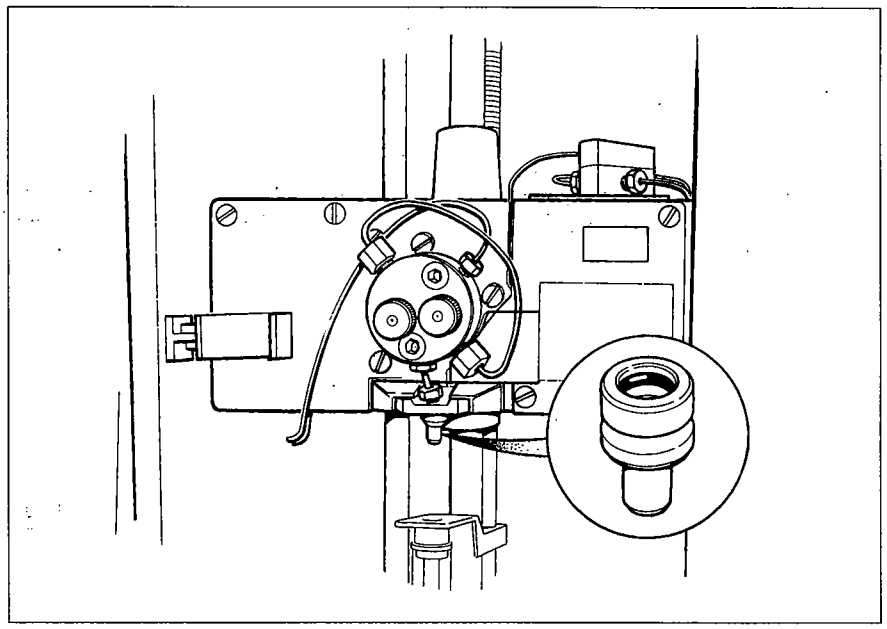


Fig. 2.13. Prefilter

The standard prefilter has a diameter of 3.2 mm. A prefilter of 0.8 mm diameter is available as an accessory.

The frit should be cleaned regularly in an ultrasonic bath as described in Section 6.14.

2.12 Automatic column holder

A wide range of columns has been specifically designed for SMART System. In addition, FPLC and HPLC columns with a length up to 300 mm can be fitted by using the Column Adaptor.

Installation of the column is a simple automatic procedure. The µPrecision Column is placed onto the column connector and held upright by the column support (Fig. 2.14). The user registers the type of column in SMART Manager. The automatic drive is then initiated and the column holder with the injection valve is driven downwards onto the column. A sensor detects and maintains the force required to keep the column firmly seated. For the protection of the operator, the motor drive will not function if the upper door of the µSeparation Unit has not been closed.

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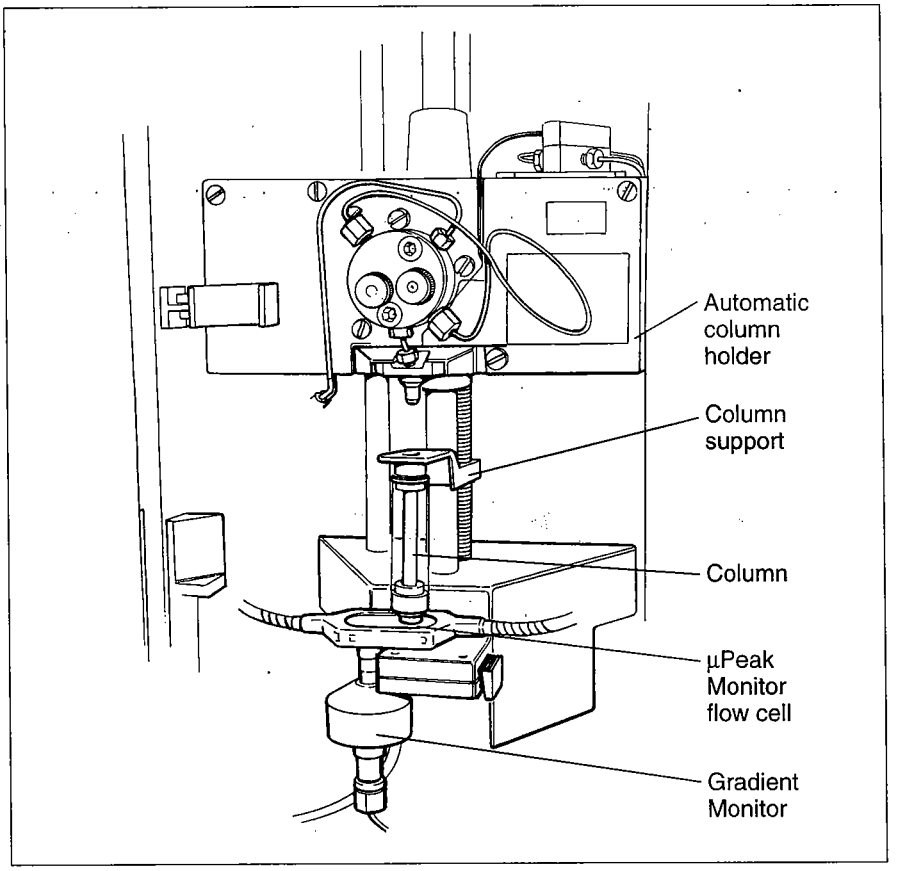


Fig. 2.14. Column installation

2.13 Temperature control

The µSeparation Unit can be cooled to +4 °C at an ambient temperature of +21 °C. The temperature is individually set for each run in the Run/Control window.

A Peltier element in combination with cold water cools the µSeparation Unit. Reproducible temperature control with an accuracy of ± 1 °C is achieved.

For effective cooling the Separation Unit, a constant supply of circulating water at +5 °C should be available from a circulating bath (min. 300 W). The user should note that if the ambient room temperature is high in combination with high humidity the efficiency of the cooling will be reduced.

If the cooling is used constantly, it is recommended to turn off the cooling for half an hour every 24 hours to avoid ice formation on the Peltier element. For defrosting see Section 6.23.

Note: It should be remembered that the buffer viscosity increases as the temperature is lowered. This can increase back pressure in the column by up to 50 %.

A fan located inside the µSeparation Unit continuously circulates cooled air. The fan switches off automatically when the door of the µSeparation Unit is opened.

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2.14 Ready lamp indicator

Inside the µSeparation Unit there are two indicator lamps. The green lamp indicates that the µSeparation Unit is switched on and ready. If the green lamp flashes, communication throughout the system has been interrupted. The yellow lamp indicates that the µSeparation Unit is in operation and a run is in progress. A flashing yellow light indicates a fault in the system and the screen displays an error message.

2.15 µPeak Monitor

2.15.1 Overview

The µPeak Monitor is a variable wavelength monitor for detection in the wavelength range 190 - 600 nm. In SMART System it is placed on the µSeparation Unit with the µPrecision Pump on top of the monitor. The µPeak Monitor consists of a main unit, optical fibres and a flow cell (optical pathlength 3 or 10 mm, cell volume 0.7 or 2 µl). The flow cell is placed at the column outlet.

The main unit consists of the lamp unit, the monochromator unit, detector electronics and control electronics. The lamp is a xenon flash lamp with a continuous spectrum in the UV-vis region. The monochromator includes a holographic stigmatic grating and a stepper motor for wavelength selection between 190 - 600 nm in steps of 1 nm. Wavelength changes can be programmed at any time during a run. Up to three wavelengths can be monitored simultaneously.

The detector electronics are connected to the monochromator and the flow cell via optical fibres. The control electronics which include SMART System processor core and software, handle data collection, processing and communication with other SMART System units.

The µPeak Monitor has extensive programming capabilities with all operator communication taking place through SMART Manager. The programmable operating parameters include wavelengths, autozero and averaging time, which can be programmed for manual or automatic operation.

2.15.2 Optical system

The optical system consists of a xenon flash lamp, condenser, block filter, grating, a beam splitter, the flow cell and a detection unit (Fig. 2.15). The entire system is precisely aligned in the factory and requires no further adjustment by the operator.

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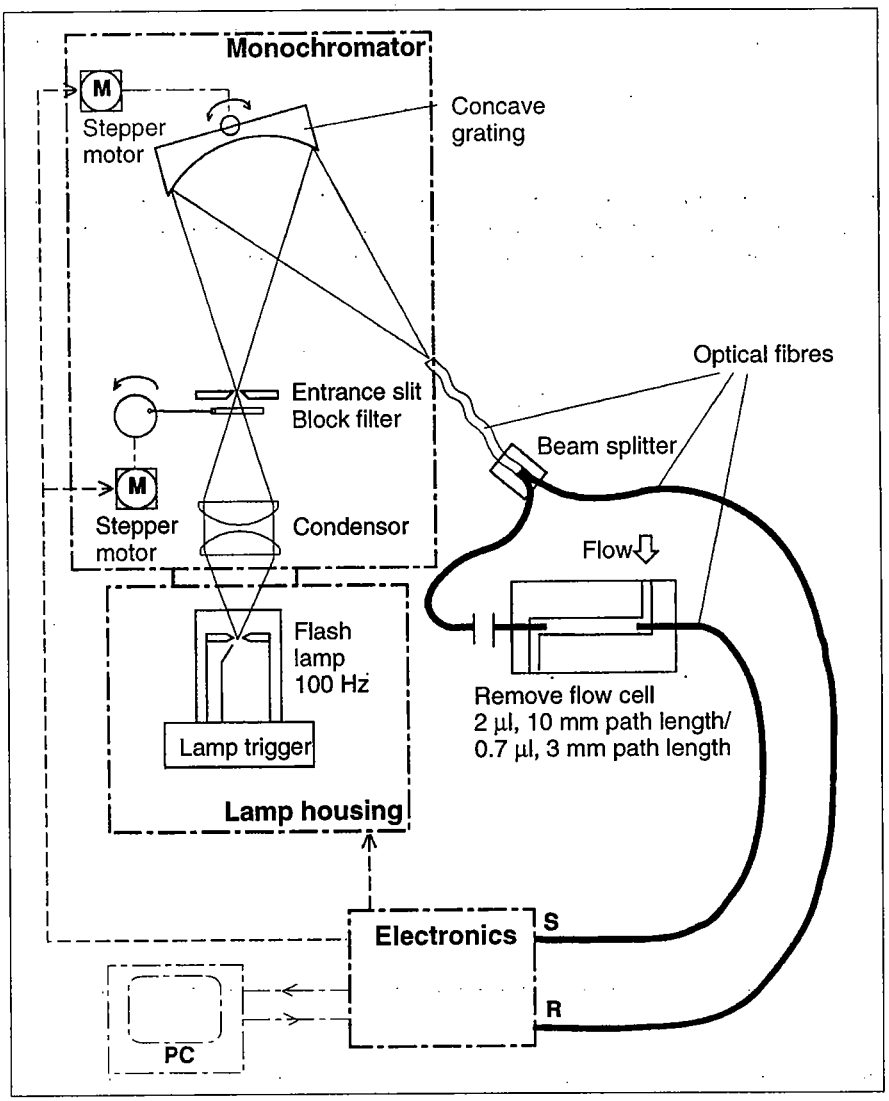


Fig. 2.15. Schematic diagram of the µPeak Monitor

2.15.3 Monochromator

The xenon flash lamp in the monochromator has an expected lifetime of >4 000 hours. It provides a high intensity light throughout the operating range. The light from the lamp passes through a condensing system to a concave aberration-corrected holographic grating. Monochromatic light from the grating is directed to an optic fibre, the grating is turned automatically by a stepping motor.

For wavelengths in the range of 360 - 600 nm, a blocking filter is automatically moved into the light path to filter out unwanted light, before entering the monochromator.

2.15.4 Beam splitter

Before entering the flow cell, the monochromatic beam is split in a beam splitter, with 50 % of the light passing through the sample in the measuring cell and 50 % being directed through the reference channel. Two photodiodes with identical characteristics monitor the signal levels of the measuring and reference beams.

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2.15.5 Flow cell

Two flow cells with different path lengths are available: a 3 mm cell with a volume of 0.7 µl and a 10 mm cell with a volume of 2 µl. The flow cells are quartz with a titanium housing.

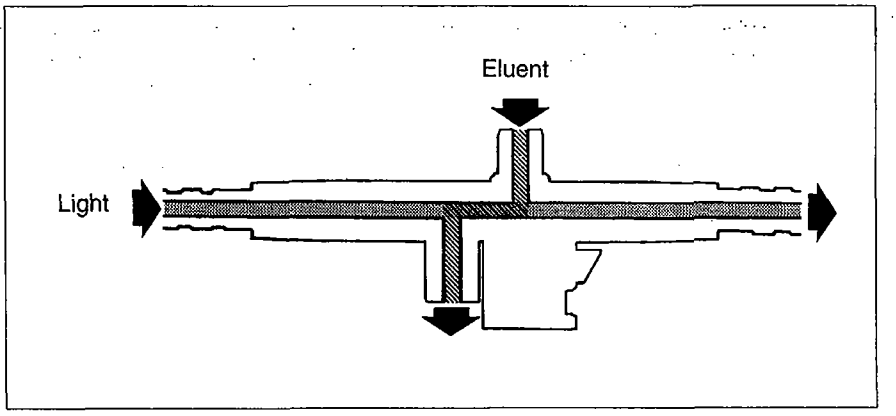


Fig. 2.16. Light and eluent flows in the flow cell

2.16 µPeak Monitor functions

2.16.1 Wavelength(s)

The operator has an option to measure at one, two or three wavelengths simultaneously in the wavelength range 190-600 nm. Wavelength changes can be programmed at any specified time/volume. When more than one wavelength is chosen the first wavelength selected will be the primary wavelength which will be used for peak fractionation and for the analogue recorder output.

2.16.2 Autozero

An autozero function sets the detected absorbance to zero at any programmed time/volume. It is recommended to use the autozero function after wavelength changes in a method.

2.16.3 Autorange

The autorange function allows for high resolution throughout the entire Absorbance range. Thereby the need to preset a fixed absorbance range before the run is eliminated. It also eliminates the risk of loosing high absorbance peaks when working at high sensitivity.

The autorange function is fully automatic and needs not to be considered by the user. At method start, the amplification of the signals from the sample and the reference photodiodes, are individually set to feed maximum signal levels into the two 16-bit D/A converters. The D/A converters produce a resolution of the absorbance in a maximum 64 000 data points, which gives an extremely high resolution even at high sensitivity.

2.16.4 Averaging time

For filtering the noise of the digital signal an averaging time can be set. The averaging time is the time interval used for calculating the moving average for the absorbance signal. A long averaging time will smooth out noise efficiently, but it will also distort the peaks.

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Because of this the averaging time should be as short as possible. The averaging time is automatically set by the software to fit the column installed in the system but it is also possible to select another value.

2.16.5 Analogue recorder output

At the back of the monitor there is a 1 V Recorder output for connection of an analogue recorder. The absorbance value for full scale deflection can be selected in SMART Manager, Run/Control or in the Method Editor.

2.17 Monitor UV-M II

Monitor UV-M II is a high sensitivity, fixed wavelength detector, consisting of an Optical Unit and a Control Unit. In SMART System, it is placed alongside the µSeparation Unit.

The eluent from the column flows through the Column Connector to the UV-M II Optical Unit which is positioned directly under the column (Fig. 2.17).

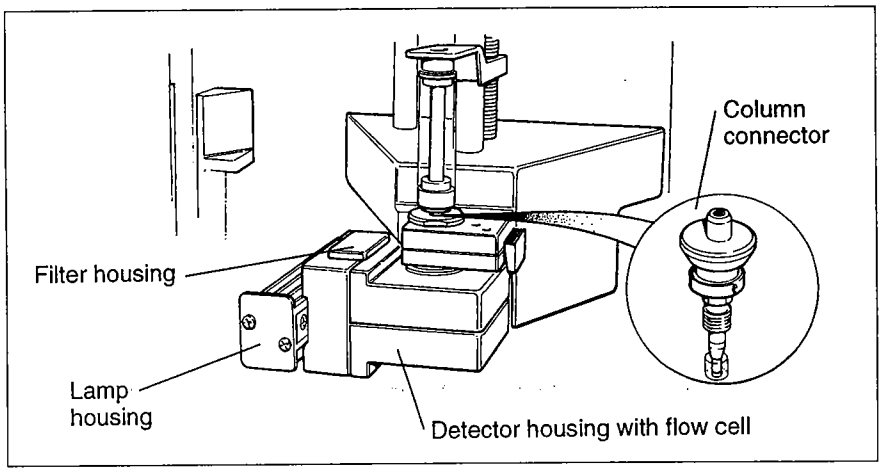


Fig. 2.17. Column connection to UV-M II detector housing

The Optical Unit comprises three parts:

- the lamp housing
- the filter housing
- the detector housing with the flow cell

On delivery, the Optical Unit is fitted with a mercury lamp for operation at 254 nm and 280 nm. A zinc lamp for operation at 214 nm is available as an accessory. Filters for detection at 214 nm, 254 nm and 280 nm are supplied. Filters for 313 nm, 365 nm, 405 nm, 436 nm and 546 nm are available as accessories. The wavelength and the filter are selected manually on the Optical Unit.

The flow cell supplied with SMART System is a high precision cell with a 5 mm path length.

The UV-M II Control Unit has a sensitivity range from 0.001 to 2 AUFS. For the 5 mm cell, this produces an optical density of 0.002 to 4 OD. The absorbance range is set manually on the Control Unit before the start of the run.

A time constant function for noise reduction can be set to 0.1, 1 or 10 seconds. The UV-M II has a preset value of 1 s. For optimum results, it is recommended that the time constant should be set as near as possible to 1/16th of the narrowest peak width in seconds. By

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pressing the RANGE buttons together, the TIME CONSTANT which is active will flash.

The detector output can be adjusted to zero. By pressing the AUTOZERO button for 2 seconds, the LED illuminates to indicate that the baseline is zeroed. If the LED flashes, the baseline is not stable.

The UV lamp can be turned on or off independently of the mains supply by pressing the LAMP ON/OFF button for 2 seconds.

SMART Manager must be informed of all parameters manually selected on the Control Unit. For example, to ensure that the scaling of the UV signal is correct, SMART Manager must be informed of the AUFS range selected on the Control Unit. The wavelength and time constant must also be entered in the User Supplied field in Run/Control configuration.

2.18 Gradient monitor

From the UV monitor flow cell, the column eluent passes directly through the gradient monitor flow cell (Fig. 2.18), where the conductivity of the eluent is monitored throughout the run.

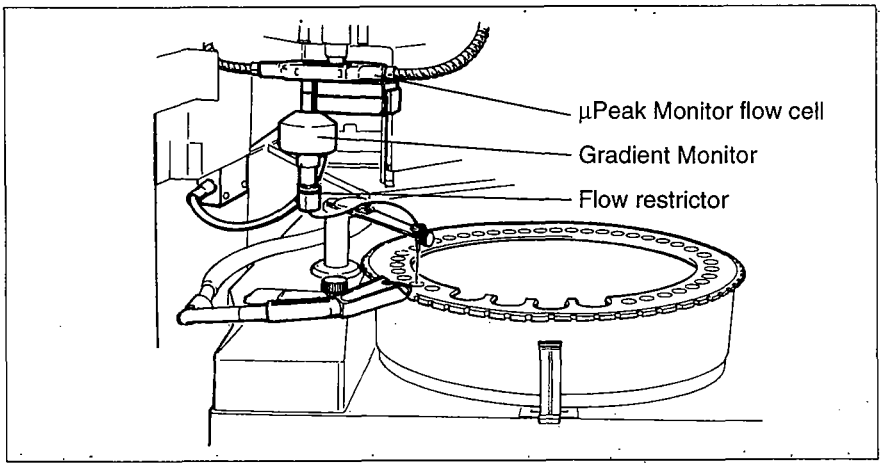


Fig. 2.18 Gradient monitor

This monitor is equipped with a 1.5 µl conductivity cell with platinum electrodes for monitoring salt buffers and organic solvents containing a counterion.

There are 4 precalibrated conductivity ranges stored in SMART Manager. However the gradient monitor can be calibrated for any buffer with a simple software controlled calibration procedure. To obtain 0 - 100 % response for your eluents it is recommended to perform a calibration. Conductivity is temperature dependent, so a calibration should be performed at the temperature set for the run.

2.19 Flow restrictor

A flow restrictor is positioned in-line following the gradient monitor. This helps maintain a constant back pressure of 0.7 MPa which prevents the formation of air bubbles in the flow cells.

To minimise the effects of band broadening, the internal volume of the restrictor is only 1.5 µl.

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2.20 µFraction collector

From the gradient monitor, the eluent passes through a Fused Silica tubing to the µFraction Collector (Fig. 2.18) where it is either collected or diverted to waste. Parameters relating to the collection of fractions are controlled by the Separation Method or can be controlled manually in the Run/Control window.

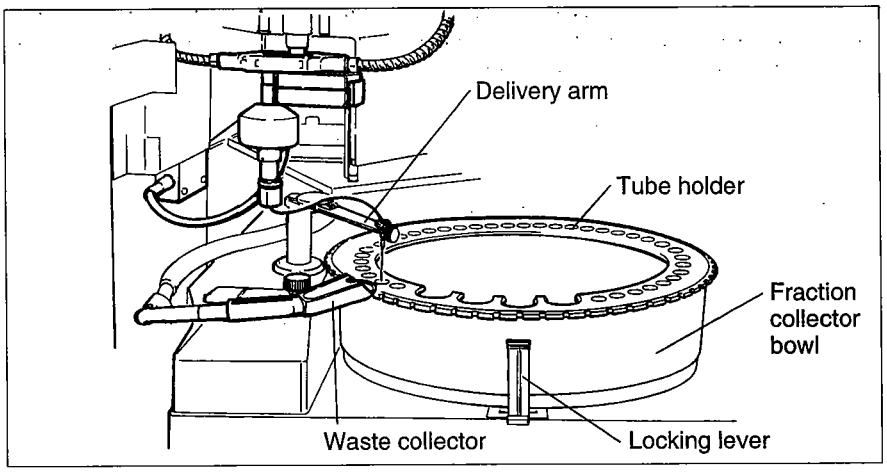


Fig. 2.19. µFraction collector

The µFraction collector is designed to collect small fractions, down to 5 µl, with an accuracy of ± 0.5 µl.

Collection tubes are held in a tube holder fitted to a circular bowl. Three types of tube holder are available; each holds a different number of tubes. These are:

- 48 x 0.5 ml sample tubes and 4 x 24 ml tubes
- 60 x 0.5 ml sample tubes
- 41 x 1.5 ml sample tubes

The 24 ml tubes can be used as waste collection tubes during sample injection or after a run if the general waste position for the collection arm is not in use.

The fraction collector bowl is motor driven and in the drop mode, gives a tube change in less than 0.15 seconds. This ensures that no eluent is lost between changes. All tube holders have a cut-out pattern on the outer edge which enables a light emitting diode at the back of the fraction collector to read the tube position. This allows the fractions to be collected in any sequence.

The fraction collector bowl can be inserted at any position. By means of a magnetic sensor, located at the rear end of the fraction collector, and a magnet located at the underside of the tube holder, the bowl automatically rotates to the start position when the locking lever is engaged.

There are two collection modes which are dependant on the volume of the fraction. In drop mode, the delivery needle is in the upper position while dispensing. In wipe mode, the delivery needle is in the lower position, in contact with the inner wall of the tube while dispensing. In this mode, a small part of the drop can be collected. Wipe mode should only be used for fractions less than 250 µl when collected in 0.5 ml tubes.

When fractions are not being collected, the delivery arm moves to the waste position. The waste tubing joins the waste outlet from the

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injection valve and exits from the µSeparation Unit into a collection vessel or drain.

2.21 System Power Supply

SMART System is supplied with power at 24 V DC from a single System Power Supply which hooks onto the rear panel of the µSeparation Unit (Fig. 2.20). The MultiNet cable distributes power from the Power Supply to SMART System modules. The System Power Supply has an automatic voltage switch and can be connected to any mains output between 100-120 V~ and between 220-240 V~ with a frequency between 50 and 60 Hz.

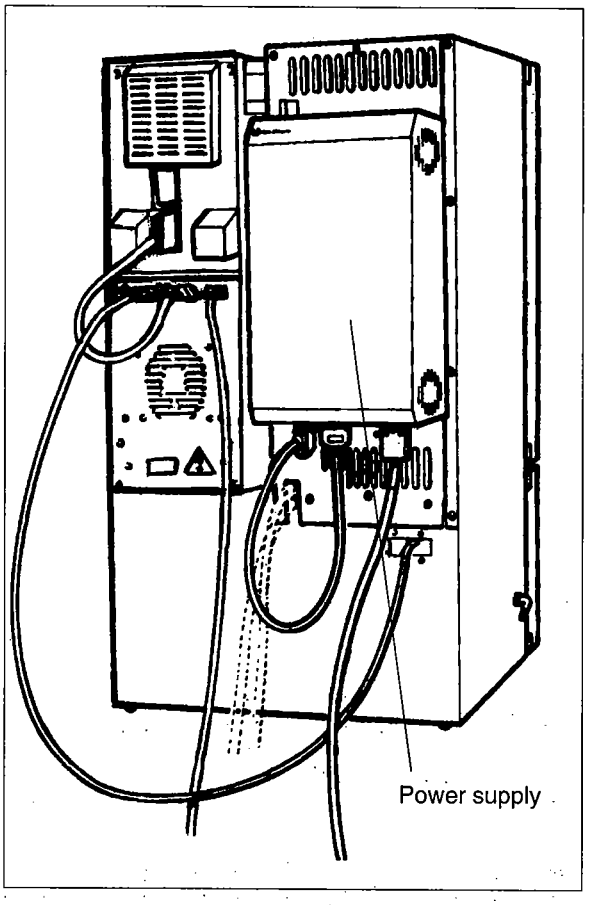


Fig. 2.20. System Power Supply

The MultiNet cable also forms the communication network between the computer and SMART System modules. A separate signal cable is supplied for the analogue signal from the UV-M II Monitor.

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Auxiliary output, SMART® System

INSTRUCTIONS

A new socket, Auxiliary output, has been added to the µSeparation Unit of SMART® System. A TTL-pulse can be sent out via this socket to activate auxiliary equipment, for example an autosampler. The polarity and length of the pulse sent out from SMART System can be set in SMART Manager. The socket, a 9 pole connector, is located on the back of the µSeparation Unit. Figure 1 shows the pin configuration.

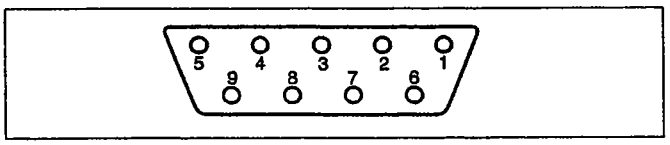


Fig. 1. Pin 3 is Output (positive = leading edge; negative = trailing edge). Pin 5 is 0 V.

New menus in SMART Manager

Run/Control

Use the dialog box for USER SUPPLIED CONFIGURATION to set the polarity of the auxiliary output to positive (default) or negative and the length of the signal to 0.2 (default), 0.4, 0.6, 0.8 or 1.0 seconds. Confirm the values with OK. These settings remain in the memory until new values are set.

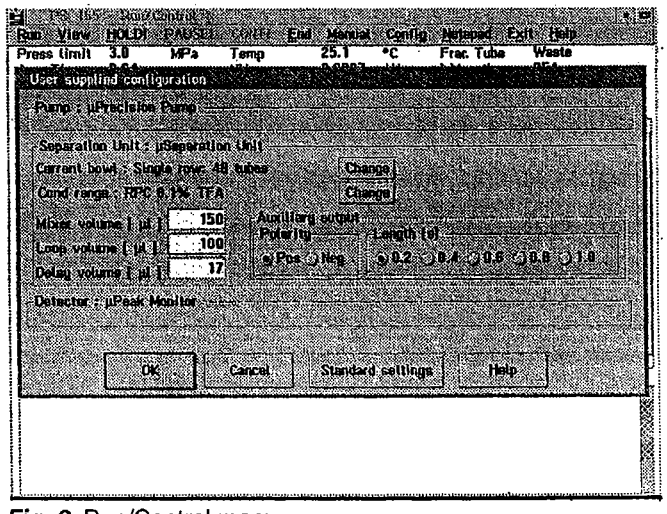


Fig. 2. Run/Control menu.

Check the TTL-pulse requirements in the instructions of the auxiliary instrument you wish to connect.

An auxiliary instrument can be activated with the command AUXILIARY OUTPUT under the menu MANUAL, SEP UNIT.

Method Editor

There is a new instruction, AUXILIARY OUTPUT, in the menu SEP UNIT. This activates auxiliary equipment at a specified time/volume in a method.

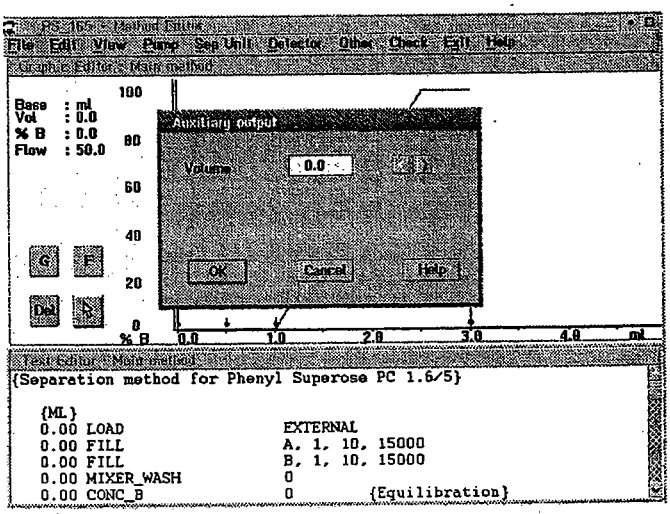


Fig. 3. Method Editor menu.

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Accessories, SMART® System

INFORMATION

New accessories for connecting SMART System to a mass spectrometer

The Flow Splitter (used when connecting SMART System to a mass spectrometer and fractions are collected and analysed in parallel) and Dummy Restrictor (used instead of the flow restrictor when a capillary with a small inner diameter is connected after the Gradient Monitor) are two new accessories used when connecting SMART System to a mass spectrometer. More information about this application is available in the Technical Note, "Electrospray LC-MS using SMART® System", Code No. 18-1104-38.

Accessory	Code No.
Flow Splitter	18-1103-79
Dummy Restrictor	18-1103-78

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New Unpacking and Installation procedures for SMART™ System

INSTRUCTIONS

The packing of SMART System has been redesigned. The system is pre-assembled before shipping. Please disregard the instructions in System Handbook, section 3.2. Instead follow the instructions below to unpack the system.

1. Cut off the straps around the box.
2. Open the top of the cardboard box (fig. 1). Remove the Packing List and then remove the foam packing on the top.

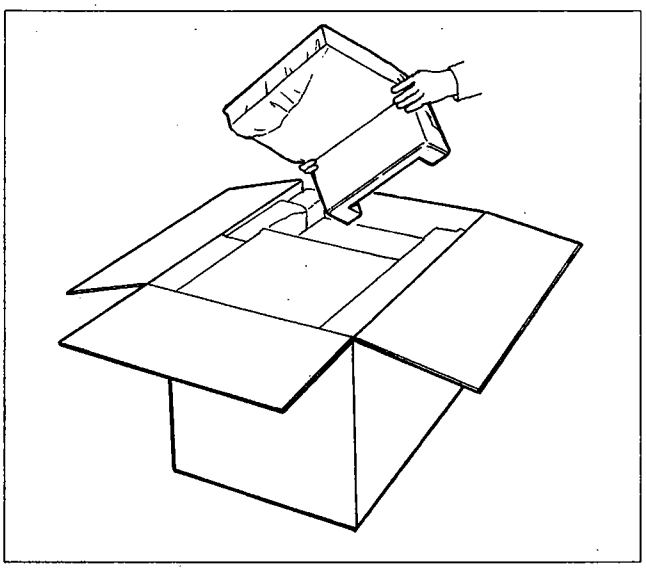


Fig. 1. Open the cardboard box.

3. Remove the System Handbook, SMART Manager and Solvent Reservoirs from the package.
4. Lift off the cardboard hood (fig. 2).

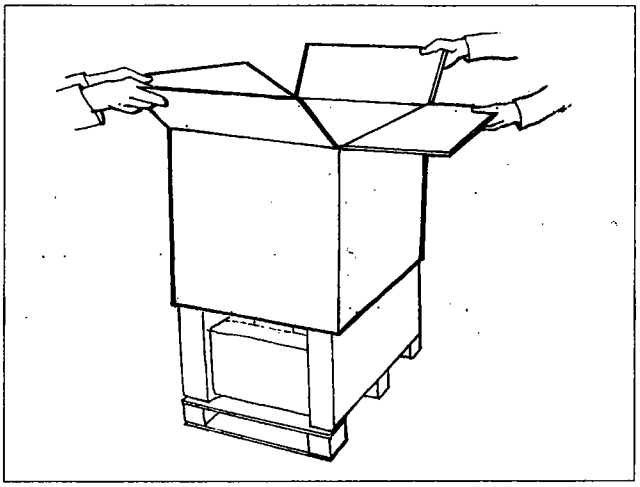


Fig. 2. Remove the cardboard hood.

Note: Do not remove any more of the wrapping until the system is placed on a bench.

5. Lift the system onto a bench, with two persons holding the red lifting straps on each side of the system (fig. 3).

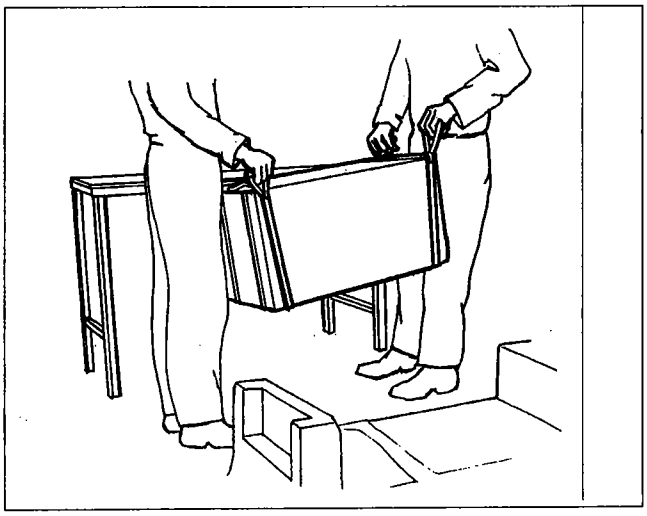


Fig. 3. Lift the system onto a bench.

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13. Twist and pull out the plastic bag with the fraction collector bowl together with the foam packing (fig. 9). Be careful not to damage the fraction collector arm on the left hand side.

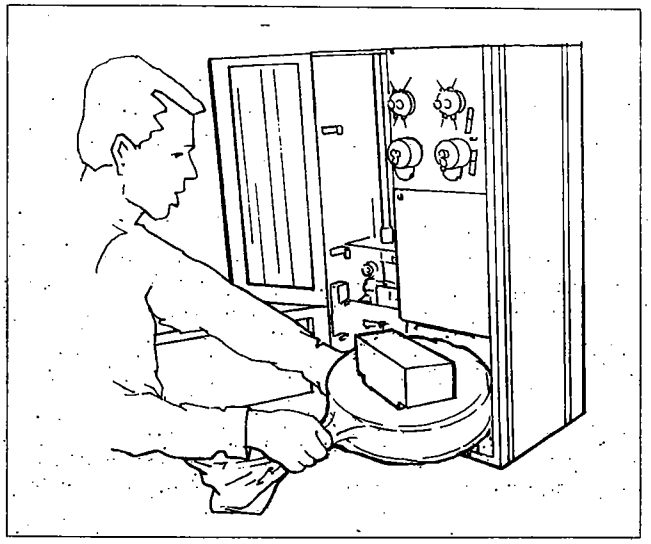


Fig. 9. Twist and pull out the fraction collector bowl.

14. Remove the second accessories bag located at the back of the lower part of the µSeparation Unit.

15. Remove the plastic bag from the fraction collector bowl. Replace the bowl and fasten the lever.

16. Check the system and the contents of the accessories bags against the Packing list.

17. Connect the fractionation capillary to the fractionation arm and secure it with the securing screw (fig. 10). Do not over tighten. Adjust the capillary tip according to the instructions in the System Handbook, section 6.14.

    Note: Do not twist the glass capillary, it should form a smooth arc pointing outward from the Gradient Monitor. Also, make sure the signal cable does not obstruct the movement of the capillary during fractionation.

    In the Test Certificate supplied with the system, a delay volume is given for the Gradient Monitor and fractionation capillary. This volume must be programmed in the Run/Control section of SMART Manager. The procedure is described in section 4.3.4 in System Handbook.

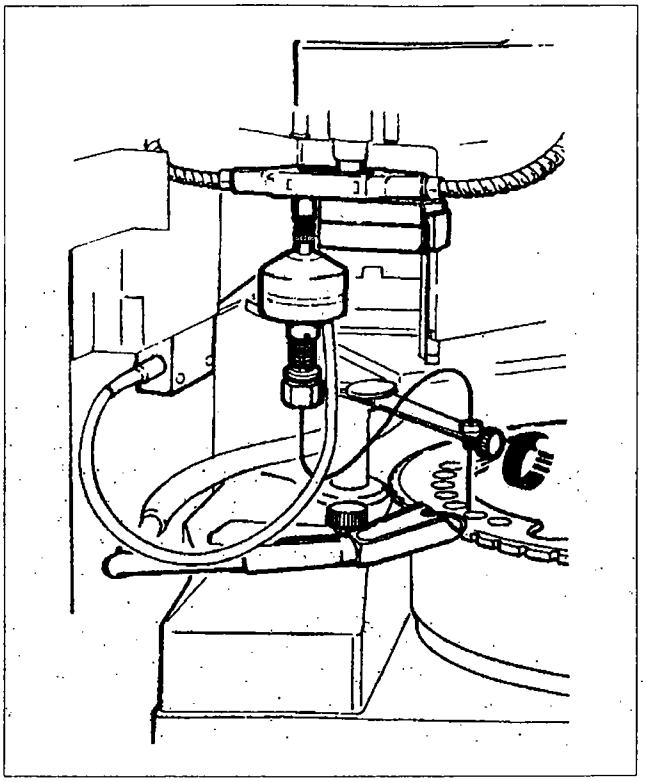


Fig. 10. Connect the fractionation capillary.

18. Make sure the waste outlet on the side of the µSeparation Unit is pointing down. Put the tubing into a waste container. The tubing end must be lower than the waste outlet to avoid over-flow. Avoid bends on the tubing that can cause air traps. If preferred the waste tubing can be removed and the waste outlet can drain directly into a waste container (fig. 11).

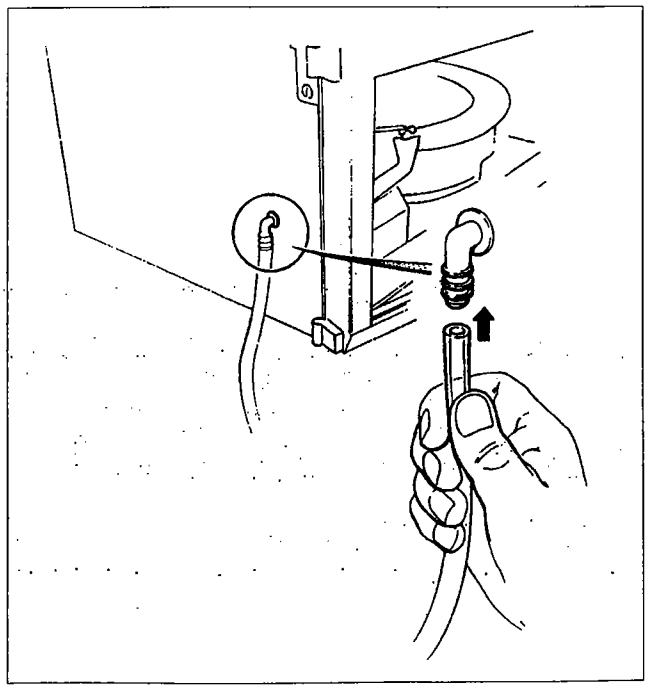


Fig. 11. Fit the waste tubing.

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6. Tilt the system carefully to an upright position (fig. 4).

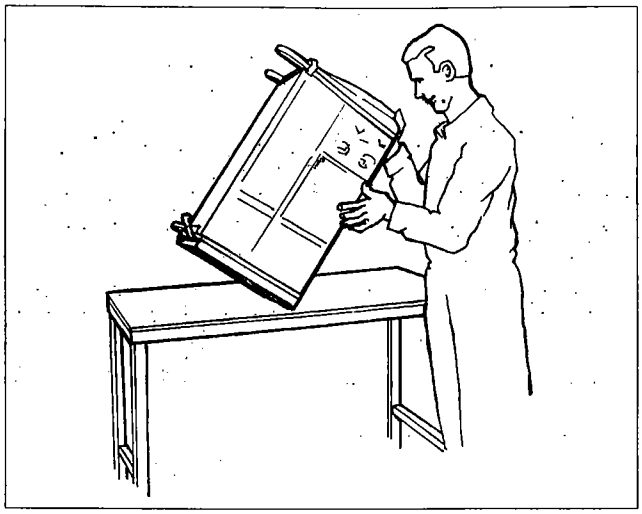


Fig. 4. Tilt the system to an upright position.

7. Remove the lifting straps.

8. Remove the outer plastic films around the system. Cut the films with the scissors resting against the straps underneath to avoid scratching the system (fig. 5).

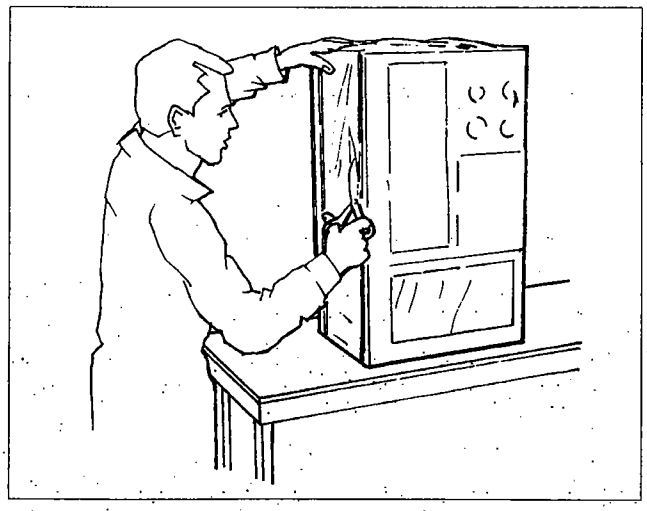


Fig. 5. Cut the plastic film.

9. Cut off the straps holding the system together.

10. Remove the thin sheets of foam rubber from the sides of the system. Carefully remove the foam rubber sheet from between the µSeparation Unit and the µPrecision Pump. Lift the foam rubber straight up. (fig. 6)

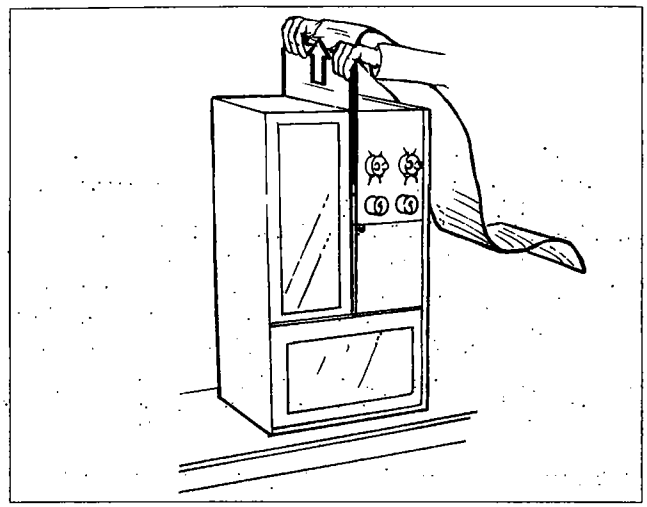


Fig. 6. Remove the foam rubber sheets.

11. Open the upper door on the µSeparation Unit and remove the accessories bag (fig. 7).

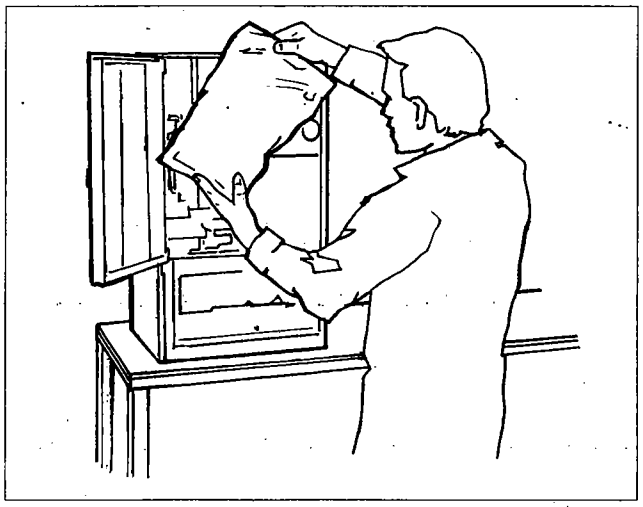


Fig. 7. Remove the accessories bag.

12. Open the lower door on the µSeparation Unit. Release the locking lever of the fraction collector.

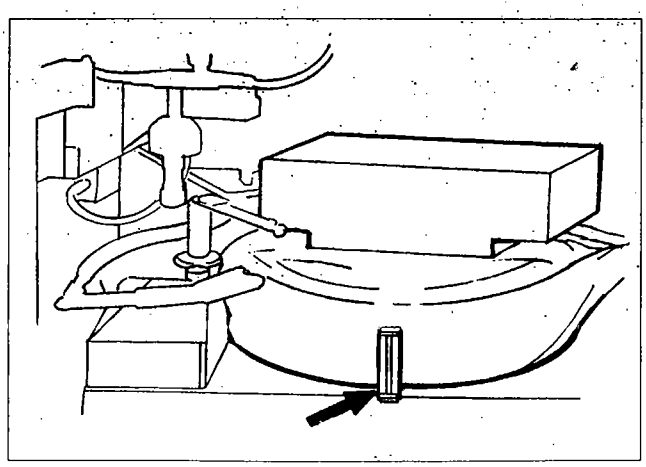


Fig. 8. Release the locking lever of the fraction collector.

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19. If you want to cool the system, connect the water inlet and outlet tubing at the back of the Separation Unit to a circulating water bath. For the MultiTemp II replace the connectors on the MultiTemp with the connectors supplied.

    Remove the tubing connectors at the rear of MultiTemp II with a wrench 11/16". Do not bend, shake or twist the inlet and outlet tubes from MultiTemp II. Put the sealing rings on the new Tubing Connectors. Secure them with the clamps supplied.

20. Install SMART™ Manager according to the instructions in the System Handbook, section 3.3. Connect the Computer Cable to the computer.

22. Select the appropriate mains cable and connect it to the power supply.

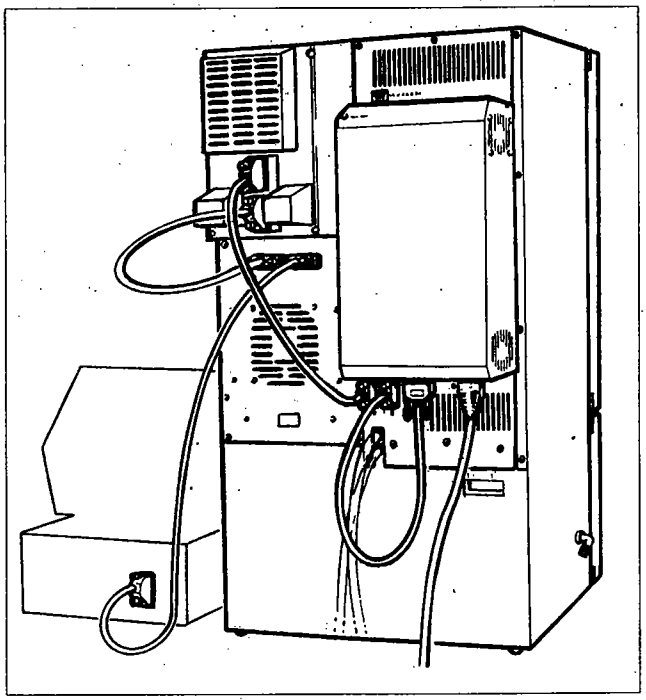


Fig. 12. Connect the mains cable.

Note: Before you install your column according to the instructions in section 4.3.1, remember to install the prefilter supplied in one of the accessories bags. Push it onto the column fitting.

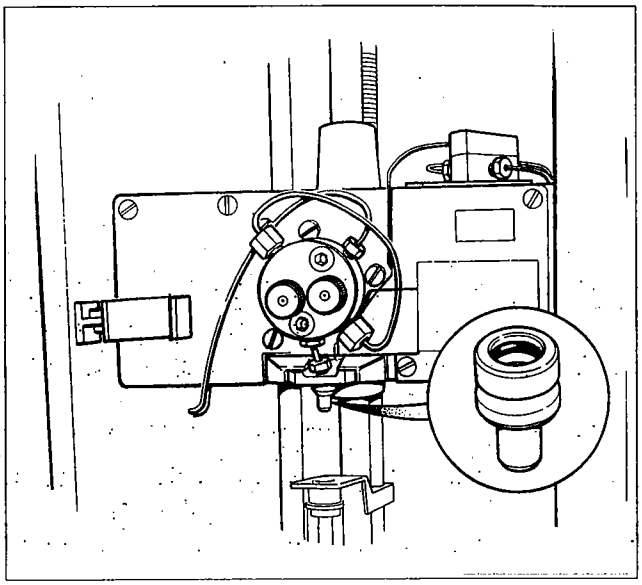


Fig. 13. Prefilter.

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3. Unpacking and installation

3.1 Site requirements for SMART System

SMART System should be installed on a stable laboratory bench. The minimum space required for the system, including the computer, is 60 x 120 cms. A free height of 105 cms above the bench is also required (Fig. 3.1).

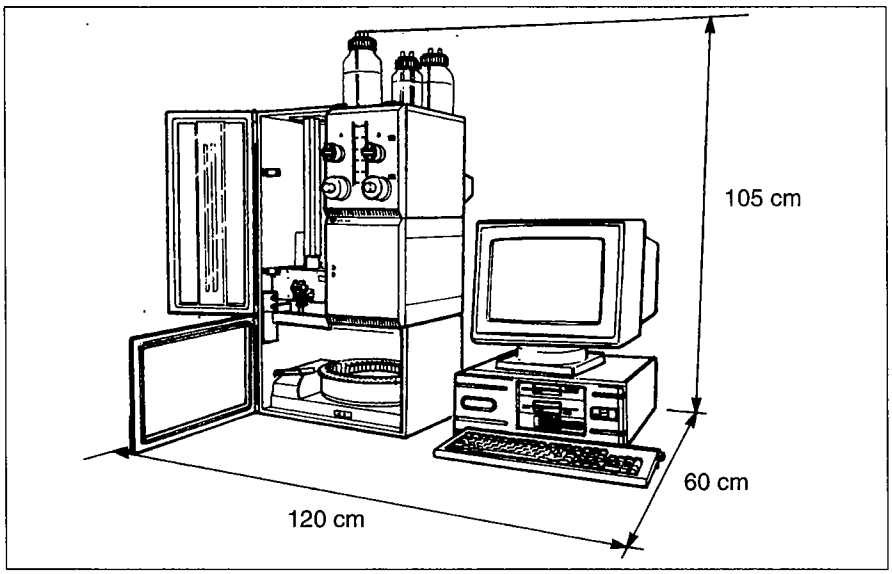


Fig. 3.1. Dimensions of SMART™ System

Two mains power points are required, one for SMART System and one for the computer. If the Monitor UV-M II is a part of the system another mains power point is required. All power points should be connected to a protected ground.

There is no fuse in SMART System. The power supply has an automatic voltage switch-over and can be connected to any mains output between 100-120 V~ and between 220-240 V~.

For effective cooling of the µSeparation Unit, a constant supply of circulating water at +5 °C should be available from a circulating bath. For more details, refer to Section 2.13.

3.2 Unpacking SMART System, µPeak Monitor

To unpack the system please follow the instructions below:

1. Cut off the straps around the box.
2. Open the top of the cardboard box (Fig. 3.2). Remove the Packing List and then remove the foam packing on the top.

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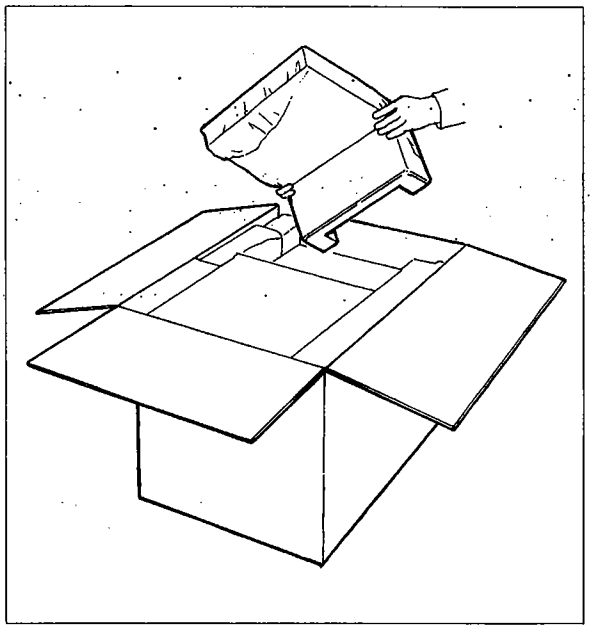


Fig. 3.2. Open the cardboard box.

3. Remove the System Handbook, SMART Manager and Solvent Reservoirs from the package.
4. Lift off the cardboard hood (Fig. 3.3).

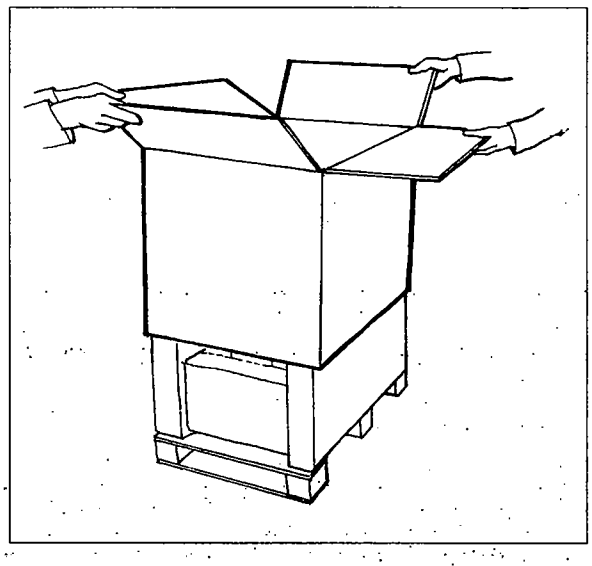


Fig. 3.3. Lift off the cardboard hood

Note: Do not remove any more of the wrapping until the system is placed on a bench.

5. Lift the system onto a bench, with two persons holding the red lifting straps on each side of the system (Fig. 3.4).

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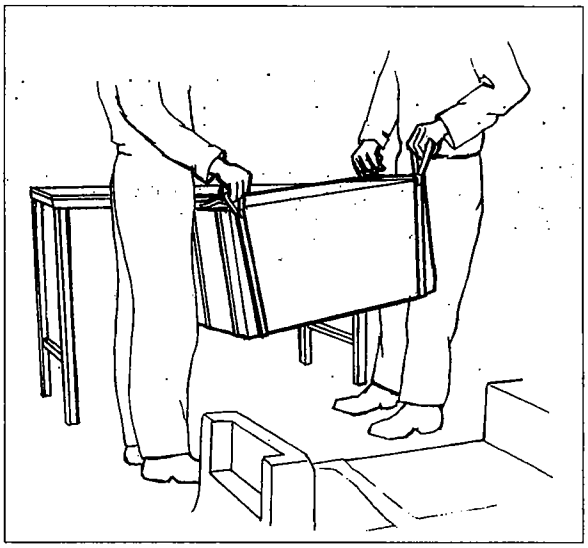


Fig. 3.4. Lift the system onto a bench

6. Tilt the system carefully to an upright position (Fig. 3.5).

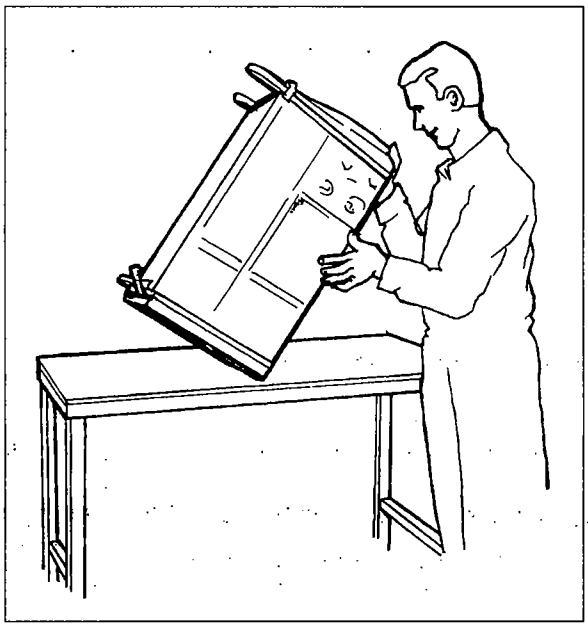


Fig. 3.5. Tilt the system to an upright position

7. Remove the lifting straps.
8. Remove the outer plastic films around the system. Cut the films with the scissors resting against the straps underneath to avoid scratching the system (Fig. 3.6).

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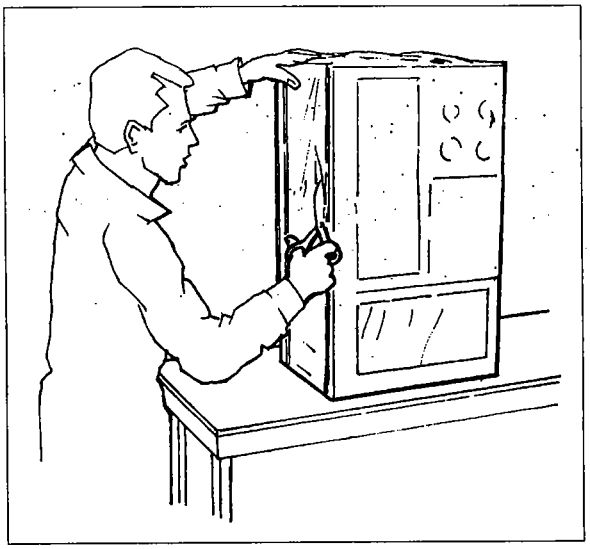


Fig. 3.6. Cut the plastic films

9. Cut off the straps holding the system together.

10. Remove the thin sheets of foam rubber from the sides of the system. Carefully remove the foam rubber sheet from between the µSeparation Unit and µPrecision Pump. Lift the foam rubber straight up (Fig. 3.7).

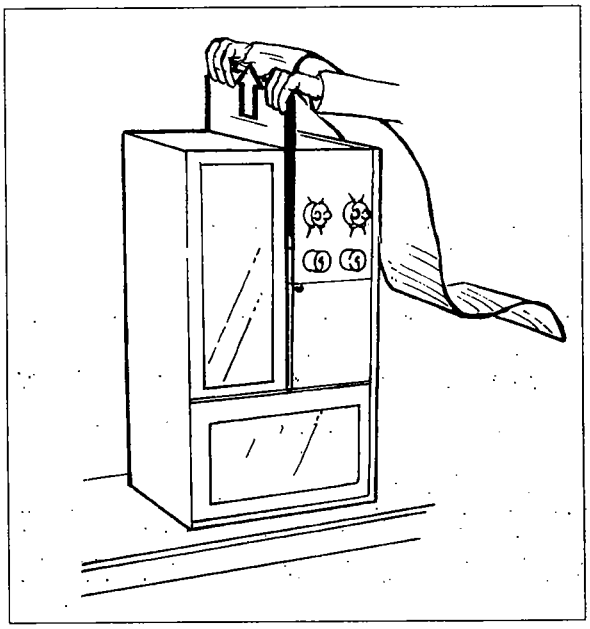


Fig. 3.7. Remove the foam rubber sheets

11. Open the upper door on the µSeparation Unit and remove the accessories bag (Fig. 3.8).

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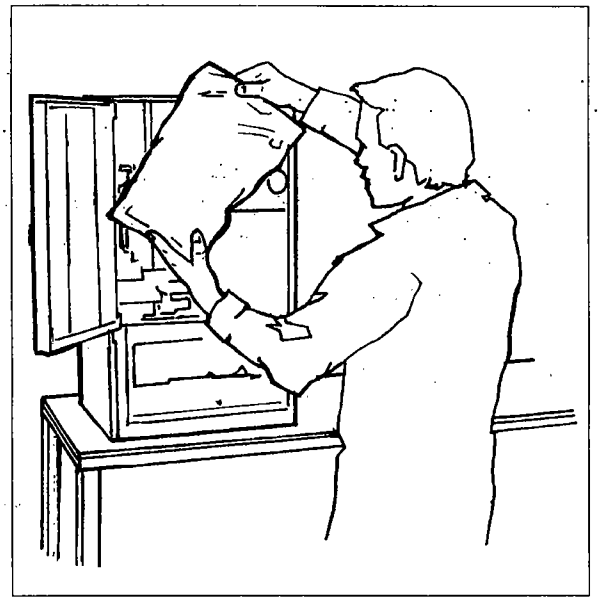


Fig. 3.8. Remove the accessories bag

12. Open the lower door on the µSeparation Unit. Release the locking lever of the fraction collector (Fig. 3.9).

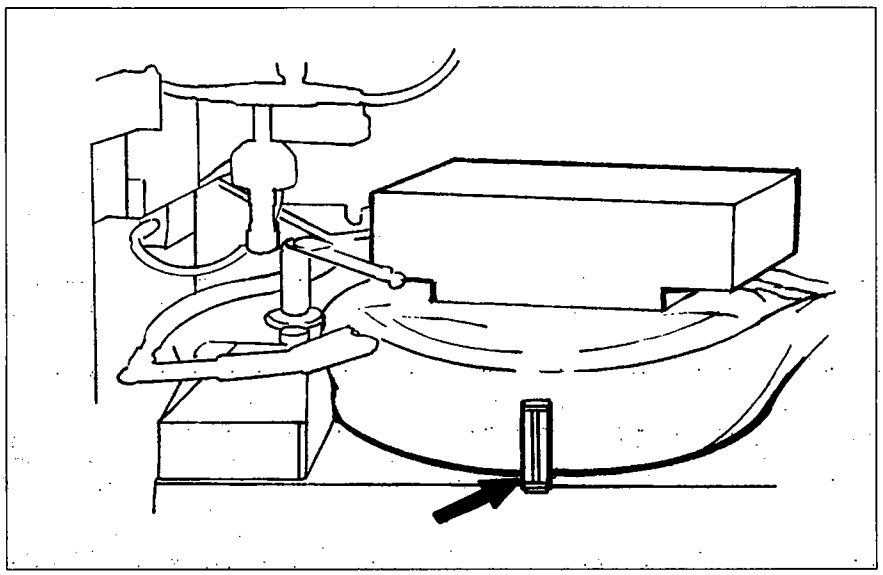


Fig. 3.9. Release the locking lever of the fraction collector

13. Twist and pull out the plastic bag with the fraction collector bowl together with the foam packing (Fig. 3.10). Be careful not to damage the fraction collector arm on the left hand side.

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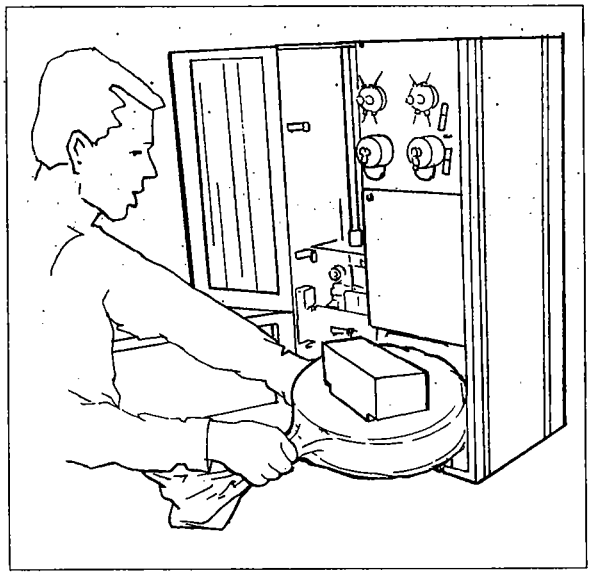


Fig. 3.10. Twist and pull out the fraction collector bowl.

14. Remove the second accessories bag located at the back of the lower part of the µSeparation Unit.

15. Remove the plastic bag from the fraction collector bowl. Replace the bowl and fasten the locking lever.

16. Check the system and the contents of the accessories bags against the Packing List.

17. Connect the fractionation capillary to the fractionation arm and secure it with the securing screw (Fig. 3.11). Do not over tighten. Adjust the capillary tip according to the instructions in Section 6.20.

    Note: Do not twist the glass capillary, it should form a smooth arc pointing outward from the Gradient Monitor. Also, make sure the signal cable does not obstruct the movement of the capillary during fractionation.

    In the Test Certificate supplied with the system, a delay volume is given for the Gradient Monitor and fractionation capillary. This volume must be programmed in the Run/Control section of SMART Manager. The procedure is described in Section 4.3.4.

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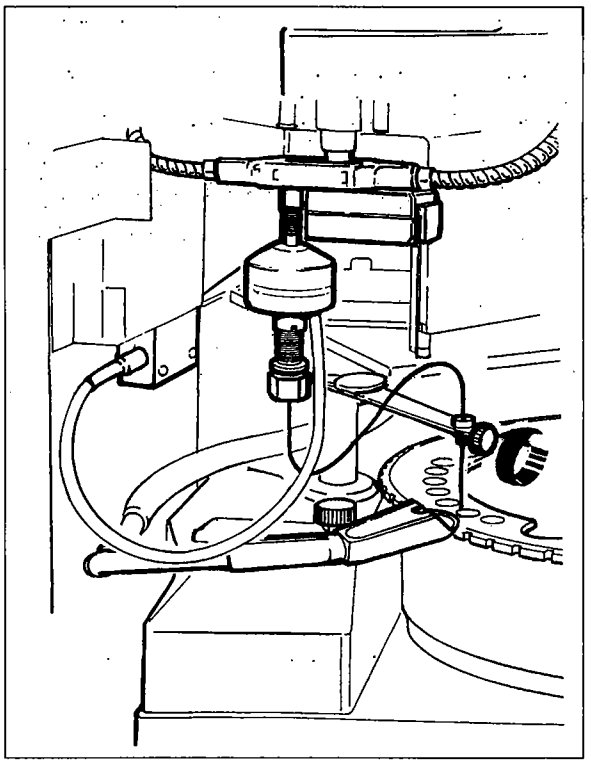


Fig. 3.11. Connect the fractionation capillary

18. Make sure the waste outlet on the side of the µSeparation Unit is pointing down. Put the tubing into a waste container. The tubing end must be lower than the waste outlet to avoid overflow. Avoid bends on the tubing that can cause air traps. If preferred the waste tubing can be removed and the waste outlet can drain directly into a waste container (Fig. 3.12).

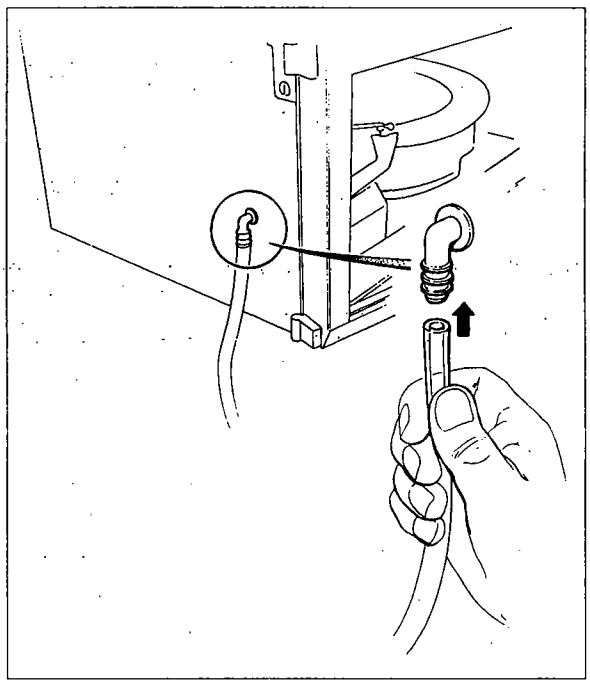


Fig. 3.12. Fit the waste tubing

19. If you want to cool the system, connect the water inlet and outlet tubing at the back of the µSeparation Unit to a circulating water bath. For the MultiTemp II replace the connectors on the MultiTemp with the connectors supplied.

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Remove the tubing connectors at the rear with a wrench 11/16". Do not bend, shake or twist the inlet and outlet tubes from MultiTemp II. Put the sealing rings on the new Tubing Connectors. Secure them with the clamps supplied.

20. Push it upwards onto the column fitting (Fig. 3.13).

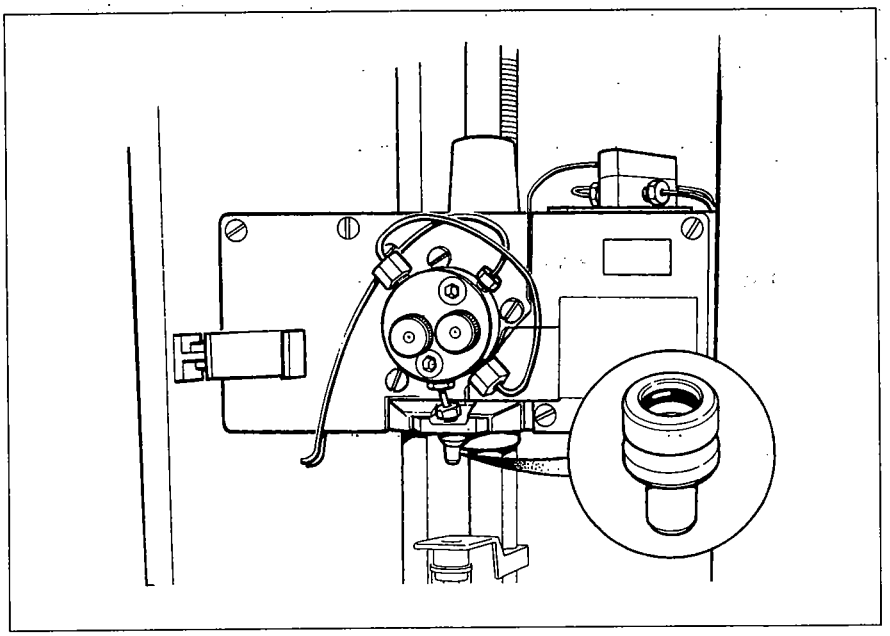


Fig. 3.13. Fitting the Prefilter

Assembly of SMART System is now complete.

3.3 Installation SMART System, Monitor UV-M II

3.3.1 Unpacking

SMART System, UV-M II is delivered in two wooden cases which are secured together. These cases can be opened using heavy duty handtools, such as a big screwdriver and a hammer. It is recommended that all packing materials should be retained if onward transport of the instrument is expected.

One of the cases contains the µSeparation Unit and the other case contains the µPrecision Pump, UV-M II Monitor and the System Power Supply. Follow the procedure below to install the system.

Separate the two cases and open each box. Check the contents against the Packing Lists supplied. Retain the Packing Lists and the test certificate.

1. Open the case containing the µSeparation Unit. Remove the packing material.
2. From the same case, remove the Fraction Collector and the accessories pack. Lift out the µSeparation Unit and place it on the bench (Fig. 3.14).

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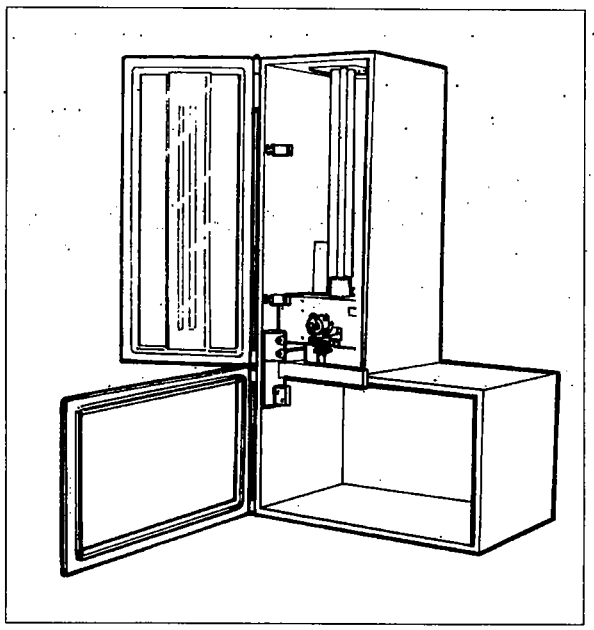


Fig. 3.14. µSeparation Unit

3.3.2 Installation of µPrecision Pump

1. Unpack the µPrecision Pump and place it on top of the µSeparation Unit. Slide the Tubing Holder onto the top of the pump at the front. Unwind the rinse tubings from the pump cylinders.

2. Remove the protective plugs from the port marked OUT on each pump inlet valve and from the On-line filters at the top of the µSeparation Unit. Connect the two outlet tubings between the OUT ports and the On-line Filters on the top of the µSeparation Unit. Use the shorter of the two tubings to connect to inlet valve A and connect the other end to any of the On-line filters.

   To connect this tubing securely, push it firmly into the port and, at the same time, tighten the hexagonal connector firmly with the fingers and then very gently with the plastic wrench supplied.

3. Remove the protective plugs from the ports 1 and 4 on each inlet valve. Connect an inlet tubing to the ports.

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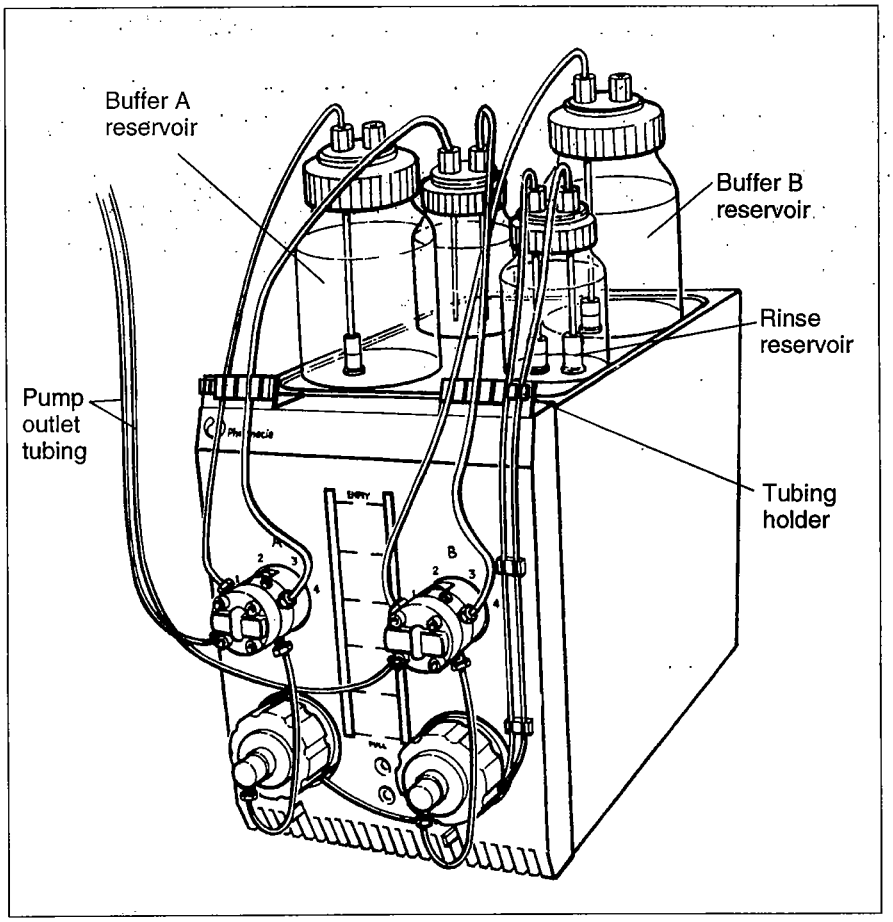


Fig. 3.15. The fluidic connections on the pump

4. Unpack the 500 ml solvent reservoirs supplied and place them on the pump. Remove the caps.
5. Remove the filters from the inlet tubings, position 1 on each valve, by slightly turning the red filter ends anti-clockwise until you can pull out the tubing.
6. Thread the tubing through the caps and replace the filters. Place the tubing in the 500 ml solvent reservoirs.
7. Repeat this for the rinsing tubing connected to the cylinders and place both tubing in the 250 ml rinse reservoir.

3.3.3 Installation of System Power Supply

1. Unpack the System Power Supply and hook it onto the back of the µSeparation Unit (Fig. 3.16). Locate the mains cable appropriate for the power supply in the laboratory. Connect one end to the mains inlet socket on the underside of the Power Supply and the other to the mains supply but do NOT switch on.

   Note: There is no voltage selector on the power supply since it has automatic voltage adjustment for voltages within the range specified.

   If it is necessary to change the plug on the mains cable, ensure that the colour coding is followed:

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- GREEN/YELLOW to EARTH terminal
- BROWN to LIVE terminal
- BLUE to NEUTRAL terminal

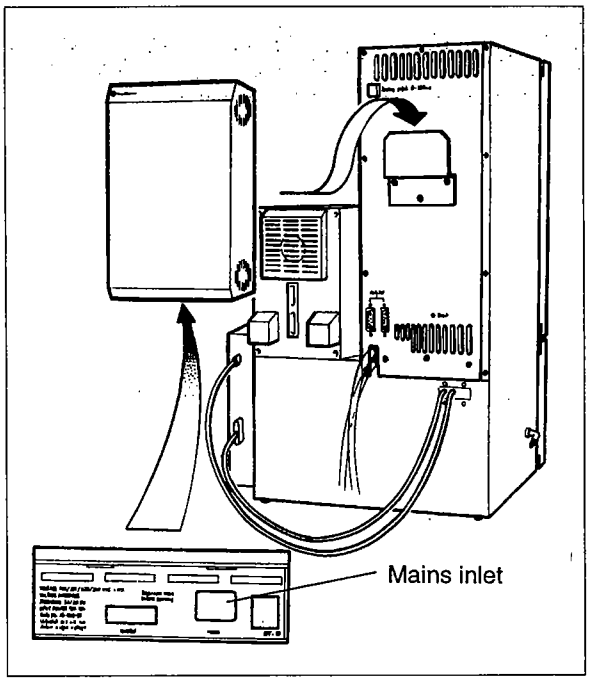


Fig. 3.16. Connecting the Power Supply

3.3.4 Installation of Monitor UV-M II

1. Locate and unpack the UV-M II Control Unit. Attach the front panel for vertical operation (Fig. 3.17). Attach the four feet.

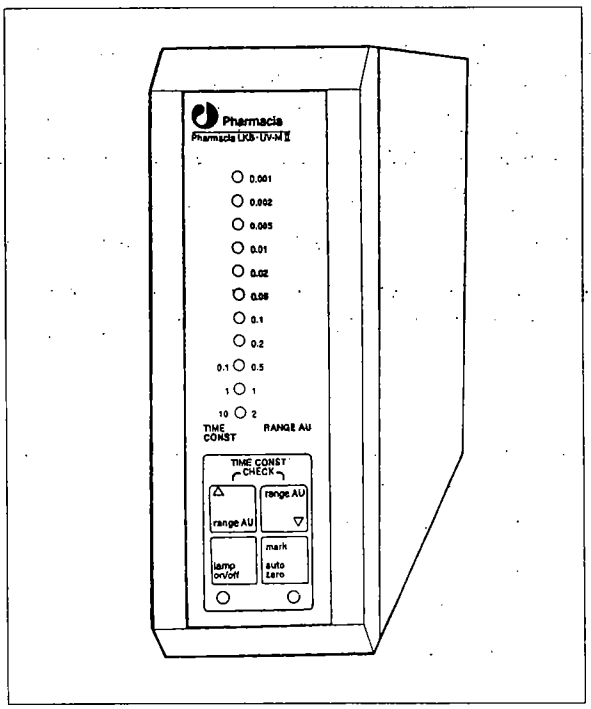


Fig. 3.17. UV-M II Monitor

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2. From the accessories supplied with the monitor, select the correct fuse for the voltage in the laboratory. For 100 - 120 V, use a 400 mA SB fuse; for 220 - 240 V, use a T 200 mA L fuse. Discard the unwanted fuse kit immediately.

   To fit the fuse, use a screwdriver to open the fuse/voltage selector housing (Fig. 3.18).

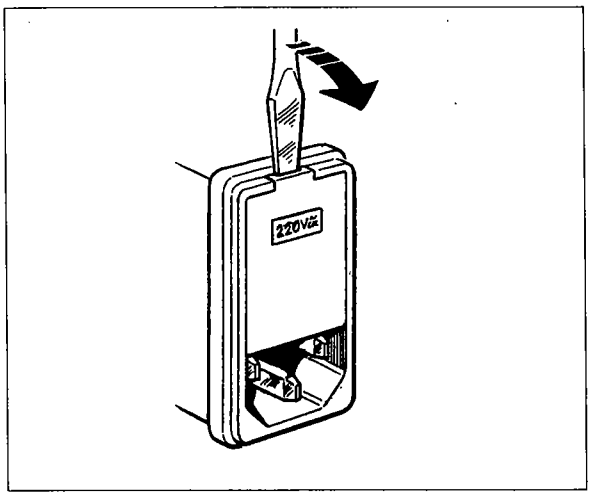


Fig. 3.18. Opening the fuse/voltage selector housing

   Insert the correct fuse holder and fuse into the right-hand position (Fig. 3.19).

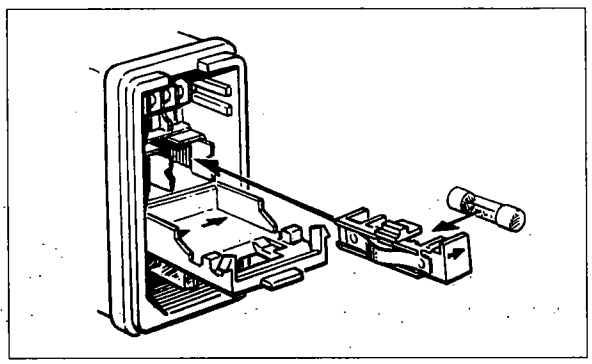


Fig. 3.19. Inserting the fuse holder

3. Remove the rotary voltage switch, select the correct voltage and place the switch into the instrument with the correct voltage showing (Fig. 3.20). Select 220 if the mains is 230 V~. Close the cover. Make sure the correct voltage is shown in the window.

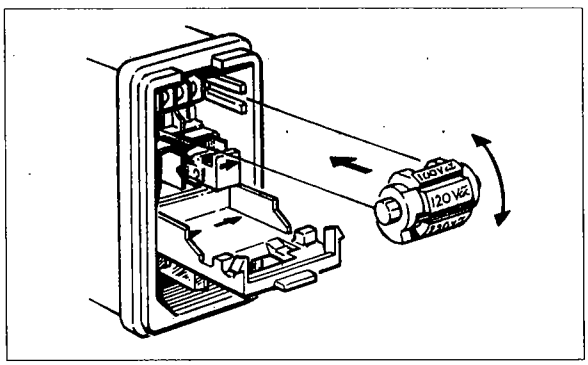


Fig. 3.20. Replacing the voltage switch with the voltage still visible

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4. Connect the cables from the Optical Unit to the Control Unit (Fig. 3.21). Connect the Optical Unit cable to the socket marked "OPTICAL UNIT"; connect the lamp cable to the socket marked "LAMP".

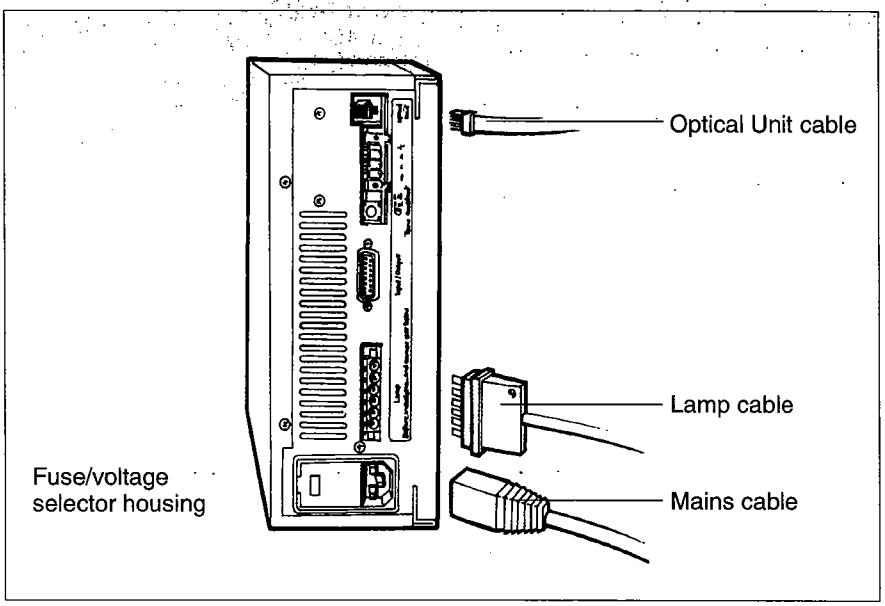


Fig. 3.21. Cable connections on the monitor back panel

5. Locate the mains cable appropriate for the mains supply in the laboratory and insert it into the mains inlet socket of the UV-M II Monitor.

6. Locate the Cable UV-M II which is packed with the accessories. Plug the end with the connector into the socket at the top of the µSeparation Unit (Fig. 3.22). Connect the green 4 pole connector onto the other end of the cable so that the red lead connects to 100 mV and the black lead connects to 0 (zero). Connect the green connector into the socket marked 0-100 mV.

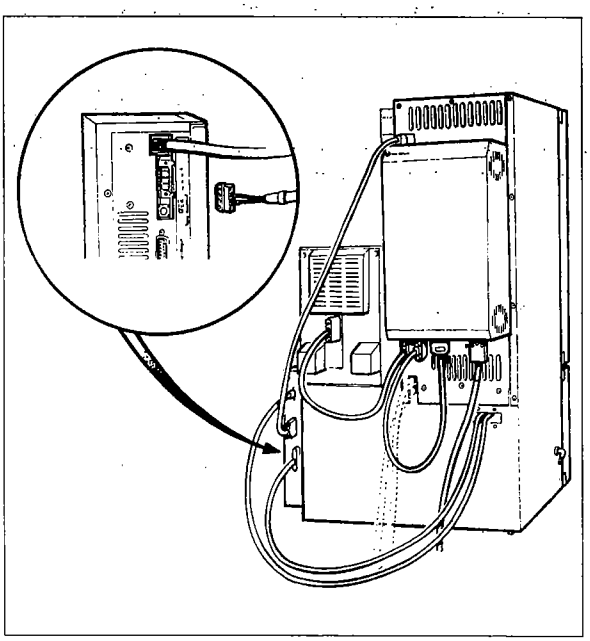


Fig. 3.22. Connecting the Cable, UV-M II

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3.3.5 Connection of MultiNet Cables

1. Locate the MultiNet Cable which is delivered in the accessories pack. Connect one end into the MultiNet socket on the System Power Supply. Connect the other end into one of the MultiNet sockets on the µSeparation Unit.
2. Locate the MultiNet cable supplied with the µPrecision Pump. Connect one end to Separation Unit and the other end to the µPrecision Pump.

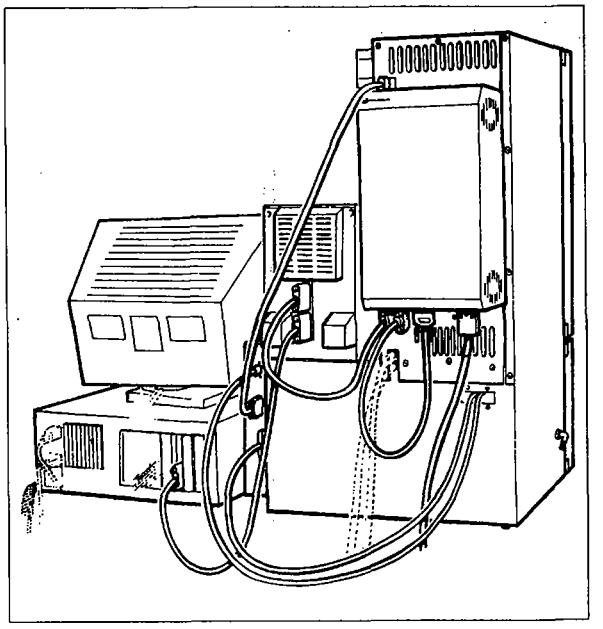


Fig. 3.23. MultiNet connections

3.3.6 Installation of the Fraction Collector

1. Unpack the Fraction Collector. To remove the bowl, release the lock by pulling it forwards (Fig. 3.24). Push the fraction collector assembly partly into the µSeparation Unit. Connect the strap cable connector to the socket in the upper left of the unit and secure it by tightening the two screws (Fig. 3.24, inset). Pull the excess cable forward to lie on top of the Fraction Collector.

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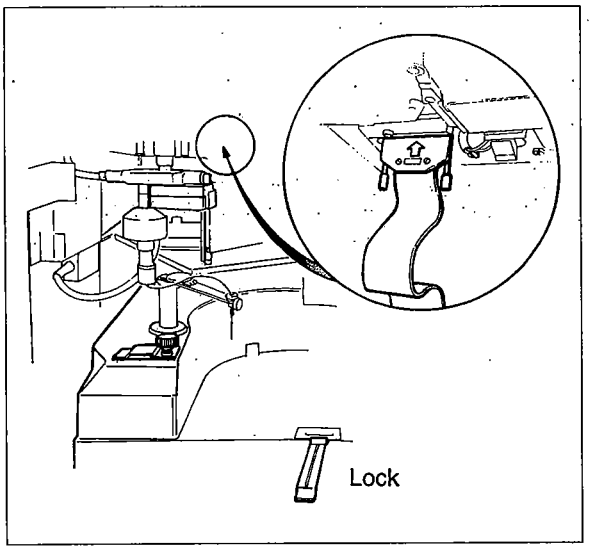


Fig. 3.24. Fitting the fraction collection

2. Push the Fraction Collector assembly completely into the µSeparation Unit. Insert the bowl into the Fraction Collector assembly and secure it with the lever.

3. Fit the fraction waste collector (Fig. 3.25). Use the adjustment screw to position the collector at an angle which allows drainage.

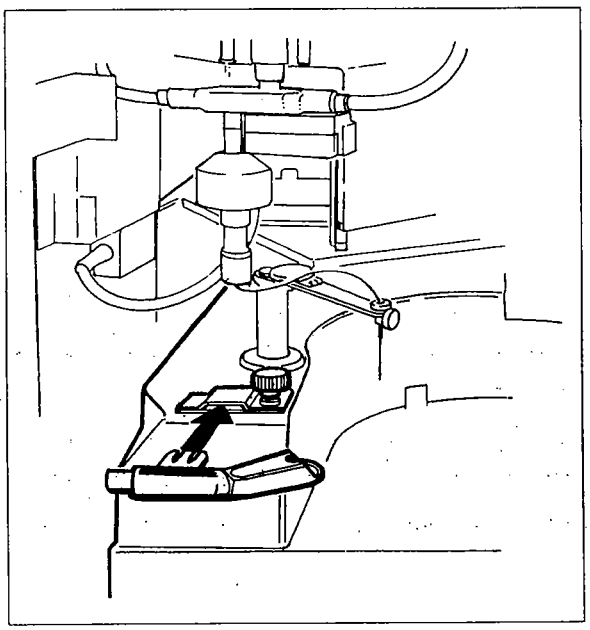


Fig. 3.25. Installing the waste collector

4. During transport the fraction collector arm is pushed down. To elevate it, turn on the power on the System Power Supply. The arm will come up and the fraction collector bowl will rotate to the start position. Turn off the power.

5. Place the delivery needle in the delivery arm and secure it with the retaining screw. The height of the delivery needle is adjusted after installation of SMART Manager, see Maintenance, Section 6.20.

In the Test certificate supplied with the system, a delay volume is given for the Gradient Monitor and fractionation needle. This volume must be programmed in the Run/Control section of SMART Manager. The procedure is described in Section 4.3.4.

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3.3.7 Connection of cooling water and waste tubing

1. If you want to cool the system, connect the water inlet and outlet tubings at the back of the µSeparation Unit to a circulating bath. For the MultiTemp II replace the connectors with the connectors supplied.

   Remove the tubing connectors at the rear of MultiTemp II with a wrench 11/16" (17 mm). Do not bend, shake or twist the inlet and outlet tubes from MultiTemp II. Put the sealing rings on the new Tubing Connectors and fasten the connectors to the inlet and outlet tubes.

   Thread the supplied clamps onto the cooling tubings of SMART System. Thread the cooling tubings onto the connectors. Secure them with the clamps.

2. Locate the waste tubing in the accessories pack. Fit it onto the waste outlet on the left side of the µSeparation Unit (Fig. 3.26). Turn the waste outlet to point downwards and put the tubing into a waste container. The tubing end must be lower than the waste outlet to avoid over-flow, and avoid bends on the tubing that can cause air traps. If preferred, the waste outlet can drain directly into a waste container.

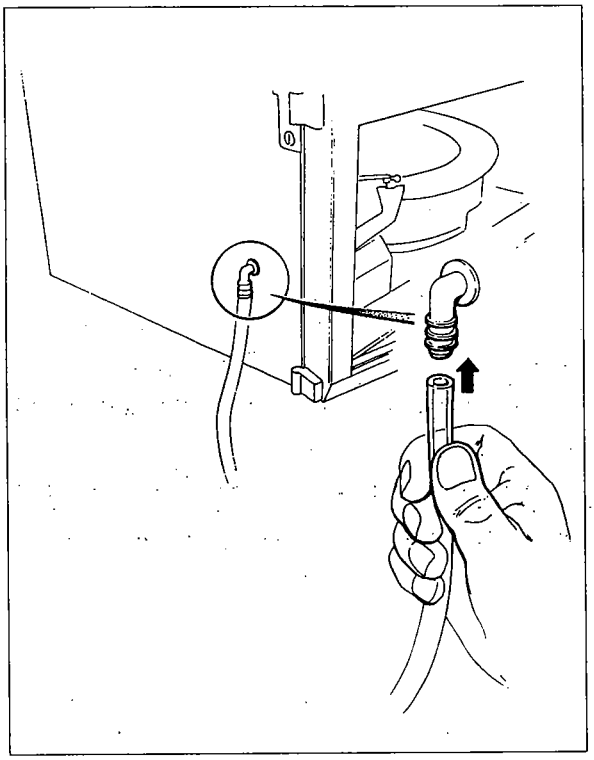


Fig. 3.26. Fitting the waste tubing

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3.3.8 Connection of Prefilter

1. Locate the Prefilter in the accessories pack. Push it upwards onto the column fitting (Fig. 3.27).

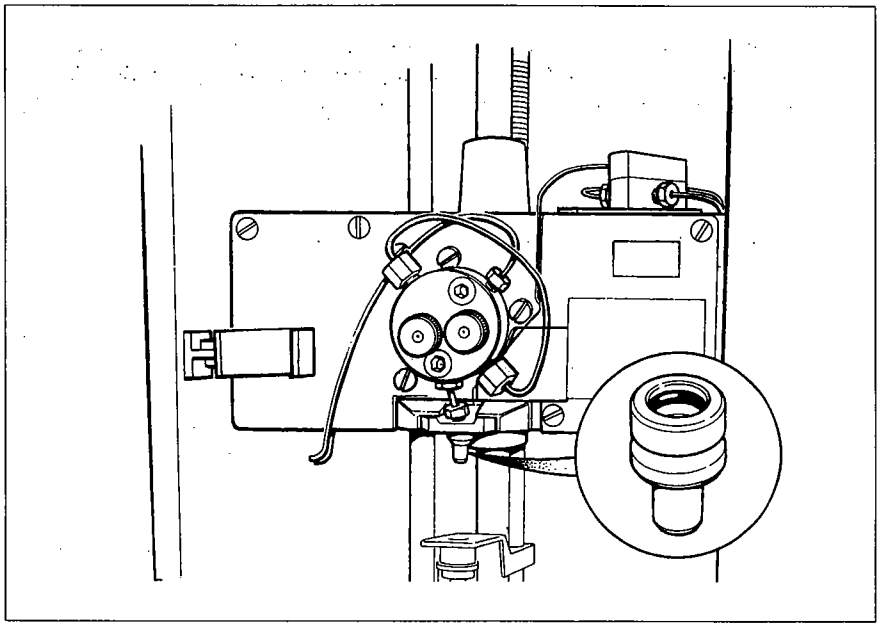


Fig. 3.27. Fitting the Prefilter

The sample injection loop and all other connections within the µSeparation Unit are already connected on delivery. There is a protective plug in the internal loop fill port on the injection valve. Leave this in place at all times.

Assembly of SMART System is now complete.

3.4 Installation of SMART Manager, AT

3.4.1 Installation of µControl Board, AT

Before unpacking the µControl Board, please read the Licence agreement for acceptance of the product.

1. For the specification of computer to run SMART Manager, refer to Section 9. Assemble the computer alongside SMART System.
2. Unpack the µControl Board and the MultiNet cable. Examine the board to check that the switches are correctly set (Fig. 3.28).

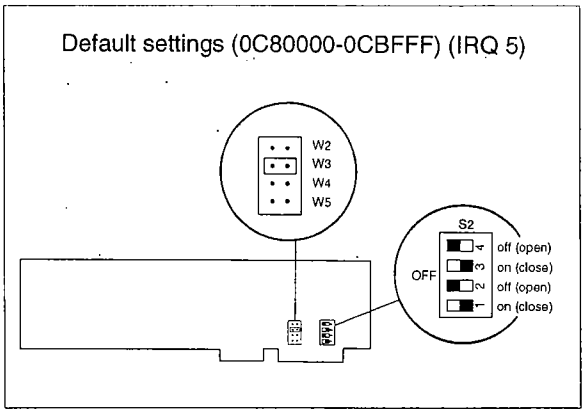


Fig. 3.28. Default settings on MultiNet board

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3. Ensure that the computer is switched off.
4. The MultiNet board can be installed in any empty slot. Refer to the instruction manual supplied with the computer for installation instructions. Ensure that the board fits firmly into the connector before replacing the panels.
5. Connect the MultiNet cable into the socket on the board (Fig. 3.29). Connect the other end into the µPeak Monitor or the µPrecision Pump if you have a Monitor UV-M II in the system.

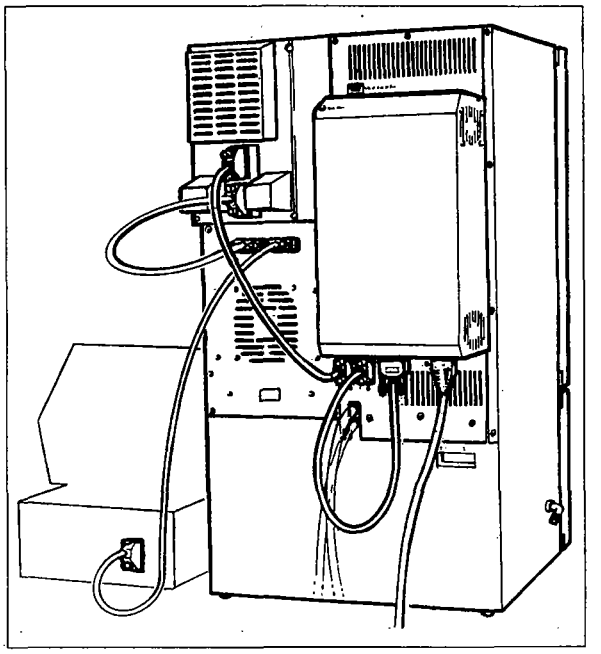


Fig. 3.29. MultiNet connections, computer to µPeak Monitor

3.4.2 Installation of SMART Manager, AT

Since SMART Manager operates within the Presentation Manager environment, OS/2 operating system must already be installed on the hard disk before attempting to install SMART Manager.

The mouse should already be installed as described in the manual supplied with the mouse.

A Registration card is supplied with SMART Manager. Please read it carefully before completing it and returning it to Pharmacia LKB Biotechnology AB. It is important that ownership of SMART Manager is registered so that we can inform you of future update versions of the program.

1. Switch on the computer and allow it to load the resident OS/2 program.
2. Access OS/2 Full Screen.
3. Insert Disk 1 into Drive A and type A: <ENTER>, type INSTALL E <ENTER>. The installation program begins. Answer any questions requested by the screen promptly and allow the program to run to its conclusion.

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3.5 Installation of SMART Manager, PS 2

3.5.1 Installation of µControl Board, PS 2

Since SMART Manager operates within the Presentation Manager environment, OS/2 operating system must already be installed on the hard disk before attempting to install SMART Manager.

The mouse should already be installed as described in the manual supplied with the mouse.

Before unpacking the µControl board, please read the Licence agreement for acceptance of the product.

Before you install the µControl Board make sure that you have a working copy of the IBM Personal System/2 Start diskette and SMART Manager diskette 1 available.

1. Unpack the µControl Board and the MultiNet Cable.
2. Ensure that the computer is switched off.
3. The µControl Board has no switches to be set as the configuration is software controlled. It can be installed in any empty slot in the computer. For installation of the board see the instruction manual supplied with the computer. Remember to ensure that the board fits firmly into connector before replacing the panels.
4. Connect one end of the MultiNet cable to the socket on the board and the other end to the µPeak Monitor or the µPrecision Pump if you have a Monitor UV-M II in the system.
5. Before you turn on the computer, insert the IBM Start Diskette in Diskdrive A.

   (If you forget this step a fault message refers to the IBM instruction manual.)
6. Turn on the computer.
7. The screen displays the IBM logotype. Press <ENTER> to continue.
8. A message appears that a configuration error in the adapter has occurred with a question if you want an automatic configuration. Press N.
9. The main menu is displayed. Choose <Copy a function> from the menu.
   Press <ENTER>
10. Put in the SMART Manager diskette 1 in Diskdrive A. The diskette contains the description file for the µControl Board which has to be copied to the IBM Start diskette.
    Press <ENTER>
11. Replace the SMART Manager diskette with the IBM Start diskette.
    Press <ENTER>
    Press <ENTER> to continue.
12. The main menu will appear again. Choose <Configuration>.
    Press <ENTER>
13. Choose <Automatic configuration> from the configuration menu.

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The configuration for the µControl Board is now completed. Remove the start diskette and restart the computer.

3.5.2 Installation of SMART Manager, PS 2

A Registration card is supplied with SMART Manager. Please read it carefully before completing it and returning it to Pharmacia LKB Biotechnology AB. It is important that ownership of SMART Manager is registered so that we can inform you of future update versions of the program.

1. Switch on the computer and allow it to load the resident OS/2 program.
2. Access OS/2 Full Screen.
3. Insert Disk 1 into Drive A and type A: <ENTER>, type INSTALL E <ENTER>. The installation program begins. Answer any questions requested by the screen prompt and allow the program to run to its conclusion.

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4. Operation

4.1 Introduction

This chapter describes the user interface of SMART Manager and general programming principles. It also describes how to use different parts of the program and the basic functions. Some of the more complex functions are described in detail to facilitate the use of them. Other functions are not described at all in this chapter, for a complete description of each menu, please see Section 5, Screens and Menus.

4.2 Getting started

4.2.1 User interface

SMART Manager runs as an application under OS/2 Presentation Manager (PM). Techniques for working with SMART Manager are basically the same as for any Presentation Manager application, as described in your OS/2 documentation, with the exception that SMART Manager runs exclusively as a full screen application.

A brief summary of the essential features of working with Presentation Manager applications is given below. Refer to your OS/2 documentation for more details.

You use the mouse and keyboard to work with SMART Manager.

Icons

Click once with the mouse to select an icon. Click twice in rapid succession to open or activate the icon. Icons cannot generally be selected or opened with the keyboard.

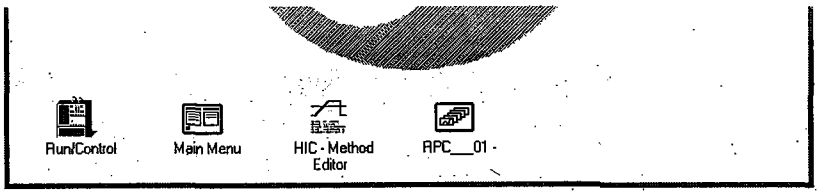


Fig. 4.1. Icons

Windows

If you have several windows open at the same time, click anywhere in an inactive window to make it active. If you click on a command or option button, the function will be activated.

Menus

Mouse: Click once on a menu bar command to pull-down the command list under the menu. Click once on a command in the list to activate the command.

Keyboard: Press the Alt key to activate the menu bar. Either use the arrow keys to move to the desired command name and press Enter or Press the underlined letter in the command name to pull-down the command list under the menu. Either use arrow keys to move to the desired command and press Enter or press the underlined letter in the command name to activate the command.

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A menu command shown in grey text cannot be activated because of the current program context.

Some commands in the command lists have a short command which is activated by pressing a letter together with the Control key. In the pull-down menus are the different short command letters listed.

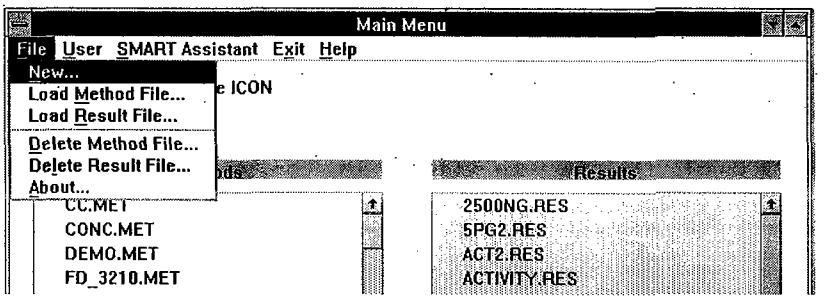


Fig. 4.2. Menus

List boxes

Mouse: Click on the arrows or drag the scroll box to scroll the contents of the list box. Click once on an item in a list to choose the item.

Keyboard: Use the PageUp/PageDown or arrow keys to scroll in a list box after selecting the list box with Tab. You can select items from a list box with arrow keys.

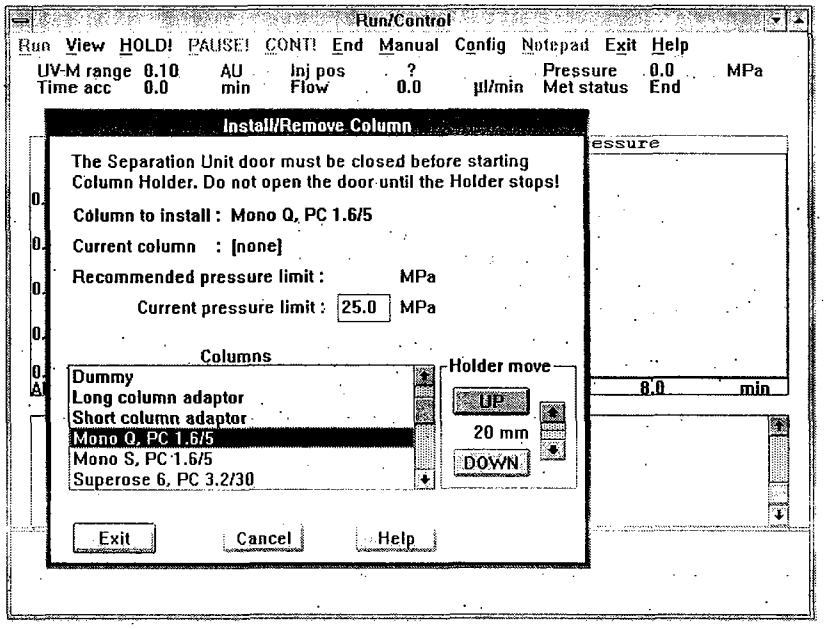


Fig. 4.3. List boxes

Pull-down lists

Some lists are implemented as pull-down lists to economise on space in the display. These are marked by an arrow at the right of the entry field. Click on the arrow to pull-down the list, and then select the required item from the list.

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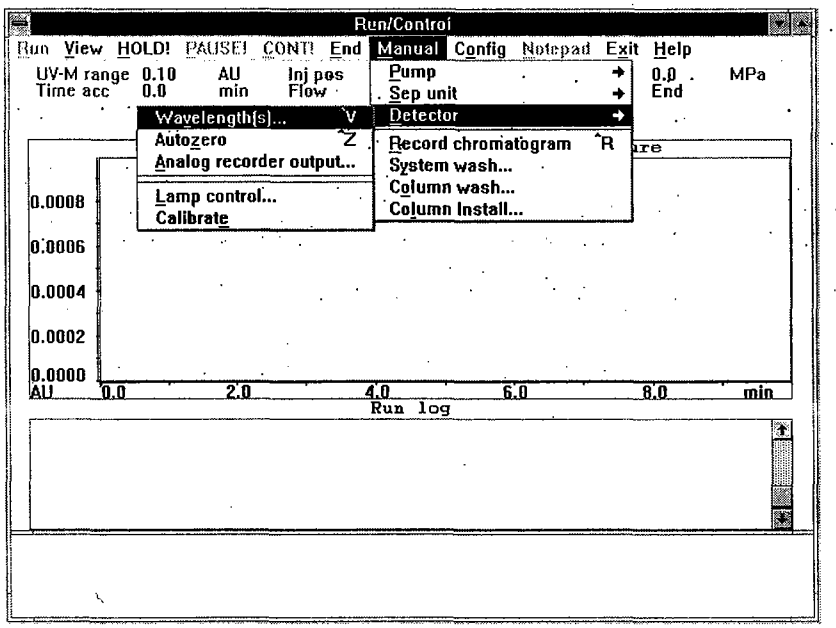


Fig. 4.4. Pull-down list

Option buttonsand check boxes

Mouse: Click once with the mouse on option buttons or check boxes to switch between "on" and "off" state.

Keyboard: Move between changeable fields (all types) using Tab or arrow keys, and switch option buttons and check boxes on or off by pressing the space bar.

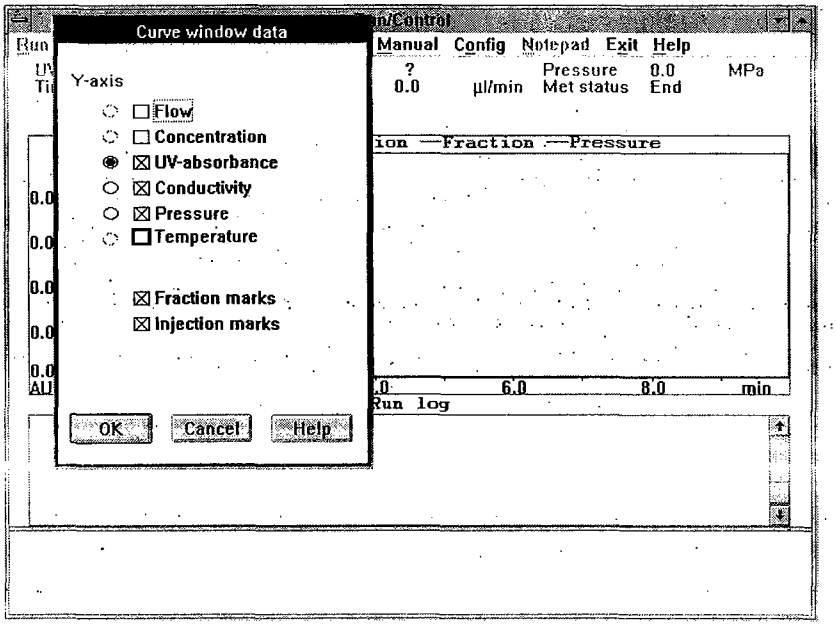


Fig. 4.5. Option buttons and check boxes

Command buttons

Mouse: Click once on command buttons to activate the respective command.

Keyboard: Press Alt together with the underlined letter in a command to activate the command on a button. In many dialogue boxes one

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button, the default command, is normally marked with a heavier border than the others. Press Enter to activate this command. Use the arrow keys to select a different button as the active command.

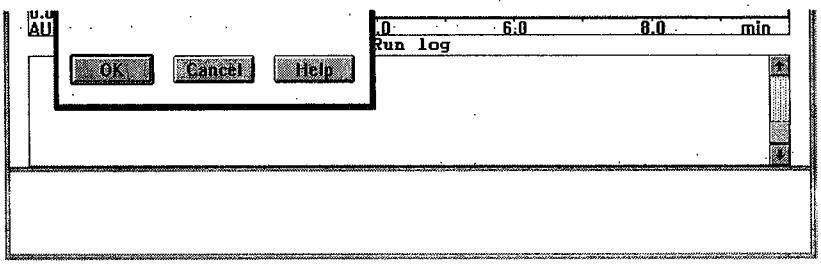


Fig. 4.6. Command buttons

4.2.2 Starting SMART Manager

To start up SMART System and SMART Manager and establish communication, it is important to remember to turn on the power on the System Power Supply and wait until the green indicator lights on the instruments start to flash before turning on the computer. If the start up is done incorrectly the green indicator lights will continue to flash on the SMART System instruments, indicating that communication has not been established.

Your installation may be configured to start SMART Manager automatically each time you switch on or re-boot the computer. If this is not the case, double-click on the SMART Manager program group in the Desktop Manager window. Double-click on SMART Manager in the program group window to start the program.

4.2.3 Resetting SMART System and SMART Manager

If the communication in the system is interrupted and the system has to be restarted you can **not** use the <Control>, <Alt>, <Delete> keys. The power to both the computer and the system must be switched off. If possible use the Shut-down function in the desktop window before switching the computer off.

When you restart the system remember to turn on the power on SMART System before turning on the computer, otherwise communication will not be established.

4.2.4 Logging in

When you have started SMART Manager the introductory screen is displayed with an option to log-in to the program or to look at an overview or the program. Click on the LOGIN button.

The login dialogue box presents a list of users. If SMART System has not been used before only the Library directory will be displayed.

Double-click on your user name, or type in your user name and then click on OK. If a new user name was entered, a confirmation box is displayed, click on YES to confirm your user name.

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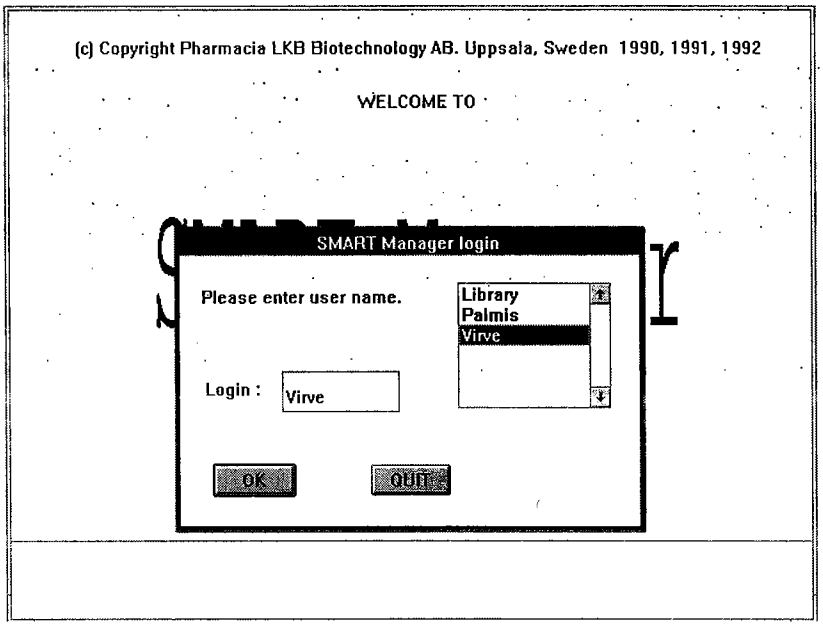


Fig. 4.7. Login window

4.2.5 Main Menu window

The Main Menu window is the directory of all saved files. Two list boxes are shown displaying Method files and Result files. From the Main Menu window you also have access to all parts of the software.

- Method Editor window to create a method
- Run/Control for manual system operations and for running programmed methods
- Result files for evaluation of your results
- SMART Assistant for help in choosing running conditions and separation strategies

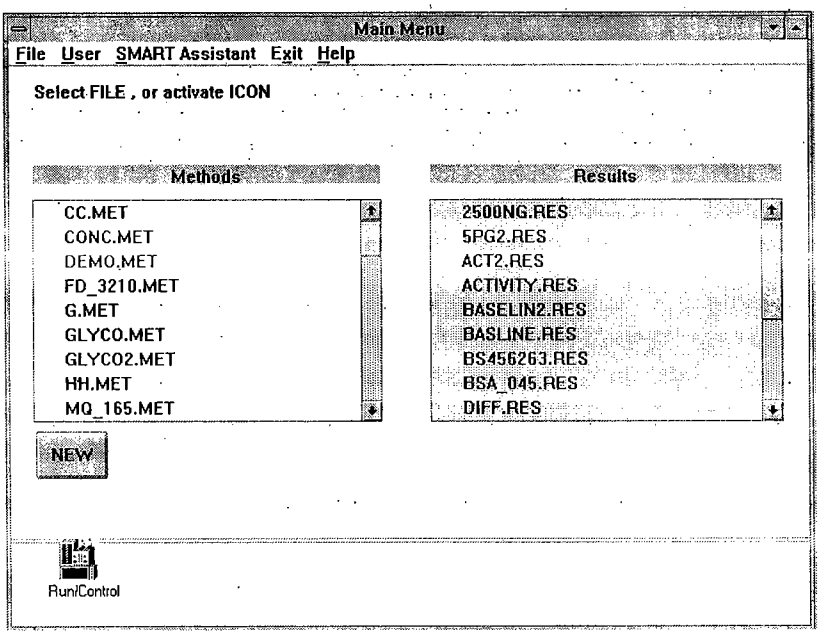


Fig. 4.8. Main Menu window

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4.3 Manual system control

To access the manual system control instructions, double-click on the Run/Control icon. The Run/Control window is displayed.

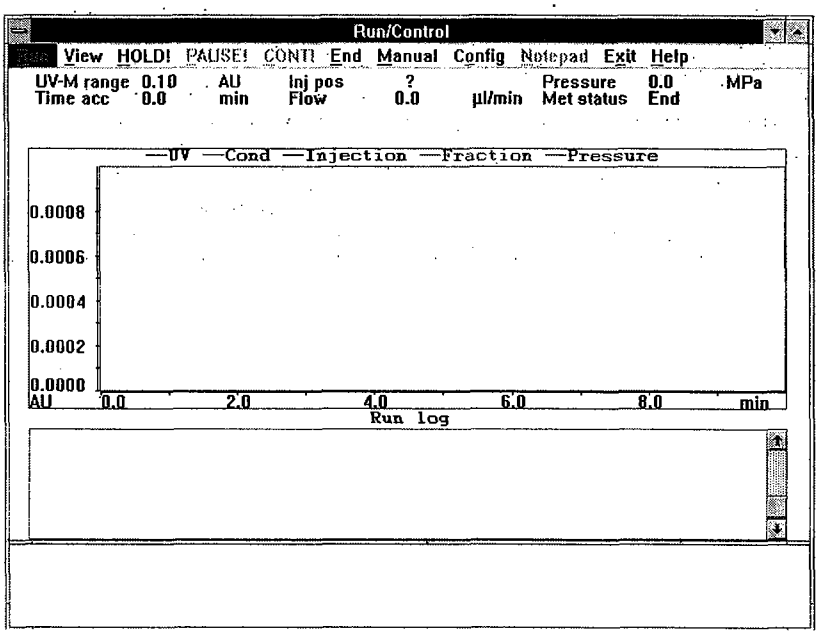


Fig. 4.9. Run/Control window

The following section will describe simple manual system control preparing for a run by:

- installing a column
- washing the system
- making a column wash
- programming the delay volume
- changing the Run Data and Curve Window Data
- exit from Run/Control

For detailed information about the functions and functions not mentioned here see Chapter 5.

4.3.1 Installing a column

Under the menu Manual you will find most functions related with manual system control and the instruction for Column installation at the bottom of the list.

1. Click on COLUMN INSTALL and you will see the Install/Remove Column dialogue box. By scrolling the Columns list box you will see all column options. For a system wash choose the DUMMY from the list. In the dialogue box you can also change the preset pressure limit.

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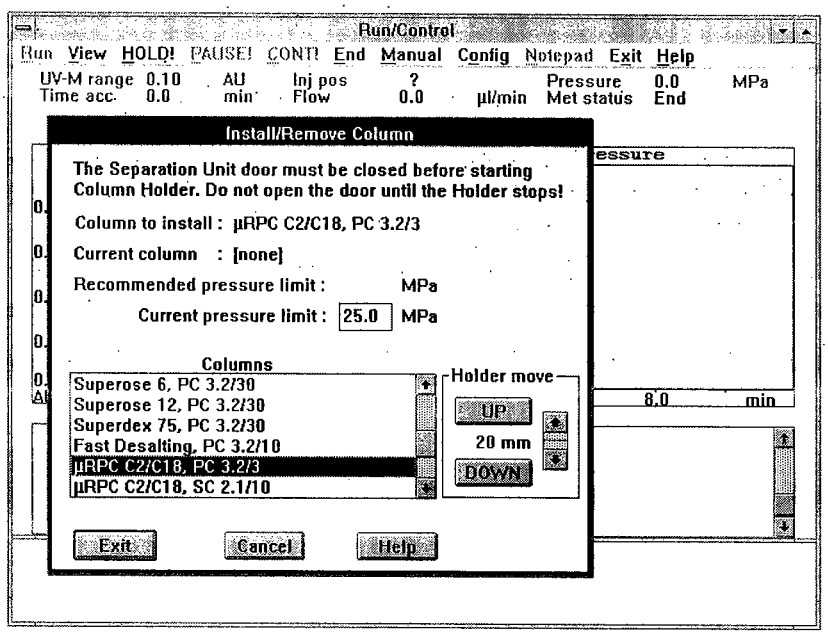


Fig. 4.10. Install Column window

2. Click on the UP command button. The automatic column holder will move up to allow installation of the dummy column.
3. Open the door to the µSeparation Unit and fit the lower end of the column onto the column connector.
4. Slide the column support onto the top of the column. Close the door to the µSeparation Unit and click on DOWN in the column installation window. The automatic column holder will move down until the column is firmly seated.

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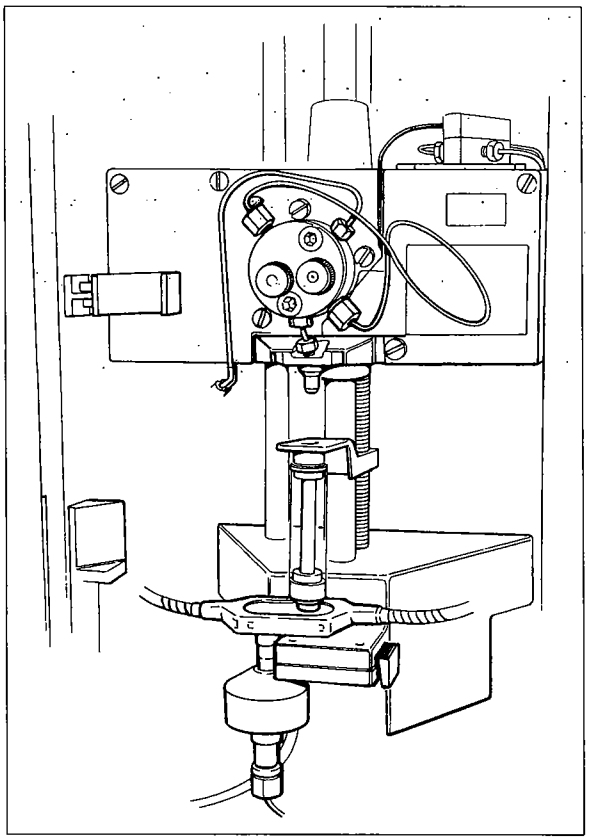


Fig. 4.11. Fitting the column support

5. In the dialogue box you can also set or change the upper pressure limit for your column. If you have chosen a column from the list box, a recommended pressure limit will set automatically. Click on EXIT.

4.3.2 Washing the system

1. To fill the pumps and wash the pump cylinders use the pump wash function. Click on MANUAL, PUMP, WASH. Select the inlet and outlet positions for the pump valves with the option buttons in the Wash Pump dialogue box. Make sure you have the inlet tubings immersed in the liquid and that the chosen outlet positions on the valves have an outlet tubing to a waste reservoir. Click on OK to start the automatic wash procedure.

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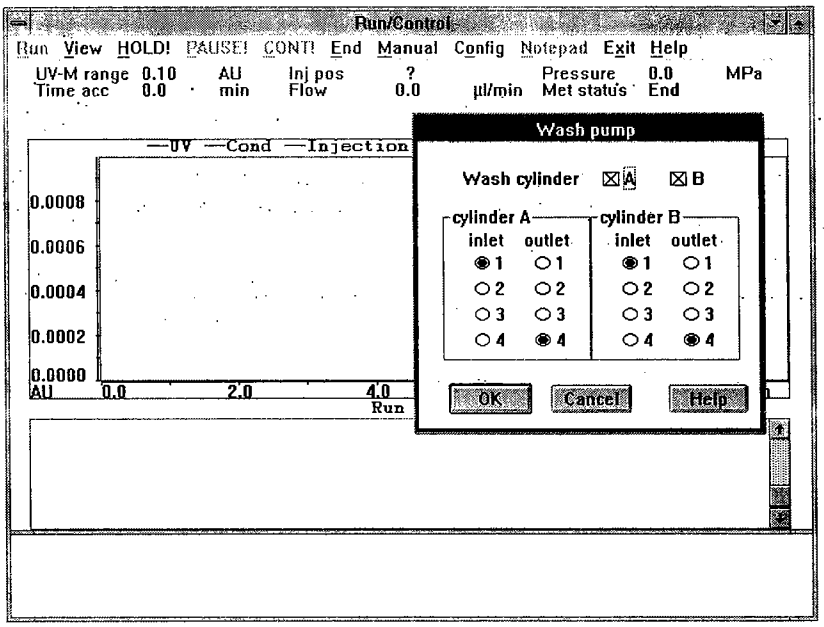


Fig. 4.12. Wash pump window

2. The next step is to wash the tubings and let the waste go out through the waste position on the injection valve. It is important to avoid washing through the UV-Monitor flow cell initially, as this is the most sensitive part for blockage. Click on MANUAL, SEP UNIT, INJECTION VALVE. In the dialogue box, click on the option button for the waste position.

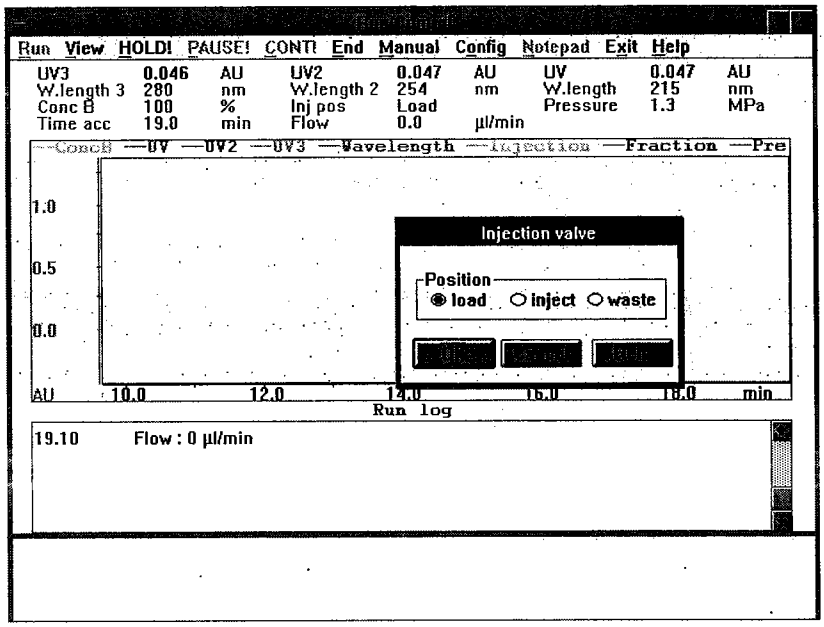


Fig. 4.13. Injection valve window

3. Start the pump flow. Click on MANUAL, PUMP, FLOW and type in 1000 µl/min in the Flow dialogue box. Click on OK.

4. Set the concentration of B to 50 %. Click on MANUAL, PUMP, CONC B and type 50 in the dialogue box.

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5. After 1 minute change the injection valve to inject position. Click on MANUAL, SEP UNIT, INJECTION VALVE and click on the option button for the inject position. Leave the pumps running for a couple of minutes. To stop the flow click on END in the Menu bar.

6. If you have a µPeak Monitor in the system you can set the wavelength while you are washing the system. Click on MANUAL, DETECTOR, WAVELENGTH(s) and type in the desired primary wavelength. If you want additional wavelengths click on the check boxes and type in the values.

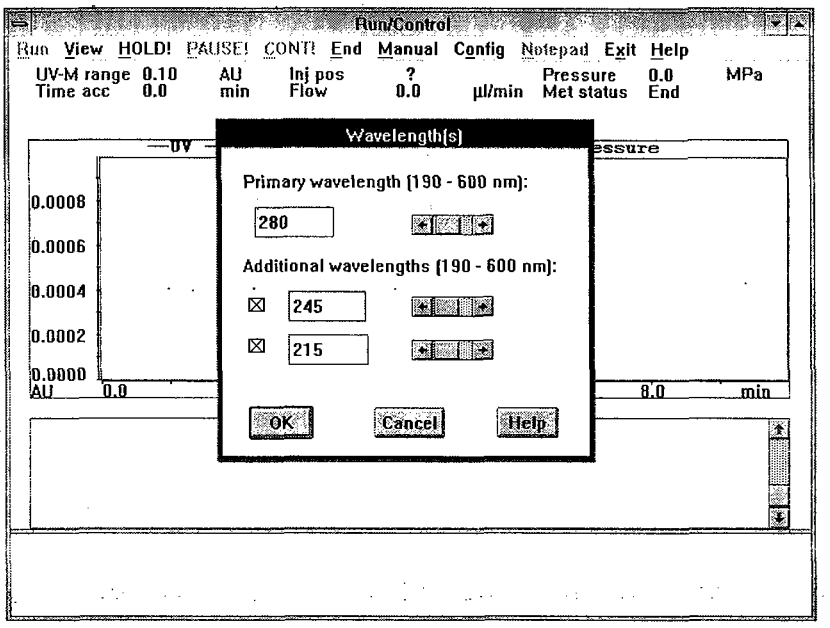


Fig. 4.14. Wavelength(s) window

7. Click on MANUAL, DETECTOR and AUTOZERO for adjustment of the baseline.

8. If you have a Monitor UV-M II you must set the sensitivity range on the monitor and in the USER SUPPLIED CONFIGURATION window under the menu CONFIG, and make an Autozero by pressing the Autozero button on the monitor.

4.3.3 Making a column wash

For the column wash you should follow the recommendations given in the Column Instructions enclosed with the column, particularly the recommended flow rate.

1. Wash the pumps with your eluents. Use the MANUAL, PUMP, WASH instruction.

2. Install your column according to the description above and make sure the arrow indicating the flow direction is pointing downwards.

3. To wash the column use the COLUMN WASH instruction under MANUAL. With this function you can easily change between washing liquids. Enter the flow rate in the dialogue box. Enter the recommended volume for the first eluent and the

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appropriate pump inlet position 1-4. You might have to change the cylinder with the option button if you want to start the wash with eluent from cylinder A.

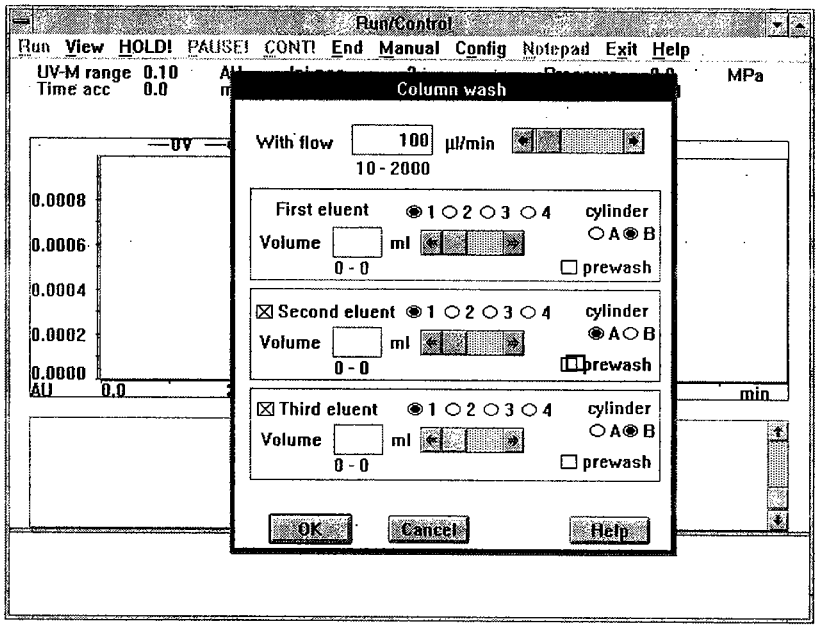


Fig. 4.15. Column wash window

4. Click in the check box for the Second Eluent. Select the appropriate pump inlet valve position and cylinder. Repeat this for the third eluent.

5. Use the prewash option if old eluent should be washed out from the pump cylinders before washing the column.

4.3.4 Programming a delay volume

For fraction collection of small fractions the delay volume (the volume between the outlet of the flow cell to the tip of the fractionation capillary) can be very critical for pure fractions. If you program the delay volume into SMART Manager you will have a delay from when a peak start is recognised until the actual tube change according to the programmed delay volume. This means that you have exactly the fraction indicated on the UV-curve in your tubes. In SMART System this volume is measured for every individual system and stated in the Test Certificate enclosed with the system.

To program the Delay volume enter the RUN/CONTROL part of SMART Manager. Under the menu CONFIG, select USER SUPPLIED CONFIGURATION. In the dialogue box under Separation Unit enter the delay volume stated in the Test Certificate. The value will be saved in SMART Manager.

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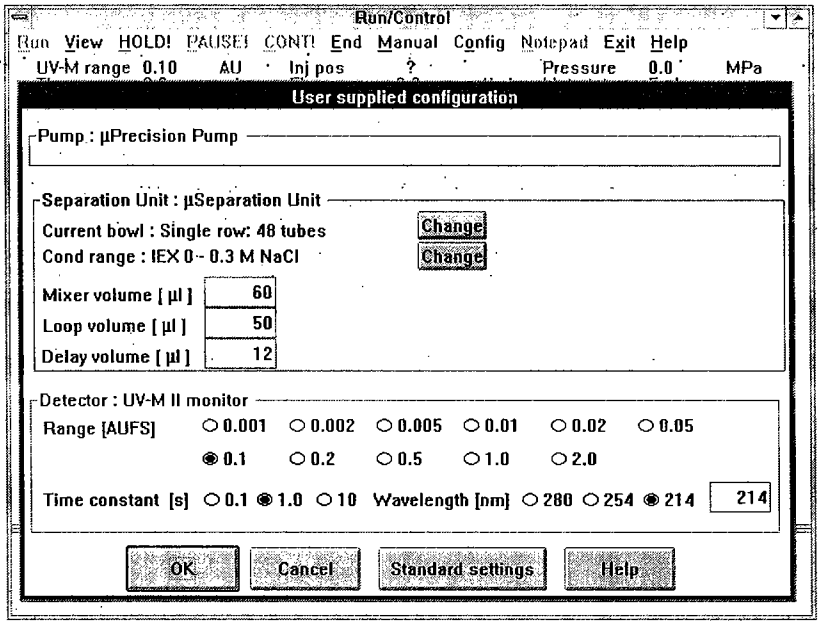


Fig. 4.16. User supplied configuration window

4.3.5 Changing the Run Data and Curve Window Data

If you want to change the standard information displayed in Run/Control, select Run data RUN DATA under the pull-down menu VIEW. In this dialogue box you can choose what parameters you want to have in the numerical display at the top of the screen.

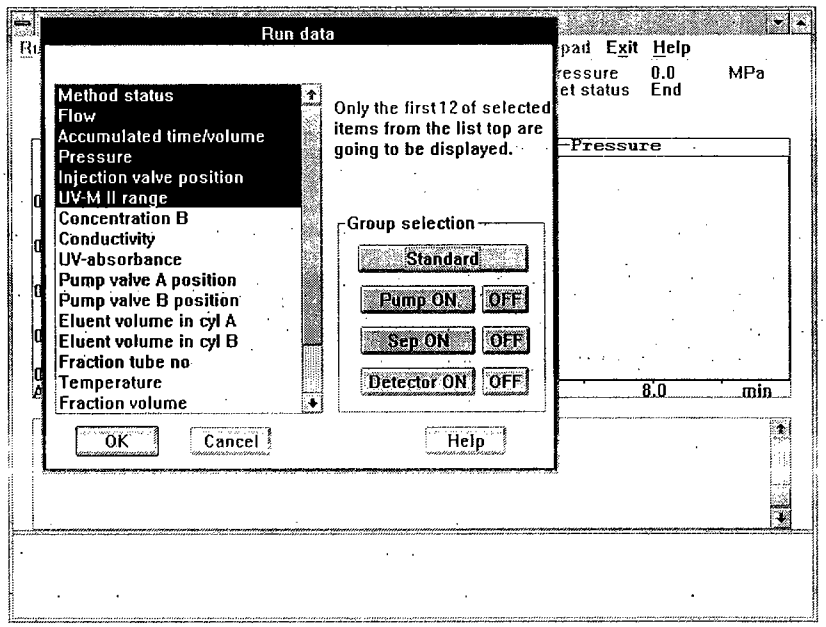


Fig. 4.17. Run data window

With the function CURVE WINDOW DATA, you can select the graphs you want to have displayed by clicking in the check boxes. You can also select what unit you want to have displayed on the y-axis by clicking on the option buttons. You can also change the unit by clicking on the y-axis.

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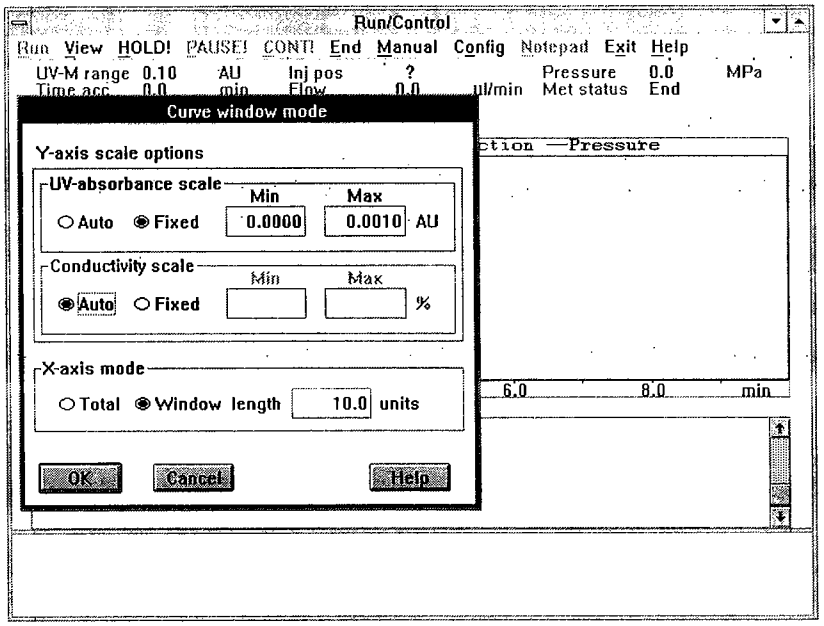


Fig. 4.18. Curve window mode

4.3.6 Exit from Run/Control

To exit from Run/Control use the pull-down menu EXIT and you will come back to the Main menu. If a flow was running, it will continue even when you exit Run/Control. This enables you to equilibrate a column while programming a Separation Method.

4.4 Programming a method

4.4.1 Entering the Method Editor

Before starting to define the method, plan the framework of the method with flow rates, gradient parameters and other important

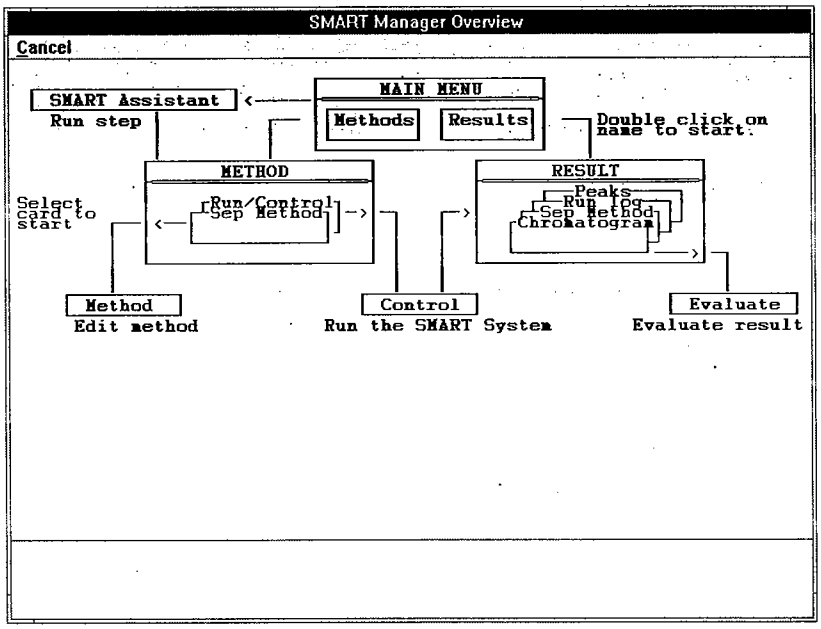


Fig. 4.19. SMART Manager overview

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To create a new method, choose NEW from the Main Menu. Type in a name for the new method with a maximum of eight characters. Confirm the name by pressing OK. The Method file contents window is displayed with the Run/Control card and the Separation Method card.

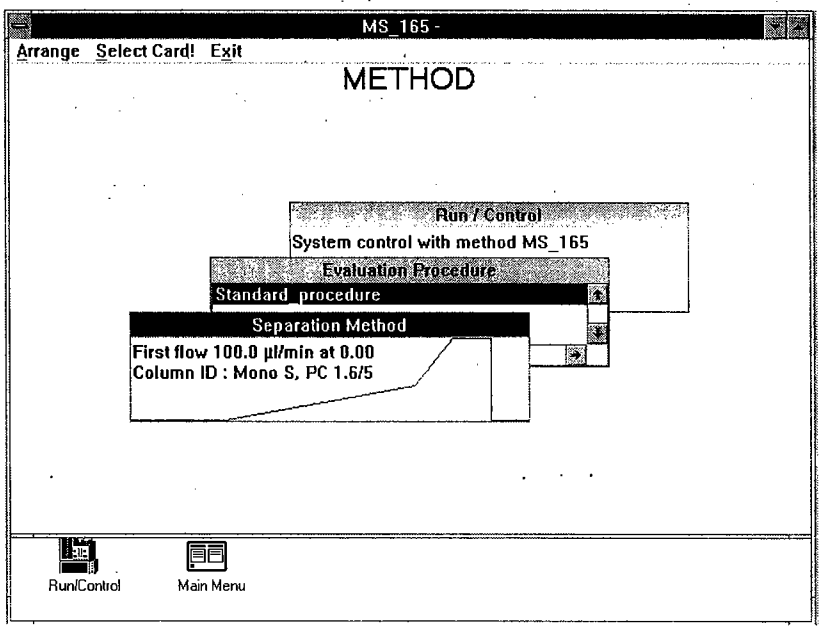


Fig. 4.20. Method file contents window

Double-click on the SEPARATION METHOD card.

The first window to appear when a new method is created is the Chemistry window. In this window you can type in information about the sample and buffers used in the run. This information will be stored with your method and can be printed with your results.

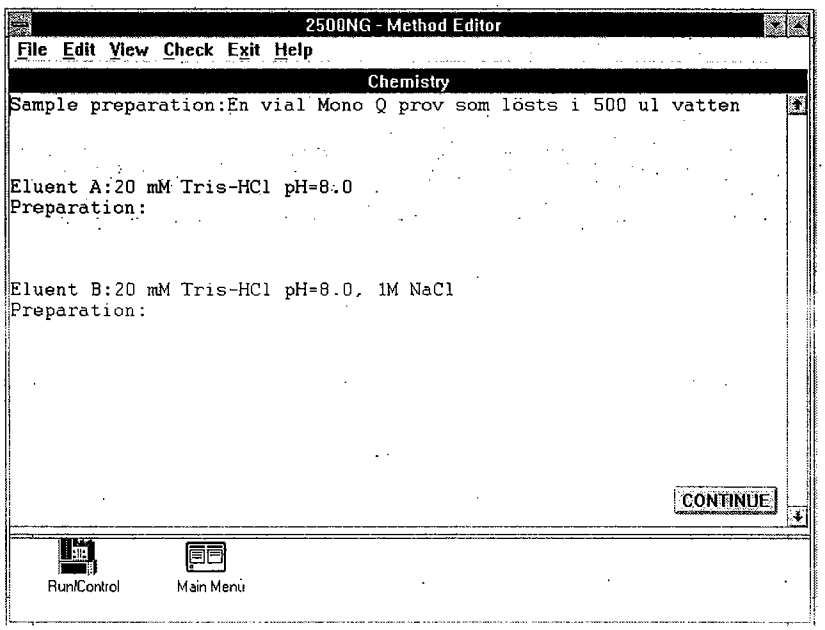


Fig. 4.21. Chemistry window

When you press Continue, the Configuration window appears. Here you define the system configuration. If you want to program the

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method in volume base, click on Min under method. In the dialogue box that appears you can change to millilitre base. If you click on Any column at the line for Column ID, a List box with all Precision Columns will appear. When you choose a column from the list the upper pressure limit for that particular column is automatically set in the system. Under the heading Instruments, you define the detector you have in the system. To change from UV-M II click on the name and in the dialogue box that appears you can change to µPeak Monitor.

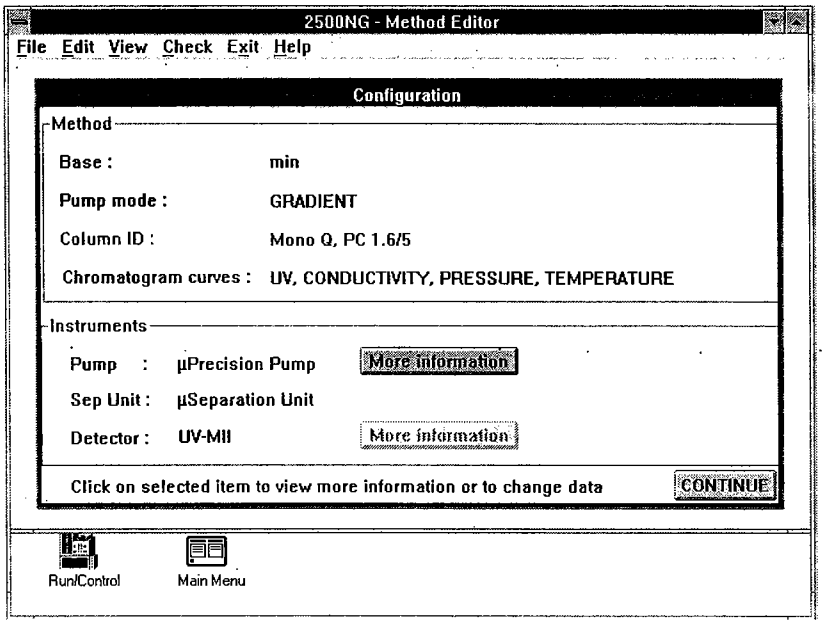


Fig. 4.22. Configuration window

4.4.2 Method Editor window

The method editor is divided into two panels, a graphic editor (top) and a text editor (bottom). You can enter instructions in a method in three different ways:

- use the graphic editor panel, for flow rate and gradient instructions only
- use the instruction commands in the pull-down menus
- type instructions directly in the text editor panel

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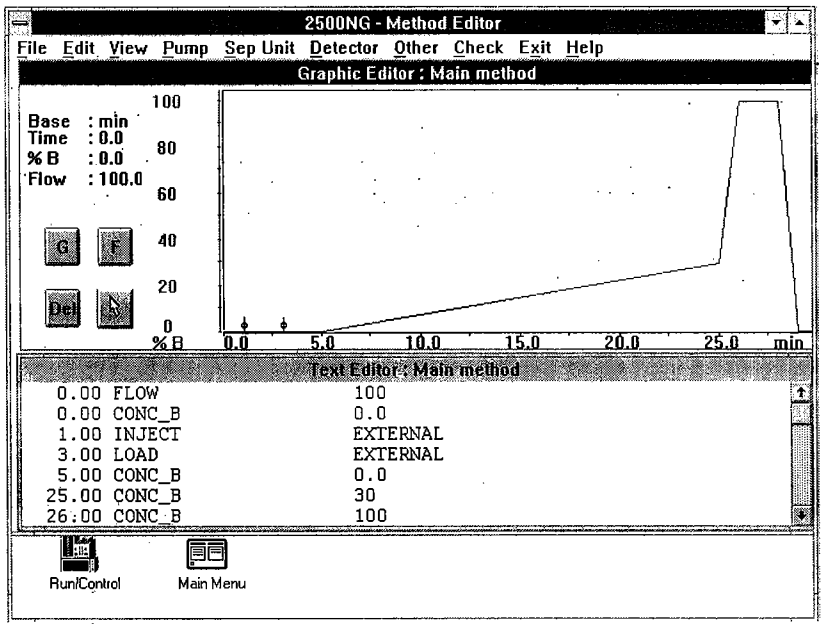


Fig. 4.23. Method Editor window

4.4.3 Reading the graphic editor panel

The graphic editor panel provides a visual overview of flow rate and gradient during the method. Events such as valve operation are shown as event markers. You can use the black vertical drag bar to read gradient values. To use the drag bar, point the cursor near the y-axis and press and hold the mouse button. The cursor changes into a hand and, by keeping the mouse button depressed, the vertical drag bar can be dragged to any position in the graphic editor panel.

The y-axis of the panel shows either flow rate (µl/min) in green or eluent composition (%B) in red. Click on the axis label to change between these two. Use the up and down arrows at the top left of the graph panel to change the scale of the y-axis.

4.4.4 Using the graphic editor

Use the flow and gradient buttons on the left of the graphic editor panel to set control points for pump operations. The button marked with F is the flow button and the button marked with G is the gradient button. Click on the appropriate button and move the cursor into the graph area. The cursor changes to an arrow with the letter for the function. To add a new control point, click at the desired coordinates. The numerical indicators on the left of the graph area display the cursor coordinates to help you place the cursor with precision. The time resolution is 0.5 minutes when using the graphic editor.

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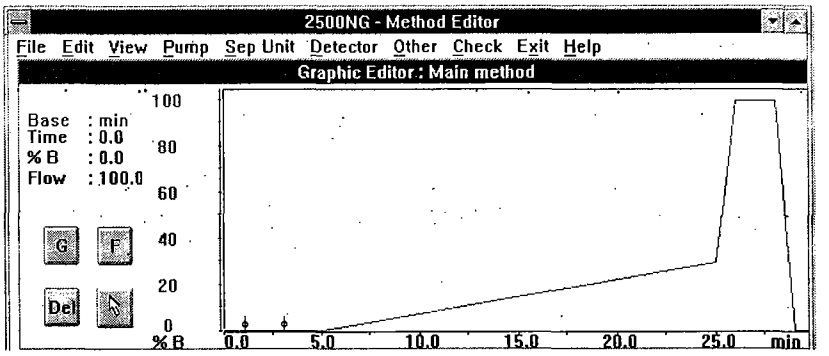


Fig. 4.24. Graphic editor window

You can also depress the mouse button anywhere in the graph area and then drag the cursor to the desired position.

To terminate operation with the command buttons, click on the button marked with an arrow.

To move a control point, click on the point with the cursor and drag it to the new position.

To delete a control point, choose the deletion tool from the left of the graphic panel and click on the control point to be deleted. The deletion tool operates on both flow rate and gradient control points. If there is more than one instruction at a given control point, a dialogue box appears asking which instruction should be deleted. Mark the instruction and click DELETE.

Note: If you move the cursor out beyond 25 minutes or 4 ml the time/volume axis will be displaced to the left to display later times. The axis is displaced back to the right when you move the cursor towards shorter times. The graphic editor panel always displays a "window" of 30 min or 5 ml, depending on the method base.

4.4.5 Using the pull-down menu instructions

The pull-down menus PUMP, SEP UNIT and DETECTOR in the Method Editor menu include lists of instructions for control of the system. Clicking on a command opens a dialogue box in which the parameters for the instruction can be entered. The dialogue boxes for the various instruction groups are basically similar. Common features are:

Time/volume

for setting the time or volume for the instruction according to the method base. Type in the required value or use the arrow buttons to scroll up or down.

Option buttons/check boxes

for switching a function on or off.

Command buttons

OK adds the instruction to the method.

Cancel closes the dialogue box.

Help provides on-line help for the currently selected instruction.

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4.4.6 Using the text editor

Instructions can also be typed directly in the Text Editor window. Instruction must be typed using the correct syntax. For example, to enter 0 % B at time or volume Zero, type the following:

0.00 CONC_B 0.00

Normally, a method instruction is written according to the following guide-lines:

- a single method instruction is written with a single statement on each line with a space between the time/volume, the instruction and the value
- several statements can be written on the same line providing they are separated by a semi-colon ";"
- reserved words are written either with uppercase or lowercase letters
- comments can be written anywhere in the method providing they are enclosed within parentheses { }

For a complete list of commands and allowed values see Appendix 4.1.

To delete an instruction line, position the cursor at the left margin of the text editor window alongside the line to be deleted. Press <CTRL> together with <ENTER>.

The graphic editor window is updated by clicking on Check, Check And Update Graphs.

4.5 Creating a method

In this example you will program a separation method with gradient elution. The method has isocratic elution at 100 % A for the first 5 minutes. The gradient is a 20 minute linear gradient from 0-100 % starting after 5 minutes, followed by isocratic elution at 100 % B from 25 to 30 minutes. At the beginning of the method the µPeak Monitor instructions are programmed, with detection at 280 and 215 nanometers. The injection valve is set in load position at the start of the method, and after 4 minutes set to the inject position and set back to the load position after 6 minutes. The method also has instructions for fraction collection starting after 5 minutes.

4.5.1 Creating the gradient

Click on the button marked G in the Graphic Editor window. Point the cursor at the bottom left corner of the graph and check that the displayed values at the top left shows Time 0 and %B 0. Click on the mouse to fix the break point. Move the cursor along the x-axis to Time 5.0, %B 0 and click on the mouse. Move the cursor to the next break point at Time 25 and %B 100 and fix the value by clicking on the mouse. Move the cursor to the last break point at Time 30, % B 100 and fix the value.

To set the flow rate to 100 µl/min, click on the button marked F and move the cursor to the arrow-up button. Click on the button until 100 µl/min is shown on the y-axis. Point the cursor at 100 and check that the displayed values show Time 0 and Flow 100 µl/min, then click on the mouse.

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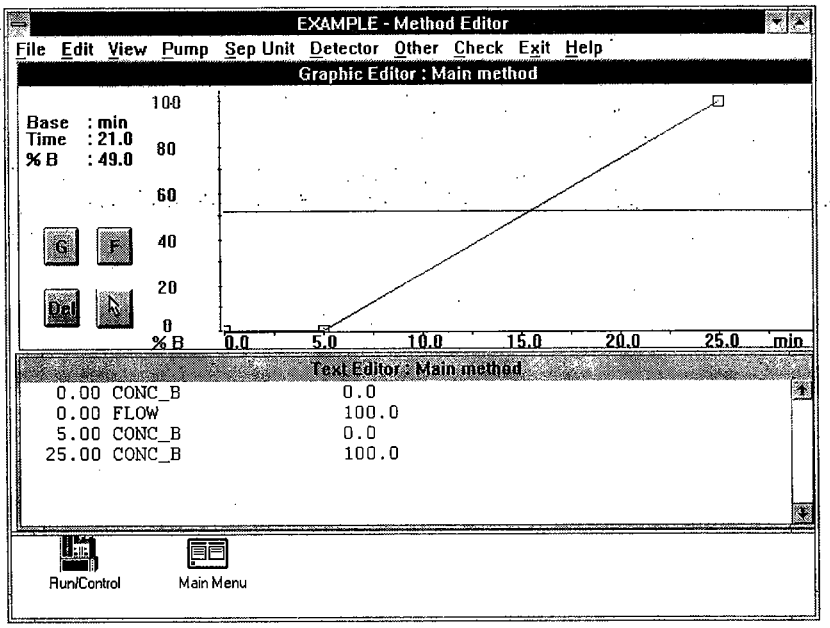


Fig. 4.25. Creating the gradient

4.5.2 Programming µPeak Monitor instructions

A list of instructions specific for the monitor is displayed in the pull-down menu DETECTOR, but only if µPeak Monitor has been chosen in the system configuration. To choose µPeak Monitor, click on VIEW, CONFIGURATION. Under Instruments, click on UV-M II and change to µPeak Monitor in the dialogue box. Wavelength settings and Autozero for the Monitor UV-M II must be set manually on the monitor before a run.

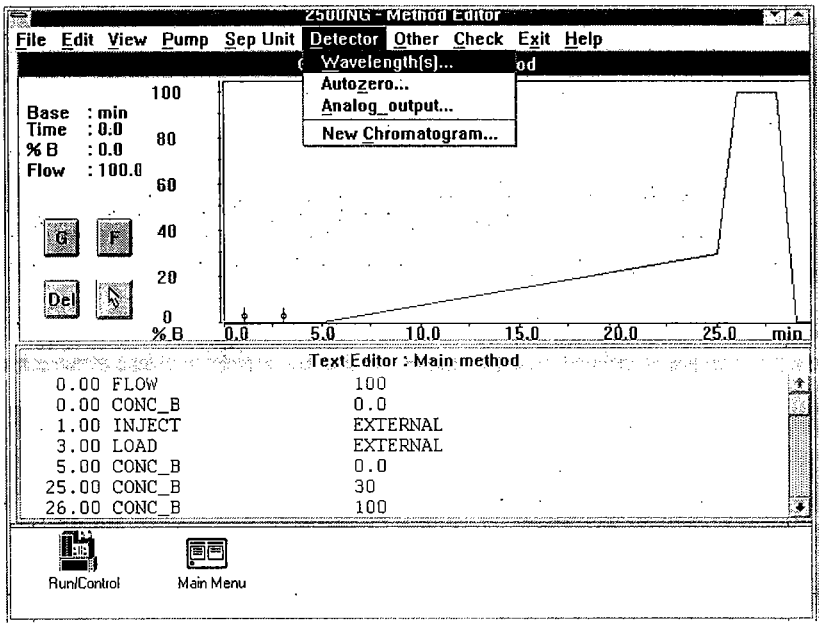


Fig. 4.26. Pull-down menu Detector

To set the monitor instructions click on WAVELENGTHS under the menu DETECTOR, and the dialogue box for the wavelength settings appears. Set the Time by using the Arrow buttons or by typing in the

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value. Set the Primary wavelength to 280 nm the same way. To choose a second wavelength click in the check box to activate it and set the wavelength to 215 nm. Click on OK to confirm the settings.

Choose AUTOZERO from the DETECTOR pull-down menu and set the time to 0.1 with the arrow buttons.

4.5.3 Programming the injection valve positions

Under the pull-down menu SEP UNIT, choose the instruction INJECTION VALVE. A dialogue box for setting the valve positions appears. Set the Time to 0 and check that the valve is in Load position and click on OK. Choose INJECTION VALVE again from the pull-down menu and at Time 4 set the valve in Inject position by clicking on the option button. Repeat this and set the valve position to Load again at Time 6 when the sample has been applied to the column.

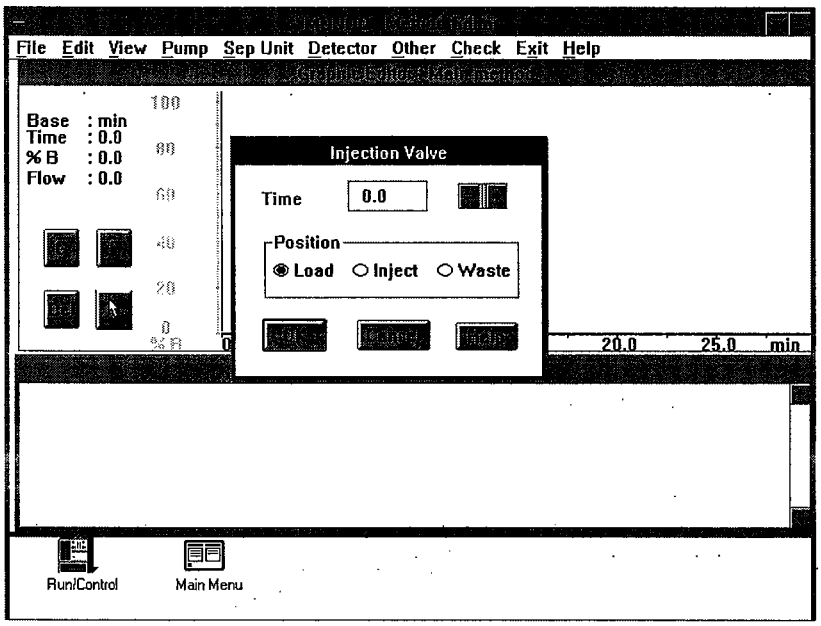


Fig. 4.27. Injection valve window

4.5.4 Programming fraction collection

Under the pull-down menu SEP UNIT, choose the instruction FRACTIONATION. In the dialogue box, set the time to 5 using the arrow buttons or type the value. Click in the box for Fraction Size and type 50. Click in the box for Start At Tube and type 1.

Click on the option button Down for the needle position, which means the fractionation capillary will go down in the bottom of the tube and wipe off the fraction. Click on OK to confirm the values.

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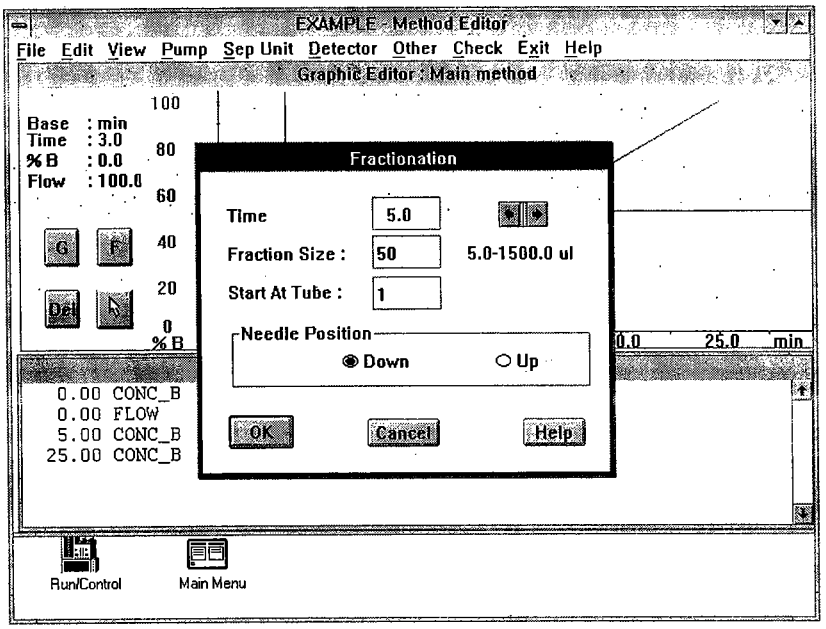


Fig. 4.28. Fractionation window

4.5.5 Saving the method

To save a new method, choose EXIT WITH SAVE under the pull-down menu EXIT. If you want to save the method without leaving the method editor, you can use the instruction SAVE under themenu FILE.

When you want to save an edited method under a new name, use FILE SAVE AS. In the dialogue box that appears you can type in the new name.

4.5.6 Using library methods

Under the user name Library in the Main Menu, there are pre-programmed separation methods for different types of Precision Columns, different system configurations and wash procedures that you can use as they are, or modify and save under your own user name. To save a library method under your user name, select one of the methods in the Main Menu by double-clicking on the name. In the File Contents window select the Separation Method card. Select FILE, SAVE AS in the Method Editor and enter a name for the method. Confirm the new name by clicking on OK. Exit the Method Editor with EXIT WITH SAVE.

See Appendix 5.1 for a short description of each method.

4.5.7 Leaving the Method Editor

Before leaving the Method Editor the created method can be checked for errors and invalid instructions. Under the menu heading CHECK, click on CHECK AND UPDATE GRAPHS. If the method is not correct a warning message will appear. This function also updates the graphic editor if instructions have been typed in the text editor.

To Exit from the Method Editor click on EXIT, EXIT WITH SAVE.

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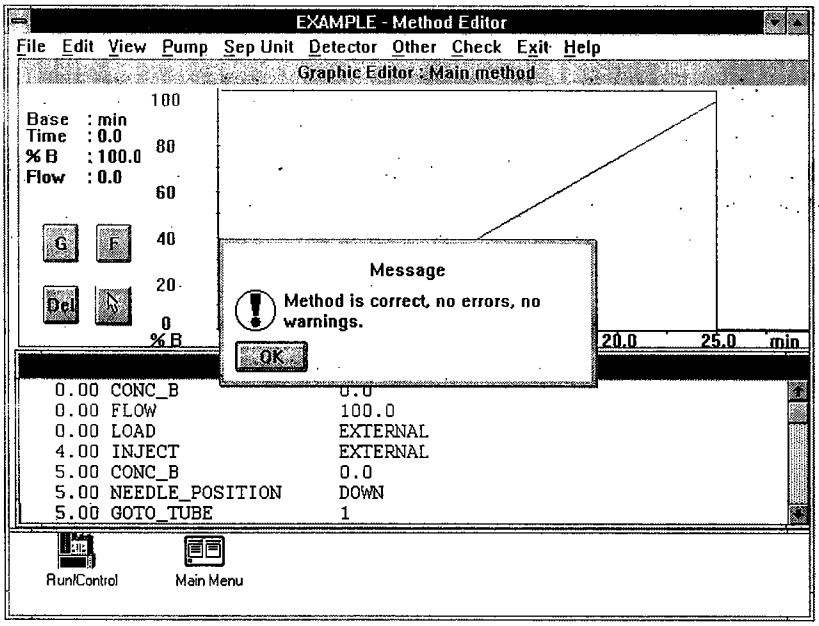


Fig. 4.29. Checking the method

4.5.8 Making the run

In the File contents window, double-click on the Run/Control card and you will transfer your method to Run/Control.

If you have a Monitor UV-M II in your system you must set the output range. Under the pull-down menu CONFIG, click on User supplied configuration USER SUPPLIED CONFIGURATION. In the dialogue box under DETECTOR set the Absorbance range, Time constant and Wavelength for the monitor and check that the settings on the monitor are the same.

To start the method, click on the pull-down menu Run. In the dialogue box your result file will automatically be named with the first 6 characters from the method name, followed by a 2 digit number to identify the result file. If you want to save your results under another name you can type in the new name followed by 01.

Click on RUN and the method will start.

4.6 Editing the method

4.6.1 Opening a method

Double-click on the method name in the Main Menu. Double-click on the SEPARATION METHOD card in File contents window.

Alternatively click on the pull-down menu Select card, which will change the pointer into a hand. Point the hand on the Separation method card and click on the card.

4.6.2 Using the graphic editor

Use the graphic editor to add new break points or move or delete existing break points for the gradient. Click on the button marked G and move the cursor and click on the coordinates you want add or move.

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To delete a breakpoint, click on the button marked Del then click on the coordinate you want to remove.

The flow rate can be changed in the same way with the flow rate tool.

To add instructions other than gradient and flow rate, use the pull-down menus. You cannot use the pull-down menus to delete or change the instructions.

4.6.3 Using the text editor

In many cases, it is simplest to change instructions by editing the commands directly in the text editor. However, this requires that you follow the method control syntax, see Appendix 4.1.

To delete an instruction line, place the cursor at the left margin of the text editor window alongside the line to be deleted. Press CTRL together with ENTER.

To add an instruction, place the cursor directly behind the line after which you want to add the instruction, press ENTER and type in the new instruction.

To change the value of an instruction, place the cursor after the old value, use the Backspace button to erase it, then type in the new value.

4.6.4 Checking syntax

If you have used the text editor to make changes in your method, the changes might not appear immediately in the graphical presentation. Use CHECK AND UPDATE GRAPHS in the method editor menu to check that the syntax of your changes is correct and to update the graphical display.

4.7 Peak fractionation

The Peak Fractionation instruction in the Method Editor can be used to collect a peak in a separate tube, or to divide a peak into many small fractions. The eluate between peaks can be collected in fractions or go directly to waste. Peaks can be detected either by level function or slope detection. Both methods will be described below.

Level detection is encouraged when the elution profile is not known. When using level detection, run a blank before setting the parameters.

Slope detection is always recommended in the case of a gradient with a drifting baseline. When using slope detection, run at least a blank and preferably a sample.

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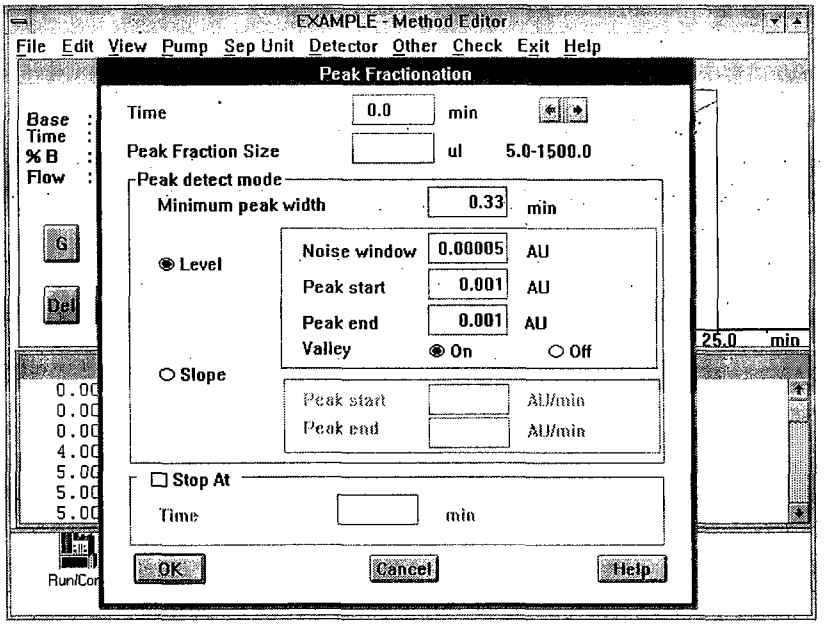


Fig. 4.30. Peak Fractionation window

4.7.1 Peak fractionation by level detection.

To collect peaks by level detection you must enter values for the following seven parameters:

- Time/Volume
- Peak fraction size
- Minimum peak width
- Noise window
- Peak start
- Peak end
- Valley (on or off)

Time/Volume

The programmed time/volume at which the peak fractionation is to begin.

Peak fractionation size

The programmed peak fractionation value is the size of the peak fractions to be collected. This value can differ from the programmed fractionation value. If each peak is to be collected in a separate tube, the programmed peak fractionation size should be larger than the expected peak. To prevent overflow in the tubes, set peak fraction size to the volume of the tubes. A peak larger than this will then be split into several fractions.

Minimum peak width

The programmed Minimum Peak Width value is the minimum distance between peak start and end. The value must always be smaller than the narrowest peak. If a peak start is detected, the program will not start checking for a peak end until the minimum peak width time has elapsed. This function prevents tube changes due

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to noise spikes. Use the default value during a blank or first sample run.

Noise window

To determine the noise window value and the peak start and end, run a blank run if the default values cannot be used. You can measure the noise from the blank run by calling up the corresponding result file. To do this, minimise the method editor screen, bring up the Main Menu screen and select the blank run result file. Double-click on the CHROMATOGRAM card, this will bring you into evaluation. Your chromatogram should now appear on the screen. Once in Evaluation, zoom in a section of the baseline where you estimate you have the highest noise level. To do this, point the cursor on this part, then press and hold the mouse button. The cursor will change into a pointing finger. By moving the finger you will see a green dotted line, this indicates the area you are enlarging. Release the mouse button and the enlarged area will appear on the screen. The noise level you want to measure is the difference between the highest and lowest y-axis value (AU). To read the coordinates double-click on the xy box in the upper left hand corner of the chromatogram. The box will change and have the numerical values of the x- and y-axes. Point the cursor near the y-axis, press and hold the mouse button. The cursor will change into the shape of an open hand which you can move along the enlarged area of the chromatogram and read the noise level in the box.

Note: Make sure the colour of the digits in the coordinates box is the same as the colour of the corresponding curve displayed on the screen. If several curves are displayed, select the values of the desired one by pointing inside the coordinates box and pressing the mouse button once. Two quick clicks or a double click will reset the xy format of the box.

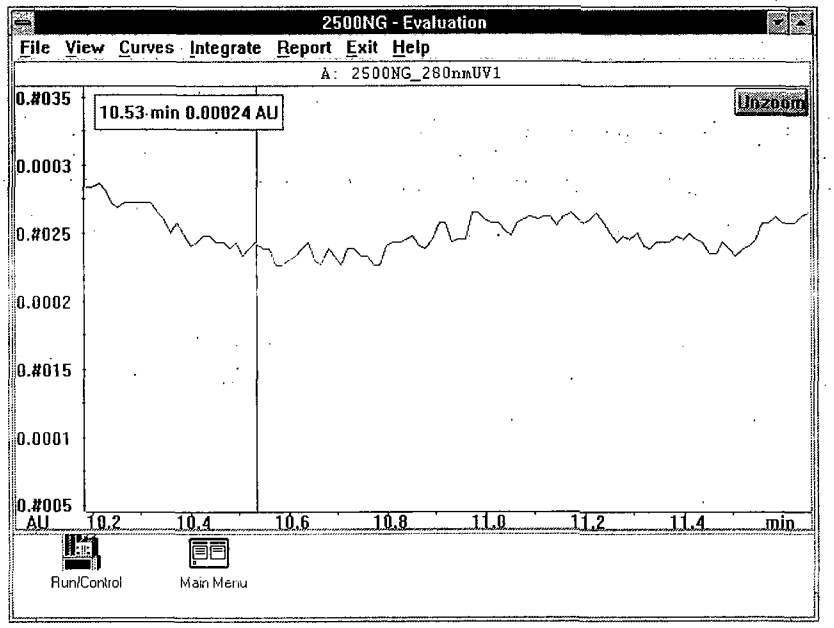


Fig. 4.31. Reading the noise level

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Decide the noise level. When you enter the noise level for the peak fractionation, enter twice the value of the measured value.

Peak start/Peak end

A general recommendation to determine the peak start and peak end values is to have the lowest peak start and peak end levels at 2 % of the expected maximum peak height. However, it is also important to check the baseline drift from the blank run. The levels must be set above the highest baseline point to avoid collection of "noise". If a sample or blank run was not done, use the default values.

Valley

When Valley instruction is On, a tube change will occur for double peaks above the peak end value. A valley is recognised by the program if both of the following events occur:

1. The absorbance goes 2 % below the peak maximum absorbance.
2. The absorbance rises 2 % above a valley.

To enter the above peak fractionation instructions in your method, open the Separation Method located in the Main Menu screen, select the Separation Method card in the File Contents window. In the Method Editor you will find the PEAK FRACTIONATION instruction under the heading SEP UNIT. Enter the desired values in the dialogue box.

To stop Peak Fractionation locate the instruction PEAK FRACTIONATION OFF under the Sep Unit menu.

Note: For methods containing peak fractionation with level detection, use an auto zero command before the fractionation starts so that the baseline level is always correct.

Return to the method editor screen by exiting evaluation and double clicking on the method editor icon at the bottom of the screen. Enter all commands relating to peak fractionation into the method. When entering the noise level for peak fractionation, enter a value twice the measured value.

A method with the above settings will fractionate the peaks in separate tubes and the elute between the peaks will go to waste. If you want to collect the elute between peaks, FRACTIONATION instructions must be programmed. Enter values for Fraction size, Start at Tube, Needle Position Down/Up to start at least 0.1 minutes before the Peak Fractionation.

4.7.2 Peak fractionation by slope detection

Peak fractionation by slope detection is useful when the chromatogram has a large baseline drift. The most accurate way to determine the slope values is to first run a sample with a similar profile as the one you will be working with, then measure the slopes of peaks of interest. If this is not possible a, use one fifth of the expected maximum slope (AU/min) as Peak Start and one tenth of the maximum absorbance/min (AU/min) as Peak End.

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To collect peaks by slope detection, you must enter values for the following parameters

- Time/Volume
- Peak fraction size
- Minimum peak width
- Peak start
- Peak end

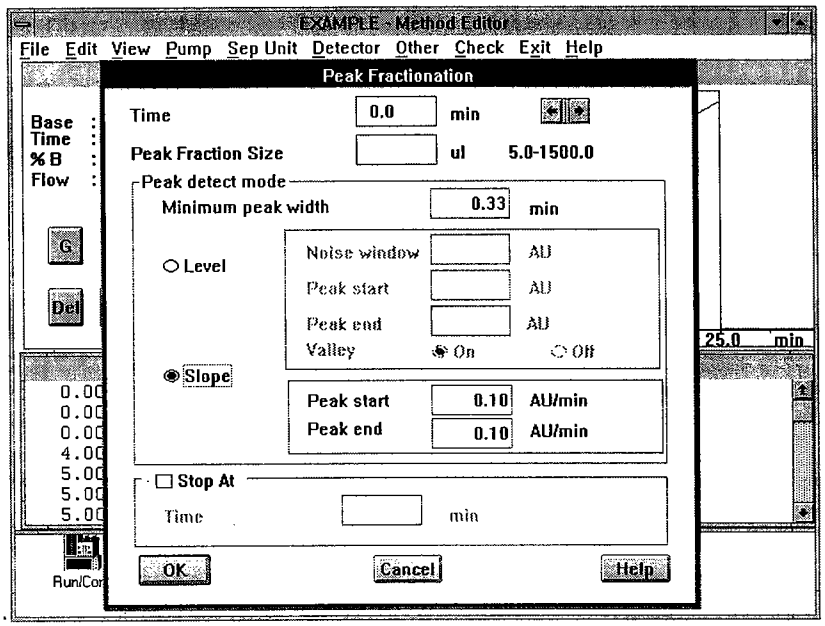


Fig. 4.32. Peak fractionation window

Time/Volume

The programmed time or volume at which the peak fractionation is to begin.

Peak fraction size

The programmed peak fractionation value is the size of the peak fractions to be collected. This value can differ from the Fraction size programmed in Fractionation. If each peak is to be collected in a separate tube, the programmed peak fraction size should be larger than the smallest expected peak. To prevent overflow in the tubes, set peak fraction size to the volume of the tubes. A peak larger than this will then be split into several fractions.

Minimum peak width

The programmed Minimum peak width value is the minimum distance between peak start and end. This value must always be smaller than the narrowest peak. This function prevents tube changes due to noise spikes. For more details see Chapter 5.

Slope peak start/Peak end

1. To measure the slope at peak start and peak end, open the result file from a test run of the sample by double-clicking on the Chromatogram card in the Main Menu. Select the

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instruction DIFFERENTIATE under the heading CURVES. Select the UV curve to be differentiated. Select where to store the curve. Click OK. The differentiated curve will appear on the screen.

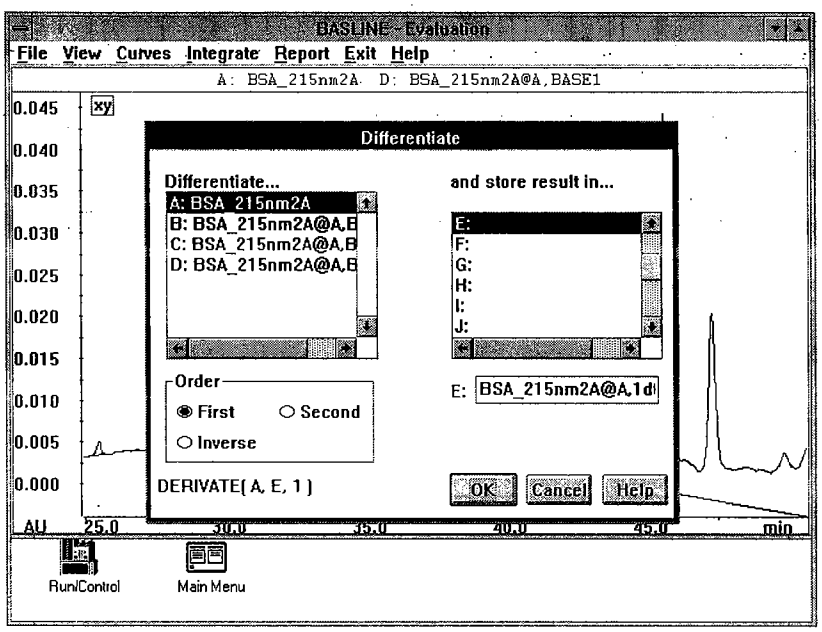


Fig. 4.33. Differentiate window

2. Select the instruction SELECT WINDOW CONTENTS under the heading VIEW. In the dialogue box, select the differentiated curve and the original UV curve. Then find the maximum amplitudes of the selected curves by using the zoom function and the drag bar. Point the cursor at the top left corner of the area you want to enlarge, press and hold the mouse button and move it to the bottom right corner of the area. Release the mouse button. The enlargement will appear.

   Double-click on the **xy** box in the top left hand corner of the chromatogram. The numerical values for the x and y coordinates will appear. Make sure the values in the box have the same colour as your differentiated curve. Point the cursor at the drag bar under the box and hold the mouse. Drag the bar along the UV curve to the area of interest. Place the bar at the start of the largest UV peak and read its absorbance. Then find the maximum range of the differentiated curve by activating the corresponding figures in the coordinates box. Read the maximum positive and negative slope values.

3. Make CURVE WINDOW LAYOUT to display both curves. Select Fixed Scales for the x and y axes. Set the x value to the length of the run. Click on OK.

   In the Set fixed Scale On Y-axis window, set the y values for the UV curve to a negative value of 40% of the highest peak absorbance and a positive value of 60%. Set the y axes values for the differentiated curve as Other to -30% (minimum) of the range estimated above and 70% (maximum). Click on OK. The differentiated curve should appear separated from and directly under the UV curve. You may have to try other values to get

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reasonable amplitudes and positioning of the curves on the screen.

4. Normally you have to zoom in (enlarge) a part of both curves to read the derivative. Activate the y axis scale to show the differentiated values by pointing at the y-scale. Place the bar at the start of the smallest peak wanted and read the derivative value in the coordinates box. A slightly smaller value will function as a Peak Start value for peaks of this size or larger. Then place the bar at the end of the same peak. Use the numerical value of the differentiated curve as the peak end value, ignoring the minus sign.

To enter the instructions in your method, open the SEPARATION METHOD card in the Main Menu screen and then select the Separation Method card in the File Contents window. In the Method Editor, you will find a PEAK FRACTIONATION instruction under the heading Sep Unit Enter the time to start Peak Fractionation, Fraction Size, Slope Detection Mode and the measured values for Peak Start and Peak End.

To stop Peak Fractionation, locate the instruction PEAK FRACTIONATION OFF under the Sep Unit menu.

A method with the above settings will fractionate the peaks in separate tubes and the elute between the peaks will go to waste. To collect fractions between peaks, select the function FRACTIONATION under the heading Sep Unit. Program the start of the fractionation at least 0.1 minutes before you start Peak Fractionation.

4.8 Evaluation

4.8.1 Introduction

SMART Manager evaluates results. This evaluation can be made very simple using the default parameters or more complex by optimizing the integration parameters. In this section, both the easy and some of the more complex curve operations are described. For a complete description of each menu see Section 5.

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4.8.2 Loading a Result File

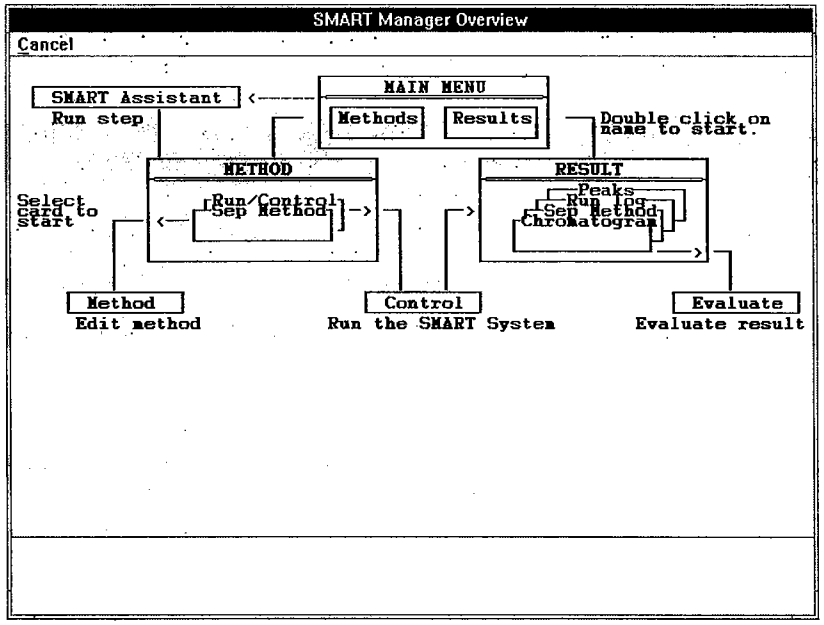


Fig. 4.34. SMART Manager overview

In the Main Menu select the Result file containing the results you want to integrate. The Result File Contents window will display a SEPARATION METHOD card, a RUN LOG card and a CHROMATOGRAM card.

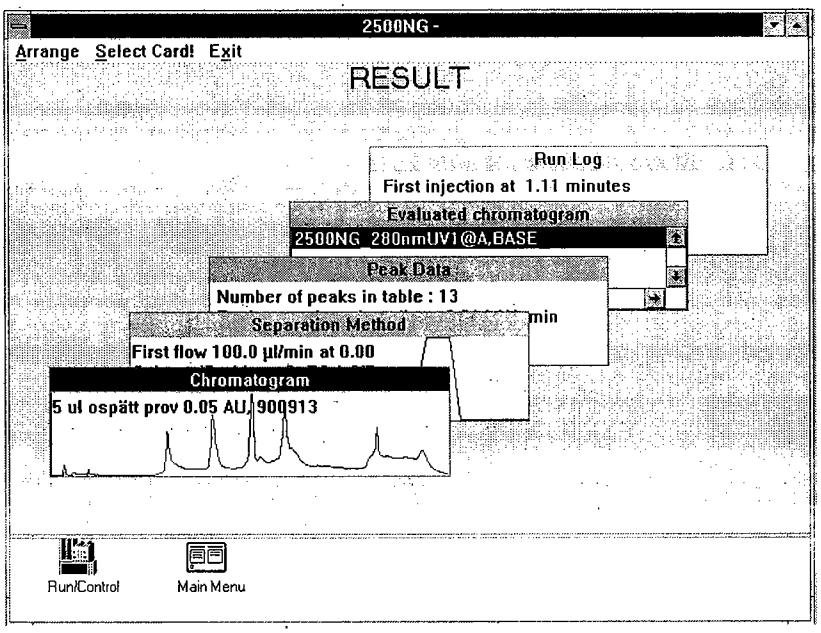


Fig. 4.35. Result File Contents window

To select the Chromatogram card, click on it and then double-click on it to open the result file. The chromatogram is displayed with all the recorded signals in the Evaluation window.

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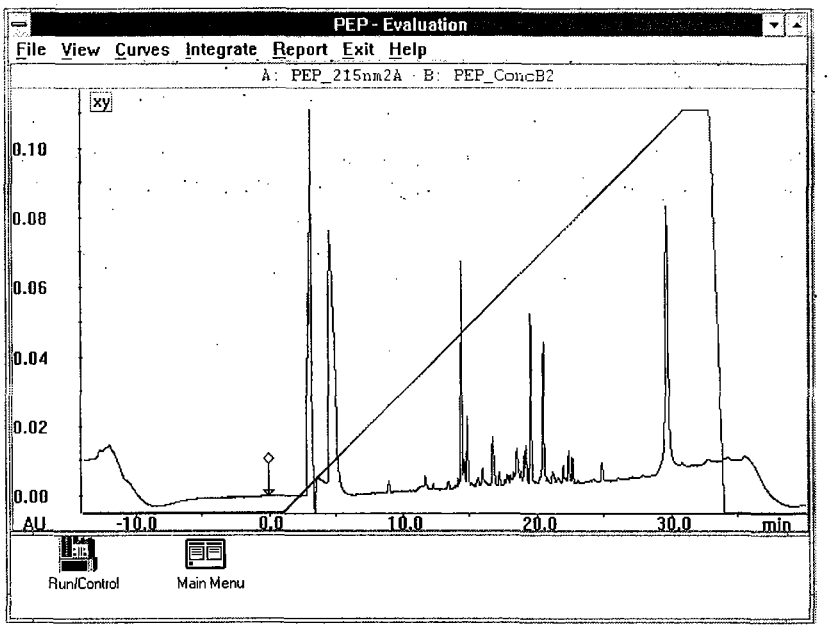


Fig. 4.36. Evaluation window

4.8.3 Changing the display

If you want to reduce the number of curves displayed, click on VIEW, SELECT WINDOW CONTENTS. In the list box all highlighted curves are displayed, so click on the curves you want to remove.

To change the units displayed on the y-axis, click on or to the left of the axis. If you want to change the base on the x-axis, click on VIEW, CHANGE BASE TO. The base can only be changed before integration or any other curve operation. After integration, the option disappears from the menu. You can also rescale the y- and x-axes by selecting VIEW, CURVE WINDOW LAYOUT. When you select Fix scaling of the axes a new dialogue box appears where you can enter the left and right limits for the x-axis and minimum and maximum values for the y-axis.

To make the evaluation more precise, all parts of the curve can be enlarged. Press on the mouse button and hold it down on a corner of the area to be enlarged. The cursor changes to a pointing finger with a box attached to it. By keeping the mouse button depressed and moving the mouse, the box can be extended to surround the area you want to enlarge. When the button is released, the display will show the enlarged area, together with an "Unzoom" command button. Clicking this button will show the original display.

4.8.4 Calculating a baseline

To integrate the results, you must first create a baseline. Under the menu INTEGRATE, select CALCULATE BASELINE. In the list box Calculate Baseline from, select the wavelength you want to integrate. In the right list box, the first empty position where the result is stored will be marked.

In the dialogue box, you will have precalculated values for the baseline calculation. When you create the baseline for the first time, accept these values and confirm your operations with OK. On the

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screen you will see the calculated baseline drawn through the baseline points.

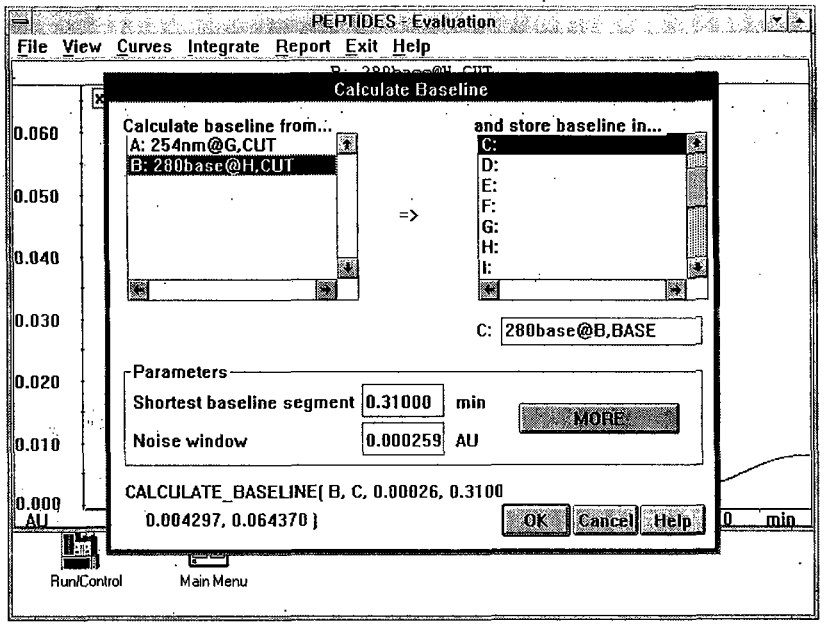


Fig. 4.37. Calculate baseline window

If you are satisfied with the baseline, you can integrate the peaks. If you would like to change the baseline, see Section 4.8.7 Optimizing integration.

4.8.5 Integrating results

Under the menu INTEGRATE, select PEAK INTEGRATE. In the dialogue box, select the UV curve for which you have calculated the baseline and accept the peak table. Under the heading Baseline, the baseline created above will be selected as default. Confirm the selection by pressing OK.

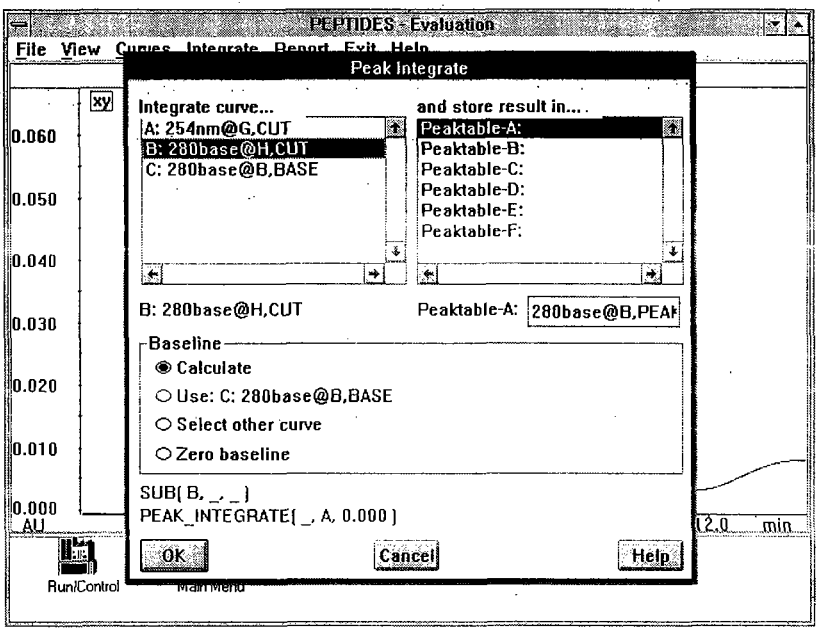


Fig. 4.38. Peak Integrate window

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The Evaluation window will have a panel with the chromatogram at the top and a panel with the peak table at the bottom.

4.8.6 Adapting the peak table

To specify what information to include in the Peak Table, select Peak Window Layout under the menu View. In the list box, you can select the functions you want to include in the Peak Table.

4.8.7 Optimizing integration

Integrating peaks in SMART Manager is divided into two steps: calculating the baseline and calculating peak areas. For accurate calculation of peak areas, a correct baseline is crucial.

There are four ways of calculating the baseline;

1. The ideal baseline is a blank run under the same conditions as the sample and used as baseline for peak integration.
2. Using the Calculate Baseline instruction for automatic calculation of the baseline, which in most cases is a very accurate measurement. See Fig. 4.39.

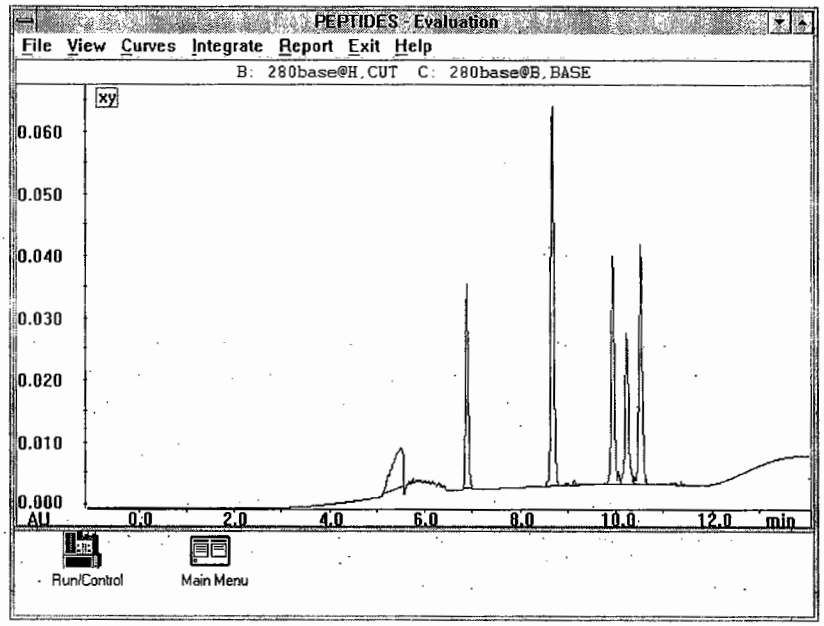


Fig. 4.39. Calculated baseline

3. Changing the baseline parameters used in the CALCULATE BASELINE instruction to better fit the curve.
4. Manually edit a baseline.

The parameters SMART Manager uses for baseline calculation are:

Shortest baseline segment, which is a measurement of the shortest relatively flat segments of the UV-curve between peaks. This is considered as baseline, measured in minutes or millilitres.

Noise window, which measures the noise of the UV-curve.

The combination of the Shortest baseline segment and Noise window can be illustrated as a rectangular box in which the UV-curve has to fit to be identified as a baseline segment. See Fig. 4.40.

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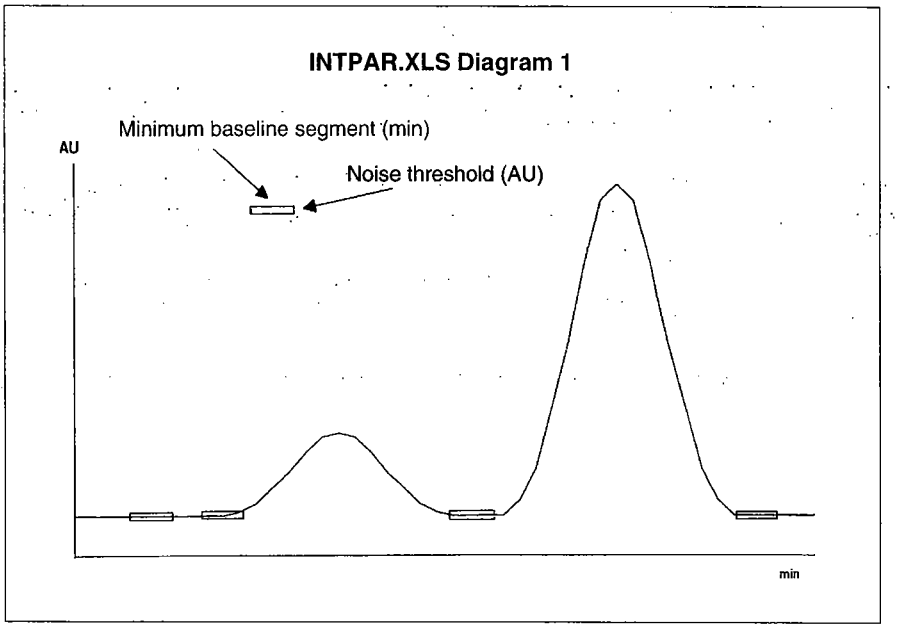


Fig. 4.40. Baseline box

In addition to the above mentioned parameters there are two more;

Maximum baseline level, which is the highest acceptable absorbance value for the baseline.

Slope limit, which measures the maximum slope of the baseline in AU/min.

The combination of Shortest baseline level, Noise window and Slope limit can be illustrated as a tilted rectangular box in which the UV-curve has to fit to be identified as a baseline segment. See Fig. 4.41.

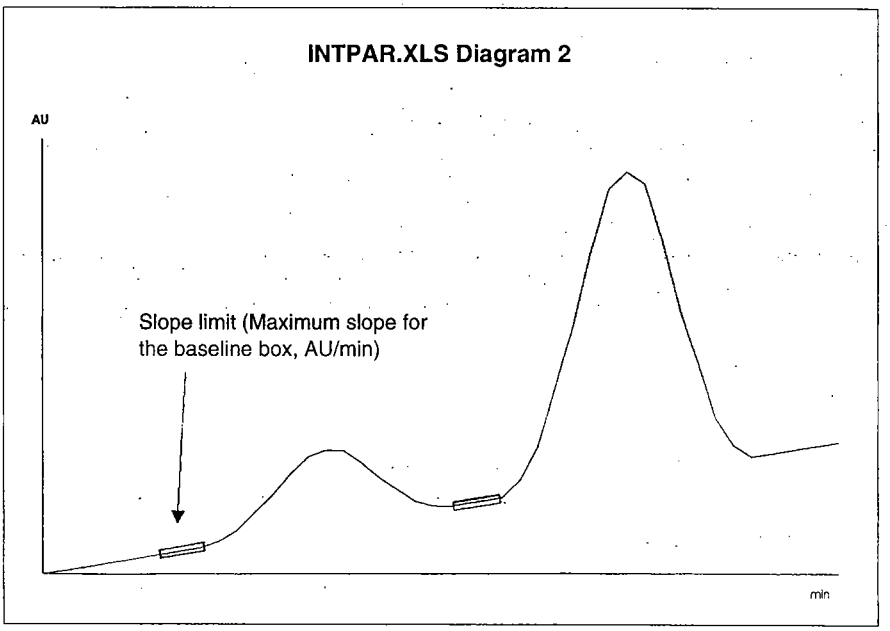


Fig. 4.41. Slope limit

When optimizing the baseline calculation, very often a change of the slope limit will give the expected result. An example of the effect of a too high slope limit causing up-slopes of the peaks to be recognised as baseline segments follows.

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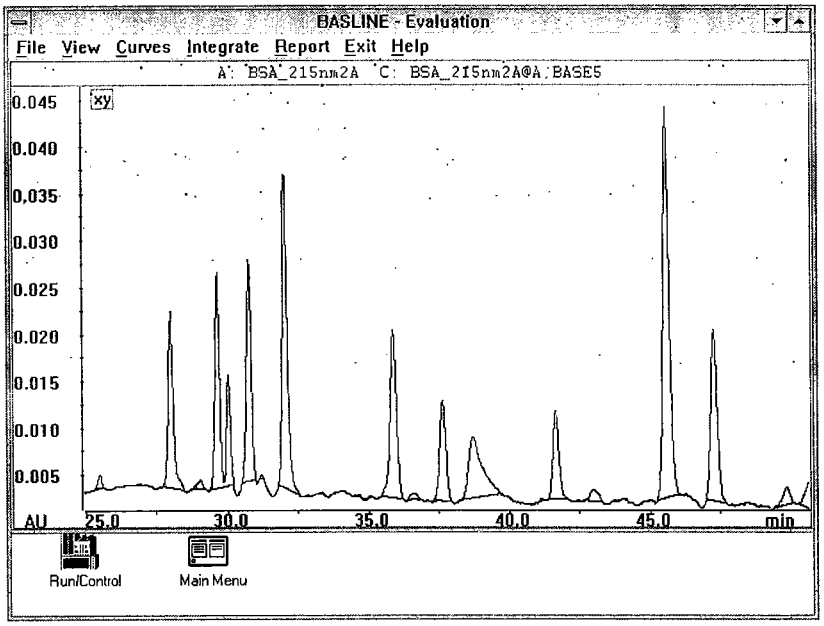


Fig. 4.42. Too high slope limit

Problems may also arise from a combination of a too long Shortest baseline value in combination with a too high Slope limit as illustrated in Fig. 4.43.

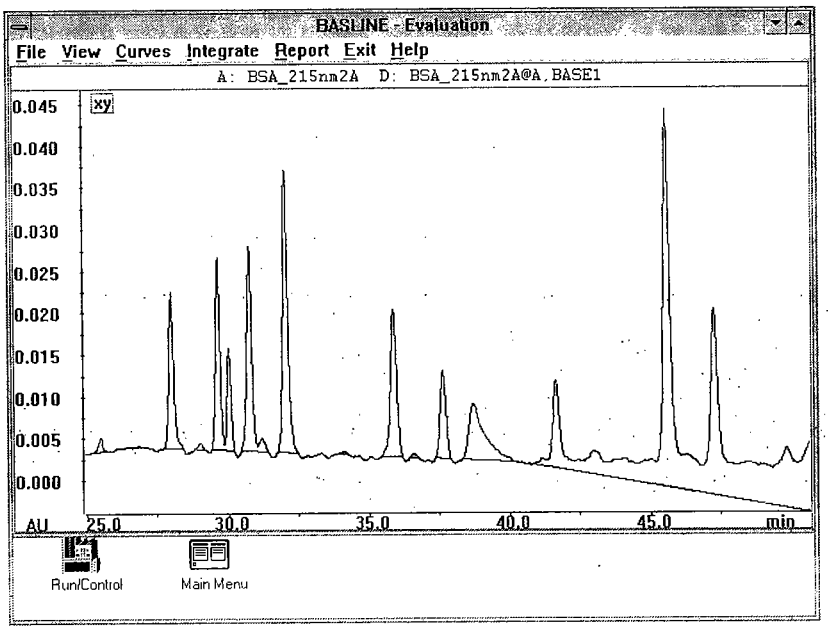


Fig. 4.43. Too long Shortest baseline segment and too high Slope limit.

Measuring the Shortest baseline segment

If you are uncertain of the length of the shortest baseline segment, you can always measure it directly on your chromatogram. Double-click on the **xy** box in the left corner of your chromatogram. Point the cursor on the bar that appears, click on the left mouse button and keep it down while you move the bar to a part of the curve where you think the shortest baseline segment is. Measure the length of the baseline segment. The numerical value for the position of the bar is

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displayed in the box. Enter the length in the calculate Baseline dialogue box.

Measuring the noise level

To measure the noise level, you can zoom in a part of the baseline. Point the cursor on the part of the baseline where you want to measure noise and click on the left mouse button. Keep the button down while drawing a box around the area you want to enlarge. On the y-axis you can read the absorbance values. The difference between the highest and the lowest values gives you the noise window. Enter the value in the Calculate Baseline dialogue box.

Measuring of Slope limit

To measure the slope of the baseline, select the instruction DIFFERENTIATE under the heading CURVES. Your chromatogram will be differentiated. To read differentiated values of the baseline, double-click on the xy box in the top left hand corner of the chromatogram. The numerical values for the x and y coordinates will appear. To read the slope values, make sure the coordinates box shows the black differentiated values. Point the cursor at the drag bar under the box and press the mouse button. Hold down the mouse button and move the drag bar along the UV curve. In the coordinates box you can read the calculated numerical slope values for the baseline.

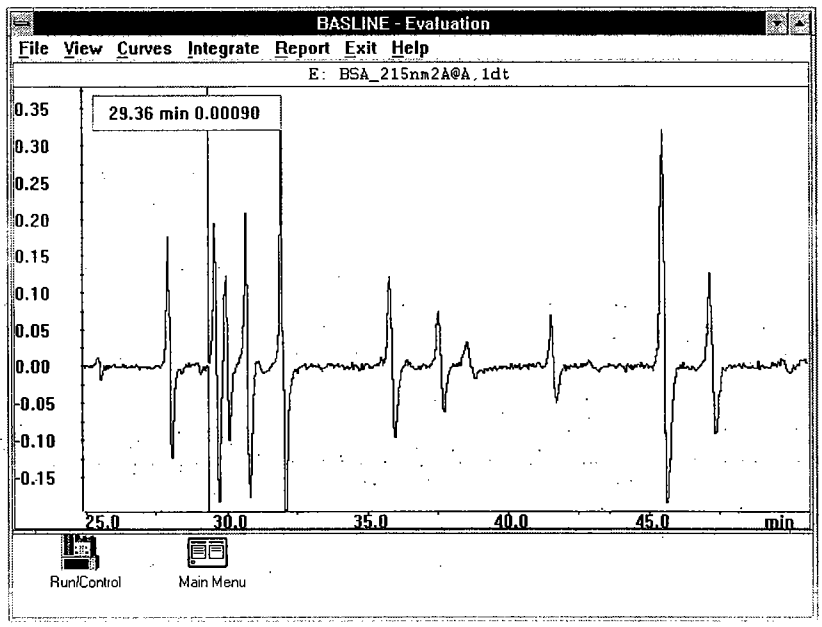


Fig. 4.44. Reading the slope limit

Enter the highest value of the baseline as the slope limit in the dialogue box.

The changes made to the baseline calculation can be saved and used in an evaluation procedure as described below.

4.8.8 Saving results

To save the baseline and peak table, use FILE, SAVE and mark what you want to save in the list box. Click on OK to confirm.

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4.8.9 Making a report

Before generating a report, select the report contents by clicking on REPORT, Specify Report Contents SPECIFY REPORT CONTENTS. In the dialogue box you select what to include, and if you click on the Sub item button you have a list of sub items to select for each item. For more information on the specific items, see section 5.5.3.

To generate a report, select GENERATE REPORT under the heading REPORT. In the dialogue box you have a choice to send the report to a printer or preview the layout on the screen.

Select Screen under the heading "Send report to" and click on OK. The Report will then be shown on the screen.

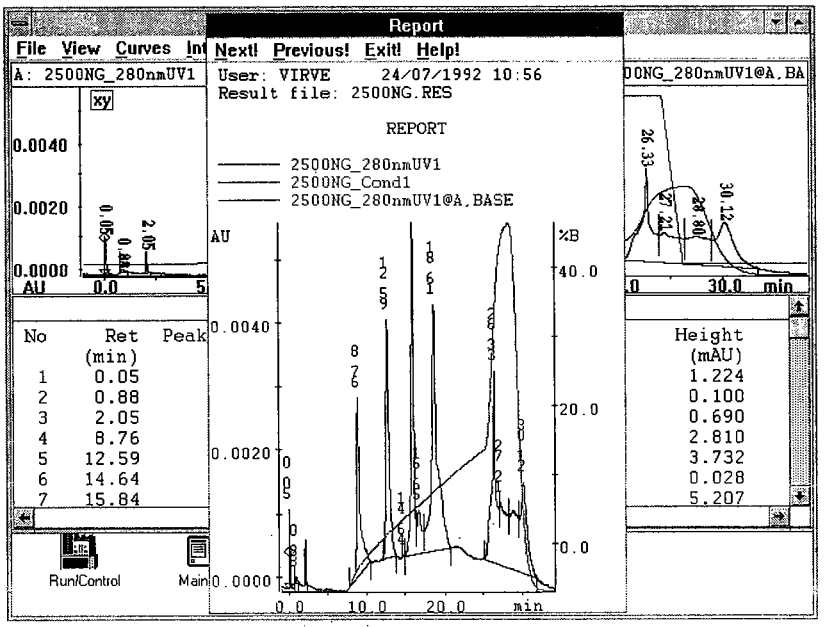


Fig. 4.45. Report window

4.8.10 Storing an evaluation procedure

When you have created an evaluation procedure, like the integration procedure above you can store it and include it in your method. Select EVALUATION PROCEDURE under the heading FILE. The entire procedure, with all the steps will be displayed. Select FILE, SAVE and select the method file in which you want to save the evaluation procedure. Type a name for the procedure. Select EXIT from the menu.

4.8.11 Using an evaluation procedure in a method

In the Main Menu, select the method file in which you saved the evaluation procedure above. In the File Contents window select the SEPARATION METHOD card. In the Method Editor menu select OTHER, CALL EVALUATION PROCEDURE. In the dialogue box program a time/volume when you want to start the integration. The instruction CALL EVALUATION PROCEDURE will appear in the text editor panel. Every time the method is run the results will be automatically evaluated according to the created procedure.

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4.8.12 Comparing curves

In the Evaluation part of SMART Manager you can compare results from different result files. Up to 8 curves can be displayed in one window and up to three windows on the screen.

Select a Result file from the Main Menu and open the CHROMATOGRAM card. In Evaluation, click on FILE, OPEN, CURVE. To the left in the dialogue box you have the result files generated under your own user name. You can also open files under another user name by clicking on the option button OTHER USER. When you have selected the result file, select the curve you want to open in the middle table, and in what curve memory you want to place it. Then click on TRANSFER.

To select which curves should be displayed, click on VIEW, SELECT WINDOW CONTENTS. To separate the curves for an easier comparison select SHIFT, SHIFT AMPLITUDE under the heading CURVES.

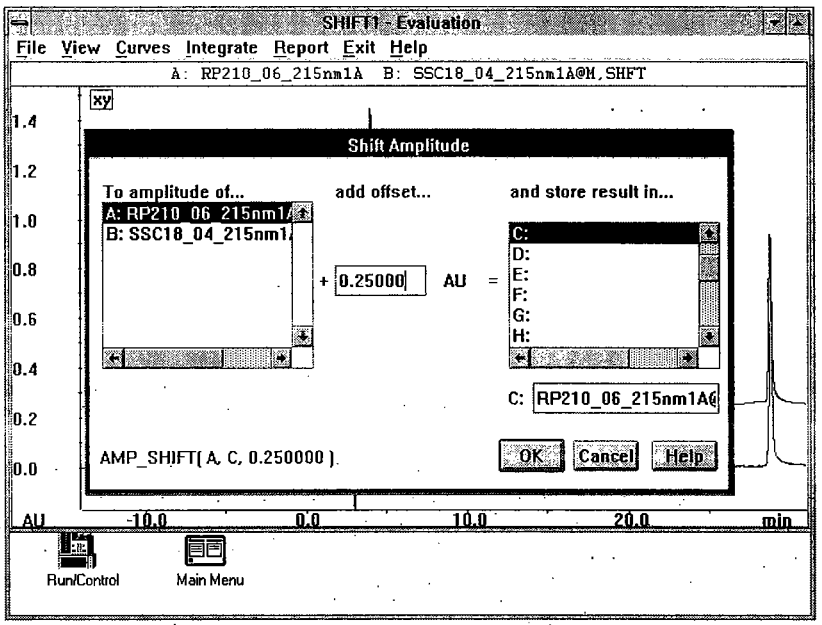


Fig. 4.46. Shift Amplitude window

In the left list box select one of the curves and add an offset in absorbance units to the curve. Select curve position for the result, click on OK.

The result may look like in Fig. 4.47.

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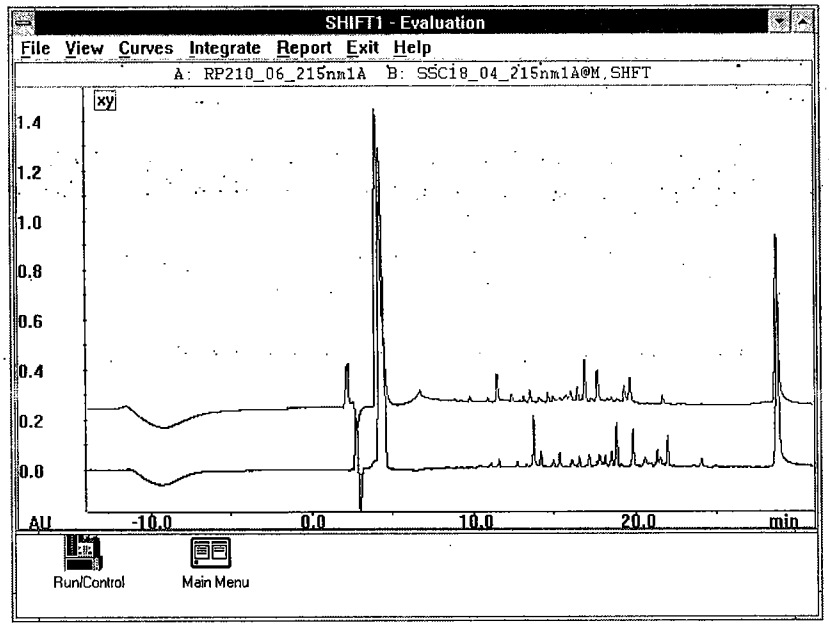


Fig. 4.47. Shift amplitude

4.8.13 Subtracting baseline

If you want to subtract a blank run from your result start with opening your result file from the run. In Evaluation, click on FILE, OPEN, CURVE. Select the Result File for the blank run and the UV curve and place it in an available curve position as your result. Click on TRANSFER. Select CURVES, SUBTRACT. In the dialogue box, select the result file from the run, subtract it with the blank run and select position to place it in. Use VIEW, SELECT WINDOW CONTENTS to select the curves to be displayed.

4.8.14 Mirror image

You can also compare curves and display a curve as a mirror image of the other curve. If needed, open the curves you want to compare using, FILE, OPEN CURVE. Under the menu CURVES, select SHIFT, MULTIPLY AMPLITUDE. In the right hand list box select one of the curves and multiply it with a factor -1.0. Select a curve position to store it in and click on OK.

Use VIEW, SELECT WINDOW CONTENTS to select the curves to be displayed. To further improve the display of the curves you can use the function SHIFT, SHIFT AMPLITUDE for the second curve.

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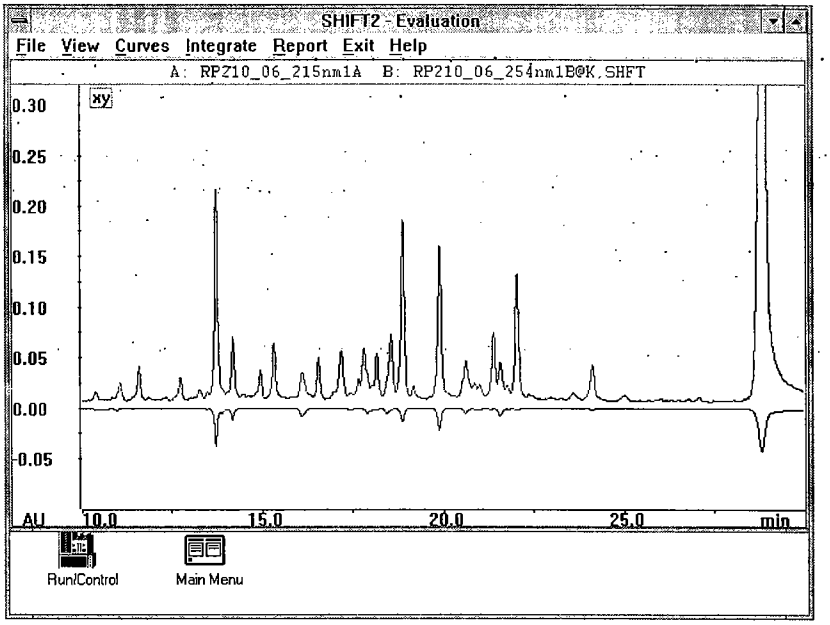


Fig. 4.48. Mirror image

4.8.15 Calculating absorbance ratios

Ratios between absorbance curves give useful information about peak purity or peak identity. The divide function can be used when you have a result file with the run detected at more than one wavelength with µPeak Monitor.

Before dividing the curves you must make sure that both chromatograms have a baseline close to 0 AU. This can be achieved with baseline subtraction. To create a baseline use the instruction CALCULATE BASELINE under the menu INTEGRATE, and if necessary EDIT BASELINE, or you can subtract a blank run from your chromatogram. To subtract the created baseline select SUBTRACT under the menu CURVES. In the dialogue box select the UV curve and the baseline.

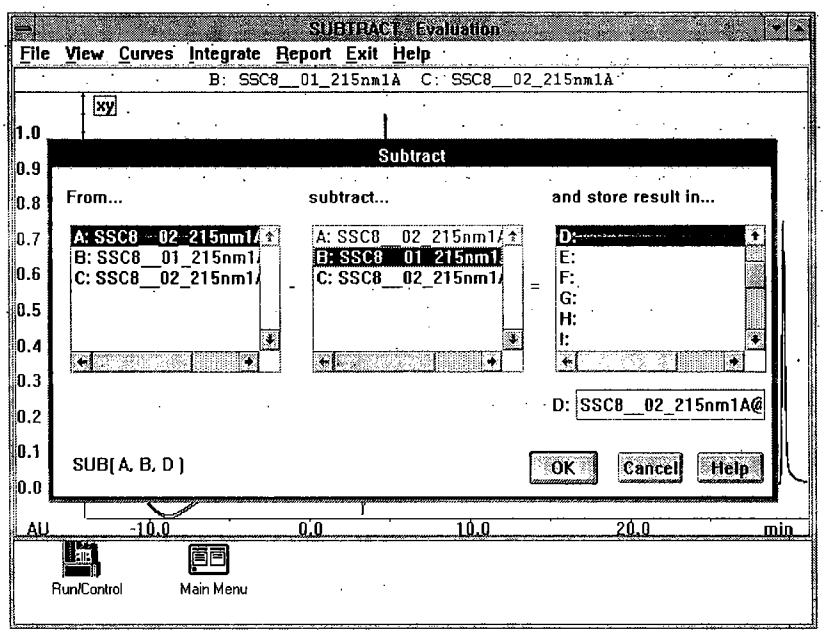


Fig. 4.49. Subtract baseline window

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If you want to subtract a blank run you must first open the curve and transfer it to the result file of the UV curve. Select FILE, OPEN CURVE. Select the result file which has the blank run and then select the UV curve for the blank. Select an empty curve position to place the curve in. Click on TRANSFER.

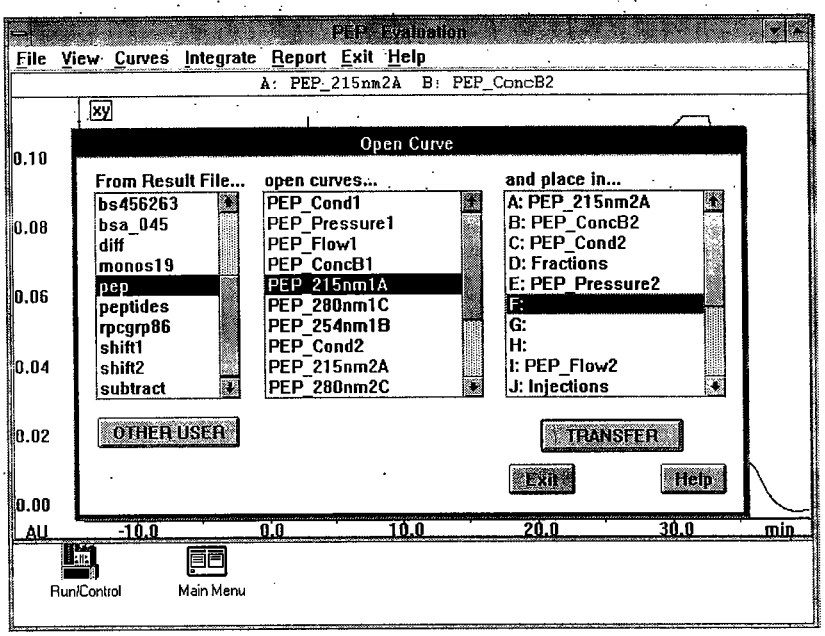


Fig. 4.50. Open curve window

After this you can select SUBTRACT under the heading CURVES and subtract the blank run from the UV curve as described above. The result appears as in Fig. 4.51.

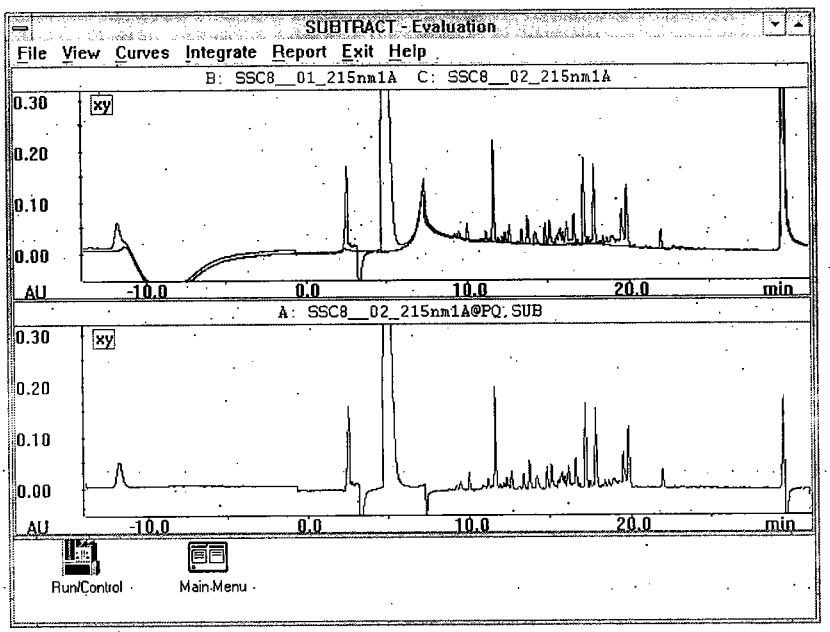


Fig. 4.51. Subtract baseline

When you have achieved two chromatograms with zero baseline select DIVIDE under the heading CURVES. In the dialogue box, select the curves to be divided and where to store the result. If you

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want to use a threshold value mark the check box. Accept the default values for thresholds or enter new values.

The ratio between the curves appears as in Fig 4.52.

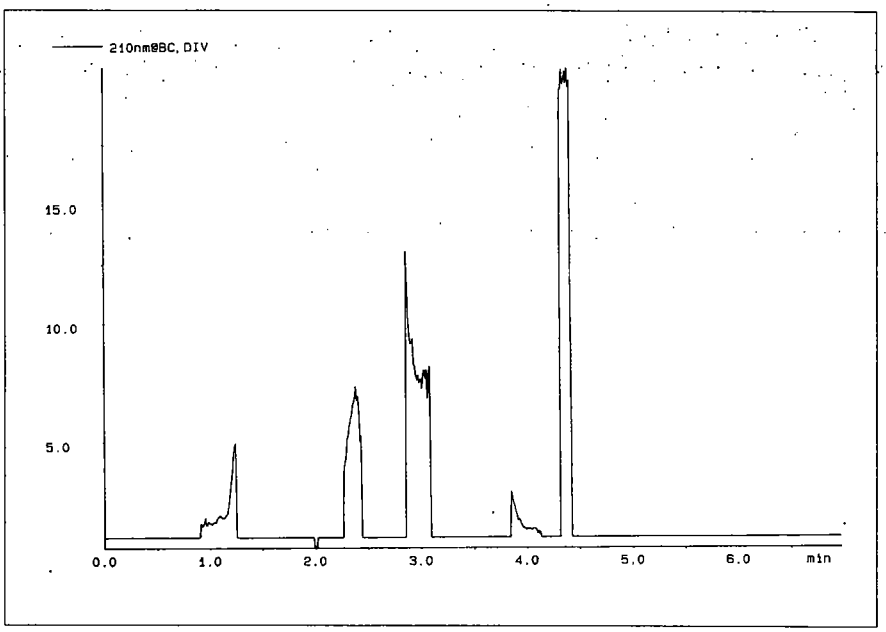


Fig. 4.52. Ratio between curves

The curves can be filtered to get a cleaner picture. Use the function SMOOTH under the heading CURVES. Select the Median filter in the dialogue box which filters noise that appears as spikes or in a small area of the curve.

The resulting ratio can then be used for peak identification as different compounds have a specific ratio between absorbances at different wavelengths.

The ratio can also be used to check peak purity. If the ratio is constant throughout a peak, the peak is probably pure. If the peak has shoulders after the division it is most probably contaminated. For more details, see Chapter 10, section 9.

4.8.16 Creating histograms

A histogram can be created of the UV-absorbance in the fractions or by entering values from an external measurement, e.g. a radioactivity measurement or activity measurement of the fractions. A condition for both is that the fractions have been collected using the Fractionation or the Peak Fractionation instruction in SMART Manager.

4.8.17 Creating histogram with UV-absorbance in fractions

In Evaluation select CURVES, FRACTION HISTOGRAM. In the left list box select the UV-curve of your choice, and from the middle box the fraction marks and in which curve memory to store the histogram at the right. Click on OK. The calculated histogram will appear. To save the curve click on FILE, SAVE CREATED HISTOGRAM. Select the curve position in which you want to save the histogram and click on OK. Select FILE, CLOSE WINDOW. The

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created histogram will appear in the window. To save the result with your result file, click on FILE, SAVE. In the left list box highlight CREATED HISTOGRAM. The result file from which the original curve was taken will be highlighted in the right list box.

4.8.18 Creating a histogram from external measurement of fractions

If you have measured the activity in your fractions, you can create a histogram or activity curve that can be overlaid on the original chromatogram.

In the Main Menu select the Result File, and in the File Contents window open the Chromatogram card. Select CURVES, CREATE HISTOGRAM. A dialogue box appears in which you can set the scale for the y-axis and change the scale for the x-axis.

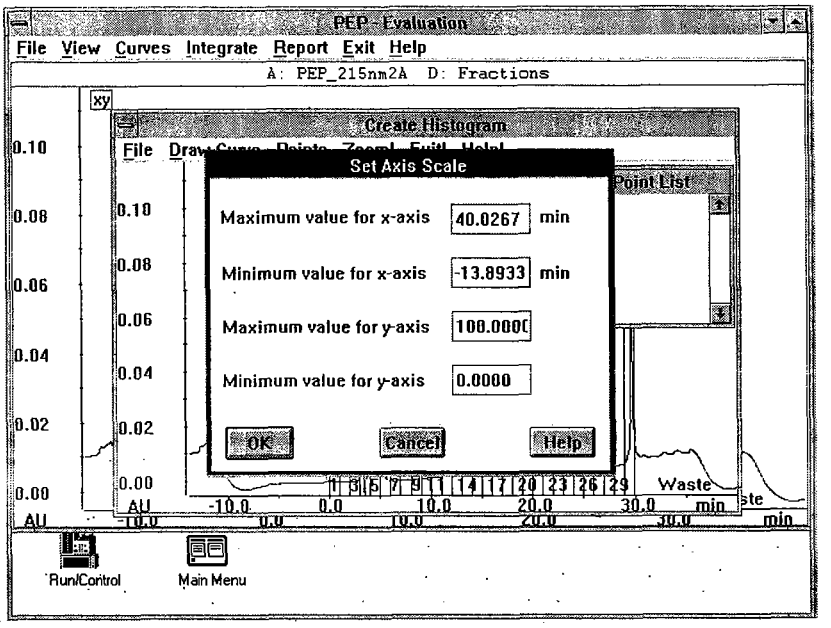


Fig. 4.53. Set Axis Scale window

To enter your activity measurements you can either:

1. Move the cross cursor to the desired x- and y coordinate and click on the left mouse button. A data point marked with a cross will appear. Continue to enter all points as required.
2. Select POINT, SET ACTIVITY POINTS. A list box will appear with the number of the fraction and box to enter the activity value. To proceed to the desired fractions click on the NEXT button. Continue to type in all the activity values required in this way.

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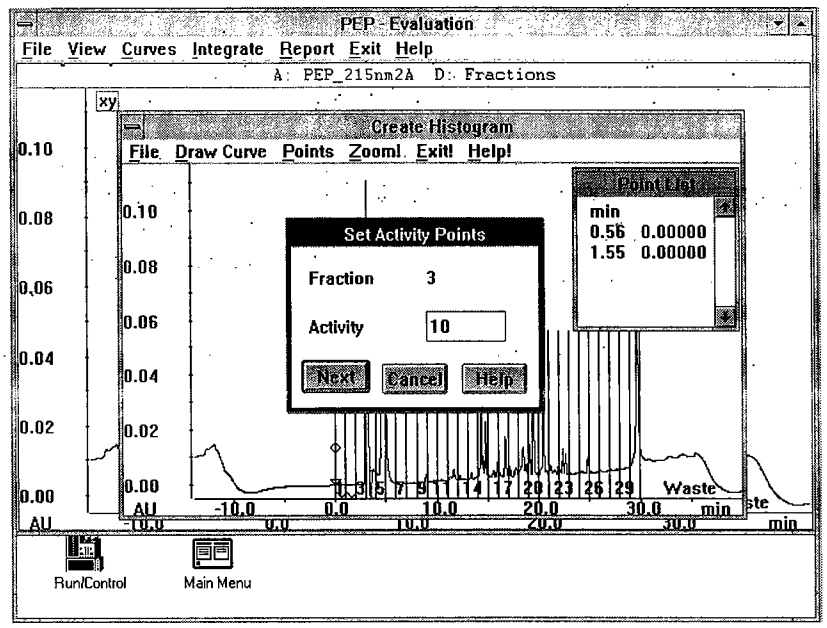


Fig. 4.54. Create Histogram window

3. Select POINTS, SET POINT. In this dialogue box you can enter the activity values by specifying the Retention time and the Amplitude for your activity.

To delete points you can use either of the three functions, under the menu POINTS, DELETE POINT which only deletes a specified point, DELETE LAST POINT or DELETE ALL POINTS.

When you have entered all the desired points, select DRAW CURVE and either DRAW SPLINE, DRAW THROUGH POINTS or DRAW HISTOGRAM.

To save the curve click on FILE, SAVE CREATED HISTOGRAM. Select the curve position in which you want to save the histogram and click on OK. Select FILE, CLOSE WINDOW. The created histogram will appear in the window. To save the result with your result file, click on FILE, SAVE. In the left list box highlight CREATED HISTOGRAM. The result file from which the original curve was taken will be highlighted in the right list box.

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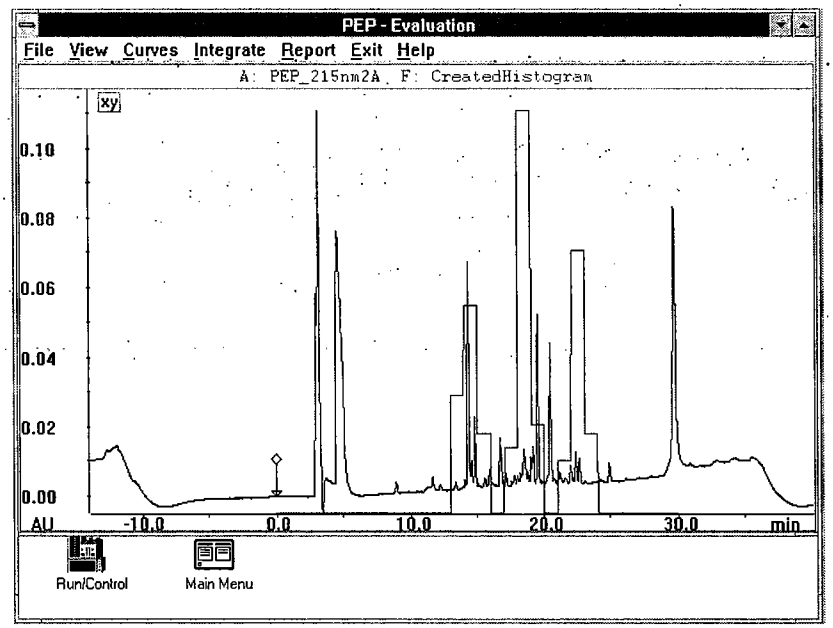


Fig. 4.55. Created histogram

4.9 SMART Assistant

4.9.1 Introduction

SMART Assistant is a knowledge information system which provides the user with help in identifying a suitable separation strategy to solve a specific chromatographic problem. Techniques and media are recommended and a Separation Method is created. The information given is dedicated to SMART System so that all techniques, media and methods suggested by SMART Assistant are suitable for use with the system. The information pathway is illustrated in figure 4.56.

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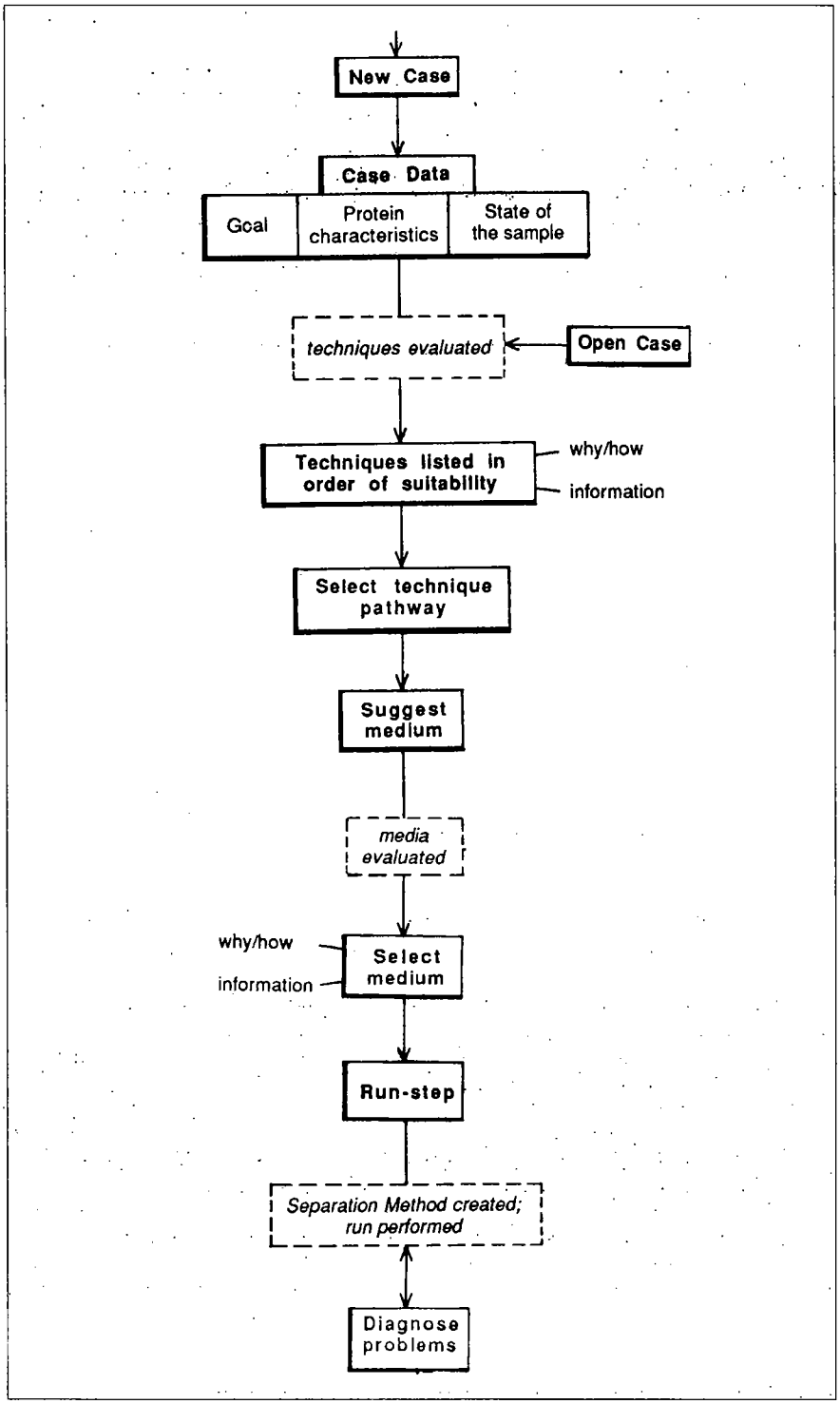


Fig. 4.56. SMART Assistant Information Pathway

SMART Assistant is accessed from SMART Manager Main Menu. The user defines the goal of the separation and, if desired, a preferred separation technique. If the sample is to remain active during the separation, this should also be specified.

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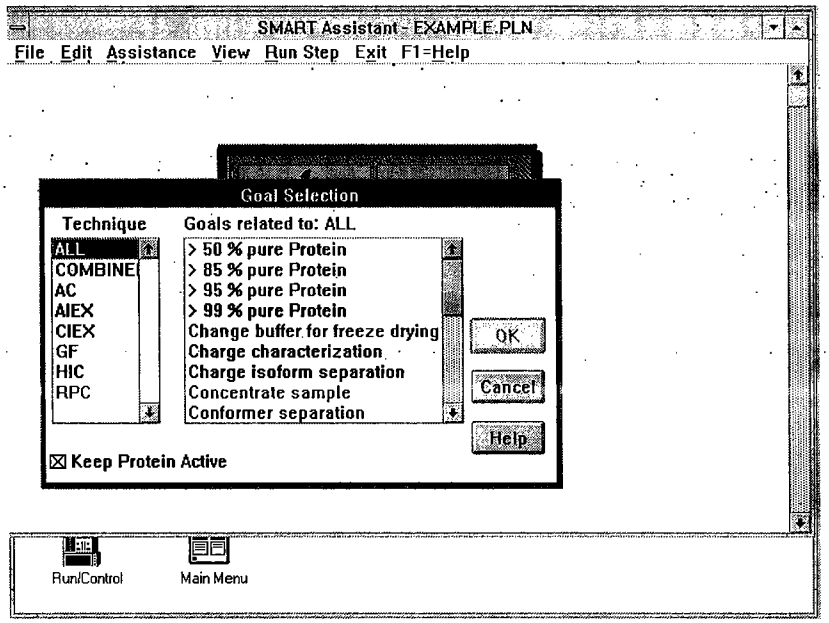


Fig. 4.57. Goal selection window

Further information to be entered relates to the characteristics of the sample and includes the type of protein and its ligand, the molecular weight, isoelectric point and other physical properties, if these are known. The sample volume, its total protein content and its target protein concentration should also be entered. Using this information, SMART Assistant suggest a suitable technique or series of techniques and what medium to use. A complete Separation Method is created, and the method can be transferred to SMART Manager and run. If the results of this are unsatisfactory, the user can return to SMART Assistant and ask for help in diagnosing the cause of the problems.

To learn about how SMART Assistant can help you, follow the steps below. The example describes a hypothetical series of events which are representative of a laboratory situation. SMART Assistant is described in more detail in Screens and Menus, in Section 5.6 of this Handbook.

4.9.2 Using SMART Assistant

The example

A soluble glycoprotein in 350 µl of a patient's serum has to be isolated and purified to 99 % purity. It is known that the sample contains 45 µg protein and that 35 % of the total protein is the glycoprotein component. The molecular weight of the glycoprotein is 85 000, the pI is 4.3 and it is rather hydrophilic. In order to keep the glycoprotein stable, the pH must be below 5.2 and there must be 20 mM salt present.

In SMART Manager Main Menu, click on START ASSISTANT in the pull-down menu SMART ASSISTANT. In the introductory screen select FILE, NEW CASE in the menu or click on GO. Enter the name. A Goal Selection dialogue box appears. Under the heading Technique select Combined. You will notice that the list box next to it changes. Select >99 % pure protein in the list box for goals related to: Combined Techniques.

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In the Protein Characteristics dialogue box you enter the known characteristics of your sample. In this example scroll the list box under Protein and highlight "Glycoprotein". You will see glycoprotein in the Protein field, and in the ligand field Lectin is suggested as a suitable ligand.

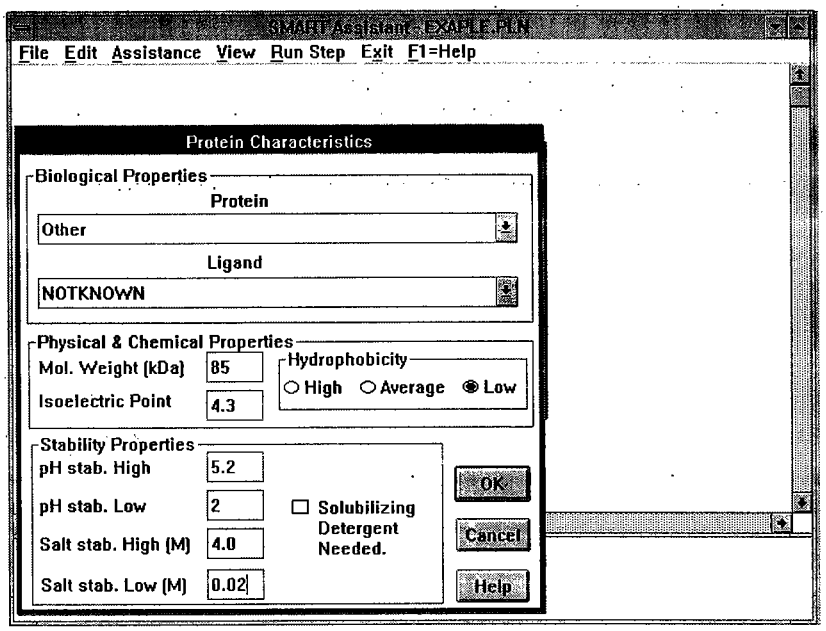


Fig. 4.58. Protein characteristics window

Enter the following known properties in the dialogue box:

- Molecular weight (kDa): 85
- Isoelectric point: 4.3
- Hydrophobicity: low
- pH stability high: 5.2
- pH stability low: 2
- Salt stability high (M): 4.0
- Salt stability low (M): 0.02

In the State of sample dialogue box enter the following:

- Volume µl: 350
- Total protein µg: 45
- Present purity (% (w/w) total protein: 35

The possible techniques are evaluated by SMART Assistant and are presented on screen in order of suitability. At the top of the list, AIEX (anion exchange) is presented in a green box, which indicates this is the most suitable technique to reach the goal set for this sample. A yellow box indicates it is a possible technique; techniques in red boxes should be avoided.

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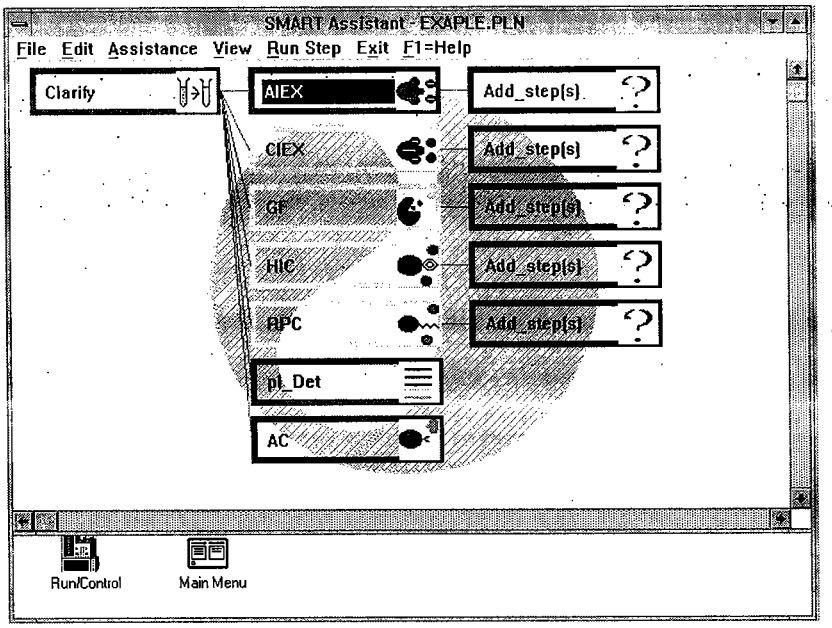


Fig. 4.59. Recommended techniques

Under the heading VIEW choose WHY/HOW and you can read about Anion exchange as a technique in the appearing window. To close the window, click on EXIT in the dialogue box.

Under the menu ASSISTANCE, choose SUGGEST MEDIUM. SMART Assistant suggests Mono Q as the medium and recommends a buffer exchange prior to the run.

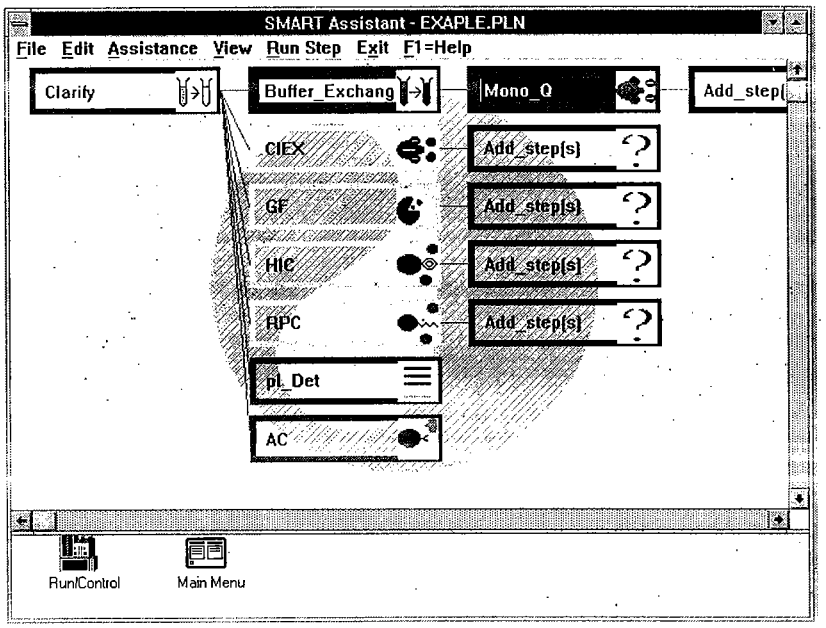


Fig. 4.60. Suggest medium

To find out more about the buffer exchange or the Mono Q run, highlight the box and use the instruction WHY/HOW under the menu VIEW. You will find detailed information about the running conditions.

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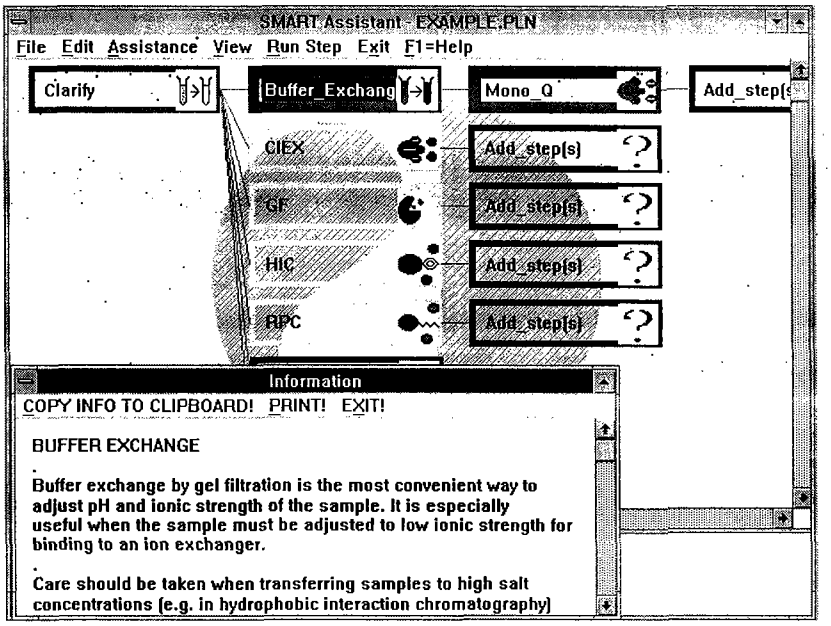


Fig. 4.61. Information on buffer exchange

A separation method for the suggested column will be created when you use the instruction Run step RUN STEP, TRANSFER TO SMART. Type a name for the method. If you click on the button OPTIONS, you can select to include a pump wash before method start or to include a Automatic Evaluation in your method. In this dialogue box, you can also program your system configuration. Click on OK.

The created method will appear in the File Contents window in SMART Manager. You can either check the method in the Method Editor or Run it directly.

Assume that the run was made and that you wish to continue with another step. Exit the File Contents. Highlight the Add Step box in SMART Assistant. Select SUGGEST TECHNIQUE under the menu ASSISTANCE. A dialogue box describing the current state of sample appears. Click OK and you will have a new list with suggested techniques of which RPC and GF are in green. Select RPC and ASSISTANCE, SUGGEST MEDIUM. A µRPC C2/C18 column is suggested.

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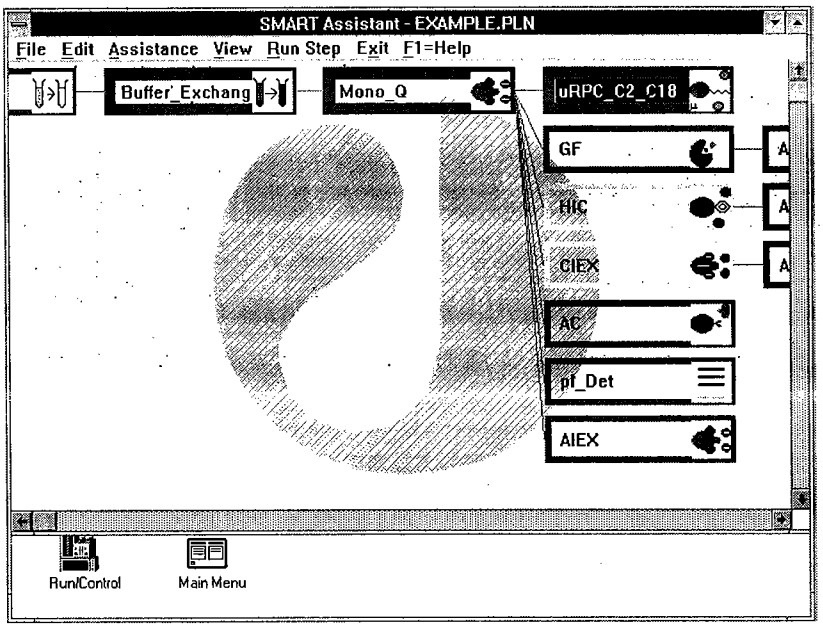


Fig. 4.62. Selected path

The method can be transferred to SMART Manager as described previously.

To check the state of the sample after the final purification step, select END SAMPLE RESULT under the menu EDIT.

4.9.3 Using Rewind Technique

Rewind Technique is a function in the EDIT menu which allows the user to go back some steps so that a different pathway can be chosen to achieve the aim of the separation. Steps can be retraced so that other pathways can then be selected.

By highlighting one of the steps and then clicking on REWIND TECHNIQUE under the menu EDIT, the path is forced to end at the highlighted technique. The user can then choose to follow a different pathway.

Example

Instead of running the recommended techniques in the order suggested by the above example, change to a different order.

On the pathway of recommended techniques and media, highlight Mono Q. Click on EDIT, REWIND TECHNIQUE. All techniques to the right of Mono Q are removed, leaving only a single Add Steps box.

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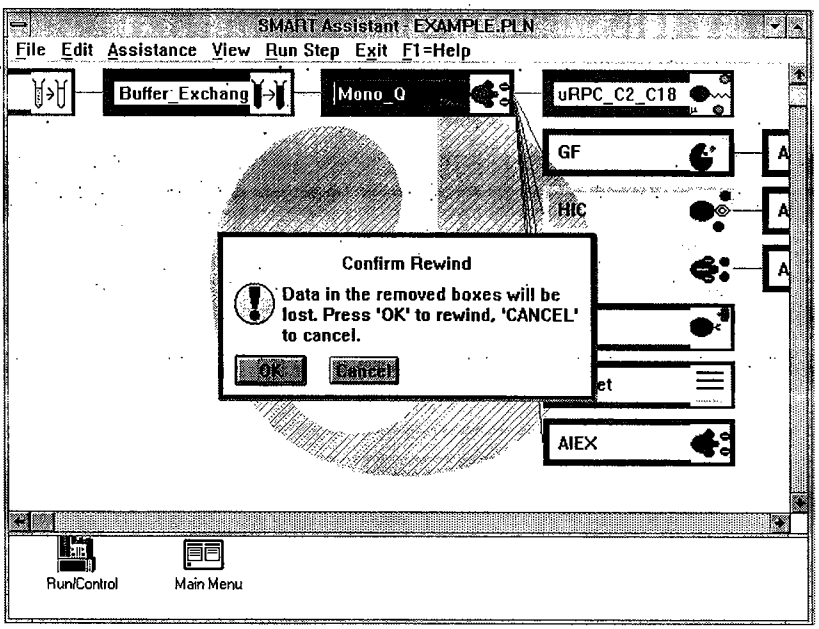


Fig. 4.63. Rewind technique

A new order can be selected using the Add Steps box. Highlight Add Steps box. Click on ASSISTANCE, SUGGEST TECHNIQUE. After the Current State of the Sample box is displayed, a list of techniques presented, in order of recommendation.

In the previous example, two techniques were recommended. Instead of RPC choose Gel Filtration this time. Highlight GF and click on ASSISTANCE, SUGGEST MEDIUM. A list of possible gel filtration media appears. Choose Superose 12 at the top of the list and click on OK. SMART Assistant suggests a concentration step before the gel filtration step as the sample volume is too large to apply directly.

Highlight Concentration and choose WHY/HOW under the menu VIEW. You can see a suggestion to use the Mono Q column for the concentration step. If you select RUN STEP, TRANSFER TO SMART, a method will be created with a step gradient to elute the sample in a smaller volume.

After the concentration step you can continue by highlighting the Superose 12 box and then clicking on RUN STEP, TRANSFER TO SMART to run the method created for the Superose 12 column.

4.10 Using Superloop

Superloop is supported in the two holders on the left inside the µSeparation unit. Superloop can be filled before connection by pouring in the sample or after connection by filling with a pump or syringe. The former method, which is described below, does not require an extra pump and prevents the back pressure obtained during filling with a syringe.

1. Disassemble Superloop and clean it.
2. Connect the tubings to the inner end pieces (one 10 cm and one 40 cm or 75 cm).
3. Insert the moveable seal (with the sealing ring down) from the upper end of the loop.

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4. Insert the upper inner-end piece (with the long tubing) on top of the moveable seal and push both down.

5. Screw on the outer upper-end piece.

6. Turn Superloop upside down.

Note: To reduce background disturbances when working at high sensitivity, flush Superloop with buffer A before filling it with sample.

7. Carefully pour the sample into Superloop. Do not fill above the line indicating "O" volume.

8. Insert the lower inner-end piece and turn it until the slot aligns with the countersink in the glass tube (about 5 mm left of "O"). Insert the protective jacket. Tighten by screwing the outer end piece into place.

9a. If Superloop has been partly filled with sample and contains air, proceed with step 10. Otherwise proceed with step 9b.

9b. If Superloop is essentially air free, return Superloop to its normal position and install it in the µSeparation Unit and connect it to the injection valve. The short tubing should be connected to the lower right connection on the injection valve, while the long tubing should be connected to the upper left connection. Superloop is now ready for use.

10. If Superloop contains air after it has been filled with a sample, install Superloop upside down in the µSeparation unit. Connect the long tubing to the upper left position on the injection valve. Do not connect the short tubing. Switch the injection valve to the inject position and start the pump at maximum flow rate (2 ml/min). Continue at this flow rate until no air or only a small air bubble is visible in Superloop. The flow rate can be reduced for the last part. Return Superloop to its normal position and connect according to step 9b.

It should be noted that the pressure limit for Superloop is 4 MPa (40 bar, 580 psi). The flow rate has to be adjusted for this limit and the maximum pressure limit for the system should be set accordingly.

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Appendix 4.1

Method instructions

Before each instruction time/volume has to be entered

Instruction syntax	Parameters	Allowed values	Units (if any)
FLOW *flow*	*flow*	0-2000	µl/min
CONC_B *conc*	*conc*	0-100	%B
FILL*cyl_id*, *inlet*, *volume*, *flow*	*cyl_id*	A or B	
	inlet	1, 2, 3 or 4	
	volume	0-10 (10 ml cyl)	ml
		0-20(20 ml cyl)	ml
		0-50 (50 ml cyl)	ml
	flow	0-15000 (allcyl)	µl/ml
EMPTY *cyl_id*, *outlet*, *volume*, *flow*	*cyl_id*	A or B	
	outlet	1, 2, 3 or 4	
	volume	0-10 (10 ml cyl)	ml
		0-20 (20 ml cyl)	ml
		0-50 (50 ml cyl)	ml
	flow	0-15000 (10 ml cyl)	µl/min
		0-30000 (20 ml cyl)	µl/min
		0-75000 (50 ml cyl)	µl/min
Pump_WASH *cyl_id*, *inlet*, *outlet*	*cyl_id*	A or B	
	inlet	1, 2, 3 or 4	
	outlet	1, 2, 3 or 4	
LOAD		INTERNAL or EXTERNAL could be used but not necessary	
INJECT		INTERNAL or EXTERNAL could be used but not necessary	
WASTE			
FRACTION_SIZE *volume*	*volume*	5-1500	µl
FRAC_STOP			
GOTO_TUBE *position*	*position*	1-60	
NEEDLE_POSITION *position*	*position*	UP or DOWN	
LARGE_TUBE *position*	*position*	A, B, C or Z	
PEAK_FRACTION_SIZE *volume*	*volume*	5-1500	µl

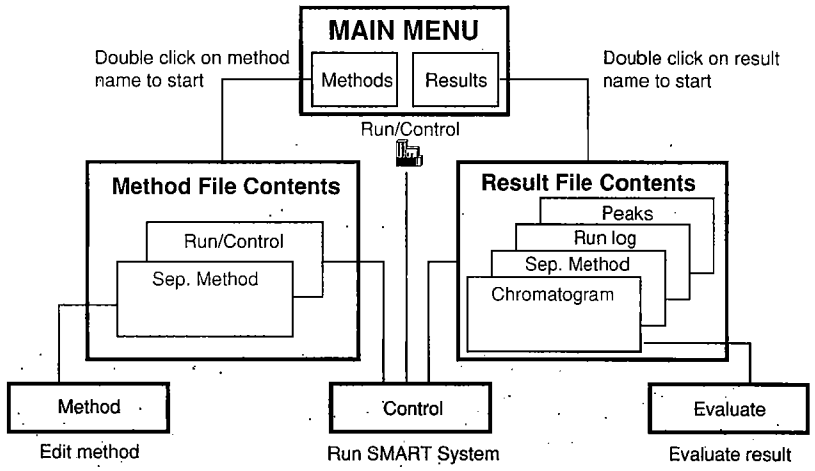
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Instruction syntax	Parameters	Allowed values	Units (if any)
PEAK_LEVEL *peakwidth*, *noise*, *start*, *stop*, *valley*	*peakwidth*	0.00-60.00	min
	noise	0.000-0.1000	AU
	start	0.000-32.000	AU
	stop	0.000-32.000	AU
	valley	ON or OFF	
PEAK_SLOPE *peakwidth*, *start*, *stop*	*peakwidth*	0.00-60.00	min
	start	0.000-32.000	AU/min
	stop	0.000-32.000	AU/min
PEAK_OFF			
MIXER_WASH *conc*	*conc*	0-100	%B
HOLD			
LOOP *loop_no*	*loop_no*	1-99	
END_LOOP			
CALL_SUB_METHOD *name*	*name*	Any submethod name	
CALL_EVAL_PROC *name*	*name*	Any evaluation procedure name	
NEW_CHROMATOGRAM			
WAVELENGTH *wl1*	*wl1*	190-600	nm
DUAL_WAVELENGTH *wl1*, *wl2*	*wl1*	190-600	nm
	wl2	190-600	nm
TRIPLE_WAVELENGTH *wl1*, *wl2*, *wl3*	*wl1*	190-600	nm
	wl2	190-600	nm
	wl3	190-600	nm
AUTOZERO			
ANALOG_OUTPUT *range*, *offset*	*range*	0.000-2.500	AU
	offset	0-100	%

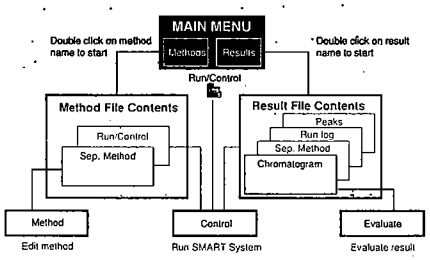
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SMART®Manager/Short instructions

SMART Manager overview



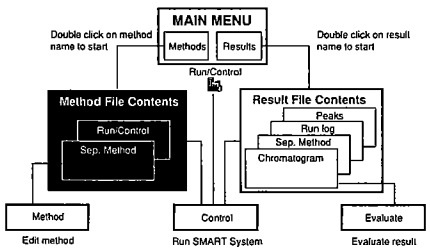
Main Menu



◂SMART Manager▸ Main menu

File	User	SMART Assistant	Exit	Help
New...	Change...	Start Assistant	Shutdown SMART Manager	Help for Help...
Load method file...			Resume SMART Manager	Extended Help...
Load result file...				Keys Help...
Delete method file...				Help Index...
Delete result file...				
About...				

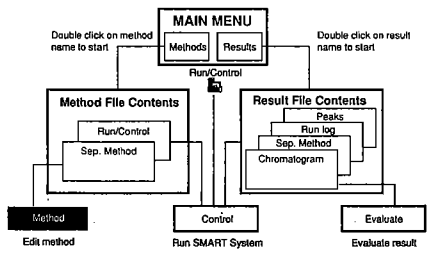
Method File Contents



◂SMART Manager▸ <Method name>

Arrange	Select card !	Exit
Tile...		Exit file contents
Cascade...		Resume file contents

Method Editor



◂SMART Manager▸ <Method name> Method editor

File	Edit	View	Pump	Sep Unit
Clear...	Copy...	Chemistry	Conc B...	Injection Valve...
Save As...		Configuration	Flow...	Goto tube...
Save		Main Method	Fill...	Fractionation...
Print...		Sub Methods...	Empty...	Frac Stop...
Eval Procedures...			Pump Wash...	Large tube...
				Peak Fractionation...
				Peak Fractionation Off...
				Mixer Wash...

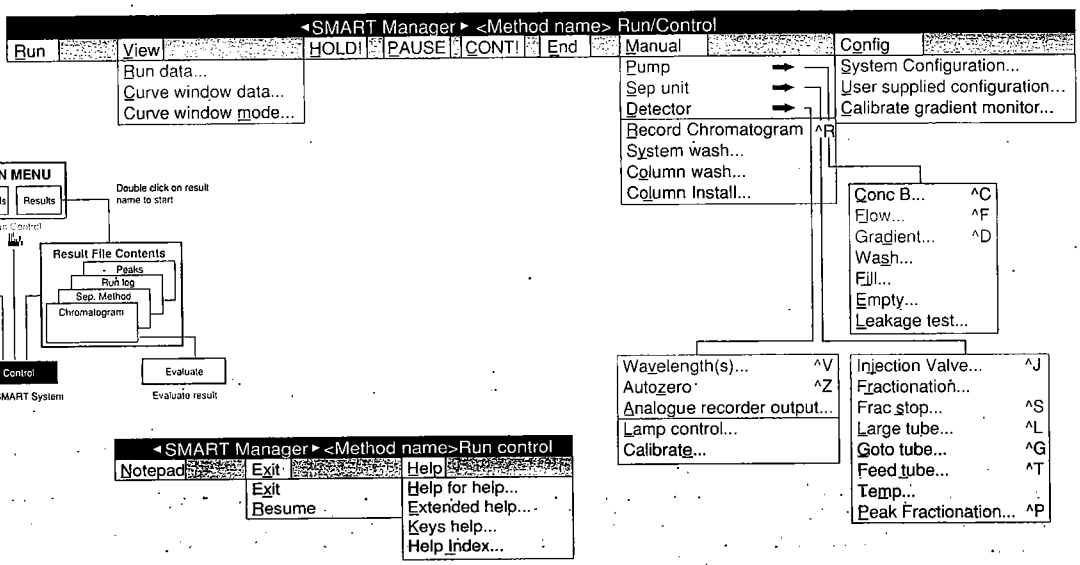
◂SMART Manager▸ <Method name> Method editor

Detector	Other	Check	Exit	Help
Wavelength(s)...	Hold...	Check And Update Graphs	Exit with Save	Help for Help...
Autzero...	Call Sub Method...	Update Graphs	Exit without Save	Extended Help...
Analogue recorder Output...	Loop...		Resume Editor	Keys Help...
New Chromatogram...	End loop...			Help Index...
	Call Eval Procedure...			

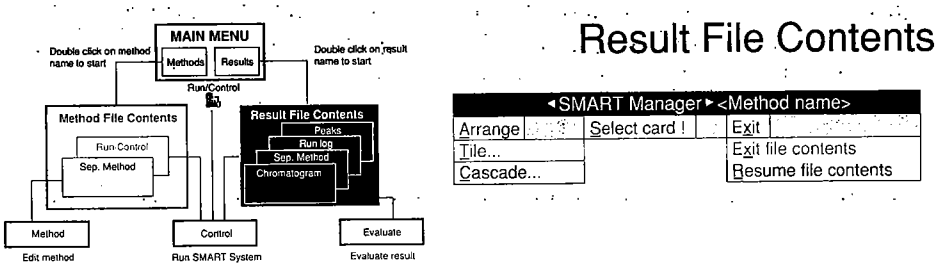
Pharmacia Biotech

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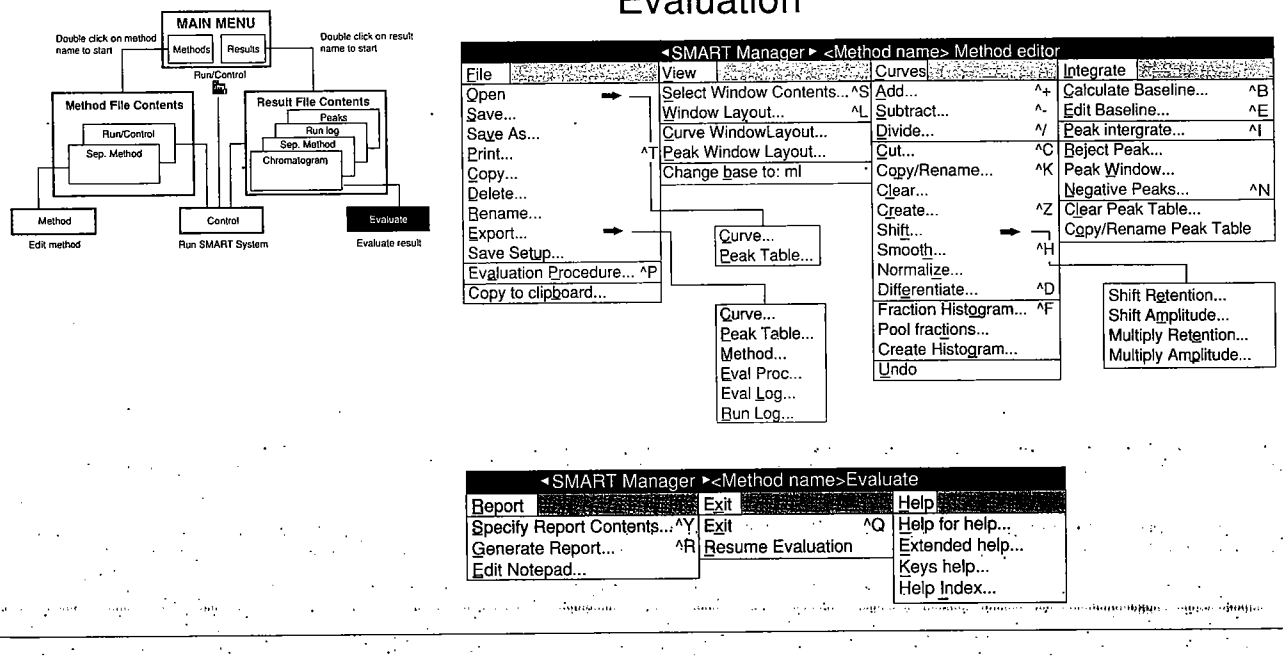
Run/Control



Result File Contents



Evaluation



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5. Screens and menus

5.1 SMART Manager system philosophy

5.1.1 Overview

This section of the handbook explains the concept of SMART Manager, the program which controls SMART System and handles data acquired from a chromatographic run.

SMART Manager is designed for all types of chromatographic separations on SMART System; from those separations requiring a simple run to those separations requiring automatic separations and evaluations.

A chromatographic run on SMART System can be controlled by a set of programmed conditions called a Separation Method created by the user. When the Separation Method is started, it runs automatically according to the programmed conditions. However, it is possible to make temporary changes in a running Separation Method using the Run/Control Manual commands. All events of the run, both programmed and temporary, are recorded in the Run Log. A complete chromatographic separation can be run using only manual commands.

The resulting Chromatogram can be evaluated and integration performed. The manual evaluation is recorded by SMART Manager and used to form an Evaluation Procedure which can be recalled later and used with a Separation Method for the automatic evaluation of the results of chromatographic runs.

5.1.2 User directories and file names

When first accessing SMART Manager, the user enters his name which is "logged-in". The program reserves space, called a directory, on the hard disk for that user. All files, both Method and Result Files which are created subsequent by this user are stored in this directory. All files are listed in the Main Menu and show the name of the user who created them. Method Files and Result Files can be accessed at any time by any user. However, the user can only make changes to his own files.

When a new Separation Method is created, it is given a file name by the user and is saved in that user's directory. A Separation Method file is given the extension .MET. Information generated by the run, such as the Chromatogram can be stored under an automatically created file name, which consists of the first six letters of the method name and two figures (01, 02 ...). It is also possible to enter a new file name. If a new name is entered, the consecutive runs with the same method, will have the six first letters from the new name, followed by two figures. Data resulting from the run takes the extension .RES

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5.2 Login, Main Menu and File Contents

5.2.1 SMART Manager Welcome screen and Login

In SMART Manager, each user has his own directory where all his files are stored. It is accessed first by clicking on LOGIN on the Welcome Screen and then clicking on the specific USER NAME in the displayed list box (Fig. 5.1). A new user logs in by typing his name in the text box. The Login directory has a user called Library, which contains preprogrammed methods. The methods are described in Appendix 5.1. Confirmation can be given either by clicking on the OK button or by pressing <ENTER>. The Main Menu of files created is then displayed.

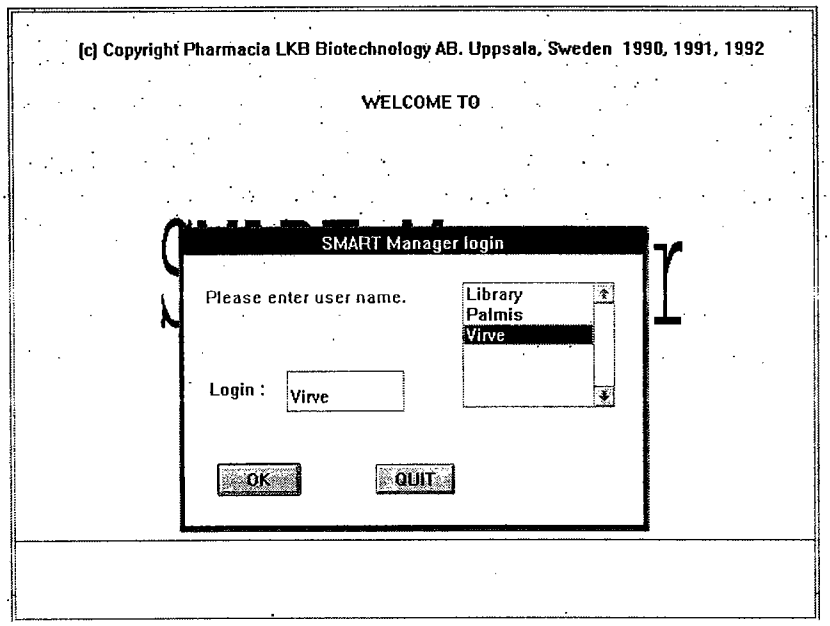


Fig. 5.1. Login window

5.2.2 Main Menu window

The Main Menu is the directory of files currently saved. Two list boxes are shown, one displaying Method Files and the other displaying Result Files (Fig. 5.2). Files created by the current user are at the top of the list. These are followed by files created by other users with the user's name shown in brackets. Method Files and Result Files can be accessed by any user.

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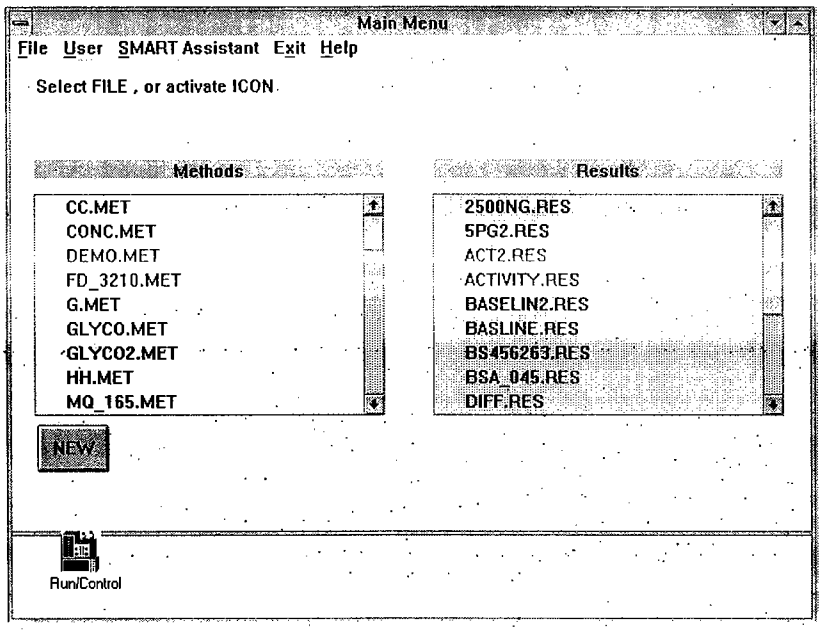


Fig. 5.2. Main Menu window

A command button marked NEW is located in the lower left of the screen. By clicking on this button, a new Separation Method can be created.

In the Main Menu window, a file from either the Method or the Result list boxes can be opened by highlighting it and then either double-clicking on it or clicking on FILE, LOAD METHOD FILE.

5.2.3 Main Menu pull-down menus

File

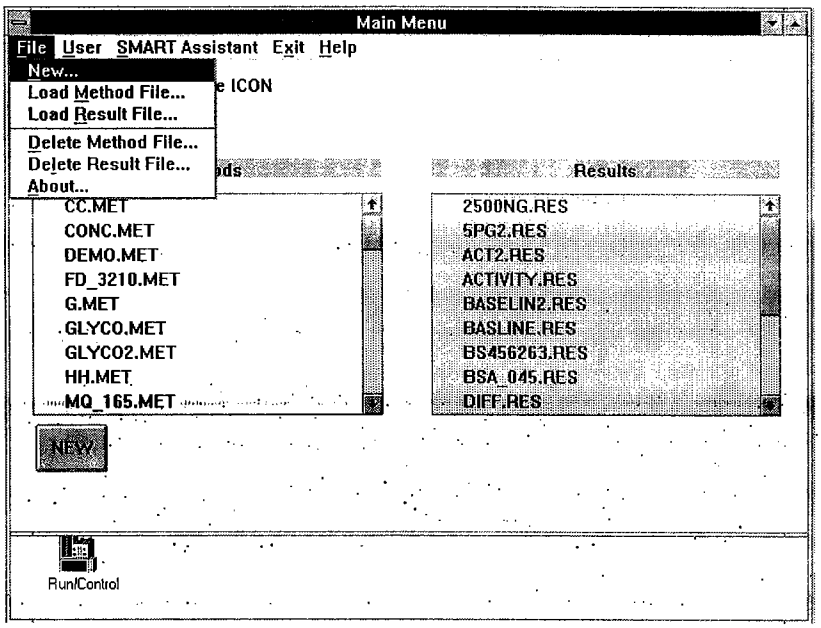


Fig. 5.3. Pull-down menu File

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- New
  The current user can create a new method using a new file name. This has the same function as clicking on the New command button in the lower part of the screen. By giving the method a name and confirming it with OK you enter the File Contents window with the card box. Here you can select if you want to enter the Method Editor or the Run/Control part of SMART Manager.
- Load Method File
  The highlighted Method File is loaded and you enter the File contents window with the cards to enter the Method Editor or the Run/Control part of SMART Manager. Any user can open any Method File.
- **Load Result File**
  The highlighted Result File is loaded and you enter the File Contents window with the cards for the Chromatogram, Separation Method, Peak Data table, Evaluated Chromatogram and the Run Log displayed. The number of cards displayed depends on what operations have been performed on the result in the Evaluation part of SMART Manager. Any user can open any Result File.
- **Delete Method File**
  The highlighted Method File will be deleted from the list after confirmation from the user. (Only files from the logged in user.)
- **Delete Result File**
  The highlighted Result File will be deleted from the list after confirmation from the user. (Only files from the logged in user.)
- User
  If User is selected it is possible to change to another user name in the Login list box or to a new user name. A warning message will be displayed with a information that any changes made to Method or Result Files under the current user name will not be saved automatically. By clicking on the Cancel command button, you can return to the Main Menu to access Method or Result Files to be saved. By clicking on the OK command button in the warning message box, similar window as the Login list box will be displayed with names of all users except the current one. The user name can be changed by clicking on the new name and on the OK button or alternatively by double-clicking on the wanted user name. A new user name can be created by writing the new name in the edit box, then clicking OK.

SMART Assistant Main Menu SMART Assistant

- Start Assistant
  This function accesses SMART Assistant which is a knowledge and information base dedicated to SMART System. It is designed to assist the user in finding the most appropriate technique and medium for specific chromatography applications. Then, together with SMART Manager, SMART Assistant automatically creates a Separation Method for each proposed step. For more detailed information see Section 5.6.

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Exit

- **Shutdown SMART Manager**
  This function exits from the Main Menu and SMART Manager.
- **Resume SMART Manager**
  This function resumes SMART Manager.

Help

- **Help for Help**
  This function describes how to use the Help command.The Help choice appears on the menu bar of every window including the associated Help choices listed below.
- **Extended Help**
  This function provides general information about a window. It also gives information about each menu choice and the instructions listed under that menu. All menu choices are listed at the end of the Extended Help dialogue box. When you select a specific menu a new dialogue box will appear with all the functions listed at the end of the dialogue box. You can also use the help index described below to access help for a specific instruction.
- **Keys Help**
  This function describes how to use the keyboard for specific tasks.
- **Help index**
  This function contains a complete list of all instructions you can get help for.

5.2.4 Method and Result File Contents window

The window structure of the Method File Contents and the Result File Contents is similar. The title bar shows the name of the open file. Data such as Chromatogram and Separation Method relating to the file are presented as cards stacked one behind the other.

In the Method File Contents (Fig. 5.4), a Separation Method card and a Run/Control card are always present. If an Evaluation Procedure has been stored with the Separation Method, an Evaluation Procedure card may also be present.

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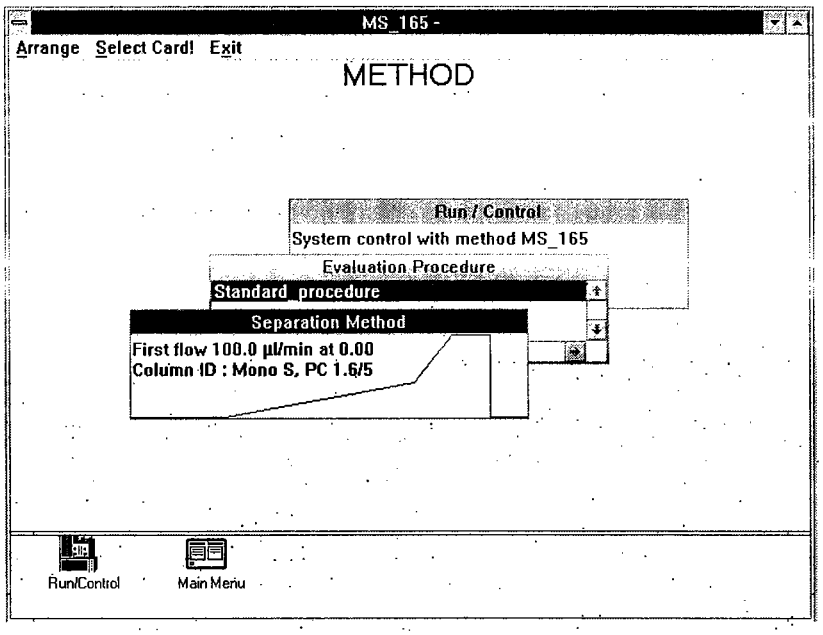


Fig. 5.4. Method File Contents window

A Result File is generated by running a Separation Method or making a manual run with the Record Chromatogram function activated. The Result File Contents window (Fig. 5.5) contains the Separation Method, the Chromatogram and the Run Log cards. After evaluating the Chromatogram and storing the results, the Peak Data with the results of the integration and the Evaluated chromatogram are displayed as cards in the Result File Contents.

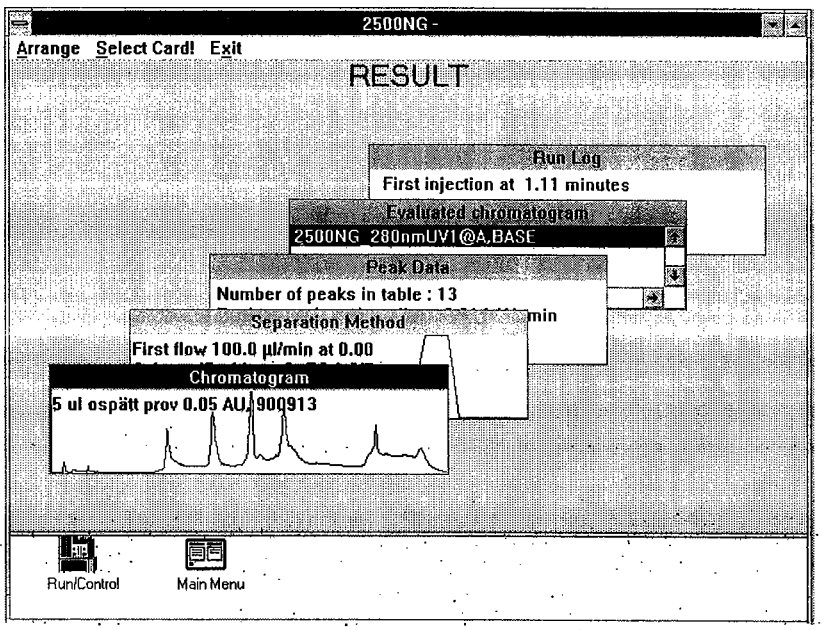


Fig. 5.5. Result File Contents window

Each card can be activated and brought into the foreground by clicking on it once. Brief information on the data held is then visible on the face of the card. The data card can be opened by double-clicking on it or alternatively by using the Select card instruction in the menu.

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5.2.5 File Contents pull-down menus

Arrange

- Tile
  The data cards displayed can be rearranged across the screen so that the information on all cards is visible simultaneously. A card can be opened by double-clicking on it or using the select card instruction.
- Cascade
  The cards are displayed as a stack one behind the other. A card can be activated by double-clicking on it or using the select card instruction.

Select card

This function allows one card to be selected. By clicking on the function, the cursor changes to a pointing finger which can then be used to indicate the card to be opened.

Exit

- **Exit file contents**
  This function exits the File Contents window and returns to the Main Menu.
- **Resume file contents**
  This function resumes the File Contents window.

5.3 Method Editor

5.3.1 Introduction

The chromatographic separation of the sample can be performed in SMART System according to a Separation Method created by the user. The Separation Method is the interface between SMART Manager and SMART System and it contains the following information:

- the chemistry of sample preparation and the eluent
- the configuration of the system required to run the Separation Method
- a single Main Method
- as many Sub Methods as required

The Method Editor is activated from the Method File Contents window by opening the Separation Method card.

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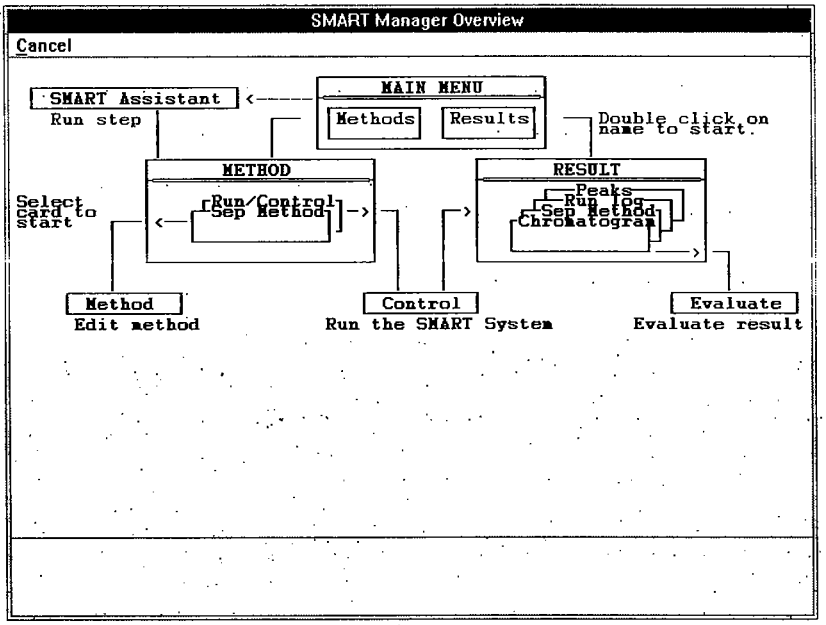


Fig. 5.6. SMART Manager overview

If a new Separation Method is being created, the Chemistry screen is displayed first, followed by the Configuration screen.

In the Chemistry window, information about sample preparation and eluents can be typed in.

The Configuration section contains specifications related to the Separation Method such as what column is used and what curves are being saved. It also contains specifications related to the instrumentation such as what monitor is used in the system.

If an existing Method is being edited, the Main Method showing the graphic editor window and the text editor window are displayed directly and the Chemistry and Configuration windows can be displayed using the View pull-down menu.

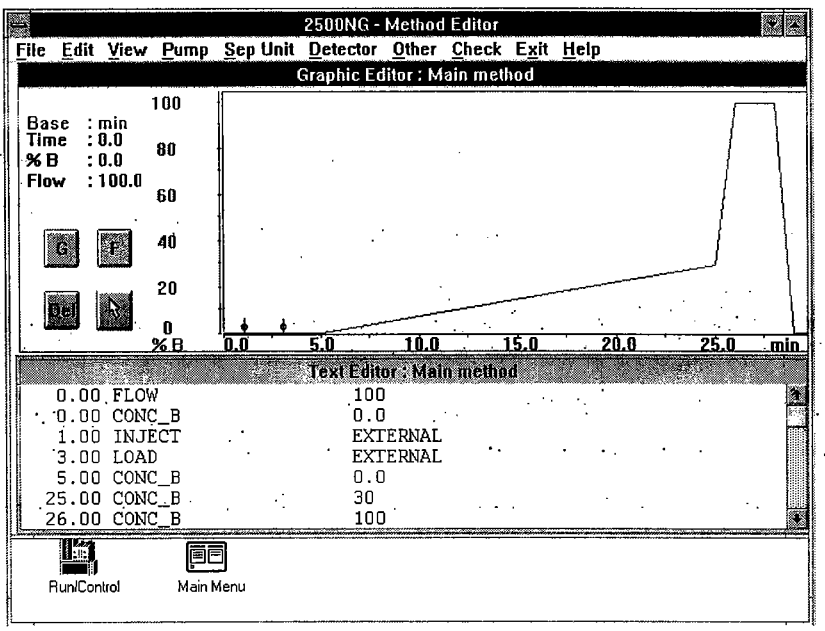


Fig. 5.7. Method Editor window

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The Method Editor is divided into two panels, a graphic editor (top) and a text editor (bottom). Instructions can be entered in three different ways:

- by using the graphic editor, for flow rate and gradient instructions only
- by using the instruction commands in the pull-down menu
- by typing in the instructions in the text editor panel

How to use the different alternatives to enter instructions is described in Section 4.4.3 - 4.4.6.

If the method is built up using the graphic editor window, corresponding written instructions appear directly in the text editor window. This list of instructions is constantly updated on a time or volume basis as further parameters are selected and added. This helps the new user to quickly become familiar with the format of the instructions which are used in the text editor window and which are accepted by the program. If the text editor window is updated or modified, the effect of the change can be visualised on the graphic editor window by using the menu instruction Check and Update Graphs one of the menu options.

5.3.2 Chemistry window

The Chemistry window is used to record the preparation procedures for the sample and the eluents which are used in the current Separation Method (Fig. 5.8). The headings relate to Sample preparation and preparation of Eluents A and B. The headings can be changed and new headings can be written by the user. Information typed in the Chemistry window is saved with the Separation Method and can be printed with the results.

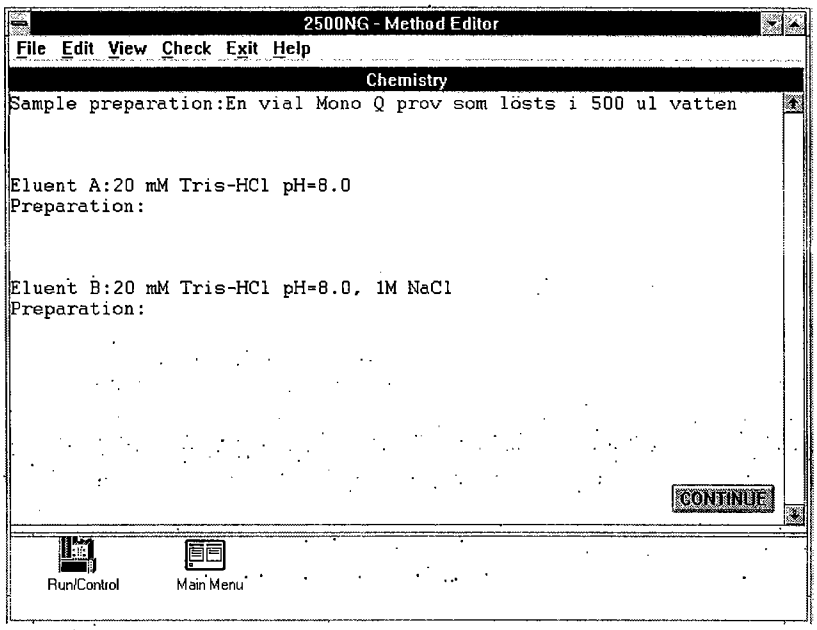


Fig. 5.8. Chemistry window

The Chemistry window is accessed directly when a new Separation Method is being created. To access this window when an existing Separation Method is being edited, click on VIEW, CHEMISTRY.

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To exit from the Chemistry window, either use the command button marked CONTINUE or click on VIEW, followed by the desired choice.

5.3.3 Configuration

The Separation Method Configuration is the specification of the components within SMART System which are required to run the current method. In addition to the equipment and functional parts required, it also includes basic information on the Separation Method.

The Method Configuration takes part in two checking procedures. The Separation Method is checked against the Method Configuration and if they are incompatible, the user is warned that the method is not correct. The Method Configuration is also automatically compared with the actual configuration of the system before running the method, and if these are incompatible, the run will not start and the screen will display an error message. The monitor and pump cylinder selection must be correct to run a method. Wrong column configuration gives only a warning.

The Configuration window is accessed from the Chemistry window by clicking on the CONTINUE command button. Alternatively, it can be accessed by clicking on VIEW, followed by CONFIGURATION. The Configuration window displays two groups of instructions, Method and Instruments (Fig. 5.9).

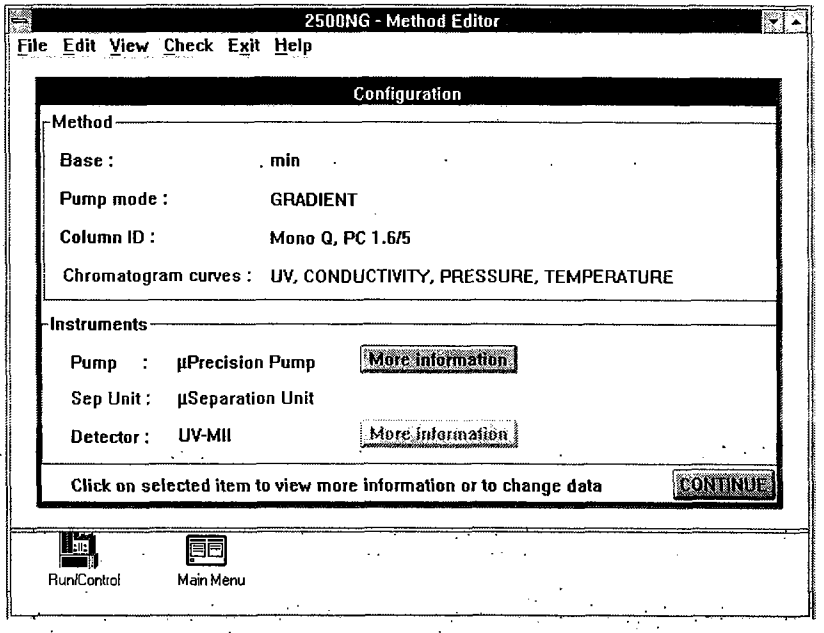


Fig. 5.9. Configuration window

Method

By clicking on the individual selection (min, GRADIENT, etc.), a dialogue box is displayed showing the options available.

- Base
  The instructions in the Separation Method can be programmed in either time (min) or volume (ml) base.

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- **Pump mode**
  The pump can either run in Gradient or Continuous mode. In Gradient mode both pump cylinders are pumping simultaneously to produce the programmed gradient. In Continuous mode the two cylinders alternate to deliver the same eluent for isocratic separations.
- **Column ID**
  This function defines the column type for the Separation Method. A range of columns is displayed in a list box. The default is Any column. The column selected defines the maximum system pressure, a pre-programmed value for the columns listed in the dialogue box. If µPeak Monitor is selected the column ID also defines the averaging time for the Monitor signal.
- **Chromatogram Curves**
  In this dialogue box the curves to be recorded stored in the Result File are selected. The UV curve is always recorded. All curves can be showed on the screen during run, but only the ones selected here will be recorded.

Instruments

Of the instruments listed in the Instrument field the detector is the only one that can be changed, by clicking on the monitor name.

Clicking on the "MORE INFORMATION" button makes further options available.

µPrecision Pump Information

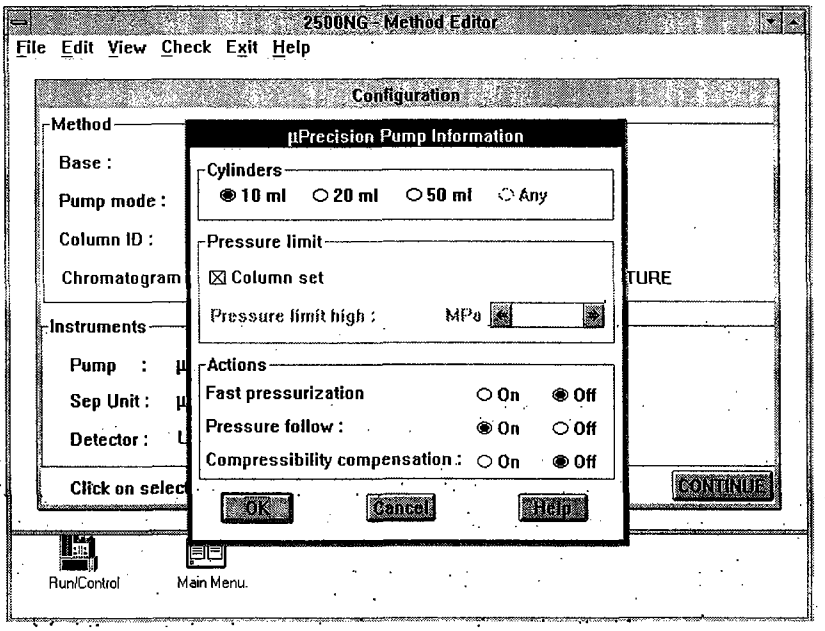


Fig. 5.10. µPrecision Pump information window

Cylinders

The type of pump cylinder used for the Separation Method is specified. Available cylinder sizes are 10 ml, 20 ml and 50 ml.

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Pressure limit

For the security of the system, the maximum system pressure limit for the run is specified. If this limit is reached during the run, SMART System will pause automatically. The desired maximum pressure limit can be entered or, by clicking on the COLUMN SET function, the system uses the maximum pressure limit for the column specified under Column ID.

Note: It is important to remember that the operating temperature of the system has an effect on system pressure since liquids become more viscous at lower temperatures and cause an increase in the system pressure.

Actions

See also Section 2.7.

- **Fast Pressurisation**
  If this function is activated, the column is rapidly pressurised to a stable target pressure. This shortens the time for the system to reach the final run pressure. The target pressure calculated by the program takes in account the type of column and the selected flow rate.
- **Pressure Follow**
  This function maintains the system pressure in the non working cylinder, when only one cylinder is pumping. This ensures that the gradient composition is accurate at the start of the gradient.
- **Compressibility Compensation**
  The compressibility of an eluent is always measured after pump wash, by comparing the resulting compressibility curve to a known standard curve. When the Compressibility Compensation is activated this measurement helps to maintain an accurate flow rate during gradient formation under conditions when system pressure varies during the run, such as reversed phase gradients with viscosity changes.

µSeparation Unit

No further information is required.

Detector

To select either µPeak Monitor or Monitor UV-M II click on the monitor name after Detector. A dialogue box with the monitor choice will appear. When µPeak Monitor is selected the MORE INFORMATION button will become active.

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µPeak Monitor Information

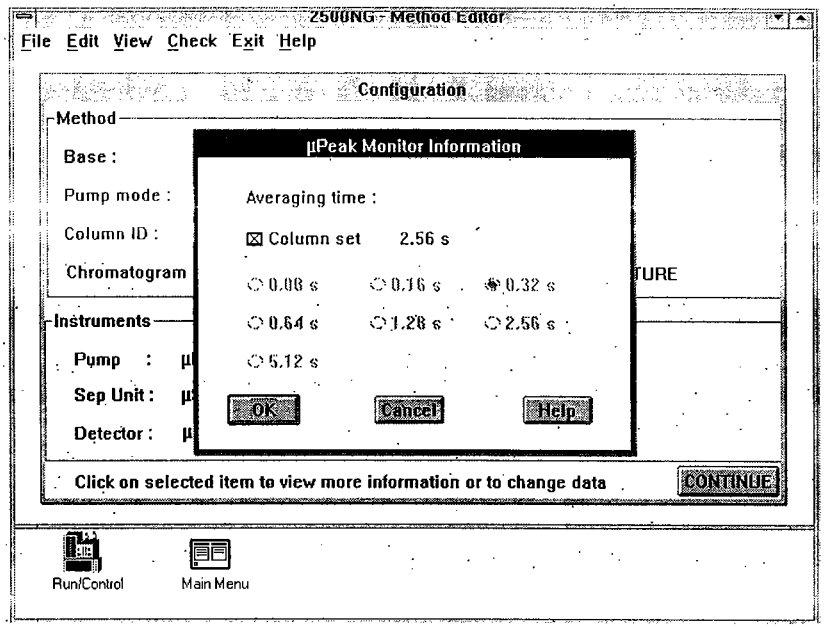


Fig. 5.11. µPeak Monitor information window

In this window an averaging time for the µPeak Monitor can be set. The averaging time is used for filtering the digital signal from the monitor. The averaging time is the time interval used for calculating the moving average for the absorbance signal.

A long averaging time will smooth out noise more efficiently, but also distort the peaks. Because of this, the averaging time should be as short as possible.

For detectors with analogue signals a Time constant is more commonly used for filtering noise. The following table contains approximate values for time constants corresponding to certain averaging times, as well as the minimum peak width at half height for which this averaging time should be used.

Averaging time (sec)	Time constant (sec)	Min peak width (sec)
5.12	2,0	32
2.56	1.0	16
1.28	0.5	8
0.64	0.2	3.2
0.32	0.1	1.6
0:16	0.05	0.8
0.08	0.03	0.5

Normally it is recommended to use the value selected by SMART Manager which is related to the type of column installed.

5.3.4 Main Method

The Main Method screen has two windows, the graphic and the text editor window (Fig. 5.12). It is accessed from Configuration by clicking on the CONTINUE command button or by clicking on VIEW, MAIN METHOD or by selecting a method in the Main Menu.

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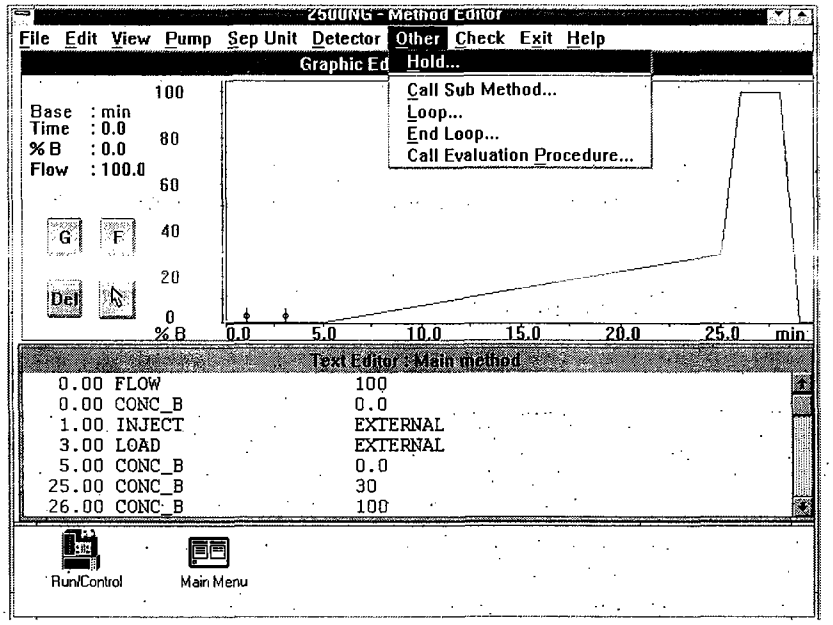


Fig. 5.12. Main Method window

The graphic editor window gives a graphic display of the current main Separation Method or Sub Method. If a saved method is being edited, the profile of the separation conditions is shown with the base, either time or volume, indicated on the x-axis.

The name of the current Separation Method is shown at the top of the window. In the upper left margin, the base (min or ml) and the current values for time or volume, % B and flow rate are displayed. By clicking on the y-axis, the user can switch between the y-axis scales indicating the flow rate in µl/min and the concentration in % B.

Using the graphic editor window, a Separation Method is created using the pull-down menus only or using the command buttons in conjunction with the pull-down menus. The command buttons are marked G for Gradient and F for Flow. By clicking on G, for example, the gradient function is activated and a gradient breakpoint can be entered. This function remains active until "arrow "button is pressed. The F button for flow rate operates in a similar way. A breakpoint can be erased by clicking on the Del button. Instructions for the injection valve, fraction collector etc are programmed using the pull-down menus.

The time or volume of the event can be selected either by typing it in or by using the vertical drag bar. This is activated by clicking on the black line near the y-axis, and then pulling it to the time or volume of the event. Using the drag bar, the smallest steps which can be entered are 0.5 minutes or 0.1 millilitres. A more detailed description of how to use these functions when creating a Separation Method is given in Section 4.4.4.

Following a change in the graphic editor window, the text editor window is automatically updated. Following a change in the text editor window, the graphic editor window is updated by accessing the pull-down menu, CHECK, UPDATE GRAPHS.

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5.3.5 Method Editor pull-down menus

File

The File pull-down menus (Fig. 5.13) are used to organise the current Separation Method file.

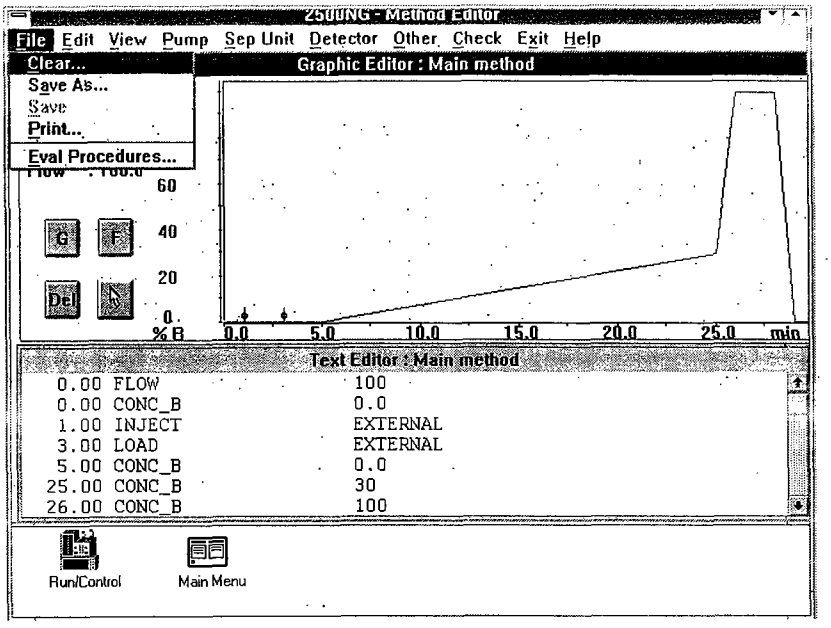


Fig. 5.13. Pull-down menu File

- **Clear**
  Specified parts of the current Separation Method can be cleared. This can be either the Main Method together with any Sub Methods or the Chemistry. No confirmation is required before the request is executed.
- **Save As**
  The current Method can be saved under another name. This function allows an existing Method to be edited and then stored under a new file name. The Method can be renamed using this function. The name can have a maximum of eight characters. The program checks and requests another name if there is already a file saved under the same name.
- **Save**
  The current method can be saved under the current file name. The previous file is overwritten and there is no request for confirmation before the request is executed.
- **Print**
  The current method can be placed in the printer spooler queue. The user must enter the information which is to be printed. This can be Chemistry; Configuration, Main Method and/or Sub Method.
- **Evaluation Procedure**
  The evaluation procedures created in the Evaluation part of SMART Manager and saved in the method file are listed in the dialogue box. Evaluation procedures can be renamed or deleted.

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To rename an Evaluation Procedure, highlight its name in the list box, then click on the Rename Eval Proc command button. Enter the new name in the dialogue box and click on OK.

To delete an Evaluation Procedure, highlight its name in the list box, then click on the Delete Eval Proc command button. In the dialogue box displayed you can confirm the deletion or cancel.

Edit

The Edit pull-down menus help to edit the Separation Method when using the text editor window.

- **Copy**
  The selected window is copied into the clipboard and can be used in other software programs.

View

The View pull-down menu (Fig. 5.14) is used to select the part of the current Separation Method which is to be displayed. When a new method is being created, the display defaults to the Chemistry window. When an existing Separation Method is being edited, the display defaults to the Main Method. Only one of the four options can be displayed at any one time.

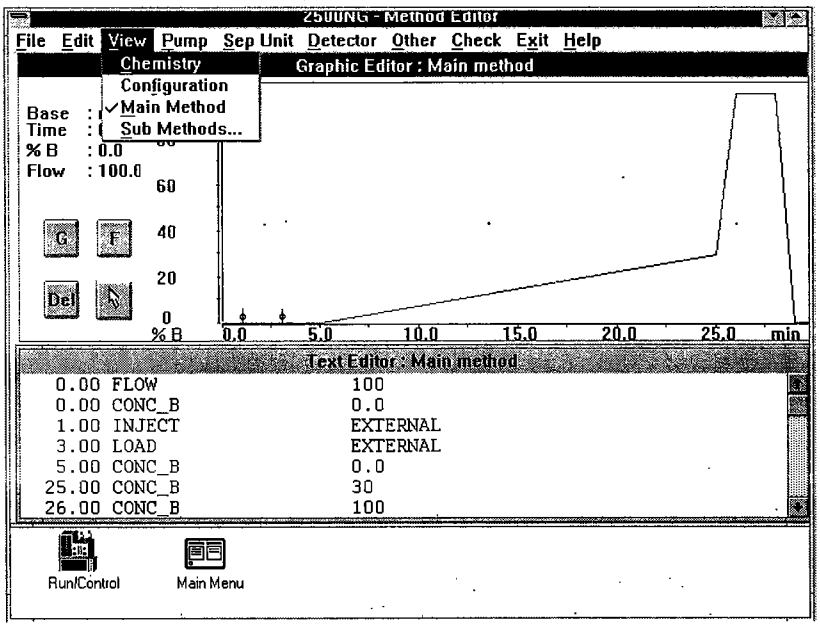


Fig. 5.14. Pull-down menu View

- **Chemistry**
  The Chemistry text editor window which shows the description of the sample preparation and the eluents as entered by the user is displayed.
- **Configuration**
  The window showing the Configuration of the system for the current method is displayed.
- **Main Method**
  The Main Method is displayed showing the graphic and text editor windows.

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- **Sub Method**
  This function is used to create a new sub method or to edit an existing Sub Method. Sub Methods can also be renamed or deleted.

  To create a new Sub Method, click on the New Sub Method command button, enter a name with a maximum of eight characters and click OK. Click OK in the Sub Methods dialogue box and program the new Sub Method in the Method Editor window. To save the Sub Method together with the Main Method use FILE, SAVE.

  To rename a Sub Method, highlight the name in the list box and click on the Rename Sub Method command button. Enter the new name in the dialogue box and click on OK.

  To delete a Sub Method, highlight the name in the list box and click on the Delete Sub Method command button. Confirm it by clicking on OK.

Pump

The Pump pull-down menu is used to select the pump parameters for the run (Fig. 5.15).

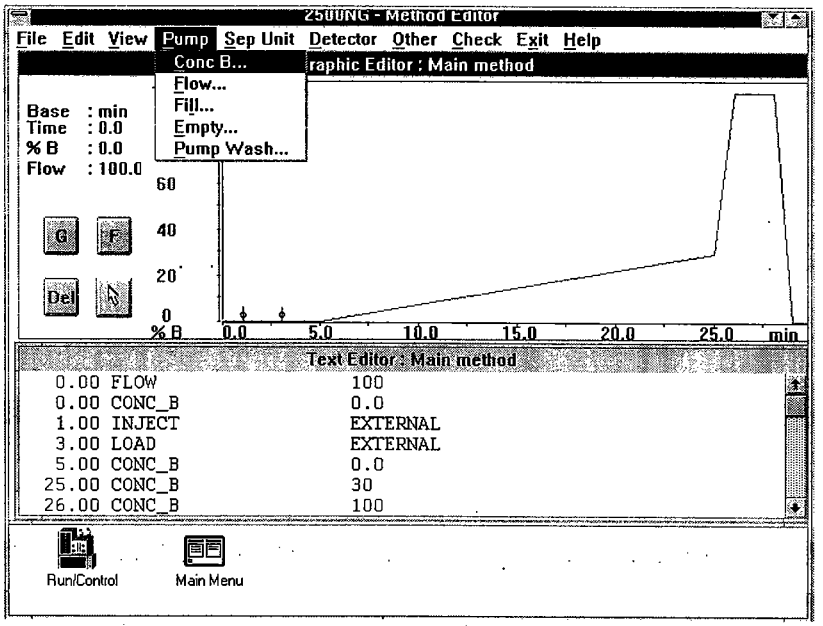


Fig. 5.15. Pull-down menu Pump

- **Conc B**
  This function allows the composition of the gradient to be selected for the current time or volume in the Separation Method. The window displays the breakpoint time or volume and the concentration of eluent B in %.

  Note: The Conc B parameter is only available when gradient mode is selected in Method Configuration.

- **Flow**
  This function allows the selection of the flow rate at specified time or volume in the Separation Method. The time or volume is displayed together with the flow rate. The valid flow rate

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range of 0 - 2 000 µl/min is also displayed. The user can enter a new flow rate and time or volume.

- **Fill**
  This function programs the filling of pump cylinders A and/or B at the specified time or volume. For the selection of the eluent, the inlet valve position is defined as positions 1, 2, 3 or 4. The maximum volume of the cylinder is displayed and the volume to be filled is entered. The filling flow rate can be normal, 15 000 µl/min or low, 5 000 µl/min. Low is used with viscous liquids. The system is paused while the cylinders are being filled.

- **Empty**
  This function programs the emptying of pump cylinders A and/or B at the specified time or volume. The outlet position for the emptying is defined as either positions 1, 2, 3 or 4. The volume to be emptied can be selected. The flow rate for emptying can be normal, 15 000 µl/min (10 ml cylinders), 30 000 µl/min (20 ml cylinders), 75 000 µl/min (50 ml cylinders) or low, 5 000 µl/min (10 ml cylinders), 10 000 µl/min (20 ml cylinders), 25 000 µl/min (50 ml cylinders). Low is used when the eluents are viscous. The system is paused while the pump is emptied.

- **Pump Wash**
  This function programs the washing of pump cylinders A and/or B at a defined time or volume. Both the inlet and the outlet for the wash liquid must be specified for both pump cylinders. This function is used when the eluent is to be changed. The system is paused while the pump is being washed.

  Following the wash, the pump performs an automatic test on the compressibility of the liquid in the cylinder and this information is retained by the program and used if Compressibility Compensation is selected in Method Configuration.

Sep Unit

This pull-down menu (Fig. 5.16) is used to select the operational parameters for the µSeparation Unit. It includes instructions for sample injection, fraction collection and mixer washing.

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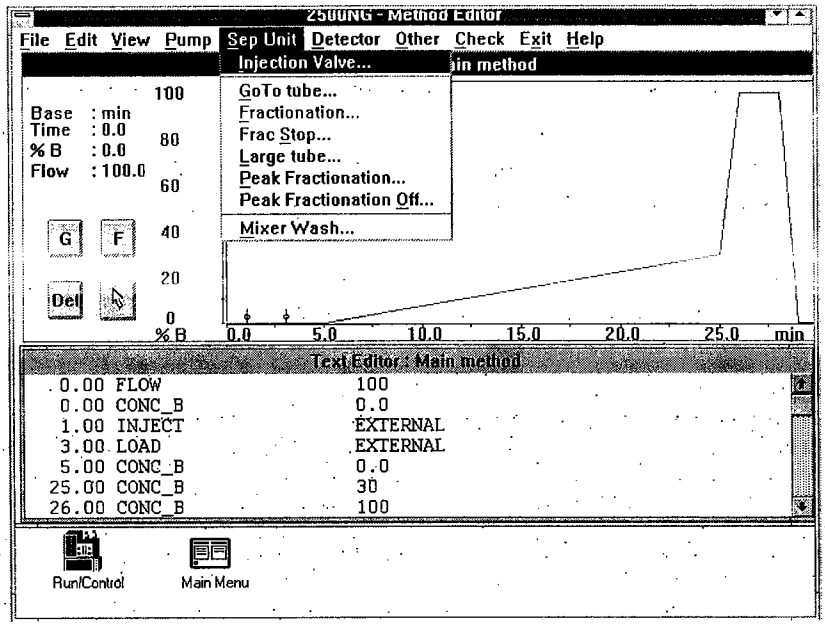


Fig. 5.16. Pull-down menu Sep Unit

- **Injection Valve**
  The injection valve can be switched to the load, inject or waste position at the time or volume defined.

  For SMART Systems with an injection valve with an internal sample loop the load and inject instructions must be typed in the Text Editor window.

  To set the valve in load or inject position for the internal loop, type:

  0.00 LOAD INTERNAL or

  0.00 INJECT INTERNAL

- **GoTo Tube**
  This function positions the fraction collector at the selected collection tube at the defined time/volume. SMART Manager allows for the delay volume between UV monitor and fraction collector before making the change.

- **Fractionation**
  This function sets the parameters to control fraction collection at the time or volume defined. For all programmed instructions, the delay volume is taken into account. The following instructions can be programmed:

  a) Fraction size
  This selects the required size of the fraction to be collected at the specified time or volume.

  b) Start at tube
  This function sets the tube number for the start of fraction collection. This function should not be used if the GoTo Tube function has already been used.

  c) Needle position
  This sets the position of the fraction collector delivery arm to Up or Down. With the arm in the Up position the fractionation capillary will be just above the tube.

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Complete drops can be collected. With the arm in the Down position the fractionation capillary touches the bottom of the tube, and a smaller quantity, or part of a drop, can be collected. Fractions below 250 µl should be collected with the arm in the Down position.

- **Frac Stop**
  This instruction programs the end of fraction collection at the specified time or volume. The fraction collector arm moves to the external waste position after taking account of the delay volume.
- **Large Tube**
  This function position the fraction collector at the larger tubes, A,B,C or Z , for Tube Holder 48/8, at the defined time or volume. This function is used for the collection of large volume fractions or for waste collection. The delay volume is allowed for.
- **Peak Fractionation**
  The Peak Fraction instruction can be used either to collect peaks in single tubes or to divide peaks into smaller fractions. Fractions between peaks will go to waste. To collect the fractions between peaks the Fractionation function must be programmed. In the Fractionation instruction the needle Up or Down can also be selected. The program takes account of the delay volume.

  For a more detailed description of using the Peak Fractionation function see Section 4.7.

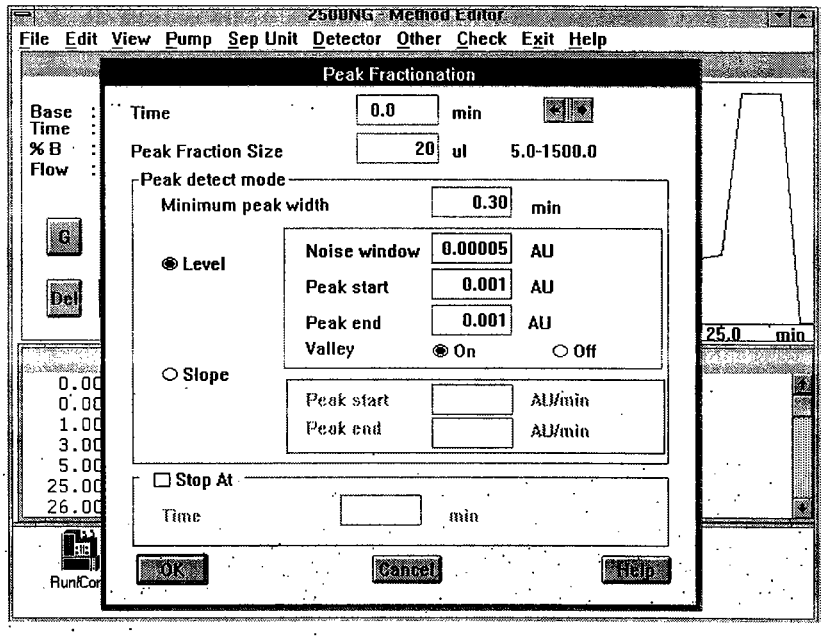


Fig. 5.17. Peak Fractionation window

a) Peak fraction size
This function defines the size of the fraction to be collected within the peak in µl. If the object is to collect many small fractions over a peak, the peak fraction size should be smaller than the narrowest peak. If the object is to collect peaks in a single tube, the peak fraction size should be larger than the widest peak.

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b) Minimum peak width
For collection with peaks identified by level or slope the Minimum Peak Width is the minimum distance between peak start and peak end. The programmed value should be smaller than the narrowest peak.

c) Level/Noise window
The programmed Noise window will protect from accidental peak start and end detection depending on noise.

d) Level/Peak start
A peak start is detected when the absorbance is above the programmed peak start level. For the program to recognise a peak start the absorbance level must also be above the lowest absorbance value since last peak, plus twice the noise window.

e) Level/Peak end
A peak end is detected when the absorbance is below the programmed peak end level. For the program to recognise peak end the absorbance level must also be below the highest absorbance value in the peak minus, twice the noise window.

f) Level/Valley
When the Valley function is On, absorbance valleys can be detected above the Peak Start level. The fraction collector will move to a new tube. The criteria for identification of a valley is, that the absorbance level is 2 % below the detected maximum absorbance level minus the noise window, and that the absorbance increases 2 % plus the noise window.

g) Slope/Peak start
A peak start is detected if the slope of the absorbance is higher than the programmed value for more than 1/10 of the programmed minimum peak width.

h) Slope/Peak end
A peak end is detected when the slope of the absorbance is steeper than -1.2 times the programmed value and flatter than the programmed value for more than 1/10 of the minimun peak width.

e) Stop at
If activated, this field defines the end of fraction collection by peak detection. This will not effect ordinary fraction collection if active. If a Stop at instruction is programmed, there is no need to use the Peak Fractionation Off instruction.

- **Peak Fractionation Off**
Peak fractionation is stopped at the time or volume specified.

- **Mixer Wash**
This function sets the concentration of the gradient in the mixer at a specified time or volume. The function can be used at the start of a gradient run so that all tubings up to the mixer and the mixer itself are filled with eluent at the composition required. It is not necessary to use this function when

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consecutive runs are using the same composition of eluent. The system will be paused while the mixer is being washed.

Detector

This pull-down menu (Fig. 5.18) is mainly used to select the instructions for the µPeak Monitor. The instruction New Chromatogram is used for both monitors.

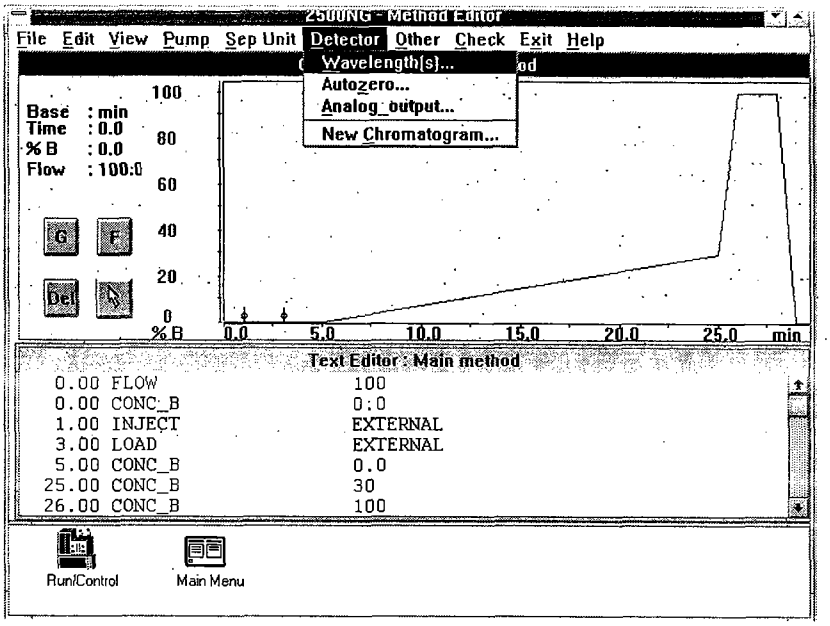


Fig. 5.18. Pull-down menu Detector

- **Wavelength(s)**
  This function is used to set the wavelength(s), 190-600 nm, for µPeak Monitor. One, two or three wavelengths can be monitored simultaneously. For highest sensitivity, a single wavelength should be used.

  Some eluents have a rapidly increasing absorbance at lower wavelengths, e.g. methanol at 215 nm will change with 30 mAU per nm, which causes a higher noise level detecting at dual or triple wavelength. During detection with dual or triple wavelength the rapid change in absorbance demands an exact positioning of the grating when switching between the different wavelength positions. With the design in µPeak Monitor the stepper motor can reposition the grating within 0.75 µm which means a wavelength reproducibility better than 0.05 nm. In case of the methanol at 215 nm this means a noise less than 1.5 mAU.

  Detection at the selected wavelength(s) will start at the time/volume specified. When a wavelength change takes place in a separation method, a New Chromatogram command will be executed, so data from before and after the change will be stored in separate chromatograms but in the same result file.

  The primary wavelength is the wavelength used for peak fractionation and may also be sent to the analogue recorder output for analogue recording. This wavelength is included in the 'Regular Curves' diagram in the evaluation report. Curves

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for all selected wavelengths can be printed afterwards in the evaluation report using the 'Free Choice Curves' option.

The curves will automatically be given names consisting of the eight last letters of the result file name, followed by an underscore, the wavelength, the current chromatogram number and a letter, A, B, or C, designating whether the chromatogram is from the primary, second or third wavelength.

Example: MonoQ12_254nm1A for primary wavelength

MonoQ12_254nm1B for second wavelength

MonoQ12_254nm1C for third wavelength

If no wavelength instruction is programmed in the method, the monitor will use the previously set wavelength(s).

- **Autozero**
  This function is used to set the absorbance of the wavelength(s) to a reference zero baseline at a specified time/volume.

- **Analogue output**
  This function is used to set the full scale absorbance range for the analogue recorder output of the primary wavelength. If the primary wavelength is changed during the run, a downward mark will be made on the recorder output.

  The Zero absorbance level can be set to a percentage of full scale, to make it possible to register the signal even if the signal drifts below zero.

  To handle unexpectedly large signals, the signal to the recorder is inverted (mirrored) when it reaches the upper limit of the scale. Levels below the lower limit are truncated.

- **New chromatogram**
  This function instructs the program to store the current results as a new chromatogram within the current Result File. A new subfile is opened for data storage.

  The data is stored with a sampling frequency of 5 Hz up to 4000 data points (approximately 13 minutes). When the maximum data points have been reached the program erases every other data point and the sampling continues at 2.5 Hz. For very long runs when high data resolution is required, it is recommended to use the New chromatogram instruction in the method.

Other

These pull-down menus include the settings of various additional instructions for the control of the chromatographic run (Fig. 5.19):

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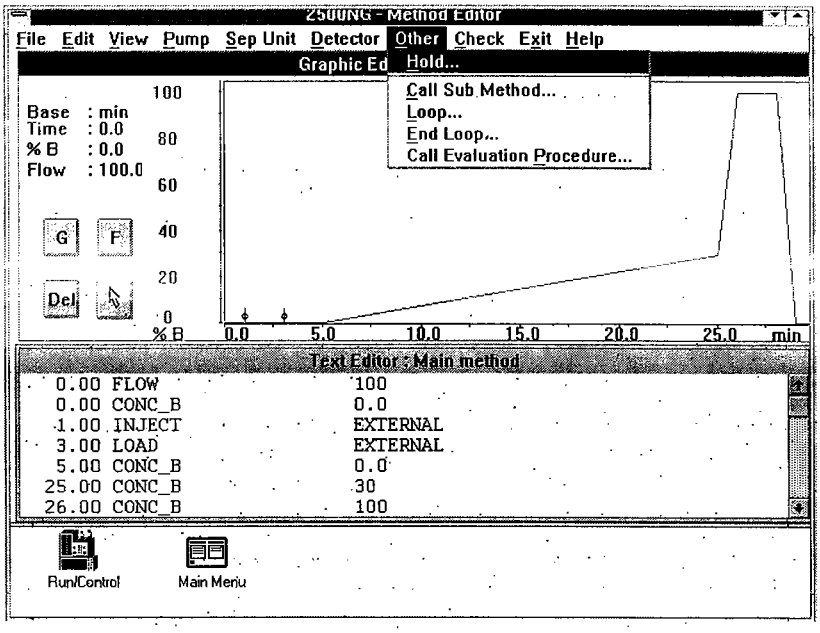


Fig. 5.19. Pull-down menu Other

- **Hold**
  This function sets the system in HOLD. The pumps continue to deliver eluent at the current flow rate and concentration but the base (time or volume) is not updated until the run is continued. A hold is cancelled by the user manually activating CONT! in Run/Control.

- **Call Sub Method**
  This function allows a Sub Method to be called and inserted in the Separation Method at the current time or volume. The user selects the desired Sub Method from a list of available Sub Methods.

- **Loop**
  This function programs the start of a loop in the Separation Method at the current time or volume. From 1 to 99 loops can be selected. A single Separation Method can contain up to 6 Loop and End loop instructions. Loops within a loop should be avoided.

- **End Loop**
  This function programs the end of the loop at the current time or volume in the Separation Method.

- **Call Evaluation Procedure**
  This function allows an Evaluation Procedure to be called at the current time or volume. The user selects the desired Procedure from the displayed list of names. Data recorded after the Call Evaluation Procedure instruction will be stored in a new chromatogram, but in the same result file.

Check

Check allows the system to perform an automatic check on the created Separation Method to ensure that it contains no instructions which are invalid and cannot be accepted by SMART System (Fig. 5.20).

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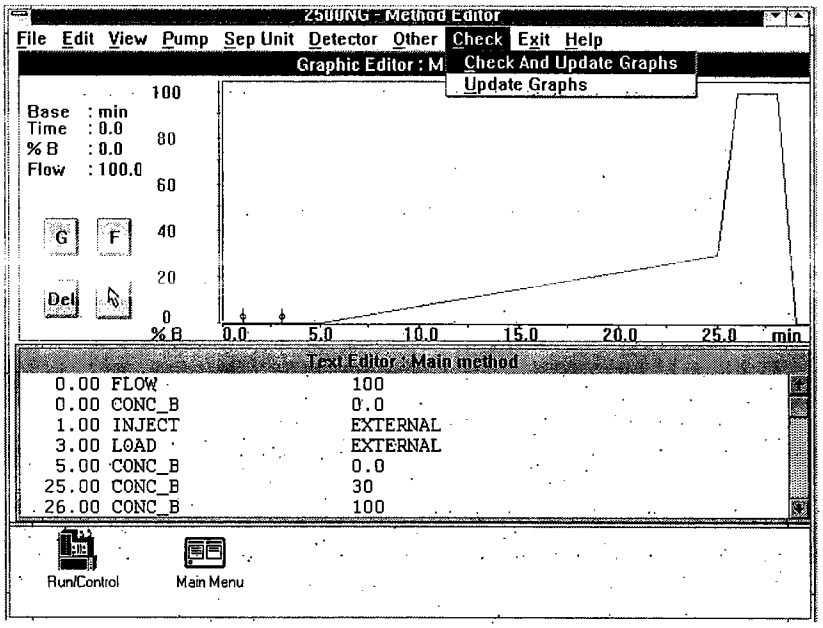


Fig. 5.20. Pull-down menu Check

- **Check And Update Graphs**
  This function checks the Separation Method to ensure that all instructions are valid and can be understood by the system. A check is also made to ensure that the Method Configuration and the Separation Method are compatible. The result of this test is displayed in a separate window. If any part of the Separation Method is found to be incorrect, the graphic window is cleared and an error message is given. In addition, the user is advised of the volume of eluents A and B required for the run. The graphic editor window is updated automatically when the method is correct.

- **Update Graphs**
  This function updates the graphic editor window to correspond to the text editor window.

Exit

This function allows work with the Separation Method to be terminated with or without saving the method (Fig. 5.21).

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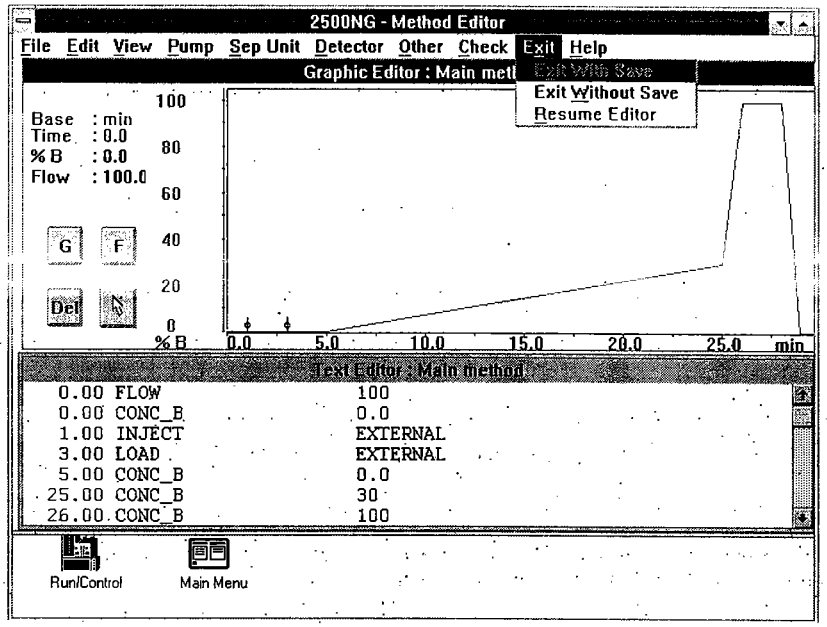


Fig. 5.21. Pull-down menu Exit

- **Exit With Save**
  This function exits the Method Editor and returns to the File Contents window. The Separation Method is saved under the current file name. If the Method Editor has been entered from a Result File this instruction will be inactive. To save an edited method in a Result File, use the File, Save As function.
- **Exit Without Save**
  This function exits the Method Editor and returns to the File Contents window without saving the Separation Method.
- **Resume Editor**
  Work with the Separation Method window can be resumed.

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Help

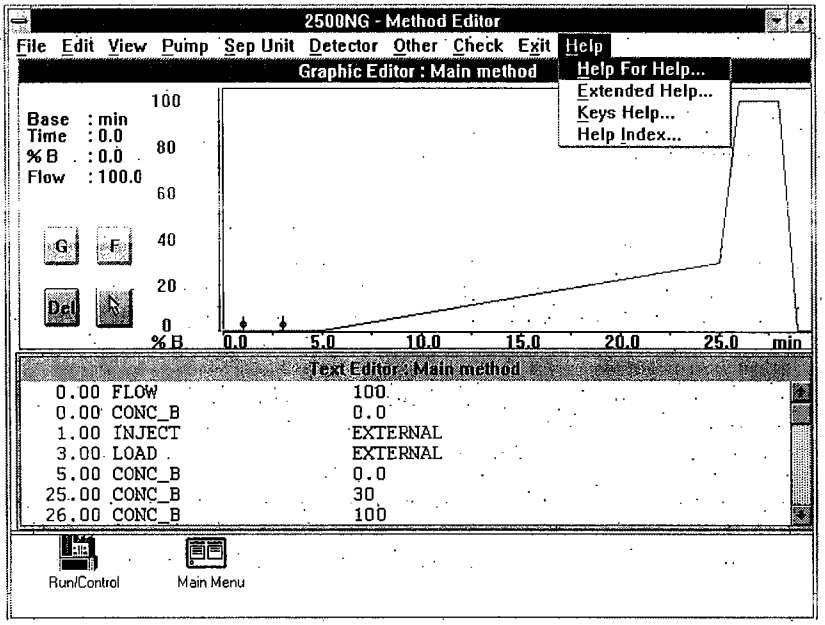


Fig. 5.22. Pull-down menu Help

- Help for Help
  This function describes how to use the Help command.
- Extended Help
  This function provides general information about the Method Editor. More detailed information is given for the Chemistry, Configuration and Main Method window when selected in the dialogue box.
- Keys Help
  This function describes how to use the keyboard for specific tasks in the Method Editor.
- Help index
  This function contains a complete list of all instructions in the Method Editor you can obtain help for.

5.4. Run/Control

5.4.1 Run/Control introduction

Run/Control provides the environment for starting a run using a specified Separation Method or for manual control of the system. In addition, a pause or a hold in the run can be made and the run can be restarted or terminated completely. Manual changes to the current system parameters can also be made.

The Run/Control window gives a graphic presentation of the progress of the separation. The display can be configured to show selected curves resulting from the separation. For example, the chromatogram and the conductivity signal can be displayed. It is a graphic environment with easy to use functions and commands to control the run and make changes to the Separation Method. A log of the events which occur during the run is recorded in the Result File.

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5.4.2 Run/Control window

The Run/Control window is displayed by double-clicking on the Run/Control card in the Method File Contents. Alternatively, for manual runs, the Run/Control window can be activated by double-clicking on the Run/Control icon.

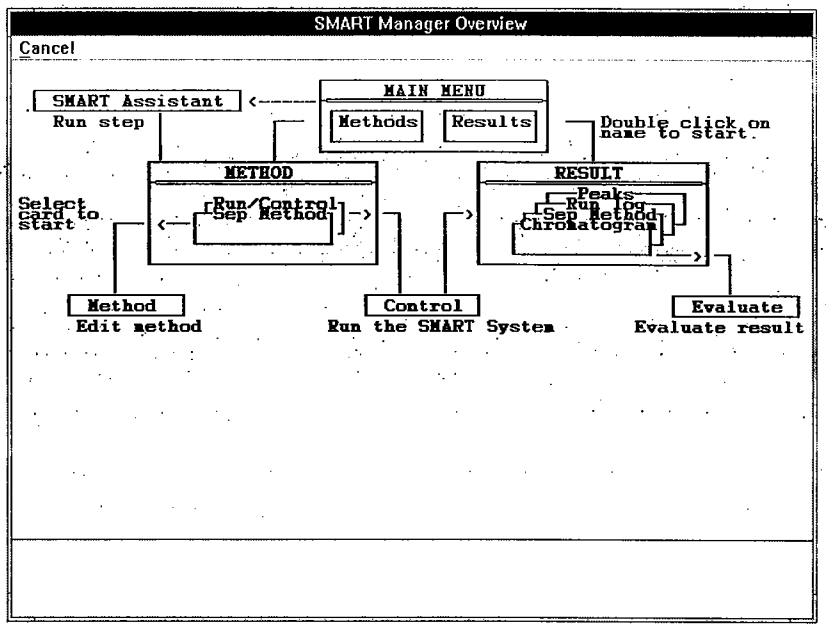


Fig. 5.23. SMART Manager overview

The curve window in the Run/Control window displays the progress and results of the run. The x-axis is scaled according to the elapsed time or volume of the current Separation Method. The scale on the y-axis refers to the result curves, such as the UV absorbance, the conductivity signal or the system pressure. During a run, the y-axis units can be changed by clicking to the left of the y-axis. The range of the y-axis is as default automatically changed according to the incoming data unless fixed scaling is selected in Curve window mode. Events relating to fraction collection and sample injection may also be displayed graphically.

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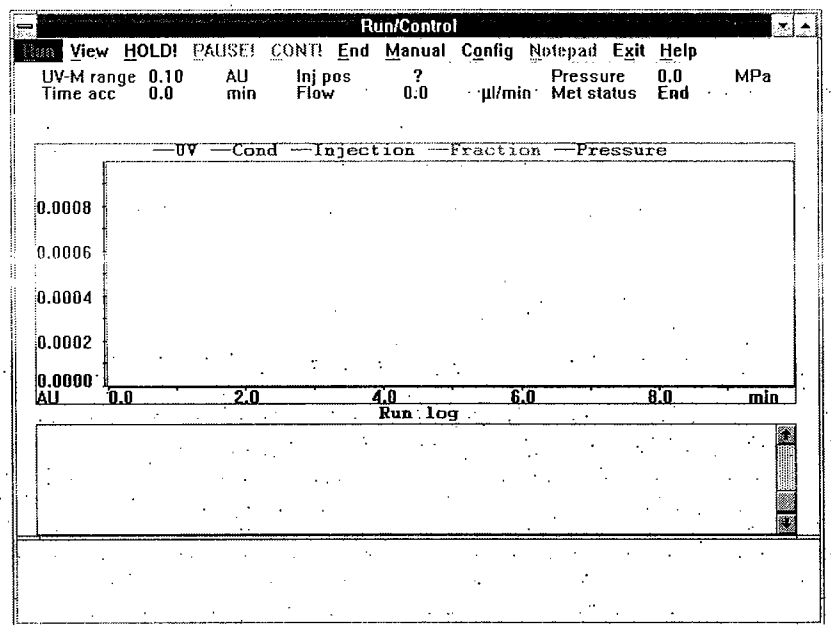


Fig. 5.24. Run/Control window

In the run data area above the graphic display, the current values for up to 12 parameters, such as method status, accumulated time or volume, UV absorbance, fraction tube, injection valve position and flow rate can be displayed.

The lower area of the screen contains the Run Log where all events, system warnings and errors are recorded. Both the events programmed in the Separation Method and the changes made by the operator during the run are recorded. During the run, the operator can access Notepad and add further comments as required.

5.4.3 Run/Control pull-down menus

Run

Run is used to start a run according to the current Separation Method. Before starting the run, the user is asked to specify the name of the Result File where the results of the run are to be stored. The six first letters from the current Method File name followed by two figures is shown as default. The user can change to a new name with a maximum of 8 characters. The user can also enter any comments on the run.

Before the run starts, the system compares the actual current configuration of the system with the configuration specified in the Separation Method. If these are incompatible, the run cannot start and a "Configuration warning" is given. Either the Method Configuration or the system must be changed before the run can start.

When the run ends, SMART System is automatically reset to a defined state.

View

The data to be displayed graphically and numerically during the run are selected (Fig. 5.25).

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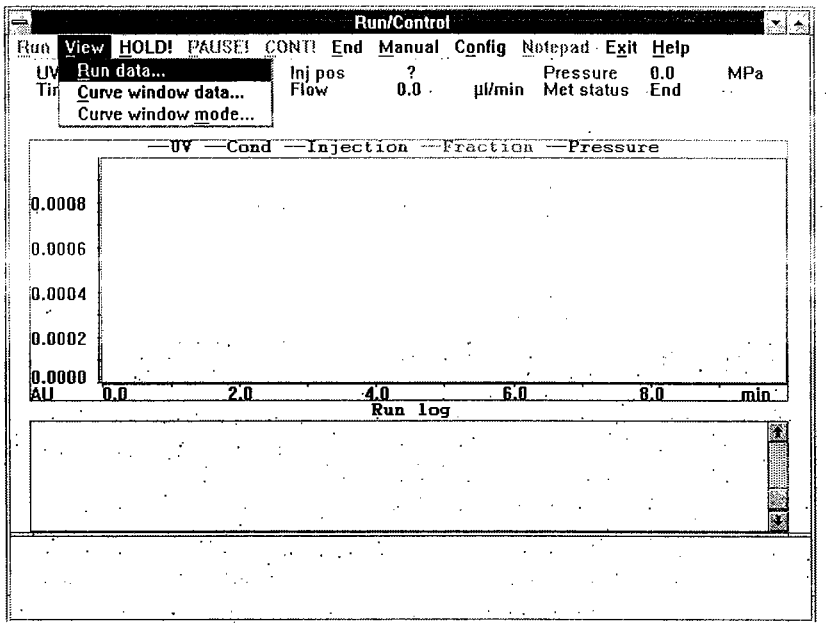


Fig. 5.25. Pull-down menu View

- **Run data**
  This function allows the selection of the numerical data for display above the graphic display. The variables can be selected individually or in groups as follows:
  - Standard - is a preset selection of parameters
  - Pump ON - includes all pump parameters
  - Pump OFF - excludes all pump parameters
  - Sep ON - includes all parameters relating to the µSeparation Unit
  - Sep OFF - excludes all parameters relating to the µSeparation Unit
  - Detector ON/OFF - includes all parameters relating to the detector

The maximum number of parameters which can be displayed is 12. These are chosen from the following list:

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Parameter	Units	Default
Method status		on
Flow	µl/min	on
Accum. time/volume	min, ml	on
Pressure	MPa	on
Inj. valve position		on
UV-M II range	AU	on
Concentration B	% B	off
Conductivity	% B	off
Wavelength	nm	off
Wavelength 2	nm	off
Wavelength 3	nm	off
UV-absorbance	AU	off
UV-absorbance 2	AU	off
UV-absorbance 3	AU	off
Averaging time	s	off
Pump valve A position	stop,1, 2, 3, 4	off
Pump valve B position	stop, 1, 2, 3, 4	off
Eluent volume cyl. A	µl	off
Eluent volume cyl. B	µl	off
Fraction tube no		off
Temperature	° C	off
Fraction volume	µl	off
Pressure limit	MPa	off
Column holder		off
Record chromatogram	On/Off	off

- **Curve window data**
  This function allows the selection of curves and data for the graphic display. The parameters can be chosen from the following list:

Parameter	Units
Flow	µl/min
Concentration B programmed	% B
UV absorbance	AU
Conductivity	% B
Pressure	MPa
Temperature	° C
Fraction marks	Tube no
Injection marks	

  By clicking on an option button, the y-axis values for one curve can be selected for display as default. The y-values of all other curves selected can be displayed and toggled by clicking in the left margin of the Run/Control window.

- **Curve window mode**
  Scaling on the x- and y-axis can be selected for the window display:

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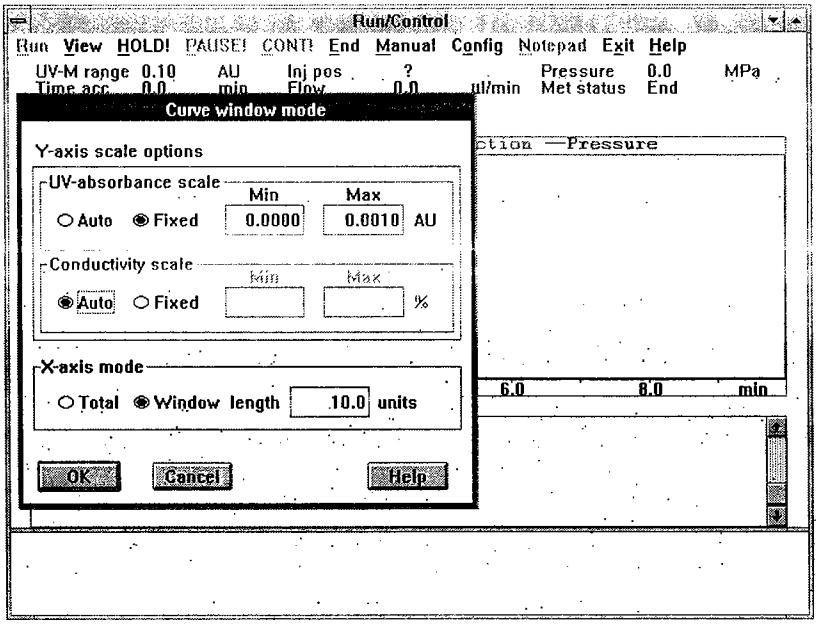


Fig. 5.26. Curve window mode

Y-axis scale options

UV Absorbance Scale can be auto when it is set according to the actual signals, or fixed, when the user enters the minimum and maximum absorbance values. If µPeak Monitor is used different scales can be selected for the different wavelengths. The Conductivity Scale can be similarly set.

X-axis mode

The x-axis can be either Total, where the curve is rescaled during the run so that the complete curve is always displayed, or Window, where the curve is not rescaled but is displayed in a moving window similar to a chart recorder. The current signal is always visible in scale. When Window is selected, the user also enters the length required.

HOLD

When hold is activated the pump continues isocratically at the flow rate and gradient composition of the current breakpoint. The chromatogram time/volume (accumulated) is updated but the method time is not updated. Hold is cancelled by clicking on CONT or END.

Note: Hold status can also be initiated by a programmed instruction in the Separation Method.

PAUSE

This function pauses the run. The pump stops (flow rate zero). Accumulated time and program time are not updated. Pause is cancelled by clicking on CONT or END. The PAUSE function is not available during manual operation. The µPeak Monitor lamp will not be switched off during PAUSE.

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CONT

This function continues a run which is in hold or paused at current time.

END

This function ends the run. The results of the interrupted run (partial run) can be saved when running a method and if Record Chromatogram is on in manual.

At the end of a run, SMART System is automatically reset to a defined state. The run cannot be continued.

Manual

This menu (Fig. 5.27) allows changes to be made to the values set in the current Separation Method or functions to be set manually. The change takes place and continues at the new value until the parameter is changed again either manually or by a further instruction in the Separation Method. The changes are valid only for the current run and do not modify the Separation Method in any way. All changes are recorded in the Run Log.

In addition to changing the running conditions, the column can be installed using the Manual option.

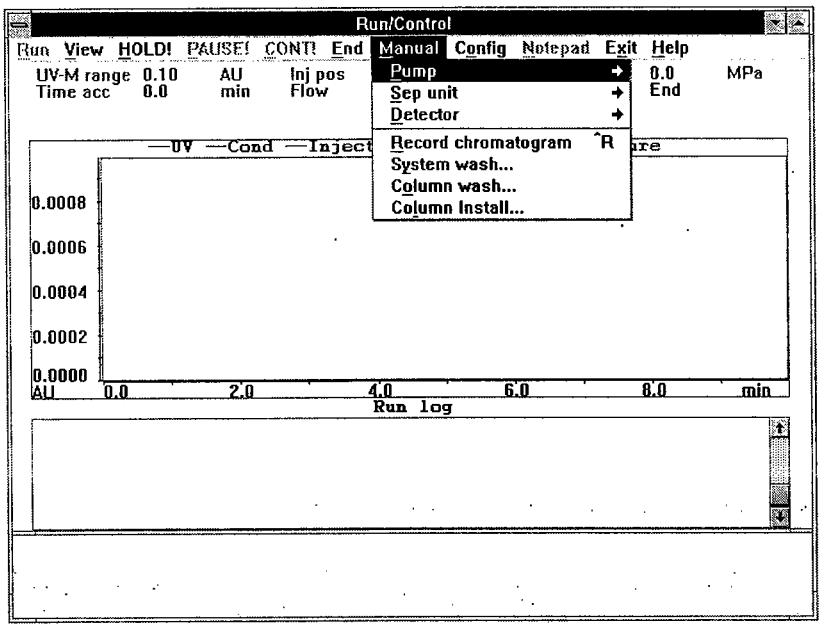


Fig. 5.27. Pull-down menu Manual

- **Pump**
  This option allows new values to be set for the pump functions. Conc B, Flow, Gradient, Wash, Fill, Empty and the Leakage test can be initiated (Fig. 5.28).

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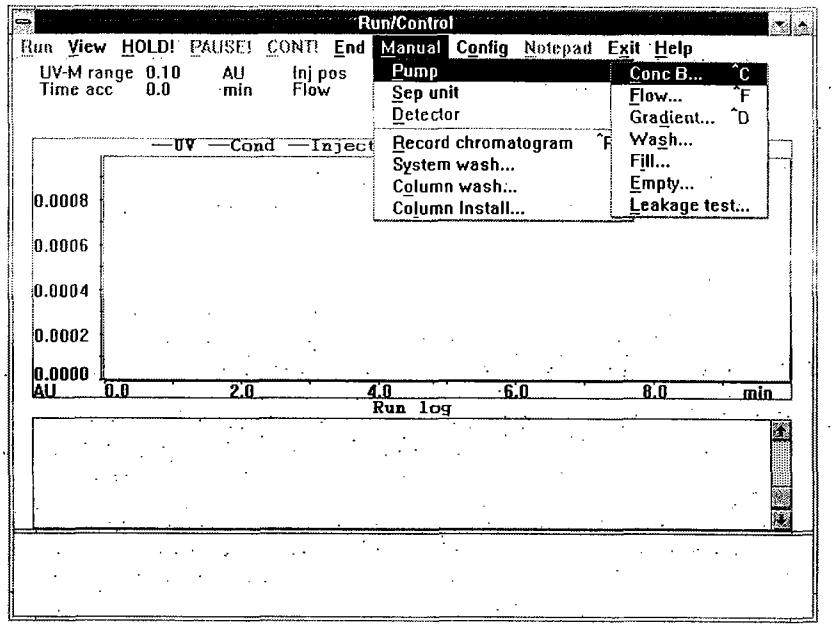


Fig. 5.28. Pull-down menu Pump

Conc B

The current concentration of eluent B is shown; a new concentration can be entered.

Flow

The current flow rate is shown and a new value within the available range can be entered.

Gradient

The current concentration of eluent B is displayed. Using this function, the user can change to another concentration which should be attained within a time specified by the user.

Wash

This function is used for washing of pump cylinders A and/or B. Both the inlet and the outlet port for the wash liquid must be specified for both pump cylinders. This function is used when the eluent is to be changed. The system automatically calculates the compressibility factor for the eluent after a completed wash procedure.

Fill

Pump cylinders A and/or B can be filled using an eluent from inlet 1 (default), 2, 3 or 4. The current volume in each cylinder is displayed. The filling flow rate can be normal, 15 000 µl/min or low, 5 000 µl/min. Low is used with viscous liquids. To perform a manual pump fill during a Separation method the run must be paused first.

Empty

Pump cylinders A and/or B can be emptied and the contents of the cylinders can be directed through outlets 1, 2, 3 or 4 (default). The current volume of eluent in the cylinders is displayed. The flow rate for emptying can be normal, 15 000 µl/min (10 ml cylinders), 30 000 µl/min (20 ml cylinders), 75 000 µl/min (50 ml cylinders) or low, 5 000 µl/min (10 ml cylinders), 10 000 µl/min (20 ml

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cylinders), 25 000 µl/min (50 ml cylinders). Low is used with viscous liquids. To perform a manual pump empty during a Separation method the run must be paused first.

Leakage test

Pump cylinders A and/or B can be tested for leaks as a regular test of pump function. The inlet valve is automatically switched to the closed position and the cylinder is pressurised to a user defined pressure. If this pressure cannot be maintained without adjustment of the piston position, a leakage has been detected and will be measured. If the piston position is maintained the test will stop after 10 minutes.

When an accurate reading of the leakage has been obtained, the test will stop automatically and the leakage in µl/min is displayed for each cylinder tested. If the colour of the background to the value is green, the leakage is small and no action needs to be taken. If the background colour is yellow there is a small leakage that may effect the result, if running at very low flow rate. Repeat the leakage test and if the colour remains yellow and you are using low flow rates, clean or replace the piston seal, (see Section 6.4). However, if the background colour is red, the leakage must be corrected by cleaning or replacing the piston seal (see Section 6.4) or by remaking the titanium tubing connection between the cylinder and the inlet valve (see Section 6.15).

The Break button on the Leakage Test window can be used to stop the test at any time.

- **Sep Unit**
  Changes to functions relating to the µSeparation Unit (Fig. 5.29).

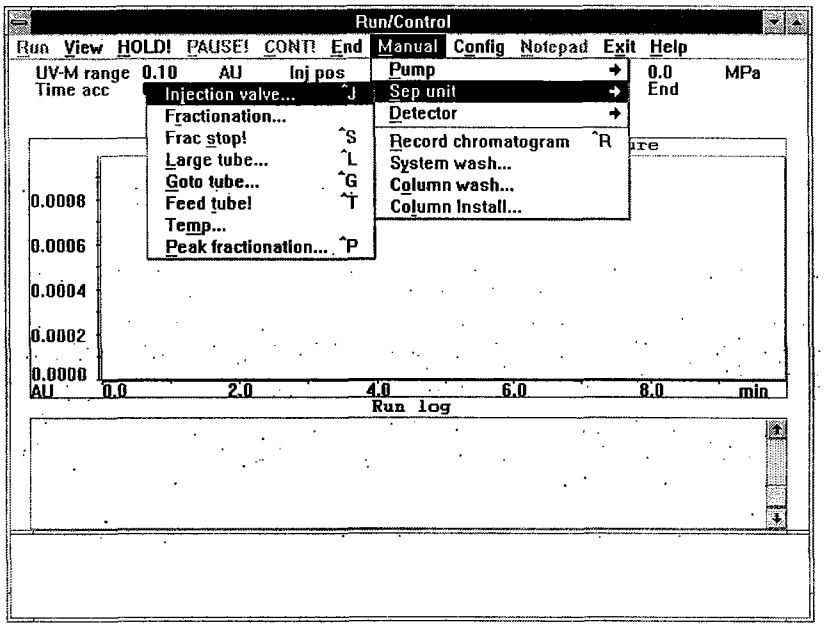


Fig. 5.29. Pull-down menu Sep Unit

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Injection Valve

The injection valve can be switched to the load, inject or waste position.

For SMART Systems with an injection valve with an internal sample loop, the valve positions for the internal loop can be set by using the load position for inject and the inject position for load. The waste position is the same.

Fractionation

This function sets the parameters to control fraction collection. For all programmed instructions, the delay volume is taken into account. The following parameters can be set:

a) Fraction size
This selects the required size of the fraction to be collected.

b) Start at tube
This function sets the tube number for the start of fraction collection. To change the tube position during fractionation the Go to tube function should be used.

c) Needle position
This sets the position of the fraction collector delivery arm to Up or Down. With the arm in the Up position the fractionation capillary will be just above the tube. Complete drops can be collected. With the arm in the Down position the fractionation capillary touches the bottom of the tube, and a smaller quantity, or part of a drop, can be collected. Fractions below 250 µl should be collected with the arm in the Down position.

Frac stop

The fraction collector stops after the delay volume has passed. The needle moves into the waste position.

Large Tube

Large fractions are diverted into tubes A, B, C or Z (Tube Holder 48/8) after the delay volume has passed.

Go to tube

The fraction collector moves to the specified tube after taking account of the delay volume.

Feed tube

The fraction collector feeds one tube after allowing for the delay volume.

Temp

The current temperature in the µSeparation Unit is shown together with the current target (set) temperature. Option buttons allow cooling to be switched on or off. When activated, a new target temperature can be selected from the range ambient (maximum 30 °C) to +4 °C.

Note: Before activating cooling, refer to Section 2.13 regarding the temperature of the cooling water being circulated around the µSeparation Unit.

Peak fractionation

The Peak fractionation can be set On or OFF. The Peak

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Fraction instruction can be used either to collect peaks in single tubes or to divide peaks into smaller fractions. If only Peak Fractionation is used, eluate between peaks will go to waste. To collect the eluate between peaks the Fractionation function must be programmed. In the Fractionation instruction the needle Up or Down can also be selected.

The program takes account of the delay volume.

For a more detailed description of using the Peak Fractionation function, see Section 4.7.

a) Peak fraction size
This function defines the size of the fraction to be collected within the peak in µl. If the object is to collect many small fractions over a peak, the peak fraction size should be smaller than the narrowest peak. If the object is to collect peaks in a single tube, the peak fraction size should be larger than the widest peak.

b) Minimum peak width
For collection with peaks identified by level or slope, the Minimum Peak Width is the minimum distance between peak start and peak end. The programmed value should be smaller than the narrowest peak.

c) Level/Noise window
The programmed Noise window will protect from accidental peak start and end detection caused by noise

d) Level/Peak start
A peak start is detected when the absorbance is above the programmed peak start level. For the program to recognise a peak start, the absorbance level must also be above the lowest absorbance value since last peak, plus twice the noise window.

e) Level/Peak end
A peak end is detected when the absorbance is below the programmed peak end level. For the program to recognise a peak end, the absorbance level must also be below the highest absorbance value in the peak minus, twice the noise window.

f) Level/Valley
When the Valley function is On, absorbance valleys can be detected above the Peak Start level. The fraction collector will move to a new tube. The criteria for identification of a valley is, that the absorbance level is 2 % below the detected maximum absorbance level minus the noise window, and that the absorbance increases 2 % plus the noise window.

g) Slope/Peak start
A peak start is detected if the slope of the absorbance is higher than the programmed value for more than 1/10 of the programmed minimum peak width.

h) Slope/Peak end
A peak end is detected when the slope of the absorbance is steeper than -1.2 times the programmed value and

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flatter than the programmed value for more than 1/10 of the minimun peak width.

e) Stop at
If activated, this field defines the end of fraction collection by peak detection. It does not effect ordinary fraction collection if active.

- Detector
This menu (Fig. 5.30) has functions relating to the µPeak Monitor.

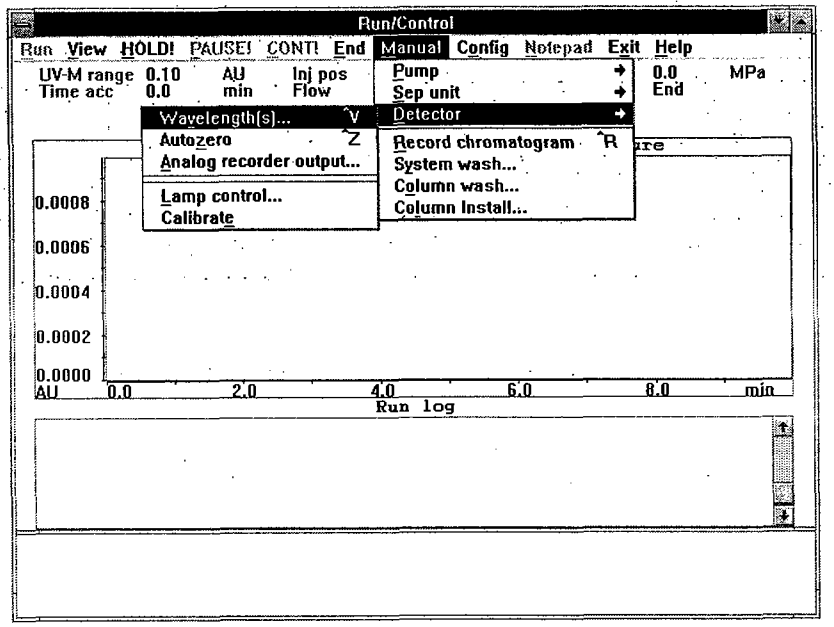


Fig. 5.30. Pull-down menu Detector

Wavelength
This function is used to set the wavelength(s), 190-600 nm, for µPeak Monitor. One, two or three wavelengths can be monitored simultaneously. For highest sensitivity, a single wavelength should be used.

Some eluents have a rapidly increasing absorbance at lower wavelengths, e.g. methanol at 215 nm will change with 30 mAU per nm, which causes a higher noise level detecting at dual or triple wavelength. During detection with dual or triple wavelength the rapid change in absorbance demands an exact positioning of the grating when switching between the different wavelength positions. With the design in µPeak Monitor the stepper motor can reposition the grating within 0.75 nm which means a wavelength reproducibility better than 0.05 nm. In case of the methanol at 215 nm this means a noise less than 1.5 mAU.

Detection at the selected wavelength(s) will start at the time/volume specified. When a wavelength change takes place in a separation method, a New Chromatogram command will be executed, so data from before and after the change will be stored in separate chromatograms but in the same result file.

The primary wavelength is the wavelength used for peak fractionation and may also be sent to the analogue recorder

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output for analogue recording. This wavelength is included in the 'Regular Curves' diagram in the evaluation report. Curves for all selected wavelengths can be printed afterwards in the evaluation report using the 'Free Choice Curves' option.

The curves will automatically be given names consisting of the eight last letters of the result file name, followed by an underscore, the wavelength, the current chromatogram number and a letter, A, B, or C, designating whether the chromatogram is from the primary, second or third wavelength.

Example: MonoQ12_254nm1A for primary wavelength

MonoQ12_254nm1B for second wavelength

MonoQ12_254nm1C for third wavelength

If no wavelength instruction is programmed in the method, the monitor will use the previously set wavelength(s).

If Record Chromatogram is activated it is not possible to change wavelength(s).

Autozero

This function is used to set the of the absorbance of the wavelength(s) to a reference zero baseline at a specified time/volume.

Analogue recorder output

This function is used to set the full scale absorbance range for the analogue recorder output of the primary wavelength. If the primary wavelength is changed during the run, a downward mark will be made on the recorder output

The Zero absorbance level can be set to a percentage of full scale, to make it possible to register the signal even if the signal drifts below zero

To handle unexpectedly large signals, the signal to the recorder is inverted (mirrored) when it reaches the upper limit of the scale. Levels below the lower limit are truncated.

Lamp control

The lamp can be manually switched On or Off. When high sensitivity is required, it is recommended to switch on the lamp to stabilize the detection system. It is not possible to switch off the lamp when data from a manual run or a Separation Method is displayed on the screen.

Calibrate

This function is used to start a wavelength calibration. Normally this is not necessary, since the monitor is automatically calibrated when the system is started. If the system has not been switched off for a long time a Wavelength not Calibrated warning might occur and the Calibrate instruction can be activated. It is not possible to use the Calibrate instruction during run.

- **Record chromatogram**

This function turns on the Record Chromatogram function when activated . This allows the results from a manual run to be saved. After completing the manual run, Record

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Chromatogram is activated a second time, then the user must specify a Result File where the chromatogram is to be saved. This function allows the data to be stored in a Result File when no Separation Method is currently running.

- System wash
  This function allows a wash or partial wash of the system to be initiated. The pump cylinder and inlets for the first and second eluents can be selected. The wash can be either through the injection valve to waste or through the Dummy Column and flow cell to waste. The system wash takes a long time, especially for larger pump cylinders. For quick washing of the system it is recommended to use the library methods SYSW10.MET, SYSW20.MET or SYSW50.MET for the different cylinder sizes.

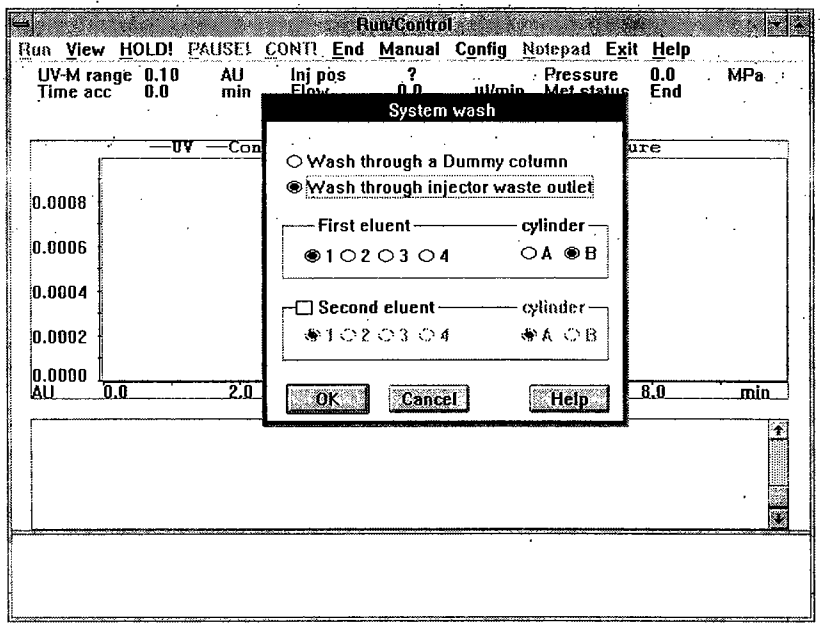


Fig. 5.31. System Wash window

It is not possible to activate the system wash function while running a Separation Method or recording the chromatogram from a manual run.

- Column wash
  This function initiates a column wash. First the flow rate is specified, then up to three eluents can be set. The duration volumes of each eluent can be chosen. Each eluent (100 %) is pumped through the column in the specified order at the specified flow rate. A prewash can also be initiated to prime the pump cylinder(s) before the run.

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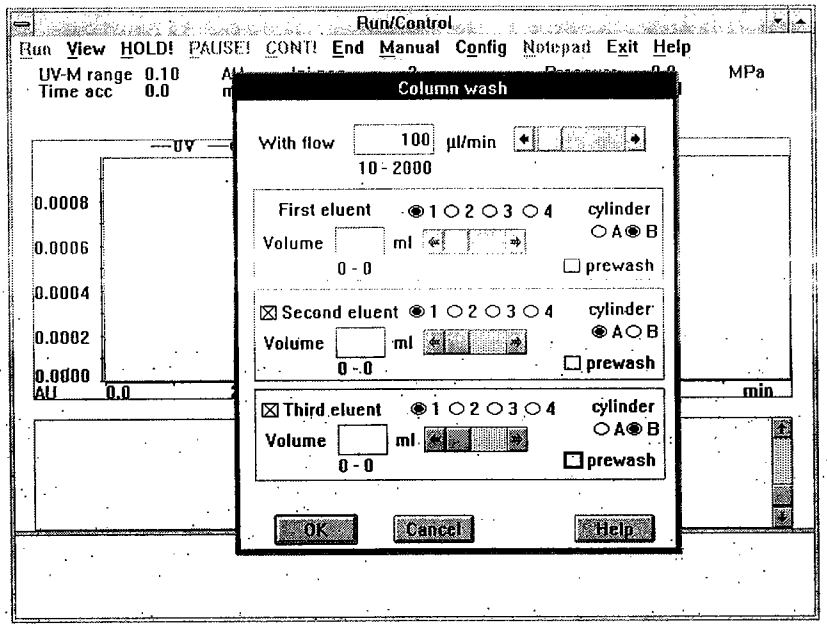


Fig. 5.32. Column Wash window

It is not possible to activate the column wash function while running a Separation Method or recording the chromatogram from a manual run.

Note: The user should follow the recommendations in the Column Instructions before initiating a wash.

- **Column Install**
  This function allows the column to be installed or removed.

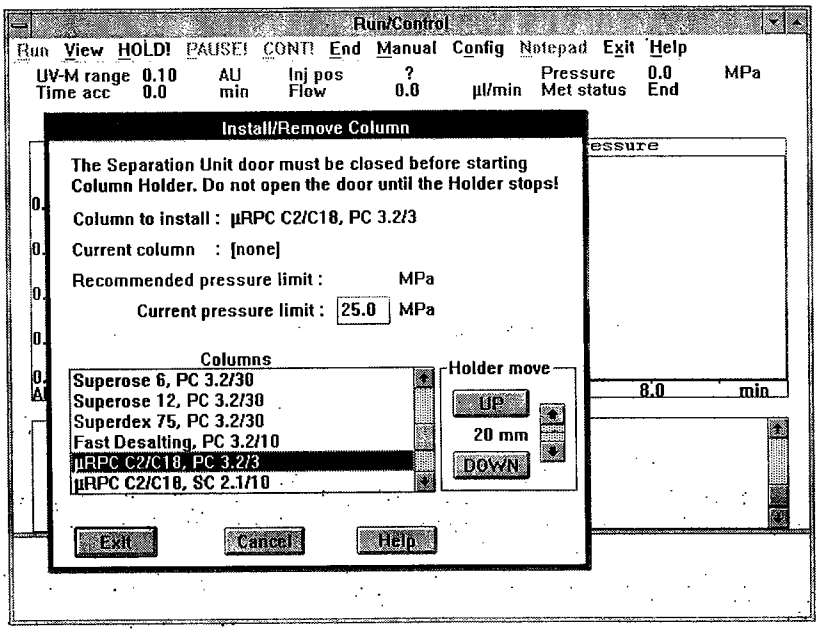


Fig. 5.33. Column Install window

In the dialogue box the available columns are listed. If a column is chosen from the list box the recommended pressure limit will be displayed. The pressure limit can be changed in the Current pressure limit edit box.

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The pressure limit set will also be used when a Separation Method is run, if the Column Set option was selected in the method configuration. If a numerical value for the pressure was entered in the method configuration, this value will be used instead. For the µPeak Monitor the averaging time will also be set according to the column installed, if the Column Set option has been selected in the Method Editor, Configuration.

The column holder can then be moved up or down by clicking on the UP or DOWN button respectively. This function cannot be used when a Separation Method is running.

Config

SMART System has the ability to recognise some component parts of the system and to read their current internal status. For example, the system can automatically recognise the type of pump cylinders which are fitted. The system must also be informed of the current status of other components, but since the system is not able to recognise them, the operator has to enter their configuration manually. For example, the mixer volume and type of fraction collector tube holder must be entered manually. It is particularly important that the system is informed of the current operating parameters of the UV-M II Monitor; these must be entered by the user.

The Configuration entered in the method is the description of the system and its components which are required to run that Separation Method. This is entered in the Configuration window in the relevant Separation Method. Before a run is started, the system makes an automatic check to ensure that the actual current system configuration is compatible with the configuration entered in the Separation Method. This check ensures that the system can run the method.

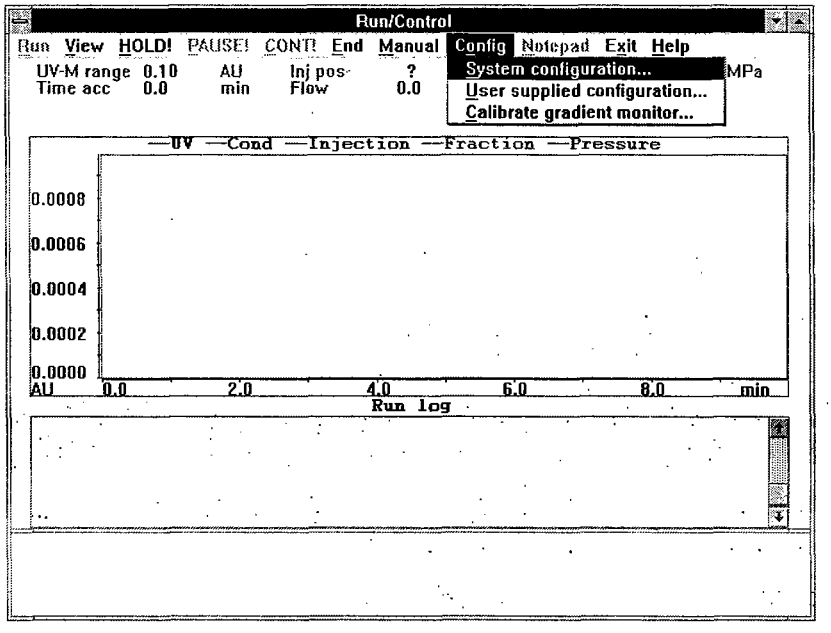


Fig. 5.34. Pull-down menu Configuration

- **System configuration**
  This option shows the actual System Configuration. These values are automatically recognised by the system.

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- User supplied configuration
  The user must supply this additional information about the system (Fig. 5.35).

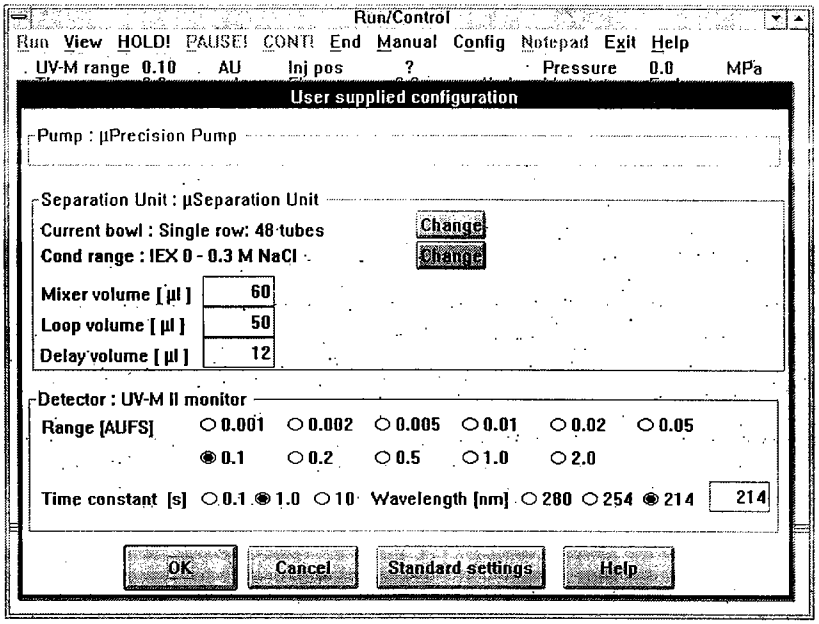


Fig. 5.35. User Supplied Configuration window

Fraction bowl
: This specifies the type of tube holder fitted to the fraction collector.

Conductivity range
: This specifies the range for the calibration of the gradient monitor. Four conductivity ranges are precalibrated. All conductivity ranges can be recalibrated using the SMART calibration option button or the Calibrate gradient monitor instruction under the Config menu.

Mixer volume (µl)
: This specifies the current volume of the mixer.

Loop volume (µl)
: This specifies the volume of the sample loop fitted to the injection valve.

Delay volume (µl).
: This is the dead volume of the system, from the detector to the fraction collector. The exact volume is measured for each system and stated in the Test Certificate enclosed with the SMART System. This value has to be entered in the Delay volume edit box.

Detector range (AUFS)
: This should be the same as the absorbance range set on the UV-M II detector.

Time constant (seconds)
: This should be the same as the time constant set on the rear panel of the UV-M II detector.

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Wavelength (nm)
This should be the same as the wavelength set on the UV-M II detector.

Note: It is important to ensure that the detector settings are entered correctly.

- **Conductivity calibration**
  This function allows calibration of the conductivity ranges. Before activating the conductivity calibration the pump cylinders must be filled with the right eluents and the Dummy column installed in the column holder.

  A new name can be entered for the conductivity range.

Notepad

Notepad allows the user to make notes during a run. Comments entered are saved together with the Result File.

- **Edit**
  This function allows the marked text to be edited, copied or moved.
- **Exit**
  This function exits from the notepad.

Exit

- **Exit**
  This function exits from the Run/Control. If a Separation Method has been run and a Result File been generated, a dialogue box will appear with a choise to exit to the Method File Contents or the Result File Contents. If no Result File has been generated it exits to the File Contents window or Main Menu depending on whether Run/Control was entered by clicking on the icon or by selecting a method.
- **Resume**
  This allows to resume the Run/Control window.

Help

- **Help for Help**
  This function describes how to use the Help command.
- **Extended Help**
  This function provides general information about the Run/Control. More detailed information is given for each menu in Run/Control.
- **Keys Help**
  This function describes how to use the keyboard for specific tasks in the Run/Control.
- **Help index**
  This function contains a complete list of all instructions in the Run/Control you can obtain help for.

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5.4.4 The Run Log

The Run Log in the lower area of the screen continuously records details of all events in the run. Programmed events as well as manual changes are recorded. System warnings and errors are recorded to simplify trouble shooting.

The Run Log is saved in the Result File together with the Method and Chromatogram. Although it is possible to inspect the Run Log, this documentation cannot be modified.

5.5 Evaluation

5.5.1 Evaluation overview

The results of a chromatographic run can be evaluated in one of the following ways:

- by interactive graphic manipulation of the UV absorbance curve
- by an automatic evaluation procedure

The two evaluation methods are closely related. As the user evaluates the chromatogram manually using the interactive graphic display, each step is recorded by the program. This record or log of the evaluation forms the basis of the Evaluation Procedure which can be saved and later recalled for the automatic processing of the results from other runs.

5.5.2 Evaluation window

The Evaluation part of SMART Manager can be entered directly from Run/Control if a Result File has been generated or by selecting a Result File from the Main Menu and the Chromatogram card in the File Contents window.

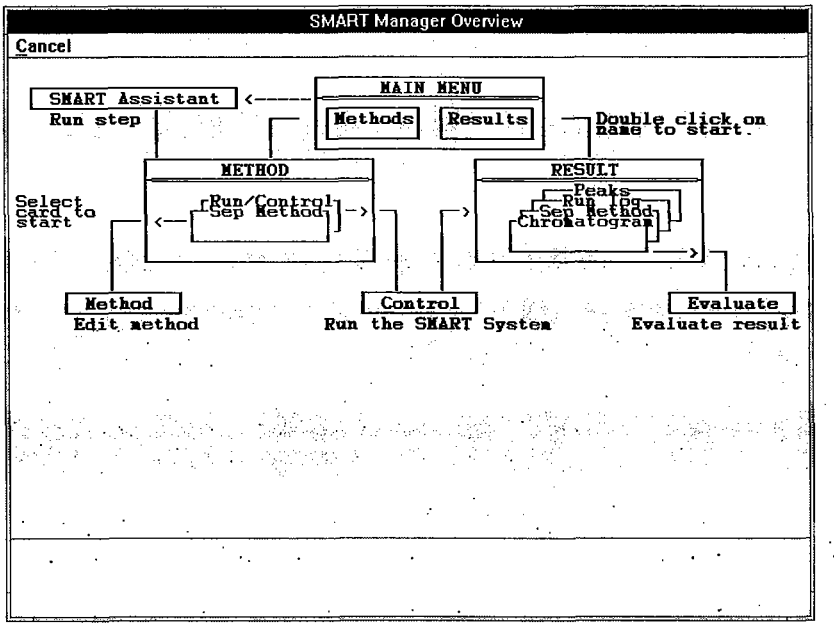


Fig. 5.36. SMART Manager overview

For manual evaluation, the screen display for the chromatogram is extremely flexible, with the operator able to choose a layout from several different options (Fig. 5.37). The display selected can be

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changed whenever necessary and can include other result curves for reference and comparison. If the Chromatogram has been previously evaluated and integration performed, the Peak Data Table is also available.

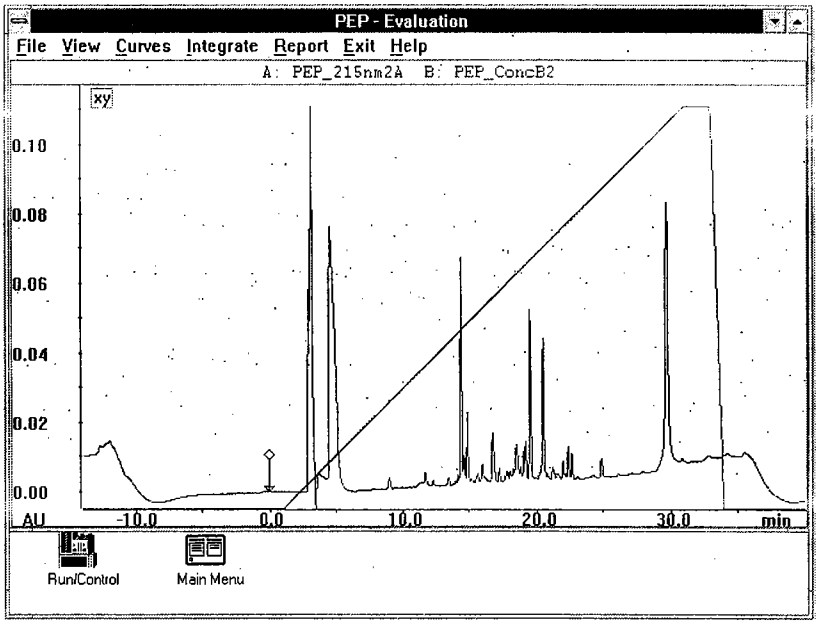


Fig. 5.37. Evaluation window

To simplify the process and to make the evaluation more precise, all parts of the curve displayed on the screen can be enlarged. By pressing and holding the left mouse button on a corner of the area to be enlarged, the cursor changes to a pointing finger with a box attached to it. By continuing to depress the mouse button while moving the mouse, the box can be extended to completely surround the area to be enlarged. By releasing the mouse button, the area in the box is fixed and magnified (zoomed). Once "zoomed", the curve can be unzoomed by clicking on the Unzoom box in the top right of the window.

The unit shown on the y-axis is changed by clicking on or to the left of the y-axis.

5.5.3 Evaluation pull-down menus

File

The File pull-down menus (Fig. 5.38) are used to organise the Result File.

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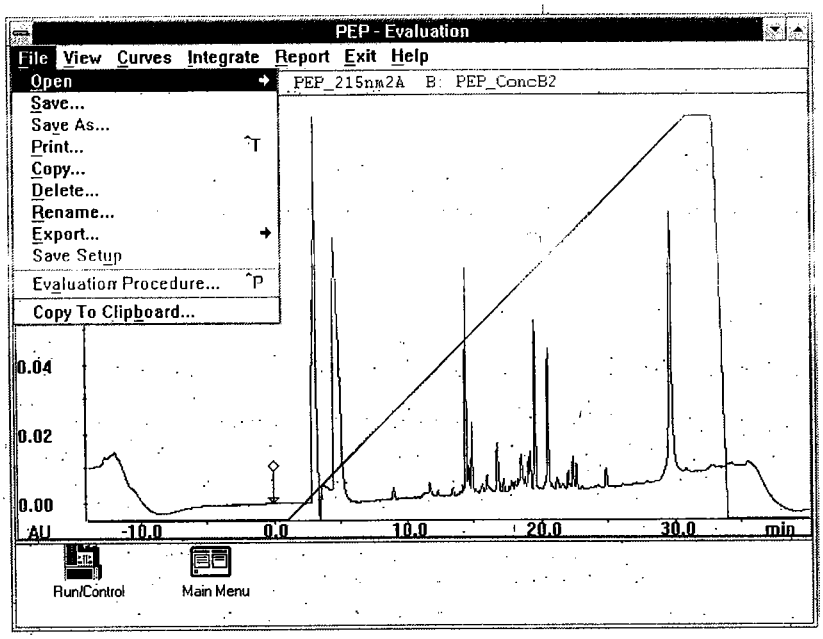


Fig. 5.38. Pull-down menu File

- **Open**
  This function allows a curve or peak table to be opened and transferred into a curve memory for display in the evaluation window. The curve or peak table to be opened can be selected from the current Result File, from another Result File belonging to the current user or from another user's file.

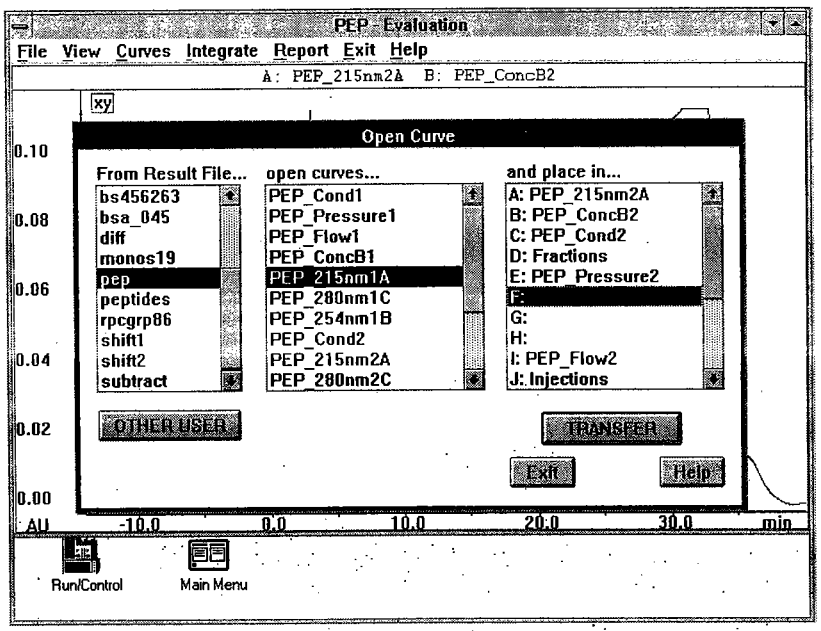


Fig. 5.39. Open curve window

- **Save**
  This function is used to save curves or peak tables in the current result file. The curves and peak tables can be selected in the list box. A screen promptly warns if a curve or peak data of that name already exists in the Result File.

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- Save As
  This function saves the curves or peak tables from the evaluation under another Result File name entered by the user. The result curves can be stored in the current Result File, in another existing Result File or in a new Result File. If curves and tables are saved in an existing result file they will be added to the file contents.
- Print
  This function sends the currently displayed curve or peak data to a printer. The user can specify the part of the screen window to be printed and the printer parameters.
- Copy
  This allows copying of a Result File to a new or existing Result File. If copying to an existing result file, the old contents of the file will not be available. A Result File can also be copied from another user.
- Delete
  This allows a Result File to be deleted. Confirmation must be given. The current Result File and other users' files cannot be deleted.
- Rename
  This allows a Result File to be renamed. The user selects the file to be renamed from the list and then types in the new name. The current Result File and other users' files cannot be renamed.
- Export
  Information from SMART Manager can be exported to other software, such as spreadsheet or graphic programs. Two formats are available for the exported files, ASCII and WKS.

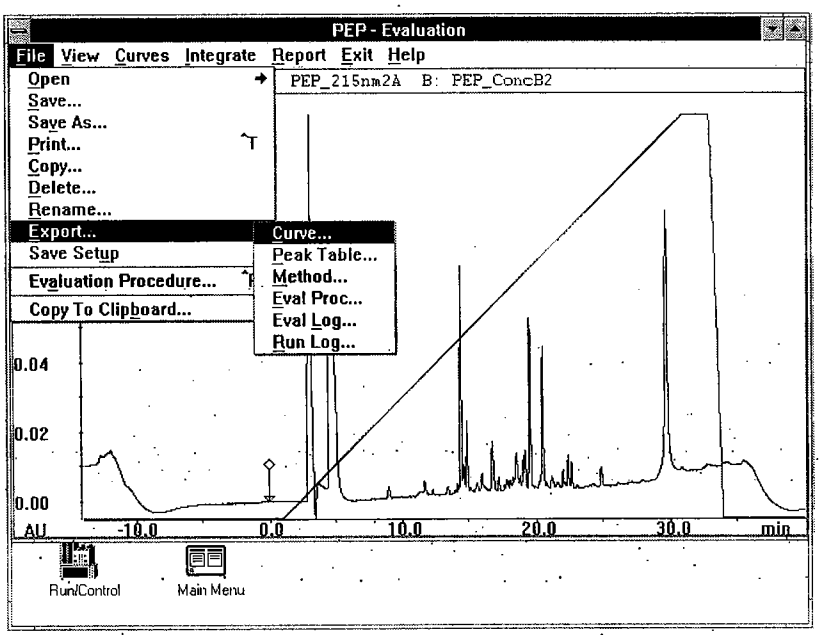


Fig. 5.40. Pull-down menu Export

Export curve
Curves or part of curves can be exported. In the dialogue box the curve to be exported, export format and where to export

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the curve is selected. The exported file is placed in the User Directory if a path name is not included in the file name.

To export a part of the curve, the limits can be set in the edit boxes under Cut Curve or by using the CUT GRAPHICALLY option button. When Cut Graphically is selected a window with the curve appears and the limits can be set with vertical drag bars on each side of the window.

At the bottom of the Export Curve dialogue box the number of data samples to be exported can be set. If all data points are to be exported the reduction factor should be set to 1. If high resolution is not needed in the exported file, a higher reduction factor can be selected. The number of data samples in the exported file will be displayed.

File formats: An exported ASCII file will have two columns, one containing retention time/volumes and one containing the corresponding curve amplitudes. An exported WKS file, which can be used by spreadsheet programs will contain the curve name in the top left cell (A1) and two columns of data. The B column will contain the retention data, the C column will contain the corresponding curve amplitudes, both starting on the row 2 in the spreadsheet.

Export Peak table
This function is used to export a peak table. In the dialogue box the peak table to be exported, the export format and where to export the peak table can be selected. The exported file is placed in the User Directory if a path name is not included in the file name.

File formats: An exported ASCII file will contain those columns that have been specified using Peak window layout under the menu View. An exported WKS file contains the whole peak table independently of what is specified in Peak window layout.

Export Method
This function is used to export the current Separation Method. In the dialogue box the part of the method, export format and where to export the file can be selected. The exported file is placed in the User Directory if a path name is not included in the file name.

Export Eval Proc
This function is used to export an Evaluation Procedure in the current Result File. In the dialogue box the Evaluation Procedure, the export format and where to export the file are selected. The exported file is placed in the User Directory if a path name is not included in the file name.

Export Eval Log
This function is used to export the Evaluation log in the current Result File. In the dialogue box the format and where to export the file are selected. The exported file is placed in the User Directory if a path name is not included in the file name.

Export Run Log
This function is used to export the Run Log in the current Result File. In the dialogue box the export events (Occurrences, Warnings and Errors), the export format and where to export

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the file can be selected. The exported file is placed in the User Directory if a path name is not included in the file name.

- **Save setup**
  This function saves user specific settings as parts of the curve window layout, the Peak Window Layout and the Specify Report Contents.

- Evaluation Procedure
  This function accesses the Evaluation Procedure. As default the evaluation log is shown in the evaluation procedure window. The Procedure is identified by a name and contains the following functions:

  File

  New clears the evaluation procedure window and gives the possibility to enter a new procedure name.

  Open loads an Evaluation Procedure selected from the Method File belonging to the current user or from another user's Method File.

  Save saves the current Evaluation Procedure under a new file name in an existing Method File.

  Print prints the current contents in the evaluation procedure window.

  Open log opens the log of the current evaluation.

  Export is used to export the contents of the evaluation procedure window to an ASCII or WKS file. In the dialogue box the format and where to export the file are selected. The exported file is placed in the User Directory if a path name is not included in the file name.

  Rename And Delete is used to rename or delete Evaluation procedures stored in the current Result or Method file.

  Run

  This function runs the Evaluation Procedure on the current Result File. A warning is given that running an Evaluation Procedure will change data.

  Exit

  This function terminates the Evaluation Procedure window.

- **Copy to clipboard**
  This function copies the currently displayed curves or peak data to the clipboard. The user specifies which part of the window to be copied.

View

The View pull-down menu is used to select the items and layout for the screen display (Fig. 5.41).

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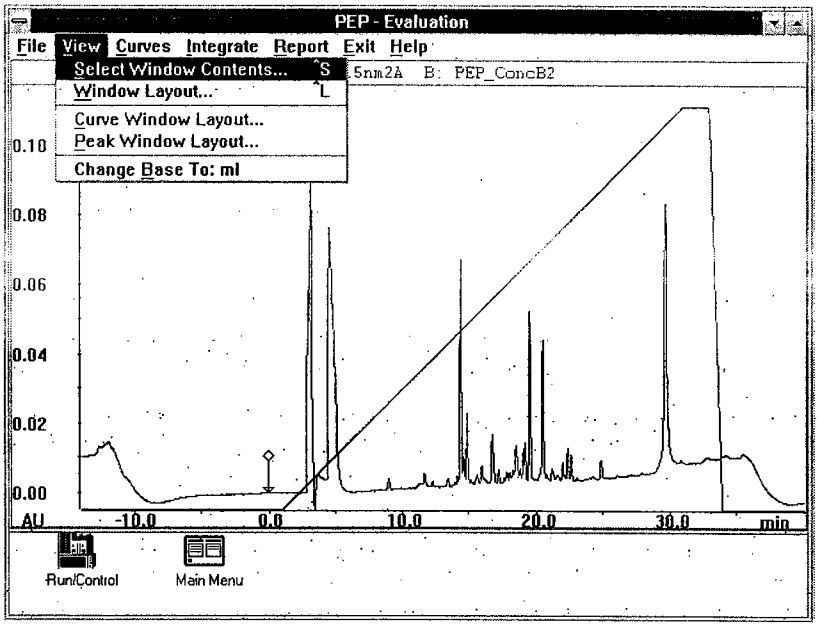


Fig. 5.41. Pull-down menu View

- **Select Window Contents**
  This allows the user to select the curves and peak data to be displayed in each screen window. For example, a selection can be made from the signals saved in the current Result File. These can be UV, conductivity, programmed % B, flow rate, fraction marks, injection valve events, pressure and temperature. The user can select up to eight curves from the list for display in the window.

- **Window layout**
  This function allows selection of the layout of the windows on the screen. Several options are displayed, including a single curve window, two curve windows, three curve windows, one curve displayed in one large and one small window, one curve window together with a peak data window, two curve windows and a peak data window, peak data window alone.
  Alternatively, by choosing "free layout" the user can determine the number of windows and the percentage of the screen to be used by the different curves and peak data.

- **Curve window layout**
  On both the x- and y-axis, the scaling can be either automatic (default) or fixed. In Automatic, SMART Manager sets the scale according to the total run time or volume, and the y-axis is scaled according to the signal in the curve.

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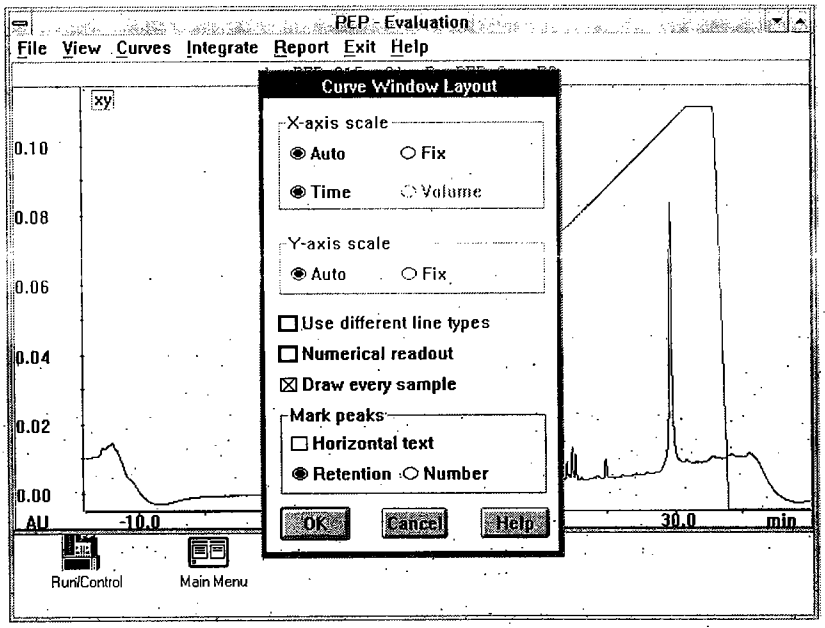


Fig. 5.42. Curve window layout

If Fixed is selected, another dialogue box is displayed where the user enters the left and right limits for the x-axis and the maximum and minimum values for each unit on the y-axis. If UV-absorbance curves from several wavelengths are evaluated, up to three different y-axis scales can be used. Any additional curves will have the same scale as the first specified curve, at the top of the dialogue box. Curves detected at the same wavelength will always have the same scale. The scale is always the same for all curve windows.

Different curves are displayed in different colours. In addition, each curve can be displayed with a different line type.

The numerical readout box can be activated to give the X and Y-magnitude at the current cursor position. The readout box can also be activated by clicking on the xy-icon in the upper left corner of the curve window. By clicking on the numerical read out box in the curve window, the read out shifts between the different curves on the screen.

With the field marked Draw every sample activated, the curves draw every data point sampled. This function gives better data resolution, and ensures that the highest peak point is visible. However, the curve is drawn more slowly when this function is activated.

The peaks identified for integration can be indicated by either the retention time (default) or the peak number, and the text can be written horizontally or vertically.

- Peak window layout
  This function selects the peak data columns to be listed in the peak table (Fig. 5.43). Data is only available for either time or volume depending on the currently used base.

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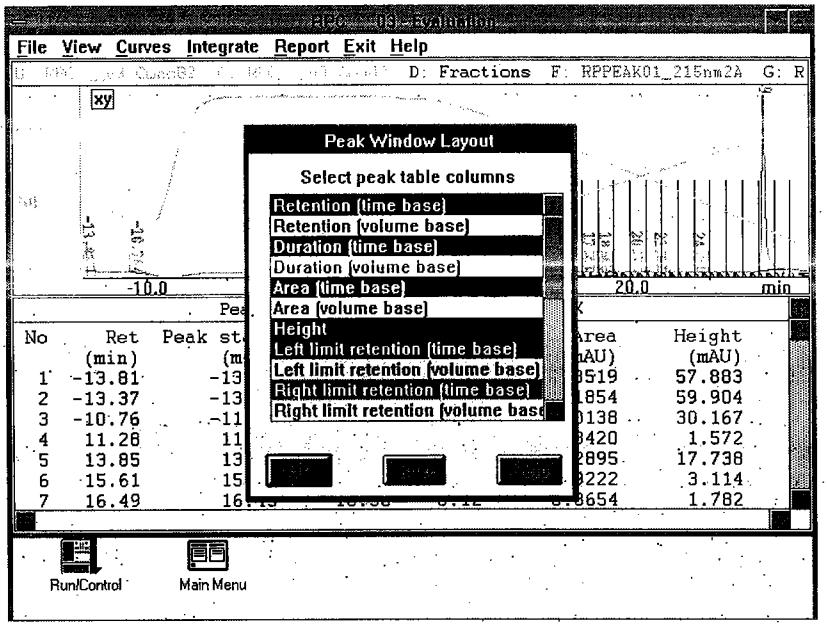


Fig. 5.43. Peak Window Layout

The user can select from the following information:

Retention time base
- current retention time

Retention volume base
- current retention volume

Duration (time base)
- duration of the peak in minutes

Duration (volume base)
- duration of the peak in millilitres

Area (time base)
- peak area using time as the base

Area (volume base)
- peak area using volume as the base

Height
- height from the baseline to the peak maximum (Fig. 5.44)

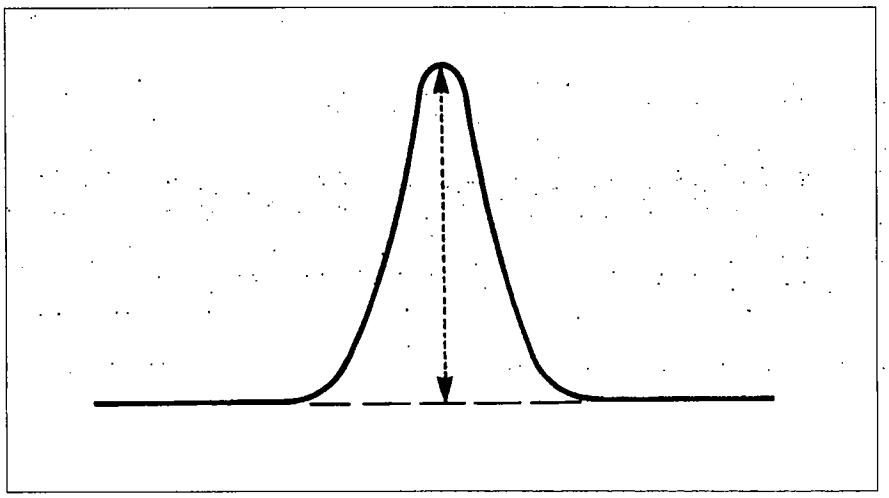


Fig. 5.44. Height from baseline to peak maximum

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Left limit retention (time base)
- retention time at the peak start

Left limit retention (volume base)
- retention volume at the peak start

Right limit retention (time base)
- retention time at the peak end

Right limit retention (volume base)
- retention volume at the peak end

Width at half height (time base)
- width in minutes at half height

Width at half height (volume base)
- width in millilitres at half height (Fig. 5.45)

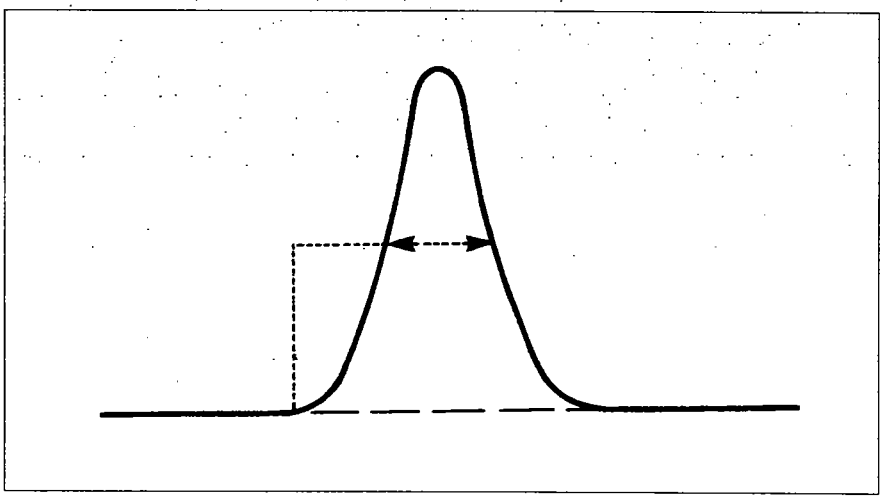


Fig. 5.45. Width at half height

Percent of total area (time base)
- percentage of total area above the baseline (time)

Percent of total area (volume base)
- percentage of the total area above the baseline (volume)

Percent of total peak area (time base)
- percentage of total peak area which is integrated

Percent of total peak area (volume base)
- percentage of the total peak area which is integrated

Type of peak limits
- drop is selected for unresolved peaks; not drop selected for resolved peaks (Fig. 5.46)

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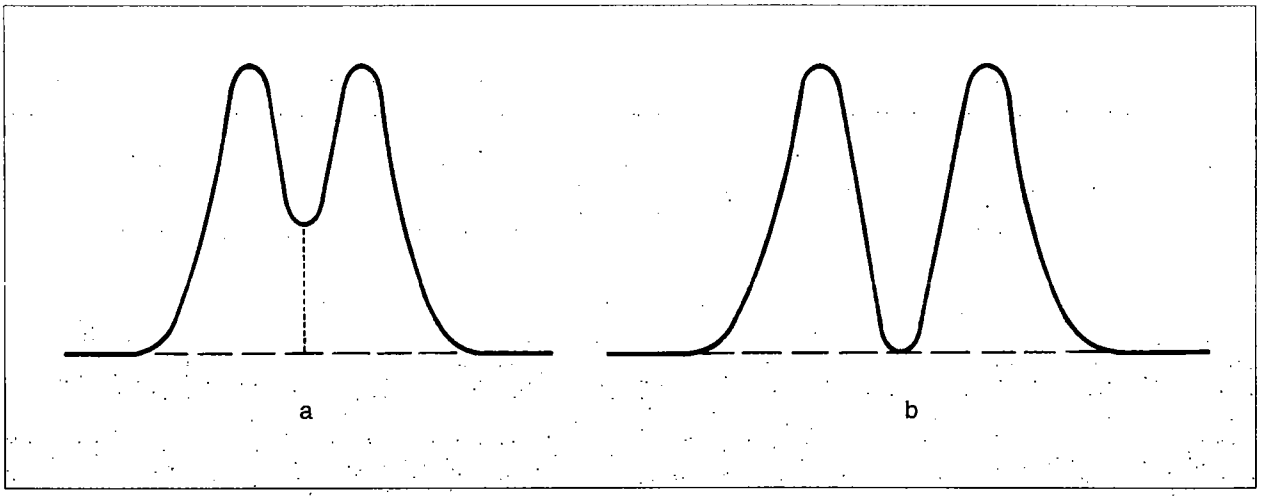


Fig. 5.46. Type of peak limits: a) drop, b) not drop

Height at left limit
- height in AU above the baseline at the peak start (Fig. 5.47)

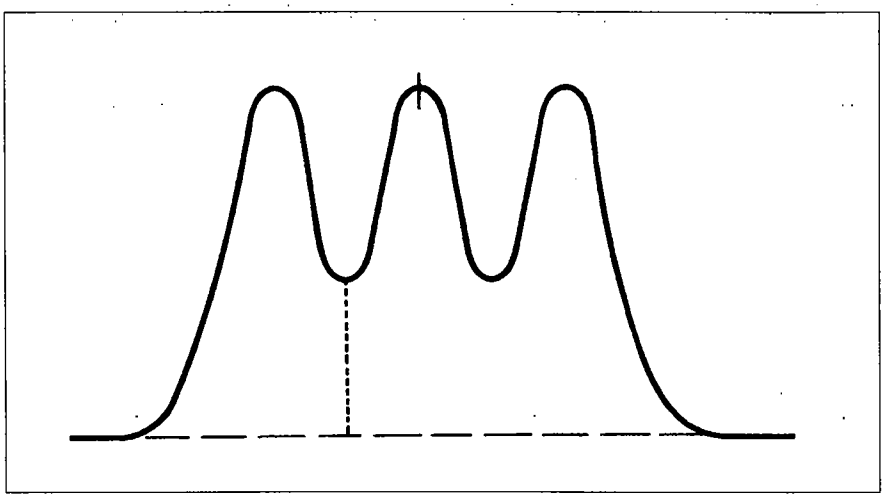


Fig. 5.47. Height at left limit

Height at right limit
- height in AU above the baseline at the peak end (Fig. 5.48)

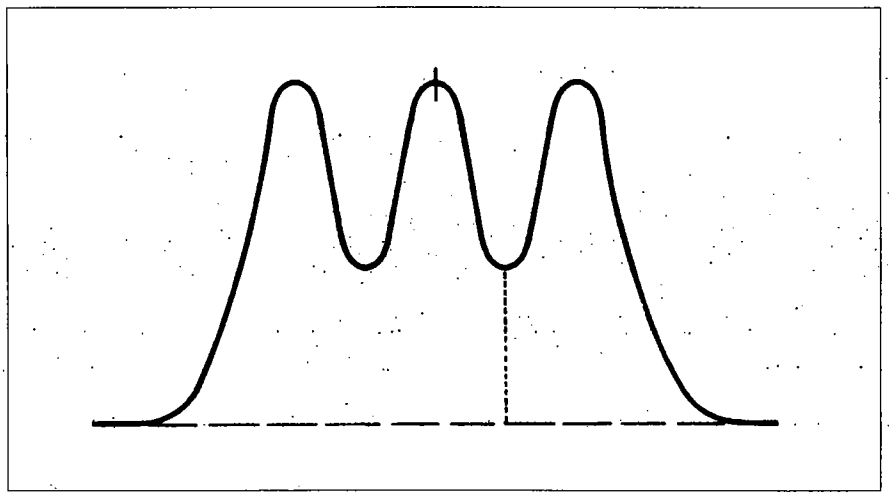


Fig. 5.48. Height at right limit

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Fraction tube i.d.
- number of fraction collection tube at peak start, maximum and end.

Baseline height
- height of the baseline at the peak maximum, peak start and peak end (Fig. 5.49)

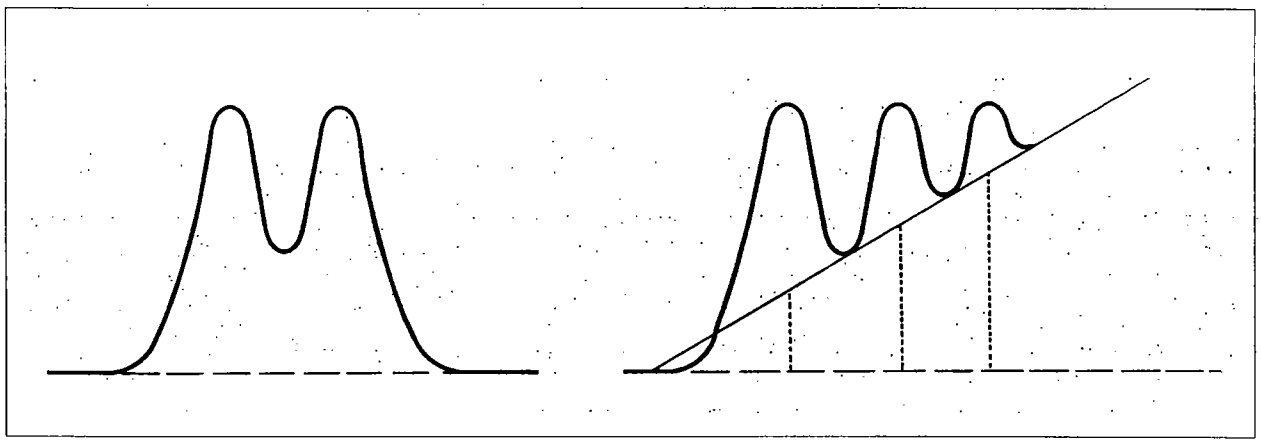


Fig. 5.49. Baseline height

Programmed gradient height
- programmed gradient output for a specific peak maximum, start and end (Fig. 5.50)

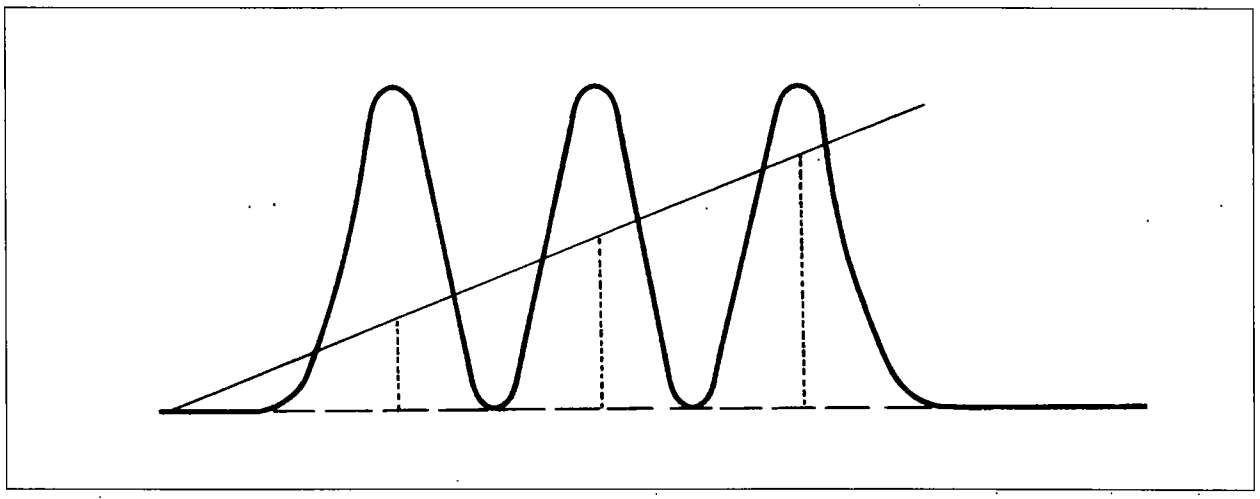


Fig. 5.50. Programmed gradient height

Conductivity height
- conductivity curve output for a specific peak maximum, start and end (Fig. 5.51)

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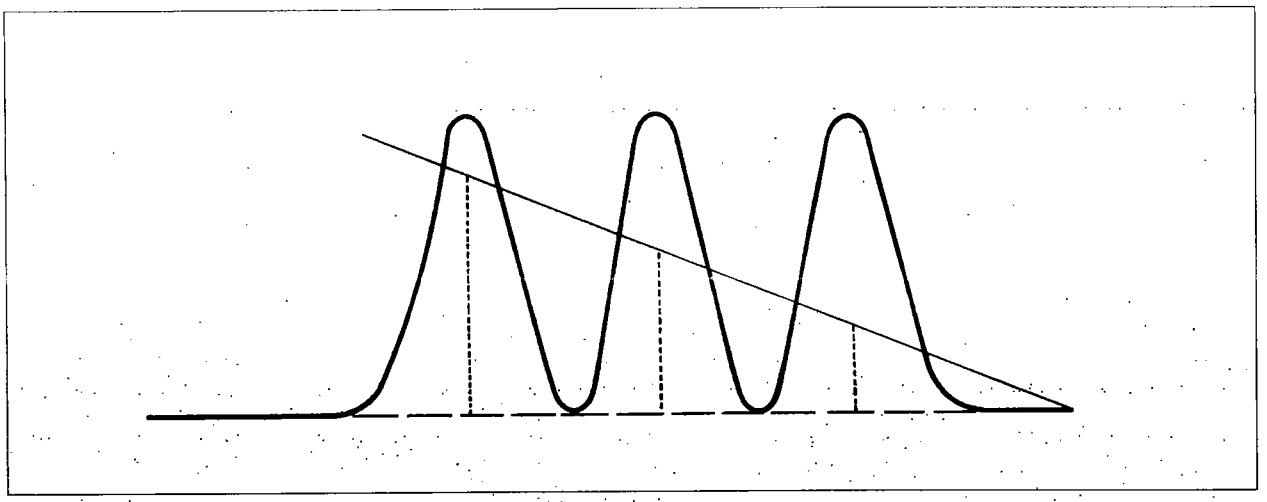


Fig. 5.51. Conductivity height

- **Change base to min/ml**
  This function allows the user to select the base for integration. This change can only be made before integration or before any other curve operation. After integration or any other calculation, the option disappears from the menu.

Curves

The Curves pull-down menu can be used before integration to handle the curve data in a number of ways (Fig. 5.52). Curves resulting from these processes are identified by new file names and can be displayed in the specified window as Curves A - Q. In most cases, a further window appears when the functions described below are activated. The resulting curves are stored in a temporary curve memory. To store the curves in the Result File, the function File, Save or Save As must be used.

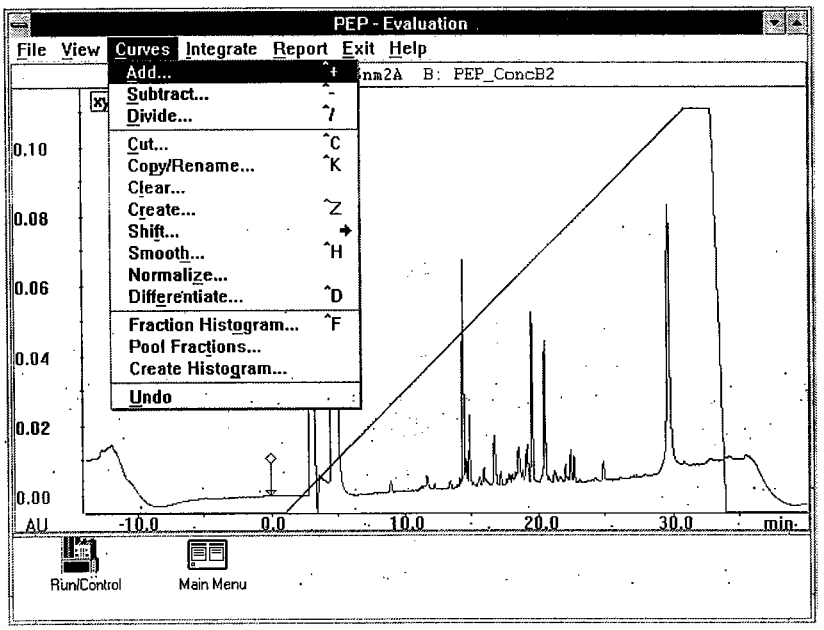


Fig. 5.52. Pull-down menu Curves

- **Add**
  This function adds two curves together to create a third curve

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which is stored under a specified curve name. The operator can either accept the default curve name or enter another. This function can be used to obtain a view of how the results of a run on a complex mixture might appear, if chromatograms from less complex separations are added.

- Subtract
  This function subtracts one curve from another curve to create a third curve which is stored under a specified curve name. The operator can either accept the default curve name or enter another. By subtracting the results of a blank gradient run from the results of a run with a sample, the interference caused by the gradient can be removed.

  How to subtract a baseline is described in Section 4.8.13.

- Divide
  This function divides one curve by another curve to create a third curve which is stored under a specified curve name. The operator can either accept the default curve name or enter another. This function can be used when investigating the ratio between two curves when studying peak purity and peak identity. The user can enter threshold values for the curves to be divided. If the amplitude of one of the curves is below the threshold the ratio is set to 1 in that point.

  How to calculate absorbance ratios is described in Section 4.8.15.

- Cut
  This function allows part of a curve to be extracted from the complete curve. This function can be used when studying a small part of the curve or to remove any part of a curve which is not required when comparing two curves. The part of the curve cut out can be saved under another name, using the Save As function.

  When Cut is activated, the user can specify the cut position either by positioning the left and right drag bars or by clicking on the left and right x-position co-ordinates and typing in the new value. Clicking on OK initiates the cut.

- Copy/Rename
  This function copies a curve from one curve memory to another. The user specifies the curve names of the source and destination curves or can accept the default destination curve name. This function will not effect the curve names in the stored result file.

- Clear
  This function clears a curve from the curve memory, not from the Result File. The user specifies the curve name to be removed.

- Create
  This function allows a baseline to be created from points which are specified by pointing and clicking with the mouse or by typing in the x- and y-values of each point. The inserted points can be shown in a Points list box. The Create Curve window (Fig. 5.53) also displays the x- and y-values at the current position of the cursor.

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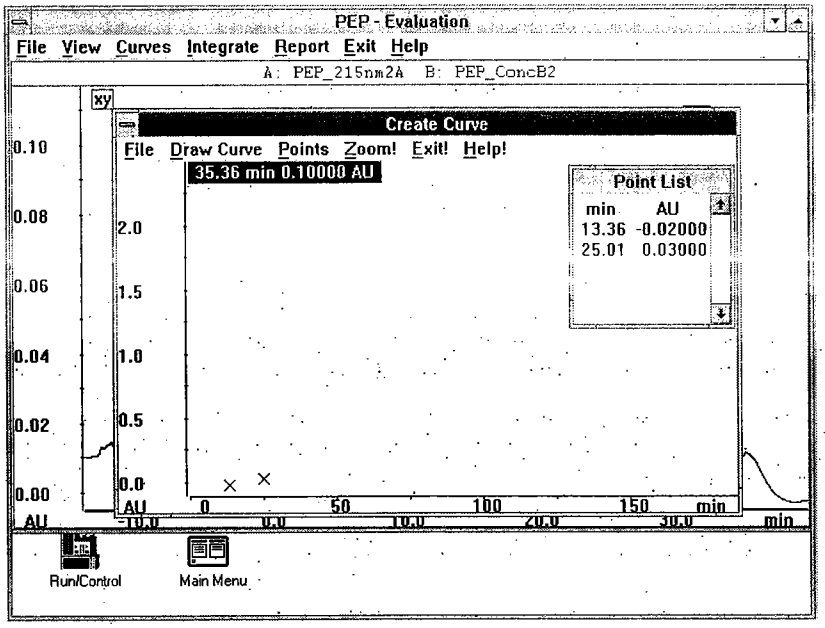


Fig. 5.53. Create Curve window

The following pull-down menus are available;

File

Open Help Curve 1 opens a specified help curve to assist the user when creating other curves. Help curves are selected from the curves in the curve memory.

Open Help Curve 2 opens another specified help curve.

Clear Help Curve 1 clears the help curve 1.

Clear Help Curve 2 clears the help curve 2.

Set Axis Scale allows the user to type in the minimum and maximum values required on the x- and y-axis. It is important to have the correct values on both axis as the default values can be high, which means that help curves run at high sensitivity appears as a straight line if the y-axis is not rescaled.

Save Created Curve saves the created curve. The user can accept the default name or type in his own choice. This function only stores the curve in the temporary curve memory. To store the curve in the Result file, the function File, Save must be used.

Close Window closes the Created Curve window. If the curve is not saved, a warning is given.

Draw Curve

Draw Spline calculates and draws a spline curve between the marked points. This is a smooth curve which may not pass through all points.

Draw Through Points calculates and draws through the marked points.

Clear Drawn Curve deletes the drawn curve.

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Points

View Point List displays the box showing the list of all marked points in minutes or in millilitres.

Set Point sets a new point by displaying a box where the x- and y-values (time or volume and amplitude) of each point can be entered by the operator.

Delete Point deletes the point specified by the user. The point to be deleted is selected on the point list and then Delete point is selected. The time or volume and the amplitude of the marked point is displayed for the user's confirmation. Points can also be deleted by double-clicking on the point in the point list.

Delete Last Point deletes the last point entered.

Delete All Points deletes all points and the interpolated curve after confirmation by the user.

Zoom

Zoom changes the cursor to a pointing hand, which can be used to magnify a part of the curve. Place the cursor in the corner of the area to be magnified, and keep the mouse button down. Mark the area to be magnified and release the mouse button.

- **Shift**

  This function moves and changes the specified curve in both the x- and y-directions, that is in the retention and the amplitude scale. Shift retention and shift amplitude are used for moving the curve, while multiply retention and multiply amplitude are used to multiply the curve by the required factor. The shift offset value and the multiplication factor is entered by the user.

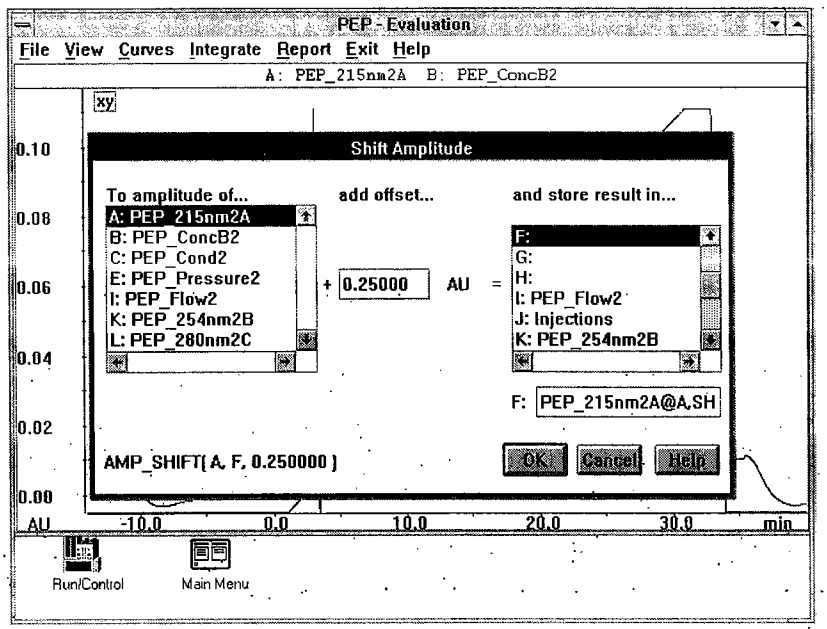


Fig. 5.54. Shift amplitude window

Shift can be used when two curves are being compared on the screen. A useful application for multiplication is to correct the absorbance signal when the incorrect detector settings for the UV-M II monitor, have been entered in the User Supplied

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Configuration. The Multiply amplitude can also be used to make a mirror image of a curve, by multiplying the curve with -1.

How to use Shift Amplitude and Multiply Amplitude is described in Section 4.8.12 and 4.8.14.

- **Smooth**
  This function smooths the specified curve with a filter selected from Moving average, Autoregressive or Median.

  Moving average
  This is a simple way of filtering regular noise. The width in minutes is set to either light for a small width and light filtering or hard for a larger width and harder filtering. The program calculates the average value of the signal within that time.

  Autoregressive
  This is a more efficient filter for regular noise. A filter constant value is set; the higher the constant, the more efficient the filtering.

  Median
  This filters occasional noise such as air bubbles. It should not be used for periodic noise. A width which is more than twice the width of the noise peak should be entered. The program calculates a medium value of the signal within that time.

- **Normalize**
  Normalize allows a specified curve to be normalized to another curve when comparing the two curves. The scaling can be changed in all directions manually or automatically using the Normalize function.

  The curve to be normalized is displayed within a frame which is used for manual normalization. By clicking on an area within the frame and keeping the mouse button depressed, the curve in the frame can be moved to any position in the window. It is then fixed in that position by releasing the mouse button. In addition, the four sides of the frame can be used individually to change the size of the curve. By passing the cursor over one side of the frame, it changes to a double-headed arrow. By clicking and keeping the mouse button depressed, the active side of the frame can be moved to another position to re-size the curve.

  The following pull-down menus are available:

  File

  Open Help Curve opens a specified help curve which is to be compared with the curve to be normalized.

  Clear Help Curve clears the help curve.

  Save Normalized Curve saves the normalized curve in a temporary curve memory. The user can either accept the default name or type in his own choice. To save the curve in the Result File, the function File, Save must be used.

  Close Window closes the Normalize Curve window. If the curve is not saved, a warning is given.

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Normalize

Highest peak normalizes the retention time of the specified curve on the highest peak. The highest peak in AU in the normalized curve is given the same retention time as the highest peak in the help curve.

Move

This function moves the specified curve in relationship to the help curve. By clicking on Move, a hand cursor appears in the centre of the rectangle. The hand can be moved to any position required and can then be fixed by clicking once again.

Size

This function allows the size of the specified curve to be changed.

- **Differentiate**
  This function calculates and shows the derivative of the curve specified and stores the result in a temporary curve memory, under the default curve name or a name entered by the user. The 1st (default) and 2nd order derivative and the inverse derivative can be calculated.

  The inverse derivative is used to compare the amount of sample resulting from different runs. The height of the curve is equal to the accumulated area above zero. The inverse derivative is equivalent to the mathematical integration of the curve.

  The 1st and 2nd order derivatives are used to study the peak shoulders when searching for impurities.

  The Differentiate function can also be used to determine the slope limit for baseline calculation, see Section 4.8.7. It an also be used to calculate the slope of peaks for Peak Fractionation with slope detection, see Section 4.7.2.

- **Fraction histogram**
  This function automatically creates a bar diagram of the specified curve with the bars starting and ending at the fraction marks.

  When using this function on the UV absorbance curve, the total UV absorbance in each fraction is displayed. When used on the conductivity curve, the salt concentration in each fraction can be calculated.

- **Pool fractions**
  This function allows visualising fractions that has been pooled together. A new fraction mark curve is created. To visualise the effect of this on the absorbance, another fraction histogram can be created using the new pooled fraction mark curve.

- **Create histogram**
  This function creates a histogram from any data entered by the user within each fraction. It can be used to store and present activity data together with the chromatogram. The fraction mark record is selected and the Create histogram window is displayed. Points can be entered and the histogram calculated using the Draw Curve function.

File

Open Help Curve 1 opens a specified help curve to assist

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the user when creating the histogram. Help curves are selected from the curves already stored in the Result File. Help curve 1 is fraction marks.

Open Help Curve 2 opens another specified help curve. Help curve 2 is the UV curve.

Clear Help Curve 1 clears help curve 1.

Clear Help Curve 2 clears help curve 2.

Set Axis Scale allows the user to type in the minimum and maximum values required on the x- and y-axes.

Save Created Histogram saves the created histogram in a temporary curve memory. The user can accept the default name or type in his own choice. To store the created histogram in the Result File, the function File, Save must be used.

Close Window closes the Created Curve window. If the curve is not saved, a warning is given.

Draw curve

Draw Spline calculates and draws a spline curve between the marked points. This is a smooth curve which does not pass through all points.

Draw Through Points calculates and draws through the marked points.

Draw Histogram calculates and draws a histogram using the marked points.

Clear Drawn Curve deletes the drawn curve or histogram.

Points

View Point List displays the box showing the list of all marked points in minutes or in millilitres.

Set Point sets a new point by displaying a box where the x- and y-values (time or volume, and amplitude) of each point can be entered by the operator.

Delete Point deletes the point specified by the user. The point to be deleted is selected on the point list followed by Delete point. The time or volume and the amplitude of the marked point are displayed for the user's confirmation. Points can also be deleted by double-clicking on the point in the point list.

Delete Last Point deletes the last point marked.

Delete All Points deletes all points and the interpolated curve after confirmation by the user.

Set Activity Point sets a point in each fraction. The point is entered numerically.

Zoom

This function allows any part of the Create Histogram window to be enlarged. This allows the points marking the new curve or histogram to be selected more accurately. By clicking on Zoom, the cursor changes to a pointing finger which is used to draw a box around the

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area to be enlarged. When the mouse button is released, the area is magnified. After enlarging, clicking on Unzoom returns the area to its original size.

- Undo
  This function cancels the last operation in the curve menu.

Integrate

The Integrate function performs the integration calculation according to the parameters set by the user (Fig. 5.55), or with the settings automatically given by the program.

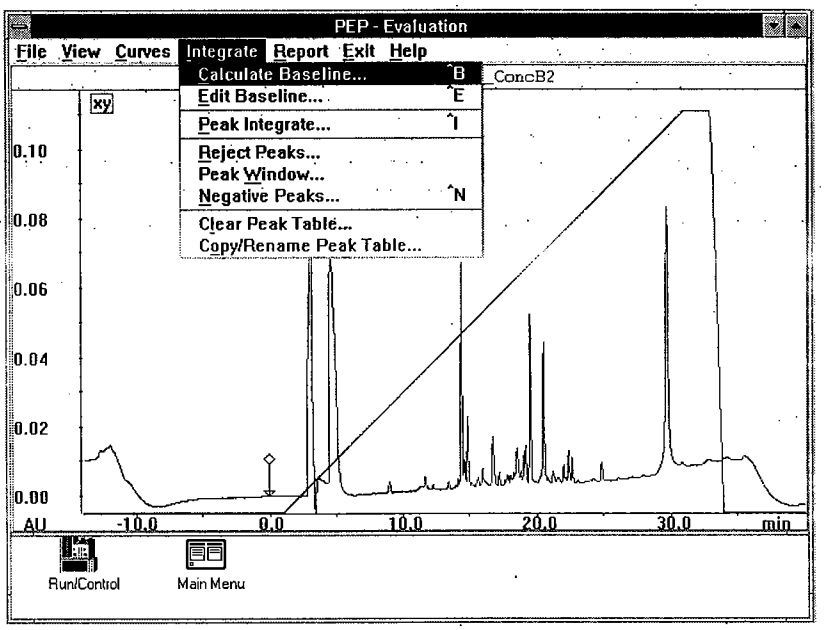


Fig. 5.55. Integrate window

- Calculate baseline
  A baseline can be calculated for the specified UV absorbance curve. The parameters used for the calculation are the shortest baseline segment in minutes or millilitres and the noise window in AU. Suitable values for all parameters are suggested by the program.

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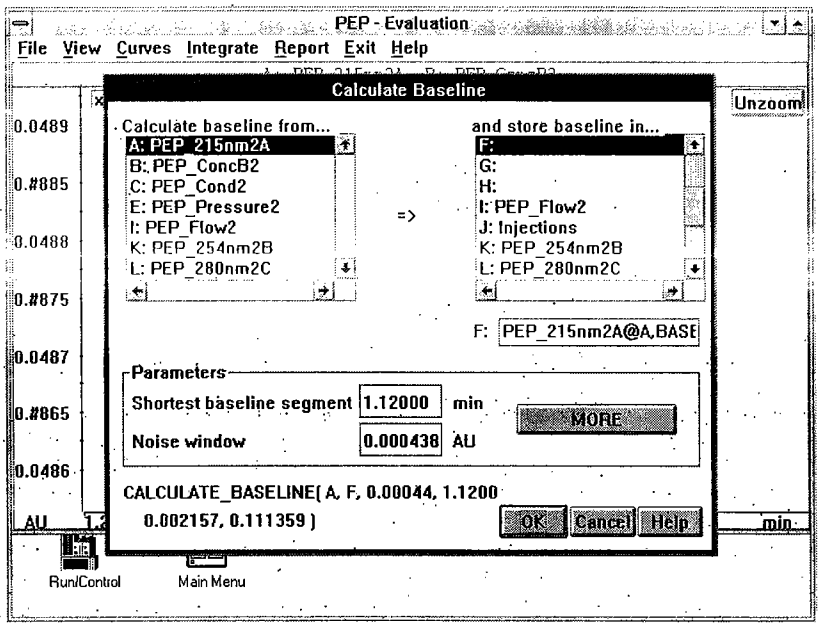


Fig. 5.56. Calculate Baseline window

Further parameters can be changed by clicking on the MORE button.

Maximum baseline level is the highest acceptable absorbance value for the baseline. The default value calculated by the program can be accepted for most applications except when the peaks are very wide and flat.

Slope limit is for the baseline the highest acceptable slope in AU/min.

A search on the chromatogram identifies baseline points according to the selected limits. The baseline is calculated as a smooth curve through all the fixed baseline points.The calculated baseline is stored in the curve memory. To store the baseline in the Result File, the function File, Save must be used.

Fore a more detailed description of the baseline parameters, please see Chapter 4, Section 4.8.7.

- Edit baseline
  The calculated baseline can be changed using the Edit Baseline window. Baseline points can be deleted or added by clicking on the curve or by using the Points list box.

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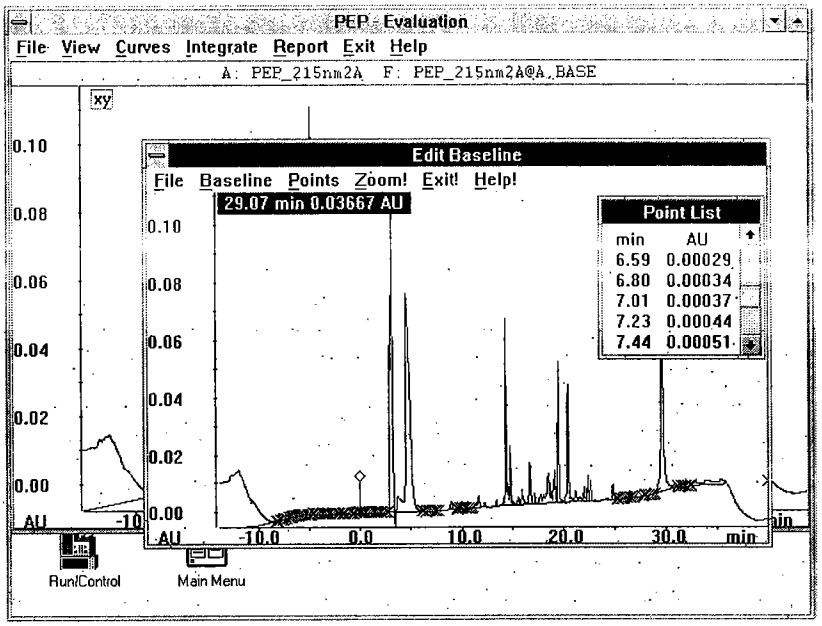


Fig. 5.57. Edit Baseline window

The following pull-down menus are available:

File

Open Help Curve 1 opens a help curve to assist the user when creating other curves. Help curve 1 is as default the UV curve from the Result File.

Open Help Curve 2 opens a second help curve. Help curves are already stored in the current Result File. Help curve 2 is as default the calculated baseline.

Clear Help Curve 1 clears help curve 1 from the screen.

Clear Help Curve 2 clears help curve 2 from the screen.

Set Axis Scale allows the user to specify the minimum and maximum values for x- and y-axes.

Save Edited Baseline saves the baseline curve in the curve memory at a selected position, under either the default name or under a new name entered by the user.

Close Window closes the Modify Baseline window

Baseline

Draw Baseline calculates and draws the baseline curve. This produces a smooth curve which may not pass through all the marked points.

Clear Baseline clears or deletes the newly drawn curve.

Points

View Point List displays the box listing the values of all marked points.

Set Point sets new points by displaying a box where the x- and y-values can be entered by the user. Points can also be added by clicking directly at the desired position on the screen.

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Delete Point allows a point on the points list to be specified by the user and to be deleted. The point can be specified either by highlighting on the list box or by typing in. A point can also be deleted by double-clicking on it in the point list.

Delete Last Point deletes the last entered point.

Delete All Points deletes all points and the interpolated curve after confirmation by the user.

Zoom

This function allows any part of the newly created curve to be enlarged. By clicking on Zoom, the cursor changes to a pointing finger. The area to be enlarged is then identified by drawing a box around it while keeping the mouse button depressed. When the mouse button is released, the box is magnified. After enlarging, Unzoom returns the curve to the original size.

- **Peak Integrate**

  This function performs integration on the curve specified by the operator. The user also specifies a name for the resulting peak table.

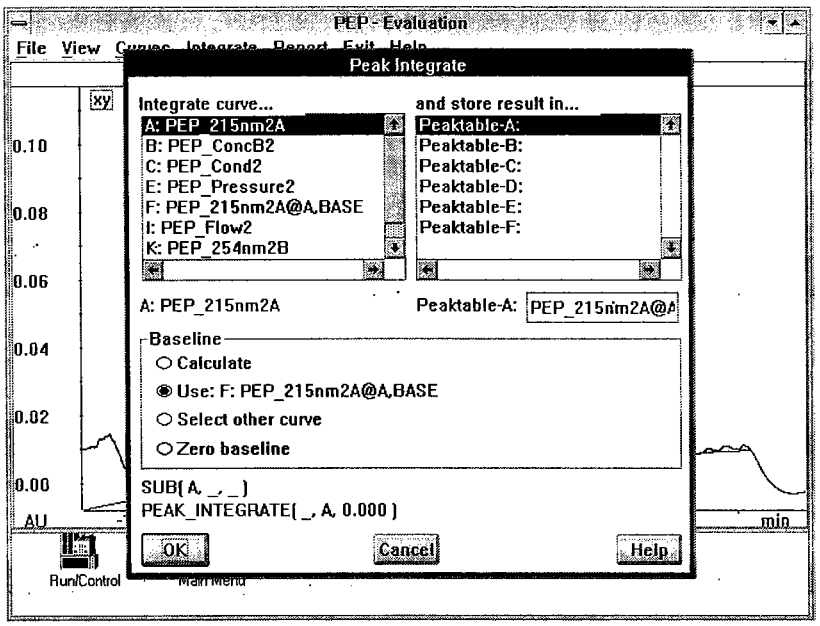


Fig. 5.58. Peak Integrate window

The baseline is selected from the following options:

Calculate creates a new baseline using the same procedure as in the Calculate Baseline function.

Use uses the latest baseline created in the Calculate Baseline function.

Select other curve uses the curve already created using the Calculate Baseline function or another curve, such as a curve generated by a blank run. The curve of choise is selected from the curve memory list box that appears after clicking OK in the Peak integrate window.

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Zero baseline uses a straight line located at 0 absorbance units as baseline.

After performing the integration, the curve is redrawn showing the peaks identified by number or retention time in the peak search as selected in View, Curve Window Layout. A Peak Data Table is produced with all items chosen in View, Peak Window Layout. These include the peak number, retention time, left limit, right limit, duration, area and height listed for all peaks identified.

- **Reject Peaks**
  This function sets the peak size limits for identification by the program during integration. The parameters are:

  Height is at least sets the minimum peak height in AU

  Width is at least sets the minimum width at baseline of the peak in minutes or millilitres

  Area is at least sets the minimum area (AU x minutes or AU x millilitres) of the peak

  Peak area is one of a number of the largest sets the total number of peaks to be identified

  If integration has been performed previously, the Peak Integrate window appears again so that integration can be performed using the the reject peak limits. Peaks outside these size limits are rejected and do not appear on the Peak Data Table.

- **Peak Window**
  This function selects a part of a curve on which peak integration is to be performed.

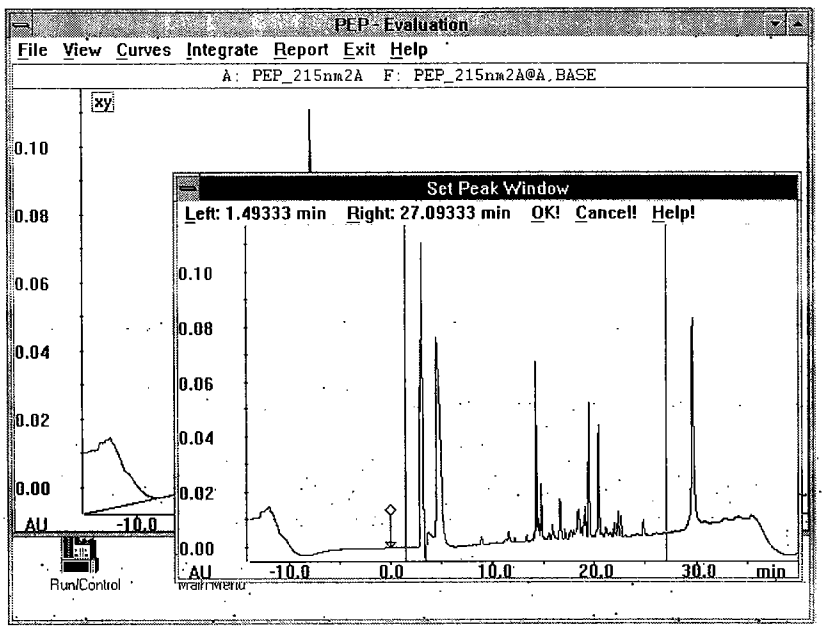


Fig. 5.59. Peak window

In the Peak Window the curve is selected. The Set Peak Window screen is displayed and the part of the curve to be integrated is selected by using the drag bars or by typing in the minimum and maximum values. This function can be used when individual parts of the chromatogram are to be integrated

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separately using different integration parameters. After setting the part of the curve to be integrated, the Peak Integrate window appears so that integration can be performed.

- Negative Peaks
  With this function it is possible to select if negative peaks should be accepted or not during integration.

Report

The Report function allows selected data to be sent to the printer, plotter or screen display. The user selects the items to be included in the report.

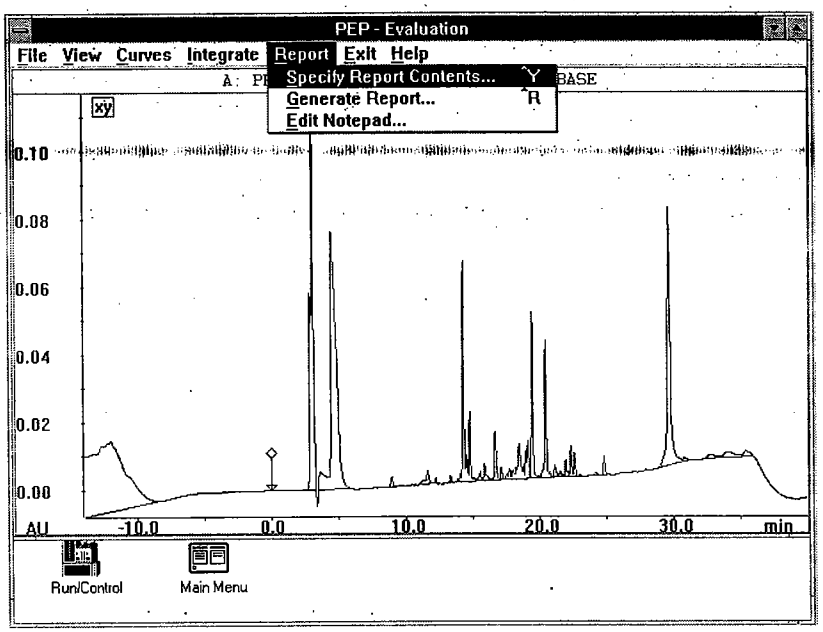


Fig. 5.60. Pull-down menu Report

- Specify Report Contents
  This function selects the items to be included in the report (Fig. 5.61). Sub items can be selected for each item. The available sub items will appear in the right hand list box when clicking on the Sub items option button.

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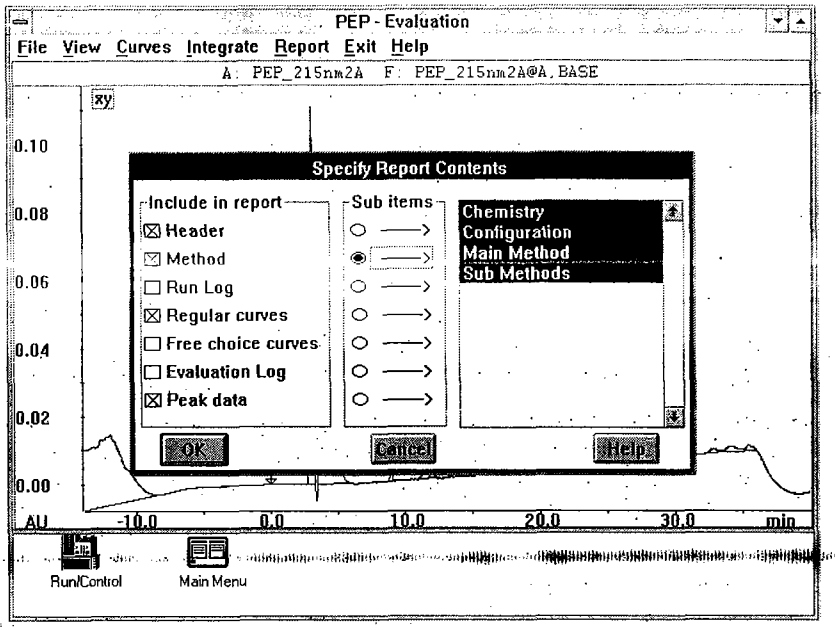


Fig. 5.61. Specify Report Contents window

Items can include the following:

Header (default) including the sub items current user, run user, current date and time, run date and time, report title, name of Result File, Method name, frames and notepad

Method (default) including the sub items Chemistry, Configuration, Main Method, Sub Methods

Run Log including the sub items occurrences, warnings and errors

Regular curves (default) including the sub items Chromatogram, gradient conductivity, programmed gradient, fraction marks, baseline, programmed flow

Free choice curves, the curves of choise are selected from the curve memory when generating a report

Evaluation Log

Peak data (default)

- **Generate report**
  This function sends the report to the printer, plotter or screen. The user can specify a name or heading for the report. There is an option to review the default items. It is possible to change the default items using this option. The items that can be reviewed and/or changed are regular curves and peak table.

  If the report is sent to the screen, the Report window is displayed. The menu headers are as follows:

  Next displays the next Report window, Chromatogram Peak Data Table, Run Log, Method and Evaluation Log

  Previous displays the previous Report window

  Exit terminates the Report window

  If the report is sent to a printer/plotter the printer properties window will appear.

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- **Edit Notepad**
  This option accesses the notepad for editing comments to be included in the report. The Notepad is automatically saved with the Result File.

Exit

- Exit
  This function terminates the evaluation and returns to the Result File Contents screen. Confirmation is required.
- **Resume evaluation**
  This function resumes to the evaluation process.

Help

- **Help for Help**
  This function describes how to use the Help command.
- Extended Help
  This function provides general information about Evaluation. More detailed information is given for each menu in Evaluation.
- **Keys Help**
  This function describes how to use the keyboard for specific tasks in Evaluation.
- **Help index**
  This function contains a complete list of all instructions in the Evaluation you can obtain help for.

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5.6 SMART Assistant

5.6.1 SMART Assistant overview

SMART Assistant is a knowledge and information base dedicated to SMART System. It is designed to assist the user in finding the most appropriate techniques, media and generating suitable methods to reach the goal for the separation.

SMART Assistant asks the user about the separation goal and the characteristics of the target protein and about the sample solution. Then the user gets a suggestion on an appropriate separation strategy. Different chromatographic techniques and combinations of techniques are suggested and ranked in order of suitability. The user selects a separation pathway and can then get suggestions on appropriate chromatographic media. The user can get information on why the SMART Assistant recommends or not recommends a technique or media and what characteristics the different media and columns have.

When the user has decided which media and column to be used, SMART Assistant creates a Separation Method to be transferred to SMART Manager where it can be run and/or edited. The user can also get advice on how to prepare the buffers needed and how to configure SMART System prior to run.

If problems occur during the separation the user can ask SMART Assistant for help to diagnose the problems and to obtain suggestions how to proceed.

In Section 4.9.3 there is an example of a typical way to work with SMART Assistant.

5.6.2 SMART Assistant screens

SMART Assistant is entered from the Main Menu, SMART Assistant pull-down menu. When selecting Start Assistant the introductory screen appears (Fig. 5.62). A new case is started either by clicking on Go or by selecting FILE, NEW CASE from the menu.

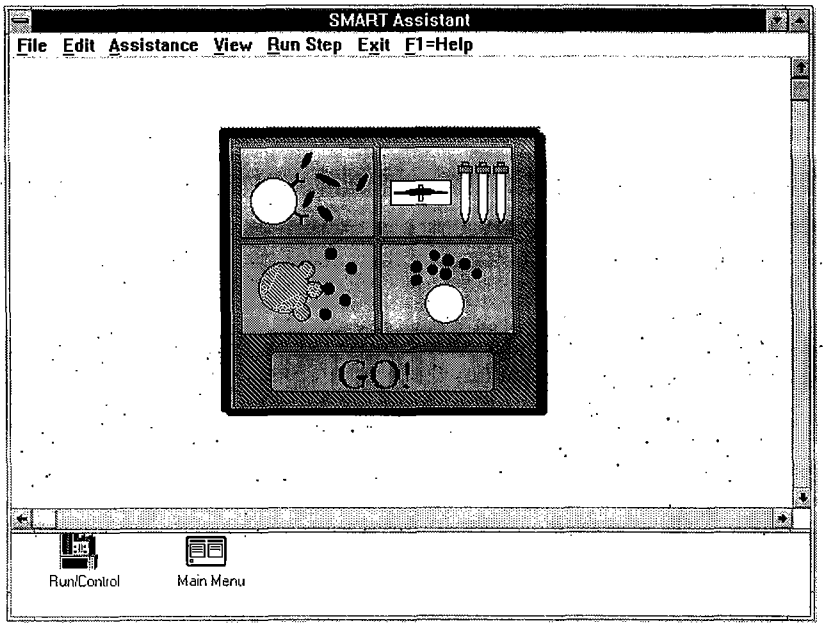


Fig. 5.62. SMART Assistant introductory screen

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Goal Selection Window

For each new case study, the operator enters the aim of the separation in the Goal Selection Window (Fig. 5.63). The Technique list box lists 8 options, each one relating to a different list of goals found in the Goal list box. The selection in the technique list box decides what goals can be obtained with the separation, it does not influence the techniques the SMART Assistant will recommend as suitable. The goals relating to each technique are listed below.

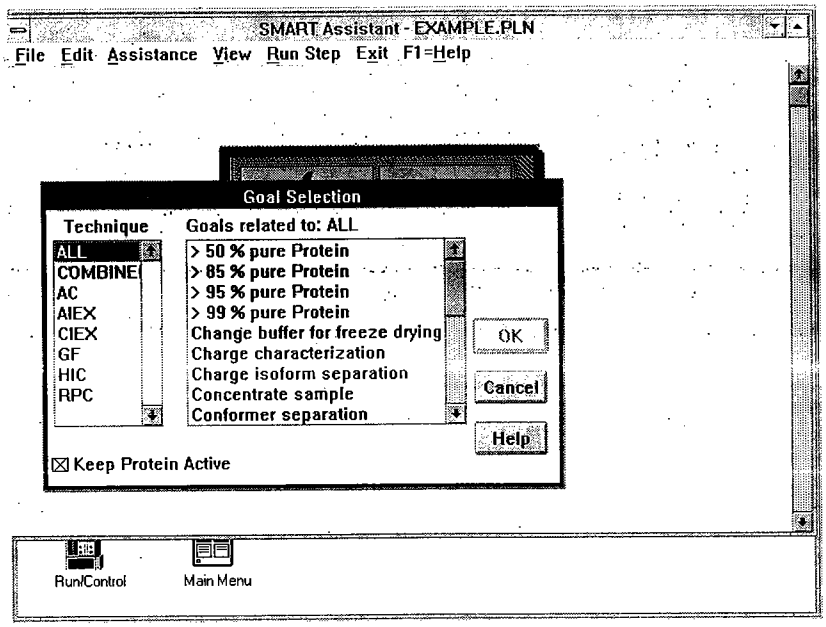


Fig. 5.63. Goal Selection window

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The table below shows all separation goals possible in SMART Assistant and for which technique the selection is available.

Available goals	Technique selection
>50 % pure protein	ALL, COMBINED
>85 % pure protein	ALL, COMBINED
>95 % pure protein	ALL, COMBINED
>99 % pure protein	ALL, COMBINED
Change buffer for freeze drying	ALL, GF
Charge characterization	ALL, AIEX, CIEX
Charge isoform separation	ALL, AIEX, CIEX, RPC
Concentrate sample	ALL, AC, AIEX, CIEX, HIC, RPC
Conformer separation	ALL, HIC, RPC
Fractionate and purify	ALL, AC, AIEX, CIEX, GF, HIC, RPC
Hydrophobicity characterization	ALL, HIC
MW determination of target protein	ALL, GF
Protein digest fractionation	ALL, AIEX CIEX, GF, HIC, RPC
Purity determination of target protein	ALL, AIEX, CIEX, GF RPC
Removal of undigested protein	ALL, AC AIEX, CIEX, GF, RPC
Remove acidic contaminants	ALL, AIEX, CIEX
Remove basic contaminants	ALL. AIEX, CIEX
Remove dimers or polymers	ALL, GF
Remove high MV proteins	ALL, GF
Remove low MV proteins	ALL, GF
Remove salt from sample	ALL, GF, HIC, RPC
Size characterization	ALL, GF

Abbreviations:
AC: affinity chromatograpy
AIEX: anion exchange chromatography
CIEX: cation exchange chromatography
GF: gel filtration
HIC: hydrophobic interaction chromatography
RPC: reversed phase chromatography

In addition, if the protein is to be kept active during the separation, the KEEP PROTEIN ACTIVE box is selected.

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Protein Characteristics window

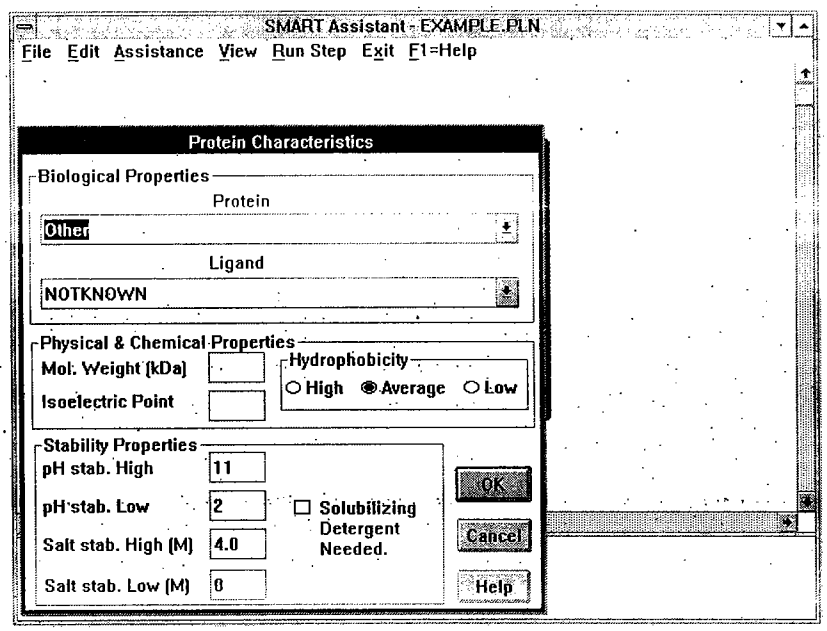


Fig. 5.64. Protein Characteristics window

In this window, the user enters the protein and ligand (if known), together with the following physical and chemical properties and stability characteristics (Fig. 5.64):

molecular weight
isoelectric point
hydrophobicity (High, Average, Low)
pH stability high
pH stability low
salt stability high (M)
salt stability low (M)
need of solubilizing detergent

Note: If either the molecular weight or the isoelectric point of a protein is not known, the field can be left blank.

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State of Sample window

In this window, the user defines the state of the sample (Fig. 5.65).

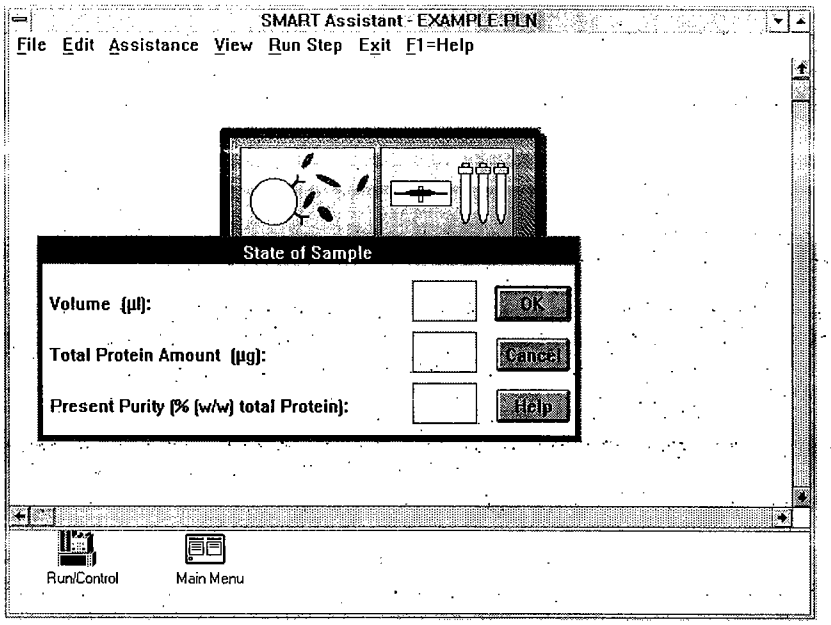


Fig. 5.65. State of Sample window

The following information is entered:

- volume in µl
- total protein amount in µg
- present purity (% (w/w) total protein)

Listing of techniques

All available chromatography techniques are evaluated by the program and are then listed in order of preference for the stated aim of the study (Fig. 5.66). Those with a green border are recommended for this application, those with a yellow border are acceptable while those with a red border are unsuitable for use and should be avoided. A box with a blue border indicates that an additional step can be evaluated later.

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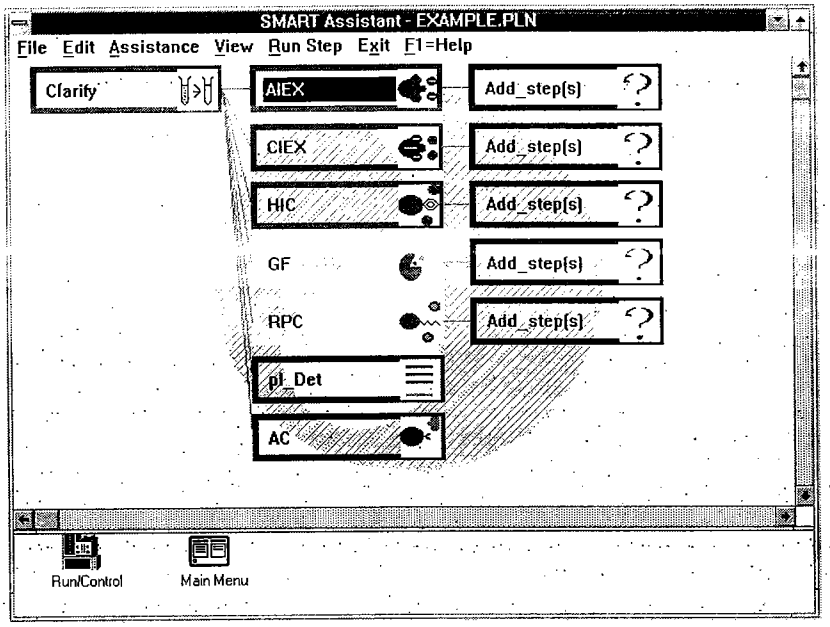


Fig. 5.66. Listing of techniques

The user can obtain further information on each of these techniques either by double-clicking on the technique to access the Actions on the Box window (see below) or by using the menu headers, View or Assistance (see Section 5.6.3).

Actions on the box

By double-clicking on the selected item in the list of techniques, the "Actions on the Box" window is displayed (Fig. 5.67). This window is the main pathway for the interaction with SMART Assistant by the user. The most commonly used action and information commands are available in this window. The options become available when they are relevant to the highlighted item.

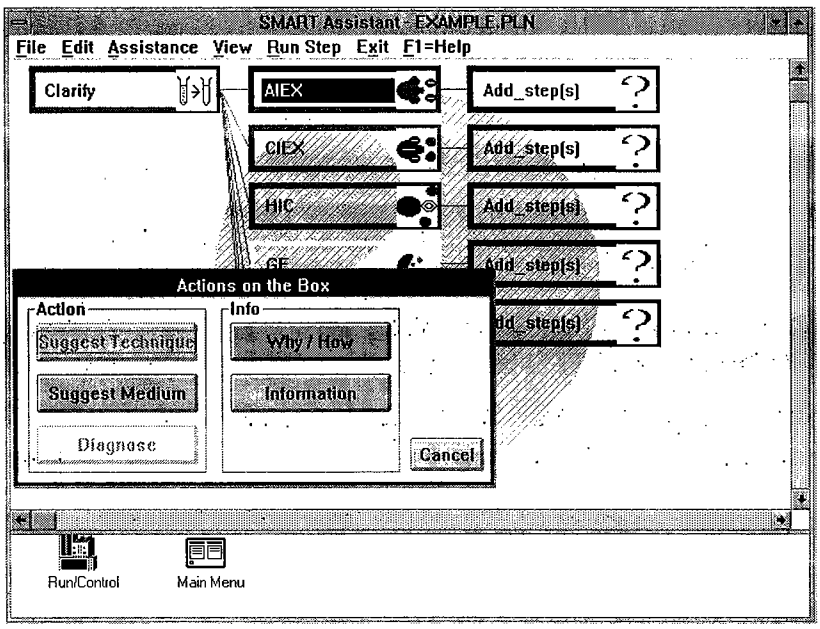


Fig. 5.67. Actions on the Box window

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Suggest Technique requests SMART Assistant to recommend a suitable technique which will accomplish the aim of the separation and is used for the blue boxes for additional steps.

Suggest Medium requests SMART Assistant to recommend a medium for use with the selected technique. If several media are available for the highlighted technique, a window with these will appear. The different media will have colour coding, (green, yellow or red), to indicate the recommendation level. A media is selected by highlightening it and selecting OK in the menu.

Diagnose requests SMART Assistant for the possible cause of and remedy for problems which occurred during the run. This function can be used after the run if the separation has been unsuccessful.

Why/How requests SMART Assistant for information on the reason why the highlighted technique or medium has been recommended. Reasons for choosing the selected medium and a detailed description of the elution method is provided together with details of buffer mixing. The disadvantages of the unsuitable techniques are also given

Information requests SMART Assistant for information on the highlighted technique or the medium. The complete specification of the medium is given

Recommended medium

When a medium is selected, its suitability for the stated goal is shown as an inner frame within the original border of the box. The same colour coding is used as denotes the level of suitability of techniques. A box of mixed colours will result where the recommendation level of the technique is different from that of the medium.

5.6.3 SMART Assistant pull-down menus

The most commonly used commands are described in the section above. This section will give a complete description of all available commands in SMART Assistant.

File

The File pull-down menus are used to load, save and print files (Fig. 5.68).

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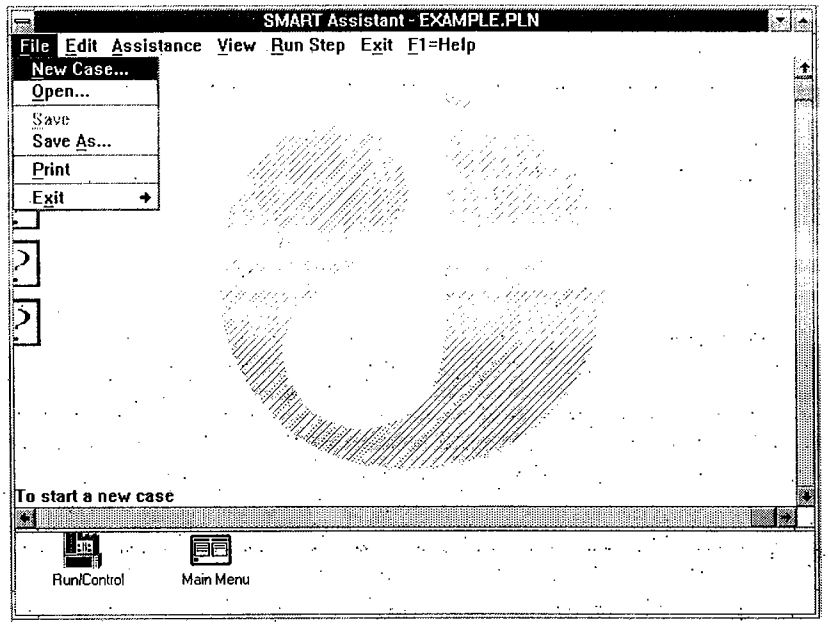


Fig. 5.68. Pull-down menu File

- **New Case**
  A new case study for SMART Assistant is opened. If the previous case has not been saved, a warning is given that this information will be lost.
- **Open**
  A previous case study which has already been saved can be opened. The directory is specified in addition to the file name.
- **Save**
  The current case study is saved under the current file name specified by the user.
- **Save As**
  The current case study can be saved under another file name specified by the user.
- **Print**
  The strategy on the screen can be printed.
- **Exit**
  Work with SMART Assistant is terminated and the screen returns to SMART Manager Main Menu.

Edit

These pull-down menus comprise functions which relate to editing of the recommended series of techniques.

- **Select path**
  This function removes all alternative paths, except the on highlighted by the user. The final step or the preferred pathway must be highlighted prior to selection of this function.
- **Rewind technique**
  The screen returns to a previous step in the pathway and all steps following the highlighted step are deleted.

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- User Notes
  A note pad is accessed where the user can record comments or notes relevant to the current selected technique. This has standard editing facilities of cut, copy, paste, undo and select all. Clicking on exit terminates work with the notepad. Comments made in the notepad are saved with the strategy (Fig. 5.69).

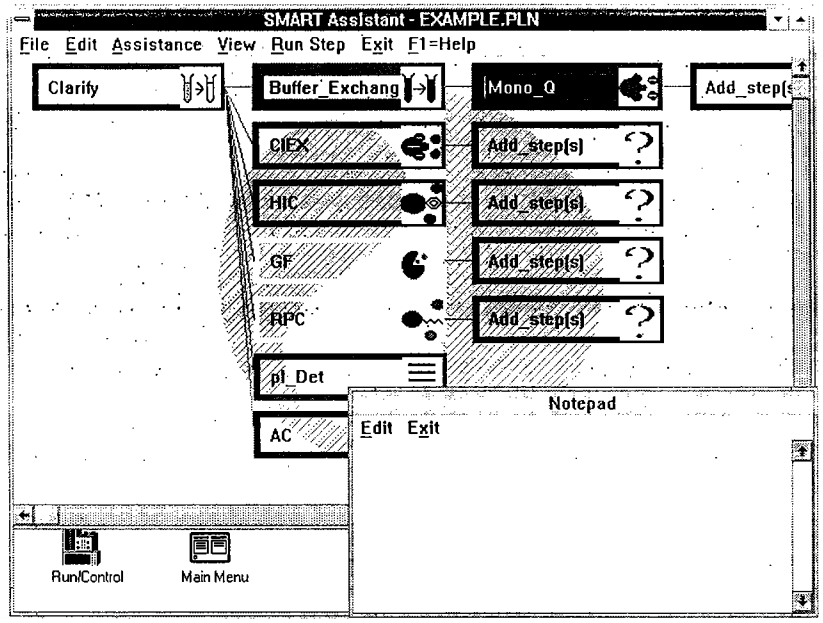


Fig. 5.69. User notes

- End Sample Result
  The user can enter the result after the final step in the separation, if it differs from the result received by SMART Assistant. If the results from the preceding steps are less than expected, this option has the effect of making SMART Assistant add another step in the pathway.

Assistance

The Assistance pull-down menu provides help in various ways. This option has a similar function to the "Actions on the Box" window. The options become available when they are relevant to the highlighted item. A technique or medium can be evaluated and presented and the cause of problems can be found.

- Suggest technique
  This option requests SMART Assistant to recommend a suitable technique which will accomplish the aim of the separation and is used for the blue boxes for additional steps.
- Suggest medium
  Suggest Medium requests SMART Assistant to recommend a medium for use with the selected technique. If several media are available for the highlighted technique, a window with these will appear. The different media will have colour coding, (green, yellow or red), to the recommendation level. A media is selected by highlightening it and selecting OK in the menu.

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- Diagnose
  This option requests SMART Assistant for the possible cause of and remedy for problems which occurred during the run. This function can be used after the run if the separation has been unsuccessful (Fig. 5.70).

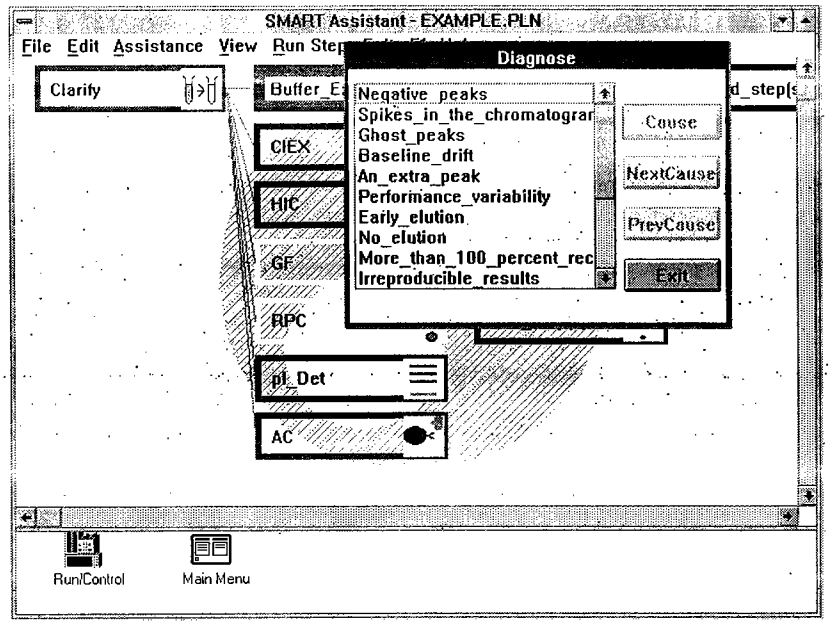


Fig. 5.70. Diagnose window

View

This pull-down menu accesses further details on the highlighted option. Some functions in this option are similar to the "Action on the Box" window.

- **Why/How**
  Using this option, the user can request information on the reason why the highlighted technique or medium has been recommended. Reasons for choosing the selected medium and a detailed description of the elution method is provided together with details of buffer mixing. The disadvantages of the unsuitable techniques are also given (Fig. 5.71).

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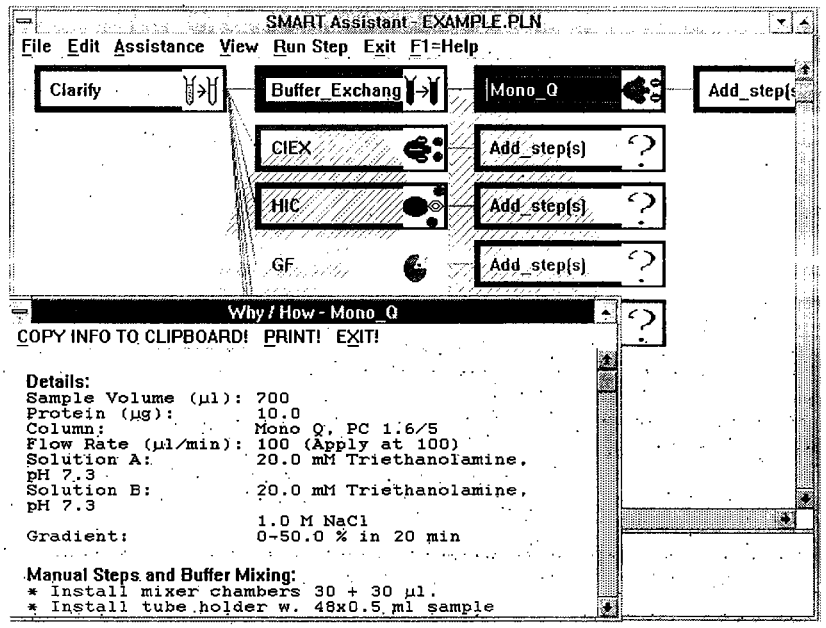


Fig. 5.71. Why/How window

- **Information**
  This option gives detailed information on the highlighted technique or the medium. The complete specification of the medium is given (Fig. 5.72).

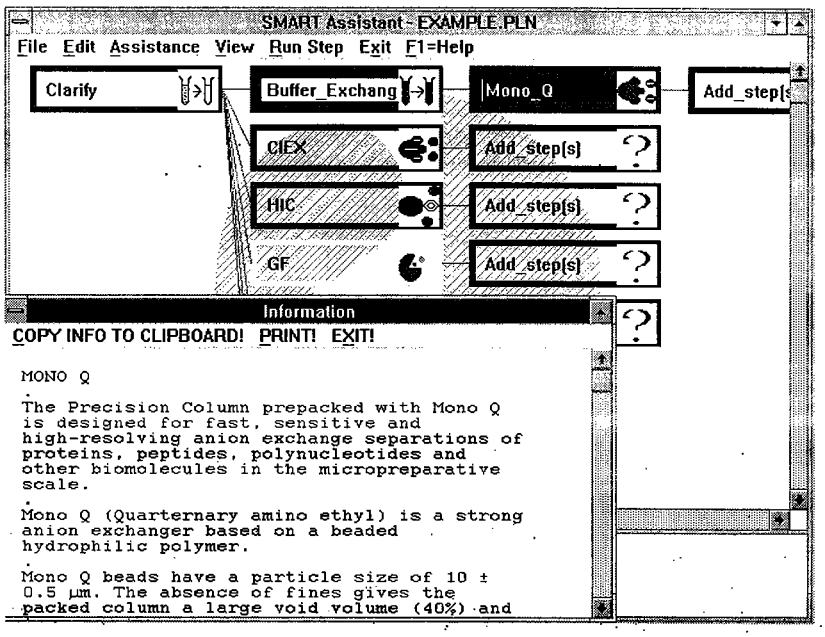


Fig. 5.72. Information window

- **Status**
  This option gives information on the current state of the sample and, where applicable, on its expected state of purity after the marked step has been performed.

- **Case data**
  This option gives information on the case study. This is the information presented by the operator at the beginning of the

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study. It includes the sample, its characteristics and type and the aim of the study (Fig. 5.73).

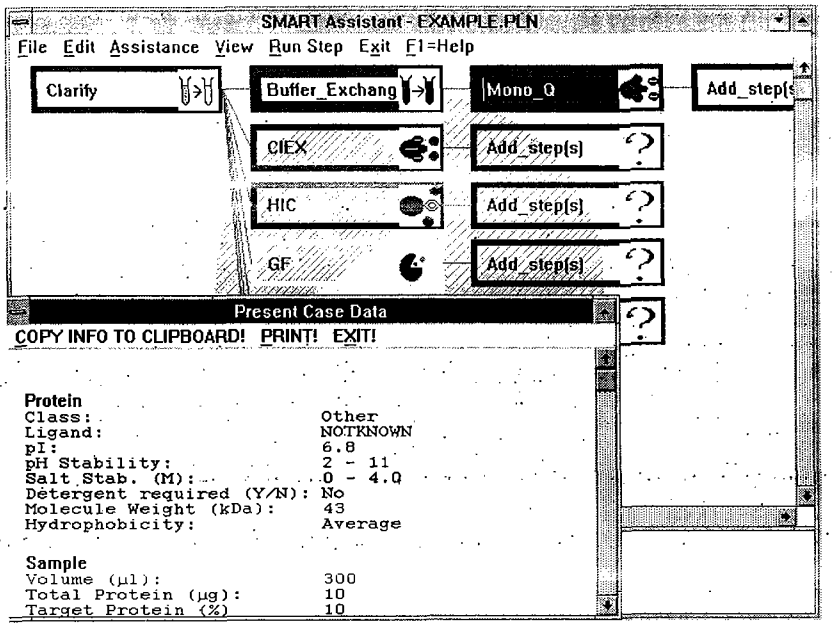


Fig. 5.73. Case Data window

- Summary
  This option is only available in the menu during a multistep separation. The function is used to get a list of all steps in the selected pathway and to show the derived volume and total amount of protein after each step.

- Monochrome
  This option configures the screen for a monochrome display. Different line types are used instead of colour to indicate a technique which is recommended (continuous line), acceptable (broken line) or not recommended (dotted line). For steps not yet defined, a dot-dash line is used.

Run step

This pull-down menu is the interface with SMART Manager for the creation of a suitable Separation Method for each step in the recommended pathway.

- **Transfer to SMART**
  This option creates a Separation Method for the marked step in the recommended pathway. The Save SMART File window is displayed on the screen. The user enters the name of the method. It is stored in SMART Manager in the same way as any other Separation Method.

  The OPTIONS button gives the user a possibility to control the content of the generated method, such as Pump Wash before method start and an Automatic Evaluation at the end of the run (Fig. 5.74). The user can also specify the pump cylinder size, method base and which monitor to use. As default the option configuration is the same as for the previously transferred method except for pump cylinder size, which is as default the size most suitable for the method.

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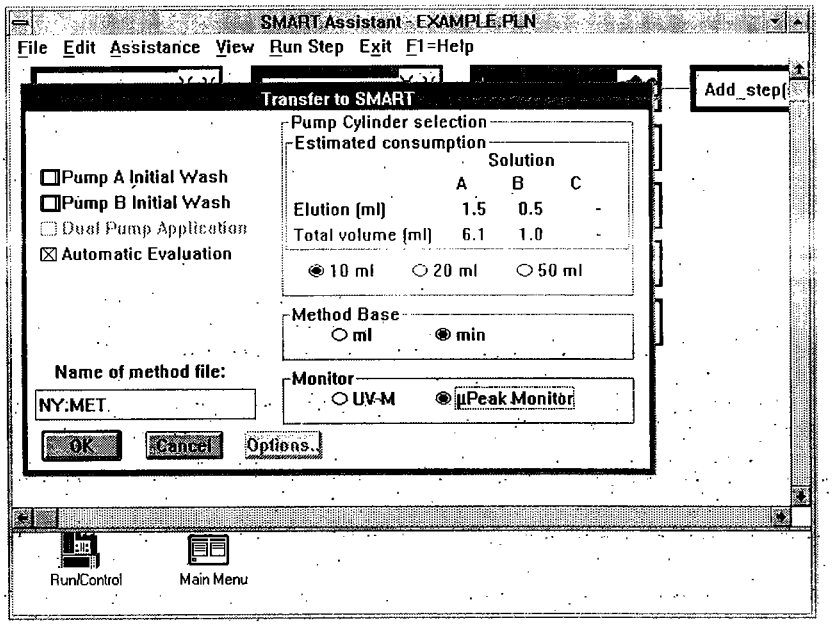


Fig. 5.74. Transfer to SMART, Options window

The method is transferred to the File Contents window of SMART Manager the method can be run or edited as any other method in SMART Manager.

Help

This pull-down menu gives help with what to do next and with the preparation of buffers for the run.

- **What to do now**
  This option offers relevant advice to the user on the next step in the process of determining a satisfactory separation technique (Fig. 5.75).

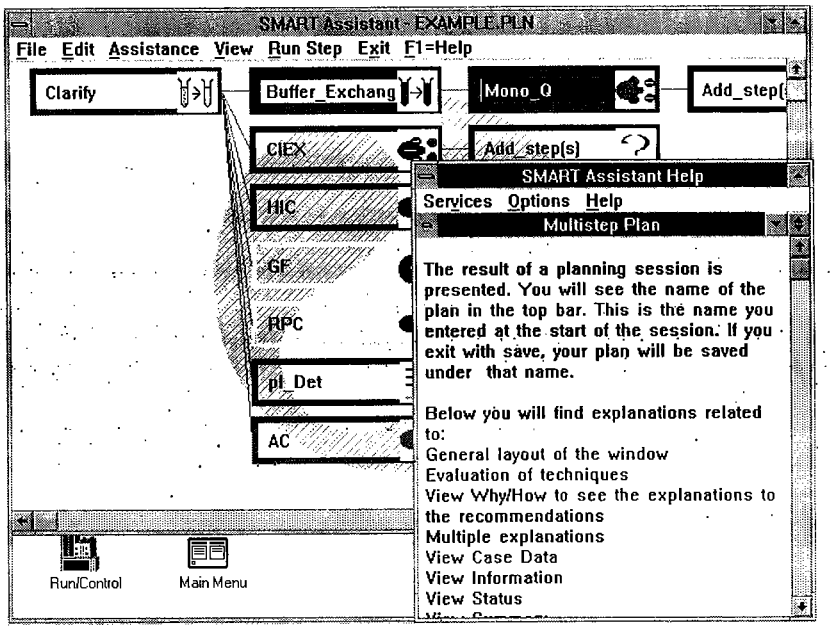


Fig. 5.75. What to do now window

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- Buffer mixing
  This option informs the user of how to prepare buffers for the separation (Fig. 5.76). The components of the buffers and the amounts to be used are given.

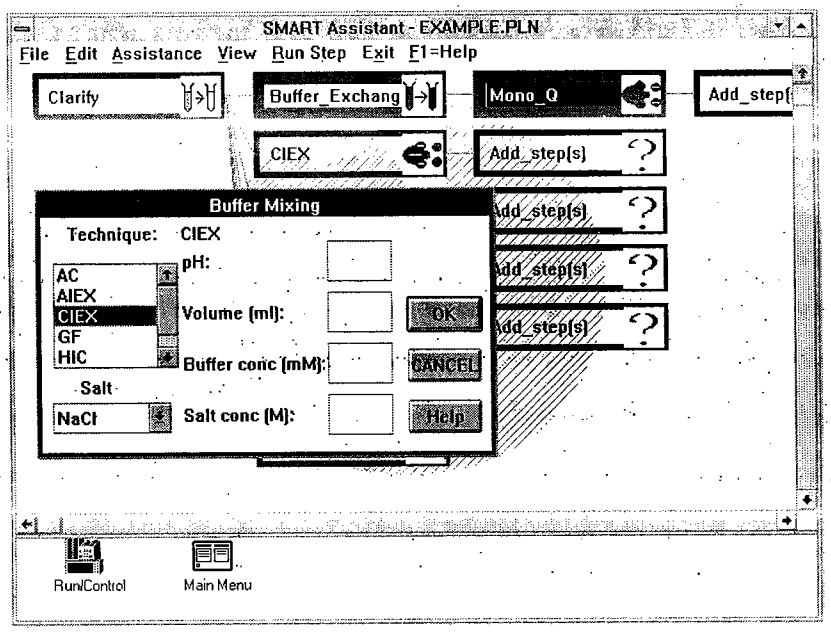


Fig. 5.76. Buffer mixing window

- About
  This option gives a brief overview of the aim of the SMART Assistant. It includes the copyright notice and the program version number.

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Appendix 5.1

Appendix for Library methods

CONC.MET	is a method for concentration of large sample volumes. The method is described in Method Manual, Section 7.2
FD_3210.MET	is a standard method for desalting on the Fast Desalting PC 3.2/10 column
MQ_165.MET	is a standard separation method for the Mono Q PC 1.6/5 column
MS_165.MET	is a standard separation method for the Mono S PC 1.6/5 column
NHSCOUPL.MET	is a method for coupling a ligand to the NHS-activated Superose® PC 3.2/2. The method is described in the Method Manual, Section 8.5.2.
NHS_322.MET	is a standard separation method for the NHS-activated Superose PC 3.2/2 column
PS_165.MET	is a standard separation method for the Phenyl Superose PC 1.6/5 column
PUMPW10.MET	is a method for a quicker wash than using the MANUAL, PUMP WASH command, of the 10 ml pump cylinders to be used for example when changing between similar eluents
PUMPW20.MET	is a method for a quicker wash than using the MANUAL, PUMP WASH command, of the 20 ml pump cylinders to be used for example when changing between similar eluents
PUMPW50.MET	is a method for a quicker wash than using the MANUAL, PUMP WASH command, of the 50 ml pump cylinders to be used for example when changing between similar eluents
R18S2110.MET	is a standard method for the Sephasil C18 SC 2.1/10 column
R18_2110.MET	is a standard separation method for the µRPC C2/C18, SC 2.1/10 column
R18_323.MET	is a standard separation method for the µRPC C2/C18, PC 3.2/3

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R8S2110.MET	is a standard separation method for the Sephasil C8 SC 2.1/10 column
S12_3230.MET	is a standard separation method for the Superose 12 PC 3.2/30 column
S6_3230.MET	is a standard separation method for the Superose 6 PC 3.2/30 column
SYSW10.MET	is a much quicker system wash for 10 ml cylinders, than using the MANUAL, SYSTEM WASH command
SYSW20.MET	is a much quicker system wash for 20 ml cylinders, than using the MANUAL, SYSTEM WASH command
SYSW50.MET	is a much quicker system wash for 50 ml cylinders, than using the MANUAL, SYSTEM WASH command
UPEAKGRD.MET	is an example of a gradient separation method using the µPeak Monitor functions
UPEAKISO.MET	is an example of an isocratic separation method using the µPeak Monitor
X75_3230.MET	is a standard separation method for the Superdex 75 PC 3.2/30 column

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Appendix 5.2

Short key instructions for Smart Manager

Run/Control:

Ctrl key	Instruction
C	Conc %B
F	Flowrate
D	Gradient
J	Injection valve
S	Frac stop
L	Large tube
G	Goto tube
T	Feed tube
P	Peak fractionation
R	Record chromatogram
V	Wavelengths
Z	Autozero

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Evaluation:

Ctrl key	Instruction
T	Print
P	Evaluation procedure
S	Select window contents
L	Window layout
+	Add
-	Subtract
/	Divide
C	Cut chromatogram
K	Copy/rename
Z	Create curve
H	Smooth
D	Differentiate
F	Fraction histogram
B	Calculate baseline
E	Edit baseline
I	Integrate
N	Negative peaks
V	Specify report contents
R	Report
Q	Exit

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6.23 Defrosting the µSeparation Unit 50

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New maintenance procedures for 10 ml pump cylinders

INSTRUCTIONS

The pump piston seal and the rinsing seal for 10 ml cylinders have been redesigned for pumps with serial number 1460 or higher.

Note: The design of the 20 and 50 ml cylinder is unchanged and the instructions in the System Handbook are still valid.

The spare parts for the new 10 ml cylinders have the following Code Numbers:

Part	Code No.
Sealing ring, 10 mm	18-1037-54
Rinsing seal (2/pack)	18-1037-55
O-ring, 13.1 x 1.6 mm	18-1001-59
Rinsing tubing	18-1037-56

Replacing the pump piston seal and rinsing seal

The lifetimes of the rinsing seal and the piston seal are approximately the same and we recommend that you change the rinsing seal when you change the piston seal. The rinsing seal can also be changed separately.

Wear on the piston seal can be indicated either by the loss of system pressure or by the conductivity signal being different from the programmed gradient. If either of these faults occur, first check that all tubing connections are tight and not leaking. If no leakage is found in the connections, run the leakage test on cylinders A and B (see Section 6.3 in the System Handbook). If there are indications that the piston seal on one of the cylinders is worn and leaking, follow the instructions below to replace it.

Wear on the rinsing seal or a damaged O-ring is indicated by the appearance of liquid in the drip tray under the pump.

Before disassembling the faulty pump cylinder, ensure that you have the spare parts specified above available.

1. Remove the rinsing tubing and inlet tubing from the reservoirs.
2. Click on the CONTROL icon to activate the Run/Control window. Click on MANUAL, PUMP, EMPTY and select Cylinder A or Cylinder B, to empty the pump cylinder that is leaking.
3. Disconnect the titanium tubing from the port on the pump cylinder and from the connection on the inlet valve.

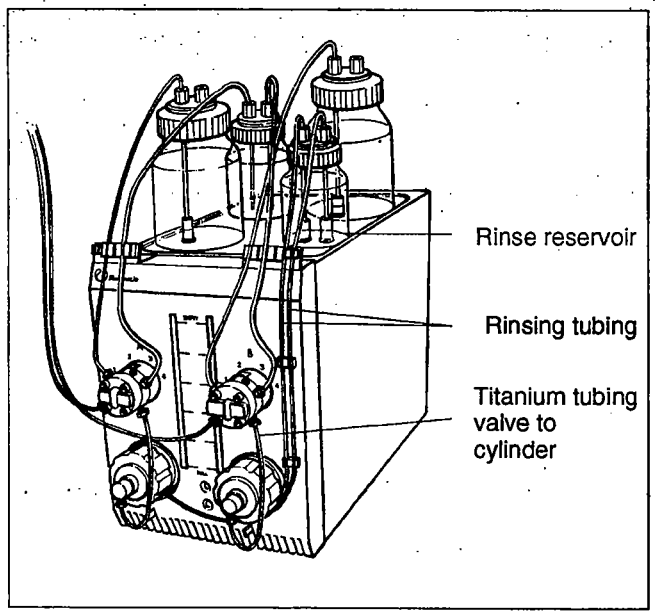


Fig. 1. µPrecision Pump.

4. Unscrew and completely remove the cylinder retaining nut. To release the cylinder, push the lever under the cylinder down and pull out the cylinder from the pump housing.

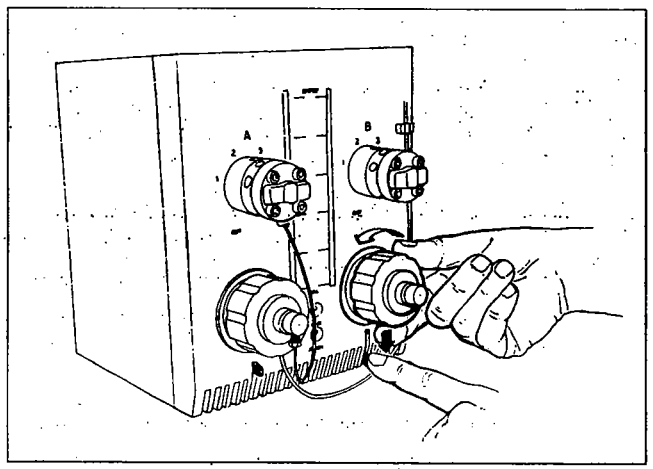


Fig. 2. Removing the cylinder retaining nut.

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5. To change the rinsing seal, pull it off from the back of the cylinder. It has a tight fit, you will have to pull it quite hard. Remove the rinsing tubing from the rinsing seal.

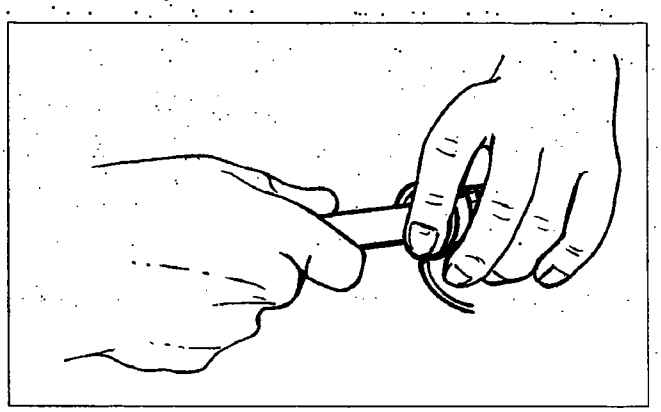


Fig. 3. Removing the rinsing seal.

If only the rinsing seal needs replacing, continue with step 10.

6. Carefully pull the piston out of the cylinder and examine the piston seal for damage and dirt. If the seal is only dirty, wipe it clean and refit the piston according to steps 9-17 below.

7. To remove a damaged sealing ring, grip it firmly with a pair of pliers and twist it off.

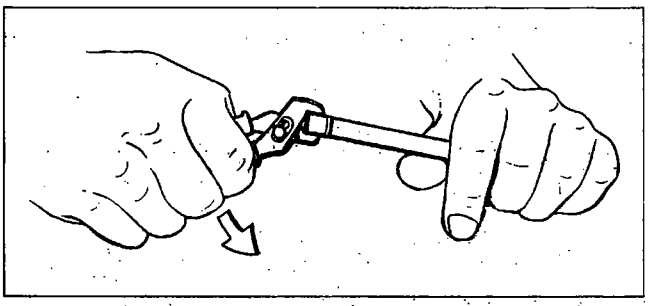


Fig. 4. Removing the sealing ring.

8. Please use the supplied mounting ring when replacing the sealing rings. Place the mounting ring on the table. Insert the sealing ring with the spring facing the table. Push the piston into the sealing ring. Remove the mounting ring.

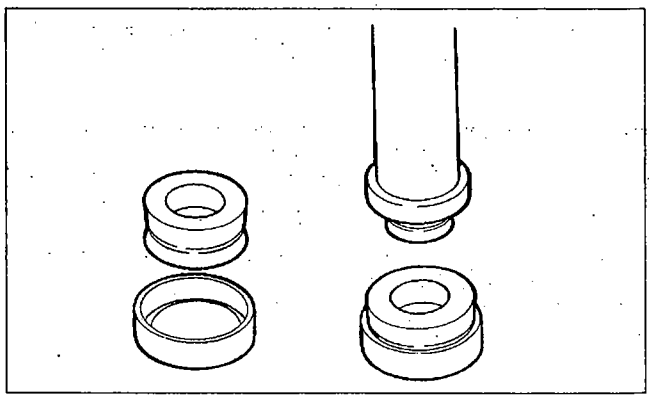


Fig. 5. Replacing the sealing ring.

9. Check that the piston, piston seal and cylinder are clean. Carefully insert the piston into the cylinder.

10. Check the O-ring on the piston for damage before refitting the rinsing seal. Replace the O-ring if required.

11. Put the rinsing tubing onto the tube sticking up from the rinsing seal. Thread the tubing through the hole in the rinsing seal.

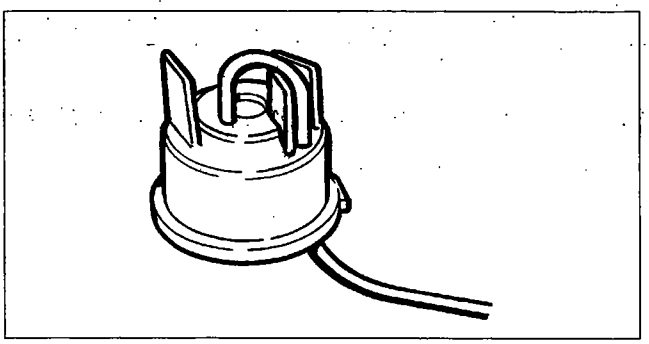


Fig. 6. Fitting the rinsing tubing.

12. Refit the rinsing seal. Wet the O-ring and push the rinsing seal onto the piston. As it has a tight fit you will have to push it hard.

13. Refit the cylinder into the pump. Make sure that the raised edge on the rinsing seal fits into the slot in the pump housing.

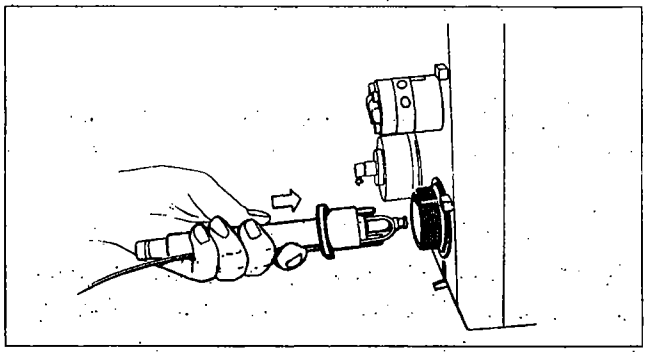


Fig. 7. Refitting the cylinder.

14. Push the cylinder assembly into position in the pump housing.

15. Refit the cylinder retaining nut and tighten it firmly.

16. Reconnect the titanium tubing between the inlet valve and the port on the pump cylinder. Replace the rinse and inlet tubing into the reservoirs.

17. Click on the CONTROL icon to activate the Run/Control window. Click on MANUAL, PUMP, WASH to wash out the pump. Perform the leakage test again to check that the fault has been corrected.

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6. SMART System maintenance

6.1 General maintenance

SMART System comprises precision instruments which will give many years of trouble-free running providing that the following recommendations are observed. Instructions for maintenance and running conditions for Precision Columns are given in the individual Column Instructions.

Checklist for general maintenance

- Check that there is enough eluent in the reservoirs
- Always filter the eluents
- Clean On-line filter frit and prefilter frit regularly
- Rinse the system with water after using salt buffers
- Check the tubings for leakage
- Always use piston rinse
- Wipe the instrument regularly with a damp cloth. Let the instrument dry completely before use.

Before the start of each run, always ensure that there is an adequate supply of eluent in the reservoirs. Never allow the µPrecision Pump to run dry since this will cause premature wear on the piston seals.

Always check that the solvent filters are covered by eluent in the reservoirs. If air is accidentally drawn into the cylinders, it will be detected by the automatic air detection check which is performed after filling the cylinders. The pump will stop and a screen warning will be given.

To protect all components of SMART System, it is essential that the liquids passing through the system are clean and pure. To prolong the life of the column and to prevent blockages, buffers should be passed through a 0.22 µm filter and samples should be free from particulate material. Solvent filters should be renewed at regular intervals. The frits in the µSeparation Unit On-line Filter and in the Prefilter should be cleaned regularly in an ultrasonic bath to prevent blockage. It is particularly important to keep the Monitor UV-M II flow cell clean and free of particles.

If the system is to be left unused after using salt solutions, it is most important to flush the system thoroughly with distilled water to prevent the formation of crystals. Never allow the UV flow cell to dry out if the eluent contains dissolved salts, proteins or other solutes of low volatility. Install the Dummy Column and flush through the entire system using the System Wash function in the Manual menu in Run/Control.

Inspect SMART System visually. Perform the leakage test at regular intervals (See Section 6.3). If leakages are found, first tighten the connector further and if this fails to correct the problem, remake the connection. Instructions are given in Section 6.22.

To prevent the accumulation of buffer salts in the pump and thus prolong the life of the piston seal, it is recommended that the piston

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seal rinsing system is used at all times. Since the rinse liquid is drawn in behind the piston as eluent is delivered from the pump and then flushed out again as the cylinder is refilled, the same volume of liquid is in constant use. Because of this, the rinse liquid must be changed regularly; alternatively, 20 % ethanol can be used instead to prevent bacterial growth. All servicing should be entrusted to qualified personnel only. Please contact your local Pharmacia Biotech representative fore more service information.

6.2 Cleaning the fluidic pathways

A system clean should be performed at regular monthly intervals and when problems such as ghost peaks occur.

1. Prepare 2 M NaOH and place it in a vessel on top of the pump.
2. Remove the inlet tubings from the eluent buffer reservoirs and place them in the 2 M NaOH. The outlet tubing from the inlet valve should be placed in a waste container.
3. In Run/Control, click on MANUAL, PUMP, WASH. Choose to wash both cylinders with the NaOH.
4. After washing the pump, fit the Dummy Column into the column connector and close the µSeparation Unit door. Install the Dummy Column using the Column Install function in the Manual pull down menu of Run/Control.
5. Click on MANUAL, PUMP FLOW, start a flow rate of 1000 µl/min. Click on MANUAL, PUMP CONC B and set the concentration B to 50 %. Wash through the Dummy Column until both cylinders are empty.
6. Place the inlet tubings into distilled water and repeat Step 3 and Step 5. After the wash, remove the Dummy Column.

   If you are going to run at very high sensitivity repeat the procedure above with water, isopropanol followed by water.

6.3 Performing the leakage test

This tests the pump for leakage. It should be used if the system pressure drops and there are no indications of leakages from tubing connections throughout the system. The test can also be used before a run as a regular test of pump function.

At the start of the test, the inlet valve is automatically switched to the closed position and the cylinder is pressurised to a user defined pressure. If this pressure cannot be maintained for 10 minutes, a leakage has been detected and will be measured.

1. Check that there is some liquid in the cylinder to be tested. If not, click on MANUAL, PUMP, FILL and fill the cylinder with a small volume of eluent.
2. In the Run/Control window, click on PUMP, LEAKAGE TEST. The Leakage Test window is displayed.
3. As default, both pump cylinders are tested as indicated by a cross in the check boxes. If you want to test only one cylinder, cancel the selection of that cylinder.
4. Enter the pressure at which the cylinder is to be tested. Use a value typical for the running conditions.
5. Click on START. The test may take up to 10 minutes if the leakage is small.

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When an accurate reading of the leakage has been obtained, the test will stop automatically. The leakage in µl/min is displayed for each cylinder tested. If the colour of the background to the value is green, the leakage is small and no action needs to be taken. However, if the background colour is red, the leakage must be corrected by cleaning or replacing the piston seal (see Section 6.5 or 6.6) or by remaking the titanium tubing connection between the cylinder and the inlet valve (see Section 6.22).

If necessary, you can stop the test at any time by clicking on the Break button.

If the leakage test still shows leakage, tested at different positions in the cylinder and after replacing the sealing ring, leakage in the pump valve can be suspected, see Section 6.8.

6.4 Changing pump cylinders

Three sizes of pump cylinder are available for the µPrecision Pump, 10, 20 and 50 ml. Each cylinder is supplied complete with the cylinder support and rinsing tubing fitted.

1. Remove the rinse tubing and inlet tubing from the reservoirs.

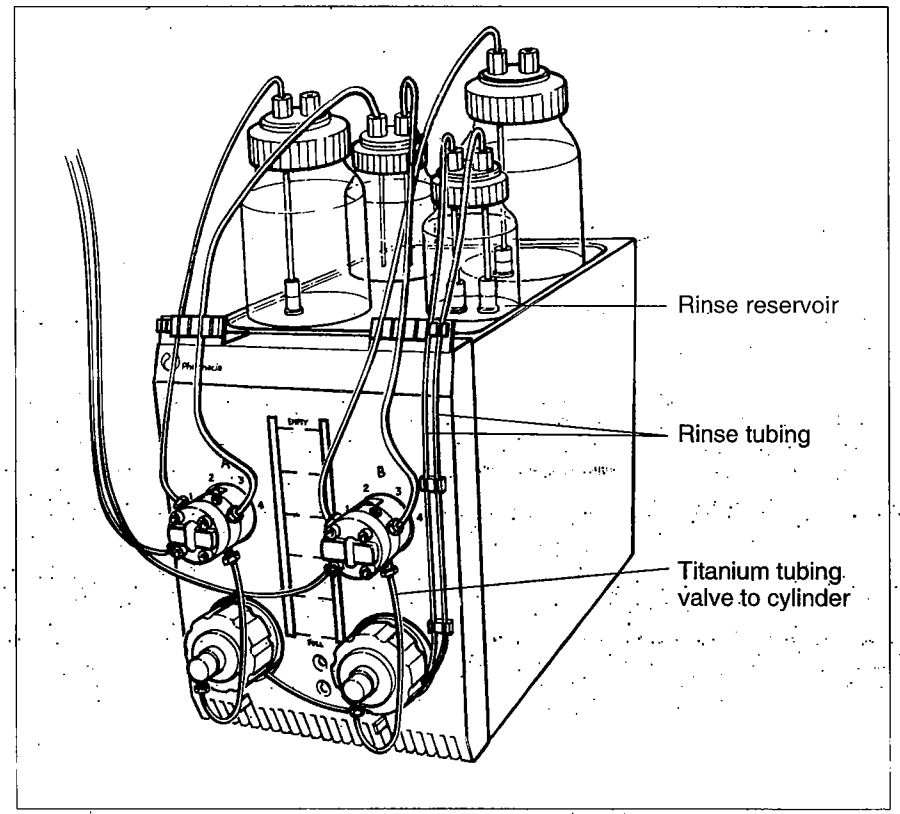


Fig. 6.1. µPrecision Pump

2. Click on the CONTROL icon to activate the Run/Control window. Click on MANUAL, PUMP, EMPTY and select, to empty the pump cylinders.
3. Disconnect the titanium tubing from the port on the pump cylinder and from the connection on the inlet valve.
4. Unscrew and completely remove the cylinder retaining nut. To release the cylinder, push the lever under the cylinder down and pull the cylinder out from the pump housing.

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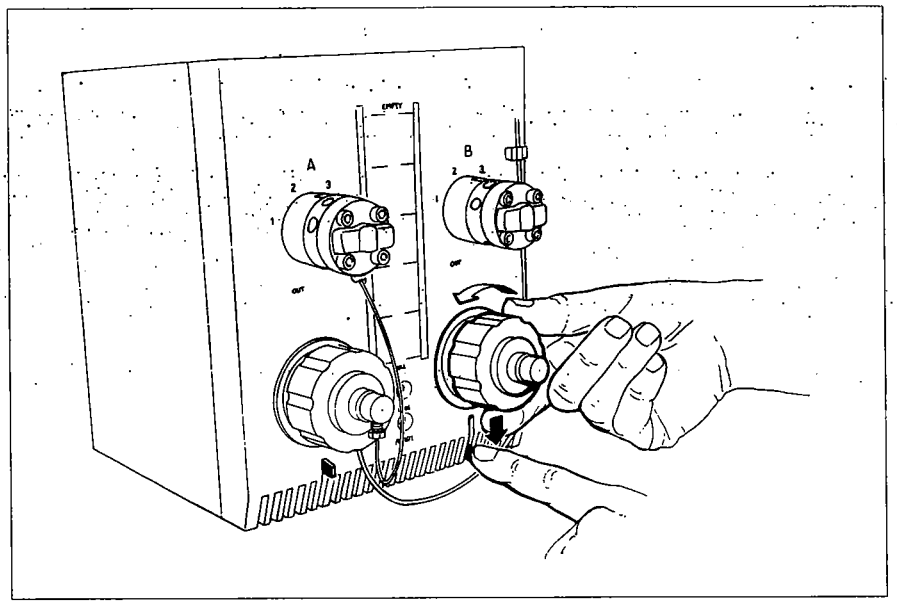


Fig. 6.2. Removing the cylinder retaining nut

5. Repeat Steps 3 and 4 to remove the other cylinder.
6. Fit the new cylinders into the pump. For 10 ml cylinders make sure that the raised edge on the rinsing seal fits into the slot in the pump housing. For 20 and 50 ml cylinders, hold the rinsing tubing in the locating channel along the side of the cylinder support and insert the cylinders into the pump housing, (Fig. 6.3). When the end of the cylinder connects into the stepper motor drive, the lever under the cylinder returns automatically to the up position.

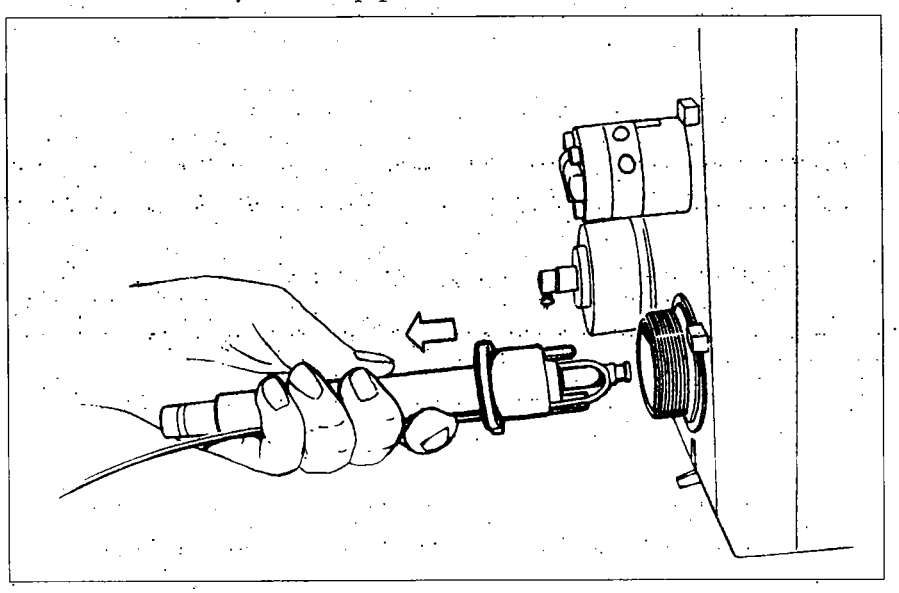


Fig. 6.3. Fitting the cylinder

7. With the rinsing tubing fitted in the slot, refit the cylinder retaining nut and tighten it firmly (Fig. 6.4).

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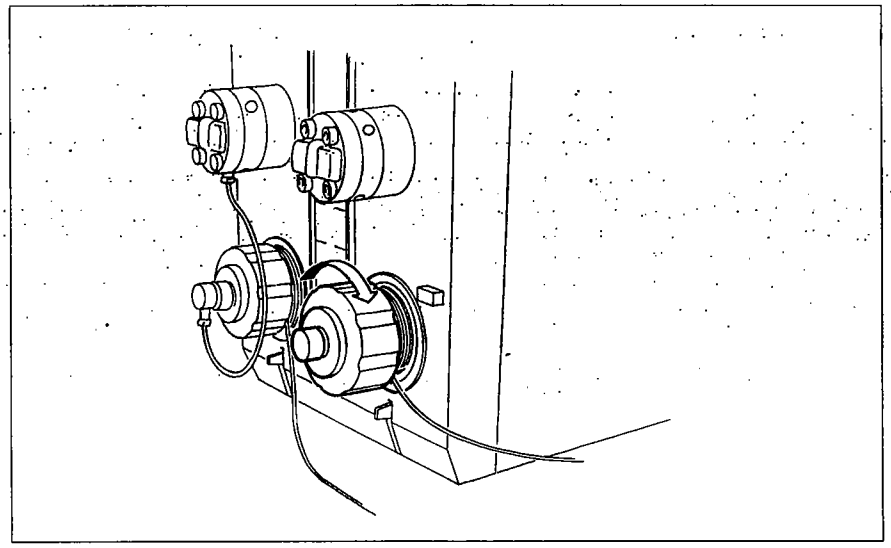


Fig. 6.4. Tightening the cylinder nut

8. On each cylinder, re-connect the titanium tubing between the inlet valve and the port on the pump cylinder. Replace the inlet and rinsing tubings into the reservoirs.
9. Click on MANUAL, PUMP WASH. Select cylinders A and B. Choose the inlet and outlet ports. Click on OK to start the pump wash.

6.5 Replacing the pump piston seal, rinsing seal and tubing, 10 ml cylinder

The lifetimes of the rinsing seal and the piston seal are approximately the same and we recommend that you change the rinsing seal when you change the piston seal. The rinsing seal can also be changed separately. The rinsing tubing does not require to be replaced as frequently.

Wear on the piston seal can be indicated either by the loss of system pressure or by the conductivity signal being different from the programmed gradient. If either of these faults occur, first check that all tubing connections are tight and not leaking. If no leakage is found in the connections, run the leakage test on cylinders A and B (see Section 6.3). If there are indications that the piston seal on one of the cylinders is worn and leaking, follow the instructions below to replace it.

Wear on the rinsing seal or a damaged O-ring is indicated by the appearance of liquid in the drip tray under the pump.

1. Before disassembling the faulty pump cylinder, ensure that you have the spare parts specified below available.
   - Sealing Ring, 10 mm, Code No. 18-1037-54 Type II
   - Rinsing seal (2/pack) Code No. 18-1037-55 Type II
   - O-ring, 13.1 x 1.6 mm Code No. 18-1001-59
   - Rinsing tubing Code No. 18-1037-56

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2. To remove the cylinder from the pump, see Section 6.4 step 1-4.

3. To change the rinsing seal, pull it off from the back of the cylinder. It has a tight fit, you have to pull it quite hard. Remove the rinsing tubing from the rinsing seal.

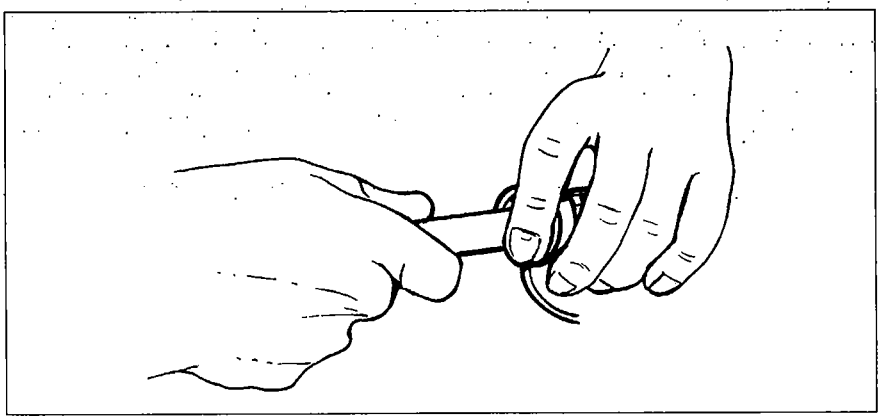


Fig. 6.5. Removing the rinsing seal

If only the rinsing seal needs replacing, continue to step 10.

4. Carefully pull the piston out of the cylinder and examine the piston seal for damage and dirt. If the seal is only dirty, wipe it clean and refit the piston according to step 7-15 below.

5. To remove a damaged sealing ring, grip it firmly with a pair of pliers and twist it off.

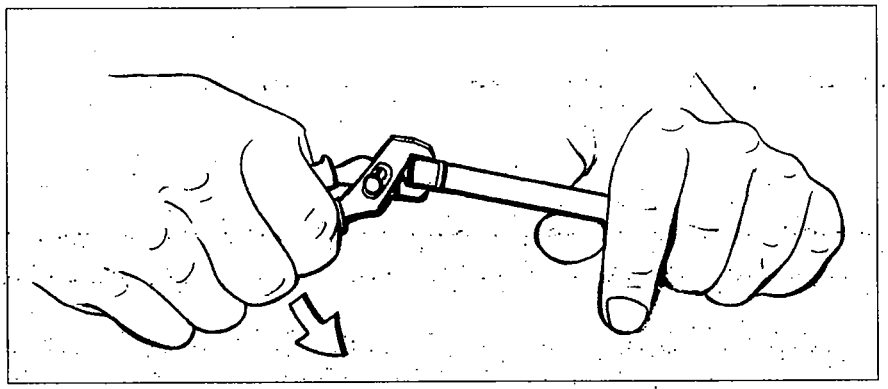


Fig. 6.6. Removing the sealing ring

6. Place the new sealing ring on a table with the spring facing the table. Push the piston into the sealing ring.

7. Check that the piston, piston seal and cylinder are clean. Carefully insert the piston into the cylinder.

8. Check the O-ring on the piston for damage before refitting the rinsing seal. Replace the O-ring if required.

9. Put the rinsing tubing onto the tube sticking up from the rinsing seal. Thread the tubing through the hole in the rinsing seal.

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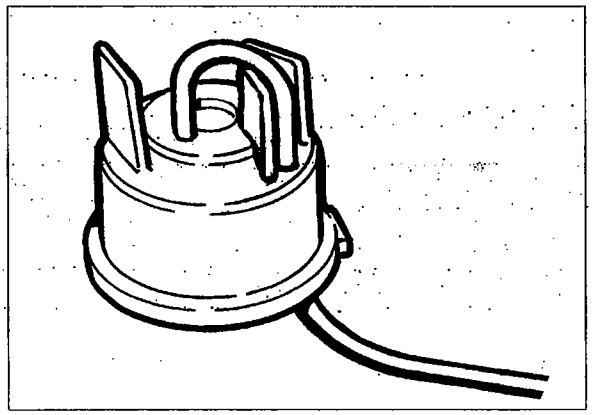


Fig. 6.7. Fitting the rinsing tubing

10. Refit the rinsing seal. Wet the O-ring and push the rinsing seal onto the piston. As it has a tight fit you have to push it hard.
11. Refit the cylinder into the pump. Make sure that the raised edge on the rinsing seal fits into the slot in the pump housing.

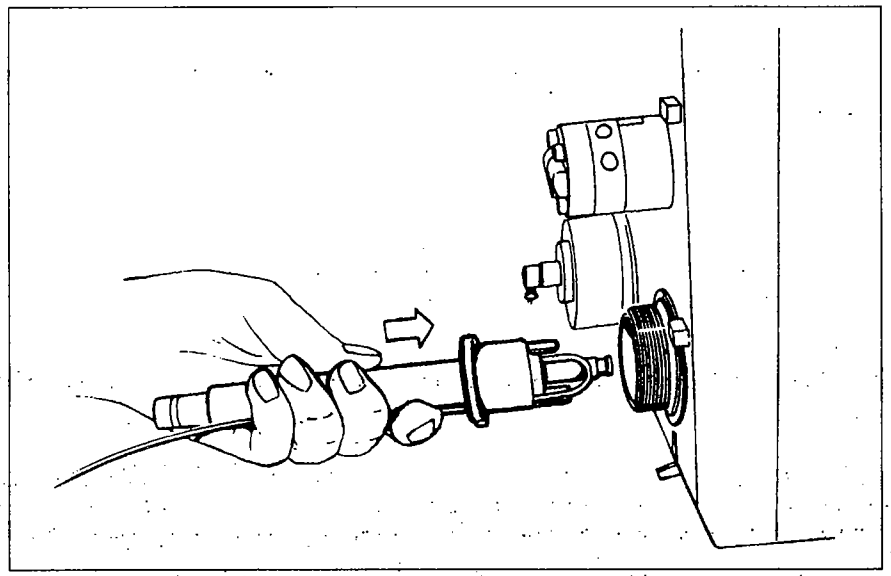


Fig. 6.8. Refitting the cylinder

12. Push the cylinder assembly into position in the pump housing.
13. Refit the cylinder retaining nut and tighten it firmly.
14. Reconnect the titanium tubing between the inlet valve and the port on the pump cylinder. Replace the rinsing and inlet tubing into the reservoirs.
15. Click on the CONTROL icon to activate the Run/Control window. Click on MANUAL, PUMP, WASH to wash out the pump. Perform the leakage test again to check that the fault has been corrected.

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6.6 Replacing the pump piston seal and rinsing seal, 20 and 50 ml cylinders

Wear on the piston seal can be indicated either by the loss of system pressure or by the conductivity signal being different from the programmed gradient. If either of these faults occur, first check that all tubing connections in SMART System are fully tightened and are not visibly leaking. If this does not correct the problem, run the leakage test on cylinders A and B (see Section 6.3 for a description of the leakage test). If there are indications that the piston seal on one of the cylinders could be faulty or worn, follow the steps below.

An indication of wear on the rinsing seal, or a damaged O-ring is the appearance of liquid in the drip tray under the pump.

1. Before disassembling the faulty pump cylinder, ensure that you have the spare parts specified below available. The code numbers of the appropriate sealing rings are given below.

   - Sealing Ring, 14 mm (20 ml cylinder)
     Code No. 18-1001-47
   - Sealing Ring, 22 mm (50 ml cylinder)
     Code No. 18-1001-66
   - Rinsing Seal, 10 mm (20 ml cylinder)
     Code No. 18-1001-51
   - Rinsing Seal, 10 mm (50 ml cylinder)
     Code No. 18-1001-51

   It is also recommended that the following two O-rings are replaced at the same time.

   - O-ring, 17.1 x 1.6 mm, (20 ml cylinder)
     Code No. 18-1001-50
   - O-ring, 25.1 x 1.6 mm, (50 ml cylinder)
     Code No. 18-1001-68

2. To remove the cylinder from the pump see section 6.4 step 1-4.
3. To remove the cylinder support from the back of the cylinder, push outwards and backwards on the two clips of the split ring of the chamber (Fig. 6.9).

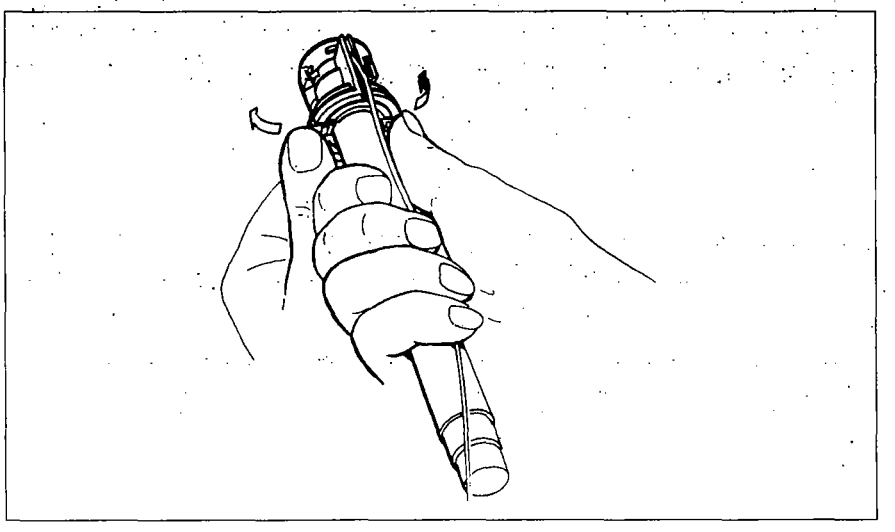


Fig. 6.9. Removing the cylinder support

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4. Carefully pull the piston completely out of the cylinder and examine the seal for damage and dirt (Fig. 6.10). If the seal is only dirty, it should be wiped clean and refitted as described in Step 7 and 11-14.

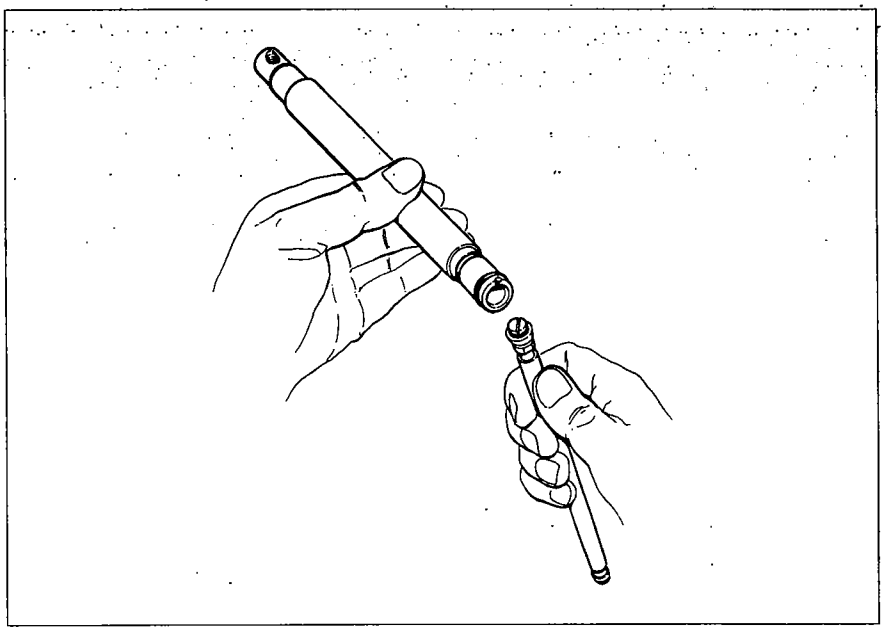


Fig. 6.10. Removing the piston

5. When the seal is damaged, remove it by holding the piston firmly in an adjustable wrench and using a screwdriver to loosen the titanium locking screw (Fig. 6.11).

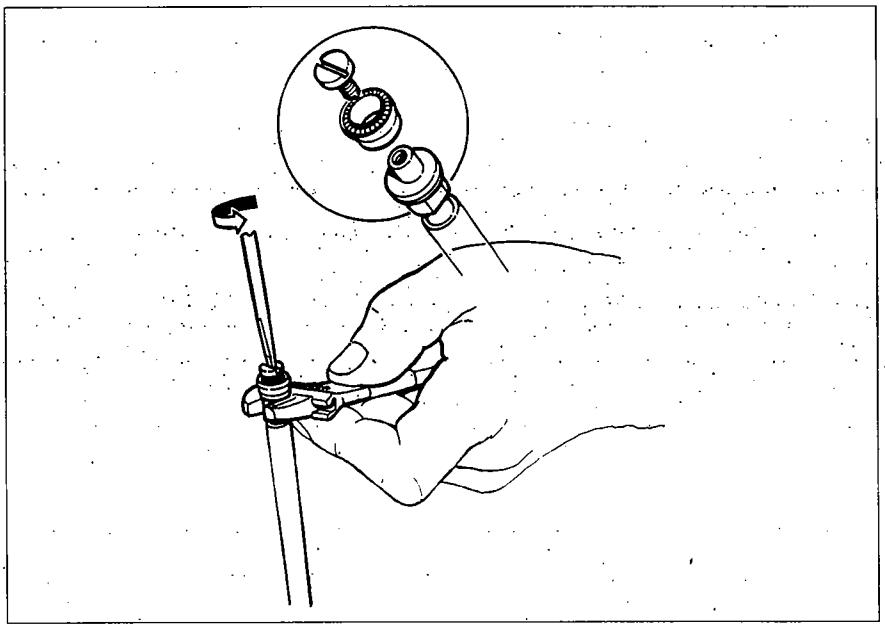


Fig. 6.11. Removing the piston seal

6. Position the new seal so that the spring is visible from the end of the piston (Fig. 6.11, inset) and then fit it onto the piston. Secure it tightly with the titanium screw.

7. Wet the piston seal with distilled water and then carefully insert the piston almost to the end of the cylinder. If necessary, gently twist the piston at the same time as pushing it inwards.

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If only the sealing ring needs replacing proceed to step 10.

8. Remove the cover plate from the cylinder support (Fig. 6.12). Remove the rinsing seal by pushing it outwards from the inside of the cylinder support. Examine the seal for wear and damage. Fit a new seal, ensuring that it is positioned with the spring facing the inside of the cylinder support.

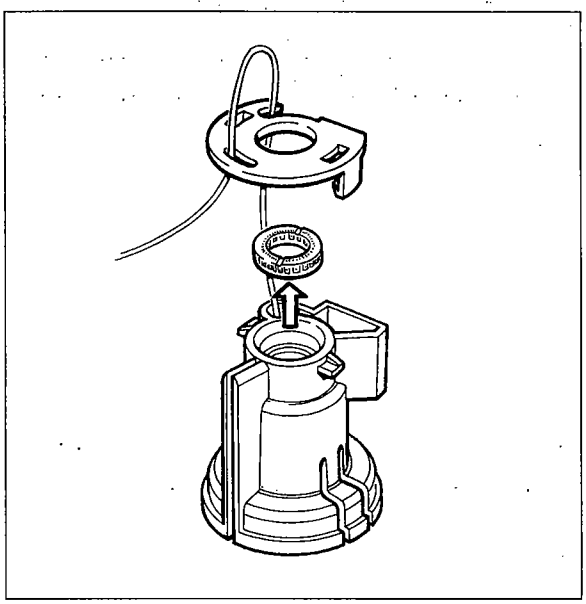


Fig. 6.12. Removing the cover plate and rinsing seal

9. Refit the cylinder support cover plate (Fig. 6.13). Refit the cylinder support onto the cylinder. While pushing the two parts firmly together, twist the assembly slightly until the two clips on the cylinder support split ring snap into position with a click, indicating that they have located into the groove on the cylinder.

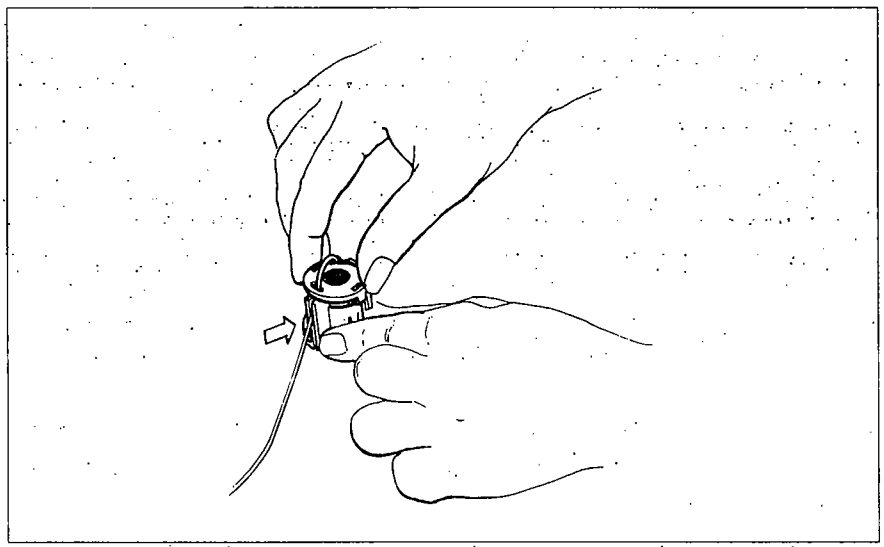


Fig. 6.13. Refitting the cover plate

10. Refit the cylinder support. While pushing the two parts firmly together, twist the assembly slightly until the two clips on the cylinder support split ring snap into position with a click, indicating that they have located into the groove on the cylinder.

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11. Hold the rinsing tubing in the locating channel in the cylinder support and refit the cylinder assembly into the pump housing (Fig. 6.14). When the end of the piston connects into the stepper motor drive, the lever under the cylinder returns automatically to the up position.

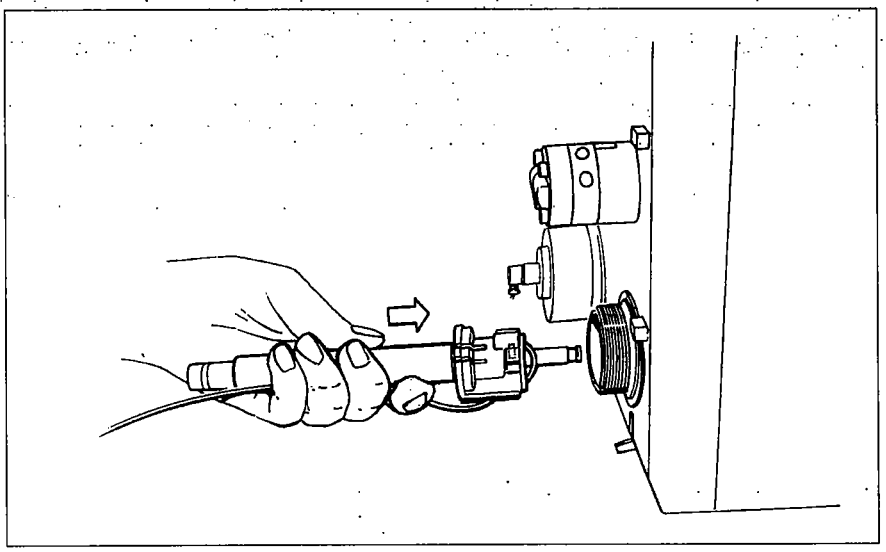


Fig. 6.14. Refitting the cylinder

12. With the rinsing tubing in the slot, refit the cylinder retaining nut and tighten it firmly.
13. Reconnect the titanium tubing between the inlet valve and the port on the pump cylinder. Replace the rinse tubings into the reservoirs.
14. Click on the CONTROL icon to activate the Run/Control window. Click on MANUAL, PUMP, WASH to wash out the pump. Perform the leakage test again to check that the fault has been corrected.

6.7 Replacing the Rinsing Tubing, 20 and 50 ml cylinders

Before starting to disassemble the pump cylinder, ensure that Rinsing Tubing (Code No. 18-1001-70) is available as a spare part. This is supplied flanged and complete with the rubber cone.

1. To remove the cylinder from the pump see Section 6.4 step 1-4.
2. Remove the cover plate from the cylinder support.
3. Use forceps to remove the rubber cone which secures the rinsing tubing in position in the cylinder support (Fig. 6.15).

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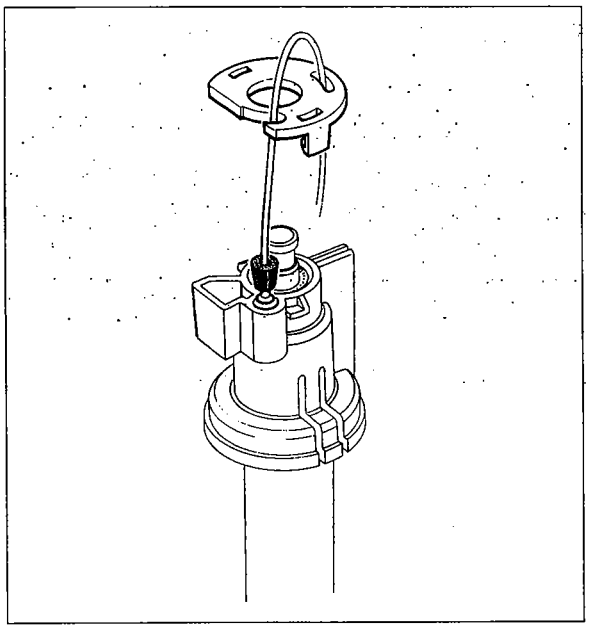


Fig. 6.15. Removing the cover plate, cone and tubing

4. Remove the inlet filter from the other end of the tubing. To do this, unscrew and separate the two pieces of the metal part. Pull out the tubing.
5. Remove the cover plate from the tubing.
6. Fit the cover plate onto the new rinsing tubing. Fit the inlet filter onto the other end.
7. Replace the rubber cone into the cylinder support.
8. Refit the cover plate to the cylinder support (Fig. 6.16).

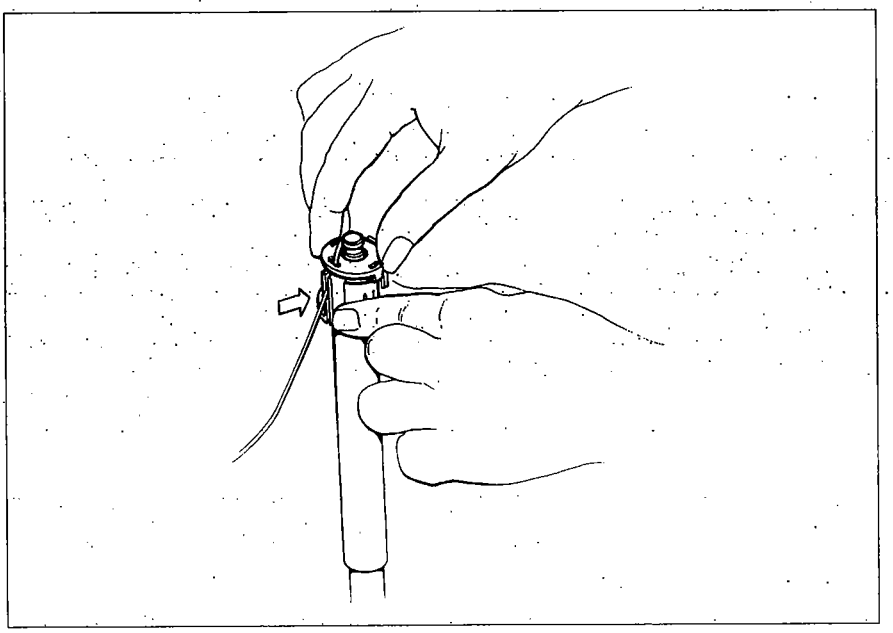


Fig. 6.16. Replacing the cover plate with locating channel indicated

9. Hold the rinsing tubing in the locating channel in the cylinder support and refit the cylinder assembly into the pump housing (Fig. 6.17). When the end of the piston connects into the stepper motor drive, the lever under the cylinder returns automatically to the up position.

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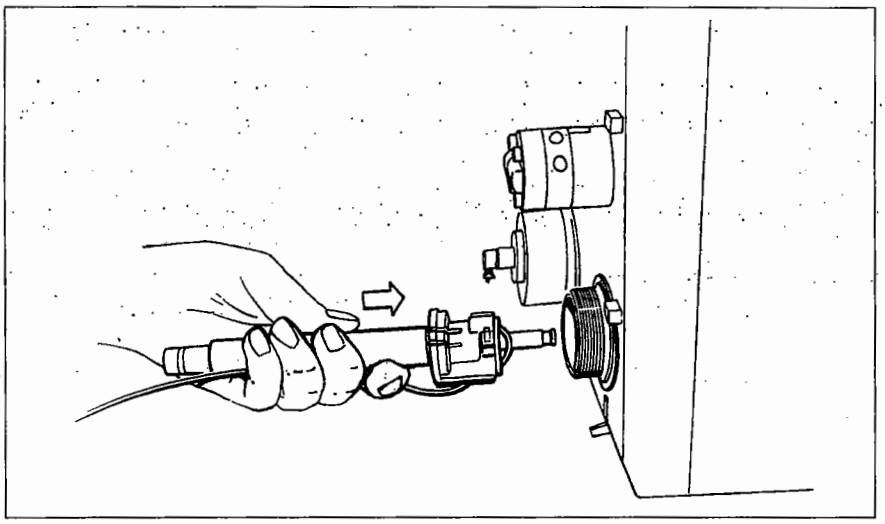


Fig. 6.17. Refitting the pump cylinder

10. With the rinsing tubing in the slot, refit the cylinder retaining nut and tighten it firmly.
11. Reconnect the titanium tubing between the inlet valve and the port on the pump cylinder. Replace the inlet tubing and the rinsing tubing into the reservoirs.
12. Click on the CONTROL icon to activate the Run/Control window. Click on MANUAL, PUMP, FILL and select to fill the pump cylinder again. Choose the cylinder and the inlet port.

6.8 Replacing the pump inlet valve channel plate

Wear on the interior plates of the valve can be indicated by the conductivity signal showing a gradient with a curve, or "knee effect" instead of a linear gradient. After many years of continuous use, it should be expected that the channel plate on the inlet valve will be worn and will have to be replaced.

Note: A non-linear gradient can also be caused by a leaking connection, a blockage in the pump outlet tubings, a leaking and worn piston seal or a failure in the pressurisation system of the cylinders. The correction of these problems is described elsewhere.

1. Before starting to disassemble the valve, ensure that there is a Channel Plate (Code No. 18-1017-69) available as a spare part.
2. Click on the CONTROL icon to activate the Run/Control window. Click on MANUAL, PUMP, FILL and select to fill the pump cylinder of the faulty valve with 1 ml of buffer through position 1.

   Note: This is the final software instruction which should be sent to the inlet valve. Do not use SMART Manager again while the valve is being disassembled.
3. Remove the inlet tubing from the reservoirs. Disconnect the inlet tubings connected to the valve to be disassembled. Disconnect the titanium tubing from both the pump cylinder and the inlet valve.

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4. The valve position number is engraved on the surface of the channel plate and it can be seen from above the valve. Check that the valve is in position 1.

5. Using a 3 mm Allen key, start to loosen each of the four Allen screws in turn (Fig. 6.18).

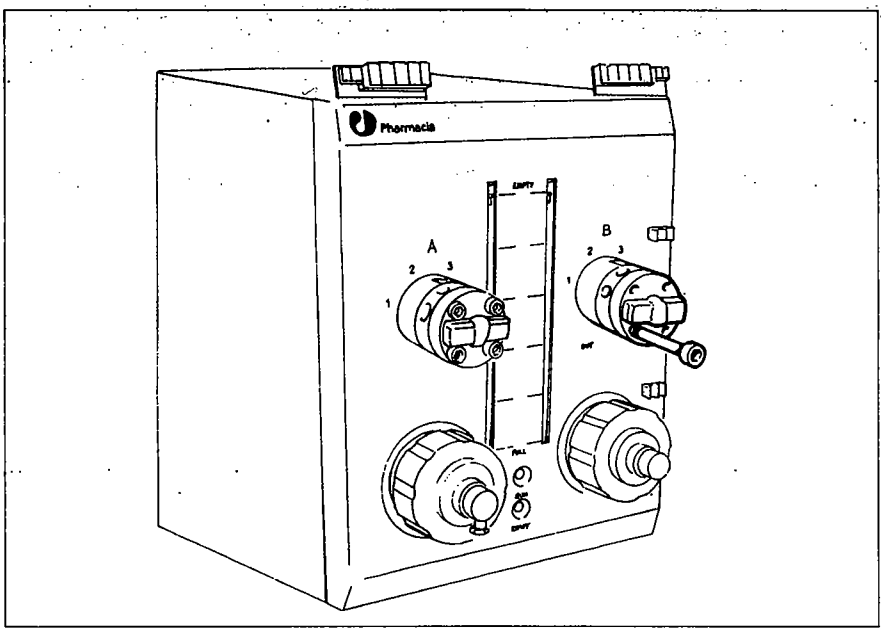


Fig. 6.18. Disassembling the inlet valve

6. Remove each screw in turn. Pull off the front part of the valve. The channel plate can be seen on the outer part of the valve. The switching plate with the channel aligned to position 1 can be seen on the inner part. If the switching plate is removed, ensure that it is replaced in the same position.

7. Remove the channel plate by levering it out gently with a screwdriver (Fig. 6.19). Turn the new channel plate so that the circular channel is visible. Fit it over the locating pins, pushing it in evenly until it is seated correctly in position.



Warning: The channel plate is coated in Krytox*, a fluoro plastic grease to ensure smooth movement. This substance is toxic and the operator is advised to wear protective gloves when handling the channel plates during this procedure. Wash your hands after any accidental contact with Krytox.

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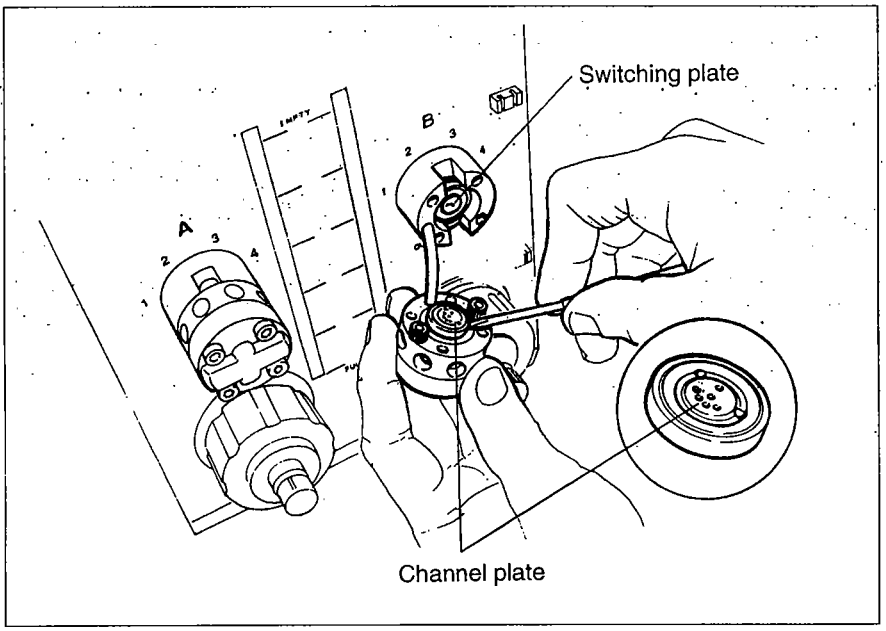


Fig. 6.19. Removing the channel plate

8. If necessary, hold the switching plate in position in the inner part of the valve while refitting the outer part of the valve. Re-tighten each of the four retaining screws in turn by a small amount until they are fully tightened.

9. Refit the titanium tubing between the valve and the pump cylinder. Reconnect the inlet tubings to the valve. Ensure that all connections are tight.

6.9 Servicing the On-line Filter

If the back pressure exceeds 0.8 MPa with a flow of water at 2000 µl/min through the waste position in the injection valve, a blockage in the On-line filter can be suspected. If tests indicate that the frit is blocked, follow the instructions below and change the filter.

1. Before disassembling the filter housing, ensure that there is an On-line Filter (Code No. 18-1800-34) available as a spare part.

2. Remove the securing clip from inside the µSeparation Unit and lift up the filter housing (Fig. 6.20). Remove the knurled screw.

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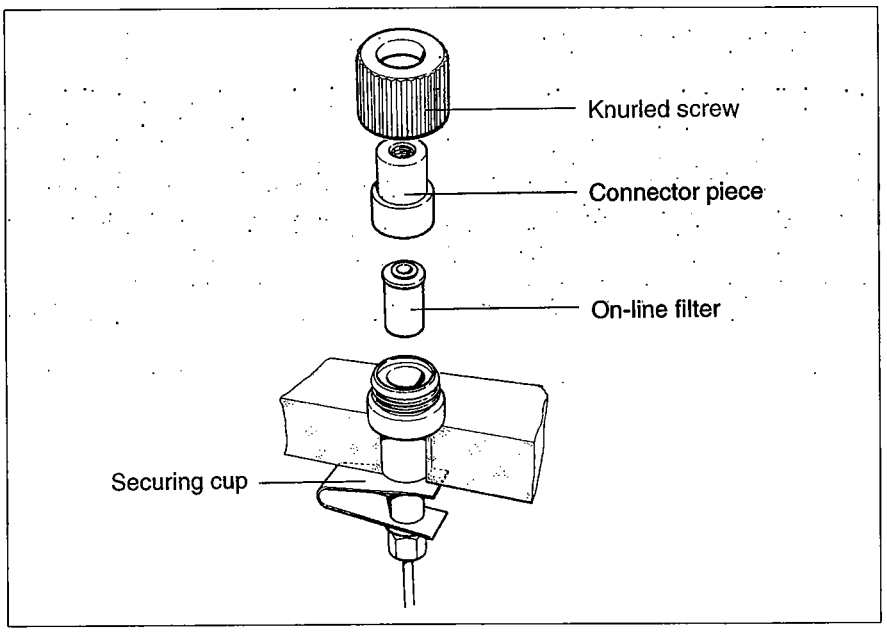


Fig. 6.20. On-line Filter

3. Remove the connector piece. Lift out the On-line Filter.
4. The filter can either be cleaned in an ultrasonic bath and then refitted or replaced with a new one. Refit the connector piece and the knurled screw. Replace the filter housing and secure it with the clip.

6.10 Changing the mixer chambers

Four chamber inserts of different volume are available for the µMixer. These are 12 µl, 30 µl, 75 µl and 150 µl. In addition, a zero volume chamber bypass can be fitted.

By using different mixer volumes for different flow rates, the mixing of the two eluents can be combined optimally. The table in Section 2.9 gives the recommended chamber size for use with different flow rates.

Note: Two 12 µl mixer chambers can not be fitted at the same time. If you use a 12 µl mixer chamber please place it in the bottom position.

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1. Loosen and remove the three Allen screws on the top of the mixer (Fig. 6.21). Lift the upper part to access the mixer chambers.

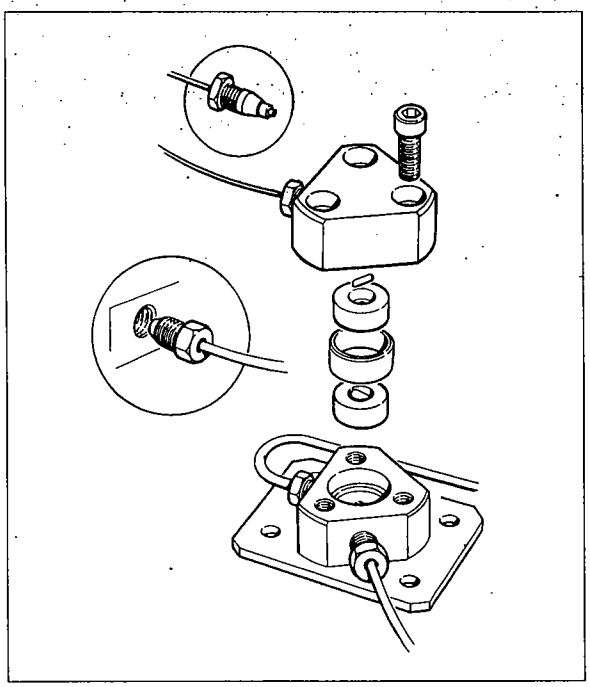


Fig. 6.21. µMixer

2. Lift the upper part and remove the two mixer chambers, together with their magnetic stirrers and the connecting piece.
3. Fit the new lower mixer chamber with its magnetic stirrer. Place the connecting piece on top and then fit the upper mixer chamber with its magnetic stirrer.

   Note: The zero volume chamber bypasses are fitted in place of the two mixer chambers.

4. Refit and fully tighten the three Allen screws.
5. In the Main Menu, click on the Run/Control icon to make manual changes to SMART System. Click on CONFIG, USER SUPPLIED CONFIGURATION. Enter the new mixer volume in the dialogue box.

6.11 Changing the sample loop

Four sample loops of different volume are supplied with SMART System. These are 10 µl, 20 µl, 50 µl and 100 µl.

1. To avoid air entering the flow path the injection valve should be turned to Waste position. In the Main Menu, click on the Run/Control icon. Click on MANUAL, SEP UNIT, INJECTION VALVE. Click on WASTE position. Click on OK.
2. Loosen the fingertight screws securing the external loop to the injection valve (Fig. 6.22). Remove the loop and store it for future use.

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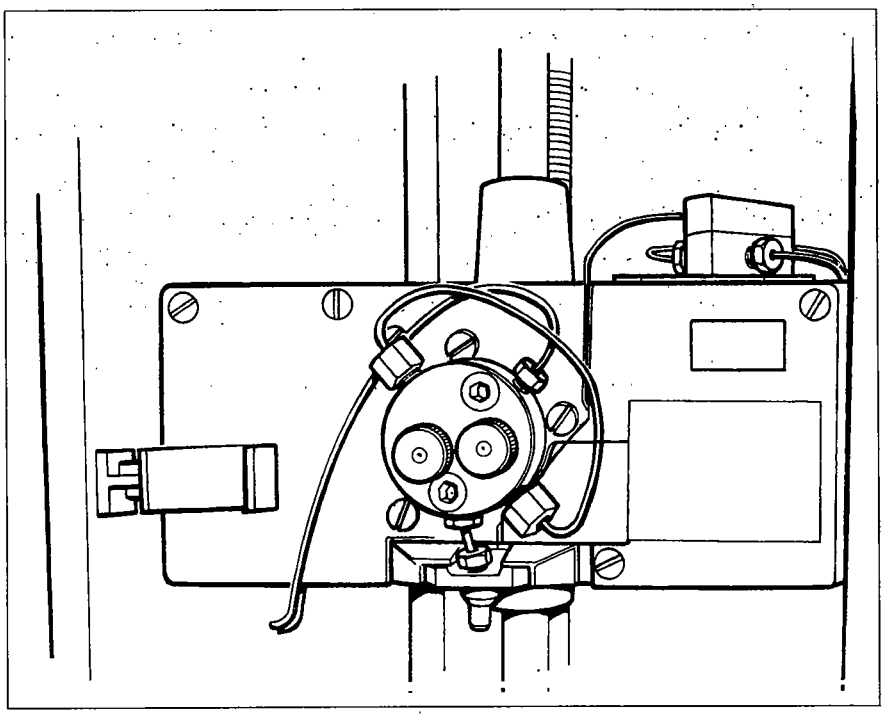


Fig. 6.22. External loop

3. Fit the new loop and secure the screws firmly with the fingers and then with the wrench supplied.
4. Click on CONFIG, USER SUPPLIED CONFIGURATION. Enter the new loop volume. Click on OK.

6.12 Replacement of injection valve fill port

The fill port will wear gradually over several years of use and will have to be replaced.

Before attempting to replace the port, ensure that there is a Fill Port (Code No. 18-1015-88) available as a spare part.

1. To remove the port, turn the outer part anti-clockwise to loosen it and then withdraw the port (Fig. 6.23).

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NEW INJ VALVE SEE SECTION 2

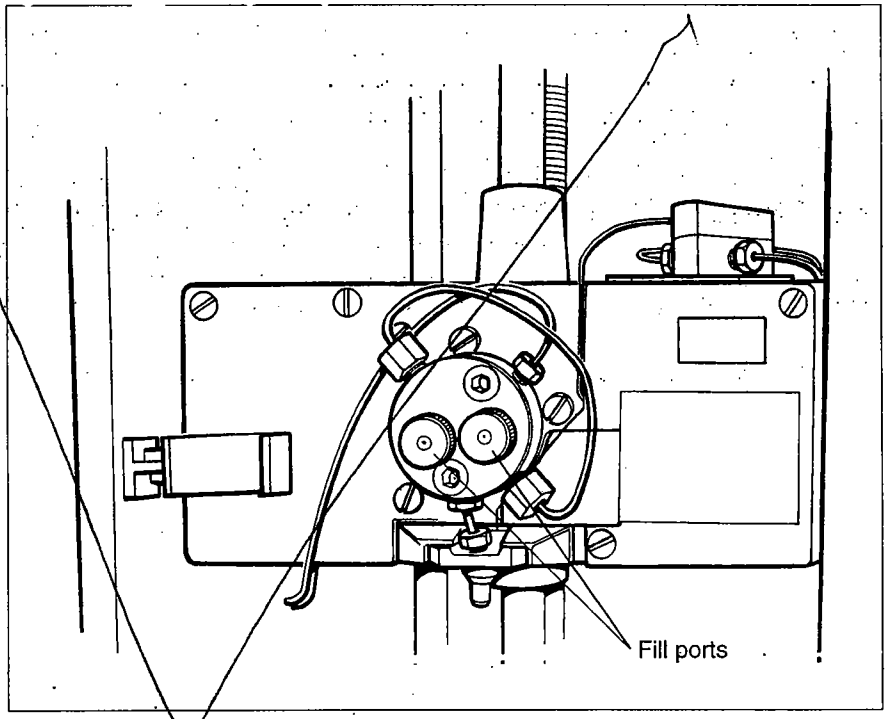


Fig. 6.23. Injection valve

2. Insert a syringe into the new port until the end of the needle reaches the end of the port.
3. With the syringe held in this position, fit the new fill port and turn it clockwise to tighten it fully.

6.9 Replacement of injection valve internal channel plate

Wear on the internal channel plate is indicated by leakage around the injection valve. To confirm the fault, set the injection valve in Load position, disconnect the sample loop and start the flow. If eluent is coming out through the sample loop ports, the channel plate is damaged. If this is not the case run eluent at high flow rate through the valve, switching it between inject and load position. If you see a difference in the back pressure for the two valve position, a damaged channel plate can be suspected.

Warning: The channel plate is coated in Krytox*, a fluoro plastic grease to ensure the smooth movement. This substance is toxic and the operator is advised to wear protective gloves when handling the channel plates during this procedure. Wash your hands after any accidental contact with Krytox.

* Krytox™ is the registered trade mark of Du Pont

Before disassembling the injection valve, ensure that the Channel Plate (Code No. 18-1015-87) is available as a spare part. In addition, in case of damage occurring during this procedure, it is a wise precaution to have Tubing, valve to column, (Code No. 18-1015-65) also available.

1. When disassembling the injection valve, disconnect the waste channel tubing and carefully bend down the valve housing. Care must be taken with the titanium tubing between the valve and the column. Do not remove the external loop unless it is one of the larger loops and is inconvenient.

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2. Use a 3 mm Allen key to loosen the two Allen locking screws in turn (Fig. 6.24). Continue to hold the valve while loosening the locking screws.

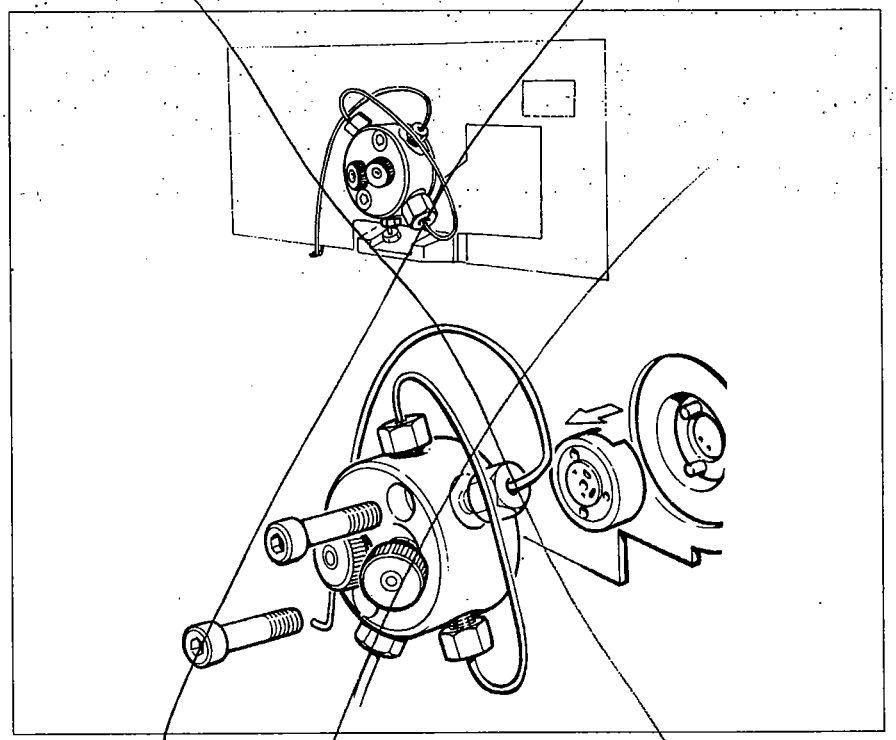


Fig. 6.24. Disassembling the injection valve

3. Gently remove the outer part of the valve, taking care not to damage the tubing connection to the column.
4. Observe the alignment of the channel plate. Use the screw supplied in the spare part package to remove the channel plate. Thread the screw into one of the holes in the old channel plate and pull out the channel plate.

   Note: Use protective gloves when handling the channel plate since there is a thin film of toxic Krytox smeared over the outer surface.

5. Ensure that the new plate is kept clean and free of scratches. Fit it in the same alignment as the old plate. Tighten the two screws alternately until a distinct stop.
6. Carefully replace the outer part of the valve. Replace the two Allen screws and tighten each in turn. Finally, use the short end of the Allen key to tighten the two screws extremely firmly.

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6.14 Changing the Prefilter Frit

The prefilter should be disassembled regularly and cleaned in an ultrasonic bath. The instructions below describe how to access the filter. If tests indicate that the frit is blocked, it can be replaced.

1. Before disassembling the prefilter, ensure that there is a frit available as a spare part.
   - Prefilter Frit, 3.2 mm; Code No. 18-1800-77
   - Prefilter Frit, 0.8 mm Code No. 18-1800-78
2. Use the Column Install function in Manual in the Run/Control window to drive the automatic column holder upwards away from the column. The prefilter can then be removed by pulling it downwards off the column fitting.
3. Loosen and remove the upper connection (Fig. 6.25). Remove the frit.

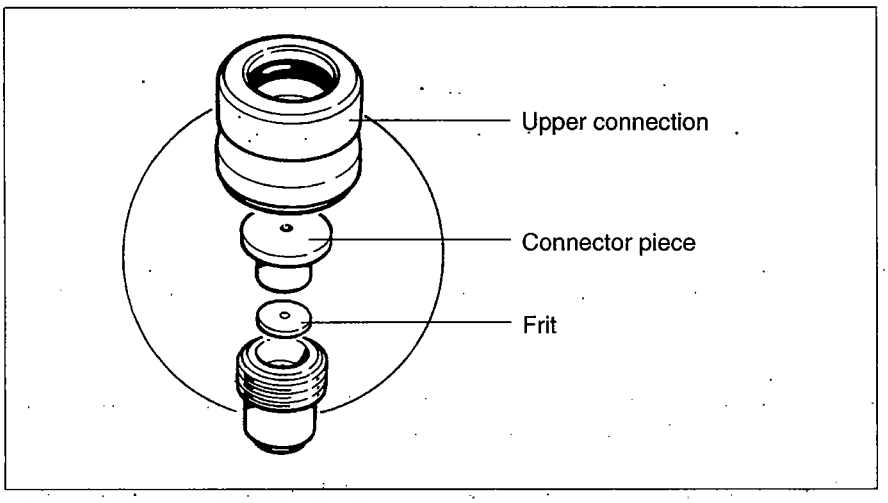


Fig. 6.25. Disassembling the Prefilter

4. The frit can either be cleaned in an ultrasonic bath before refitting or replaced with a new one. Refit the upper connection.
5. Replace the prefilter on the end of the column fitting. Re-install the column of choice.

6.15 Fitting the Column Adaptor

The Column Adaptor is required when columns other than µPrecision columns are to be used in SMART System.

Note: For safety reasons, it is important that the Column Adaptor is installed in the µSeparation Unit before fitting the column into the Column Adaptor (Fig. 6.26).

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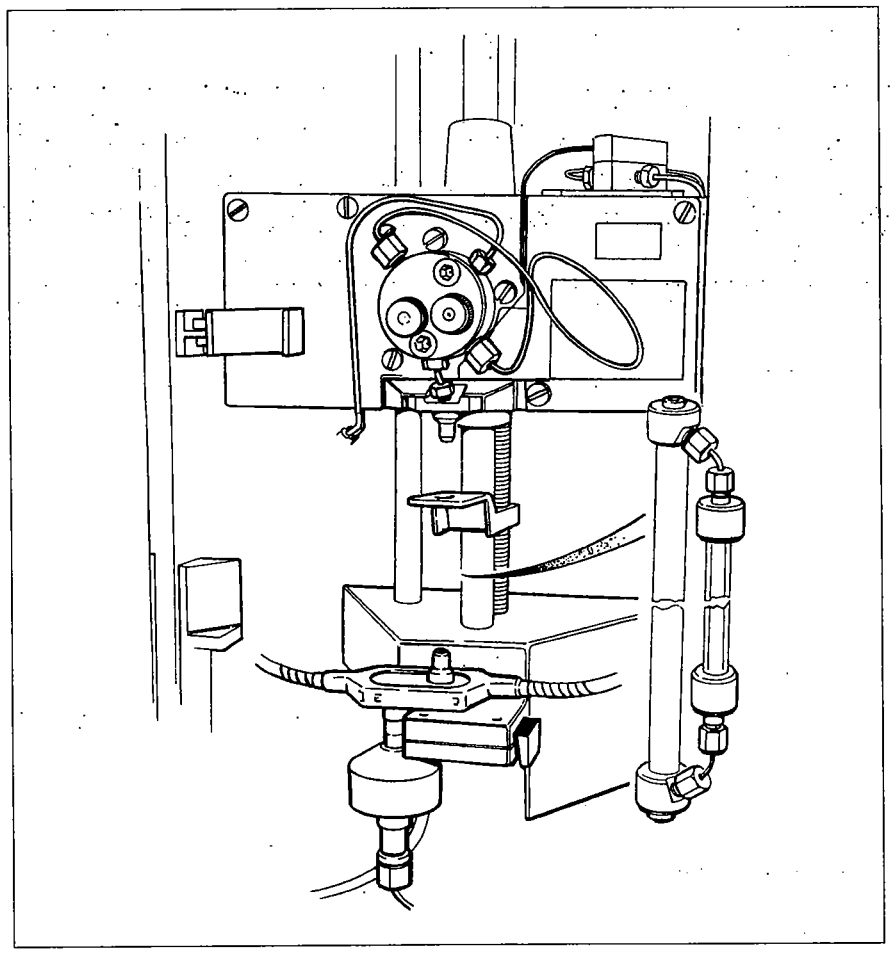


Fig. 6.26. Column Adapter

1. The Column Adaptor is delivered complete with two lengths of supporting rod. Select the most convenient length for the column you wish to install.
2. Assemble the two end pieces onto the supporting rod.
3. If necessary, click on MANUAL, COLUMN INSTALL in Run/Control. Highlight the short or the long adaptor and close the door of the µSeparation Unit. Click on UP to drive the automatic column holder upwards to make space for the Column Adaptor.
4. Fit the Column Adaptor onto the column connector.
5. Fit the column support onto the top of the Column Adaptor to hold it in position. Close the door of the µSeparation Unit.
6. In Run/Control, click on MANUAL, COLUMN INSTALL. Highlight the short or long column adaptor. Click on DOWN.
7. Connect the column of your choice to the Column Adaptor. For FPLC columns, use the Union M6 female to 1/16 inch male.
8. The column pressure limit is not set. If necessary, change this, in the COLUMN INSTALL dialogue box, to the appropriate pressure limit for the column you have installed.

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6.16 µPeak Monitor

6.16.1 Cleaning the flow cell

If unexplained baseline noise, drift or instability occur the flow cell should be cleaned according to the procedure below:

1. Install the dummy column in the system.
2. Disconnect the gradient monitor from the flow cell. Replace it with tubing.
3. Connect a large sample loop, 500 µl, to the injection valve.
4. Fill the µPrecision Pump with distilled water.
5. Fill the sample loop with a 10 % surface active detergent solution and pump approximately 300 µl through the flow cell at low flow rate, about 50 µl/min. Stop the flow and leave the detergent solution in the flow cell for 20 minutes. Start the flow and pump out the remaining detergent solution through the flow cell.
6. Fill the sample loop with isopropanol or ethanol and pump through at a flow rate of 20 µl/min.
7. Rinse the loop and flow cell with 10 ml distilled water.

6.16.2 Changing the µPeak Monitor flow cell

If the flow cell needs to be replaced, follow the instructions below.

1. Turn off the System Power Supply and disconnect the mains cable.



Warning: The Peak Monitor contains high voltage electronics. It is essential that the monitor is switched off before any covers are removed from the instrument.

2. To remove the front cover, insert a screwdriver under the front cover and pull down the black securing forks on both sides. Hold the lower end of the front cover and hook it off.

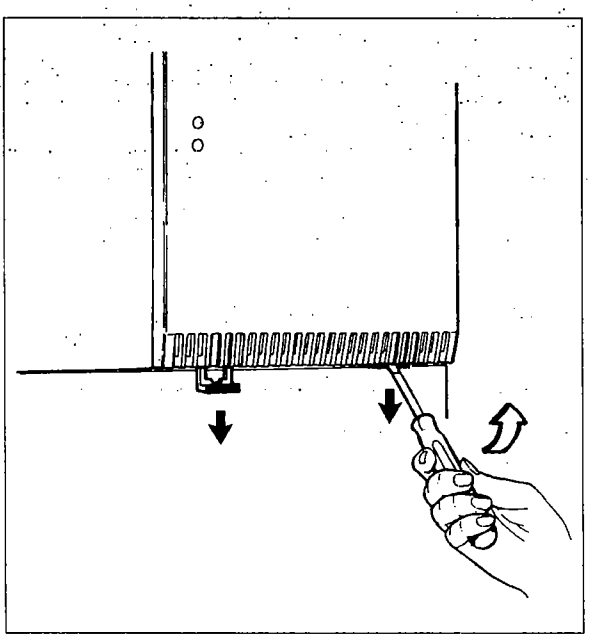


Fig. 6.27. Removing the front cover

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3. Remove the four screws on the front panel. Disconnect the cable at the top of the monitor and remove the front panel.
4. Remove the side cover:
   1. Loosen the two screws holding the side cover.
   2. Turn it 25 degrees to the right.
   3. Pull it off.

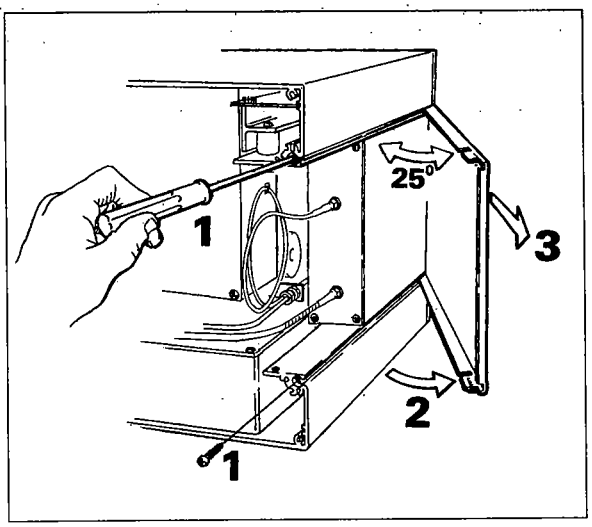


Fig. 6.28. Removing the side cover

5. Unscrew the screw on the front part of the fibre optics holder with the 3 mm Allen key supplied.

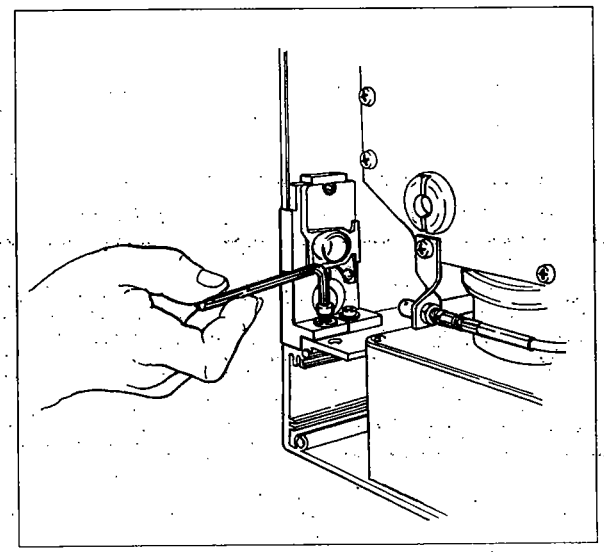


Fig. 6.29. Removing part of the fibre optics holder

6. Disconnect the optical fibre B from the flow cell splicer A. Disconnect fibre C from the detection unit D, (Fig 6.30). Pull out the fibres from the grommet and then put on the protective caps.

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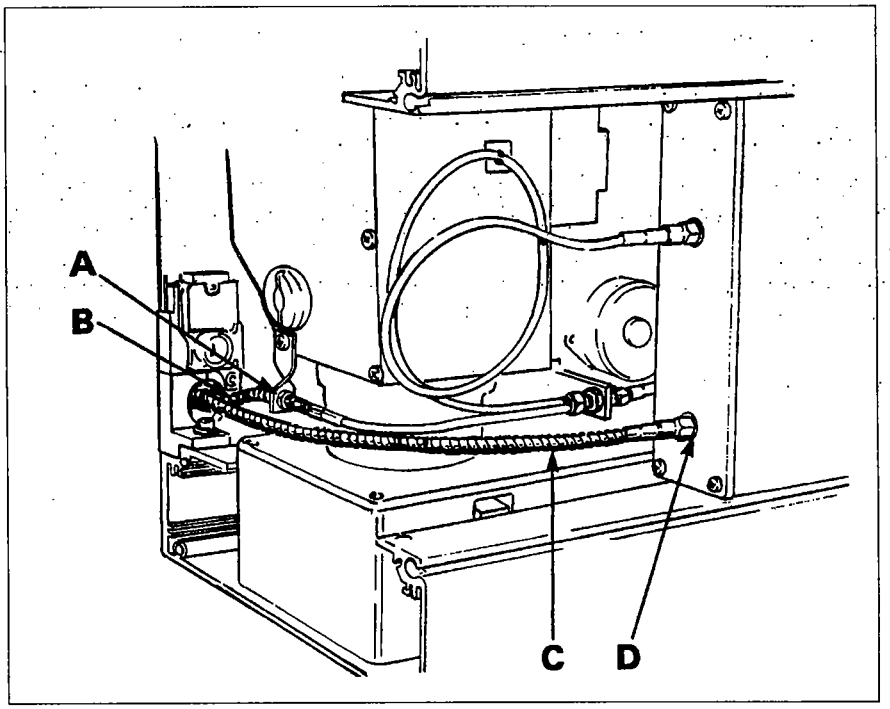


Fig. 6.30. Removing the optical fibres

7. Disconnect the fractionation needle from the fraction arm (A in Fig. 6.31).
8. Disconnect the gradient monitor signal cable from the socket (B in Fig. 6.31).

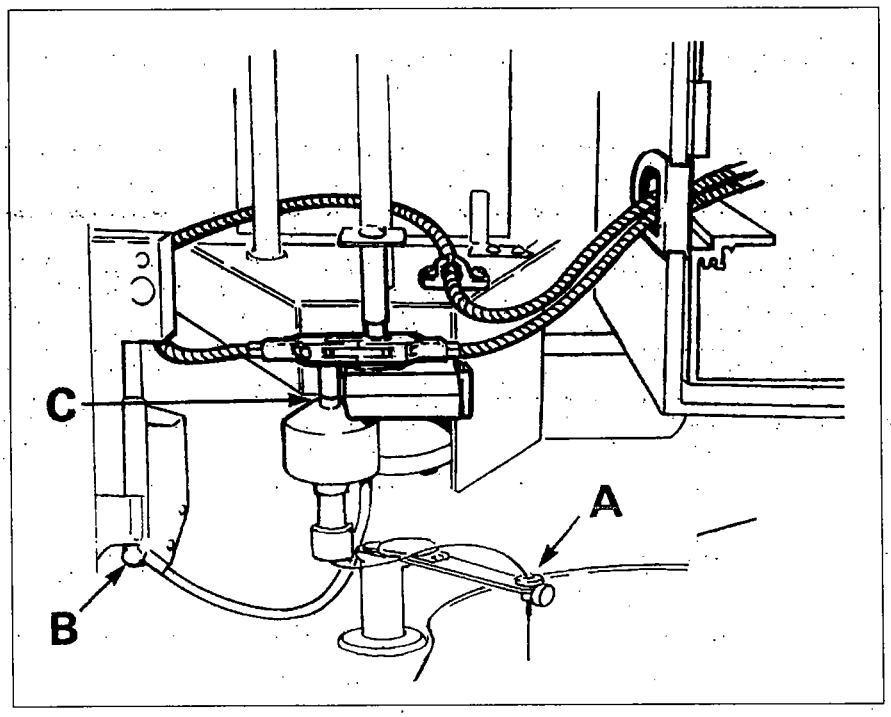


Fig. 6.31. Disconnection of fractionation needle and gradient monitor cable

9. Pull out the securing fork holding the flow cell and remove it.

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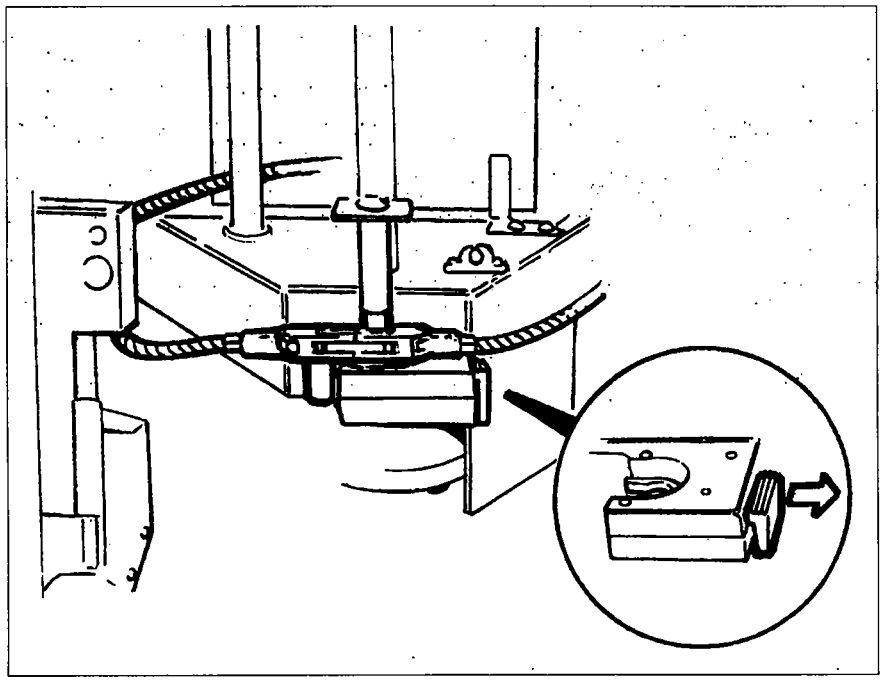


Fig. 6.32. Removing the flow cell

10. Disconnect the gradient monitor and flow restrictor from the outlet of the flow cell (C in Fig. 6.31).

11. Unpack the new flow cell and remove the red protective caps from the inlet and outlet of the flow cell. Leave the black protective caps on the end of the optical fibres.

    Note: Handle the optical fibres carefully.

12. Pull out the black securing fork (B in Fig. 6.33) and place the flow cell with the optical fibres in the holder inside the µSeparation Unit. Before securing it make sure the left hand fibre is placed behind the LED holder (A in Fig. 6.33). Push in the securing fork.

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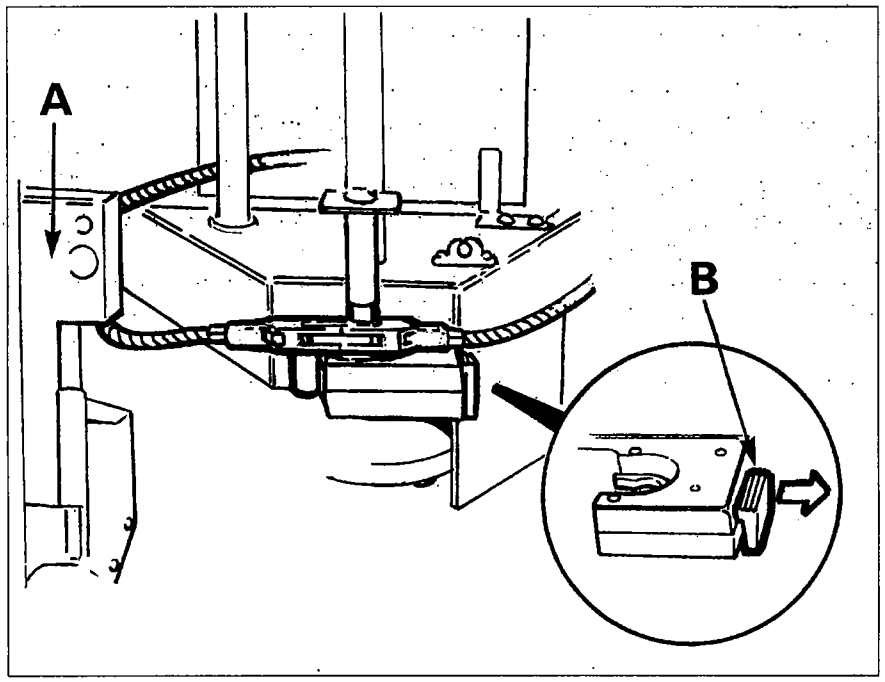


Fig. 6.33. Fastening the flow cell

13. Thread fibre B (Fig. 6.34) behind the column holder. Pull out the grommet from the side of the µSeparation Unit and thread the fibre through the upper hole in the grommet. Do not remove the black protective caps from the fibre ends!

14. Thread fibre C (Fig. 6.34) through the lower hole in the grommet. Put the grommet with the fibres back into the µSeparation Unit.

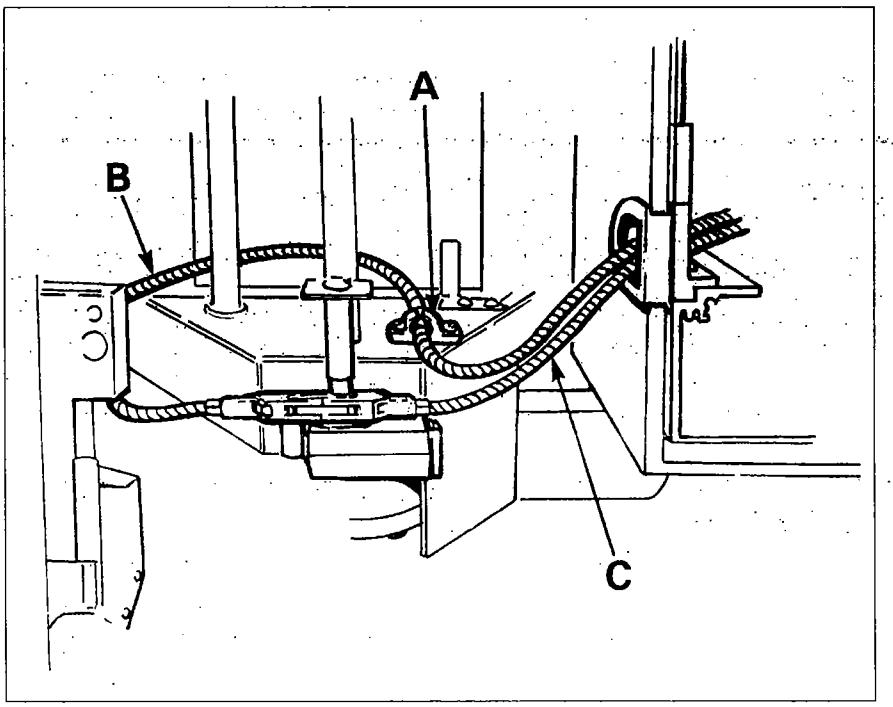


Fig. 6.34. Location of fibres in µSeparation Unit

15. Remove the protective cap from fibre B and from the flow cell splicer. Connect fibre B (see Fig:s 6.34 & 6.35) to the flow cell

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splicer (A in Fig. 6.35). Insert it gently without letting the fibre end touch the sides of the splicer. Tighten the nut fingertight.

Warning: Do not at any time touch the tips of the optical fibres with your fingers as this can result in poor monitor performance. If you accidentally have touched the fibre tip it can be wiped with 30 % isopropanol on lens paper.

16. Remove the protective cap from fibre C and from the detection unit. Connect fibre C (in Fig:s 6.34 & 6.35) to the detection unit D.

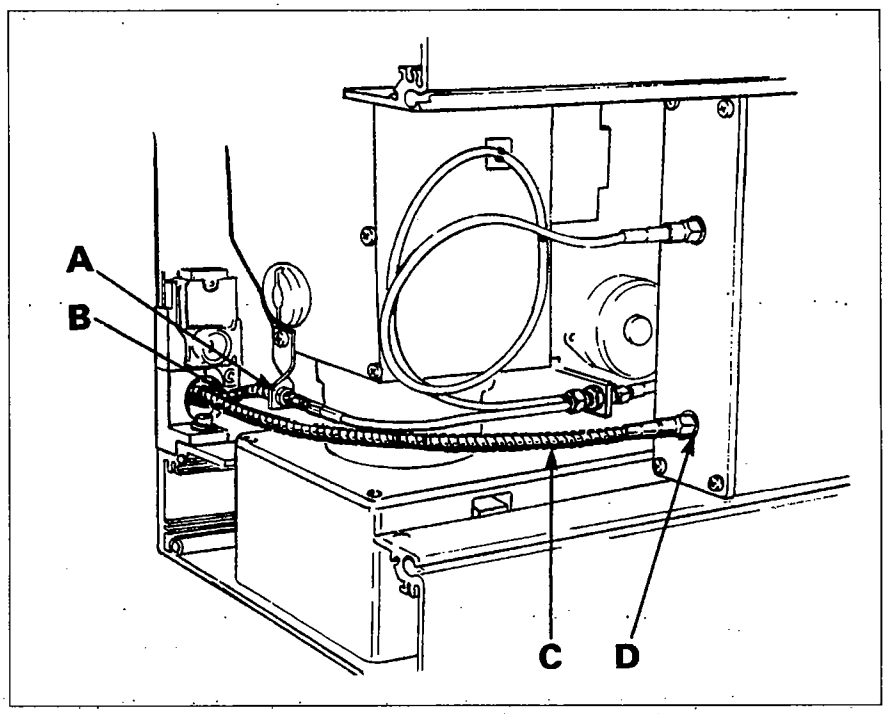


Fig. 6.35. Connections of optical fibres

Note: It is important that the fibres are connected to the correct inlets, otherwise the specifications will not be met.

17. Replace the fibre optics holder and fasten the Allen screw.
18. Replace the side cover and make sure the slits at the back of the cover fit into the pins at the back of the monitor. Secure the side cover with the screws.

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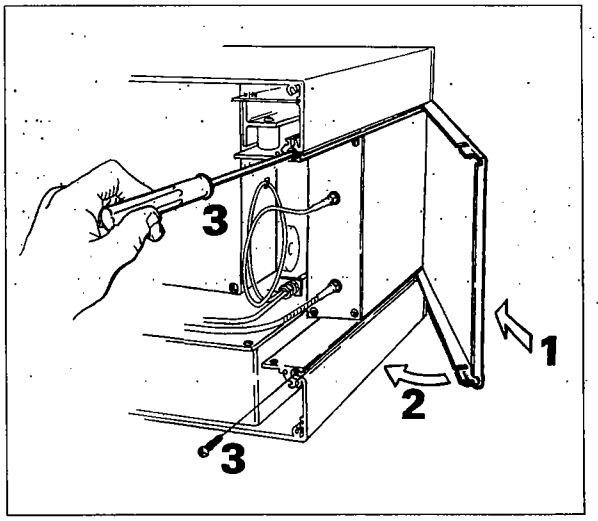


Fig. 6.36. Replacing the side cover

19. Replace the front panel and the four screws. Make sure the cable is not trapped between the monitor and the front panel.

    Note: Do not overtighten the screws.

20. Hook the front cover onto the front panel and push up the securing forks.

21. To reconnect the Gradient Monitor see Section 6.18.

6.16.3 Replacing the beam splitter

If a warning, *Reference light path error*, occurs the complete beam splitter, Code No. 18-1029-15, must be replaced.

1. Turn off the System Power Supply and disconnect the mains cable.

   Warning: The Peak Monitor contains high voltage electronics. It is essential that the monitor is switched off before any covers are removed from the instrument.

2. To remove the front cover, insert a screwdriver under the front cover and pull down the black securing forks on both sides. Hold the lower end of the front cover and hook it off.

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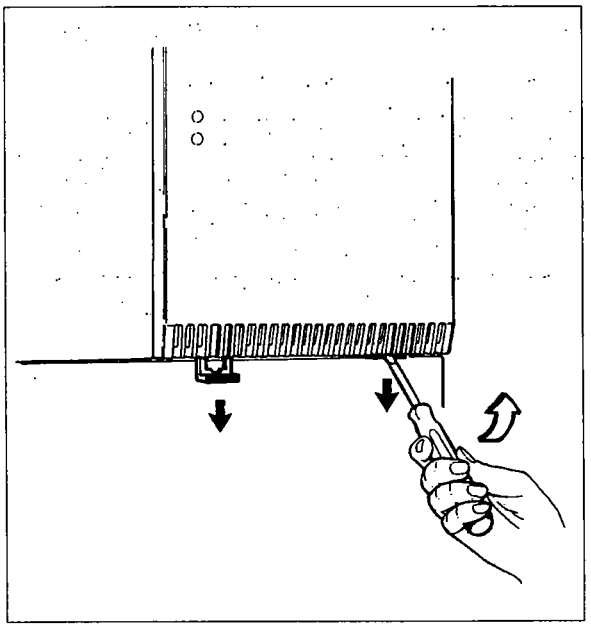


Fig. 6.37. Removing the front cover

3. Remove the four screws on the front panel. Disconnect the cable at the top and remove the front panel.
4. Remove the right side cover of the monitor;
   1. Unscrew the two screws holding the side cover.
   2. Turn it 25 degrees to the right.
   3. Pull it off.

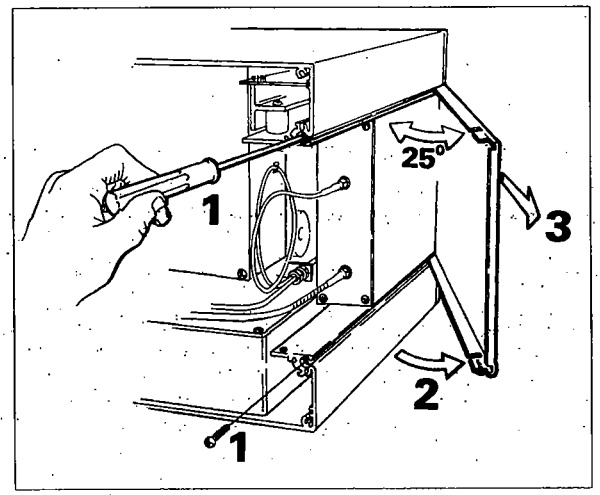


Fig. 6.38. Removing the side cover

5. Disconnect the beam splitter reference fibre in the detector unit B.
6. Loosen the fibre from the clip A on the side of the monochromator by pushing in the plastic hook with a small screwdriver.
7. Disconnect the connector with the two fibres from the beam splitter splicer C.
8. Disconnect the beam splitter sample fibre from the flow cell splicer D.

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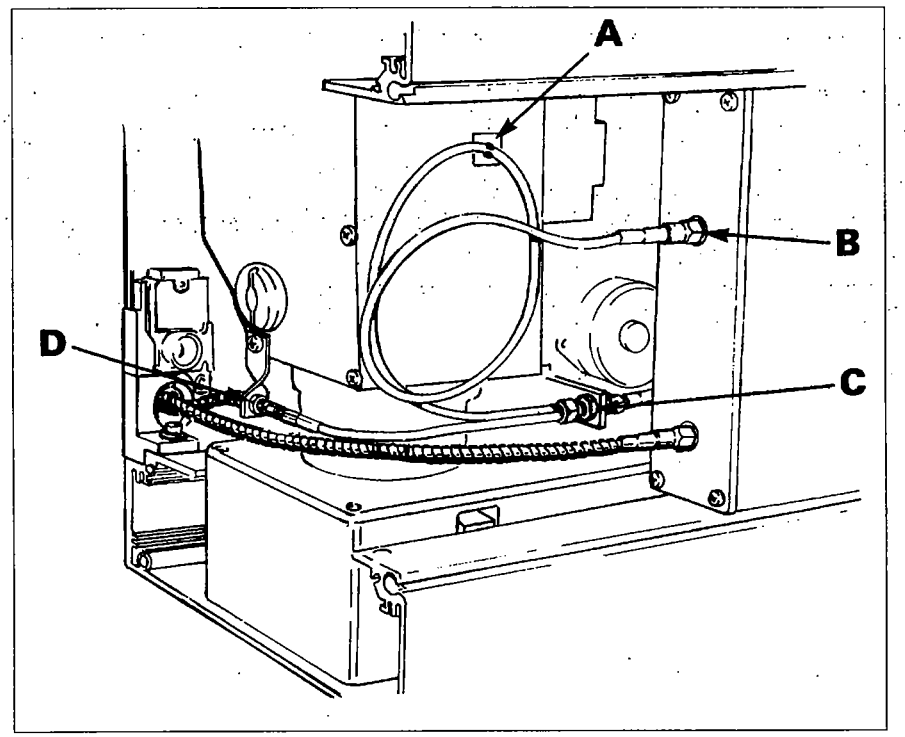


Fig. 6.39. Replacement of beam splitter

9. Remove the protective caps from the new beam splitter fibres.

   Warning: Do not at any time touch the tips of the optical fibres with your fingers as this can result in poor monitor performance. If you accidentally have touched the fibre tip it can be wiped with 30 % isopropanol on lens paper.

10. Connect the beam splitter sample fibre to the flow cell splicer D.
11. Connect the connector with the two fibres to the beam splitter splicer C. The beam splitter reference fibre should be upwards.
12. Form a single gentle loop of the beam splitter reference fibre and connect the fibre end to the detector unit B.
13. Secure the fibre loop in the clip A on the monochromator.
14. Replace the side cover and make sure the slits at the back of the cover fit into the pins at the back of the monitor. Secure the side cover with the screws.

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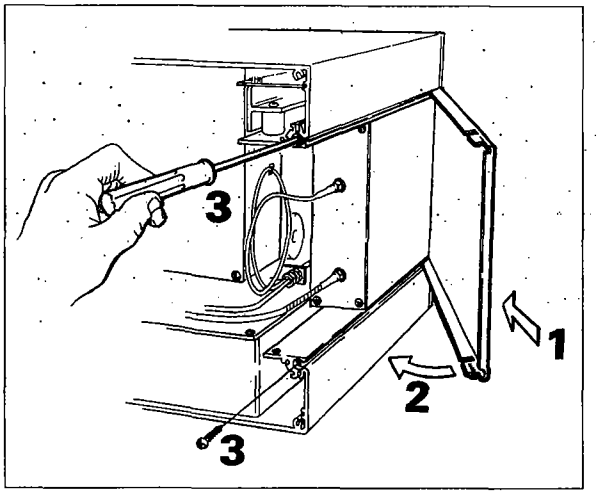


Fig. 6.40. Replacing the side cover

15. Replace the front panel and the four screws. Make sure the cable is not trapped between the monitor and the front panel.

    Note: Do not overtighten the screws.

16. Hook the front cover onto the front panel and push up the securing forks.

6.17 Monitor UV-M II

6.17.1 General maintenance for the flow cell

A clean flow cell is essential for the correct operation of the UV-M II. Follow the recommendations in Section 6.1 regarding the eluents.

If the eluent contains dissolved salts, proteins or other solutes of low volatility, ensure that the flow cell is never allowed to dry out. After use, install the Dummy Column and flush through the entire system using the System Wash function in the Manual menu in Run/Control.

Do not allow particles to enter the flow cell. The flow cell may be inspected for particles and air bubbles by removing it from SMART System and examining the light path through the window with a magnifying glass.

Instructions are given below for the removal of the flow cell from the system and for different cleaning procedures. Further details are given in the Instruction Manual supplied with the monitor.

6.17.2 Changing the flow cell

1. Switch off the Monitor UV-M II.
2. Use the Column Install function in Manual in Run/Control to raise the automatic column holder. Remove the column from the system and store as recommended in the Column Instructions.
3. Remove the delivery needle from the fraction collector arm. Disconnect the gradient monitor signal cable.
4. If necessary, remove the cable grommet and disconnect the Optical Unit cables from the back panel of the UV-M II Control Unit. Pass the cable through the back panel of the µSeparation Unit.
5. Pull out the securing fork and remove the Optical Unit (Fig. 6.41). If the larger Zn lamp is fitted, loosen the column

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connector and tilt the Optical Unit until it can be removed easily from its support.

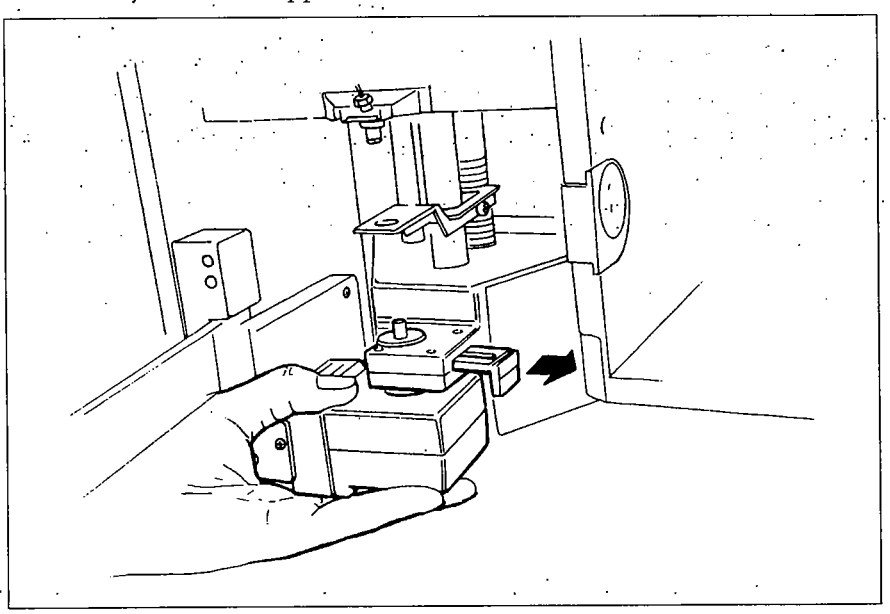


Fig. 6.41. Disconnecting the Optical Unit

6. Use a 2.5 mm Allen key to remove the two screws holding the main body of the gradient monitor to the underside of the Optical Unit (Fig. 6.42). Use the wrench supplied to loosen and remove the connector from the flow cell.

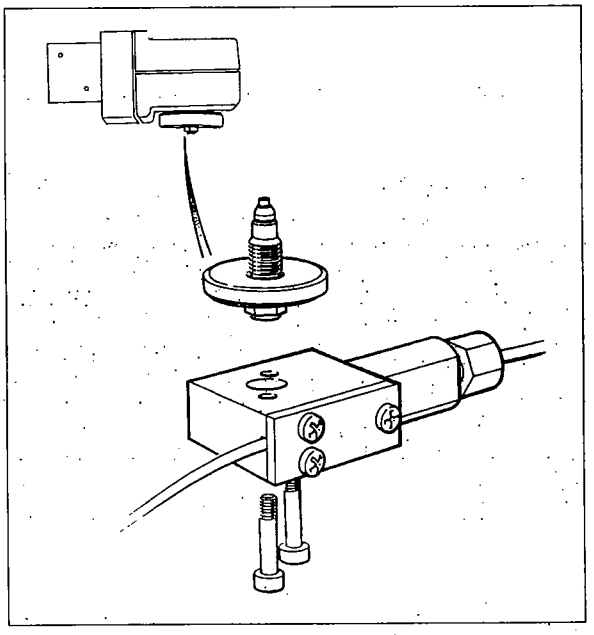


Fig. 6.42. Removing the gradient monitor

7. Loosen and remove the column connector from the flow cell. Loosen the locking screw and then pull the flow cell out of the Optical Unit (Fig. 6.43).

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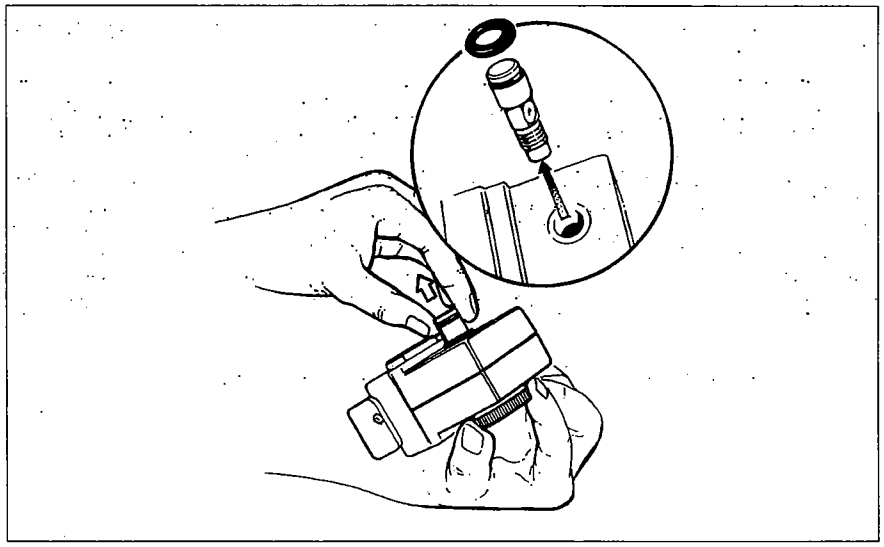


Fig. 6.43. Removing the flow cell

8. Insert the flow cell with its protective ring into the Optical Unit. Secure it in position with the locking ring on the underside of the Optical Unit (Fig. 6.44).

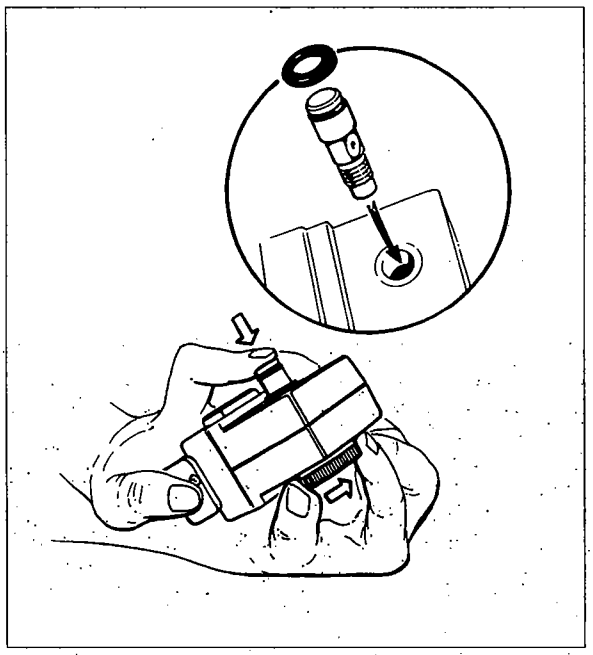


Fig. 6.44. Inserting the flow cell

9. Fit the column connector into the flow cell and tighten it slightly (Fig. 6.45)

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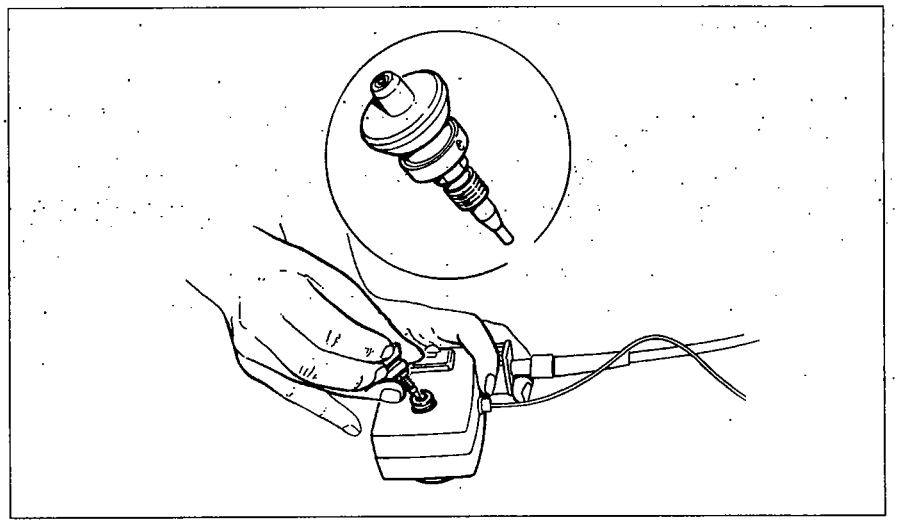


Fig. 6.45. Fitting the column connector

10. Fit the upper connection piece of the gradient monitor to the underside of the flow cell, and use the wrench supplied to tighten it firmly (Fig. 6.46).

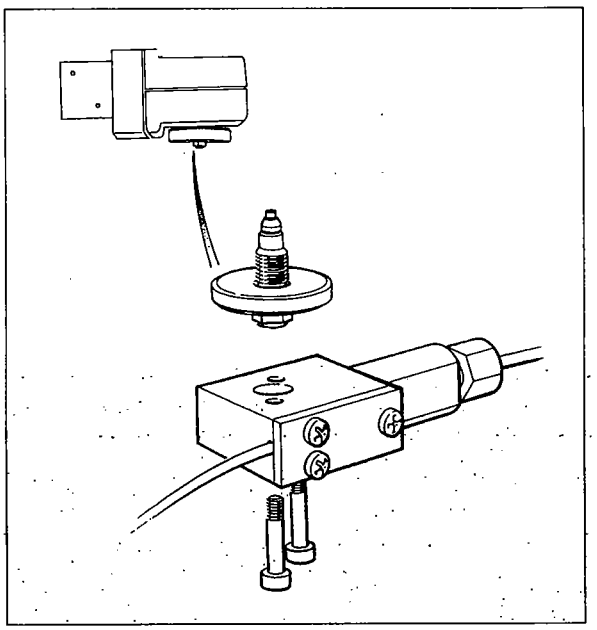


Fig. 6.46. Fitting the gradient monitor

11. Use the two Allen screws to connect the main body of the gradient monitor to the connection piece on the underside of the Optical Unit.

12. If the cables from the Optical Unit have been disconnected, pass the cable out through the back panel of the µSeparation Unit. Connect the lamp cable and the Optical Unit cable into the sockets marked LAMP and OPTICAL UNIT respectively on the back panel of the UV-M II Control Unit. Replace the cable grommet.

13. Pull out the securing fork and fit the Optical Unit (Fig. 6.47). If the larger Zn lamp is fitted, loosen the column connector and tilt the Optical Unit until it can be fitted easily into its support. Tighten the column connector firmly.

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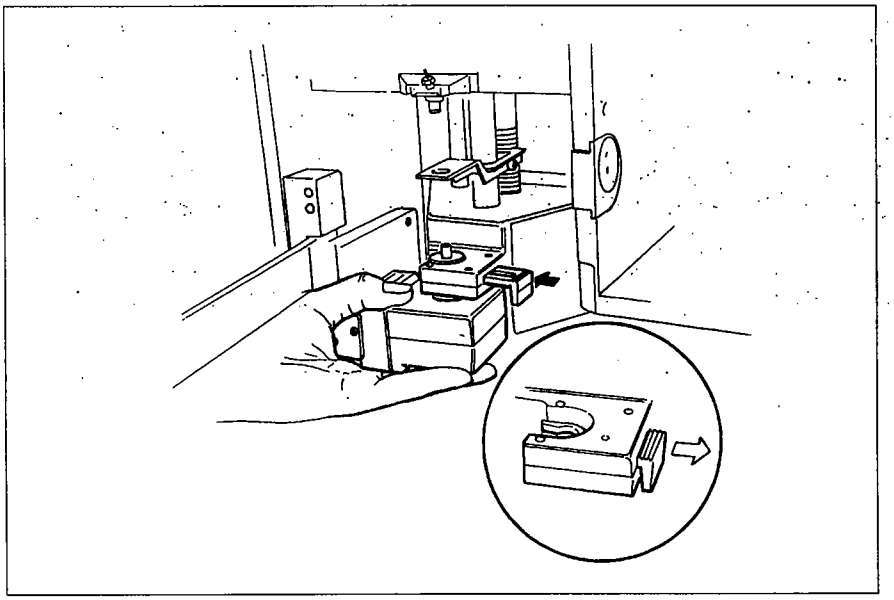


Fig. 6.47. Fitting the Optical Unit

14. Refit the delivery needle into the fraction collector delivery arm (Fig. 6.48). Reconnect the gradient monitor signal cable.

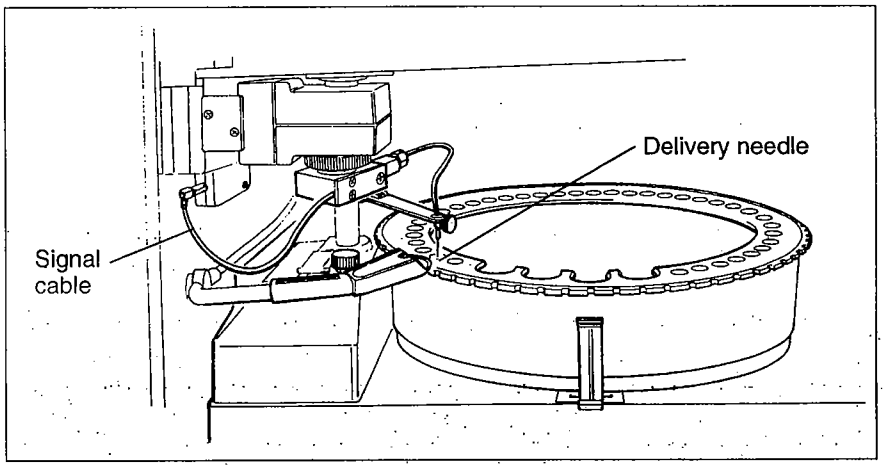


Fig. 6.48. Completing the reassembly

15. Re-install the column. Switch on the Monitor UV-M II.

6.17.3 Removing trapped particles from the flow cell

1. Remove the flow cell from the Optical Unit as described in Section 6.17.2.
2. Connect a syringe to the flow cell outlet and flush a clean solution of ethanol in distilled water (50 % v/v) through the cell in small quantities. Examine the cell from time to time to check if the particles have been washed out.
3. Rinse the cell with filtered distilled water (about 20 ml) and then replace it in the Optical Unit.
4. Reconnect the cell as described in Section 6.17.2.

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6.17.4 Removing non-particulate contaminants

Most non-particulate contaminants e.g. denatured proteins, salts etc. can be removed by flushing the cell with an appropriate solvent. Laboratory detergents of the type used to decontaminate glassware may also be used.

Oily deposits, which increase the tendency to trap air bubbles, can be removed by rinsing the flow cell first with a non-polar solvent, such as hexane, then with a polar solvent such as isopropanol and finally with distilled water or detergent.

Persistent contaminants may be removed by either of the following cleaning procedures, depending on the nature of the contaminant. Warming the cleaning medium to 40 °C may improve the result.

Cleaning with detergent:

1. Remove the flow cell from the Optical Unit as described in Section 6.17.2.
2. Fill the cell with a 10 % solution of RBS 25, Deconex or an equivalent and let it stand for at least two hours.
3. Rinse the cell with distilled water, followed by ethanol/distilled water (50 % v/v) and finally distilled water. Use at least 20 ml of each liquid.
4. Replace the cell in the Optical Unit as described in Section 6.17.2.

Cleaning with chromic acid:



Warning: Chromic acid is extremely corrosive. Wear eye protection when using it. Handle it with extreme care and treat any spilling immediately with a large excess of water.

1. Prepare fresh chromic acid by adding concentrated sulphuric acid (100 ml) to a saturated solution of sodium dichromate (3.5 ml).
2. Remove the flow cell from the Optical Unit as described in Section 6.17.2.
3. Connect a glass syringe to the outlet of the cell and carefully draw chromic acid into the cell. Do not draw acid into the syringe.
4. Allow the chromic acid to remain in the cell for 10-20 minutes.
5. Remove the chromic acid carefully without splashing and then rinse the cell with 100 ml distilled water, followed by 20 ml ethanol/distilled water (50 % v/v) and finally 20 ml distilled water.
6. Replace the cell in the Optical Unit as described in Section 6.17.2.

6.17.5 Cleaning the filters

For optimal performance, it is essential that the interference filters are clean and free of any particulate material. Do not touch the interference filters. If the filters become contaminated with dust, fingerprints or oil, proceed as described below or in the Instruction Manual supplied with the Monitor UV-M II.

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1. Remove the Optical Unit from SMART System as described in Section 6.17.2.
2. If the Zn lamp is fitted, remove the Zn lamp housing. Remove the four screws in the filter housing and separate the filter housing from the Optical Unit.
3. Carefully take the filter out of the housing without touching or scratching the filter surface.
4. Use lens cleaning tissue dipped in ethanol to clean the filter surfaces gently.
5. Place the clean filter back into the filter wheel and reassemble the filter housing in the Optical Unit.
6. Replace the cell in the Optical Unit as described in Section 6.17.2.

6.17.6 Changing the UV-M II Monitor lamp assembly

When delivered, the Optical Unit of Monitor UV-M II is fitted with an Hg lamp for operation at 280 nm and 254 nm. A Zn lamp is available for operation at 214 nm. Each type of lamp has its own housing so that the lamp and housing is replaced when changing from one type to another.

1. Switch off the mains power switch at the back of the UV-M II Control Unit. Disconnect the lamp cable and the Optical Unit cable from the back panel.
2. Remove the cable grommet on the back of the µSeparation Unit.
3. Remove the fractionation needle from the fraction collector arm. Disconnect the Gradient Monitor signal cable.
4. Pull out the securing fork and remove the Optical Unit. If the Zn lamp is fitted, loosen the column connector and tilt the Optical Unit until it can be removed easily from its support (Fig. 6.49).

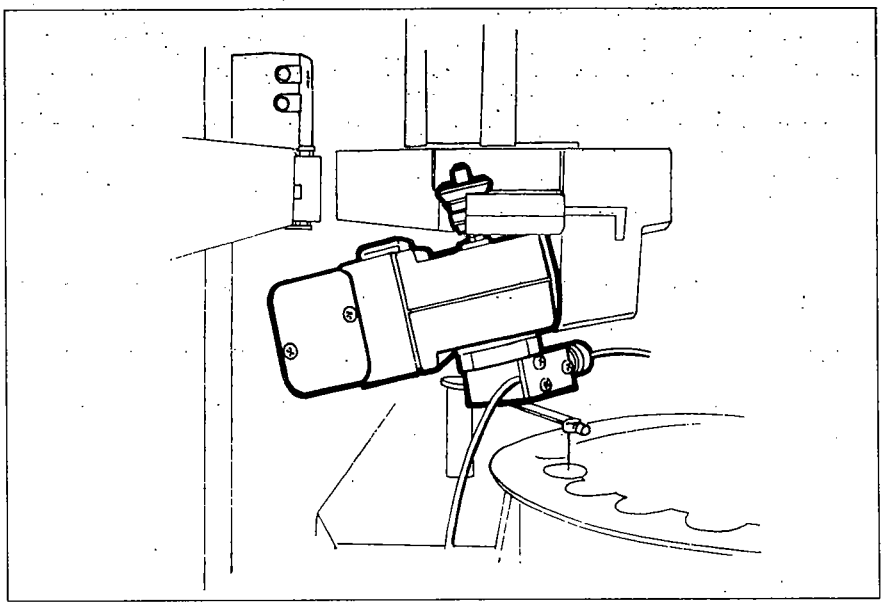


Fig. 6.49. Removing the Optical Unit with the Zn lamp

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5. Pull the cables through from the back of the µSeparation Unit.
6. On the end plate of the lamp housing, loosen and remove the two securing screws. Remove the end plate.
7. Slide the lamp housing off the Optical Unit. Refit the end plate.
8. On the lamp housing to be fitted, repeat Step 6 and remove the end plate.
9. With the light aperture facing the filter, slide the lamp housing onto the guides on the Optical Unit. Depress the two pressure pads on the filter housing in sequence as the lamp housing is being fitted.
10. Refit the end plate onto the lamp housing.
11. Slide the lamp housing firmly into position. There is a faint click when the housing is positioned correctly. The mercury lamp housing can click into two positions, one for 280 nm and one for 254 nm. The zinc lamp housing has only one position.
12. Push the cables for Optical Unit and lamp through to the back of the µSeparation Unit. Replace the cable grommet.
13. Refit the Optical Unit and push in the fork to secure it in position.

    Note: If the larger zinc lamp is fitted, loosen the column connector before fitting the Optical Unit. Tighten the column connector and then push in the fork.
14. Re-connect the lamp and Optical Unit cables to the back panel of the UV-M II Control Unit. Switch on the UV-M II.
15. In Run/Control, click on CONFIG, USER SUPPLIED CONFIGURATION. In the Monitor UV-M II field, click on the wavelength of the newly fitted lamp.

6.17.7 Changing a defective lamp

The mercury lamp, the reflective surface used for 280 nm and the zinc lamp have approximate lifetimes of 8 000 hours, 4 000 hours and 2 000 hours respectively.



Warning: To prevent injury to eyes ensure that the lamp cable is disconnected before changing a defective lamp. If the mercury lamp is broken make sure that all the mercury is removed.

1. Switch off the mains power on the monitor. Remove the Optical Unit from SMART System as described in Section 6.17.2.
2. Remove the two screws on the lamp housing plate which is attached to the lamp power cable.
3. Carefully pull the lamp out of the lamp housing.
4. Replace the defective lamp with the new lamp and insert the assembly into the lamp housing. Secure the end plate again with the two screws. Do not touch the lamp.
5. Replace the cell in the Optical Unit as described in Section 6.17.2.

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6.18 Changing the Gradient Monitor

Before removing the Gradient Monitor make sure you have a new one available.

- Gradient Monitor/Flow Restrictor, µPeak Monitor
  Code No. 18-1029-94
- Gradient Monitor/Flow Restrictor, UV-M II
  Code No. 18-1017-28

µPeak Monitor

1. Remove the fractionation capillary from the fraction collector arm. Disconnect the Gradient Monitor cable from the socket on the left hand side of the Separation Unit. Unscrew the Gradient Monitor from the µPeak Monitor flow cell.
2. Insert the short PEEK capillary supplied into the Gradient Monitor inlet. Make sure it reaches the bottom, so that no dead volume results.
3. Slide the plastic ferrule onto the capillary.
4. Carefully position the Gradient Monitor below the outlet of the flow cell. Ensure that this alignment is straight. Then carefully screw the Gradient Monitor into the flow cell. To make the final tightening, hold the cover of the Gradient Monitor housing and turn until the cover starts to slip. This will prevent overtightening.

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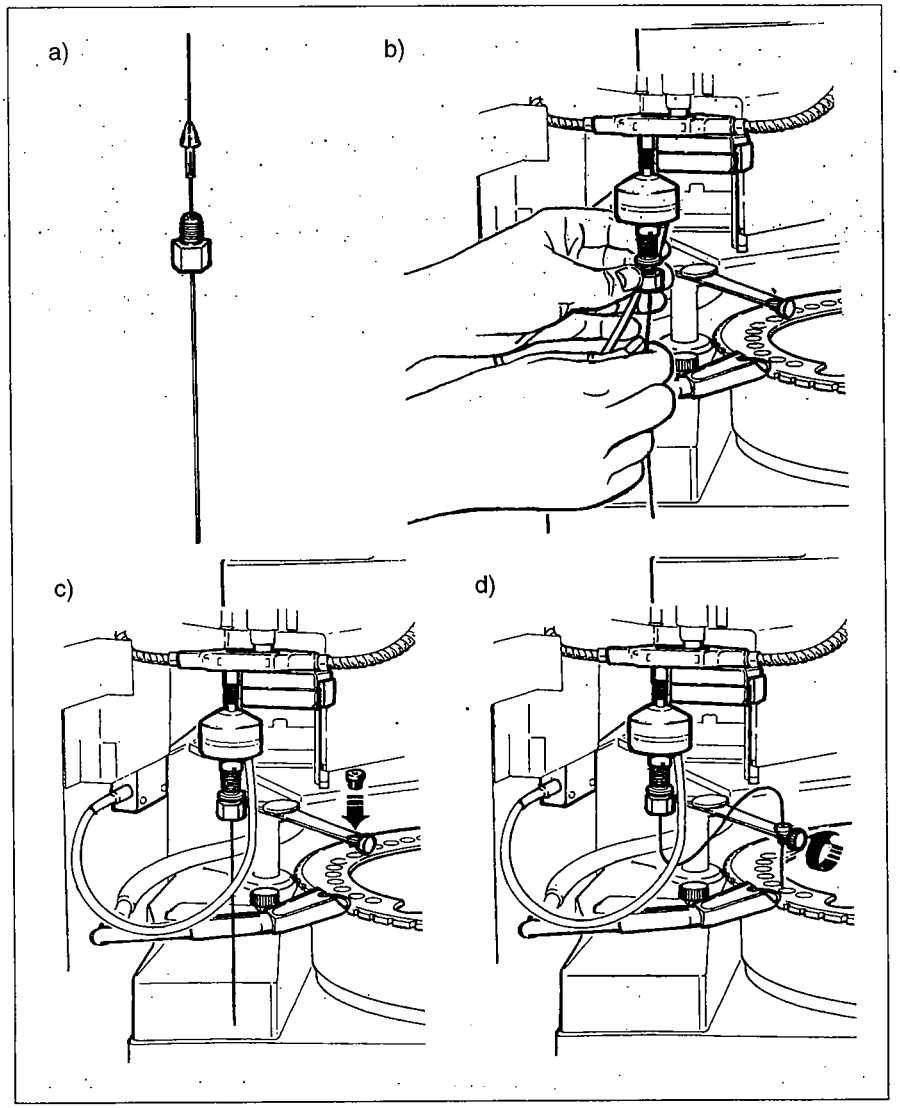


Fig. 6.50. Installation of the Gradient Monitor

Note: If the Gradient Monitor is not well aligned before it is screwed in, the threads on the Gradient Monitor inlet may be damaged, resulting in poor sealing.

5. Connect the gradient monitor signal cable to the socket. Connect the fractionation capillary to the fraction collector arm.

Monitor UV-M II

1. Remove the fractionation needle from the fraction collector arm. Disconnect the Gradient Monitor signal cable from the socket on the left hand side of the µSeparation Unit.
2. Use a 2.5 mm Allen key to remove the two screws holding the main body of the gradient monitor to the underside of the Optical Unit.

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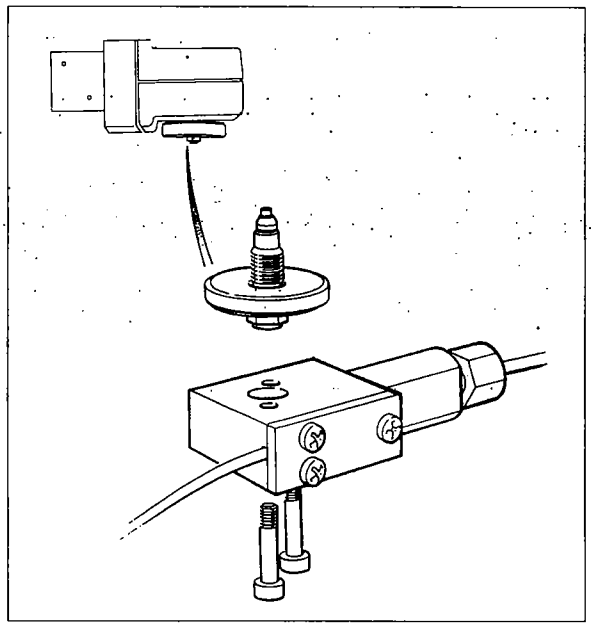


Fig. 6.51. Removing the gradient monitor

3. Connect the new gradient monitor to the Optical Unit with the Allen screws.
4. Replace the fractionation needle. Push the tubing into the flow restrictor while tightening the connector. Connect the gradient monitor signal cable to the socket.

6.20 Adjustment of fractionation capillary

The position of the fractionation capillary in the collection tube must be correctly set to ensure that precise fractions are collected, especially in Wipe Mode. In Drop Mode, the needle is in the up position and centrally aligned to the collection tube. In the Wipe Mode, the needle is in the down position in contact with the inner wall of the collection tube. Two adjustments are possible; the sideways movement of the delivery arm and the height of the fractionation capillary in the delivery arm.

1. Loosen the fractionation capillary and remove it from the fractionation arm.
2. In Run/Control, click on MANUAL, PUMP FLOW. Set the flow to 1 µl/min.
3. Click on MANUAL, SEP UNIT, FRACTIONATION. Set the fraction size to 1000 µl/min. Set the Needle position to down Mark the check box Start at tube and confirm the settings. The fraction collector arm will move to the first tube.
4. Check the sideways adjustment of the capillary. It should be positioned at the centre of the tube. To adjust it sideways slightly loosen the delivery arm locking screw at the back of the arm. Support the back of the arm while you adjust the position. Tighten the locking screw.
5. Put back the fractionation capillary into the fractionation arm. Adjust the height of the needle so it is located just above the bottom of the tube.
6. Check that the position of the capillary for waste collection is correct. Click on MANUAL, SEP UNIT, FRAC STOP. Click on OK and the needle moves over the waste collector.

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7. Check the position of the capillary once more in the tube by repeating step 2 and 3. Make further adjustments if necessary.

6.21 Procedure for locating a blockage

High back pressure can be the result of a blockage somewhere in SMART System. The following procedure is a guide to identifying the position of the blockage in the system. For typical back pressure values at different flow rates, see Table 1. Table 2 gives typical back pressure values at different system positions. Further instructions are given in the following section for the remaking of the tubing connections.

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connections.

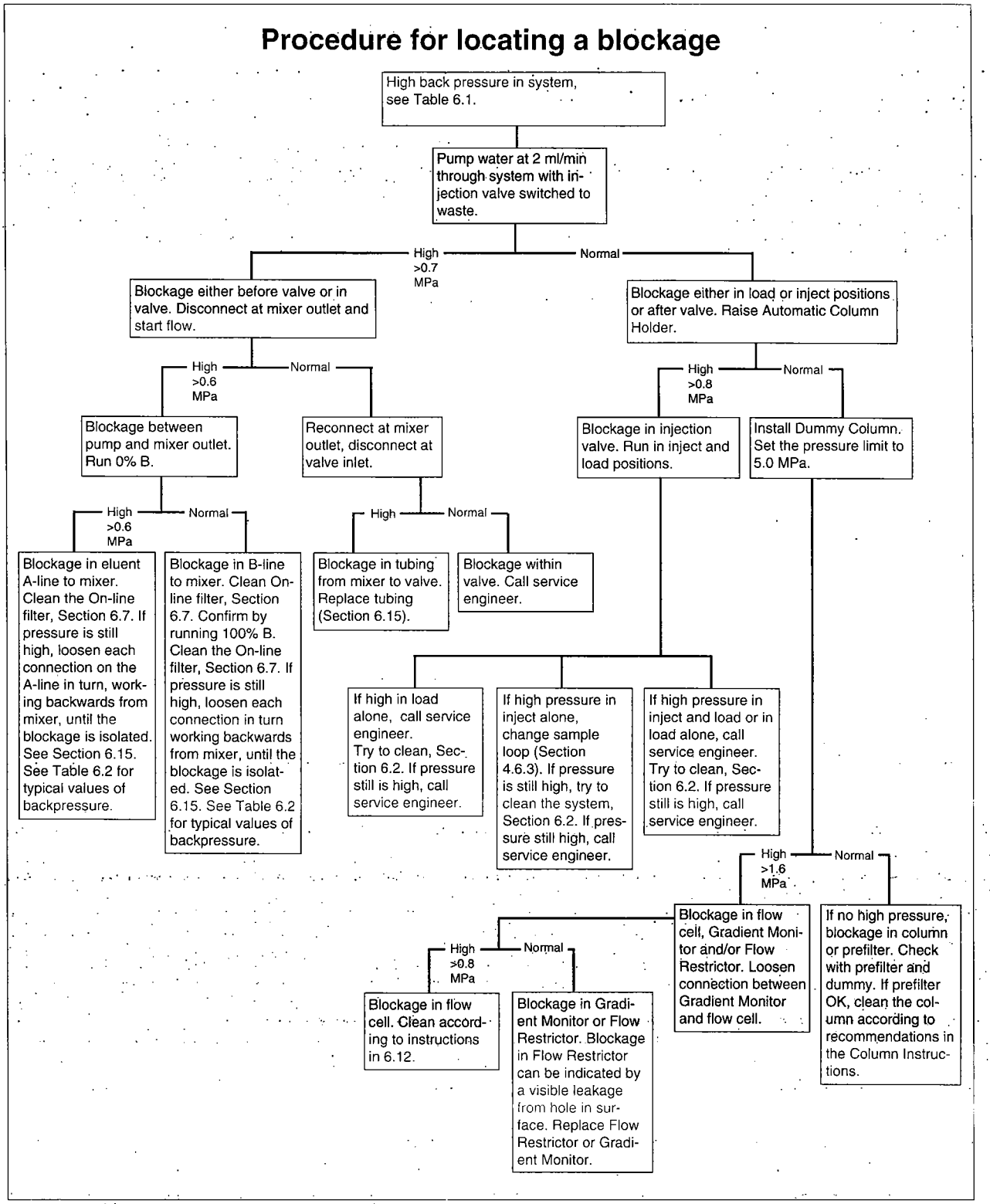


Chart for locating a blockage

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Table 1. Typical back pressure values in a complete SMART System

Flow rate (µl/min):	Back Pressure (bar) With prefilter	Without prefilter
10	7	6.5
100	8	7.5
400	10	8.5
1000	13	10
1500	16	12
2000	19	14

Note: These are approximate values, it can differ up to ± 0.2 MPa on the higher values because of difference in tubing inner diameter.

Table 2. Pressure table for trouble shooting high back pressure.

Eluent: H_2O System disconnected at	Flow rate: 2000 µl/min Back pressure MPa
Pump valve outlet	0
On-line filter inlet	0.15
On-line filter outlet	0.2
Mixer inlet	0.35
Mixer outlet	0.4
Inj valve waste position	0.5
Inj valve load position	0.55
Inj valve inject (50 µl loop)	0.6
Restrictor outlet	1.4
Frac needle outlet	1.4

Note: These are approximate values, it can differ up to ± 0.2 MPa on the higher values because of difference in tubing inner diameter.

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6.22 Remaking fluidic connections

The different types of tubing used in SMART System are illustrated in Figure 6.52.

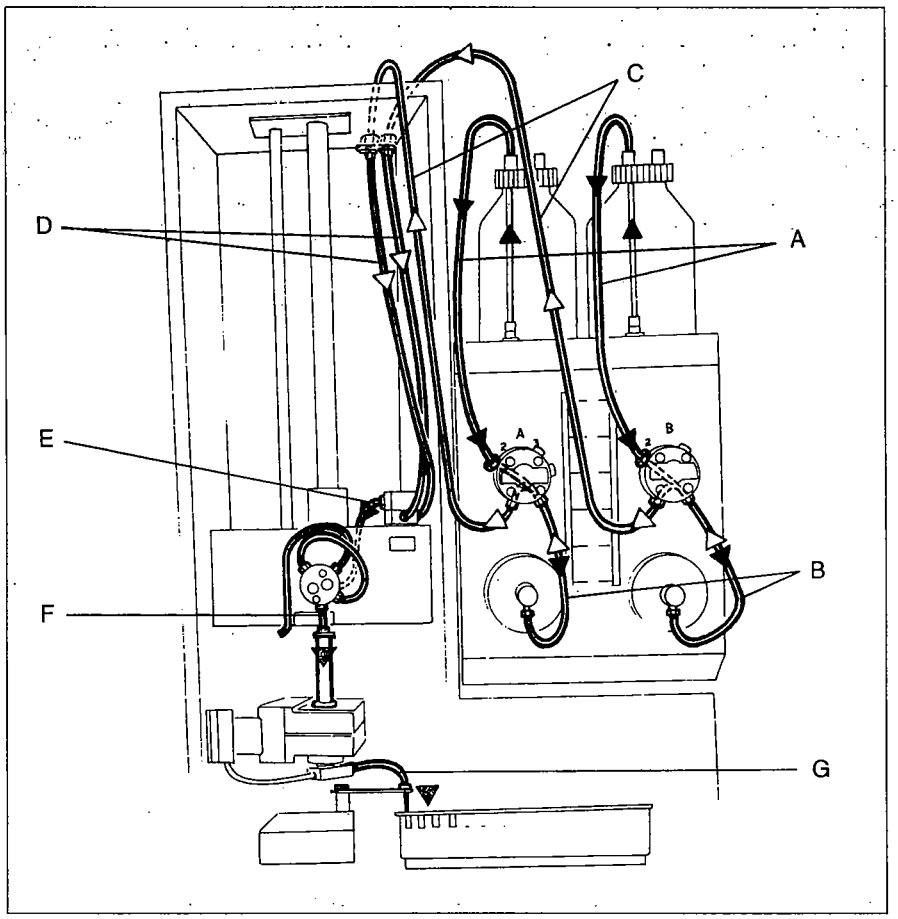


Fig. 6.52. SMART® System fluidic pathways

A. Pump Inlet Tubing (Code No. 18-1800-26)

This tubing connects the buffer reservoir to the pump inlet valve for eluent delivery. The ETFE tubing, o.d. 2.7 mm, i.d. 1.9 mm, is flanged ready for connection to the inlet valve. The connector on the valve is an M6 fitting. The replacement tubing is supplied complete with a solvent filter.

To remake this connection, push the tubing into the connector and tighten the hexagonal connector firmly, first with the fingers, then with the black wrench supplied.

B. Cylinder Tubing, Ti (Code No. 18-1012-23)

This titanium tubing connects the inlet valve to the pump cylinder. It is supplied complete with fingertight connectors for connection to both the valve and the cylinder.

To remake this connection, push the tubing into the connector and hold it firmly in position while tightening the hexagonal connector. Tighten until it is fingertight and then use a 1/4"-3/8" wrench to tighten fully.

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C. Pump Outlet Tubing, 2 x 0.7 m, (Code No. 18-1800-27) or Pump Outlet Tubing, 2 x 0.5 m (Code No. 18-1800-29)

These two pieces of PEEK tubing to connect the pump outlet the On-line Filters on the µSeparation Unit. Each piece is supplied complete with fingertight connectors on both ends.

To remake this connection, push the tubing into the hexagonal connector. Hold it there firmly while tightening the connector first with the fingers then with the wrench supplied.

D. Tubing, inlet to mixer x 2 (Code No. 18-1015-31)

These two pieces of PEEK tubing connect the On-line Filters on the µSeparation Unit with the mixer inlets. Each piece is supplied complete with fingertight connectors on both ends.

To remake this connection, push the tubing into the hexagonal connector. Hold it there firmly while tightening the connector first with the fingers then with the wrench supplied.

E. Tubing, mixer to injection valve (Code No. 18-1015-64)

The titanium tubing connecting the mixer outlet to the injection valve is supplied with a fingertight screw for connection to the mixer and a titanium compression screw and ferrule for connection to the injection valve.

To access the connector at the back of the injection valve, loosen the two screws securing the clear plastic plate and remove the plate (Fig. 6.53). The compression screw at the back of the valve is now accessible. Loosen the compression screw and remove the old tubing.

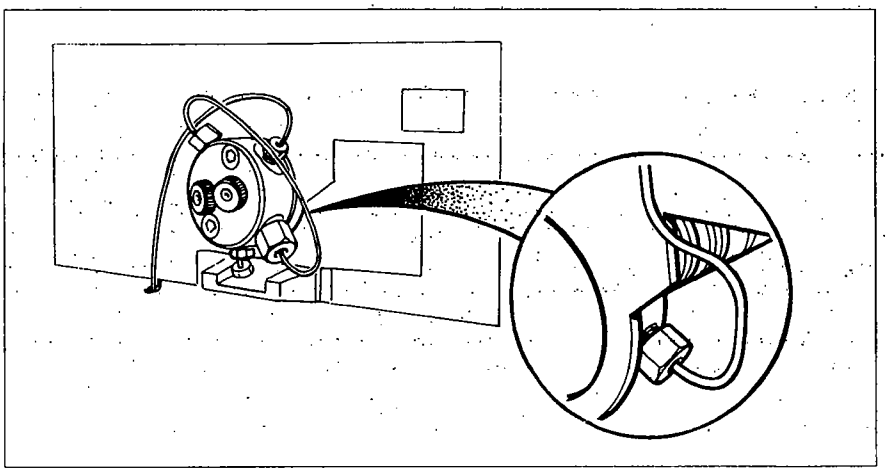


Fig. 6.53. Inlet connection on the injection valve

To fit the new tubing to the injection valve, push the tubing firmly into the connector. Hold it in this position while tightening the compression screw until fingertight. Finally, tighten with the wrench supplied.

To remake the tubing connection on the mixer, push the tubing into the hexagonal connector. Hold it there firmly while tightening the connector first with the fingers then with the wrench supplied.

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F. Tubing, injection valve to column (Code No. 18-1015-65)

This titanium tubing is supplied with titanium compression screws already fitted to both ends of the tubing. The tubing is fragile and should therefore be handled with care while fitting.

To remake this connection, push the tubing into the compression screw. Hold it there firmly while tightening the screw first with the fingers then with the wrench supplied.

G. Fractionation needle (Code No. 18-1017-26)

This spare part consists of the fractionation needle with PTFE tubing and a fingertight screw to connect to the flow restrictor. To replace this tubing, release the needle from its supporting arm and loosen the fingertight connector on the flow restrictor.

To make the connection on the flow restrictor, push the tubing into the hexagonal connector. Hold it there firmly while tightening the connector first with the fingers then with the wrench supplied. Fit the needle into the delivery arm and tighten the locking screw.

H. Fractionation capillary (Code No. 18-1000-18)

This spare part consists of five fused silica fractionation capillaries, a fingertight screw to connect to the flow restrictor, two ferrules and an adaptor to insert to the deliver arm.

To connect the capillary to the Gradient Monitor, hold the capillary about 2 cm from the end and slide the compression screw and the ferrule onto the capillary. Insert the capillary carefully into the Gradient Monitor outlet, make sure it reaches the bottom, and fasten the compression screw slightly. Hold the capillary aligned to the bottom of the Gradient Monitor outlet and tighten the compression screw firmly by finger force. Insert the fractionation capillary adaptor to the fraction collector arm if required. Insert the fractionation capillary into the adaptor and secure it with the securing screw. Do not overtighten.

6.23 Defrosting the µSeparation Unit

If you are using the cooling function continuously in SMART System, and notice that the temperature can not be maintained at the set value, it can be caused due to ice formation on the Peltier element. This can happen if the cooling is on constantly, and the humidity is very high. To defrost the Peltier element, turn off the cooling for about 30 minutes, or until the ice is melted. Make sure the waste tubing at the side of the µSeparation Until is positioned in a waste container or drain.

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7. Trouble-shooting guide

µPrecision Pump

Symptom	Possible cause	Remedy
Flashing yellow light	System in pause because of a fault	Check screen message as listed below
	Screen message: "Valve cannot find position"	Try setting the valve in another position e.g. choose Manual, Pump Fill, Pos x. If the valve still cannot find the correct position, call Pharmacia Biotech service engineer
	Screen message: "Pressure exceeded limit"	Check pressure limit set against Column Instructions and adjust if necessary Follow blockage location guide, see Maintenance, Section 6.15.
	Screen message: "Cylinder A/B is empty"	Perform pump fill; check if sufficient eluent in reservoirs
Pump stopped, yellow light not flashing	Screen message: "Air detected in cylinder A/B"	Check that the inlet filter is covered by the eluent and that there is sufficient eluent in the reservoir
		Damaged inlet tubing or bad connection. Check and replace
		In Run/Control select MANUAL, PUMP EMPTY followed by PUMP FILL
Leakage from drain outlet under he pump cylinder	Leakage from the rinsing seal	Replace rinsing seal (see Maintenance, Section 6.5)
Shadows or steaks in the rinse liquid	Laking piston seal	Perform leakage test (see Section 6.3) If the seal is leaking, disassemble the cylinder. If the seal is dirty, wipe it and reassemble the cylinder. Perform the leakage test again and if it is still leaking, replace the piston seal. See Maintenance, Section 6.4

µSeparation Unit

Symptom	Possible cause	Remedy
Condensation inside the µSeparation Unit	The doors are not shut or have been opened too often	Keep doors shut
	High humidity in the lab	Move SMART System to an air-conditioned room
Flashing yellow light on µSeparation Unit	System in Pause because of a fault	Check the message on screen

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µMixer

Symptom	Possible cause	Remedy
Mixer not rotating, as seen in window under the mixer	No flow through system	The mixer only rotates when eluent isthe being pumped through the system
	Mixer faulty	Call Pharmacia Biotech service engineer

µInjection Valve

Symptom	Possible cause	Remedy
Sample leaks out from behind injection needle	The syringe is not in the correct position	Ensure that the syringe is pushed all the way into the fill port before loading the sample
	Blockage in the fill port	Remove and check the fill port. Refit according to Maintenance, Section 6.10
	Blockage in the loop	Switch the injection valve to the inject position and flush the loop or replace it
		Ensure that all samples are free of particulate material
	When using the internal loop, valve in waste position	Turn valve to load position
	The fill port is not tightened correctly	Insert the syringe needle into the fill port and tighten the fill port

Automatic Column Holder

Symptom	Possible cause	Remedy
Automatic Column Holder does not move	Door not closed	Check that the door of the µSeparation Unit is closed
	Faulty	Call Pharmacia Biotech service engineer

Flow Restrictor

Symptom	Possible cause	Remedy
Leakage in Flow Restrictor	Blockage in fractionation needle	Check by loosening it. Replace it if necessary
	Fault in Flow Restrictor	Replace the Flow Restrictor or call Pharmacia Biotech service engineer

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µFraction Collector

Symptom	Possible cause	Remedy
Fraction Collector cannot find programmed tube position	Tube holder fitted incorrectly	Ensure that the tube holder is correctly the fitted to the bowl
	Tubes fitted incorrectly	Check that the caps on the small tubes point inwards
	Fraction Collector positioning fault	Call Pharmacia Biotech service engineer
Fractions outside the tubes	Tube holder fitted incorrectly	Check the fitting of the tube holder
	Fractionation capillary not in correct position	Check the adjustment of the capillary (see Maintanance, Section 6.20)
	Incorrect tube holder chosen in configuration	Check Run/Control User Supplied Configuration

µPeak Monitor

Symptom	Possible cause	Remedy
Yellow LED (RUN) on panel flashing	Calibration taking place at start up	Wait until start up is finished
	Fault in the system	Check displayed error message on screen
Green LED (READY) on panel flashing	Communication throughout the system interrupted	Shut down SMART Manager and restart or reboot the computer and system

Monitor UV-M II

Symptom	Possible cause	Remedy
Panel keys do not function	Lamp plug connected or disconnected with mains power on	Disconnect the mains cable for a short time
Lamp on/off LED not illuminated	Mains cable not connected	Connect mains cable
	No voltage at mains socket	Check socket by using a lamp or other electrical appliance
	Fuse blown	Replace fuse. If fuse blows again, call Pharmacia Biotech service engineer immediately
Lamp on/off LED flashing	Lamp cable not connected	Connect lamp cable
	Power malfunction on lamp	Consult Pharmacia Biotech service engineer.
Lamp on/off LED on, no signal	Control Unit not connected to µSeparation Unit	Check the connection. Ensure that the green connector snaps into position
	Wrong filter in use	Change to the correct filter
	Wrong lamp or wrong lamp position	Check that the correct lamp is installed and in the correct position
	Electronic fault	Contact Pharmacia Biotech service engineer
Autozero LED flashing	Unstable baseline	See noisy and drifting baseline, below
	Optical Unit not connected to Control Unit	Connect Optical Unit
	Flow cell not installed	Install flow cell

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System Problems

Symptom	Possible cause	Remedy
Noisy baseline	Air in flow cell	Install the Dummy Column and run eluent through the flow cell at a high flow rate. Remove the Dummy Column and place a drop of water on the flow cell to prevent air being introduced.
		Degass the eluents
		Check that the Flow Restrictor gives 0.3 MPa back pressure with the Dummy Column in the system
	Flow cell not tightened firmly in position, UV-M II	Tighten locking nut firmly
	Gradient Monitor and Flow Restrictor not tightened	Tighten (see Maintenance, Section 6.18)
	Dirty flow cell	Clean according to the instructions in Maintenance, Section 6.16.1, 6.17.3 or 6.17.4
	Dirty filter, UV-M II	Clean the filter (see Maintenance Section 6.17.5)
	Wrong lamp position, UV-M II	Check that the lamp position and the wavelength setting are the same
	Wrong filter, UV-M II	Change to the correct filter
	Aging lamp, UV-M II	Change the lamp according to the instructions in Maintenance, Section 6.17.7.
	Aging lamp, µPeak Monitor	Call a Pharmacia Biotech service representative
	Wrong mixer volume	Check recommendations in Section 2.9
Drifting baseline	Lamp not warm enough, UV-M II	The lamp must be on for at least 1 hour before use, especially when operating at high sensitivity
	Detector not warm enough, µPeak Monitor	In the Run/Control Menu, use the command MANUAL, DETECTOR, LAMP CONTROL to switch on the lamp before running methods at high sensitivity. Let the lamp be on while equilibrating columns or editing methods
	Flow cell not completely tight, UV-M II	Tighten locking nut firmly
	Dirty flow cell	Clean according to the instructions in Maintenance, Section 6.16.1, 6.17.3 or 6.17.4
	Gradient Monitor not firmly tightened	Tighten the connection between the Gradient Monitor and the flow cell (see Maintenance, Section 6.18)
	Eluents A and B give different absorbance	Contaminated buffer components. Use chemicals from another manufacturer or use higher quality Compensate for the absorbance difference by adjusting eluents A and B to slightly different pH (see Method Manual, Section 10)

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Symptom	Possible cause	Remedy
Ghost peaks	Air in eluents	Ensure eluents are degassed
	System dirty	Follow program for system clean with 2 M NaOH (see Section 6.2)
	Contamination in eluents in gradient techniques	See Method Manual, Section 10
	Sample not dissolved in start eluent	Dissolve sample in eluent A
	Dirty column	Clean the column according to Column Instructions
	Dirty sample loop	Flush through sample loop with both A and B eluents
	Contamination between internal and external loop	Ensure that the stop plug is in position in the fill port that is not in use Leave the pump running continuously at a low flow rate at 100 % B when the system is not in use
	Insufficient mixing or mixer not functioning	Check mixer operating. Check recommended mixer volume Section 2.9
Low sensitivity	Flow cell dirty	Follow instructions to clean flow cell (see Maintenance, Section 6.16.1, 6.17.3 or 6.17.4)
High back pressure	Blockage in the system	Locate the block following the procedure in Maintenance, Section 6.21
	Increased viscosity of liquids due to the use of a lower temperature	An increased back pressure of up to 50 % can be expected at 4 °C
	Use of eluent with high viscosity	Lower the flow rate or change buffer/solvent
	Dirty column	Wash according to Column Instructions
Back pressure too low	Leakage in the system	Check for visible leaks or salt precipitation at all tubing connections. Perform leakage test on the pump as described in Maintenance, Section 6.3
	Leaking Flow Restrictor	Using the Dummy Column, check that the Flow Restrictor gives 0.3 MPa back pressure. Replace Flow Restrictor if necessary

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Symptom	Possible cause	Remedy
Distorted gradient profile	Leakage in system	Check for visible leaks or salt precipitation at all tubing connections. Perform leakage test on pump as described in Maintenance, Section 6.3.
	Pressurisation not used	Select pressurisation in Separation Method configuration
	Mixer wash function not used	Select Mixer Wash function under µSeparation Unit in Separation Method
	Mixing not optimal	Wrong mixer volume used (see recommendations in Technical description, Section 2.9)
	Mixer not functioning	Check that the mixer is rotating by looking into the window under the mixer
	Conductivity signal out of range	Check the conductivity range is set correctly.
	Compressibility compensation not used	Select compressibility compensation in Separation Method configuration
Noisy gradient signal	Wrong mixer volume used	See recommendations in Technical description, Section 2.9
	Mixer not functioning	Check that the mixer is rotating by looking into the window under the mixer
	Leaking piston seal	Perform leakage test on cylinders A and B (see Maintenance Section 6.3). If the seal is leaking, disassemble the cylinder and replace the piston seal. See Maintenance, Section 6.5 or 6.6
Curve like a "knee" on gradient signal	Pressure follow not used	Select pressurisation in Separation Method configuration
	Mixer wash function not used	Select Mixer Wash function under µSeparation Unit in Separation Method
	Leakage in the connections before the mixer	Check for leaks. Tighten or remake the connections (see Maintenance, Section 6.22.)
Signal from Gradient Monitor out of range	Incorrect conductivity range selected	Check the range set
	System temperature different from 20°C	Calibrate the Gradient Monitor at the operating temperature
Gradient signal changes with flow rate	Compressibility function not used	Select compressibility compensation in Separation Method configuration
Spikes on gradient	Sample not dissolved in eluent A	Ensure that the sample is dissolved in eluent A
	Contamination from sample loop	Flush through sample loop using eluent A
Uneven fraction volumes	Leakage in the system	Check for visible leaks or salt precipitation at all tubing connections. Perform leakage test on pump as described in Maintenance, Section 6.3.

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7.1 Error and warning messages

This table includes most error and warning messages that may occur during normal use of SMART™ System. If a message that is not included in the list is shown, or if the suggested action does not solve the problem, make a note of the message, then call your Pharmacia LKB service representative. For general trouble-shooting help, see section 7 of the System Handbook or section 6 of the µPeak Monitor User Manual.

No	Message	Possible Cause	Remedy
6	"Hardware error: A/D converter"	Faulty A/D converter.	Call a Pharmacia Biotech service representative.
7/8	"Pressure exceeded limit"	1. Blockage in column, filters or system	Check pressure limit, Locate. blockage according to handbook section 6.14.
		2. Pressure limit incorrect.	
9	"Pump valve position not defined. Fill cylinder A and B, then continue."	The valve position for filling the pumps is not defined.	Perform a fill operation for both pump cylinders.
10/11	"Cylinder A/B is empty"	Pump A/B should deliver eluent, but there is no eluent left in the cylinder.	Fill pump A/B.
12/13	"Valve A/B cannot find position"	The position of valve A/B cannot be defined, possibly due to a faulty valve.	1. Repeat the operation. 2. Call a Pharmacia Biotech service representative.
16/17	"Fill operation pump cylinder A/B failed"	Mechanical problem in pump.	1. Repeat the fill operation at a lower flow rate.
			2. Call a Pharmacia Biotech service representative.
18/19	"Empty operation pump cylinder A/B failed"	Mechanical problem in pump.	1. Repeat the empty operation at a lower flow rate.
			2. Call a Pharmacia Biotech service representative.
102/ 103	"No module A/B cylinder"	Pump cylinder A/B is missing or incorrectly installed.	Check installation of pump cylinder. Handbook section 6.
104/ 105	"Cylinder A/B : Undefined ID"	The pump could not recognize the ID of the cylinder.	Check installation of pump cylinder.
106/ 107	"Pressure A/B is not calibrated"	The pressure sensor of pump A/B is not correctly calibrated.	Call a Pharmacia Biotech service representative.
108/ 109	"Leakage warning cylinder A/B"	Leakage was detected in pump A/B.	See section 6 of handbook.
110/ 111	"Air detected in cylinder A/B"	1. There is air in the the eluent bottle or tubes, or the inlet fittings are loose.	1. Check eluent bottle and tubes. Tighten the inlet fittings 2. Check the valve channel plate.
		2. The valve channel plate is loose or worn.	3. Remove air in cylinder by emptying it, then refilling.
112	"Fast pressurization is disabled. Please reinstall column"	The Fast pressurization option has been selected, but is not applicable for the installed column.	Deactivate the Fast pressurization option or install another column.

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No	Message	Possible Cause	Remedy
113	"Pressure limit is exceeded - Check column or deactivate Fast pressurization"	The set pressure limit was exceeded during fast pressurization.	1. Check the set pressure limit. 2. Check the in-line filters, prefilters, column and system for blockage. 3. Deactivate Fast pressurization.
205	"Injector valve cannot find position"	The valve is not functioning properly.	Reset the system, then repeat the operation.
231	"Automatic Column holder position sensors indicate both press and free"	One or both column holder sensors dirty or faulty.	Call a Pharmacia Biotech service representative.
248	"Bowl does not move on command"	Faulty bowl drive mechanism.	Clean bowl and drive mechanism. If this does not help, call a service representative.
249	"Bowl cannot find demanded position"	The bowl or capstan is dirty.	Clean bowl and capstan.
250	"Both fraction collector arm end-sensors indicate active"	Dirty or faulty sensors for fraction collector arm horizontal movement.	Call a Pharmacia Biotech service representative
251	"Bowl cannot find "home" position"	The tube holder is damaged, the magnet is missing or the magnetic sensor is faulty.	Check the tube holder for damages. Check the magnet.
252	"No bowl installed"	There is no bowl in the fraction collector, or the locking lever is not in the up position.	Check the installation of the bowl.
253	"Needle did not reach the up position"	The fractionation needle could not reach the up position.	Check the installation of the fractionation capillary.
254	"Needle position sensor indicates both up and down position"	The needle position sensor is dirty or faulty.	1. Shut down both system and computer. Take out the fraction bowl from the µSeparation Unit. Restart system and computer. Start SMART Manager. Then put the fraction bowl back after completed upstart. 2. Call a Pharmacia Biotech service representative
255	"Needle did not reach the waste position correctly"	The fractionation needle could not reach the waste position.	Check the installation of the fractionation capillary.
256	"Needle did not reach the collection position"	The fractionation needle could not move to the collection position.	Check the installation of the fractionation capillary.
257	"Current tube position invalid for fractionation, insert Goto tube"	A command was given to fractionate in a large tube (A-Z) or the waste position.	Insert a command to position the fraction collector at a numbered tube.
258	"Demanded tube number not found"	The bowl or capstan is dirty.	Clean bowl and capstan.
268	"Hardware error : A/D converter"	Faulty Separation unit circuit board.	Call a Pharmacia Biotech service representative.
269	"Hardware error : A/D converter timeout"	Faulty Separation unit circuit board.	Call a Pharmacia Biotech service representative.

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No	Message	Possible Cause	Remedy
279	"Hardware error : EEPROM, read error"	Faulty Separation unit circuit board.	Call a Pharmacia Biotech service representative.
290	"Conductivity calibration, high value, failed"	There is air in the buffers or in the system, or a leakage or blockage in the system.	Make sure there is no air, leakage or blockage in the system, then try the calibration again. Also, ensure that the content of the "high conductivity" cylinder is homogeneous.
291	"Conductivity calibration, low value, failed"	1. There is air or a leakage or blockage in the system. 2. Both cylinders contain the same buffer, or the buffer in the cylinder designated "l "low conductivity" actually has a higher conductivity.	Make sure there is no air in the system. Check the eluents and designation of "low conductivity", then try the calibration again.
297-299	"The PC workload is too high"	The PC is handling too many operations at once.	Close other programs. If necessary, reset the PC.
305	"Injector valve out of position"	The switching mechanism is slightly loose.	Position the injector valve using the Manual, Sep Unit, Injection Valve command. Then retry the original operation.
306	"Fraction collector error"	The fraction collector bowl did not find the "home" position during startup.	Test the fraction collector using manual commands before running a method. (Remember to start flow or set delay volume to zero.)
340	"RAM test indicates error"	Faulty RAM circuit.	Do not use the system. Call a Pharmacia Biotech service representative.
341	"PROM test indicates error"	Faulty PROM circuit.	Do not use the system. Call a Pharmacia Biotech service representative.
342	"EEPROM test indicates error"	Faulty EEPROM circuit.	Do not use the system. Call a Pharmacia Biotech service representative.
350	"Hard to find tube position, please clean bowl and capstan"	The fraction collector finds the correct position with difficulty, since the bowl or drive mechanism is dirty.	Clean the bowl and capstan.
352	"No bowl installed"	There is no bowl in the fraction collector, or the locking lever is not in the up position.	Check the installation of the bowl.
353	"Frac configuration is not allowed during fractionation"	A configuration of the fraction collector was attempted while fractionation was taking place.	Use the "Frac stop" or "End" command to stop fractionation before reconfiguring.
355	"Fill needle cannot find the down position"	The fractionation needle could not reach the down position.	Fractionation will continue, but with the needle in the up position. If the problem persists, call a Pharmacia Biotech service representative.
356	"The needle cannot go down. It is incorrectly positioned."	The fraction collector arm is not in the correct position over the tube.	Check that the arm movement is not obstructed.

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No	Message	Possible Cause	Remedy
357	"Feed Tube is not valid. Use Goto Tube or Large Tube instead"	When the current tube is A, B or Z, the Feed Tube command cannot be used.	Use the Goto Tube or Large Tube commands instead.
358	"Cooler/heater : Temperature sensor error"	The temperature sensor is not functioning properly.	Call a Pharmacia Biotech service representative.
359	"Temperature in Sep unit exceeds 40 °C"	The temperature in the separation unit exceeded the specification limit during the startup check.	Check the ambient temperature and the temperature of the cooling water.
361	"The temperature in Sep Unit is not decreasing. Check cooling water."	1. The cooling water is not cold enough 2. Ice has formed on the cooling element. 3. Ambient temperature too high.	1. Check the water temperature. 2. Defrost the cooling element by switching off cooling for 1-2 hours. 3. With a high ambient temperature, you may have to accept a higher temperature in the separation unit.
371	"Fractionation is stopped"	1. A programmed tube change command was given when the fraction collector was at a large tube. 2. This message is also shown as a reminder that fractionation was taking place if certain other errors occur.	1. Avoid manual Large tube operations while fractionation is in progress. 2. Correct other error and continue fractionation.
379	"Hardware error : EEPROM, write error"	Faulty EEPROM.	Call a Pharmacia Biotech service representative.
383	"Close the door, cooling is on"	The door to the µSeparation Unit is open and cooling is on.	Close the µSeparation Unit door.
384	"The column holder will not move, since the separation unit door is open."	A Column Install operation was attempted while the door to the µSeparation Unit was open.	Close the µSeparation Unit door, then repeat the operation.
385	"Column holder is not in press"	The automatic column holder is not in press on the column.	Check that the µSeparation Unit door is closed, then use the Column Install command to move the column holder downwards.
390	"High value of conductivity range is near the upper limit."	The calibration of the conductivity monitor failed.	Check the eluents, then calibrate again.
392	"The UV-M input is not calibrated."	The monitor input is not calibrated.	Call a Pharmacia Biotech service representative.
393	"Conductivity calibration parameters are incorrect. Recalibrate."	The conductivity monitor is not properly calibrated.	Calibrate the monitor.
394	"Conductivity calibration parameters are incorrect. Recalibrate."	The conductivity monitor is not properly calibrated. The restored parameters are incorrect.	Recalibrate the monitor or restore another parameter set.
395	"Conductivity monitor is not calibrated"	The conductivity range is not calibrated, or the calibration data is corrupted.	Calibrate the conductivity monitor range.

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No	Message	Possible Cause	Remedy
396	"No offset value for UV-MII."	UV-MII monitor input incorrectly calibrated.	Only occurs in service mode.
399	"Demanded low level sep-unit command is not implemented"	Update version numbers of different units do not match.	Call a Pharmacia Biotech service representative.
401	"Data channel overflow in µControl Board. Data to be stored OK however."	A high computing load while gathering data, which causes the real-time display of data not to be updated. The data will be correctly stored on the disk.	Avoid performing computationally intensive tasks while gathering data if you want to be able to see the data on the screen correctly in real time.
402	"Wavelength calibration failure"	The calibration procedure failed.	1. Check the optical fibre connections and repeat the operation. 2. Call a Pharmacia Biotech service representative.
404	"Low light intensity"	Both source and reference light levels are too low. 1. Fibre(s) not fitted. 2. Lamp worn out.	1. Check the optical fibre connections. 2. Call a Pharmacia Biotech service representative.
405	"Source light path error"	Low source light level. 1. Fibre(s) not fitted. 2. Dirty flow cell (or strong absorbation in flow cell). 3. Broken source fibre.	1. Check the optical fibre connections. 2. Clean flow cell. 3. Replace flow cell.
406	"Reference light path error"	Low reference light level. 1. The reference optical fibre(s) is not fitted. 2. Broken reference fibre.	1. Check the beam splitter reference fibre connection. 2. Replace the beam splitter.
407	"Mode fibre error"	No light from source or reference channel	Call a Pharmacia Biotech service representative.
408	"Lamp module failure"	Lamp failure	Call a Pharmacia Biotech service representative.
409	"Too high source light level"	Erroneous source fibre installation.	Check source fibre for correct installation.
410	"Too high reference light level"	Erroneous reference fibre installation.	Check reference fibre for correct installation.
411	"Wavelength not calibrated"	Detector not calibrated.	To calibrate µPeak Monitor, use the following commands: - Manual - Detector - Calibrate
501	"Method downloading error"	Communication between the PC and the instruments is not functioning properly.	Reset the system.

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7.2 Error Messages in Method Check

When the command "Check and update graphs" is executed, the instructions in the text editor are checked so that the method can be run in Run/Control. If the method contains errors, these must be corrected. Listed below are the different messages, what may have caused the problem and suggestions on how to proceed. The messages are listed in alphabetical order.

Message	Possible cause	Action
"A flow rate of 0 ml/min in volume base will stop the method."	The volume-based method includes an instruction to set the flow rate to 0.	In volume base, only use flow values greater than zero. If zero flow is necessary, make the method in time base.
"A Sub Method cannot call itself."	A Sub Method includes a Call command with its own name as parameter.	Delete the instruction or change the name in the parameter.
"Called Evaluation Procedure does not exist"	There is no Evaluation Procedure with this name in the current Method File, or the name is misspelled.	Check the name of the Evaluation Procedure.
"Called Sub Method does not exist"	The called Sub Method either is not in this Method File or its name was misspelled.	Check the Sub Method name.
"Call to Main Method not possible"	The name of a Main Method was included as parameter in a Call command.	Change the entered command.
"Comma not expected"	An unnecessary comma was included in the parameter list.	Check the parameter list and remove the comma.
"END_LOOP is missing"	The method includes a LOOP instruction, but no corresponding END_LOOP instruction.	Insert an END_LOOP instruction.
"Insert a GOTO_TUBE instruction before the FRACTION_SIZE instruction."	There is no GOTO_TUBE instruction, so the fraction collector cannot be positioned to start collecting.	Insert a GOTO_TUBE command.
"Instructions not in order"	The instructions are not listed in the order of their time/volume values.	Rearrange the instructions in the correct order.
"Invalid character"	An instruction or value contains a character that is not allowed.	Check the instructions and values.
"Invalid parameter type"	The entered parameter is not allowed for this instruction. For example, a letter was entered instead of a number.	Check the parameters.
"Invalid token"	1. The first line of the method does not include a time/volume value. 2. An instruction or time/volume value was misspelled.	Check the instruction and time/volume value.
"LOOP is missing"	The method includes an END_LOOP instruction, but no corresponding LOOP instruction.	Insert a LOOP instruction.

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Message	Possible cause	Action
"Missing comma"	A comma is missing in a list of several parameters.	Separate the parameters in the list with commas.
"Missing parameter"	There is no parameter for an instruction that requires it.	Insert the parameter.
"No FLOW instruction at 0.0 ml, which is necessary in volume base."	A volume-based method must include a command for a flow rate greater than zero at 0.0 ml, or the method will stop.	Insert a flow instruction at 0.0 ml.
"Parameter must be integer"	A parameter that should be an integer included a decimal.	Remove the decimal.
"Parameter must be Sub Method name"	The parameter entered for a Call command is not a Sub Method name.	Check the entered name.
"Parameter value out of range"	An entered parameter has a value outside the allowed range.	Check the parameters.
"The entered parameter is not allowed for this instruction."	An entered parameter is incorrect in the context of the instruction.	Check the parameters.
"Time/Volume-value missing"	The time/volume value is missing in this instruction, or the last parameter on the previous line is missing.	Check the time/volume value and the parameters for the previous instruction.
"Too many characters in parameter"	There are more than 32 characters in the name of the called Evaluation Procedure or Sub Method.	Check the name.
"Too many loop instructions"	The method includes more than 10 loop instructions.	Remove loop instructions.

Warning and Information Messages

The following messages provide the user with information about the method or system. It is possible to continue after the message has been acknowledged, and the method can be used in Run/Control.

Message	Possible cause	Action
"No recalculation of the method is done when shifting base."	The time/volume values in the method will not be recalculated when changing base.	
"Method is correct, no errors, no warnings."	The check of the method did not find any errors.	
"The hard disk is almost full. Free disk space immediately to avoid operating system problems."	The operating system needs a certain amount of free disk space, and current space is close to the limit.	Immediately delete files on the fixed disk or move them to diskettes. Do not continue working with SMART Manager until you have done this.

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8. Accessories

8.1 Accessories

µPrecision Pump

Code No.	Name
18-1800-26	Inlet Tubing
18-1800-20	Cylinder Assembly, 10 ml
18-1800-21	Cylinder Assembly, 20 ml
18-1800-22*	Cylinder Assembly, 50 ml*
18-1800-27	Pump Outlet Tubing, 0.7 m
18-1800-29	Pump Outlet Tubing, 0.5 m
18-1800-31	Solvent Reservoir, 250 ml
18-1800-32	Solvent Reservoir, 500 ml

*Note: For correct functionality the system with 50 ml cylinders require µPrecision Pump EPROM version 1.31 and SMART Manager version 1.4 or higher.

µSeparation Unit

Code No.	Name
18-1800-34	On-line Filter
18-1801-30	Mixer Chamber Bypass
18-1801-31	Mixer Chamber, 2x12 µl
18-1801-32	Mixer Chamber, 2x30 µl
18-1801-33	Mixer Chamber, 2x75 µl
18-1801-34	Mixer Chamber, 2x150 µl
18-1800-70	Sample Loop, 5 µl
18-1800-71	Sample Loop, 10 µl
18-1800-72	Sample Loop, 20 µl
18-1800-73	Sample Loop, 50 µl
18-1800-74	Sample Loop, 100 µl
18-1800-90	Sample Loop, 200 µl
18-1800-91	Sample Loop, 500 µl
18-5897-01	Sample Loops, 1 ml and 2 ml
19-7585-01	Superloop, 10 ml
19-7850-01	Superloop, 50 ml
18-3858-01	Union, M6 Female/1/16" Male
18-1800-75	Prefilter, 3.2 mm
18-1800-76	Prefilter, 0.8 mm
18-1800-77	Prefilter Frit, 3.2 mm] 3 per pkg
18-1800-78	Prefilter Frit, 0.8 mm
18-1800-80	Column Connector, UV-M II
18-1800-85	Dummy Column
18-1800-86	Blind Column
18-1029-14	MultiTemp II Connector
18-1015-98	Cable 4 pole, (analogue recorder cable)
18-1800-87	Fingertight Connector, 0.02" 0.5 mm
18-1800-88	Fingertight Connector, 1/16" 1.6 mm

Control Card AT 18-1037-93 requires EPROM for SMART

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Code No.	Name
18-1801-10	Vial, 0.5 ml, (1000/pack)
18-1801-11	Vial, 24 ml, (100/pack)
18-1801-13	Vial, 1.5 ml, 1000/pack)
18-1801-20	Tube Holder 48/8 tube 0.5 mL
18-1801-21	Tube Holder 41/11 1.5 mL
18-1801-23	Tube Holder 60/8 0.5 mL
18-1801-40	Syringe, 10 µl
18-1801-41	Syringe, 100 µl
18-1801-42	Injection Needle, 0.7x50 mm
18-1801-53	Column Adaptor, FPLC, HPLC
18-1015-23	Adjustment Guide

µPeak Monitor

Code No.	Name
18-1800-41	Flow Cell, 10 mm
18-1800-42	Flow Cell, 3 mm
18-1029-94	Gradient Monitor/Flow Restrictor, (µPeak Monitor)
18-1000-18	Fractionation capillary, µPeak Monitor
18-1031-73	Ferrule

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8.2 Spare parts

µPrecision Pump

Code No.	Name
10 ml cylinder	
18-1001-57	Sealing Ring, 10 mm
18-1001-59	O-Ring, 13.1 x 1.6 mm
18-1001-61	Rinsing Seal, 8 mm
Or for pumps with serial number 1460 or higher	
18-1037-54	Sealing Ring, 10 mm
18-1037-55	Rinsing seal
18-1001-59	O-Ring, 13.1 x 1.6 mm
18-1037-56	Rinsing tubing
20 ml cylinder	
18-1001-47	Sealing Ring, 14 mm
18-1001-50	O-Ring, 17.1 x 1.6 mm
18-1001-51	Rinsing Seal, 10 mm
50 ml cylinder	
18-1001-66	Sealing ring, 22 mm
18-1001-68	O-ring, 25.1 x 1.6 mm
18-1001-51	Rinsing Seal, 10 mm
18-1001-70	Rinsing Tubing
18-1012-23	Cylinder Tubing, Ti
18-1800-26	Inlet tubing, complete
18-1800-27	Pump Outlet Tubing, 0.7 m
18-1800-29	Pump Outlet Tubing, 0.5 m
~~18-1017-69~~	Channel Plate

→ replaced by 18-1111-98

µSeparation Unit

Code No.	Name
18-1015-31	Tubing, inlet to mixer
18-1800-34	On-line Filter
18-1028-85	On-line Filter Housing
18-1015-64	Tubing, mixer to valve
18-1015-65	Tubing, valve to column
~~18-1015-87~~ ← 18-1111-99 new style, one-part injector comes with distributor plate	Channel Plate, injection valve
18-1015-88	Fill Port
18-1800-80	Column Connector, UV-M II
18-1801-50	Fraction Collector Bowl
18-1017-26	Fractionation Needle (for 18-1017-28)
~~18-1017-28~~ suspended	Gradient Monitor and Flow Restrictor, UV-M II
18-1012-07	Flow Restrictor, complete (for 18-1017-28) UV-M II

µPeak Monitor - 18-1800-40

Code No.	Name
18-1029-94	Gradient Monitor//Flow Restrictor, µPeak Monitor
18-1031-37	Flow Restrictor II, complete (for 18-1029-94)
18-1029-15	Beam Splitter

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9. Technical specifications

µPrecision Pump

Flow rate range	10 - 2 000 µl/min
Increments	1 µl/min
Maximum pressure	25 MPa for 10 and 20 ml cylinders 12.5 MPa for 50 ml cylinders
Flow rate accuracy	
10 ml cylinders	± 1 µl/min or ± 2 % whichever is greater
20 ml cylinders	± 1.5 µl/min or ± 2 % whichever is greater
50 ml cylinders	± 2.5 µl/min or ± 2 % whichever is greater
Reproducibility	
10 ml cylinder	± 0.2 µl/min or ± 0.4 % whichever is greater
20 ml cylinder	± 0.3 µl/min or ± 0.4 % whichever is greater
50 ml cylinder	± 0.5 µl/min or ± 0.4 % whichever is greater
Pulsation	< 3 % for flow rate < 50 µl/min < 2 % for flow rate > 50 µl/min
Gradient accuracy	1 % for flow rate > 50 µl/min 5-1 % for flow rate 10-50 µl/min

µInjection Valve

Internal loop	0.75 µl
Sample loops	5, 10, 20, 50, 100, 200, 500, 1 000, 2 000 µl
Superloops	10 ml and 50 ml

µMixer

No. of mixer chambers	2
Standard mixer chambers	2 x 30 µl
Optional mixer chambers	12, 75, 150 µl

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µPeak Monitor

Wavelength range	190 - 600 nm
Light source	Xenon flash lamp
Spectra bandwidth	4 nm
Wavelength accuracy	± 2 nm
Wavelength reproducibility	± 0.5 nm
Linearity at 280 nm	Deviation ≤ 2 % up to 2 AU
Wavelength switch time (214-254-214)	< 500 msec
Dynamic noise single wavelength, 230 nm	< 4×10^{-5} AU typical
Dynamic noise dual wavelength, 230/254 nm	< 1.5×10^{-4} AU typical
Static drift single wavelength, 254 nm	< 2×10^{-4} AU/ °C < 2×10^{-4} AU/hour
Static drift dual wavelength, 230/254 nm	< 3×10^{-4} AU/ °C < 3×10^{-4} AU/hour
Flow cell	10 mm path length 2 µl volume 3 mm path length 0.7 µl volume
Signal output	Digital via MultiNet
Analogue recorder output	1 V full scale

Monitor UV-M II

Wavelengths	Hg lamp: 254, 280 nm Zn lamp: 214 nm
Dynamic short term noise	< 12×10^{-6} AU
Dynamic long term noise	< 8×10^{-5} AU
Dynamic drift	< 4×10^{-4} AU/hour
Total volume	10 µl (including cell)
Illuminated volume	6.7 µl
Flow cell path length	5 mm
Output ranges	0.001-2 AU

Gradient Monitor

Flow cell volume	1.5 µl

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µFraction Collector

Minimum fraction size	5 µl
Maximum fraction size	1.5 ml
Increments	1 µl
Accuracy	± 0.5 µl
Tube holders	48 positions for 0.5 ml tubes 60 positions for 0.5 ml tubes 41 positions for 1.5 ml tubes
Carry-over (wipe mode)	< 2 %

Cooling

The µSeparation Unit can be cooled to +4 °C in 3 hours at an ambient temperature of +21 °C and a relative humidity of 40 % when supplied with cooling water of +5 °C.

Operating environment

Ambient temperature	4 - 40 °C
Relative humidity	20 - 95%
Atmospheric pressure	84 - 106 kPa

System Power Supply

Mains Voltage	100 - 120/220 - 240 V~ (autorange)
Frequency	50 - 60 Hz
Power	900 VA

EMC and Safety

EMC This product meets the requirement of the EMC Directive 89/336/EEC through the harmonised standards EN 50081-2 (emission) and EN 50082-1 (immunity).

Note: This is a class A product. In a domestic environment this product may cause radio interference in which cases the user may be required to take adequate actions.

Note: The declaration of conformity is valid for the instrument when it is:

- used in laboratory locations.
- used in the same state as it was delivered from Pharmacia Biotech AB except for alterations described in the user manual.
- used as stand alone unit or connected to other CE labelled Pharmacia Biotech AB instruments or other products as recommended.

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Safety This product meets the requirements of the Low Voltage Directive (LVD) 73/23/EEC through the harmonised standard EN 61010-1.

Computer requirements

The recommended computer configuration requirement is:

RAM memory	12 MB
Fixed disk	more than100 MB
Disk drive	3.5"
Operating system	IBM OS/2 version 2.1
Processor	486 DX, 33 or 50 MHz
Math coprocessor	Yes, included in 486
Adaptor and display	VGA, colour
Printer/plotter	As supported by OS/2
Serial mouse	

SMART Manager 1.41 has been tested on Compaq DESKPRO, 486/66i

Testing has shown that the use of math coprocessor is essential for trouble-free operation of the software, not just to reduce the time needed for calculation-intensive operations.

Specifications are subject to change without notice.

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1. Introduction to micropurification

1.1 What is micropurification?

Current biological and medical research is being focused on biomolecules which are only available in extremely small quantities. This has led to a rapid development of highly sensitive micromethods for analysis and characterization of such biomolecules. To perform relevant microstructural and microfunctional studies with these often labile molecules requires an advanced purification technology. Micropurification using preparative liquid chromatography of biomolecules in the low microgram and nanogram range, has been developed to meet these needs.

1.2 SMART System for micropurification

SMART® System is especially designed for sensitive manipulation and recovery technology. It provides secure isolation of ng and µg quantities of substances with preserved biological activity and opens many new possibilities in separation and purification of biomolecules.

The key to high resolution and high recovery in micropurification is a fully integrated "miniaturized" system. Small volume flow paths in combination with small dimension columns minimize dead volumes, unwanted dilution effects and non-specific adsorption; as a result, sensitivity and recovery of sample are increased. Optimized conditions allow peak detection of just one or a few ng, and the collected fractions can be smaller than one drop (down to 5 µl). Biocompatible materials, built-in cooling and chromatography media ensure sample loss is minimized.

SMART System comprises instrumentation, small dimension columns (Precision Columns), controlling software (SMART Manager) and knowledge information (SMART Assistant).

1.3 Applications for micropreparative chromatography

Micropreparative chromatography is ideally suited to purifying samples in which the biomolecule of interest is present in ng or µg quantities. Application areas for micropreparative chromatography can be recognized by the characteristics of the biological sample used as the starting material.

One area covers purification of proteins or peptides present in extremely low concentrations in tissue or in cell culture material. To use only conventional chromatography techniques would require initial sample volumes which are either impractical or impossible to handle, or (if less starting material is used) result in an extremely diluted purified sample. However, the combination of conventional chromatography techniques for initial purification, and final micropurification with concomitant concentration is extremely powerful for this sample category. A scheme using this approach for the purification of dihydrofolate reductase, an enzyme involved in folic acid metabolism, from E.coli (wild type), is shown in figure 10.1. Figure 10.2 shows the final micropurification step, using anion exchange with Mono Q® PC 1.6/5, in which the enzyme was concentrated 20-fold (to 100 µl) and produced a distinct UV peak which coincided with the activity.

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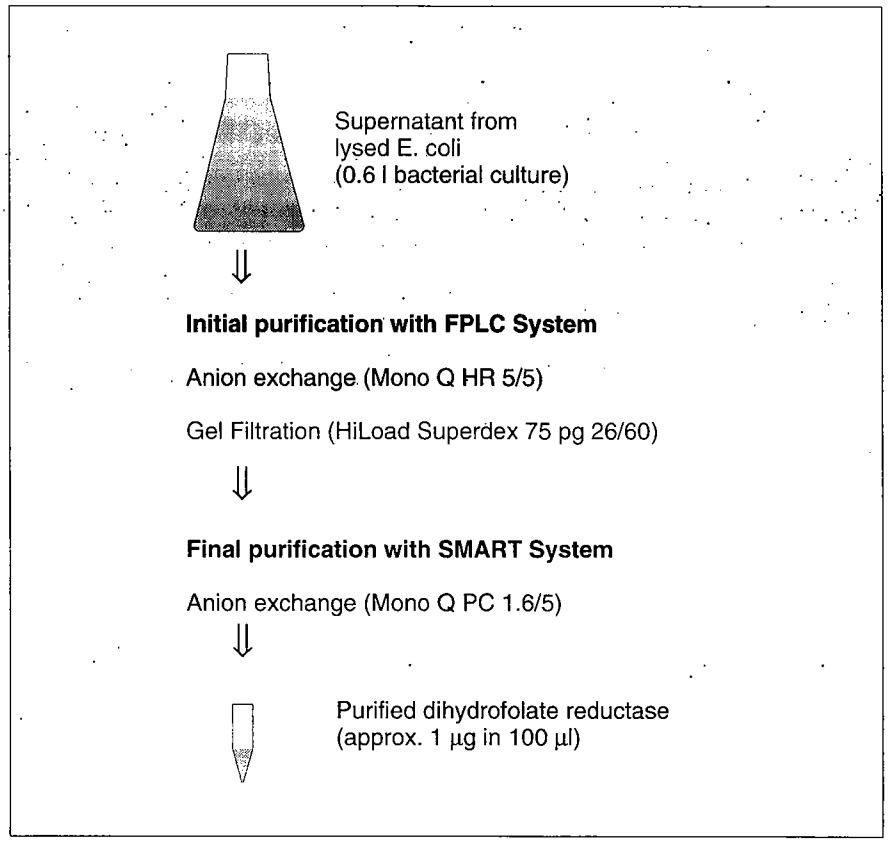


Fig. 10.1. A purification scheme for dihydrofolate reductase (DHFR) from E.coli (wild type). The result of the final micropurification step is shown in figure 10.2.

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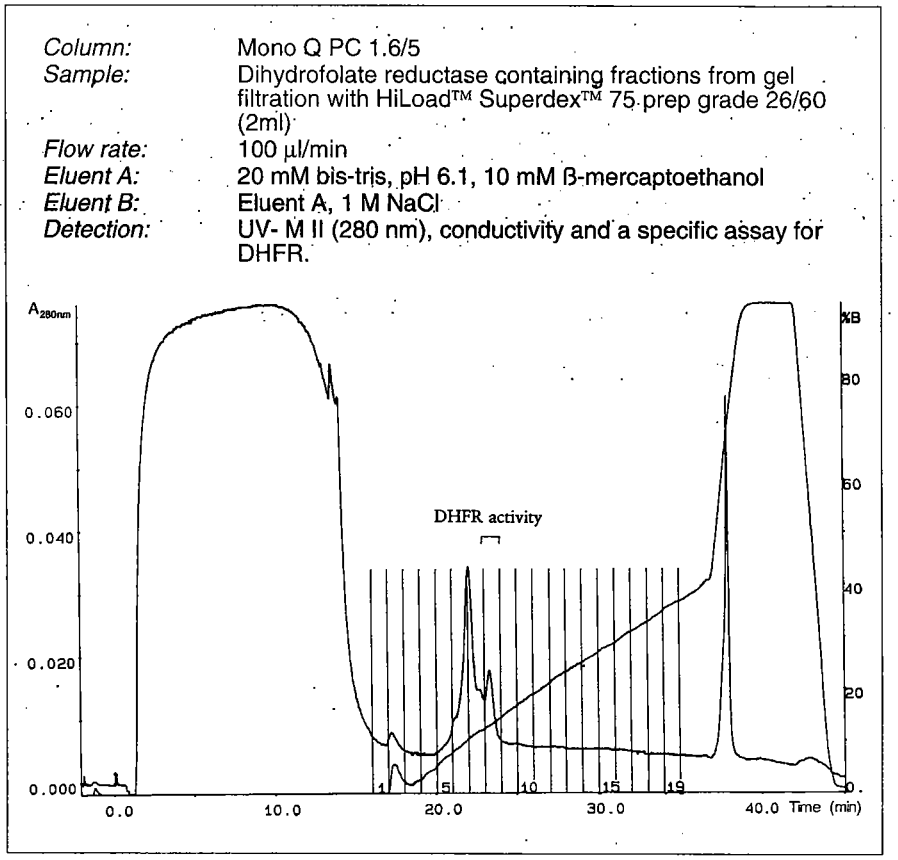


Fig. 10.2. Micropurification and trace enrichment of dihydrofolate reductase (DHFR) from E.coli using Mono Q® PC 1.6/5.
The complete purification scheme is shown in figure 10.1. Purity was checked by electrophoresis, and the identity of DHFR was confirmed by N-terminal sequence determination.

Another application area covers the purification of proteins or peptides which are available only in very small quantities. For these samples, in which the molecule of interest is not necessarily present at a low concentration, it is of utmost importance to minimize losses during the purification scheme. This sample category includes clinical material and material from individual experimental animals. Examples of this application area are gel filtration of tear proteins using only 0.75 µl of starting material (Fig. 10.3), and reversed phase chromatography of components in human cerebrospinal fluid using 20 µl of sample (Fig. 10.4).

For method development of a purification scheme, SMART System is an ideal alternative which decreases the consumption of sample and eluents. If the purification method is to be scaled up to FPLC, this is simplified by the scale factor of ten between Precision Columns (SMART System) and HR columns (FPLC).

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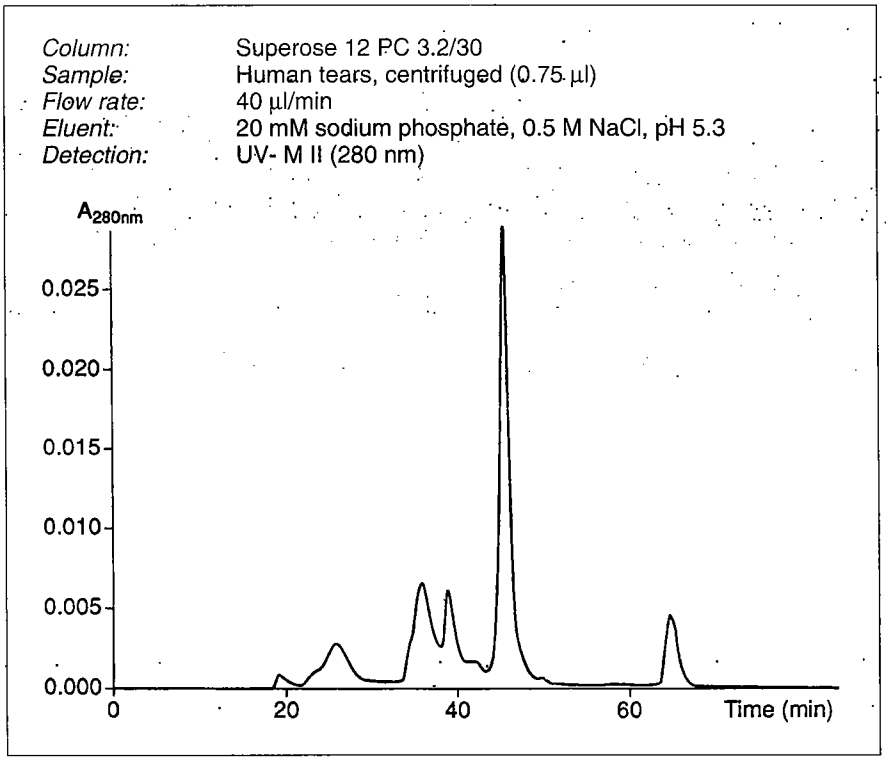


Fig. 10.3. Separation of components in human tears by gel filtration using Superose® 12, PC 3.2/30. 30 µl of sample was centrifuged to remove particulate material. The internal sample loop (0.75 µl) was used.

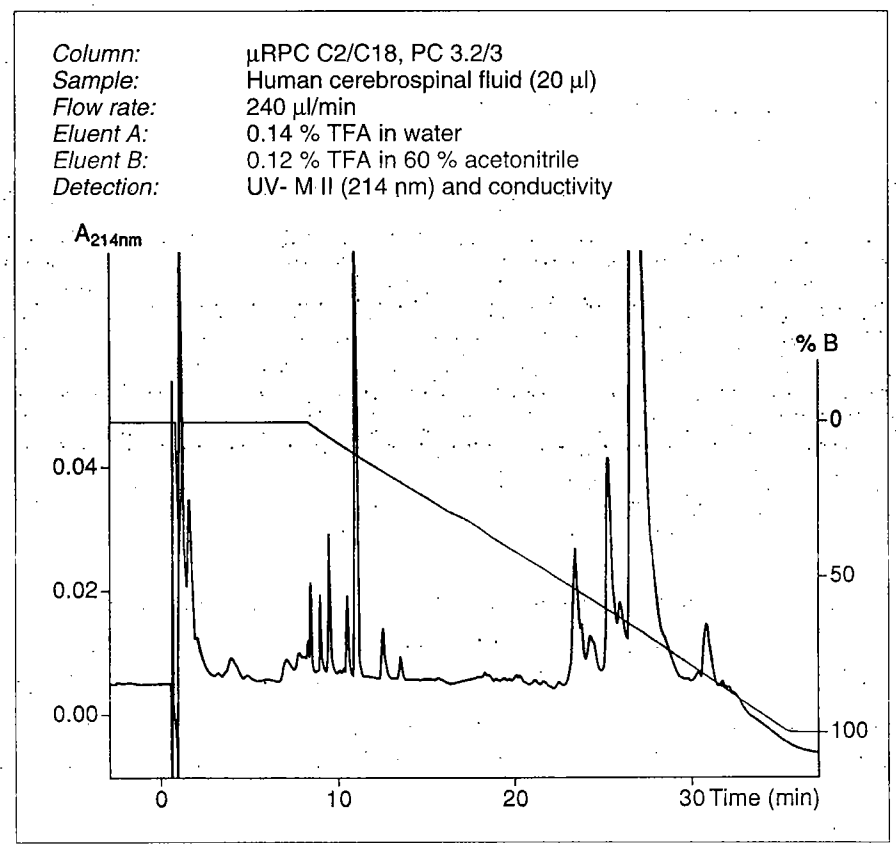


Fig. 10.4. Micropurification of components in human cerebrospinal fluid by reversed phase chromatography using µRPC C2/C18, PC 3.2/3. 20 µl of clinical sample was applied to the column, without any pretreatment. The major peak was electrophoretically identified as albumin. Other peaks were isolated for subsequent characterization by mass spectrometry and amino acid sequencing.

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A third application area includes separation of DNA. In this case, the biomolecule of interest may be present in a small volume at a relatively high concentration. For instance, a restriction enzyme digestion is usually performed in a volume smaller than 25 µl. In order to avoid time consuming concentration steps, it is desirable to maintain the nucleic acid in a volume as small as possible after the chromatographic purification step. SMART System better fulfills this requirement than conventional chromatography systems. Some examples illustrating this application area are shown in Chapter 4.

The different strategies which involve micropreparative chromatography in protein and peptide work are shown schematically in figure 10.5.

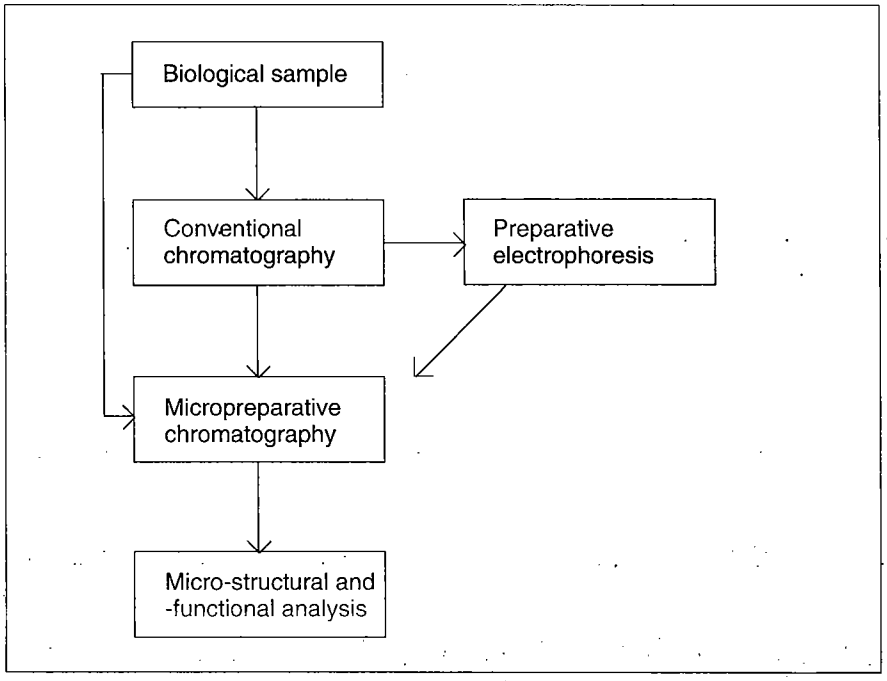


Fig. 10.5. Schematic representation of the role of micropreparative chromatography in protein and peptide work.

1.4 Detection levels for micro-structural and micro-functional characterization of proteins and peptides.

Current micromethods for the characterization of proteins and peptides are generally compatible with micropurification on SMART System, i.e. the sensitivity level is often in the pico- and nanogram range. Furthermore, an extremely powerful feature of micropreparative chromatography is the possibility to obtain a very high sample concentration. This often eliminates the need for separate concentration steps, with concomitant losses of material, before subsequent analyses. Approximate detection limits for some commonly used micromethods for protein and peptide characterization are summarized in Table 1.

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Table 1. Approximate detection limits for some micromethods for protein and peptide characterization.

	Approx. detection limit	Ref.
Purity analysis:		
SDS-PAGE, silver staining	0.3 - 0.5 ng[1]	13
Isoelectric focusing, silver staining	1 - 5 ng[1]	13
Native electrophoresis, silver staining	1 - 5 ng[1]	13
Chromatographic techniques, UV detection	1 - 5 ng[1]	
Analysis of concentration or amount:		
Amino acid analysis	< 1 pmole/aa	
UV absorption	ng range	
Structure analysis:		
Amino acid composition	< 1 pmole/aa	
Amino acid sequence(gas phase sequenator)	pmole range	
Mass spectrometry	50 pmole	
SDS-PAGE, silver staining (MW estimate)	0.3 - 0.5 ng	13
SDS-PAGE, autoradiography	pg range	
Analysis of immunoreactivity:		
Enzyme-linked immunosorbent assay	pg range[2]	
Radioimmunoassay	low pg range[2]	
Analysis of biological activity:		
Enzyme assay, radiolabelled substrate	low pg range[2]	
Enzyme assay, fluorogenic substrate	pg range[2]	
Enzyme assay, chromogenic substrate	ng range[2]	
Binding assays	pg - ng range[2]	
In vitro assays(growth factors, cytokines etc)	pg range[2]	

Footnotes to Table 1
1 Detection limit. At least 10 times more material is required for purity determination.
2 Differs by one or more orders of magnitude for different experimental systems, depending on kinetic and affinity characteristics.

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2 How to optimize sensitivity

The sensitivity of a chromatographic system can be defined as the ratio of the signal to the background noise for the peak obtained after the application of a specified amount of material. The sensitivity is dependent on a number of system and application related parameters. Critical in-built system related parameters include dead volumes, mixing capacity, pump pulsations and type of detection. An important parameter which is both system and application related is the loss of sample due to non-specific adsorption before it reaches the detector (further discussed in Chapter 3). This Chapter discusses other parameters, which are crucial for UV-detection of ng quantities of proteins.

The signal to noise ratio can be manipulated in order to increase the sensitivity. If a reduced peak volume is obtained the signal is increased (at a constant noise). Alternatively, the background noise can be lowered through the choice and handling of eluents. The signal to noise ratio can also be improved by selecting the optimal detection wavelength.

2.1 Reducing the peak volume

Important factors which reduce peak volume and consequently increase sensitivity are column diameter, gradient volume and flow rate.

2.1.1 Column diameter

Narrow columns with high efficiency (i.e. a high number of theoretical plates) are a prerequisite for micropreparative chromatography. A narrower column gives smaller peak volumes and consequently higher sensitivity, provided other factors in the system are not limiting.

Figure 10.6 compares Mono Q PC 1.6/5, diameter 1.6 mm, with Mono Q HR 5/5, diameter 5 mm. Both columns have the same length but the cross-sectional areas differ by a factor of 10. Also see figure 10.31 section 8.2, where the same comparison has been done for Superdex™ 75.

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Columns:	1. Mono Q PC 1.6/5 2. Mono Q HR 5/5
Sample:	Creatine kinase (rabbit muscle), α-amylase (Bacillus Subtilis α-lactalbumin (bovine milk), ovalbumin (hen eggwhite) mixed in proportions 20:6:6:28. A total of 2.5 µg in 50 µl.
Eluent A:	20 mM Tris-HCl, pH 8.1
Eluent B:	20 mM Tris-HCl, pH 8.1, 0.3 M NaCl
Gradient:	0-100 % B in 20 min.
Flow rate:	100 µl/min and 1 ml/min (linear flow rate 5 cm/min)
Detection:	UV- M II (280 nm)

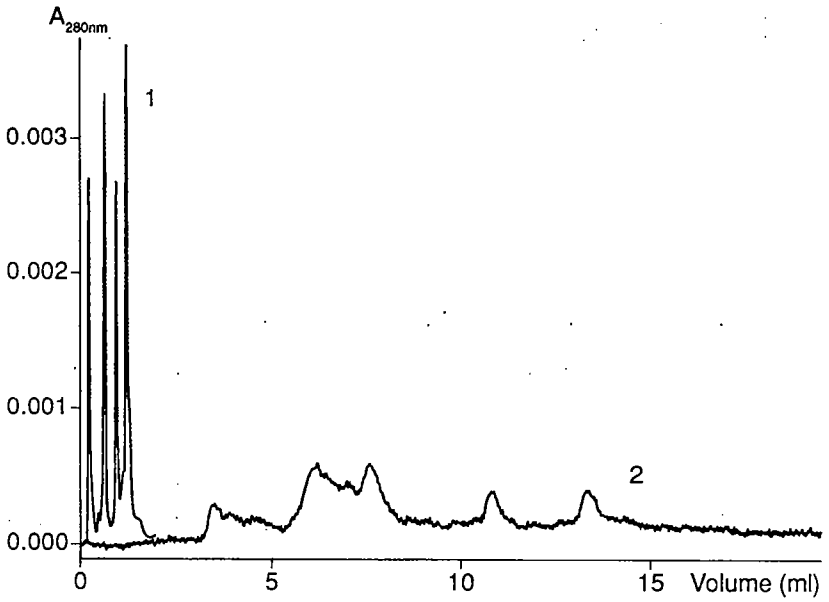


Fig. 10.6. Comparison of Mono Q® PC 1.6/5 with Mono Q® HR 5/5. The chromatograms are compared on a volume basis.

2.1.2 Gradient volume

In gradient techniques a smaller gradient volume decreases the peak volumes. For the separation on Mono Q PC 1.6/5 the gradient volume was reduced from 2 ml to 0.5 ml. This decrease is achieved by reducing either the flow rate (Fig. 10.7) or the gradient time (Fig. 10.8). Reducing the flow rate is more effective than decreasing the time since the sensitivity is increased 3.4 times compared with 2 times.

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Column:	Mono Q PC 1.6/5
Sample:	Creatine kinase (rabbit muscle), α-amylase (Bacillus Subtilis), α-lactalbumin (bovine milk), ovalbumin (hen eggwhite) mixed in proportions 20:6:6:28. A total of 2.5 µg in 50 µl.
Eluent A:	20 mM Tris-HCl, pH 8.1
Eluent B:	20 mM Tris-HCl, pH 8.1, 0.3 M NaCl
Left curve:	Flow rate 25 µl/min
Lower curve:	Flow rate 100 µl/min
Gradient:	0 - 100 % B in 20 min
Detection:	UV- M II (280 nm)

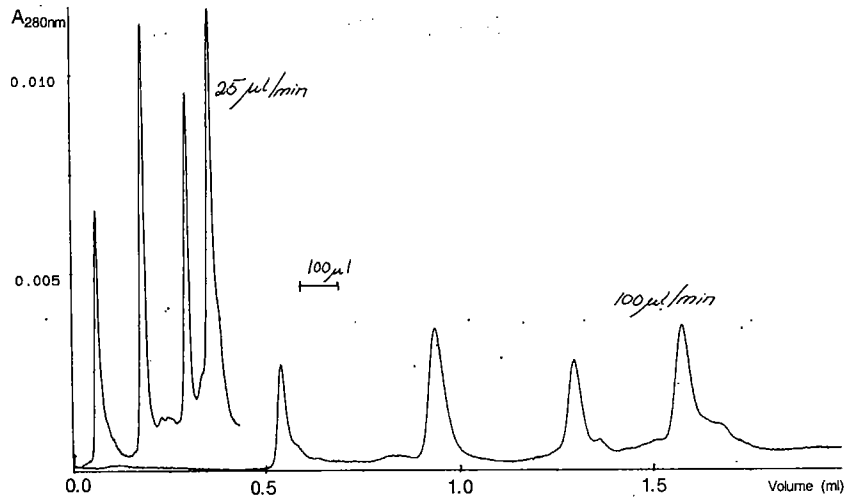


Fig. 10.7. Increase in sensitivity on Mono Q® PC 1.6/5 by decreasing the gradient volume (lowered flow rate).

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Column:	Mono Q PC 1.6/5
Sample:	Creatine kinase (rabbit muscle), α-amylase (Bacillus Subtilis), α-lactalbumin (bovine milk), ovalbumin (hen eggwhite) mixed in proportions 20:6:6:28. 2.5 µg total in 50 µl.
Eluent A:	20 mM Tris-HCl, pH 8.1
Eluent B:	20 mM Tris-HCl, pH 8.1, 0.3 M NaCl
Flow rate:	100 µl/min
Left curve:	0-100 % B in 5 min.
Lower curve:	0-100 % B in 20 min.
Detection:	UV- M II (280 nm)

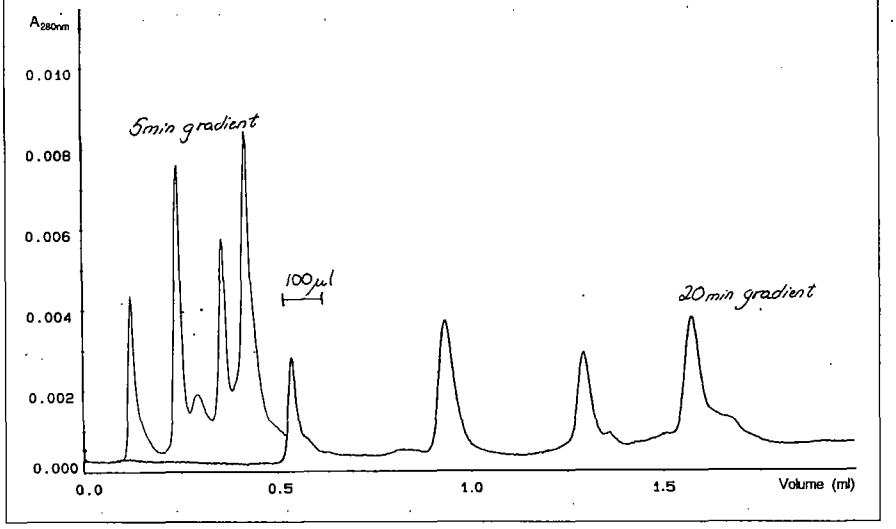


Fig. 10.8. Increase in sensitivity on Mono Q® PC 1.6/5 by decreasing the gradient volume (lowered gradient time).

2.1.3 Flow rate

For isocratic techniques a lower flow rate decreases the peak volumes. This is illustrated in figure 10.34, section 8.2, where the same sample is separated at different flow rates on Superdex 75, PC 3.2/30.

2.2 Detection wavelength

The optimal detection wavelength is a function of detector noise, absorption spectrum of the compound, and eluent disturbances. If the background noise is too high at a wavelength with high absorption (e.g. 214 nm) it could be advantageous to change to one with lower absorption (e.g. 280 nm).

2.2.1 Detector noise

The dynamic noise (determined with flowing liquid through the cell) for the UV-M II detector at three different wavelengths was estimated after magnifying the signal :

214 nm 2×10^{-5} AU

254 nm 5×10^{-6} AU

280 nm 2×10^{-5} AU

These results show that 254 nm gives the least detector noise. 214 nm and 280 nm give a comparable noise.

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2.2.2 Absorption spectrum of the compound

Proteins and peptides containing aromatic amino acid residues exhibit broad absorption spectra. The absorption ratios of four proteins were compared at different wavelengths:

Protein	A_{214nm}/A_{280nm}	A_{280nm}/A_{254nm}
Creatine kinase	20	2.5
α-amylase	8.3	2.9
α-lactalbumin	10	2.2
Ovalbumin	25	2.5

The absorptivity at 280 nm is about 2.5 times that at 254 nm. With respect to both absorptivity and detector noise the optimum detection wavelength is 214 nm, which for these proteins give a sensitivity 8-25 times higher than at 280 nm (comparable noise) and 4-12 times higher than at 254 nm.

2.2.3 Eluent disturbances

Using chemicals of the highest available purity for the preparation of eluents for micropreparative chromatography is extremely important.

UV-curve disturbances from the eluent vary depending on buffer salts, pH, additives, solvent and water quality, wavelength and column type. Figure 10.9 shows a comparison of a salt gradient (0 - 0.3 M NaCl) at three different wavelengths. The highest ghost peak was obtained at 214 nm, the lowest wavelength of the three compared, which generally gives the highest buffer disturbances. In some cases the pattern of disturbances is not reproducible even though the same chemicals and conditions are used. Furthermore, the presence of some common additives in buffers used for protein purification (e.g. some detergents, high concentrations of urea, reducing agents) precludes detection at 214 nm due to high absorbance at this wavelength.

The detection wavelength should be selected after consideration of these three factors. Thus it follows that for proteins and peptides 214 nm should be selected for optimal sensitivity, provided that the eluent disturbances can be handled (discussed below).

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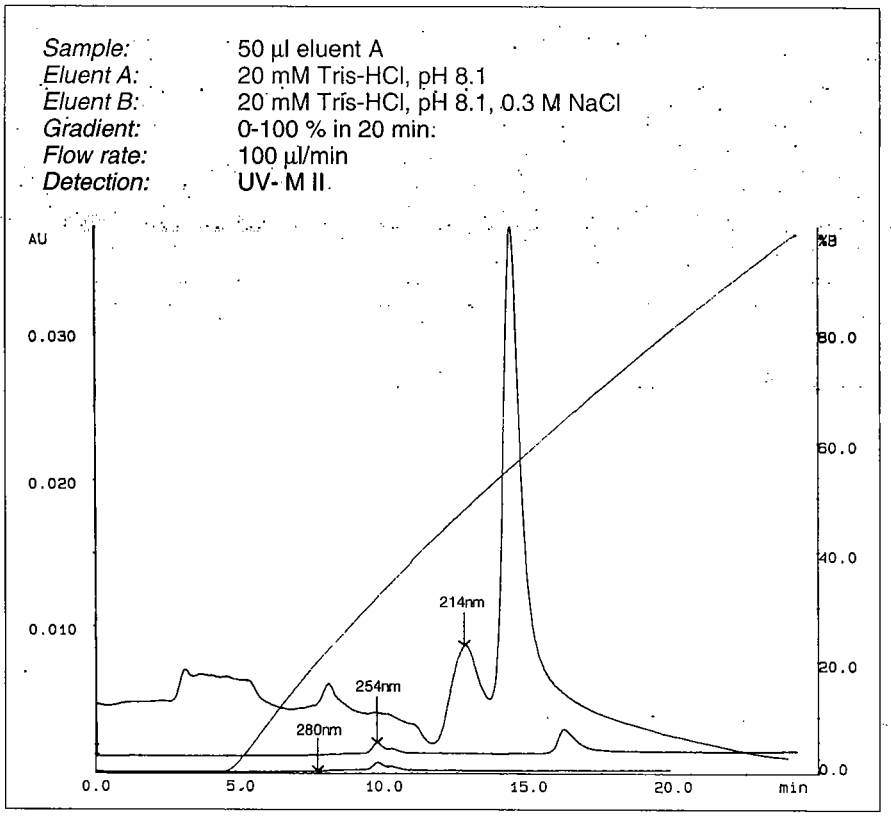


Fig. 10.9. A comparison of eluent disturbances on baselines obtained at 214 nm, 254 nm and 280 nm.

2.3 Handling of baseline disturbances

2.3.1 Ghost peaks

Eluent impurities can be trapped on a precolumn connected between the outlet of the pump which delivers eluent A, and the mixer. This column should have the same binding characteristics as the column used for the separation. The back pressure of the precolumn should be negligible in comparison with that of the system, including the column. The theoretical plate number of the column is not critical, but a high binding capacity is essential.

2.3.2 Baseline drift

A drifting baseline during gradient elution in ion exchange chromatography may be due to a difference in absorbance between eluents A and B. The difference in ionic strength between eluents A and B results in a slightly different ratio between the acid and base form of the buffer. The acid and base forms of many common buffer substances have different absorption properties at lower wavelengths, and it is therefore possible to compensate for the drifting baseline by a slight adjustment of pH in one of the buffers. This is illustrated in figure 10.10 which shows the effect of having a pH difference between eluents A and B in an anion exchange separation. To compensate for the drifting baseline a pH between 8.1 and 8.2 should have been chosen. This also demonstrates how important it is to adjust the pH carefully since a small difference can have a noticeable effect on baseline drift. The same tendency has been observed for a series of buffers monitored at 214 nm and 280 nm, but the effect is greater at 214 nm.

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A similar manipulation can correct a drifting baseline in reversed phase chromatography, where the eluents can be balanced with regard to UV absorbance by the addition of different amounts of TFA to eluent A and B. The exact amounts have to be titrated out; typically eluent A (water) may contain 0.14% TFA and eluent B (60 % acetonitrile) 0.12 % TFA. A similar compensation can be done when heptafluorobutyric acid is used as ion-pairing agent.

Note that the baseline drift can also be caused by a dirty flow cell window. Further information about buffers and eluents is found in Chapter 5.

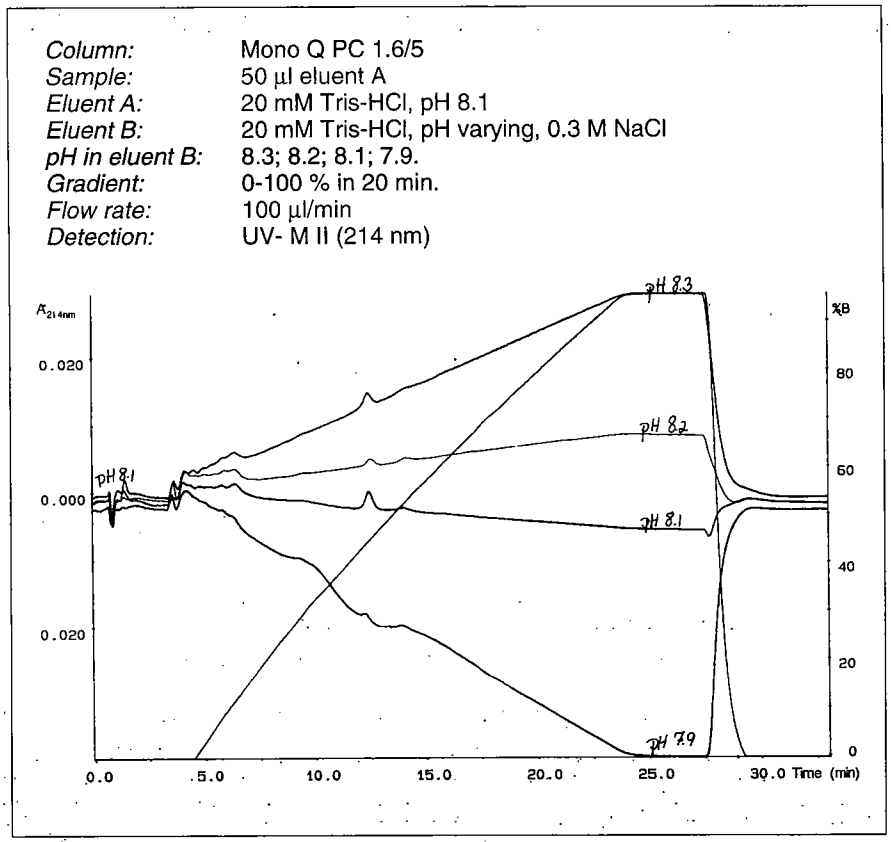


Fig. 10.10. Influence of pH differences between eluents A and B on baseline drift using Mono Q® PC 1.6/5. To compensate for the drifting baseline a pH between 8.1 and 8.2 should have been chosen.

2.3.3 Other considerations

In addition to the above, the following points relating to baseline problems with gradient techniques at high sensitivity may be considered:

1. A noisy signal can be caused by particles or an air bubble in the flow cell, an incorrect position of the flow cell (i.e. it has not been tightened properly), or an ageing lamp.
2. To control background noise blank runs should be routinely performed, especially when the system has not been used for a while.
3. A minimal time delay between consecutive runs reduces problems with ghost peaks.

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4. Keeping the system in operation as often as possible (e.g. at 10 µl/min over night, with 100 % B) reduces baseline problems, particularly those related to ghost peaks.
5. When the system has not been used for a while, or after change of column, it is recommended that eluent B is run through the system (after preservatives, such as 20 % ethanol, have been washed out with a weak eluent) until the UV signal is stable at the selected sensitivity. Equilibration with eluent A should then be performed for a specified time. This should not be longer than necessary since trace amounts of contaminants in eluent A can be enriched on the column.

2.4 Detection limits

The detection limit of a chromatographic system can be defined as the amount of injected substance needed to detect a peak twice as high as the background noise. This limit varies for different substances and different chromatography techniques. It is usually lower for isocratic techniques owing to the discussion above. Chromatograms in Chapter 8 show approximate detection levels for SMART System /UV-M II where 5 ng α-amylase are monitored on Mono Q PC 1.6/5, (Fig. 10.27); 900 pg nucleic acids are monitored on the same column (Fig. 10.28), 5 ng Cytochrome C on µRPC C2/C18, PC 3.2/3 (Fig. 10.49) and 1 ng ovalbumin is monitored on Superdex 75, PC 3.2/30 (Fig. 10.35). A detection level on SMART System/µPeak Monitor is exemplified in Figure 10.50.

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3. How to optimize recovery in protein and peptide purification

Obtaining a maximum amount of information from a minimal amount of sample is of major concern for researchers involved in microscale biochemistry. Successful microscale work requires minimal losses of both mass and activity during purification and other manipulations. As with sensitivity (Chapter 2) the recovery obtained from a chromatography system is dependent on both system and application related parameters.

System related parameters of SMART System that help maximize recovery are biocompatible wetted surfaces and reduced surface area exposed to the sample. Measurement of recovery in SMART System, and points to consider in order to obtain a high recovery in protein and peptide purification are discussed in this Chapter.

3.1 Recovery in SMART System

Mass recoveries in SMART System have been determined for different chromatographic techniques, using on-line measurement of UV absorbance and integration of peak area. On-line recovery measurements were chosen since they are rapid, allow the use of small sample volumes and are not affected by sample handling (in contrast to more sensitive techniques, e.g. using radiolabelled or fluorescent samples).

Recovery data for different gel media or columns are often shown as a linear plot of recovery against sample load. In such plots, linearity indicates a high relative recovery over the whole sample loading range tested, if it is assumed that the highest sample load gives negligible losses. This is based on the assumption that the relative loss of sample decreases with amount. A more reliable alternative is to estimate the recovery for the highest sample load experimentally. For SMART System, this was done with a blank run in which the sample passed through the system after the column had been replaced by short capillary tubing. The UV area obtained for the blank run, using a high sample load, was then used as a reference (i.e. 100 % recovery) for subsequent measurements. In order to determine recoveries for very small amounts, it was thus necessary to cover a wide range of sample loadings (covering several orders of magnitude). It should also be noted that a wide sample loading range is required to give reliable data, whereas a narrow range will most likely give too high recoveries. Furthermore, it is important to use a detection technique that is linear over the whole sample loading range.

Recovery data obtained in this way may be represented by a linear plot of recovery versus sample load. A linear plot gives the impression of high recoveries for even the lowest amounts applied, since it is very difficult to distinguish between 70 and 90 % recovery

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for sample loads that are far below 1% of the largest amounts. A double logarithmic plot is an alternative, but is difficult to analyze unless the recovery is 100 % throughout. A convenient way of illustrating recovery that accurately gives the values for low sample loadings, is to calculate the recovery for each experimental point by dividing the obtained, integrated UV area, by the theoretically obtained value (calculated from the highest sample loading). Recovery data for SMART System (and Precision Columns) have been obtained in this way. The results (shown in Chapter 8, as plots of % recovery versus sample load) indicate recoveries above 80 % at the ng level for the proteins tested.

It is important to consider what the determined recovery actually represents. The relative UV area gives information about the recovery from the column. If a measurement without a column is used as a reference, as described above, the recovery from the column relative to the recovery from a short piece of tubing is obtained. For an indication of the recovery over the whole chromatography system (including injection syringe, sample loop, column, fraction collector) a reinjection technique has been used. The collected peak was diluted with the starting buffer, and reinjected. The peak area obtained was then compared with the peak area for the first injection. These "total" mass recovery data for Mono Q PC 1.6/5 and µRPC C2/C18, PC 3.2/3 were 60-70 % and 80-90 % respectively (Chapter 8).

3.2 Recovery of mass

The recovered amount of total protein in a purification procedure is always below 100 %. Some of the losses are unavoidable, i.e. physical loss of sample solution in tubes, syringes etc. Precipitation of the molecule of interest, degradation of the sample, and non-specific surface adsorption also commonly account for the losses. In micropreparative work, particular attention must be paid to adsorption problems. All surfaces in contact with the sample, i.e. glass vessels, tubes, pipette tips, tubings in chromatographic systems, chromatographic matrices, dialysis tubings, filters and membranes are potential sites for interaction with the sample.

Losses due to such interactions may be dramatic in micropurification with small sample loading. Normally these losses are less significant at high sample loadings where the proportion of lost material is small.

3.2.1 Minimizing non-specific adsorption losses

The mechanisms by which biomolecules interact with different solid surfaces are largely unknown. Both charged and hydrophobic sites may be involved. Different proteins and peptides have different tendencies to adsorb; if the proteins and peptides are hydrophobic and/or basic they are often described as more "sticky". Below, a number of procedures or measures commonly employed to reduce non-specific adsorption are described.

Presaturation

Presaturation of potential binding sites in tubes, glass vessels and pipette tips can be done by incubation with a protein solution (e.g. 0.1 - 1 % bovine serum albumin for 5-10 min, followed by extensive washing with buffer). However, the introduction of a "foreign"

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component may affect subsequent analyses, even highly purified albumin preparations may contain traces of proteases, etc. When possible, it may be advantageous to presaturate surfaces with a purified preparation of the molecule of interest.

Introduction of a competing substance

An excess of a substance which competes with sample molecules for hydrophobic binding sites may dramatically increase recovery. Polyethylene glycol (0.05 %), isopropanol (10 %) or low concentrations of non-ionic detergent is often added to chromatographic buffers for this purpose. These compounds are also used to prevent aggregation. Presaturation with these substances is insufficient because of the reversibility of the binding, they should be added to all solutions during a chromatographic separation.

If a detergent is used for this purpose, the concentration should be well below its critical micelle concentration. Ideally, the detergent should block the non-specific binding site without participating in any other reaction (i.e. micelle formation or changes of protein structure). Only non-ionic detergents, of the highest available purity, should be used. A recommended substance for this purpose is Polysorbate 20 (e.g. Tween 20). Typically, concentrations of about 0.02 % are used, but concentrations as low as 0.005 % are effective.

By collecting fractions in tubes containing low concentrations of detergent it is possible to increase recovery from the tube, if some dilution can be accepted.

Alternatively, the addition of a carrier protein (e.g. 0.1 % bovine serum albumin) to buffers used in chromatography or to tubes in the fraction collector can reduce non-specific adsorption by competition. This is often employed when working with radioactive substances. However, the carrier protein must not bind to the gel matrix since it can dramatically reduce the available binding sites for the molecule of interest. Furthermore, the presence of a carrier protein affects the UV curve and is also likely to interfere with subsequent analyses.

Surface material

Silicone covered glassware has been used for several decades by biochemists wishing to minimize surface adsorption to reactive hydroxyl groups. Silicone reagents are available from several manufacturers (for example Surfasil from Pierce). When basic peptides are handled, plastic tubes made of polyethene (for example Minisorp tubes from Nunc) are preferred to those made of polystyrene.

Minimizing surface area

Good general advice when dealing with "sticky" biomolecules is to reduce the total surface area exposed to a sample in a purification scheme. Filtration, ultrafiltration, dialysis and lyophilization are techniques frequently known to cause considerable loss due to "harmful" surface contact. With SMART System and Fast Desalting PC 3.2/10, a rapid and high recovery alternative to microdialysis is available (see section 8.3). Furthermore, the sample

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can usually be obtained in a very small volume (typically 50-100 µl) after chromatography with SMART System, even if the applied sample volume is large, thus rendering further concentration steps unnecessary (see Chapter 7).

3.2.2 Minimizing "physical" sample loss

Some "physical" losses of sample are unavoidable in a purification scheme, but measures can be taken to keep them as small as possible. The importance of choosing tubes, syringes etc which exhibit minimal adsorption was mentioned above. Care should also be taken to choose such items from which an added volume can be quantitatively removed (i.e. have minimal dead volumes). Tubes with surfaces on which a drop rather than a film of liquid is formed are preferable. Other important aspects in this context, such as the preparation of small sample volumes for chromatography and points related to injection, are dealt with in Chapter 6.

3.2.3 Minimizing losses due to insolubility

Poor solubility of some (usually hydrophobic) peptides in protein digests is a common problem in peptide work. Such peptides can frequently be dissolved in a small volume of concentrated formic acid, or 6-8 M guanidine-hydrochloride, and then diluted to the conditions for separation (usually reversed phase chromatography).

In general, proteins have the highest solubility and stability in conditions resembling their natural environment. Therefore, when chromatography conditions differ significantly from these, problems may arise. For instance, the high salt concentrations used in hydrophobic interaction chromatography, may cause precipitation of some sensitive proteins. In these cases, a lower salt concentration in the start buffer, or an alternative purification technique, should be considered. Solubility problems may also occur at extremely low ionic strength. Other conditions under which some proteins are prone to precipitation are at extremes of pH, or at a pH close to their isoelectric point. To perform the separation at another pH may solve these problems if they occur. The solubility of proteins as a function of solvent components is presented in a review written by Schein (28).

3.3 Recovery of activity

In protein purification it is usually of interest to have high mass recovery of the substance as well as of its biological activity. This is also true of peptide purification (exceptions are peptide mapping and sequencing situations where only high mass recoveries are essential). Loss of activity when not accompanied by a loss of protein mass is due to denaturation (i.e. loss of tertiary structure), proteolytic degradation or inactivity owing to loss of cofactors, oxidation etc.

3.3.1 Minimizing loss of activity

Avoiding denaturation

Extremes of pH, high temperature, organic solvent, dilution and other conditions known to affect protein tertiary structure should be avoided when possible. If extremes of pH are necessary, for instance during elution in affinity chromatography, fractions may be collected in tubes to which a small volume of a neutralizing buffer has been

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added. Ultra-filtration and lyophilization frequently destroy the activity of labile proteins (in addition to causing mass losses), while chromatographic techniques with a concentrating effect are generally much milder. Also, extremely dilute protein solutions often lose activity rapidly, hence it is beneficial to obtain the purified protein in a concentrated form as quickly as possible.

Avoiding proteolytic degradation

Common general precautions to limit the proteolytic degradation are to work rapidly and at low temperature. It is recommended that the cooling function in SMART System is used. In many laboratories protease inhibitors are routinely added to all samples and all buffers used in the early stages of a purification scheme. The presence of proteases of different classes can be expected in complex biological samples, hence inhibitors for all or several of the different classes should be added. A suitable addition to a freshly prepared extract and to the buffers used for initial purification is :

2 - 5 mM EDTA (for metalloproteinases), 0.5-1 mM freshly dissolved phenylmethylsulfonyl fluoride, PMSF (for serine proteases, some thiolproteases and carboxypeptidases) and 0.1 µM pepstatin A (for some acid proteases) (29).

Avoiding inactivation

Many proteins can be inactivated because of specific effects on particularly reactive amino acid residues. The catalytic site of an enzyme, for example, contains amino acid residues which are abnormally reactive and therefore susceptible to modification. Such inactivation is difficult to avoid unless these particular properties are known. Equally difficult to avoid is inactivation due to loss of cofactors during purification.

More generally, proteins that contain sulfhydryl groups (free cysteins) are often prone to oxidation which may lead to a loss of activity. This can be avoided by the addition of a mild reducing agent (e.g. ß-mercaptoethanol) to the buffers.

3.4 Measurement of recovery in a purification scheme

The recovery in protein or peptide purification can be estimated by a measurement that is proportional to the mass or the biological activity of the interesting compound. Generally, mass recovery measurements give a quicker and safer estimation.

3.4.1 Measurement of mass recovery

Rechromatography and comparison of the integrated UV curves.

This technique can be used to estimate the mass recovery (of any UV absorbing substance) obtained in a single chromatographic step providing the compound of interest gives a well resolved peak. The material in this peak, or an aliquot of it, is then rechromatographed (after a blank run) under identical conditions. The percentage recovery from the system is then estimated by dividing the peak area obtained in rechromatography by that obtained in the first separation. Integration is rapidly performed with SMART Manager. The recovered amount of mass can be directly calculated if the absorbance coefficient is known. If this coefficient is unknown, an

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estimate of protein mass recovery can be made at 214 nm (1). Not only are losses on the column accounted for in this technique, but also losses which occur in fraction collection and during injection of the sample. Figure 10.11 shows how this technique was used to determine recovery for an unknown component in cerebrospinal fluid.

A "short-cut" is to dispense with the second (rechromatography) step, and use the value of the integrated peak area in the first separation as an estimate of the recovered amount (1). In this case, however, losses between the UV monitor and the fraction collector are not accounted for.

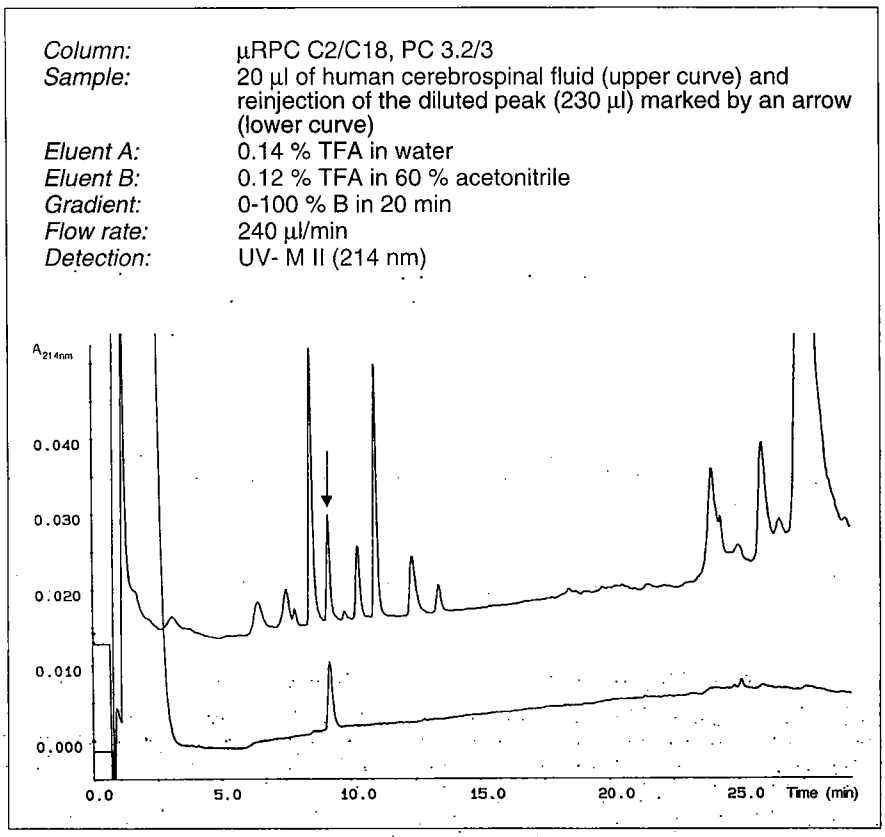


Fig. 10.11. Determining recovery on µRPC C2/C18, PC 3.2/3, by reinjection and integration of peak area of an unknown component in cerebrospinal fluid. The upper curve shows chromatography of 20 µl of human cerebrospinal fluid. A 50 µl sample loop was used. The peak marked with an arrow was collected in an 80 µl fraction (0.5 ml Sarstedt tube). This material was then transferred to a syringe. The tube was washed with 3 x 50 µl of eluent A. This liquid was also drawn into the syringe containing sample. The contents of the syringe were then mixed after the introduction of an air bubble (section 6.3), and injected into a 500 µl sample loop. Conditions for chromatography were otherwise identical for the two samples. A blank run was performed between the two sample runs. The recovery after reinjection was 78 % , as determined by integration of peak areas using SMART Manager.

With radiolabelled samples

Use of purified radiolabelled proteins or peptides is a straightforward technique for establishing recovery (by mass) of a particular component in a chromatographic step at very low sample loadings. The eluted radioactivity is compared with that of the applied sample, and the mass of the recovered amount can be directly obtained if the specific activity of the sample is known. Any free radiolabel, if

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present, must be removed (for instance with Fast Desalting, PC 3.2/10) before the experiment.

Other techniques for measuring total protein

Other techniques suitable for measuring total protein in low quantities, such as amino acid analysis, can also be used for this purpose. However, the sample is consumed during these techniques.

3.4.2 Measurement of biological activity

Measurement of enzyme activity

Enzyme assays are often used for localizing activity in a chromatogram. However, a quantitative comparison of the activity in a purified fraction with that in a crude sample is often inaccurate. The reasons for this may be the presence of activators or inhibitors in the crude sample. A careful investigation of how different factors affect the assay is usually required in order to obtain reliable quantitative data.

Receptor activity

Problems with receptor (binding) assays are generally similar to those with enzyme assays, i.e. quantitative data can often be obtained without too much effort, but factors which interfere with the binding have to be controlled to obtain quantitative results. The final step in micropurification of a nicotinic acetylcholine receptor from chicken brain, monitored by a binding assay, is shown in figure 10.12.

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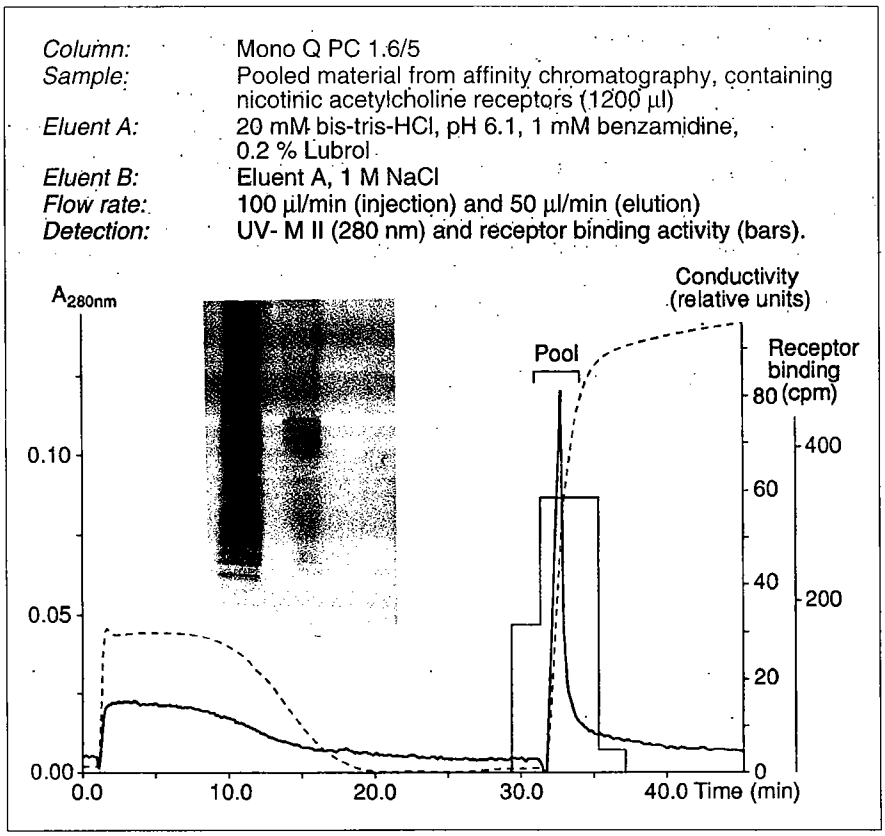


Fig. 10.12. Final purification and concentration of purified nicotinic receptor from chicken brain using Mono Q® PC 1.6/5. A micropurification scheme using SMART System was developed for nicotinic acetylcholine receptor from optic lobes in a single chicken brain in order to study subunit composition by electrophoresis. The first purification step involved affinity chromatography with bungarotoxin immobilized on NHS-activated Superose® PC 3.2/2 (Fig. 55, section 8.5). The second, and final, purification step using anion exchange chromatography (above) was performed in order to remove free nicotine from receptor protein (which otherwise interfers with the assay), and to concentrate the sample before electrophoresis. Receptor activity (bars) was measured in an assay using ^{125}I-labelled bungarotoxin. The electrophoretic analysis, monitored by silver staining (inset), was done with PhastSystem™ using PhastGel® 8-25 (reducing conditions). Left lane: Crude optic lobe extract (before affinity chromatography). Right lane: Purified, pooled material from anion exchange chromatography. The two bands correspond to subunits with approximate molecular weights of 56 K and 67 K.

Immunoreactivity

Immunoreactivity, i.e. the capacity to bind to antibodies, does not reflect the "true" activity of a protein. The presence of a functional immunogenic epitope in a purified molecule may not necessarily mean that the region responsible for the biological activity is intact. However, immunoassays, such as radioimmunoassays or enzyme-linked immunosorbent assays are extremely useful for microwork (if antibodies to the molecule of interest are available) since they are sensitive and thus consume little material (Fig. 10.13). Problems experienced when determining quantative data for immunoreactivity are similar to those when determining recovery for enzyme and binding assays.

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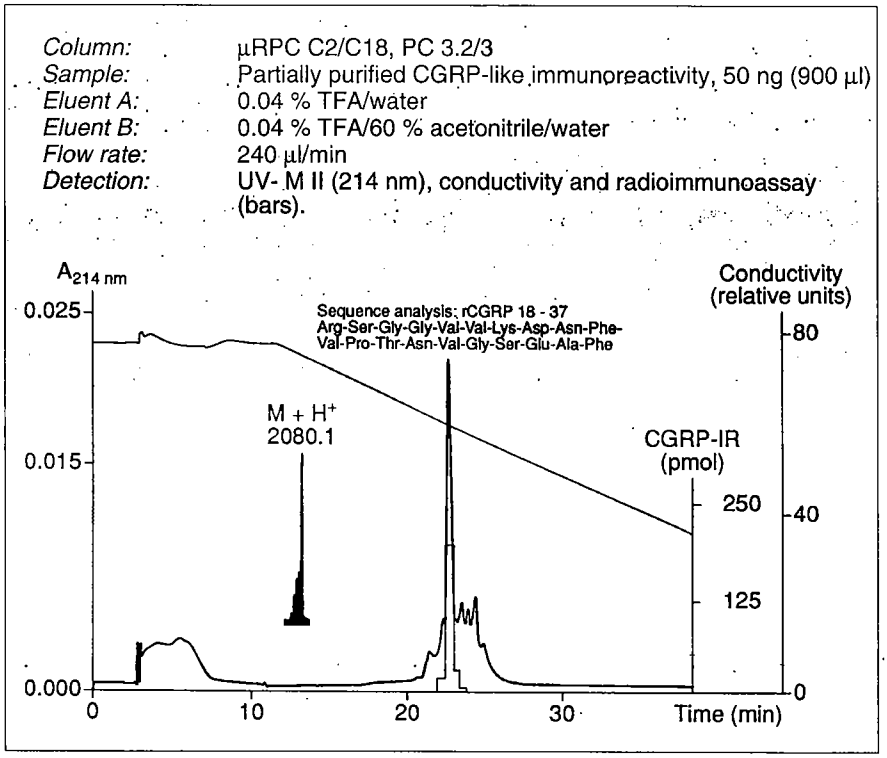


Fig. 10.13. Final micropurification step for calcitonin gene related peptide (CGRP)-like immunoreactivity using µRPC C2/C18, PC 3.2/3. A purification scheme developed for CGRP-like immunoreactivity used a combination of conventional chromatography (using gel filtration, cation exchange and reversed phase chromatography) and micropurification with SMART®System. Its aim was to perform a structural characterization of the immunoreactivity. The final micropurification step (above) was monitored by conductivity measurement (upper, downward sloping curve) and UV (214 nm). Fractions of 100 µl were collected and aliquots were analysed by a radioimmunoassay (bars). The fraction with the highest activity was analysed by mass spectrometry and gas phase amino acid sequencing (results shown in inset). The recovery of immunoreactivity was approximately 95 % (corresponding to 48 ng) in the final purification step.

3.5 Maximizing recovery through a purification scheme

General guidelines for maximizing recovery in protein and peptide purification are similar, independent of scale. However, in micropurification loss of µg levels of protein are unacceptable, while a similar loss may not even be noticed when working at a larger scale. Therefore, considerable attention has to be paid to avoiding losses in micropurification. Table 2 is essentially a summary of the suggestions in this chapter. Considerations for sample pretreatment are dealt with separately (Chapter 6).

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Table 2. Points to consider when maximizing recovery in micropurification of proteins and peptides.

- Add protease inhibitors to crude biological extracts.
- Work rapidly.
- Work at 4 °C.
- Use few steps.
- For proteins; avoid extremes of pH, high temperature and organic solvents.
- Avoid extreme dilutions of sample and extremely low salt concentrations.
- Minimize surface contact. For instance, if chromatographic resolution is good, collect one peak in one tube.
- Minimize the handling of fractions (pipettings, freezing, thawing, etc).

Avoid	Use instead
• Filtration of small sample volumes and/or diluted samples	• Centrifugation
• Microdialysis	• Fast Desalting, PC 3.2/10
• Ultrafiltration and lyophilization	• A chromatographic concentrating technique on a microcolumn

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4. Micropreparative chromatography in molecular biology

4.1 Introduction

A requirement of the modern biological laboratory is the possibility to purify DNA from cells. Cell DNA may originate from bacterial, plant or animal cells and is often required as the starting material for different recombinant DNA technologies such as restriction enzyme digestion, cloning and hybridization techniques, DNA sequencing and polymerase chain reaction (PCR). A second type of bacterial DNA often purified is plasmid DNA, which is mainly used as vectors during construction of recombinant DNA molecules.

Irrespective of which type of DNA needs to be purified, the initial steps after growth and harvesting of cells are generally as follows:

1. Enzymatic, chemical or mechanical treatment (lysis) to disrupt cell walls and membranes to release nucleic acids.
2. The resulting cell extract is then treated so that all cell components except DNA are removed. This can include protease treatment followed by phenol extraction to remove proteins, and RNAase treatment to hydrolyze RNA.
3. Concentration of the DNA containing solution.
   The DNA may now be pure enough for some applications but for others it has to be further purified. Methods for further purification of DNA include:
   a) Density gradient centrifugation, which is a commonly used method for obtaining pure plasmid DNA (27).
   b) Electrophoretic purification. This can be used for the isolation of a specific DNA fragment generated by restriction enzyme digestion of a larger DNA molecule, followed by electrophoretic separation in an agarose or polyacrylamide gel. It is also used for preparative purification of chemically synthesized oligonucleotides on a denaturing polyacrylamide gel.
   c) Chromatographic separation with FPLC or HPLC, which has become an increasingly popular method. FPLC has been used for purification of plasmid DNA (5, 17), isolation of DNA fragments generated by restriction enzyme cleavage (3, 34) and for purification of oligonucleotides synthesized in Gene Assembler® (10).

As we will show in this Chapter most of the DNA separations on FPLC can also be performed with SMART System. The separation range of SMART System covers the practical range of sample for molecular cloning, which is usually 0.5 - 10 µg.

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4.2 Applications in molecular biology with SMART System

4.2.1 Concentration of DNA

The researcher often has the DNA molecules in a large volume and needs to concentrate the sample. This concentration is usually performed by ethanol or isopropanol precipitation in the presence of salt (27), and is a convenient method when a microcentrifuge can be used. However, if there are large volumes to be concentrated, the process will be time-consuming and the risk of losing material will be apparent. A more convenient method is to use anion exchange chromatography with Mono Q PC 1.6/5 as shown in figure 10.14a. From the electrophoretic analysis in figure 10.14b, it can be shown that the DNA content in a 4 ml solution was concentrated to 70 µl. It should be noted that in contrast to standard DNA concentration methods, a simultaneous purification of the nucleic acids is achieved when SMART System is used for concentration purposes. This feature could be very useful after enzymatic treatment of DNA where the chromatographic concentration step also results in the removal of the enzyme. Another use is the concentration of DNA fragments eluted from electrophoresis gels. In addition to concentration, contaminants, which can inhibit the action of certain DNA modifying enzymes are removed from the electrophoresis gel.

Column:	Mono Q PC 1.6/5.
Sample:	0.5 µg pBR322 digested with *Eco* RI in a total volume of 4 ml.
Eluent A:	10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE), 0.5 M NaCl.
Eluent B:	TE pH 8.0, 1.0 M NaCl
Flow rate:	300 µl/min during sample loading, 100 µl/min during elution.
Gradient:	0-100 % B in 10 min.
Fraction size:	10 µl
Detection:	UV- M II

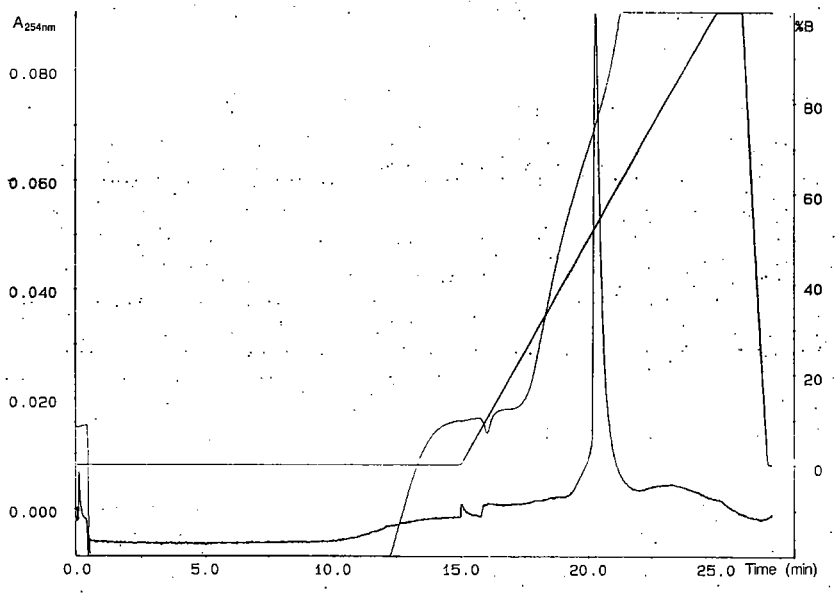


Fig. 10.14a. Concentration of DNA on Mono Q® PC 1.6/5.

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Lanes: 1) Unconcentrated DNA; 2) Fraction 15; 3) Fraction 16; 4) Fraction 17; 5) Fraction 18; 6) Fraction 19; 7) Fraction 20; 8) Fraction 21. Electrophoresis was performed by using PhastSystem ™, Phastgel® gradient 4-15 and PhastGel® native buffer strips. Prior to separation at 200 V for 60 Vh, the gel was prerun at 200 V for 80 Vh. 1 µl of each fraction was loaded onto the gel. Detection was by silver staining.

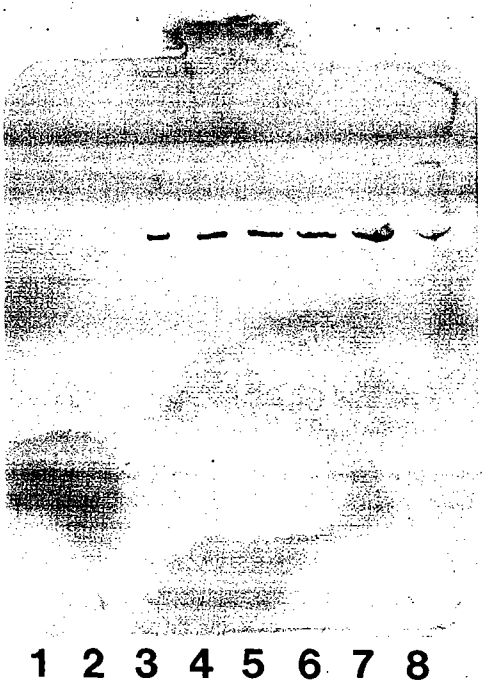


Fig. 10.14b. Electrophoretic analysis of fractions from 14a.

4.2.2 Separation of DNA fragments

A common demand in a DNA laboratory is the need to purify an inserted DNA fragment from the cloning vector which is to be used in further subcloning experiments. This kind of fragment separation is illustrated in figure 10.15a where pBR 322 restricted with *Eco* RI and *Sal* I was separated on Mono Q PC 1.6/5. The enzymatic treatment of the plasmid resulted in fragments of 651 bp and 3710 bp, where the smaller fragment represents the size of an insert and the larger one the cloning vector. The 651 bp fragment was eluted from the column in less than 20 minutes and contained in 200 µl, which is a convenient volume for alcohol precipitation and desalting in a microcentrifuge tube. The larger fragment was eluted off the column in about 30 minutes. As can be seen from the electrophoretic analysis in figure 10.15b, there is no detectable cross contamination between the two fragments as determined by silver staining.

By using this technique, it is possible to separate an inserted fragment from its cloning vector in a preparative manner in less than 20 minutes and with a recovery exceeding 95 %. This can be compared with preparative electrophoresis which is probably the most commonly used method for fragment isolation. Preparative electrophoresis involves separation of DNA fragments in an agarose or polyacrylamide gel, visualization of DNA, isolation of the piece of gel containing the desired fragment and then elution of the DNA from the gel, which rarely results in a recovery exceeding 50 % (27).

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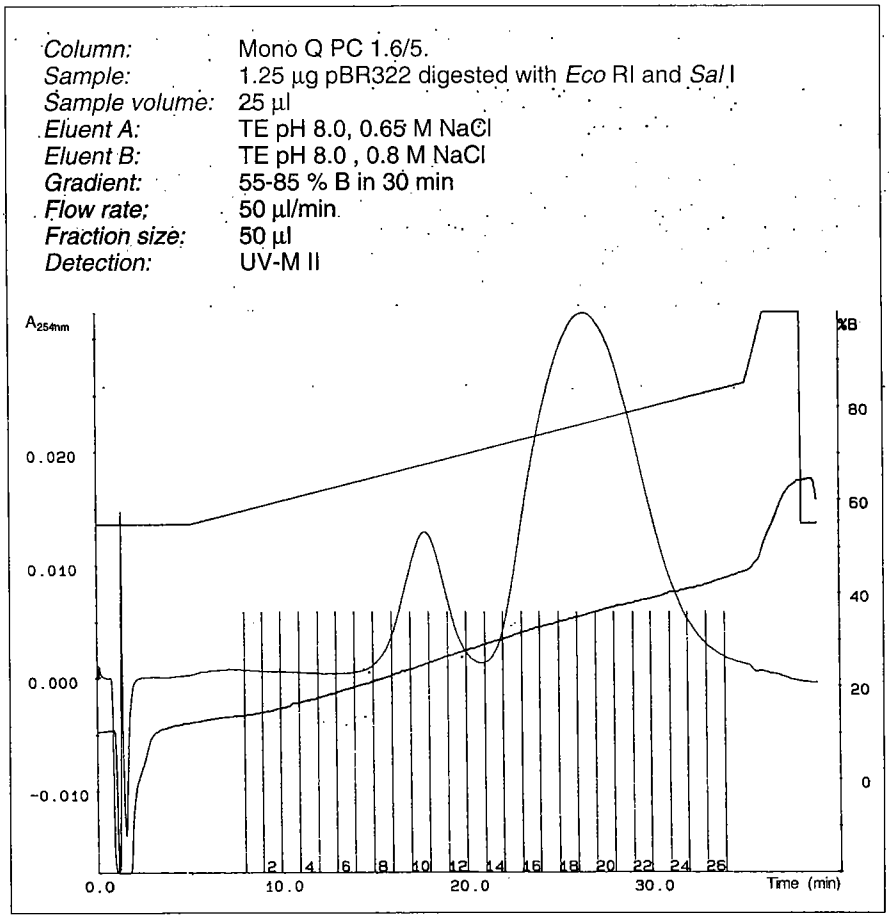


Fig. 10.15a. Separation of DNA fragments on Mono Q® PC 1.6/5.

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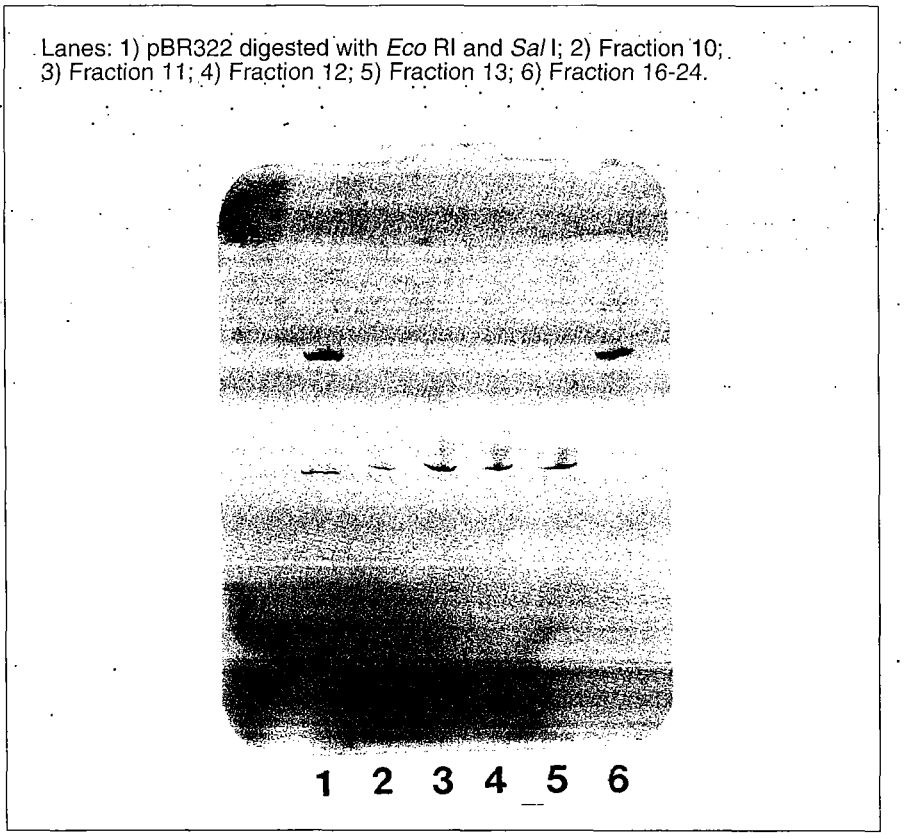


Fig. 10.15b. Electrophoretic analysis of fractions from 15a as described in the legend to figure 10.14b. Separation was for 100 Vh at 200 V.

4.2.3 Purification of PCR products

The polymerase chain reaction (PCR) is a method used for specific amplification of DNA sequences flanked by regions of known sequence (26). Although it is a relatively new technique, PCR has been used for several diagnostic purposes. Among these are genetic identification of forensic samples (13) and detection of pathogenic organisms (22). In addition, PCR is also used for many tasks in molecular cloning (27). Ion exchange chromatography with SMART™System is an alternative to conventional purification methods for PCR products by use of Mono Q PC 1.6/5 as shown in figure 10.16a.The size of the amplified product is 650 bp and it contains the gene coding for dihydrofolate reductase of Escherichia coli. After amplification, the mineral oil, which was layered on top of the reaction mixture to prevent evaporation, was removed and the contents in the tube (50 µl) was loaded directly onto the column. The separation was performed at a flow rate of 200 µl/min which resulted in a purified PCR product in less than 10 minutes. The electrophoretic analysis in figure 10.16b reveals that the main product is well separated from primers, polymerase and BSA, which was present in the reaction mixture to stabilize the polymerase. The purified product was subsequently cloned into an expression vector in order to overexpress dihydrofolate reductase (data not shown). Because of its short processing time this kind of separation is suitable not only for preparative use but also as an analytical tool to monitor the formation of an amplified product directly after a PCR.

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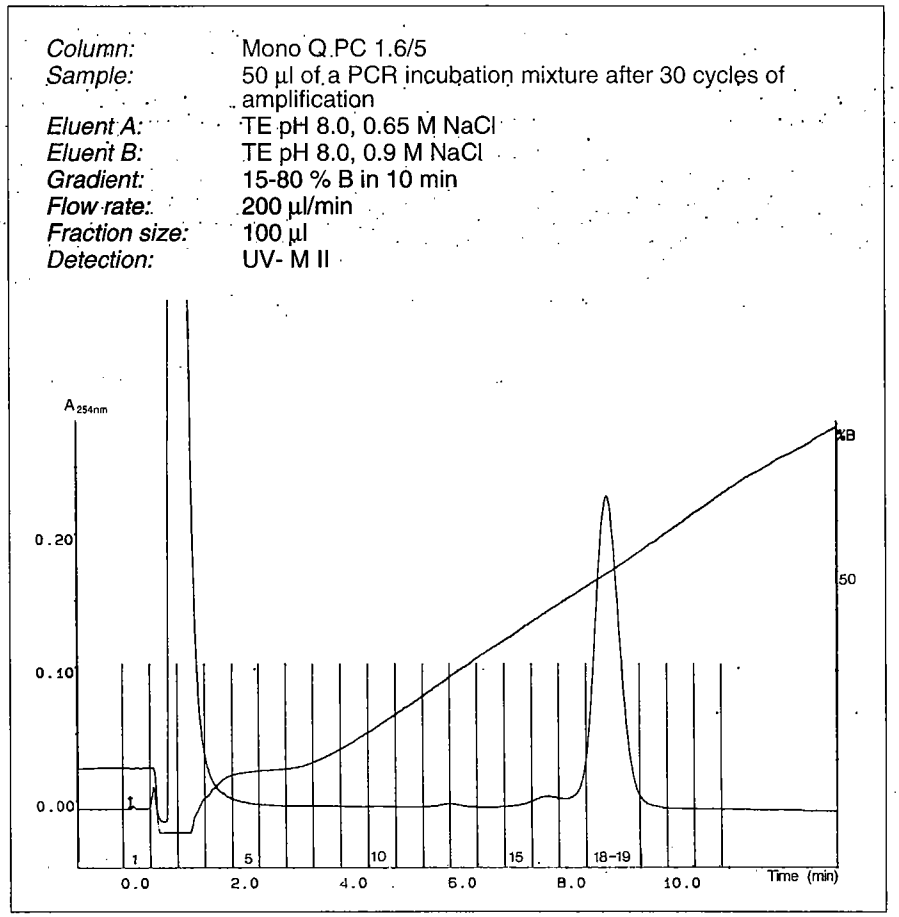


Fig. 10.16a. Purification of a PCR product on Mono Q® PC 1.6/5.

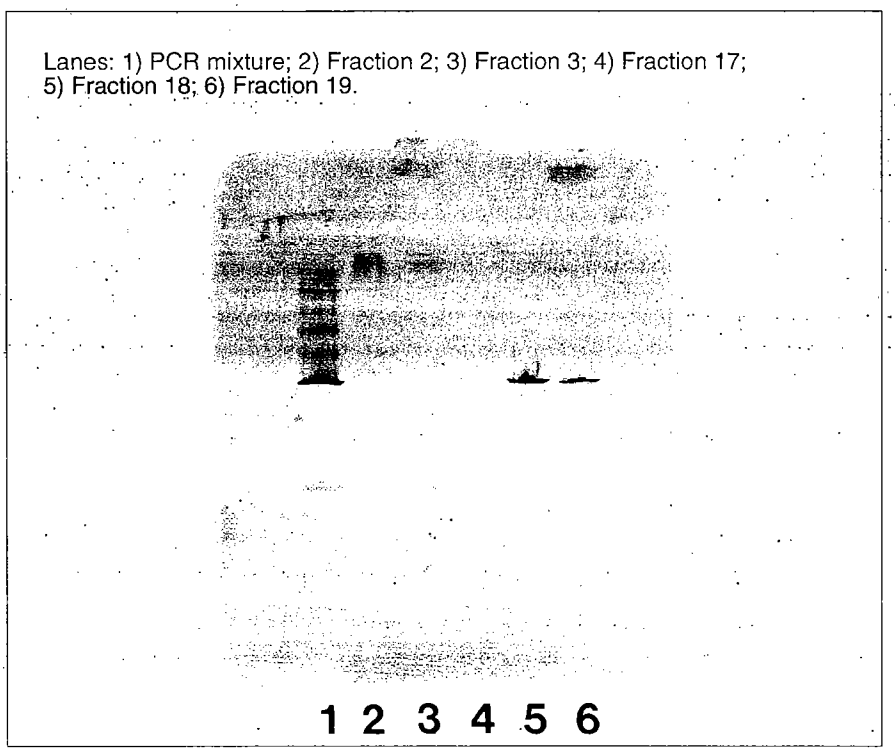


Fig. 10.16 b. Electrophoretic analysis of fractions from 16a. Conditions for electrophoresis were as described in the legend to figure 10.14b with the exception that PhastGel® 8-25 was used. Separation was for 50 Vh.

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4.2.4 Purification of plasmid DNA

In order to purify plasmid DNA it is necessary to grow the plasmid containing bacterial strain, harvest the bacteria and lyse them, and finally extract the plasmid from cell debris. If further purification of the plasmid is needed, this is usually accomplished by centrifugation to equilibrium in cesium chloride gradients containing ethidium bromide (27). However, this method is expensive, time-consuming and involves use of the toxic chemical, ethidium bromide. Because of these factors, several methods have been developed as alternatives to density gradient centrifugation. Among these are ion exchange or gel filtration chromatography with FPLC® System which give highly purified plasmid DNA (5, 17). These techniques can also be applied to SMART System which is better suited for small to middle range scale plasmid purification. Figures 10.17a and 10.18a are chromatograms of alkaline lysates containing plasmids after purification on Mono Q PC 1.6/5 and Superose 6 PC 3.2/30, respectively. Figures 10.17b and 10.18b show electrophoretic analysis on agarose gels of fractions from the chromatograms and the results from restriction enzyme cleavage.

It must be emphasized that it is necessary to break down cellular RNA prior to chromatographic separation in SMART™ System. This can be achieved with RNAase treatment or as was done here, by conducting the alkaline lysis step at 70 °C for 30 minutes.

The amount of plasmid DNA loaded onto each column (figures 10.17a and 10.18a) originated from a 1.5 ml culture of HB101 (pBR322). The yield of pure plasmid DNA was 2.5 µg and 3 µg for Mono Q PC 1.6/5 and Superose 6 PC 3.2/30, respectively. The yield of DNA obtained by using gel filtration is probably because this technique does not separate open circular (oc) plasmid DNA from covalently closed circular (ccc) plasmids. As can be seen from figure 10.17a, plasmid separation on Mono Q PC 1.6/5 separates oc from ccc DNA. The maximum loading capacities of these columns are between 50 µg and 60 µg as determined by earlier results. Thus it can be concluded that the practical separation limits for SMART System are from 1 µg to 60 µg of plasmid DNA.

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Column:	Mono Q PC 1.6/5.
Sample:	25 µl of an alkaline extract prepared by a modified Birnboim and Doly extraction procedure (5) from a 1.5 ml culture of HB101 (pBR322).
Eluent A:	TE pH 8.0, 0.5 M NaCl.
Eluent B:	TE pH 8.0, 1.0 M NaCl.
Gradient:	50-100 % B in 15 min.
Flow rate:	200 µl/min
Fraction size:	200 µl and 50 µl.
Detection:	UV- M II

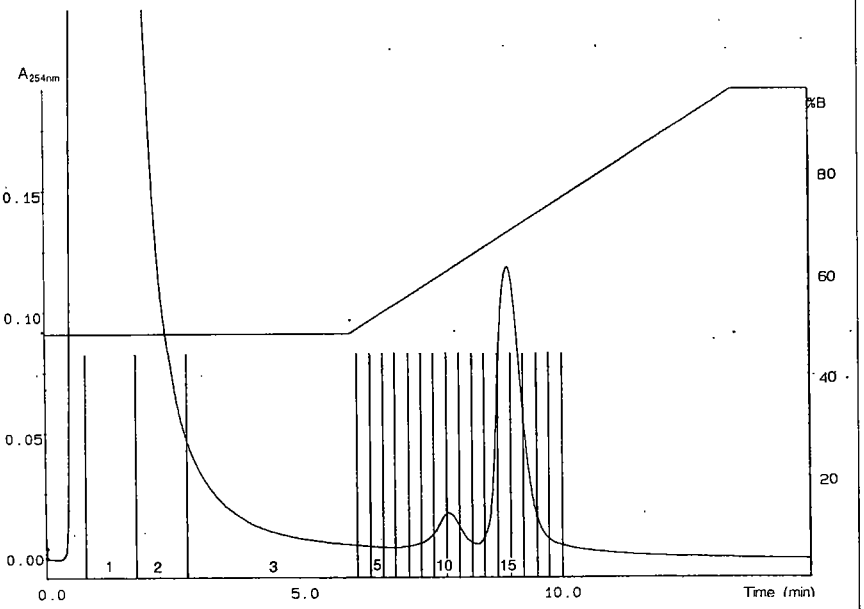


Fig. 10.17a. Plasmid purification using Mono Q® PC 1.6/5.

Lanes. 1) Crude alkaline lysate; 2) Fractions 1-2; 3) Fractions 10-12; 4) Fractions 14-18; 5) Fractions 14-18 digested with *Hinc* II. The gel was stained with ethidium bromide and nucleic acids were visualized by illumination with UV-light.

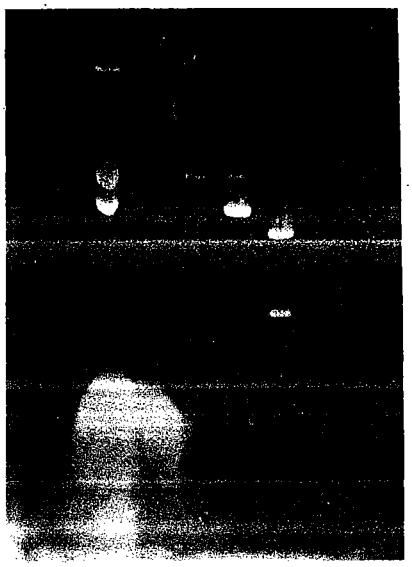


Fig. 10.17b. Electrophoretic analysis on 0.8 % agarose of fractions from 17a.

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Column: Superose 6, PC 3.2/30
Sample: 25 µl of an alkaline extract prepared from a 1.5 ml culture of HB101 (pBR322) as described in the legend to figure 10.5A.
Eluent: TE pH 8.0, 50 mM NaCl.
Flow rate: 40 µl/min during injection, 60 µl/min during elution.
Fraction size: 60 µl and 120 µl.
Detection: UV- M II

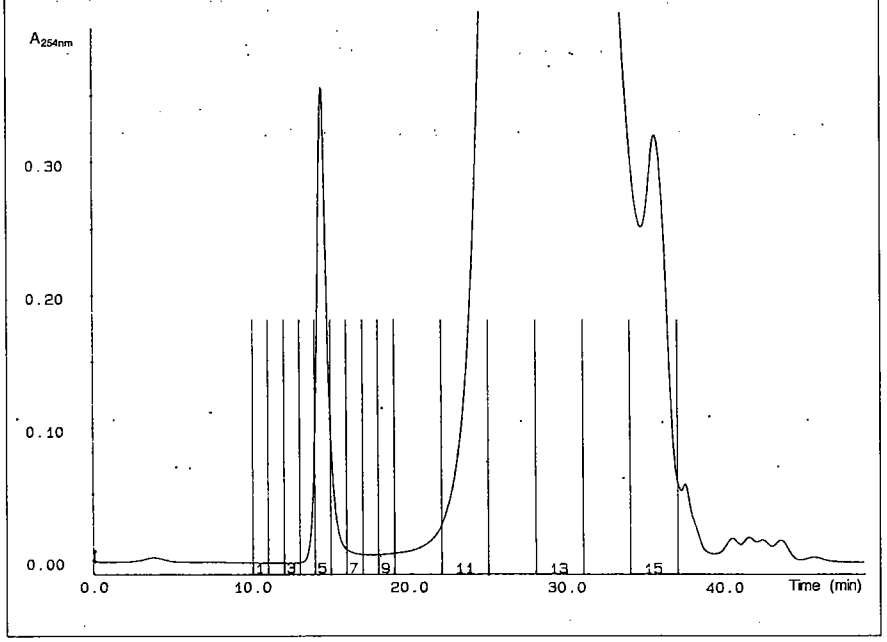


Fig. 10.18a. Plasmid purification on Superose® 6, PC 3.2/30

Lanes: 1) Crude alkaline lysate; 2) Fr 5-6; 3) Fr 5-6 digested with *Hinc* II; 4) Fr 11-15.

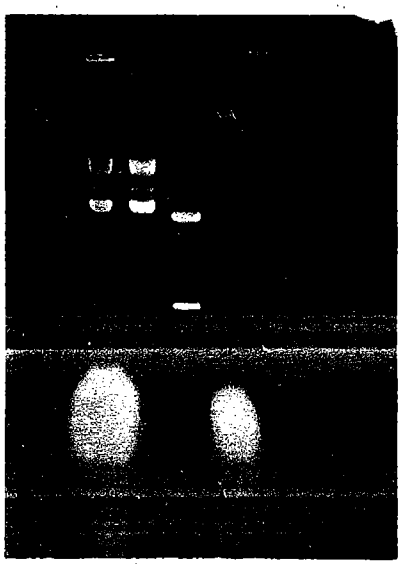


Fig. 10.18b. Electrophoretic analysis on 0.8 % agarose of fractions from 18 a.

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5. Preparation of eluents

In Chapter 2 it was stated that eluents were one of the critical factors in obtaining a good separation. The intention of this chapter is to give further guidance concerning eluents for micropurification. The following points are of particular importance.

5.1 Choice of eluent

It is important to choose an eluent in a pH range which is suitable for the chosen technique and the substance to be purified. In addition, when working at a high sensitivity setting it is necessary to check that the chosen eluent gives minimal baseline disturbances. This is particularly important when monitoring at 214 nm (as discussed in Chapter 2).

Other factors which influence the choice of eluent are the subsequent analysis or purification technique. In some cases volatile buffer systems may be preferable.

Table 3 gives suggestions on eluent compounds to choose. These are based on experience in our laboratories. Note that the quality of an eluent salt/solution differs not only between suppliers but also between batches from the same supplier.

Table 3. Buffers, salts, solvents etc. which have been used for micropurification with SMART System.

Substance	Grade	Manufacturer
Acetonitrile	LiChrosolv	Merck
Acetonitrile	HPLC	Lab-Scan Ltd
Bis-tris	p.a.	Sigma
Disodiumhydrogenphosphate	p.a.	Riedel-de-Haen
Formic acid	p.a.	Merck
HEPES		Sigma
Heptafluorobutyric acid		Sigma
Hydrochloric acid	p.a.	May and Baker Ltd
Litium chloride	p.a.	Merck
Lubrol PX		Sigma
MES		Sigma
n-propanol	p.a.	Fluka A.G.
Piperazine		Fluka A.G.
Potassiumphosphate	p.a.	Merck
Sodium chloride	p.a.	May and Baker
Sodium hydroxide	p.a.	EKA Nobel
Trifluoroacetic acid	Sequencer	Rathburn
Chemicals		
Trifluoroacetic acid	HPLC/Spectro	Pierce
Tris	p.a.	Merck
Tween 20		

Footnote to table 3. The quality of the chosen eluent salt/solution should be HPLC grade or of a corresponding quality. p.a. = pro analysi.

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5.2 Buffer preparation

The recommended concentration of a buffer is normally 20-50 mM. However, when working at higher sensitivity settings it may be advisable to lower the concentration to 5-10 mM to minimize disturbances. It is even possible (but not generally recommended) to perform ion exchange separations in the presence of trace amounts of buffer (i.e. with buffer only added for pH adjustment), in order to obtain a sample which can be directly applied to gas phase amino acid sequencing. However, a low buffer concentration increases the risk of activity loss and change of chromatographic pattern owing to pH changes.

It is generally recommended that buffers are made from the non-salt form of the buffer substance e.g. citric acid and not sodium citrate, ammonia and not ammonium chloride. This is because titration of the salt form of buffer substances results in a buffer with a higher ionic strength; this is particularly important in ion exchange techniques.

If the buffer includes salt or is used in a salt-gradient separation, the pH should be corrected with an acid or a base so that the counter-ion is the same as in the salt-gradient.

As pointed out in Chapter 2, it is possible to correct a sloping baseline obtained with Tris buffer in anion exchange chromatography by adjusting eluents A and B to different pH values. Similarly, eluents with different concentrations of TFA can be used to correct a sloping baseline in reversed phase chromatography.

Buffers should be passed through a 0.22 µm filter before use. An organic solvent resistant filter is required if the buffer contains an organic solvent. Degassing is advisable, but not always necessary. It can be done by vacuum suction, "helium bubbling" or ultrasound treatment.

Air bubble formation occurs when an organic solvent is mixed with water. To avoid the formation of bubbles during RPC separations it is recommended to use a diluted organic solvent as eluent B, for instance 60 % acetonitrile. It can be prepared without degassing by adding water on top of the organic solvent in a measuring cylinder. After a few minutes, when bubble formation has ended, the solution is slowly mixed (after mixing an ion-pairing agent is added), and no additional degassing is required.

Further information on how to prepare buffers, including recipes, are found in SMART Assistant.

5.3 Purity of the buffer

When working at high sensitivity settings it is extremely important to choose the right quality of buffer substances, salts, solvent and water to avoid unnecessary contamination. The components should be of "HPLC grade" or similar, and the water should be Milli Q* or of a corresponding quality. Always make fresh solutions to prevent bacterial growth or other contaminants. Use clean vessels and other equipment. It is recommended that everything is rinsed with clean water before use.

To check the purity of the buffer, perform a blank run and check that the baseline is stable. Preferably, the blank run should be repeated to see if it is reproducible. The blank run can be subtracted from the chromatogram in the evaluation part of SMART Manager.

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If the buffer is not pure enough one can try to purify it by adding active carbon and keeping it on a magnetic stirrer for a few hours; then filter it again. It is also possible to use a precolumn as described in Chapter 2.

* Milli Q is a registered trademark of Millipore Corp.

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6. Preparation and loading of sample

When working in the µg-ng scale it is necessary to minimize sample loss. One way is to avoid sample pretreatment, but this may raise other problems. A turbid solution (which indicates sample insolubility) should never be injected into the system. Applying dirty substances increases the risk of clogged columns or tubings. Therefore the sample may only be applied directly to the column if :

1. the volume is very small and there is a risk of sample loss due to the dead-volume of the filter. In these cases it is advisable just to centrifuge the sample before application (10 000 g for 10 min).
2. the sample is extremely diluted and there is a risk of sample loss due to adsorption to the filter.
3. the sample has previously undergone one or more chromatography steps, and is therefore estimated to be pure enough without filtration.

In all other cases the sample should be passed through a 0.22 - 0.45 µm filter. If necessary, centrifuge the sample first (10 000 x g for 10 min). Be sure to select a solvent resistant filter if the sample is dissolved in an organic solvent.

Desalting and/or buffer exchange of small sample volumes (≤ 200 µl) is ideally done with Fast Desalting, PC 3.2/10 and SMART System, where the procedure is fully controlled by monitoring the UV absorbance (sample compounds) and conductivity (salt). Further information, see section 8.3.

An alternative is NAP-5, NAP-10 or PD-10 disposable columns (Pharmacia LKB Biotechnology) where a buffer exchange of a crude sample with concomitant clarification can be rapidly performed.

6.1 Filtration

Amount of sample loss during filtration depends on:

1. Adsorption to the filter
2. The fluid retention (dead-volume) of the filter

Adsorption depends on the chemical composition of the filter, the filter area and the type of compound to be filtered. Only filters with a low binding capacity for proteins/peptides should be used. The filter should be tested for protein adsorption before a precious sample is filtered. The following procedure has been used in our laboratories:

200 µl of a standard protein (albumin or catalase), 20 µg/ml (= 4 µg), is passed through the new filter. 100 µl of the filtered sample is then run on µRPC C2/C18, PC 3.2/3 and the peak area evaluated. When three types of filter, each with an approximate area of 0.1 cm^2 were tested, sample loss due to adsorption was about one tenth of the applied sample. This demonstrates that sample amounts less than a few µg should not be filtered, whereas sample amounts larger than a few µg can be passed through a filter with an area of about 0.1 cm^2.

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The fluid retention of the filter should also be known before a small volume of a precious sample is applied. This can be determined by weighing the filter before and after filtration with water. An effective way of decreasing volume loss in the filter is to fill the syringe with air to form an air purge in front of the sample. Alternatively fill the syringe with air after injection and push that through the filter. Figure 10.19 shows how four filter types differ with respect to fluid retention, and the effect of an air purge in the syringe.

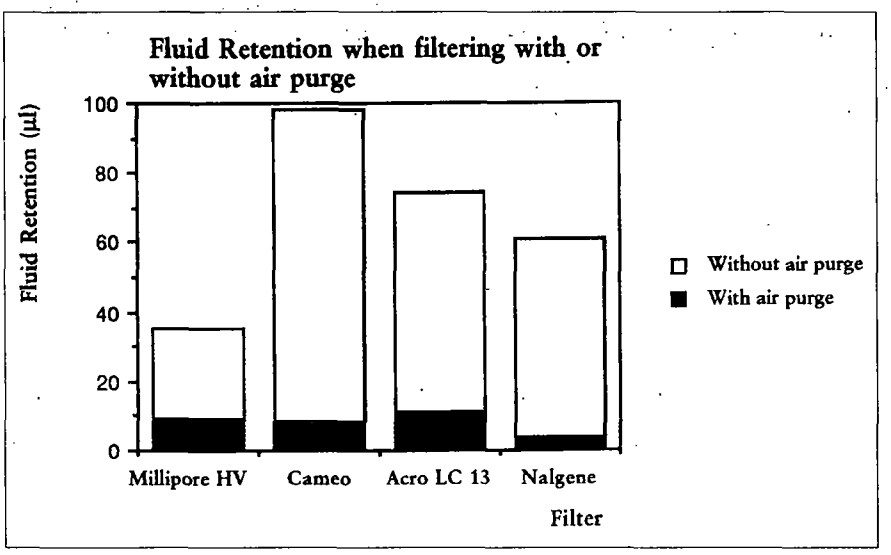


Fig. 10.19. *Comparison of four different filters with respect to fluid retention (dead volume)*
Filters
1 Millipore Millex-HV4 (0.1 cm^2).
2 MSI Cameo (0.1 cm^2).
3 Acro LC13 (1.3 cm^2).
4 Nalgene (0.07 cm^2).
All filters had 0.45 µm pore size

The effect of filling the syringe with some air before the sample is also shown. The dead volumes were determined by weighing the filters before and after filtration with water. Seven filters of each type were tested (except Acro LC 13 where five filters were tested).

Another potential risk in filtration is contamination. Leakage of UV-absorbing contaminants has been determined for different filters. 200 µl of 0.1 % TFA in Milli Q water was filtered and 100 µl of the filtered solution was run on µRPC C2/C18, PC 3.2/3. This compared with a blank gradient with a non-filtered solution run on the same column. Most of the contaminants eluted at the beginning of the gradient, but have not been identified. Figure 10.20 shows how four filter types differ in this respect. The same leakage was obtained when water (neutral pH) was passed through one of the filters. To eliminate leaking substances from the filter rinse it with ≥ 1 ml Milli Q water before use. However, this rinsing can give a dilution effect on a small sample volume.

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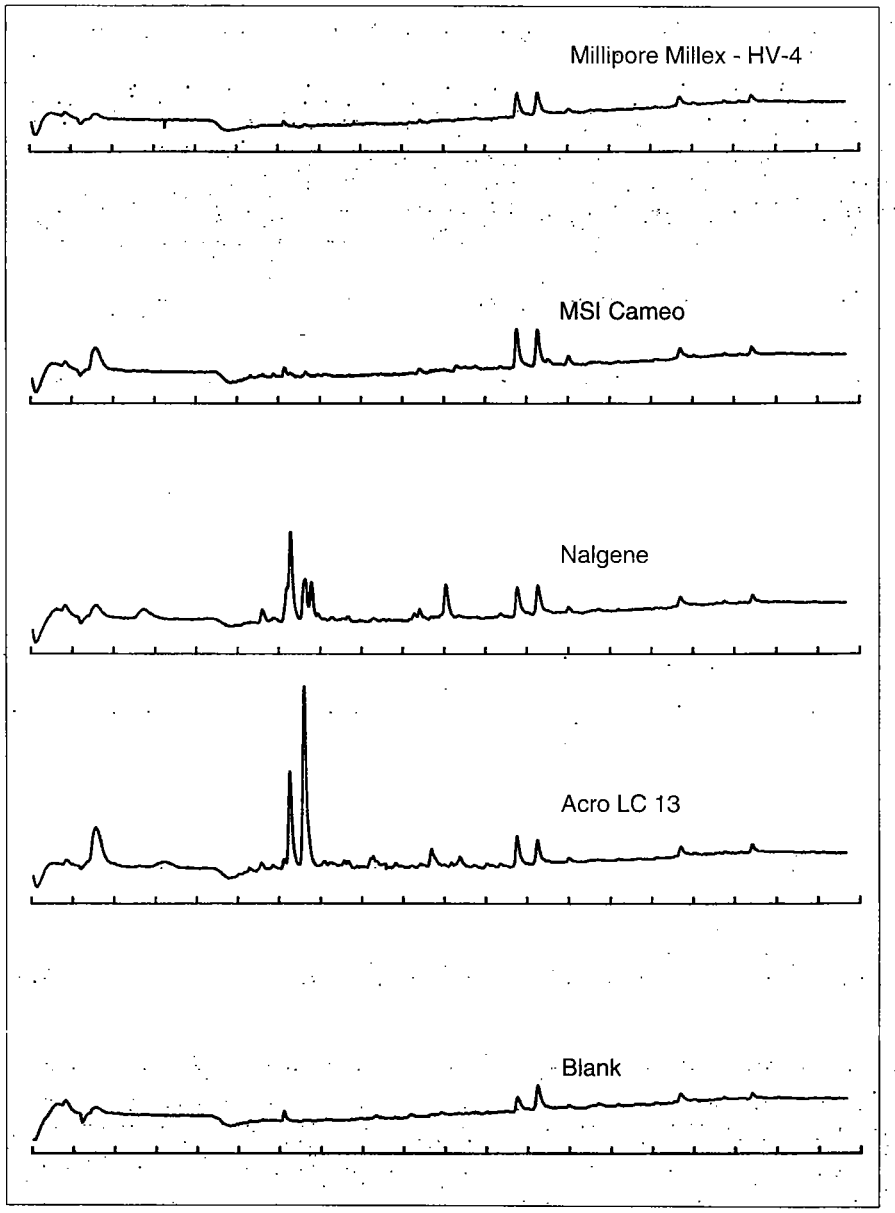


Fig. 10.20. Comparison of four different filters with respect to contamination. 100 µl of filtered 0.1 % TFA in MilliQ water was run on µRPC C2/C18,PC 3.2/3 and compared with a blank gradient with a non-filtered solution run on the same column.

6.2 Microcentrifugation

Sample volumes as small as 10-20 µl can be centrifuged to remove particulate material. Cooling minimizes evaporation. The supernatant can be recovered using the injection syringe for SMART System.

6.3 Mixing small volumes

Mixing of small sample volumes with small volumes of eluent followed by a quantitative recovery from plastic tubes is not an easy task.This problem arises when equilibrating a small volume with a suitable buffer e.g. sample pretreatment before ion exchange chromatography. In all cases where small volumes have to be mixed before chromatography, it is recommended that the mixing is performed in the syringe used for injection into the chromatographic system. The two solutions to be mixed are drawn into the syringe, followed by a small air bubble. The contents of the syringe are then mixed by turning the syringe upside-down 10-20 times.

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6.4 Sample loading

A sample is ready for injection into SMART System provided it is free of particles and the sample does not exceed the capacity of the column (see Chapter 8). The maximum volume for a one-step injection is 50 ml (for larger volumes the 50 ml Superloop has to be refilled).

To further protect the column it is recommended to use Prefilter 0.8 mm or 3.2 mm (Code No. 18-1800-75 and 18-1800-76)

It is important to consider the following:

1. Check that the sample loop and the fill port are tightly connected to avoid leakage and drainage into the injection valve.
2. "Partial filling technique" for maximal recovery: Choose a loop which has a volume larger than the sample. Partly fill the syringe with buffer, then add the sample and finally more buffer (to prevent possible sample loss in the storage tube/cup it can be rinsed with the last buffer portion). This technique can be used for all kinds of concentrating chromatography techniques where sample dilution is not critical.
3. Use glass syringes of good quality to minimize sample loss due to adsorption.
4. Use a syringe with a volume larger than the sample loop (fill it with a larger sample volume than the sample loop volume), to avoid air bubbles in the system.
5. Do not switch the valve from inject back to load position until eluent at least three times the volume of the sample loop has passed through.

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7. Trace enrichment

Large volumes of highly diluted protein and peptide solutions are commonly obtained after initial purification of biological material with Standard Chromatography. These low concentrations are often incompatible with subsequent analyses and high performance purification steps. Furthermore, many proteins quickly lose activity if they are kept at a low concentration. To enrich trace amounts of protein by conventional techniques (e.g. ultrafiltration or lyophilization) frequently results in poor recoveries (18). With SMART System, efficient trace enrichment of proteins and peptides is obtained through adsorption on microcolumns such as Mono Q PC 1.6/5 and µRPC C2/C18 PC 3.2/3. Volumes up to 50 ml (applied with Superloop™) and containing µg levels of protein have been concentrated in one step to a volume of 50 µl, i.e. a 1000 - fold concentration, with high recoveries. In addition to effective concentration, a high resolution purification is concomitantly achieved if a gradient or a series of steps are used in elution of the adsorbed material.

7.1 Important considerations for trace enrichment

7.1.1 Binding strength

The sample volume/column volume ratio is very high in trace enrichment. It is therefore essential that the molecule of interest is bound sufficiently strongly to the column, this is illustrated in figure 10.21. In quantitative terms, this means that for 50 ml of sample on a 0.1 ml column, the molecule must not be eluted by 500 column volumes of eluent A (isocratic elution). The recovery of creatine kinase (Fig. 10.21.) which elutes at low ionic strength, is minimal if applied in 50 ml. In ion exchange chromatography a stronger binding is achieved by an increase in pH (anion exchange) or a decrease in pH (cation exchange).

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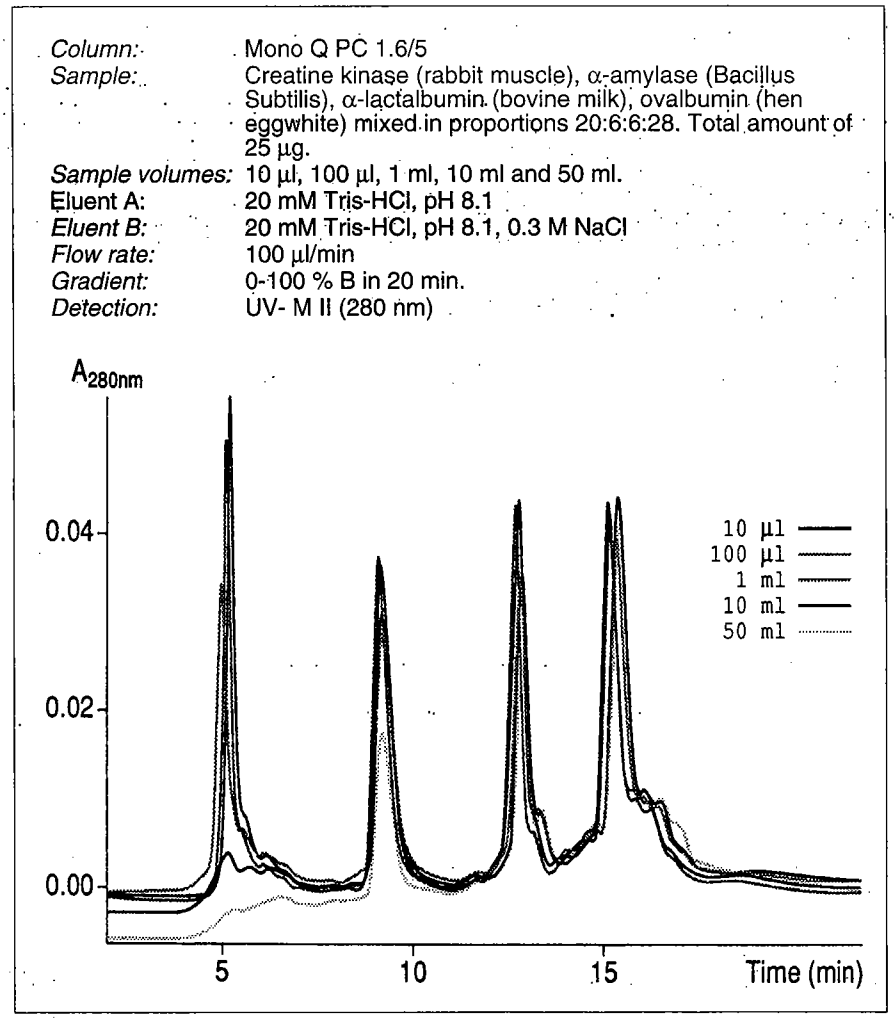


Fig. 10.21. Application of different sample volumes on Mono Q® PC 1.6/5. Gradient elution.

7.1.2 Elution mode

Elution with a gradient results in purification of the substance to be concentrated. This is shown in figure 10.21 (Mono Q PC 1.6/5) and figure 10.42 (µRPC C2/C18 PC 3.2/3). Typical peak volumes are about 100 µl. As shown in figure 10.22, elution in a single step results in even smaller peak volumes, about 50 µl on Mono Q PC 1.6/5, but there is no purification of the substance as everything is eluted at the same time.

Step-wise elution is recommended for a small peak volume and high degree of purification. If chosen, it is necessary to equilibrate the mixer with elution buffer (with the injection valve in position wash) immediately before each elution step, in order to get a sharp step. The development of a step-wise elution is checked by the conductivity monitor.

Note, because of the large sample volume, traces of contaminants in the buffer may be enriched on the column.

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Column:	Mono Q PC 1.6/5
Sample:	Creatine kinase (rabbit muscle), α-amylase (Bacillus Subtilis), α-lactalbumin (bovine milk), ovalbumin (hen eggwhite) mixed in proportions 20:6:6:28. Total amount of 25 µg.
Sample volumes:	10 µl, 100 µl, 1 ml, 10 ml and 50 ml.
Eluent A:	20 mM Tris-HCl, pH 8.1
Eluent B:	20 mM Tris-HCl, pH 8.1, 0.3 M NaCl
Flow rate:	100 µl/min
Detection:	UV- M II (280 nm)

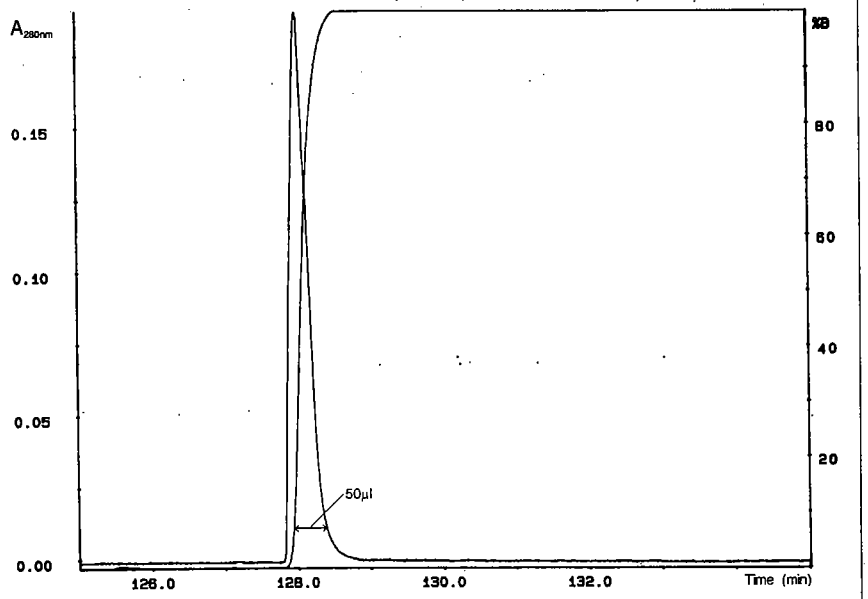


Fig. 10.22. Application of 50 ml sample on Mono Q® PC 1.6/5. Elution in one step after mixer equilibration.

Handling of the Superloop

For handling of the Superloop, see SMART System handbook, part 4.

7.2 SMART Manager method for trace enrichment

The application of 50 ml sample requires the filling of pump A five times (if the 10 ml pump cylinder is used) during the run (as for the method included in SMART Assistant) or use of both pumps, filling and pressurizing one while the other is used for the application of sample. The advantage of the second alternative is that pressure drops are avoided. This is also the type of application used in the method below. The connection to the pump valves should be the following:

Pump A	Pump B
Buffer A - valve pos. 1 Waste - valve pos. 4	Buffer B - valve pos. 1 Waste - valve pos. 4 Buffer A - valve pos. 2

Buffer A should be connected to pump B during sample application. This prevents buffer B from accidently entering the column before the elution procedure is started.

The SMART Manager method for trace enrichment is shown in figure 10.23.

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```
Main method
0.00 PUMP_WASH                  A, 1, 4
0.00 PUMP_WASH                  B, 2, 4
0.00 MIXER_WASH                 100
0.00 FLOW                       400
0.00 CONC_B                     100
0.00 INJECT                     EXTERNAL
0.00 TUBE                       A
0.00 CALL_SUB_METHOD            B_PUMP_FILL_A
0.00 CALL_SUB_METHOD            A_PUMP_FILL_B
0.00 TUBE                       B
0.00 CALL_SUB_METHOD            B_PUMP_FILL_A
0.00 CALL_SUB_METHOD            A_PUMP_FILL_B
0.00 TUBE                       C
0.00 CALL_SUB_METHOD            B_PUMP_FILL_A
0.00 CALL_SUB_METHOD            A_PUMP_WASH_B
0.00 CALL_SUB_METHOD            GRADIENT_ELUTION

SUBMETHOD: A_PUMP_FILL_B
0.00 CONC_B                     0
0.00 FILL                       B, 2, 10, 15000
8.50 CONC_B                     0
9.00 CONC_B                     100

SUBMETHOD: B_PUMP_FILL_A
0.00 CONC_B                     100
0.00 FILL                       A, 1, 10, 15000
8.50 CONC_B                     100
9.00 CONC_B                     0

SUBMETHOD: A_PUMP_WASH_B
0.00 CONC_B                     0
0.00 PUMP_WASH                  B, 1, 4
5.00 LOAD                       EXTERNAL
5.00 CONC_B                     0

SUBMETHOD: GRADIENT_ELUTION
0.00 CONC_B                     0
0.00 FILL                       A, 1, 10, 15000
0.00 MIXER_WASH                 0
0.00 NEW_CHROMATOGRAM
0.00 FLOW                       100
0.20 NEEDLE_POSITION            DOWN
0.20 GOTO_TUBE                  1
0.20 FRACTION_SIZE              50
0.20 CONC_B                     0
2.20 CONC_B                     100
2.60 CONC_B                     100
2.60 FRAC_STOP
2.60 CONC_B                     0
3.00 CONC_B                     0

SUBMETHOD: STEP_WISE_ELUTION
0.00 MIXER_WASH                 100
0.00 MEW_CHROMATOGRAM
0.00 CONC_B                     100
0.00 FLOW                       100
0.00 NEEDLE_POSITION            DOWN
0.00 GOTO_TUBE                  1
0.00 FRACTION_SIZE              25
0.50 CONC_B                     100
0.50 CONC_B                     0
1.00 CONC_B                     0
```

Fig. 10.23. SMART Manager method for trace enrichment.

Comments on figure 10.23:

The method is composed of a main part and five submethods. The programming is made in volume mode. Submethod I: (A_ pump_Fill_ B), fills pump B during delivery of eluent A for sample application. The reverse is valid for submethod II: (B_pump_Fill_A). Submethod III: (A_pump_Wash_B), changes from eluent A to eluent B in pump B while pump A is used for the application of the last part of the sample. Submethods I and II deliver 9 ml each and are used alternatively. Submethod II must be used immediately before submethod III. For example, if 42 ml of sample is applied submethods I and II are used (42/9=4.7) 4 times together. Since submethod II has to be used as the last one, submethod I has to start. Also change the volume in submethod III to 42 - (4 x 9)= 6 ml. Submethod IV is used for elution in a 2 ml gradient. Alternatively, if step-wise elution is preferred, use submethod V (Step_Wise_Elution).Flow rates should be adjusted according to the type and condition of column.

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8. Chromatography techniques using SMART® System

This chapter introduces Precision Columns and the different chromatography techniques represented using SMART System.

Precision Columns have small dimensions and good chromatographic performance, which improve separation results when working in the ng-µg range. As the cross-sectional areas of the columns are reduced, separated compounds become more concentrated and give smaller peak volumes. When combined with SMART System, the columns give high mass sensitivity. Recovery is improved by small gel bed areas and chromatographic media with minimized non-specific adsorption.

The benefits of using Precision Columns in SMART System is exemplified in figure 6 (Chapter 2), where a small amount of a protein mixture (2.5 µg) has been separated on Mono Q, PC 1.6/5 (SMART System) and Mono Q, HR 5/5 (FPLC). The scale factor of ten between Precision Columns (SMART System) and HR columns (FPLC) makes it easy to scale a separation method using any chromatographic technique represented in both systems. This is illustrated for ion exchange (Fig. 10.24) and gel filtration (Fig. 10.31).

Included in SMART Manager, the software controlling the system, is SMART Assistant. This is an information base designed to assist the user in finding the most appropriate purification scheme, provided some information about the sample to be purified is available. It can also create a proposed separation method automatically. If problems occur, they may be solved by using this information base.

Before using the columns, carefully study the recommendations in the column instructions. It is very important to follow these recommendations carefully to secure a long column life with preserved quality. The column instructions are included in the column package and should be filed in this system handbook. Further details about the different chromatography techniques can be found in different printed material from Pharmacia LKB Biotechnology AB or in chromatography textbooks (e.g. ref. 15).

8.1 Ion exchange chromatography

8.1.1 Introduction

Ion exchange chromatography is a technique which separates biomolecules according to differences in their overall surface charge and charge distribution. It is highly selective and has a high capacity. Resolution of sample components that differ by only one charge unit is attainable. The technique can be used for both purification and concentration.

In anion exchange chromatography, negatively charged groups on the surfaces of biomolecules interact electrostatically with positively charged groups covalently attached to an insoluble matrix. The reverse is true for cation exchange chromatography.

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The ion exchange columns available for SMART System are Mono Q PC 1.6/5, Mini Q PC 3.2/3, for anion exchange, and Mono S PC 1.6/5, Mini S PC 3.2/3 for cation exchange. The gel matrixes are based on 10 µm MonoBeads® and 3 µm MiniBeads® respectively. Table 4 summarizes the properties of these columns.

Table 4. Properties of Mono Q® PC 1.6/5, Mini Q® PC 3.2/3 and Mono S® PC 1.6/5, Mini S® PC 3.2/3.

Column dimensions	1.6 x 50 mm (Mono Q, Mono S) 3.2 x 30 mm (Mini Q, Mini S)
Gel volume	0.10 ml (Mono Q, Mono S) 0.24 ml (Mini Q, Mini S)
Charged group	$-CH_2N^+(CH_3)_3$ (Mono Q, Mini Q) $-CH_2SO_3^-$ (Mono S, Mini S)
Ionic capacity	0.27-0.37 mmol Cl^-/ml gel (Mono Q) 0.06-0.09 mmol Cl^-/ml gel (Mini Q) 0.14-0.18 mmol K^+/ml gel (Mono S) 0.016-0.030 mmol K^+/ml gel (Mini S)
Particle size	10 ± 0.5 µm, 3 µm
Molecular weight range	$\leq 10^7$ (Mono Q, Mono S)
Practical loading range	0.5 - 500 µg (Mono Q, Mono S) 0.5 - 200 µg (Mini Q, Mini S)
Flow rate	10 - 400 µl/min (Mono Q, Mono S) 10 - 1 000 µl/min (Mini Q, Mini S)
pH stability	2 - 12 (Mono Q, Mono S) 3 - 11 (Mini Q, Mini S)
Operational pressure limit	5 MPa (50 bar, 750 psi) (Mono Q, Mono S) 10 MPa (100 bar, 1500 psi) (Mini Q, Mini S)
Temperature range	4 - 40 °C

In figure 10.24 the Mono Q columns have been compared in order to demonstrate the scale by a factor of ten between Precision Columns (SMART System) and HR Columns (FPLC). One tenth of the sample used in the separation on Mono Q HR 5/5 was applied to Mono Q PC 1.6/5 at the same linear flow rate; the same UV-response was achieved for both runs. The chromatograms are compared on volume basis.

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Column:	Mono Q® PC 1.6/5 (top) Mono Q® HR 5/5 (bottom)
Sample:	25 µg protein (top) 250 µg protein (bottom)
Eluent A:	20 mM Tris, pH 8.0
Eluent B:	20 mM Tris, pH 8.0, 0.3 M NaCl
Gradient:	0-100 % B in 20 min
Flow rate:	100 µl/min (top) 1000 µl/min (bottom)
Detection:	UV- M II

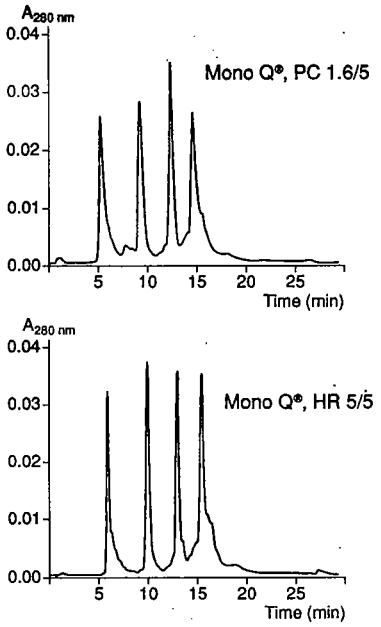


Fig. 10.24. Comparison of Mono Q® HR 5/5 (FPLC®) and Mono Q® PC 1.6/5 (SMART® System)

8.1.2 Optimizing the separation

Varying the sample volume

The influence of the sample volume on the separation has been evaluated. The same amount (mass) of four different proteins was applied on Mono Q PC 1.6/5 in different sample volumes (varied between 10 µl and 50 ml). Figure 10.21 (section 7.1) shows that the peak volume is independent of the sample volume. It demonstrates that ion exchange chromatography is very effective for concentration of highly diluted samples.

When the sample volume is large it is very important that the binding between the sample and the gel matrix is strong enough, otherwise the sample will be lost during sample application.

Varying the sample load

In figure 10.25 different amounts of proteins (varied between 250 ng and 1.25 mg) was applied to Mono Q PC 1.6/5 at a constant volume. The results indicate that the resolution is not affected when the sample amount is increased up to 25 µg. At the 250 µg level the chromatographic pattern is changed for this protein mixture. When 1.25 mg sample is applied to the column the maximum binding

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capacity for this protein mixture is reached. Depending on the amount of sample bound to the column and the binding strength of the compounds these figures differ. The practical loading range (total sample mass) of 0.5 -500 µg presented in table 4 is therefore an approximate recommendation.

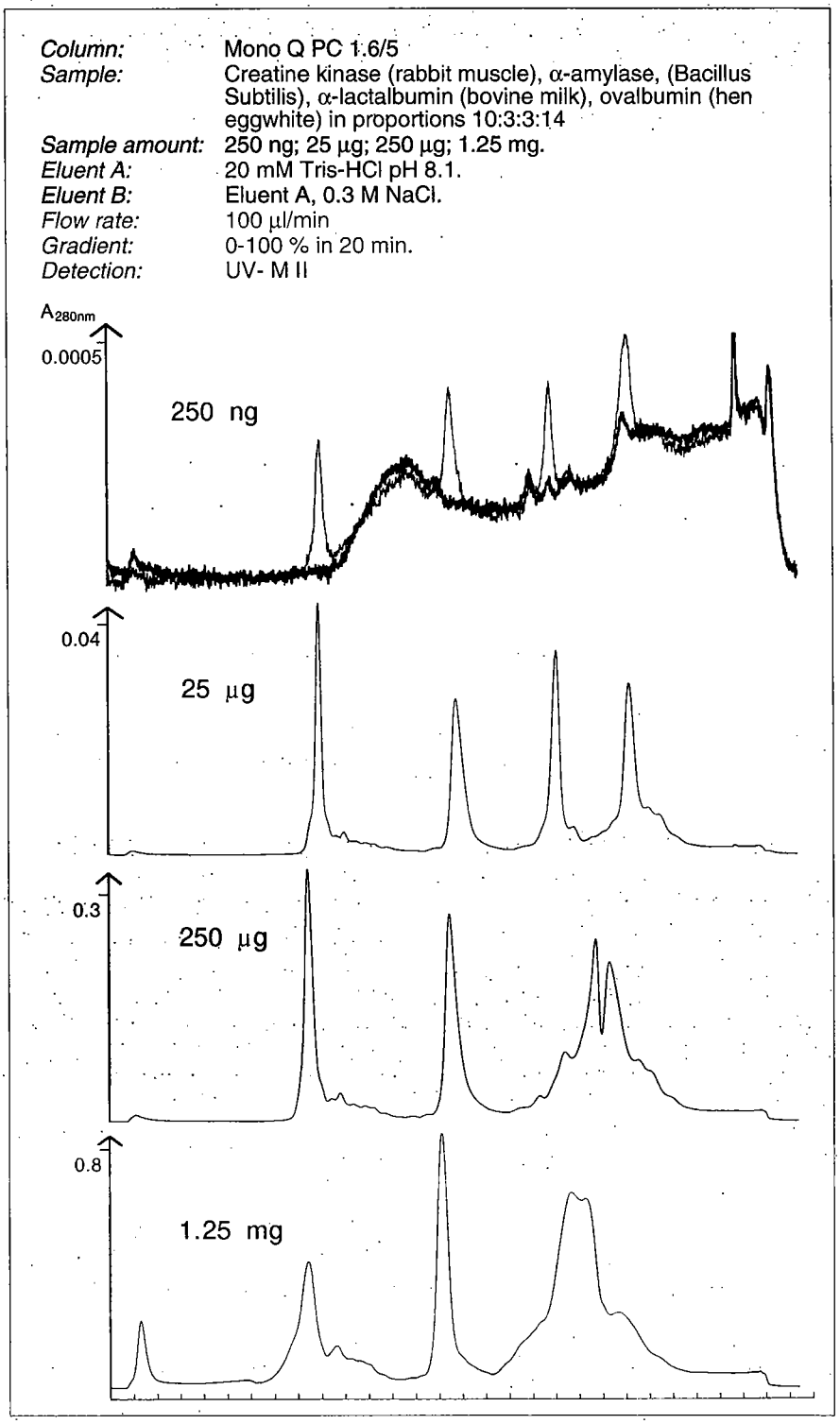


Fig. 10.25. Varying the sample load on Mono Q® PC 1.6/5.
To make the protein peak positions clearer a blank run has been performed at the lowest amount of applied sample (250 ng). The maximum capacity for this protein mixture is reached when 1.25 mg is applied (in this case a small amount of sample is found in the flow-through peak).

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Varying the running conditions

Modification of the chemical conditions (e.g. eluent composition) should be attempted first when optimizing a separation. Further information about this can be found in FPLC Ion Exchange and Chromatofocusing (8).

Another important parameter when optimizing a separation is the gradient shape. It can be modified for resolution and sensitivity by changing flow rate and gradient time (when programming in time mode) or flow rate and gradient volume (when programming in volume mode).

To optimize resolution it is most important to increase the gradient time. Figure 10.26 shows the increase in resolution by changing the gradient time from 10 to 40 minutes. This can be done either by keeping the flow rate constant (increase the gradient volume from 1 to 4 ml) or by keeping the gradient volume constant (decrease the flow rate from 100 to 25 µl/min).

The sensitivity is increased primarily by reducing the gradient volume. This is done most effectively by reducing the flow rate (see figures 7 and 8, Chapter 2).

In table 5 this discussion is summarized in terms of how to change the program to optimize the separation according to sensitivity and resolution. Both types of programming modes (time- or volume-based) are illustrated. The optimal separation method will be a compromise between resolution and sensitivity and will differ from case to case.

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Column:	Mono Q® PC 1.6/5
Sample:	830 ng creatine kinase (rabbit muscle), 250 ng α-amylase (Bacillus Subtilis), 250 ng α-lactalbumin (bovine milk), 1.17 µg ovalbumin (hen eggwhite).
Eluent A:	20 mM Tris-HCl, pH 8.1
Eluent B:	Eluent A, 0.3 M NaCl
Upper chromatogram:	Reference
Flow rate:	100 µl/min.
Gradient:	0 - 100 % B in 10 min.
Middle chromatogram:	Constant gradient volume
Flow rate:	25 µl/min.
Gradient:	0 - 100 % B in 40 min.
Lower chromatogram:	Constant flow rate
Flow rate:	100 µl/min.
Gradient:	0 - 100 % B in 40 min.
Detection:	UV- M II

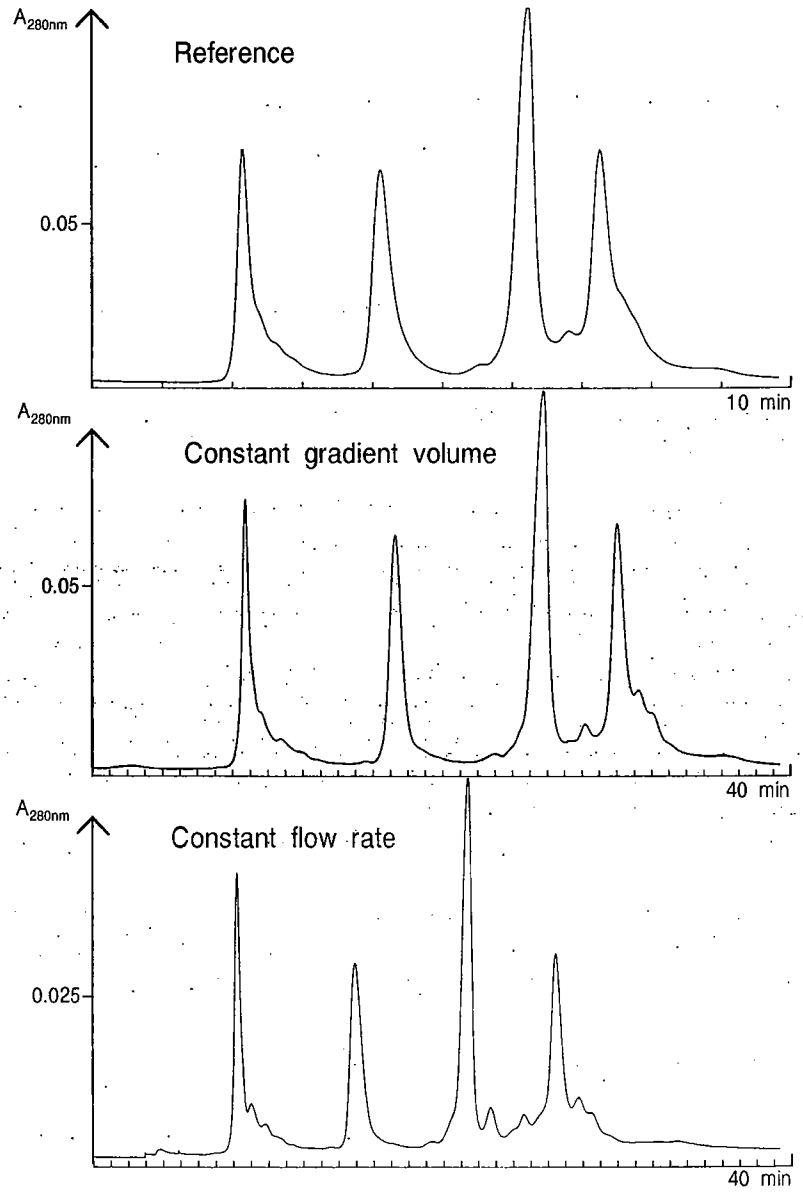


Fig. 10.26. Improving resolution on Mono Q® PC 1.6/5 by increasing the gradient time.

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Table 5. A summary of how to change the program to optimize the separation.

		Time base		Volume base	
		Gradient time	Flow rate	Gradient volume	Flow rate
High resolution	1.	+	-	0	-
	2.	+	0	+	0
High sensitivity	1.	0	-	-	-
	2.	-	0	-	0

The left side of the table illustrates the changes if time mode is used while the right side of the table illustrates the changes for volume mode. 0 = no change, - = decrease, while + = increase.

8.1.3 Detection limit

The detection limit using Mono Q PC 1.6/5 is usually in the low ng range. Figures 10.27 and 10.28 demonstrate the detection of 5 ng of α-amylase at 280 nm and 900 pg of an oligonucleotide at 254 nm.

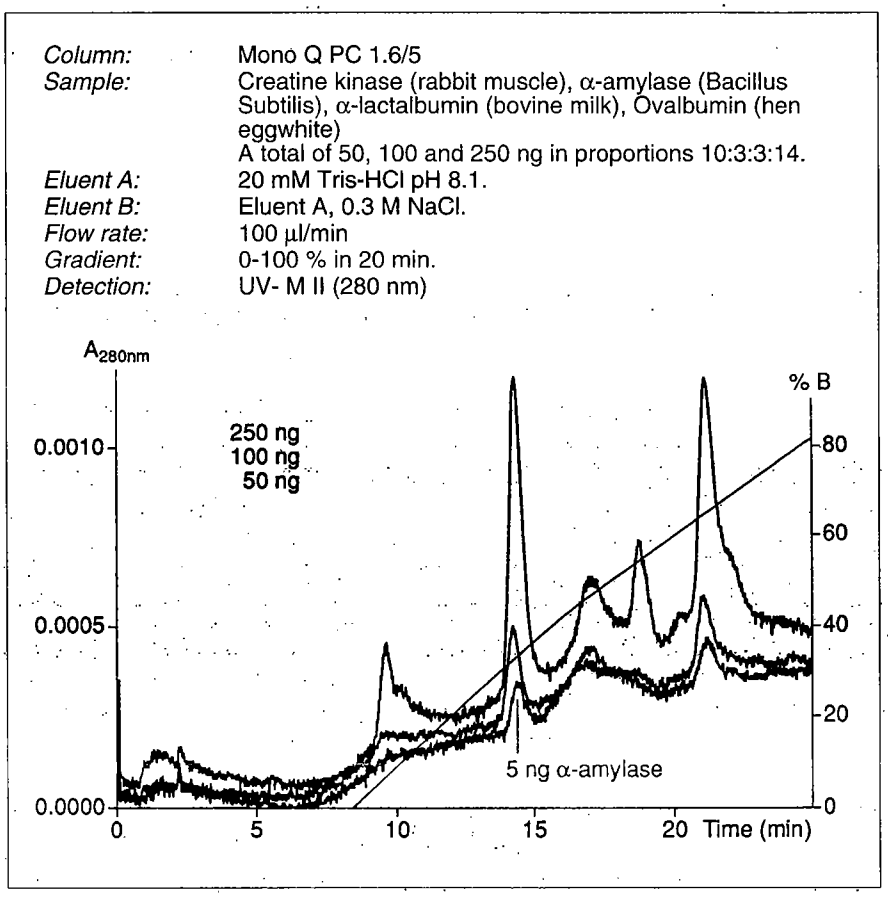


Fig. 10.27. Detection of 5 ng α-amylase on Mono Q® PC 1.6/5. Mono Q® HR 5/5 was connected in series with pump A for removal of impurities in the buffer.

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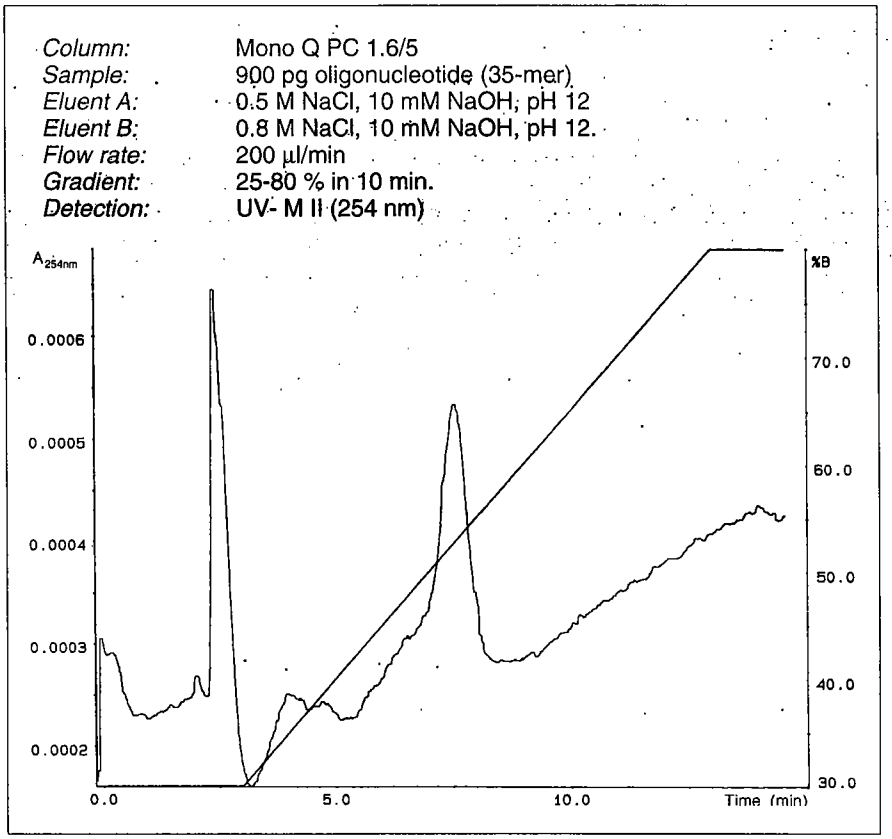


Fig. 10.28. Detection of 900 pg of an oligonucleotide on Mono Q® PC 1.6/5.

8.1.4 Recovery

Figure 10.29 shows a recovery study on Mono Q PC 1.6/5. Details about the method is described in section 3.1 (Recovery in SMART System).

First injection:

Four different amounts of α-amylase (0.1; 1; 10; and 100 µg) were loaded on the column and eluted in a gradient. The peak area was determined and compared to the area for the 100 µg injection, which was considered to be 100 % recovery.

Reinjection:

Fractions were collected from the previous runs, and diluted with eluent A. The dilution is done in order to decrease the ionic strength. The diluted fractions were loaded on the column.The UV peak area for the reinjection was compared to the UV peak area for the first injection.

The results from the first injection show that high mass recoveries are obtained, 80-100 % even at the lowest loading tested (100 ng).

The recovery from the reinjection is lower (60-80 %). This is probably due to losses in the injection syringe, fraction tubes, and sample treatment.

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Column:	Mono Q PC 1.6/5
Sample:	0.1, 1, 10, and 100 µg of α-amylase (Bacillus Subtilis)
Eluent A:	20 mM Tris-HCl pH 8.1.
Eluent B:	Eluent A, 0.3 M NaCl.
Flow rate:	100 µl/min
Gradient:	0-100 % in 10 min
Detection:	UV- M II (280 nm)
Upper curve:	Recovery calculated by comparing the UV peak area for the different loadings with the UV area for 100 µg.
Lower curve:	Recovery for a reinjected sample calculated by comparing the UV peak area with the UV area first achieved.

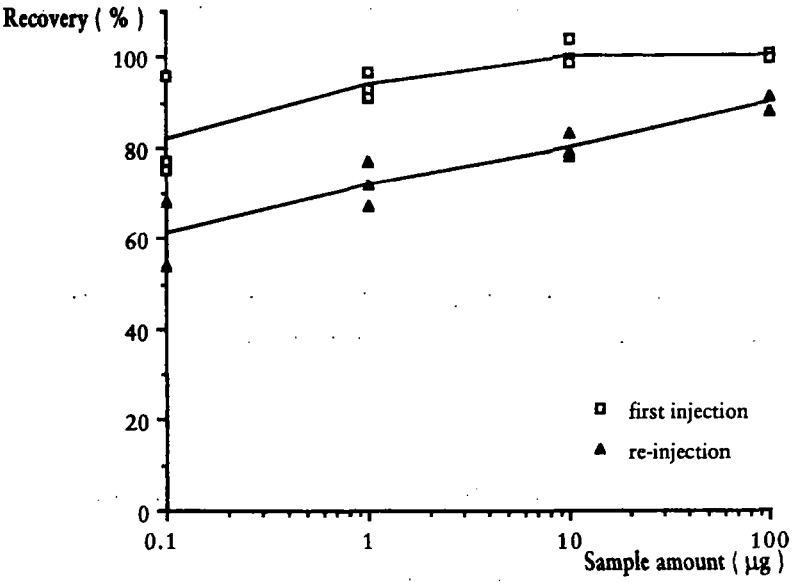


Fig. 10.29. Recovery of α-amylase on Mono Q® PC 1.6/5.

8.2 Gel filtration

8.2.1 Introduction

Gel filtration, or size exclusion chromatography, separates the molecules on the basis of size or more correctly hydrodynamic radius, which influences the ability of the molecules to enter the stationary phase. Movement into the stationary phase is governed by the internal porosity of the gel matrix, which controls the selectivity.

Available gel filtration media packed in Precision Columns, PC 3.2/30 are Superose 6, Superose 12, Superdex 75 and Superdex 200. (Also available is Fast Desalting, PC 3.2/10 which is presented separately in section 8.3.) The selectivity of these three different gel filtration media is demonstrated in the Kav plots in figure 10.30. Superdex 75 gives an unsurpassed selectivity in the molecular weight range 3 000 - 70 000, while Superose 12 has a broader separation range of 1 000 - 300 000. Superose 6 is better suited to the separation of large biomolecules, such as nucleic acids, large proteins or aggregates up to molecular weights corresponding to 5 x 10^6. (All the molecular weight ranges refer to globular proteins.)

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Eluent:50 mM Na-phosphate, 0.15 M NaCl

Sample for Superdex 75:

Protein	Mw
Blue Dextran	2 000 000
Thyroglobulin	669 000
Ferritin	440 000
Catalase	232 000
Immunoglobulin	160 000
Aldolas	158 000
Albumin	67 000
Ovalbumin	43 000
B-lactoglobulin	35 000
Chymotrypsinogen A	25 000
Ribonuclease A	13 700
Cytochrome C	12 400
Aprotinin	6 500
Vitamin B_{12}	1 355
Cytidin	243

Sample for Superose 12:

Protein	Mw
Blue Dextran	2 000 000
Tyroglobulin	669 000
Ferritin	440 000
Catalase	232 000
Immunoglobulin G	160 000
Aldolas	158 000
Albumin	67 000
Ovalbumin	43 000
B-lactoglobulin	35 000
Chymotrypsinogen A	25 000
Lysozym	23 200
Ribonuclease A	13 700
Cytochrome C	12 400
Vitamin B_{12}	1355
Cytidin	243

Sample for Superose 6:

Protein	Mw
Thyroglobulin (dimer)	1 338 000
Thyroglobulin (monomer)	669 000
Ferritin	440 000
Immunoglobulin G	160 000
Bovine serum albumin	67 000
Chymotrypsinogen A	25 000
Ribonuclease A	13 700
Aprotinin	6 500

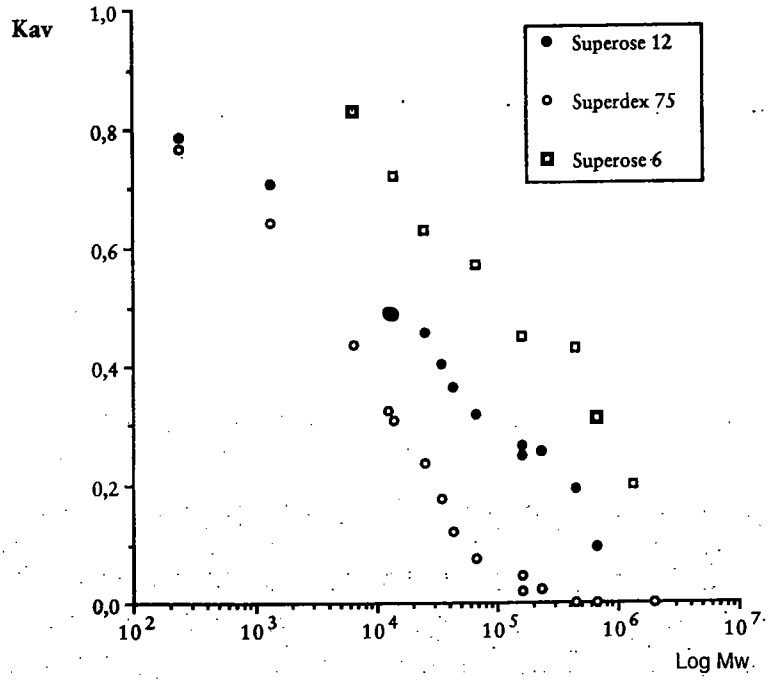


Fig. 10.30. Kav plots for Superose® 6, Superose® 12 and Superdex™ 75.

Superose 6 (13 µm) and Superose 12 (10 µm) are highly cross-linked (6 % and 12 % , respectively) agarose based media, while Superdex 75 (13 µm) and Superdex 200 (13 µm) are a composite of dextran and 12 % cross-linked agarose. Table 6 summarizes the properties of these columns.

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Table 6. Properties of Superdex™ 75, PC 3.2/30, Superdex™ 200, PC 3.2/30, Superose® 12, PC 3.2/30 and Superose® 6, PC 3.2/30.

Column dimensions	3.2 x 300 mm
Gel volume	2.4 ml
Base matrix	6 % cross-linked agarose (Superose 6) 12 % cross-linked agarose (Superose 12) dextran and 12 % cross-linked agarose (Superdex 75) dextran and 6 % cross-linked agarose (Superdex 200)
Particle size	≈ 10 µm (Superose 12) ≈ 13 µm (Superdex 75, Superose 6, Superdex 200)
Theoretical plates (N/m)	≥ 40 000/m (Superose 12) ≥ 30 000/m (Superdex 75, Superose 6, Superdex 200)
Optimal separation range (Mw for globular proteins)	5 000 - 5 x 10^6 (Superose 6) 1 000 - 300 000 (Superose 12) 3 000 - 70 000 (Superdex 75) 10 000 - 600 000 (Superdex 200)
Exclusion limit	4 x 10^7 (Superose 6) 2 x 10^6 (Superose 12) 1 x 10^5 (Superdex 75) 1.3 x 10^6 (Superdex 200)
Practical loading range	0.5 - 500 µg
Flow rate	10 - 100 µl/min
Sample volume	≤ 50 µl
Recovery	≥ 80 %
pH stability	1-14
Operational pressure limit	1.2 MPa (12 bar,180 psi), (Superose 6) 2.4 MPa (24 bar,360 psi), (Superose 12, Superdex 75) 1.5 MPa (15 bar, 217 psi), (Superdex 200)
Temperature range	4 - 40 °C

In figure 10.31 Superdex™ 75 PC 3.2/30 (SMART System) and Superdex™ 75 HR 10/30 (FPLC) have been compared in order to demonstrate the advantage with a narrow column for small sample amounts (in this case a total of 33.8 µg). Also see figure 6, Chapter 2 where the same comparison has been done for Mono Q®.

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Columns :	1. Superdex 75 PC 3.2/30 (left curve) 2. Superdex 75 HR 10/30 (right curve)	
Eluent:	50 mM Na-phosphate, 0.15 M NaCl, pH 7.0	
Sample:	1 IgG	6.1 µg
	2 Ovalbumin	6.4 µg
	3 Chymotrypsinogen A	2.9 µg
	4 Ribonuclease A	8.9 µg
	5 Aprotinin	8.4 µg
	6 Vitamin B_{12}	0.7 µg
	7 Cytidin	0.4 µg
	Total amount of sample	33.8 µg
Sample volume:	10 µl for PC 3.2/30 100 µl for HR 10/30	
Flow rate	40 µl/min for PC 3.2/30 400 µl/min for HR 10/30	
Detection:	UV- M II (280 nm)	

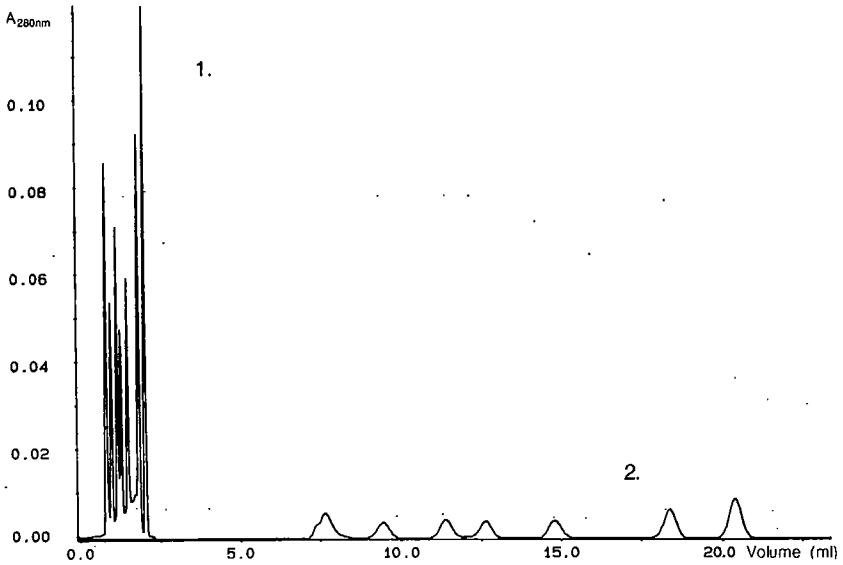


Fig. 10.31. Comparison between Superdex™ 75 HR 10/30 and Superdex™ 75 PC 3.2/30.

8.2.2 Optimizing the separation

Varying the sample volume

Figure 10.32 shows the influence of the sample volume on the separation. A smaller sample volume gives a smaller peak volume but the difference between 0.75 µl and 25 µl is not dramatic. The resolution for the 100 µl injection is poor but may be sufficient for some separations. For larger sample volumes it is preferable to use another chromatographic technique to concentrate the sample (see Chapter 7) before the gel filtration step.

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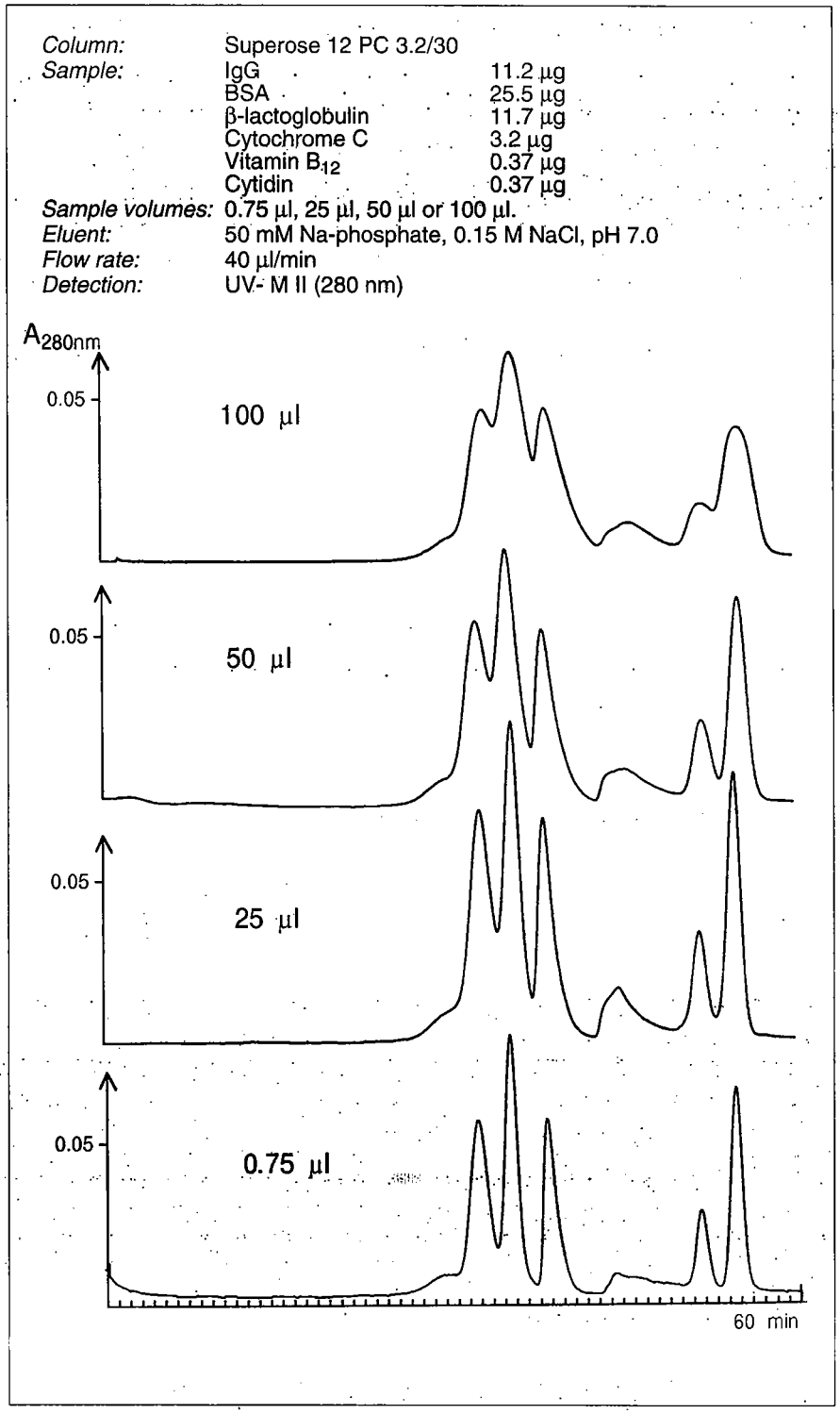


Fig. 10.32. Varying the sample volume on Superose® 12, PC 3.2/30.

Varying the sample load

In figure 10.33 it is demonstrated how the separation is affected by the sample mass applied. The results show that ≥ 1 mg total mass give broader peaks and thus lower resolution.

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The upper limit for how much sample can be loaded onto the column is often limited by the viscosity or solubility of the sample as large sample volumes can not be applied on these columns.

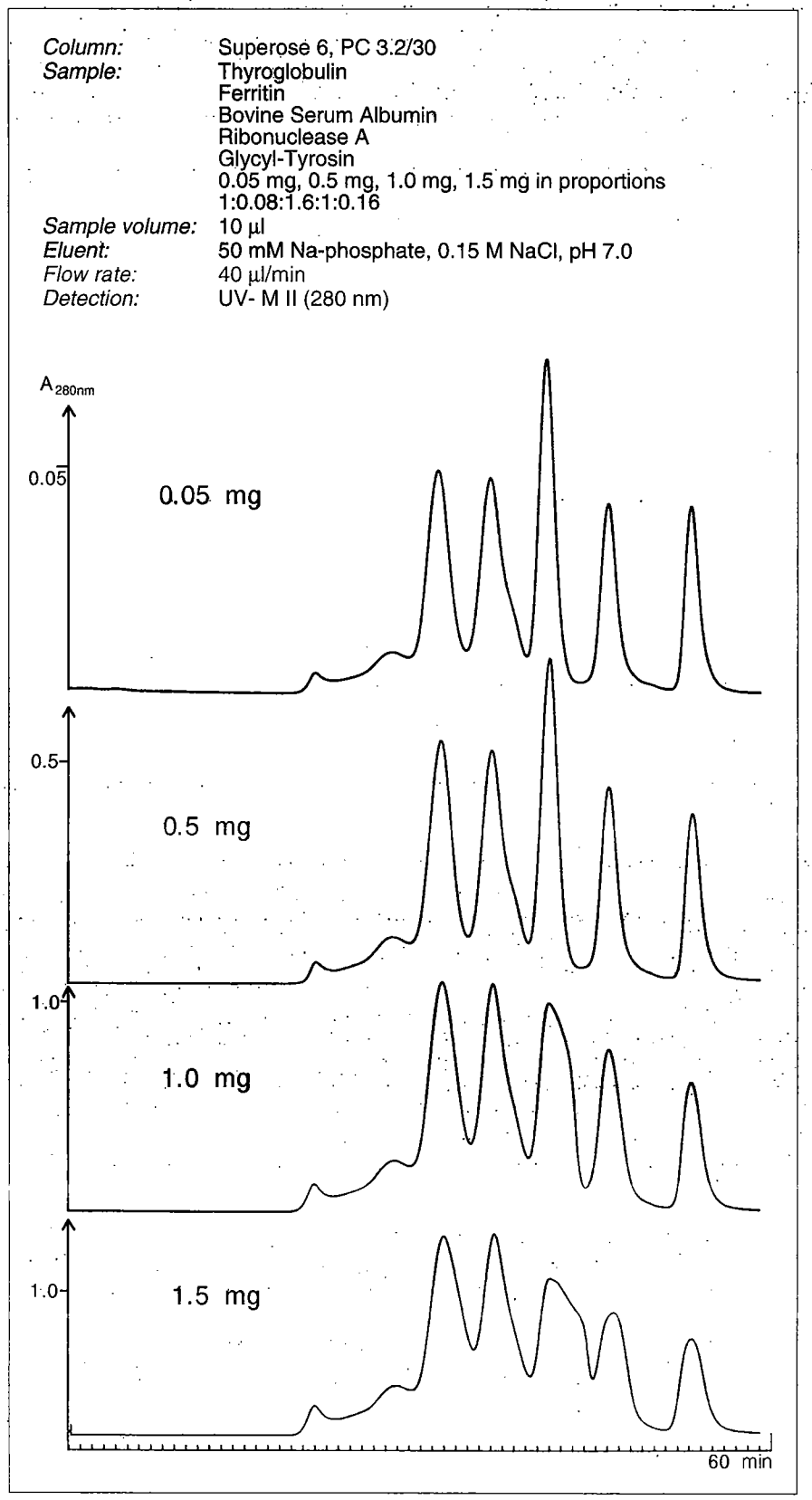


Fig. 10.33. Varying the sample load on Superose® 6, PC 3.2/30.

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Varying the flow rate

By lowering the flow rate smaller peak volumes are achieved for slowly diffusing proteins, but the separation time is increased. There is always a choice between getting excellent resolution with sharp peaks or to have short separation times. Figure 10.34 shows how the flow rate affects the separation.

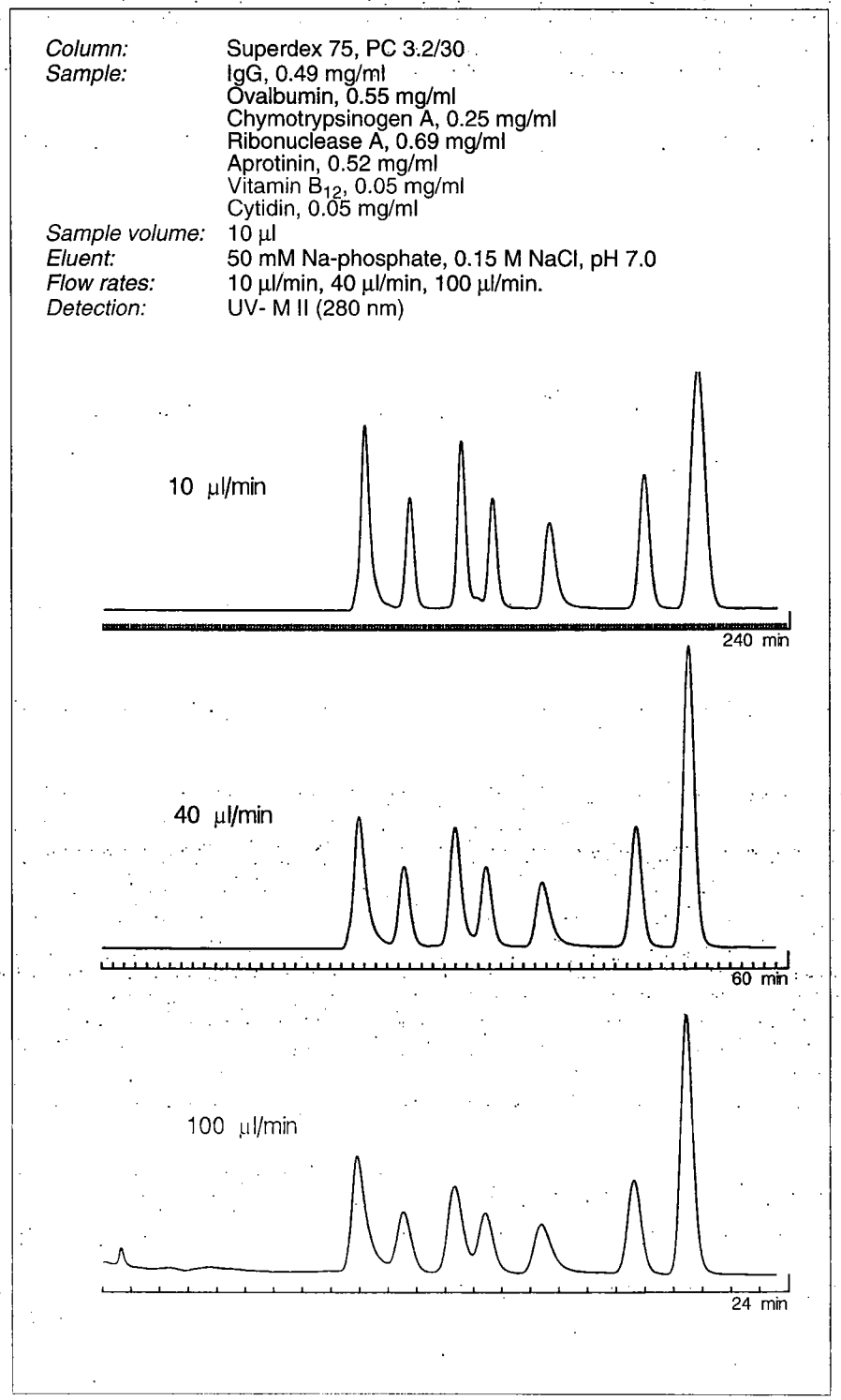


Fig. 10.34. Varying the flow rate on Superdex™ 75, PC 3.2/30.

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8.2.3 Detection limit

The detection limit for proteins on Superose 6 PC 3.2/30, Superose 12 PC 3.2/30 and Superdex 75 PC 3.2/30 is about 10 ng at 280 nm. At 214 nm the limit is about ten times lower. The detection of 1 ng ovalbumin on Superdex 75 PC 3.2/30 at 214 nm is shown in figure 10.35.

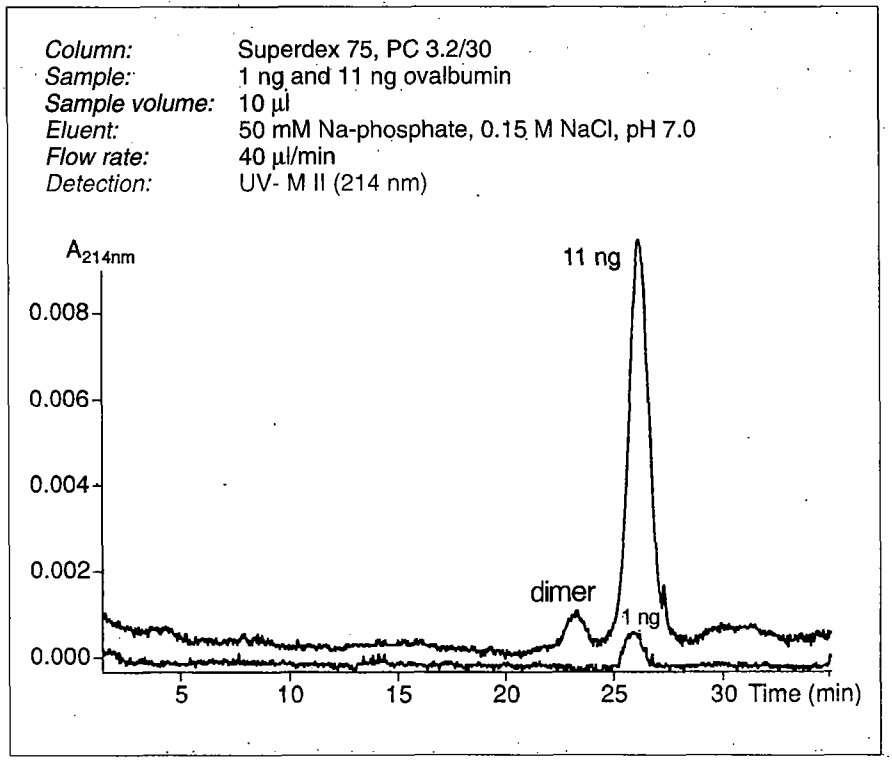


Fig. 10.35. Detection of 1 ng ovalbumin on Superdex™ 75, PC 3.2/30.

8.2.4 Recovery

Figure 10.36 shows a recovery study on Superdex 75, PC 3.2/30. Details about the method used are described in section 3.1 (Recovery in SMART System).

Four different amounts of ovalbumin were loaded on the column and eluted in a gradient. The peak areas were determined and compared to the area for the 11 µg injection, which was considered to be 100 % recovery (determined in a separate experiment).

Excellent mass recoveries have also been obtained with the Superose 6, PC 3.2/30 and Superose 12, PC 3.2/30, even in the nanogram range. The recovery for 250 ng ovalbumin, using Superdex 75, PC 3.2/30 is about 80 % , see figure 10.36. Note that the volume of the gel filtration columns (2.4 ml) is rather big compared to the other Precision Columns. To increase the recovery some salt (e.g. ≥ 0.15 M NaCl) or detergent (e.g. 0.02 % Tween 20) can be added to avoid aggregation or non-specific adsorption due to ionic or hydrophobic interactions respectively.

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Column: Superdex 75, PC 3.2/30.
Sample: 0.25, 0.5, 1.1, or 11 µg ovalbumin.
Sample volume: 10 µl
Eluent: 50 mM Na-phosphate, 0.15 M NaCl, pH 7.0
Flow rate: 40 µl/min
Detection: UV- M II (280 nm)

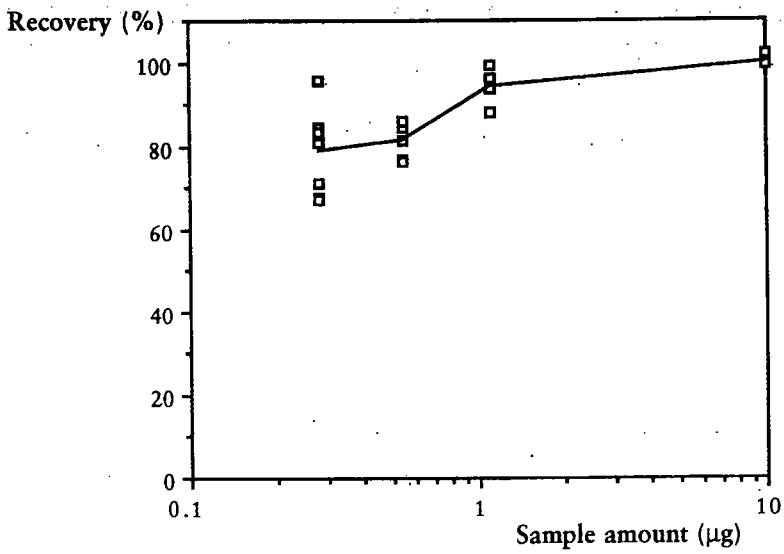


Fig. 10.36. Recovery of ovalbumin on Superdex™ 75, PC 3.2/30.

8.3 Fast Desalting

8.3.1 Introduction

Micropreparative sample handling frequently requires desalting or buffer exchange of microgram quantities of proteins in small volumes. SMART System offers a secure and rapid alternative to microdialysis. Small amounts can be detected as the dilution is relatively low.

Fast Desalting, PC 3.2/10, packed with Sephadex G-25 Superfine, a highly selective cross-linked dextran, is excellent for group separations of low Mw molecules from high Mw molecules. It has an exclusion limit of 5000 (molecules above 5000 generally elute at the void volume).

SMART system gives full control over what is occuring in a group separation. Elution of proteins and UV absorbing small molecules are traced by the UV monitor, while salts are monitored by the conductivity detector. A desalting procedure is normally completed within 2-3 minutes. The sample volume should be ≤ 200 µl. An additional advantage to alternative desalting techniques is automatic fraction collection.

The column can be used in a number of situations:

1. Desalting.
2. Exchange of small molecules and/or ions, for example, buffer exchange.
3. Removal of small molecules other than salt, e.g. removal of excess reagent after labelling a macromolecule in order to terminate a reaction.

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Table 7 summarizes the properties of Fast Desalting, PC 3.2/10.

Table 7. Properties of Fast Desalting, PC 3.2/10.

Column dimensions	3:2 x 100 mm
Gel volume	0.80 ml
Base matrix	dextran, Sephadex G-25, Superfine
Particle size (wet beads)	15 - 70 µm
Exclusion limit (MW)	5 000
Flow rate	10 - 900 µl/min
Sample volume	≤ 200 µl
pH stability	2 - 13
Operational pressure limit	1.2 MPa (12 bar, 180 psi)
Temperature range	4 - 40 °C

8.3.2 Optimizing the separation

Varying the sample volume

In figure 10.37 five different volumes were applied to the column. Bovine serum albumin in phosphate buffer with 0.5 M NaCl was desalted against an ammonium bicarbonate buffer. A "complete" desalting is achieved for volumes up to 100 µl. For 200 µl, 98 % of the protein peak still contains 0.4 % of the salt, however this is usually sufficient in most cases.

The relative dilution increases with decreasing sample volume. Table 8 shows the dilution factor for 98 % of the peak area (flow rate 600 µl/min).

Table 8. Relative dilution of different applied sample volumes. The dilution factor is determined from the sample volume related to the eluted volume (98 % of the peak area).

Sample volume	Eluted volume (98% area) (µl)	Relative dilution (µl)
200	356	1.8
100	183	1.8
50	145	2.9
20	128	6.4
10	121	12
5	99	20

Comment to table 8. The dilution is considerable using small sample volumes. The possibility to use volatile solvents for example 0.1 % TFA or as in this case, an ammonium bicarbonate buffer, facilitates volume reduction if necessary.

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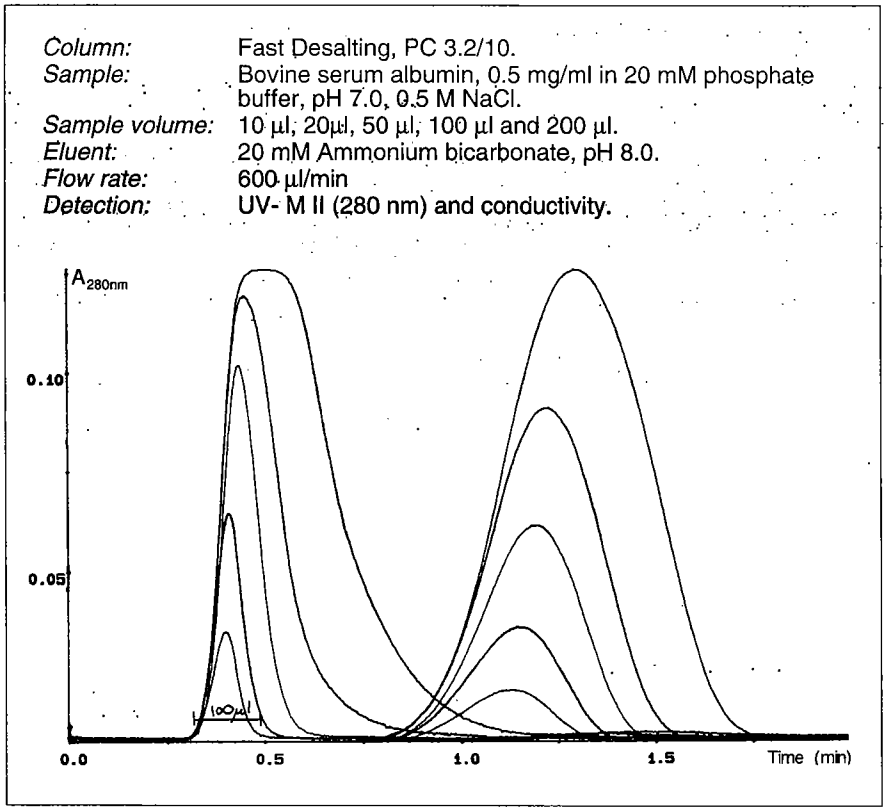


Fig. 10.37. Varying the sample volume on Fast Desalting, PC 3.2/10.

Varying the sample load

In figure 10.38 protein concentrations between 0.01 and 20 mg/ml have been used together with the 50 µl loop, i.e. amounts between 250 ng and 1 mg. The fractionation pattern is independent of the amount applied. Note that 250 ng at 280 nm are easily detected.

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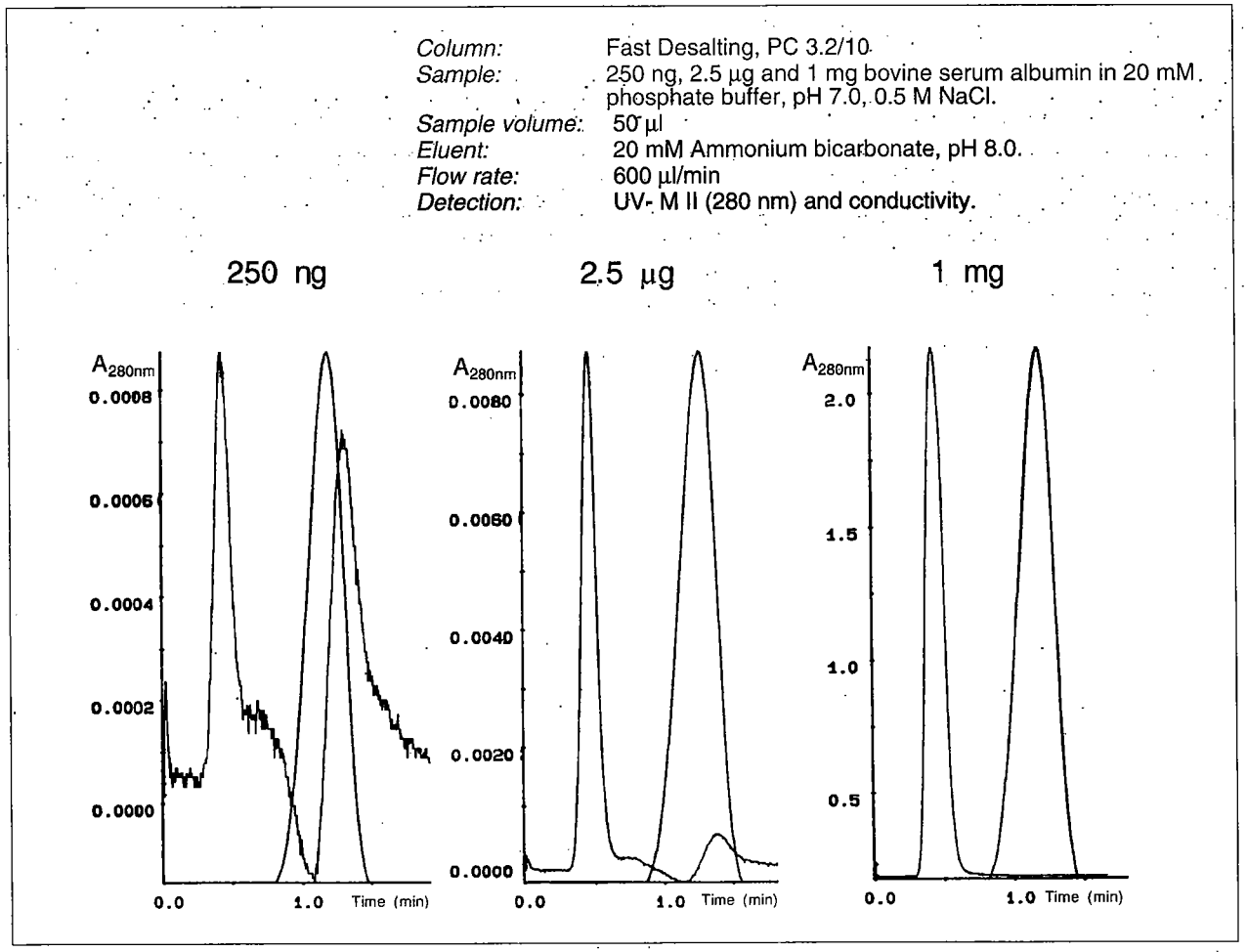


Fig. 10.38. Varying the sample load on Fast Desalting, PC 3.2/10.

Varying the flow rate

Flow rates between 50 and 900 µl/min have been tested and the result is shown in figure 10.39, which shows that the resolution is increased when the flow rate is lowered. The volume of the protein peak is independent of the flow rate which is not the case with the salt peak. This means that by reducing the flow rate the resolution between the protein peak and the salt peak is increased. (In this case a reduction from 600 to 50 µl/min increases the distance between the protein and salt by 70 µl.) However, the sample (volume 50 µl) is fully separated from the salt at 600 µl/min, so a reduced flow rate is not meaningful. For a larger sample volume, it is worthwhile to reduce the flow rate.

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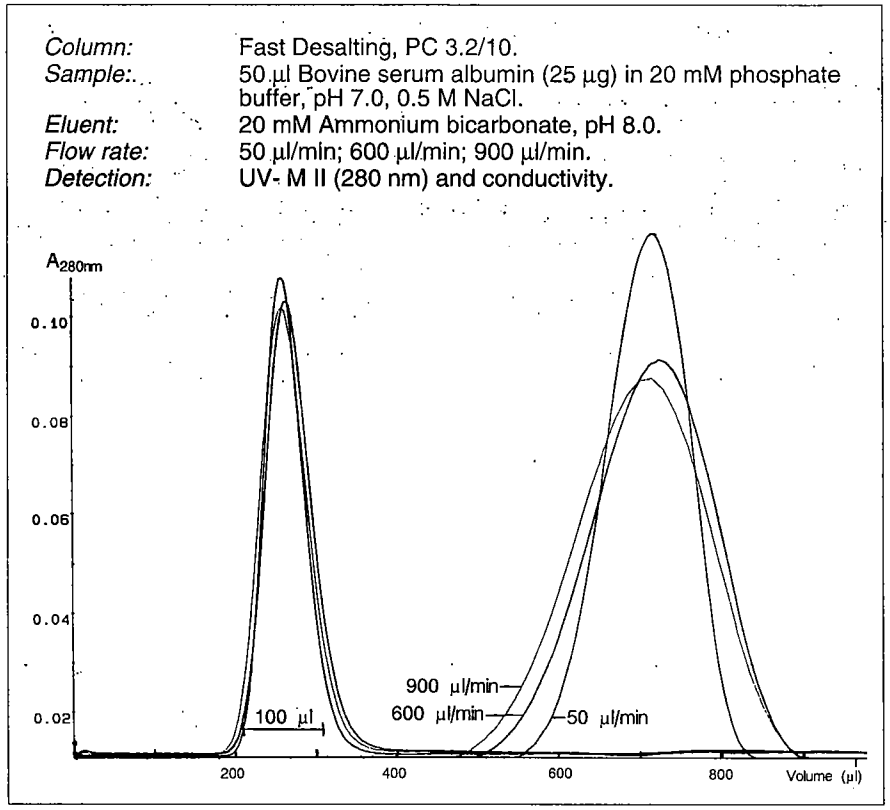


Fig. 10.39. Varying the flow rate on Fast Desalting, PC 3.2/10.

8.3.3 Desalting a reduction/alkylation mixture

Prior to sequence analysis of proteins, disulfide bonds are cleaved by reduction and alkylation under denaturating conditions. Excess low molecular compounds must then be removed before analysis. In the example below this was done by Fast Desalting PC 3.2/10 in 0.1 % TFA.

All components, were run separately at 214 nm to ensure that all the low molecular compounds can be separated from the protein/peptide chains.

Figure 10.40 shows that 4-vinylpyridine, EDTA, Tris, DTT and guanidine hydrochloride are all separated from the protein. Figure 10.41 shows the desalting of 50 µl mixture.

This example includes a range of different types of small molecules and salts. Buffers and 0.1 % TFA have been shown as eluents. Ionic strengths below 0.02 should be avoided to eliminate ionic interactions with the gel matrix.

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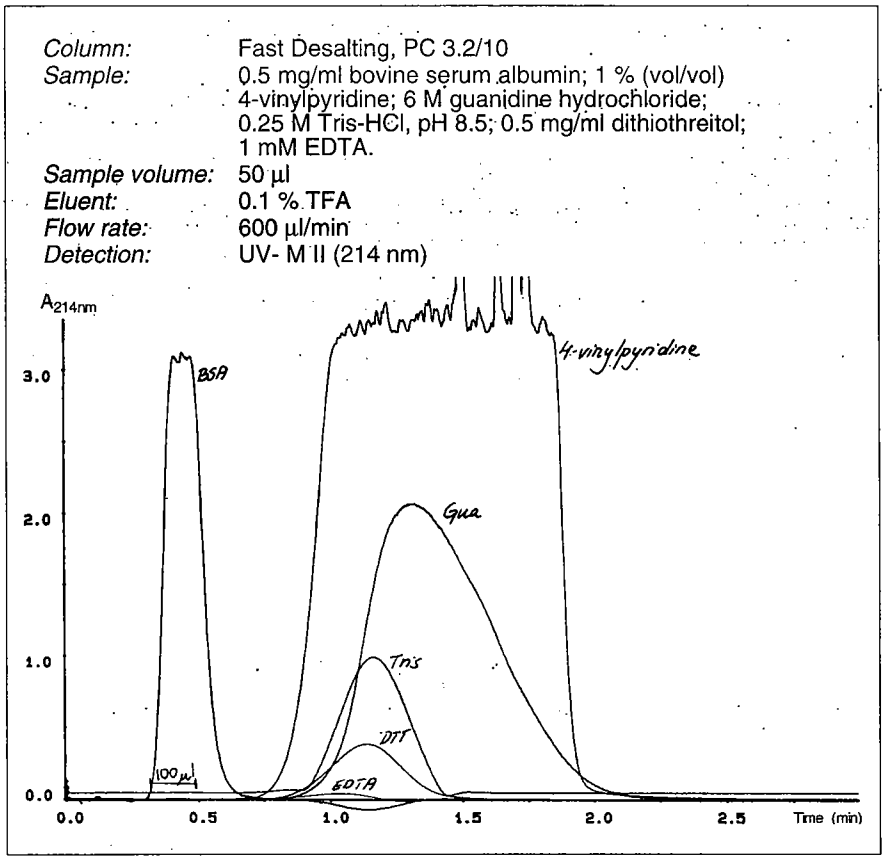


Fig. 10.40. The components in a reduction/alkylation mixture analysed separately on Fast Desalting, PC 3.2/10.

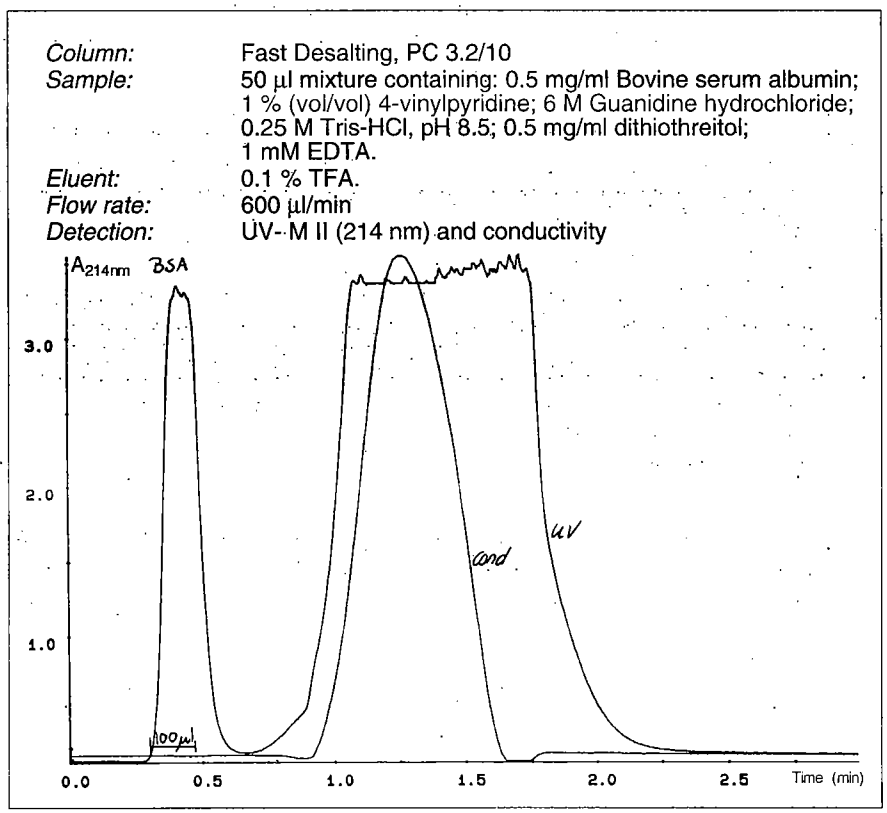


Fig. 10.41. Desalting of a reduction/alkylation mixture on Fast Desalting, PC 3.2/10.

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8.4 Reversed phase chromatography

8.4.1 Introduction

Reversed phase chromatography (RPC) is an extremely selective technique which separates molecules on the basis of hydrophobicity/polarity. It is similar to hydrophobic interaction chromatography (HIC), but RPC is usually more resolving. However, RPC involves the use of organic solvents, aggressive ions (e.g. trifluoroacetate) and a low pH which may lead to inactivation of larger biomolecules. Traditionally, RPC is used as a powerful analytical technique. For purification, RPC is most applicable for smaller biomolecules, e.g. peptides. It can be used for purification of larger biomolecules, if recovery of activity and changes in three-dimensional structure are of minor importance.

For SMART System, this technique is available with µRPC C2/C18, Sephasil C8 and Sephasil C 18 media. Table 9 lists the columns available. µRPC C2/C18 (3 µm bead size) is packed in PC 3.2/3 (glass) and SC 2.1/10 (steel) columns. The two Sephasil™ media (C8 and C18, 5 µm bead size) are also packed in the SC 2.1/10 column. Choosing the correct column for a particular application is best done by directly comparing their individual performance. However, for very high resolution separations of complex protein digests, µRPC C2/C18 SC 2.1/10 is the recommended first choice. Figure 10.42 shows a comparative study of selectivity differences between Sephasil C8, Sephasil C18 and µRPC C2/C18. Note the differences in retention time and elution pattern. Detailed lists of the column properties are found in Tables 10 and 11.

Table 9. Precision Columns for reversed phase chromatography.

Column	Column dimensions	Bed volume
µRPC C2/C18 PC 3.2/3 µRPC C2/C18 SC 2.1/10 Sephasil™ C8 SC 2.1/10 Sephasil C18 SC 2.1/10	3.2 mm i.d. x 30 mm 2.1 mm i.d. x 100 mm	0.24 ml 0.35 ml

Table 10. Main properties of µRPC C2/C18 PC 3.2/3 and µRPC C2/C18 SC 2.1/10.

Column dimensions	3.2 x 30 mm (PC 3.2/3) 2.1 x 100 mm (SC 2.1/10)
Gel volume	0.24 ml (PC 3.2/3) 0.35 ml (SC 2.1/10)
Base matrix	Silica
Alkyl chains	C2/C18
Particle size	3 µm
Pore size	120 Å
Efficiency, theoretical plates/meter (N/m)	≥ 100 000* (SC 2.1/10)
Practical loading range	ng - µg range
Recommended flow rate	≤ 1200 µl/min (PC 3.2/3) ≤ 250 µl/min (SC 2.1/10)
pH stability	2-8
Operational pressure limit	25 MPa (250 bar, 3625 psi) SMART System, 35 MPa (350 bar, 5000 psi) HPLC**

* Measured in a special test system.
** Only SC 2.1/10

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Table 11. Main properties of Sephasil C8 SC 2.1/10 and Sephasil C18 SC 2.1/10.

Column dimensions	2.1 mm x 100 mm
Gel volume	0.35 ml
Base matrix	Silica
Alkyl chains	C8 or C18
Particle size	5 µm
Pore size	100-120 Å
Efficiency, theoretical plates/meter (N/m)	> 60 000
Practical loading range	ng - µg range
Recommended flow rate	≤ 250 µl/min
pH stability	2-10*
Operational pressure limit	25 MPa (250 bar, 3625 psi) SMART System, 35 MPa (350 bar, 5000 psi) HPLC

* Avoid metal ions above pH 7.5.

8.4.2 Optimizing the separation

Varying the sample volume

The detection level is not related to sample volume, i.e. narrow peaks are obtained even if the sample volume is large (Fig. 10.42).

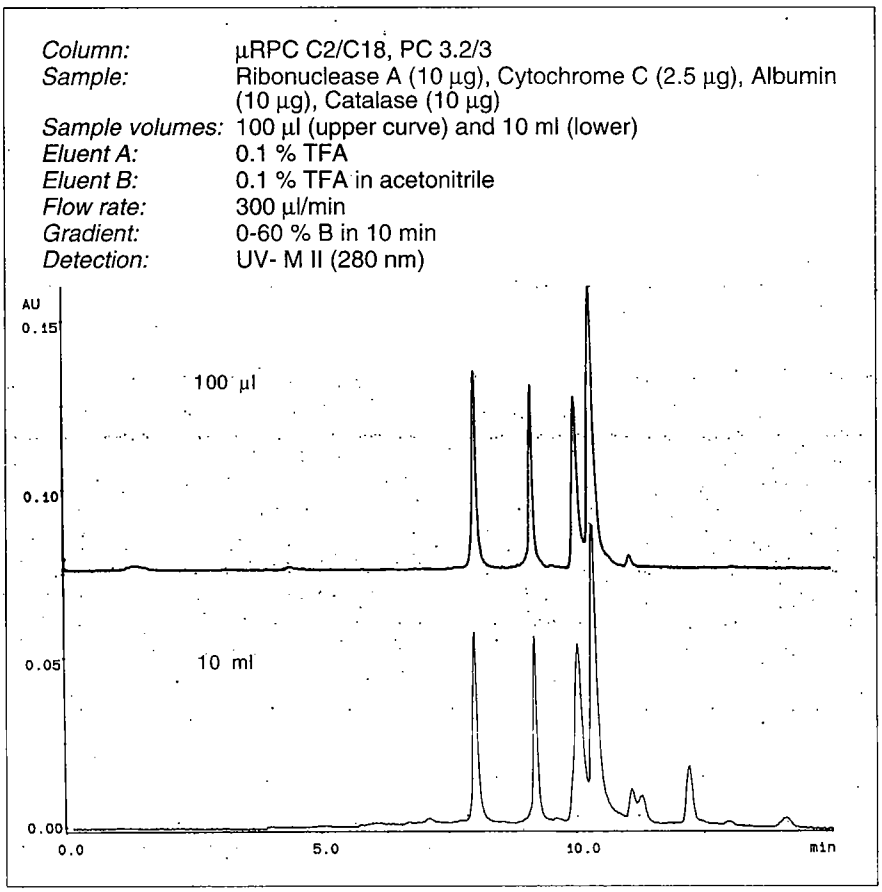


Fig. 10.42. Varying the sample volume using µRPC C2/C18, PC 3.2/3.

Varying the sample load

Different amounts of proteins (at a constant volume) were applied to µRPC C2/C18, PC 3.2/3. The results in figure 10.43 indicate that the resolution is affected for 26 µg protein mixture and at higher

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loadings the effect is more pronounced. Note that at the highest level, 1.3 mg, the maximum binding capacity is still not reached.

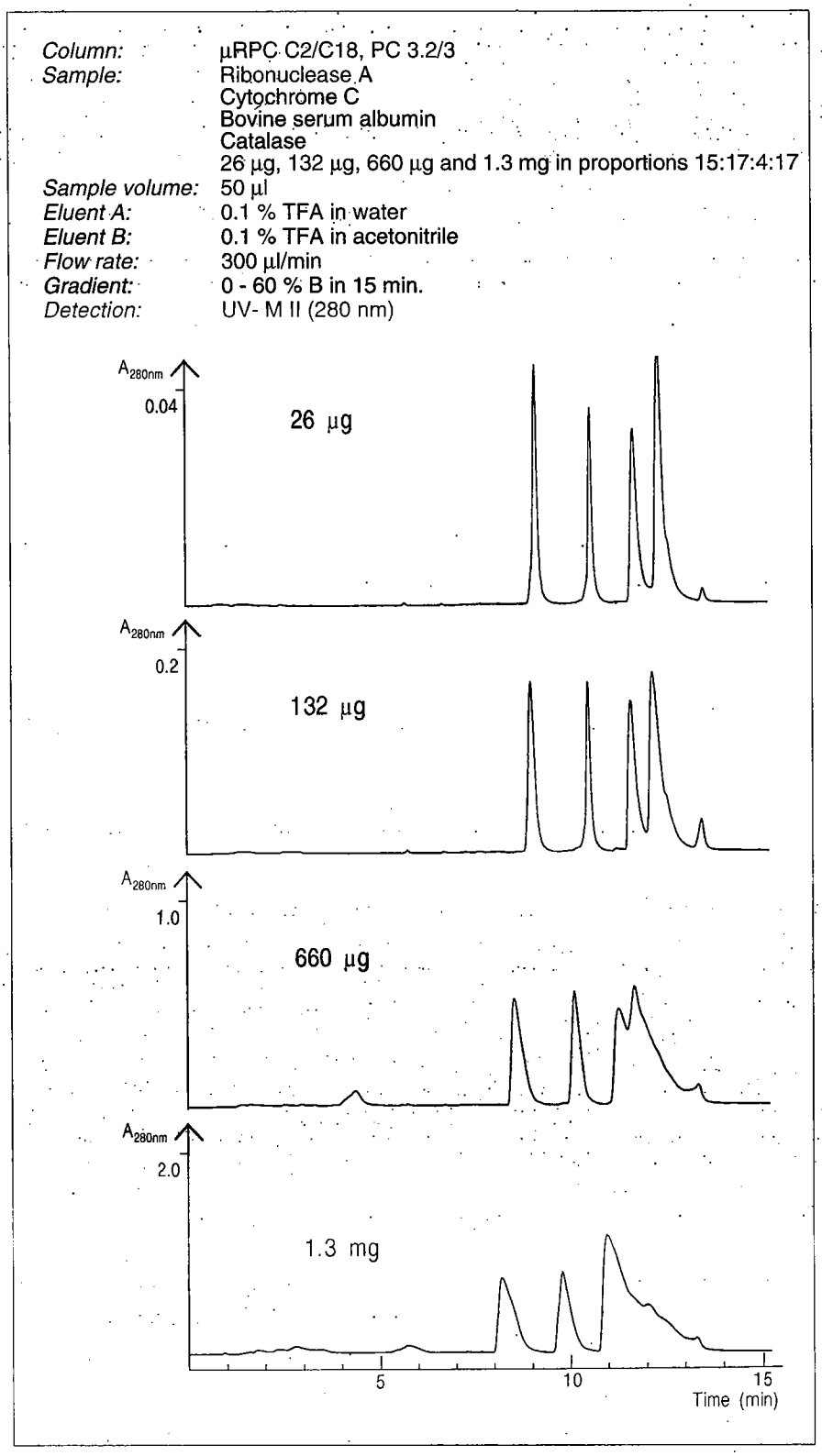


Fig. 10.43. Varying the sample load on µRPC C2/C18, PC 3.2/3.

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Varying the running conditions

When optimizing a separation (i.e. improving resolution), the first thing to change is the gradient shape. A study similar to that for ion exchange in section 8.1, has been performed for this column to show how different flow rates, gradient volumes and gradient times affect resolution and sensitivity.

The change in resolution when altering the gradient time from 7.5 to 30 minutes is shown in figure 10.44. Keeping the gradient volume constant i.e. lowering the flow rate improves the resolution. Keeping the flow rate constant i.e. increasing the gradient volume correspondingly increases the resolution further but at the expense of sensitivity.

The sensitivity is optimized by reducing the gradient volume. Figure 10.45 illustrates this effect when the volume was reduced from 9 to 2.25 ml. Reducing the flow rate gives higher resolution.

Resolution can also be greatly improved by changing the organic solvent or adding an ion-pairing agent. However, it is difficult to give any general guidelines as the effect on the resolution has to be determined empirically. The improvement made by adding ion-pairing agents, such as trifluoroacetic acid (TFA) or heptafluorobutyric acid (HFBA) is due to:

(1) formation of hydrophobic complexes between the sample molecules and the agent.

(2) a decrease in pH (below 4) which leads to ion suppression (and hence an increase in hydrophobicity) of sample molecules.

Changing the organic solvent or the ion-pairing agent may improve resolution dramatically, but the outcome is unpredictable.

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Column:	µRPC C2/C18 PC 3.2/3
Sample:	Ribonuclease A (20 µg) Cytochrome C (5 µg) Bovine serum albumin (20 µg) Catalase (20 µg)
Sample volume:	10 µl
Eluent A:	0.1 % TFA in water
Eluent B:	0.1 % TFA in acetonitrile
Upper chromatogram:	Reference
Flow rate:	300 µl/min.
Gradient:	0 - 60 % B in 7.5 min (2.25 ml).
Middle chromatogram:	Constant gradient volume
Flow rate:	75 µl/min.
Gradient:	0 - 60 % B in 30 min (2.25 ml).
Lower chromatogram:	Constant flow rate
Flow rate:	300 µl/min.
Gradient:	0 - 60 % B in 30 min (9 ml).
Detection:	UV- M II

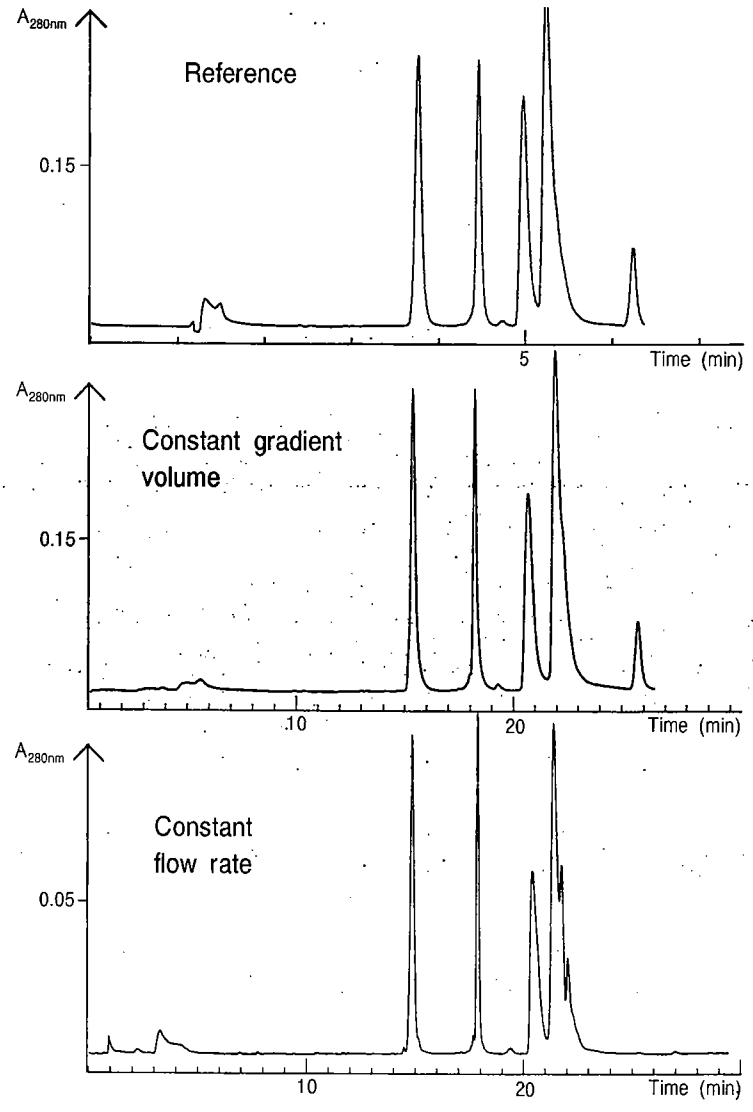


Fig. 10.44. Improving resolution on µRPC C2/C18 PC 3.2/3 by increasing gradient time.

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Column:	µRPC C2/C18 PC 3.2/3
Sample:	Ribonuclease A (20 µg) Cytochrome C (5 µg) Bovine serum albumin (20 µg) Catalase (20 µg)
Eluent A:	0.1 % TFA in water
Eluent B:	0.1 % TFA in acetonitrile
Upper chromatogram:	Reference
Flow rate:	300 µl/min
Gradient:	0 - 60 % B in 30 min (9 ml).
Middle chromatogram:	Constant flow rate
Flow rate:	300 µl/min
Gradient:	0 - 60 % B in 7.5 min (2.25 ml).
Lower chromatogram:	Lower flow rate
Flow rate:	75 µl/min
Gradient:	0 - 100 % B in 30 min (2.25 ml)
Detection:	UV- M II.

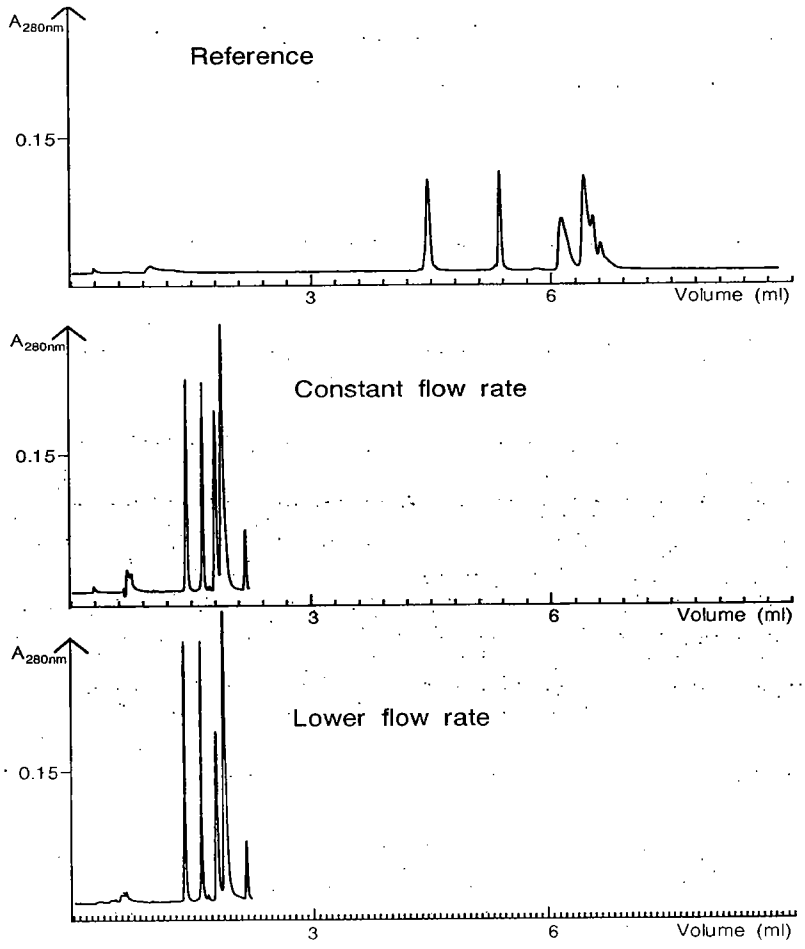


Fig. 10.45. Improving sensitivity on µRPC C2/C18, PC 3.2/3 by reducing the gradient volume. Each scale division is 1 min.

Figure 10.46 shows an optimized separation on µRPC C2/C18, PC 3.2/3 in which an extract from rat brain is separated. More than 50 different peaks of low molecular weight material are visible. Structurally similar peptides are often well resolved in RPC. Figure 10.47 shows baseline separation of several closely related

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fragments of vasopressin, a nonapeptide involved in kidney function and blood pressure regulation.

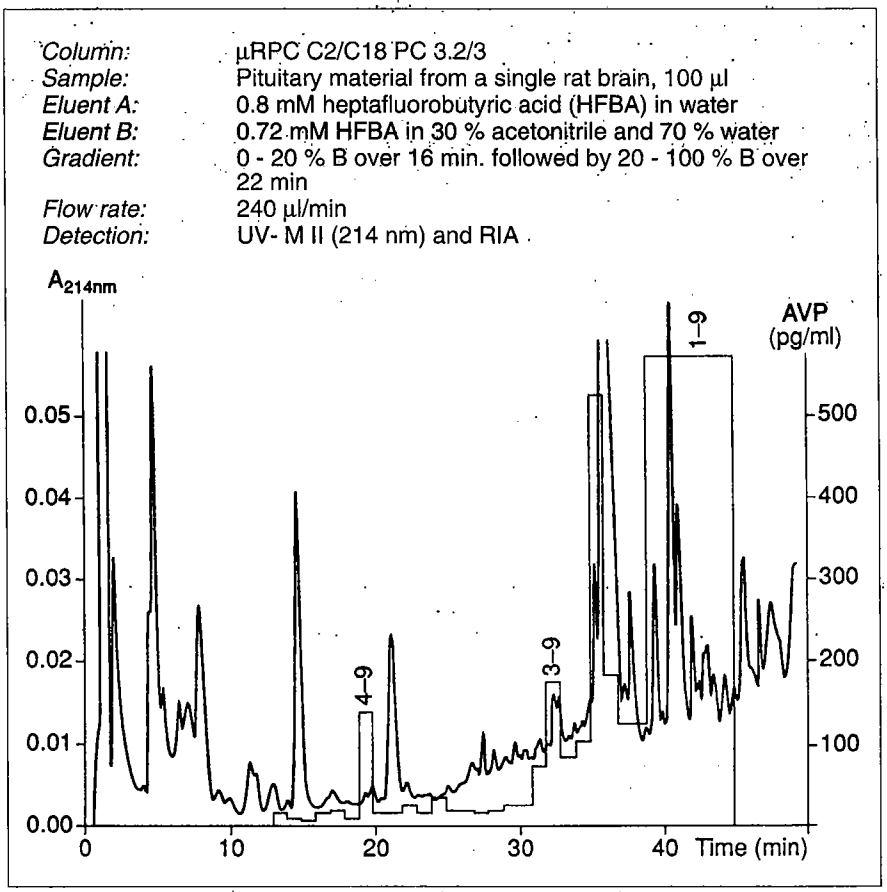


Fig. 10.46. Separation of components in rat pituitary using µRPC C2/C18 PC 3.2/3, and analysis of vasopressin immunoreactivity by a radioimmunoassay.
A rat pituitary extract (lyophilized, and dissolved in eluent A) was applied to the column, and 240 µl fractions were collected and analysed by a radioimmunoassay (bars) using antibodies directed against intact vasopressin. The material in the activity peaks (marked 4-9, 3-9, 1-9) was identified according to the elution position of synthetic vasopressin peptides (Fig. 10.45).

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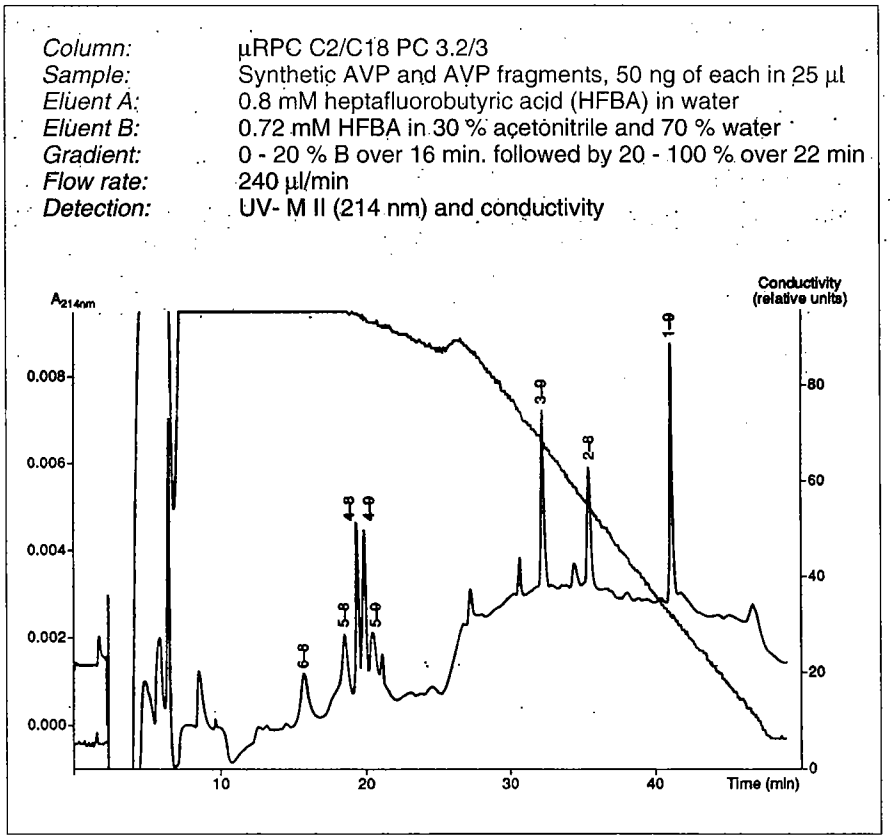


Fig. 10.47. Separation of synthetic vasopressin and closely related vasopressin peptides, using µRPC C2/C18 PC 3.2/3. 50 ng of each peptide was applied. The peptides are numbered with reference to intact vasopressin (1-9). The elution position for each peptide was determined in separate experiments.

8.4.3 Detection limit

The detection limit for proteins and peptides with µRPC C2/C18, PC 3.2/3 is in the low ng range at 280 nm, as shown in figure 10.48 and figure 10.49, where 20 ng insulin and 5 ng Cytochrome C respectively have been detected. Figure 10.50 shows a high sensitivity detection of insulin (down to 0.2 ng) with µRPC C2/C18 SC 2.1/10 and SMART System/µPeak Monitor.

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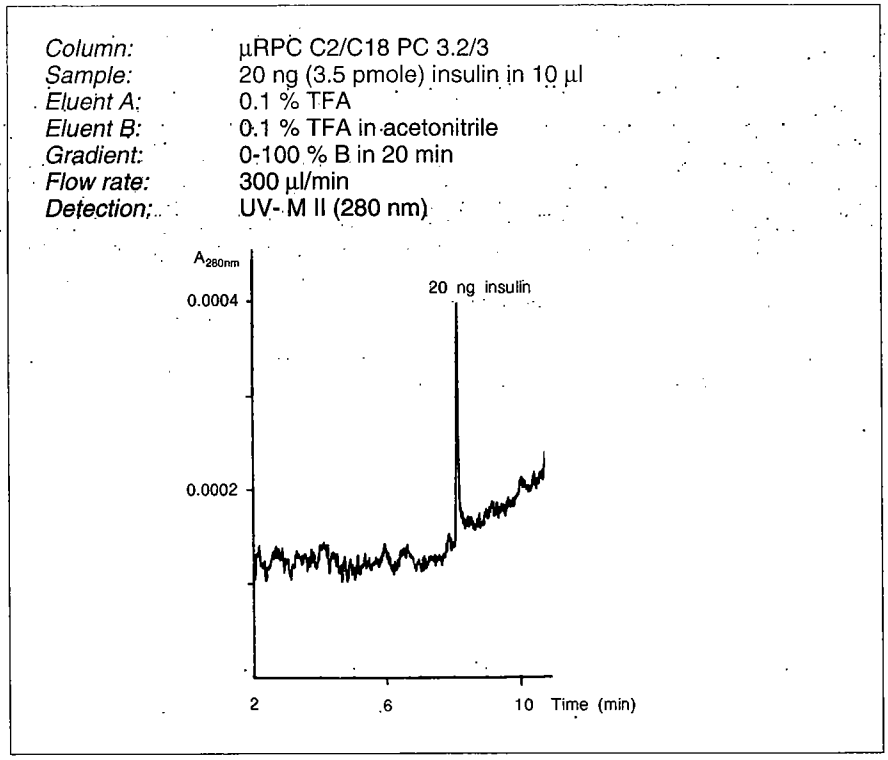


Fig. 10.48. Determination of the detection limit for insulin at 280 nm using µRPC C2/C18 PC 3.2/3.

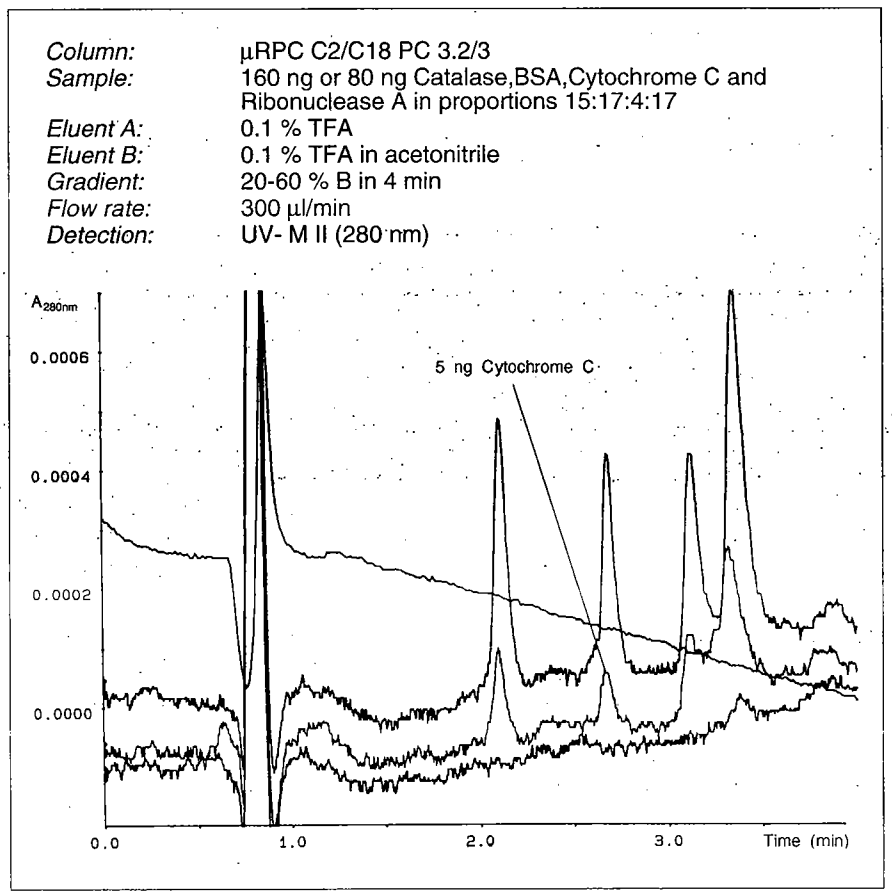


Fig. 10.49. Determination of the detection limit for Cytochrome C at 280 nm using µRPC C2/C18 PC 3.2/3. The lower curve is a blank run.

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Column:	µRPC C2/C18 SC 2.1/10
Sample:	Insulin, 1 ng/µl in 25 % eluent B
Sample volumes:	2 µl, 1 µl, 0.5 µl, 0.2 µl
Eluent A:	0.051 % TFA in MilliQ water
Eluent B:	0.050 % TFA in 60 % acetonitrile
Gradient:	25 % B for 10 min, 25-80 % B for 16.5 min, 80 % B for 15 min
Flow rate:	50 µl/min
Detection:	µPeak Monitor, 214 nm
Comment:	The chromatograms have been subtracted with a blank

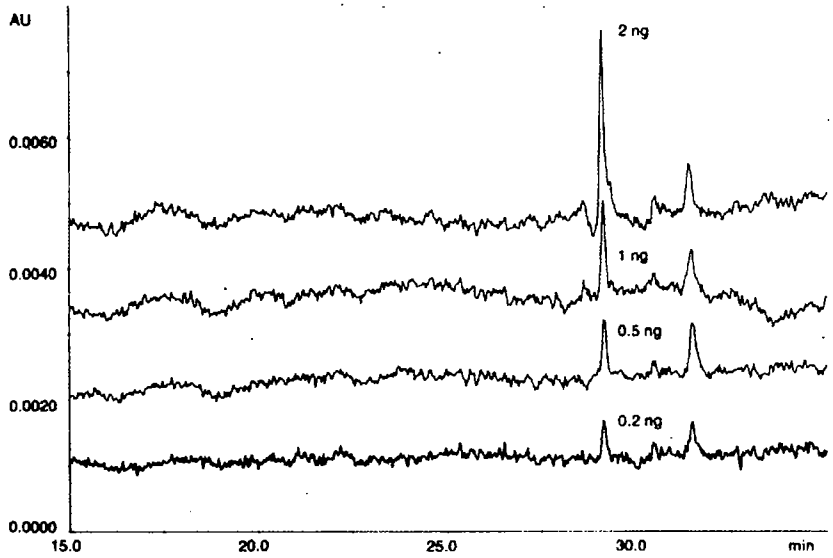


Fig. 10.50. High sensitivity detection of insulin (down to 0.2 ng).

8.4.4 Recovery

Figure 10.51 shows a recovery study on µRPC C2/C18 PC 3.2/3. Details of the method are described in section 3.1 (Recovery in SMART System).

First injection:

Four different amounts (0.1; 1; 10 and 100 µg) of Cytochrome C was loaded on the column and eluted in a gradient. The peak area was determined and compared with the area for the 100 µg injection. The recovery was calculated from the assumption that 100 % recovery is achieved for 100 µg injection.

Reinjection:

Fractions were collected from the previous runs and diluted with eluent A. The dilution was performed to decrease the concentration of organic solvent. The diluted fractions were loaded on the column.The UV peak area for the reinjection was compared with that of the UV peak area for the first injection.

The results from the first injection show that high mass recoveries are obtained, 80-100 % , even at the lowest loading tested (100 ng).

Recovery from the reinjection is lower. This is probably because of losses in the injection syringe, fraction tubes, and sample treatment.

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Column:	µRPC C2/C18 PC 3.2/3
Sample:	0.1, 1, 10, and 100 µg of Cytochrome C
Eluent A:	0.1 % TFA in water
Eluent B:	0.1 % TFA in acetonitrile
Flow rate:	300 µl/min
Gradient:	0-60 % in 10 min
Detection:	UV- M II (280 nm)
Upper curve:	Recovery calculated by comparing the UV peak area for the different loadings with the UV area for 100 µg.
Lower curve:	Recovery for a reinjected sample calculated by comparing the UV peak area with the first achieved area.

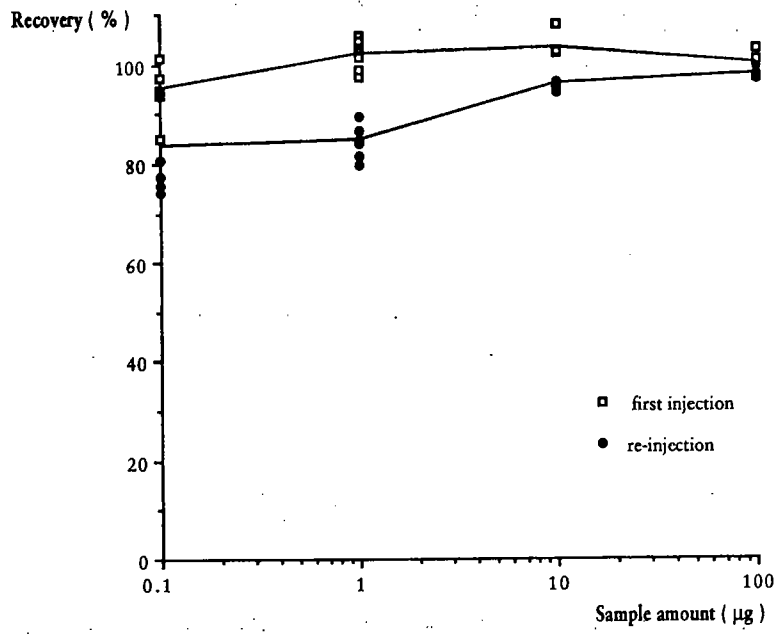


Fig. 10.51. Recovery of Cytochrome C on µRPC C2/C18 PC 3.2/3.

A recovery of 78 % of an unknown component from cerebrospinal fluid was determined by rechromatography (see Fig. 11, section 3.4). A 95 % recovery of immunoreactivity, at a sample loading of approximately 50 ng, was obtained in final purification of CGRP (Fig. 13, section 3.4.)

The amount of recovered biological activity of proteins in RPC is largely dependent on the degree which the three-dimensional structure is changed by the eluents. When a high recovery of activity is essential and RPC is used in the purification scheme, it is preferable to perform initial experiments in which the biological activity in presence of the eluents is checked.

8.4.5 Inverse gradient RPC

Removal of SDS, stain and gel components from microquantities of protein after electrophoresis and electroelution is a difficult task. This separation, which is particularly important for obtaining proteins of a sufficient purity for gas phase sequencing, can be performed by inverse gradient RPC. This technique utilizes the ability of proteins to interact strongly with certain silica-based reversed-phase packings at very high organic solvent concentrations (30). Contaminants such as SDS, Coomassie Blue and gel-related products pass straight through the column at the high concentrations of organic solvents used for

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adsorption (31). Elution is achieved by introducing a gradient of decreasing organic solvent concentration containing an ion-pairing agent (usually TFA).

Figure 10.52 shows an inverse gradient RPC experiment with µRPC C2/C18, PC 3.2/3 in which 3 µg of Cytochrome C was used as a model substance. Detection at 280 nm was chosen since the presence of TFA in eluent B only gives a steeply rising baseline at low wavelengths. To use the same column for both "standard" RPC and inverse gradient RPC may give rise to contamination problems; it is recommended to have dedicated columns for inverse gradient RPC.

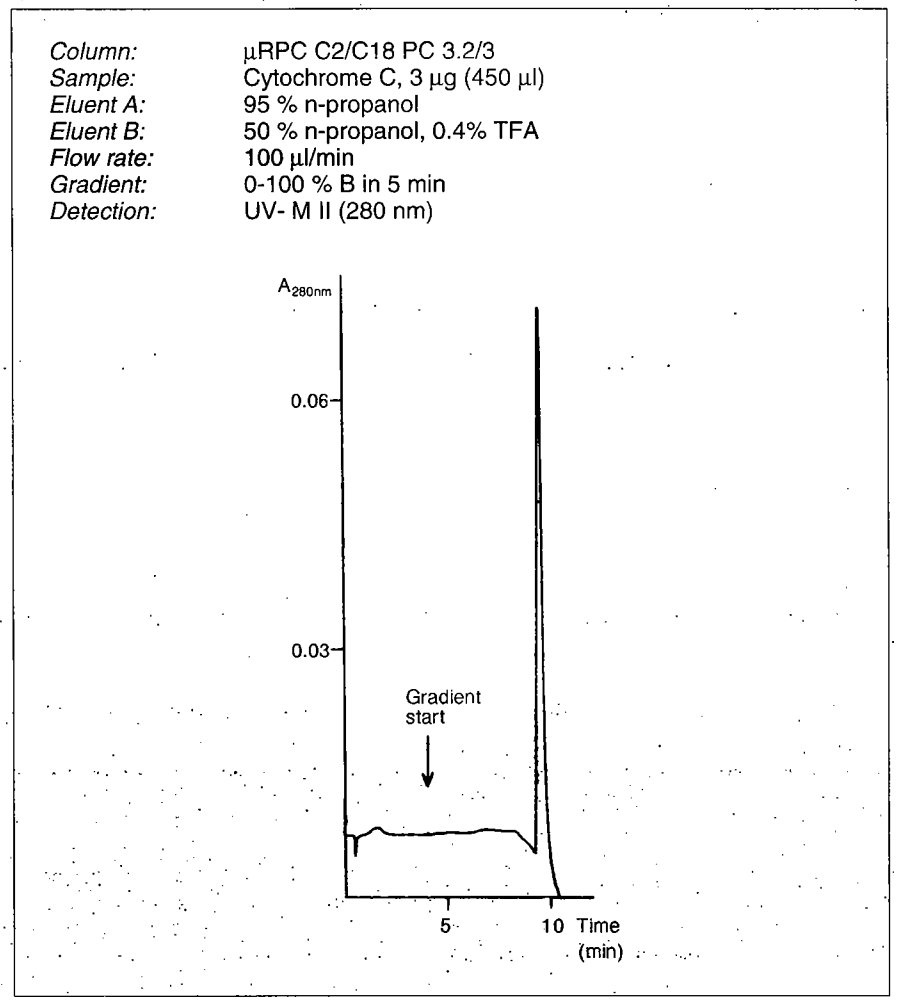


Fig. 10.52. Inverse gradient RPC of Cytochrome C on µRPC C2/C18 PC 3.2/3. 10 µl of Cytochrome C (3 µg), followed by 440 µl of eluent A, was drawn into a 500 µl syringe. The contents of the syringe were mixed after the introduction of an air bubble (see Chapter 6). The gradient start is marked by an arrow.

8.5 Affinity chromatography

8.5.1 Introduction

Affinity chromatography is a highly specific technique which separates biomolecules according to differences in molecular recognition. The basic principle is that a biospecific ligand is covalently attached to a chromatographic matrix. The immobilized ligand has a unique affinity for the substance of interest, and after binding to the ligand the substance can be selectively desorbed after the unbound material has been washed away:

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Examples of biological interactions used in affinity chromatography are:

Enzyme	Substrate analogue, inhibitor, cofactor
Antibody	Antigen
Lectin	Polysaccharide, glycoprotein, cell surface receptor
Nucleic acid	Complementary base sequence, histone, nucleic acid polymerase, binding protein
Hormone, vitamin	Receptor, carrier protein
Sugar	Lectin, enzyme or other sugar binding protein
Protein A, Protein G	Polyclonal and monoclonal IgG antibodies

In the Precision Column range affinity chromatography is available with NHS-activated Superose, PC 3.2/2, which is designed for fast and easy coupling of an optional affinity ligand. Table 12 summarizes the properties of this column.

Table 12: Properties of NHS-activated Superose®, PC 3.2/2.

Column dimensions	3.2 x 20 mm
Gel volume	0.16 ml
Activated group	N-hydroxysuccinimide (NHS)
NHS-concentration	30 µmol/ml gel
Particle size	≈13 µm
Molecular weight range	$\leq 2 \times 10^6$
Flow rate**	10-400 µl/min
Sample volume**	Not critical
pH-stability*	2-10
Operational pressure limit	1.5 MPa (15 bar, 215 psi)
Running temperature	4-40 °C

* The stability of the adsorbent prepared from NHS-activated Superose is limited by the stability of the chosen ligand.

** Factors such as recommended maximum flow rate and sample volume depend on the kinetics of the affinity system.

8.5.2 Coupling of ligand

The chemical structure of the active group on NHS-activated Superose is shown in figure 10.53. A suitable affinity ligand should have chemical properties that allow easy immobilization to a matrix. Free, primary amino groups on the ligand are required for coupling. Furthermore, the ligand should possess functional groups for coupling which are non-essential for the interaction which is being used for affinity chromatography, i.e. the binding to the substance being purified should not be adversely affected by immobilization.

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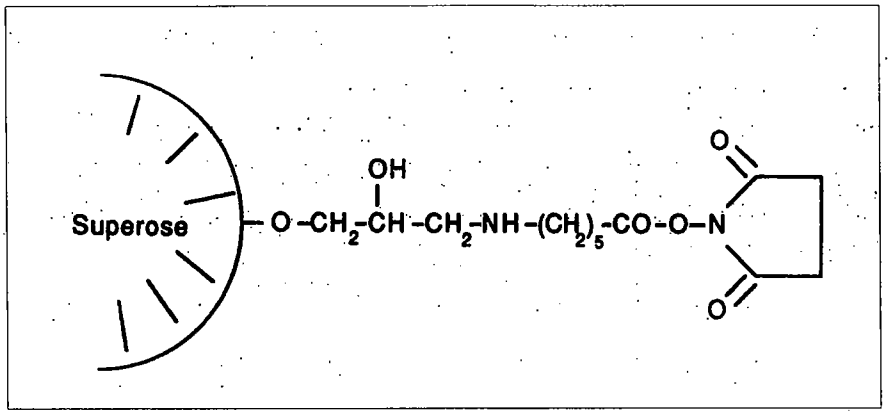


Fig. 10.53. The active group, N-hydroxysuccinimide, on NHS-activated Superose®, PC 3.2/2. Primary amino groups form an amide bond with the spacer.

The affinity pair in affinity chromatography should possess the following characteristics:

(1) The ligand must be able to form reversible complexes with the substance to be isolated or separated.

(2) The specificity must be appropriate for the planned application.

(3) The complex constant should be high enough for the formation of stable complexes or to give sufficient retardation in the chromatographic procedure.

(4) It should be easy to dissociate the complex by a simple change in the medium, and without irreversibly affecting the ligand or the substance to be isolated.

A recommended amount of coupling ligand is 0.5 - 2 mg in a concentration of 3 - 5 mg/ml. However, for very small ligands it may be necessary to increase this amount (e.g. for coupling L-lysine the recommended quantity is 24 mg in a concentration of 150 mg/ml).

A coupling procedure to NHS-activated Superose, PC 3.2/2 takes about 3 h at room temperature. Briefly, the ligand to be coupled in 200 µl of a buffer (which contains no primary amines) is injected onto the column. The flow is then stopped for about 1 h to allow time for complete coupling. Deactivation of the gel and washing of the column is then performed.

A suggested method for the coupling of a ligand to NHS-activated Superose, PC 3.2/3 is found in figure 10.54, and also in SMART Assistant.

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Ligand: 200 µl of a protein solution (approx. 3 mg/ml), in an amine-free buffer.
Eluent 1A: 1 mM HCl
Eluent 1B: 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3
Buffer 2A: 0.5 M ethanol amine, 0.5 M NaCl, pH 8.3
Buffer 2B: 0.1 M NaAc, 0.5 M NaCl, pH 4.0.

```
***** Main method *****
   0.00 PUMP_WASH            A,  1,  4    {WASHING THE PUMP}
   0.00 PUMP_WASH            B,  1,  4    {WITH 1 mM HCl AND}
                                          {COUPLING BUFFER}
   0.00 MIXER_WASH           0

   0.00 FLOW                 160          {WASHING COLUMN}
   0.00 CONC_B               0.0          {WITH 10 COL VOLUMES}
                                          {1 mM HCl}
  10.00 MIXER_WASH           100
  10.00 FLOW                 100.00
  10.00 CONC_B               0.0
  10.00 CONC_B               100.0
  10.10 INJECT               EXTERNAL     {INJECTION OF THE LIGAND}
  10.10 GOTO_TUBE            1
  12.00 FLOW                 0.00         {STAGNANT COUPLING FOR 1 h}


  15.00 PUMP_WASH            A,  2,  4    {WASHING THE PUMP WITH }
  15.00 PUMP_WASH            B,  2,  4    {DEACTIVATING BUFFERS}
  15.00 MIXER_WASH           0

  72.00 CONC_B               100.0
  72.00 CONC_B               0.0
  72.00 FLOW                 80           {5 COLUMN VOLUMES A}
  72.00 TUBE                 A            {COLLECTING EXCESS OF}
                                          {LIGAND}

  82.00 CONC_B               0.0
  82.00 CONC_B               100.0        {5 COLUMN VOLUMES B}

  92.00 CONC_B               100.0
  92.00 CONC_B               0.0          {5 COLUMN VOLUMES A}
 102.00 FLOW                 0.00         {LET IT STAND FOR 1 h}

 162.00 CONC_B               0.0
 162.00 CONC_B               100.0        {5 COLUMN VOLUMES B}
 162.00 FLOW                 80.00

 172.00 CONC_B               100.0
 172.00 CONC_B               0.0          {5 COLUMN VOLUMES A}

 182.00 CONC_B               0.0
 182.00 CONC_B               100.0        {5 COLUMN VOLUMES B}

 192.00 CONC_B               100.0
```

Fig. 10.54. Suggested programming of a coupling method for NHS-activated Superose®, PC 3.2/2 (also available in SMART Assistant). The column should be equilibrated with a storage buffer suitable for the ligand, immediately after coupling.

8.5.3 Capacity

A different coupling yield can be expected for different proteins, depending on the solubility and size of the protein and the amount of primary amino groups available for coupling. The amount of coupled protein A (a small and soluble protein) and collagen (a "difficult" protein; large, viscous, difficult to dissolve) to NHS-activated Superose, PC 3.2/2 was determined by amino acid analysis of the gel. The results are presented in table 13.

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Table 13. Coupling to NHS-activated Superose®, PC 3.2/2. 200 µl of both protein solutions (3 mg/ml) were applied.

Protein	Amount coupled to the column
Collagen (300 K)	15 µg (≈0.1 mg/ml gel)
Protein A (42 K)	260 µg (≈1.6 mg/ml gel)

The difference in amounts of collagen and protein A coupled to the gel probably depends on the extreme size of collagen (a rod-shaped, 300 K protein). A smaller protein will easily penetrate into the beads of Superose 12 and have a larger surface area available for coupling.

Up to milligram quantities of proteins can be expected to bind to NHS-activated Superose, PC 3.2/2 when non-extreme proteins have been immobilized on the column. As shown in figure 10.55, the maximum binding capacity for IgG on a column containing immobilized protein A was 1.45 mg IgG (≈9 mg IgG/ml gel).

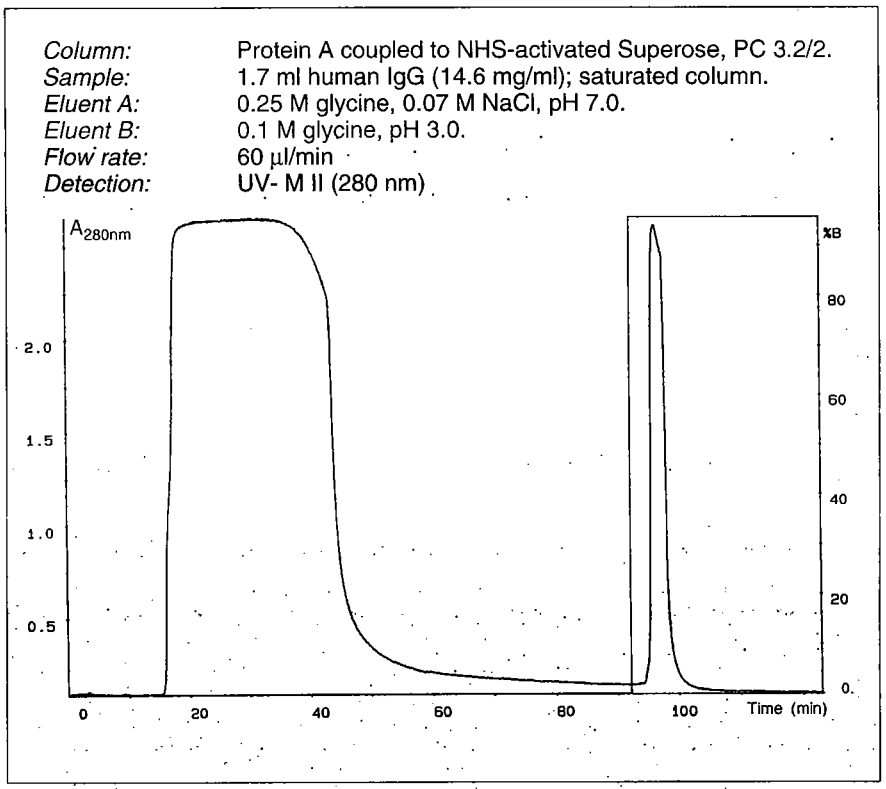


Fig. 10.55. Capacity determination of human IgG on protein A coupled to NHS-activated Superose®, PC 3.2/2. The maximum binding capacity was 1.45 mg; determined by passing an excess of IgG through the column and then measuring the amount of bound IgG, after elution.

8.5.4 Elution of bound substance

The interaction between ligand and bound substance is often due to a combination of electrostatic, hydrophobic and hydrogen bonds. Consideration of the relative importance of these three types of bond as well as substance stability will be of help in choosing suitable elution conditions. Step-wise elution is commonly used in affinity chromatography. However, when a complex biological sample has been applied to an affinity column, it is often necessary to elute non-specifically bound material in a separate step, before the substance of interest is eluted (see figure 10.56 and 10.57).

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Column:	Bungarotoxin immobilized on NHS-activated Superose, PC 3.2/2.
Sample:	Chicken optic lobe extract (300 µl, equivalent to one optic lobe), desalted
Eluent A:	50 mM sodium phosphate, pH 7.5, 1 mM PMSF, 1 mM benzamidine, 0.2 % Lubrol PX
Eluent B:	Eluent A, 1.0 M NaCl
Eluent C:	Eluent A, 0.1 mM nicotine
Flow rate:	10 µl/min (during injection) Incubation with buffer C for 30 min (flow rate 0) 25 µl/min (during elution)
Temp.:	+4 °C (controlled by the system).
Detection:	UV- M II

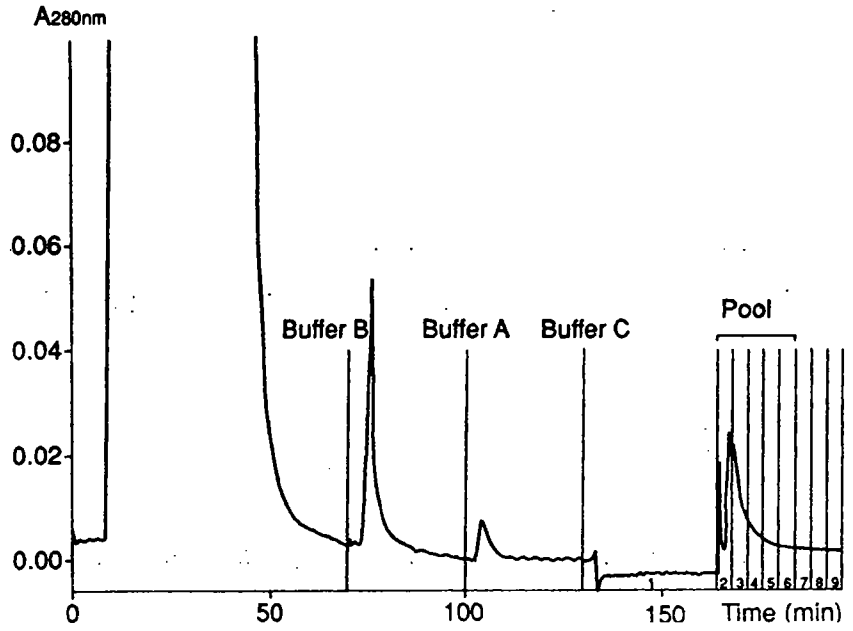


Fig. 10.56. Micropurification of nicotinic acetylcholine receptors from chicken brain using bungarotoxin immobilized on NHS-activated Superose®, PC 3.2/2.
A micropurification scheme for nicotinic acetylcholine receptor from optic lobes in a single chicken brain was developed using SMART System, in order to study subunit composition by electrophoresis. After a concentrating anion exchange step (Fig. 10.12) receptor activity was measured in an assay using ^{125}I-labelled bungarotoxin, and subunit structure was studied electrophoretically.

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Column:	Collagen type 1 immobilized on NHS-activated Superose, PC 3.2/2
Sample:	200 µl extract containing integrin collagen receptors from ^{125}I-labelled human foreskin fibroblasts
Eluent A:	10 mM Tris-HCl, 1 mM $MnCl_2$, 0.1 % Triton X-100, pH 7.4
Eluent B:	Eluent A, 50 mM NaCl
Eluent C:	10 mM Tris-HCl, 10 mM EDTA, 0.1 % Triton X-100, pH 7.4
Flow rates:	40 µl/min (0 - 1.6 ml) 160 µl/min (1.6 - 5.3 ml)
Detection:	Fractions analysed in a gamma counter
Temperature:	+ 6 °C (controlled by the system))

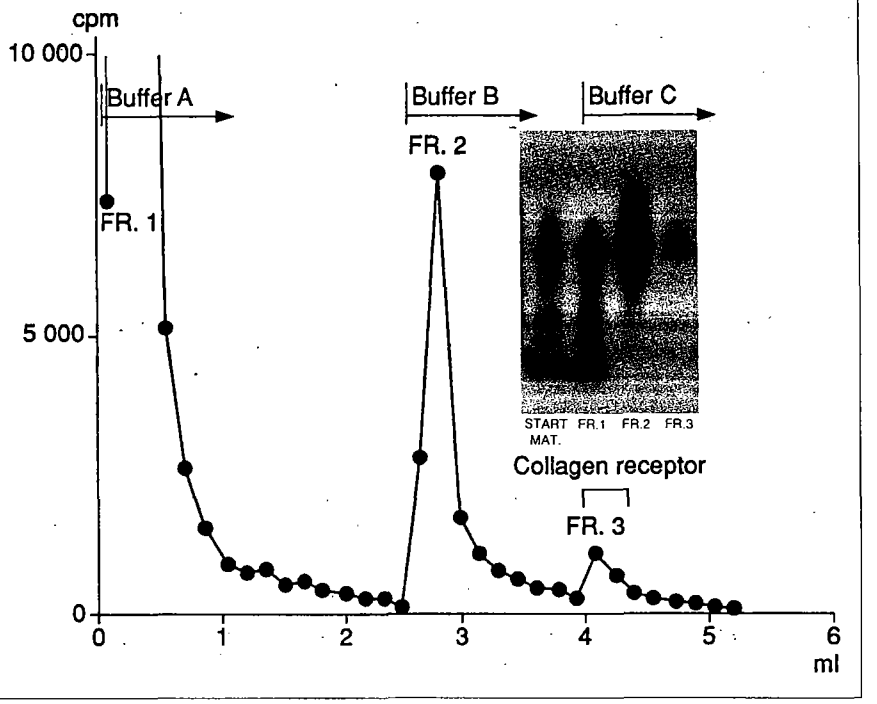


Fig. 10.57. Micropurification of ^{125}I-labelled integrin collagen receptors using collagen immobilized on NHS-activated Superose, PC 3.2/2.
Integrin collagen receptors were purified from surface-labelled human fibroblasts. The receptors were specifically eluted with EDTA (eluent C), after non-specifically bound material had been washed away with salt. Purity analysis and identification was done by autoradiography (inset) after SDS/PAGE with PhastSystem.

Frequently used elution conditions are:

1. pH change (usually a decrease, see figure 10.55). To obtain a high recovery of biological activity it is sometimes important to neutralize the fractions as soon as possible after elution. This is most easily done by pipetting a small volume of neutralizing buffer in each fraction collector tube in advance.
2. Increase in the ionic strength (e.g. 1-3 M NaCl)
3. Chaotropic salts (e.g. 1-3 M KSCN, or KI)
4. Urea or guanidine hydrochloride (4-6 M)
5. 20-40 % ethylene-glycol
6. Detergent (e.g. 1% Tween)
7. Chelating agents (e.g. 10 mM EDTA, Fig. 10.57)
8. Specific elution by competitive binding of the eluting agent to either the ligand or the protein (Fig. 10.56)

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8.6 Hydrophobic interaction chromatography

8.6.1 Introduction

Hydrophobic interaction chromatography (HIC) separates biomolecules according to differences in their hydrophobicity. As most biomolecules are hydrophobic to some degree, this technique has a broad application range. Adsorbents for reversed phase chromatography (RPC) also contain hydrophobic ligands, but the mechanism on molecular level differs from HIC. RPC can be regarded as a continuous hydrophobic phase whereas the ligands on an HIC adsorbent are interacting individually with the solutes. As a result, labile globular proteins very often denature when applied to an RPC-column, while the recovery of protein activity from a separation using hydrophobic interaction chromatography is generally high.

For SMART System the technique is available with Phenyl Superose PC 1.6/5. Table 13 summarizes the properties of this column.

Table 14. Properties of Phenyl Superose®, PC 1.6/5

Column dimensions	1.6 mm x 50 mm
Gel volume	0.10 ml
Hydrophobic ligand	R=-◎
Particle size	≈ 13 µm
Molecular weight range	≤ 2 x10^6
Maximum protein/peptide capacity	2-3 mg
Practical loading range	0.5-1500 µg
Flow rate	10-100 µl/min
pH-stability	2-13
Operational pressure limit	1.5 MPa (15 bar, 215 psi)
Temperature interval	4-40 °C

8.6.2 Optimizing the separation

Sample preparation

In hydrophobic interaction chromatography samples are applied at a high ionic strength (i.e. under conditions at which hydrophobic interaction are promoted). The most convenient way to prepare the sample is usually to mix it with an equal volume of a strong salt solution (e.g. 3 M $(NH_4)_2SO_4$). If small volumes are used, mixing in the injection syringe is recommended (Chapter 6). Since high concentrations of ammonium sulphate often cause precipitation of proteins the sample should be checked visually before being loaded on the column.

Eluents

Two factors of great importance for HIC are the type and concentration of salt used and additives which change the polarity of the solvent. The influence of different salts on hydrophobic interaction follows the Hofmeister (lyotropic) series presented in figure 10.58. Salts that promote hydrophobic interaction most are to the left in the series. The anions to the right are called chaotropic. The salts that increase the surface tension most that give the strongest hydrophobic interaction. There are also other factors which influence the hydrophobic interaction, such as protein hydration and specific interactions between the protein and the salt ions. Na_2SO_4, NaCl and especially $(NH_4)_2SO_4$ are the most utilized salts in HIC.

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Anions: PO_4^{3-}, SO_4^{2-}, CH_3COO^-, Cl^-, Br^-, NO_3^-, ClO_4^-, I^-, SCN^-
Cations: NH_4^+, Rb^+, K^+, Na^+, Cs^+ Li^+, Mg^{2+} Ca^{2+} Ba^{2+}

Fig. 10.58. Hofmeister series. Salts that promote hydrophobic interaction the most are to the left in the series.

Elution, whether performed stepwise or with a gradient, can generally be achieved in three different ways:

1. Changing the salt concentration: The elution of solutes, in order of increasing hydrophobicity, is accomplished by decreasing the salt concentration. This is the most commonly used method. In figures 10.59 and 10.60 this is the elution method used.
2. Addition of a polarity decreasing agent, like ethylene glycol (≤ 80 %) or isopropanol (lower concentrations). The addition can be made after the removal of salt or at the same time.
3. Adding detergents. Detergents work as displacers of the proteins. They have been used mainly for purification of membrane proteins.

Column:	Phenyl Superose, PC 1.6/5
Sample:	134 µl partially purified recombinant HIV reverse transcriptase, 66 µl 3 M $(NH_4)_2SO_4$ (2 µg total protein)
Eluent A:	0.5 M $(NH_4)_2SO_4$, 10 mM Tris-HCl, pH 8.0
Eluent B:	20 mM Tris-HCl, pH 8.0
Flow rate:	100 µl/min
Gradient:	0-100 % B in 8 min.
Detection:	UV- M II (280 nm)

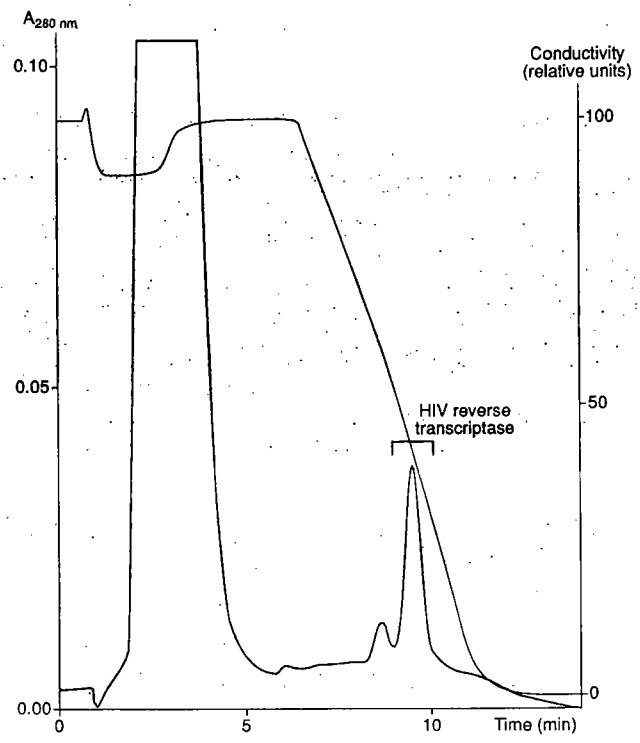


Fig. 10.59. Microscale method development for a recombinant HIV reverse transcriptase using Phenyl Superose®,PC 1.6/5.

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8.6.3 Detection limit

The detection limit using Phenyl Superose, PC 1.6/5 is usually in the low ng range. Figure 10.60 shows the detection of ng quantities of standard proteins.

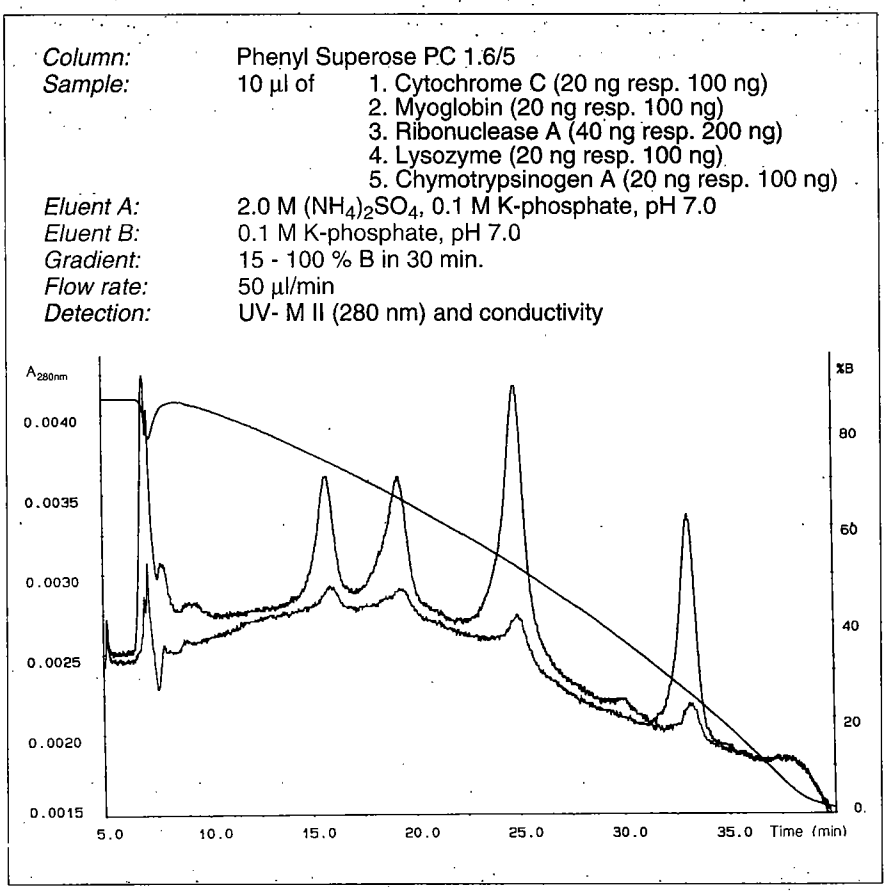


Fig. 10.60. Detection limit for proteins on Phenyl Superose®, PC 1.6/5

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9. µPeak Monitor and RPC for peptide mapping and amino acid sequence analysis

9.1 Introduction

Mapping peptides and purifying samples for peptide/protein sequence analysis requires high resolution chromatography to achieve good results. When the amount of sample available is in the µg-ng range, a chromatography system and columns optimized for small scale separations is also necessary. SMART™ System and Precision Columns fulfil both requirements and are therefore well-suited for mapping and purification work at this scale.

This Chapter describes how SMART System works within this application area. µPeak Monitor and an expanded range of Precision Columns for reversed phase chromatography (RPC) open new possibilities.

9.2 Multi-wavelength function (µPeak Monitor)

Calculating the ratio between two chromatogram curves of different wavelengths provides valuable information that helps identify peaks and assess their purity. The amino acid content of peptide fragments can be predicted and particular peptides can be identified in a mixture.

9.2.1 Illustration of the multi-wavelength function

Figures 10.61-10.64 show an example which illustrates how to work with this function.

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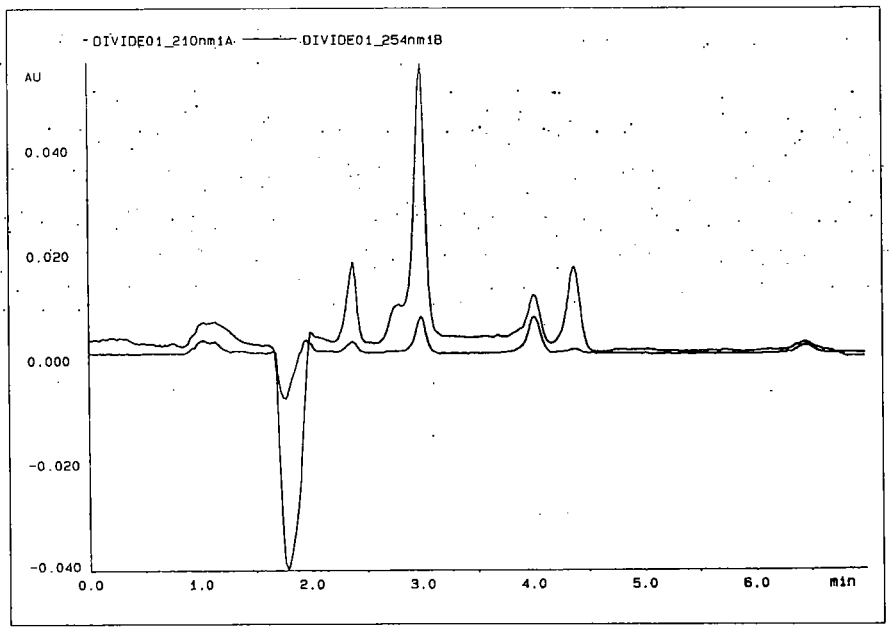


Fig. 10.61. Original chromatograms, 210 and 254 nm. When detecting at two wavelengths which are characteristic for the compound, the result could look as in this figure.

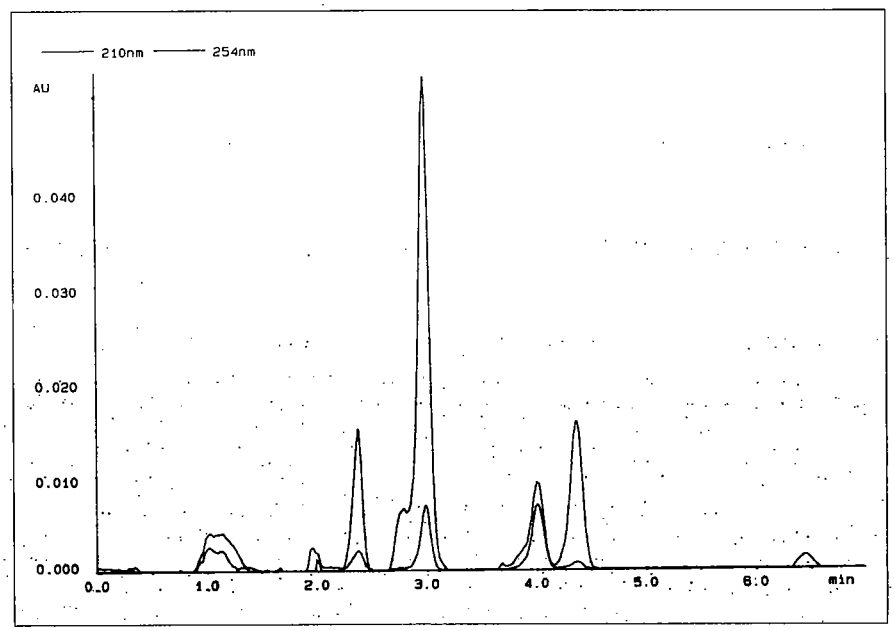


Fig. 10.62. Chromatograms after baseline subtraction. To get a useful ratio both chromatograms should have baselines close to 0 AU. This can be achieved by baseline subtraction. To create baselines use Integrate-Calculate baseline and, if necessary, Integrate-Edit baseline in Evaluation, or choose a chromatogram from a blank run as baseline. Then use Curves-Subtract to subtract the baselines from the chromatograms. The result should appear as in this figure.

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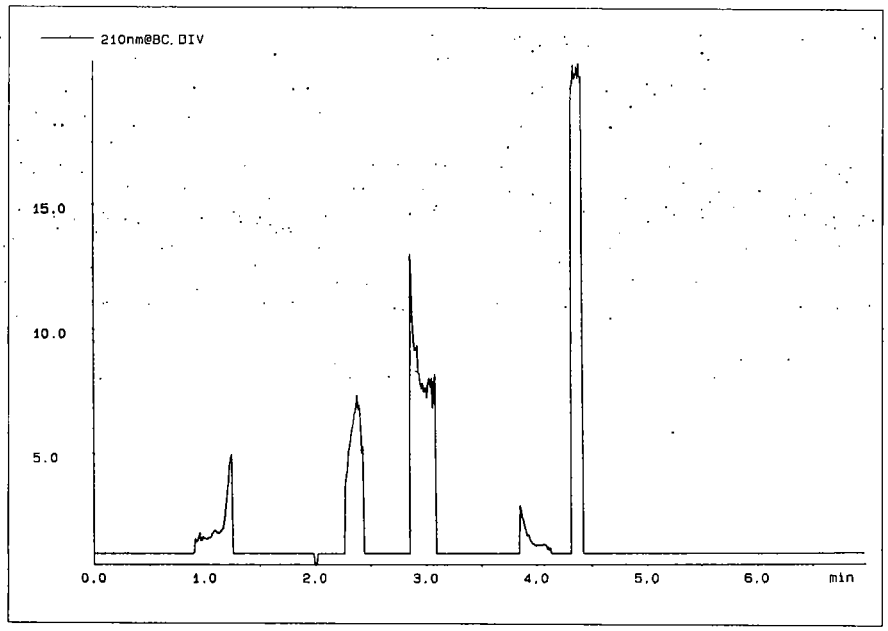


Fig. 10.63. Ratio between chromatograms from 210 and 254 nm (Threshold values set to 0.0005 AU for both curves.) When chromatograms with zero baseline have been achieved use Curve-Divide, including threshold values. Division will take place only when both absorbances are higher than their chosen threshold values. When one or both of the absorbances are lower the ratio is set to 1. (This is also valid for negative absorbances. High negative values are divided but for low negative values the ratios are set to 1.) This is done to avoid large fluctuations in ratios which are achieved at divisions between values close to zero. To get a cleaner picture the function Curves-Smooth should be used (Fig. 10.64).

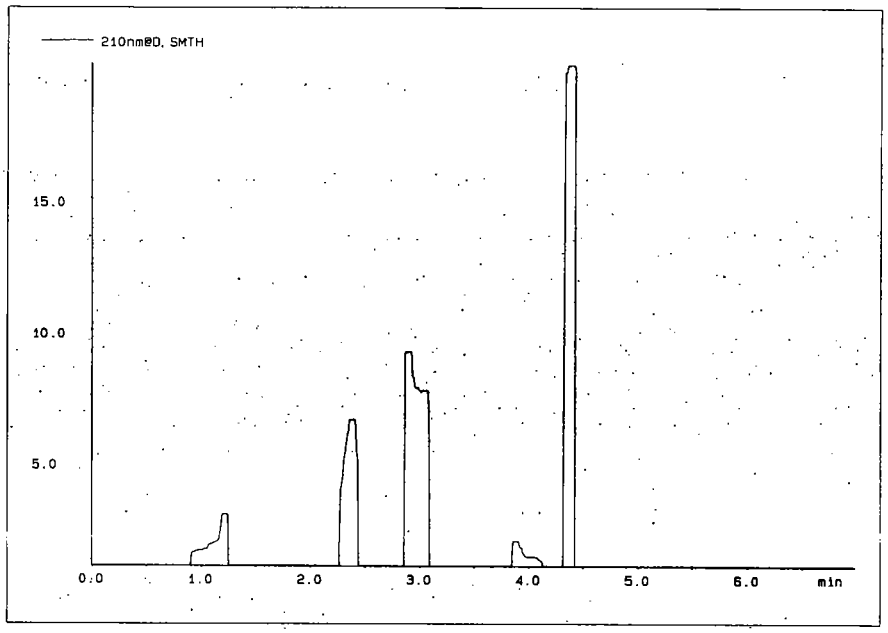


Fig. 10.64. Smoothed ratio curve (Median filter with 0.11 minutes width). The resulting ratio can then be used for peak identification. Because of differences in UV absorption spectra, different compounds have different ratios between absorbances at different wavelengths. As can be seen in Fig. 10.64 the five main compounds in the chromatogram have big differences in ratio values, ranging between 2 and 20. This could be used for identification during method development, for example, when compounds change elution order. Examining the absorbance ratios can reveal where the different compounds elute.

The ratio can also be used to check peak purity. If the ratio is constant throughout a peak, the peak is probably pure and vice versa. The ratios shown in this figure indicate that only the peak at 4.5 minutes is pure.

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9.2.2 Identification of peptides by absorbance ratio calculations

Example 1

The molar absorbances of aromatic amino acids at different wavelengths form the basis for this calculation. Table 15 lists these absorbances.

Table 15. Molar absorbances (A) of aromatic amino acids at 254 nm and 280 nm and the 254 nm/280 nm absorbance ratio (18).

	A254	A280	A254/A280
Trp	3 000	5 000	0.60
Tyr	300	1 200	0.25
Phe	180	0	
Phe+Tyr	480	1 200	0.40

Figure 10.65 shows a sample composed of five different peptides with known amino acid sequences separated on µRPC C2/C18 SC 2.1/10. To find out in which order the peptides eluted, different absorbance ratios were calculated. In addition to the A254/A280 ratio, A215/A254 and A215/A280 ratios were calculated. Since the peptide bond contributes to the absorbance at 215 nm but not at 254 nm or 280 nm, these latter ratios provide information on the peptide's length verses its content of aromatic amino acid residues.

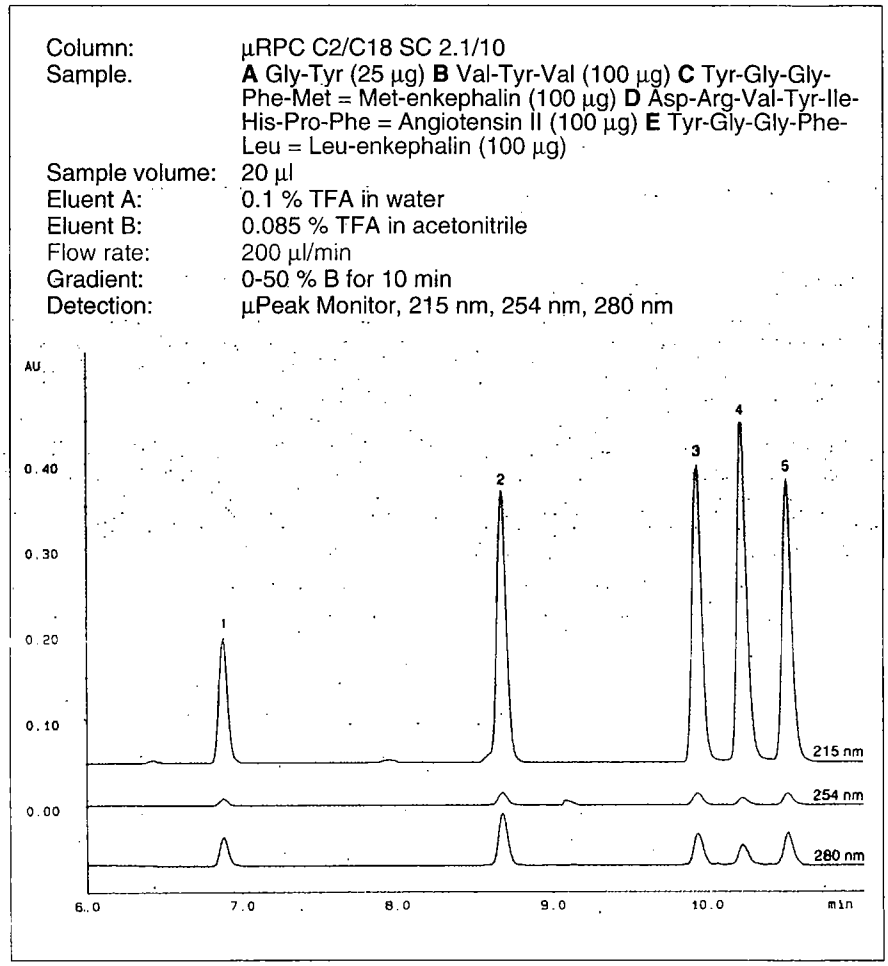


Fig. 10.65. Identification of peptides in a BSA digest by ratio calculations.

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Table 16. Absorbance (A) ratios obtained from Fig. 10.66.

Peak	A215/A254	A215/A280	A254/A280
1	23	5.8	0.25
2	28	6.9	0.25
3	33	12	0.38
4	61	21	0.35
5	31	11	0.37

Table 16 shows that peak 1 and 2 have a A254/A280 ratio = 0.25, indicating that they have one Tyr residue each (see Table 15). This corresponds to the di-peptides and tri-peptides in the mixture. Peak 1 has slightly lower A215/A254 and A215/A280 ratios compared to peak 2 indicating a shorter peptide length. Peak 1 should therefore correspond to the dipeptide (Gly-Tyr) and peak 2 to the tripeptide (Val-Tyr-Val).

Phe contributes significantly to the 254 nm absorbance so peptides containing both Tyr and Phe residues should have A254/A280 ratios around 0.4 (see Table 15). Table 16 shows that peaks 3, 4 and 5 have ratios of about 0.4, indicating a Tyr-Phe content of all three peptides.

Peak 4 has A215/A254 and A215/A280 ratios which are significantly greater than those of peaks 3 and 5 and should therefore correspond to the longest peptide in the mixture, which is angiotensin II. On the other hand, peaks 3 and 5, which have almost equal A215/A254 and A215/A280 ratios, should correspond to the two enkephalins in the mixture. However, it is not possible to distinguish between these since they only differ in the non-aromatic amino acid residues (Leu and Met).

Example 2

Figure 10.66a shows chromatography runs of two purified peptides (A and B) derived from bovine serum albumin (BSA). By multiple wavelength monitoring and ratio calculations, these peptides were identified in an enzymatic digest of BSA separated under the same conditions (Figure 10.66 b). The BSA was pyridyl-ethylated as described in (18) before being digested with Lys-C endopeptidase. Table 17 shows the absorbance ratios and retention times of the two peptides. The data strongly indicate that the two peptides A and B are identical with the fragments eluted in peaks 13 and 17.

Table 17. Absorbance (A) ratios and retention times of peptides A and B and fragments corresponding to peaks 13 and 17 of the BSA digest shown in Figures 10.68 a) and b) respectively.

	A215/ A254	A215/ A280	A254/ A280	Ret.time (min)
Peptide A	5	13	2.5	65.4
Peptide B	17	8	0.46	71.9
Peak 13	5	15	3.0	64.4
Peak 17	18	8.9	0.49	70.7

Comment to Table 17: The retention times of peptides A and B are slightly greater than those corresponding to the material in peaks 13 and 17. This is a frequent phenomenon which is presumably due to the solvent capacity of peptides in a mixture normally generating shorter retention times than when eluted separately.

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9.2.3 Checking the purity of a peak

Ratios can be used to check the purity of a peak. If a peak is pure, the absorbance spectra are the same over the whole peak and therefore the ratios should stay constant. Figure 10.67 a) shows a simulated example of that. The Figure shows chromatograms from two different wavelengths from a separation of two components. For component A the ratio between A 270 nm and A 255 nm is 3 and for component B the ratio is 2.

Column:	Sephasil C18 SC 2.1/10
Sample:	a) Purified peptides (A and B) from a BSA digest b) Pyridyl-ethylated BSA digested with Lys C endopeptidase, 70 pmol in 10 µl
Eluent A:	0.065% TFA + 1.5% B in water
Eluent B:	0.05% TFA in acetonitrile
Flow rate:	100 µl/min
Gradient:	0% B for 6 min, 0-41% B for 219 min, 41% B for 10 min
Detector:	µPeak Monitor, 215 nm, 254 nm, 280 nm

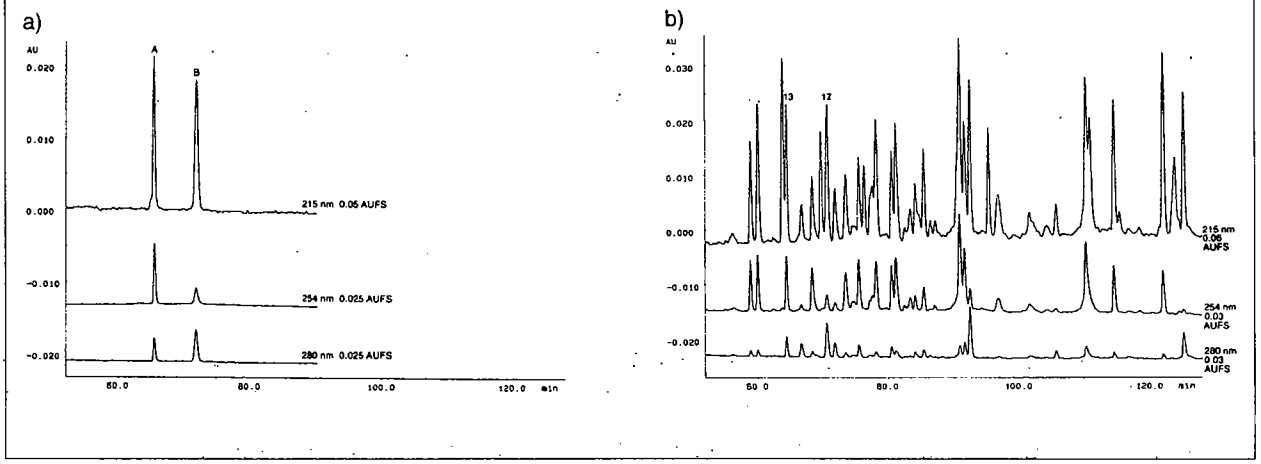


Fig. 10.66.a and b Identification of peptides in a BSA digest by ratio calculations

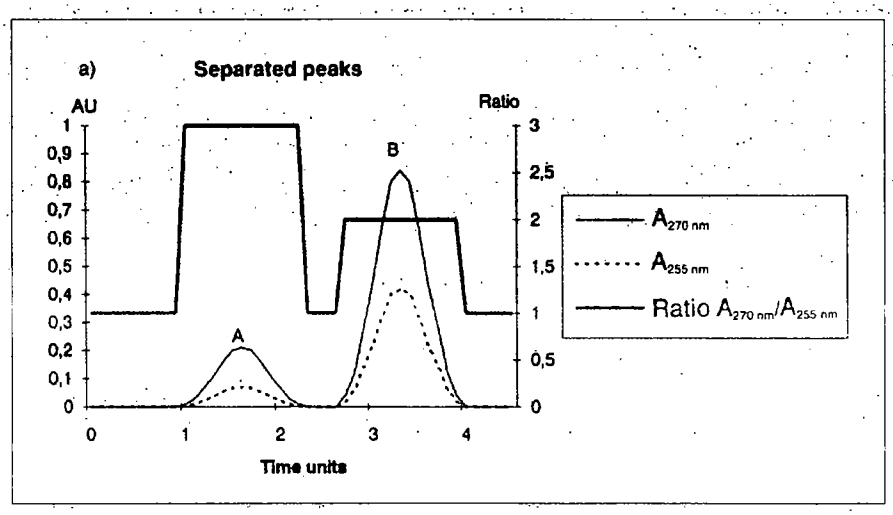


Fig. 10.67a. Simulated chromatogram of two components with differences in their absorbance spectra.

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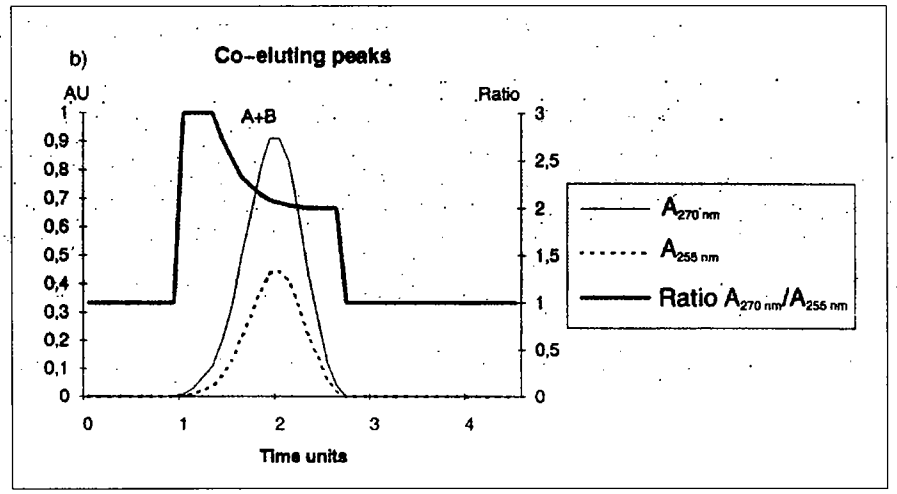


Fig. 10.67b. Simulated chromatogram of two co-eluting components with differing absorbance spectra and a small difference in retention time.

If the two components co-eluted (the small difference in retention times not showing), the corresponding chromatogram and ratios would appear as in Figure 67 b) The ratio calculation shows that at the beginning of the peak the ratio is 3, indicating that this part contains pure component A, while in the end the ratio is 2, indicating pure component B. In the middle of the peak where the ratio is changing there is a mixture of A and B.

9.3 Peak fractionation

9.3.1 Fractionation of a BSA digest

Micropreparative chromatography of complex samples that generate many peaks requires high precision fraction collection to obtain pure fractions.

A BSA digest, corresponding to 140 pmol of protein was separated on µRPC C2/C18 SC 2.1/10. Peaks were collected using the automatic peak fractionation function of SMART System. Figure 10.68 (upper chromatogram) shows this separation. The middle chromatogram shows an enlarged section with the fractions numbered. Fractions 22, 24 and 26 were re-injected after dilution with two volumes of 0.1 % TFA in water and eluted from the column with the same gradient as when eluting the BSA digest. The resulting chromatograms are shown in the lower part of Figure 10.68. Single peaks were collected from fractions 24 and 26 and the material analysed for amino acid sequence. The single peaks prove that the collection of fractions from the digest has faithfully maintained the resolution of the separation on the column.

When performing automatic peak fractionation, it is not necessary that the sample has been run previously, although this does help when setting peak volumes and peak start and end values. (See conditions for Fig. 10.68) These values can be set and changed during a separation to improve the fractionation. Fractionation can be controlled manually at any time by using the manual FEED TUBE command in SMART Manager. (Figure 10.69).

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Column:	µRPC C2/C18 SC 2.1/10
Sample:	Pyridyl-ethylated BSA digested with Lys-C endopeptidase, 140 pmol in 20 µl
Eluent A:	0.065% TFA in water
Eluent B:	0.05% TFA in acetonitrile
Flow rate:	100 µl/min
Gradient:	0% B for 6 min, 1-41% B for 160 min, 41% B for 14 min
Detection:	µPeak Monitor, 215 nm
Peak volume:	≤ 25 µl
Peak start:	12 mAU/min
Peak end:	10 mAU/min
Result of sequence analysis:	
	Fraction 24, Phe-Gly-Glu-Arg-Ala-Leu-Lys
	Fraction 26, Ser-Glu-Ile-Ala-His-Arg-Phe-Lys

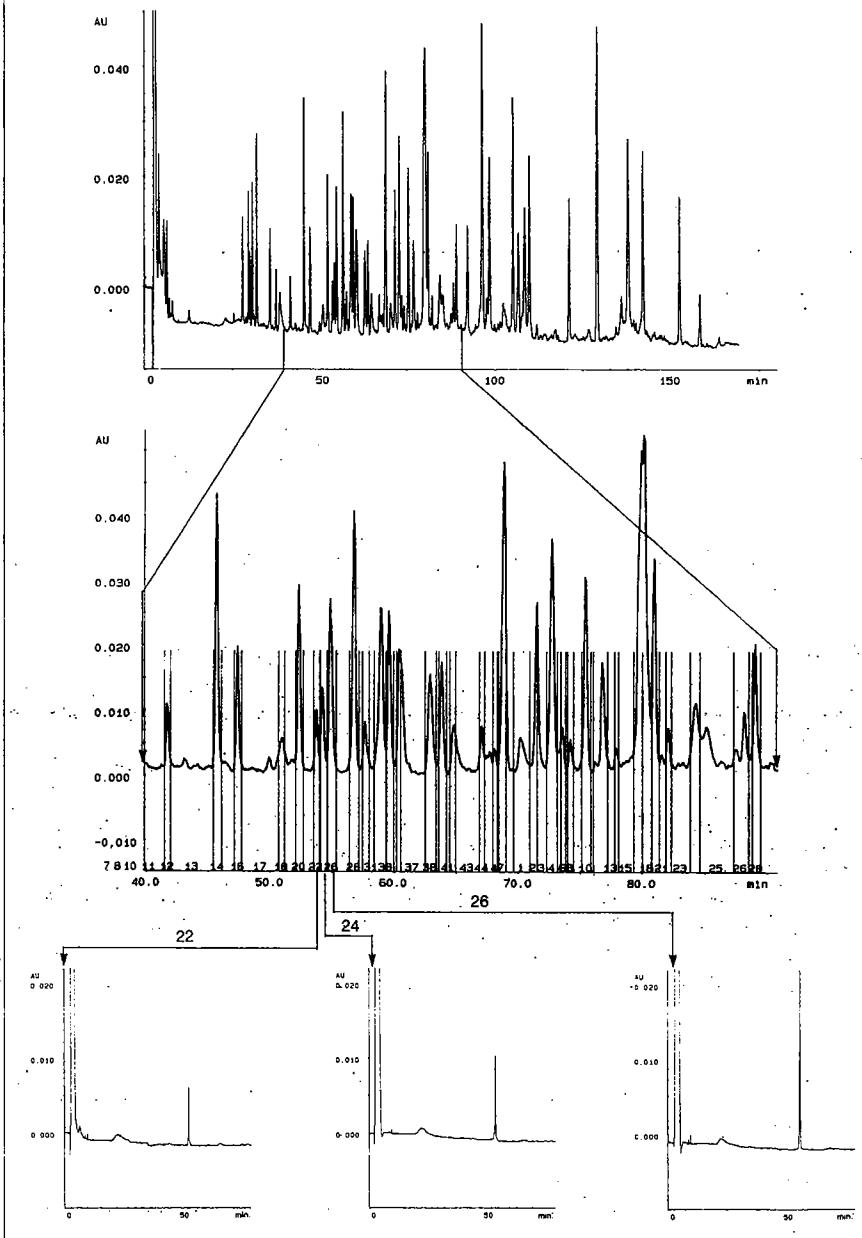


Fig. 10.68. 140 pmol BSA digest separated on µRPC C2/C18 SC 2.1/10 using automatic peak fractionation.

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9.4 Amino acid sequence analysis

The quality of sequencing results is often limited by the sample purity. SMART System, in combination with Precision Columns, makes high resolution separations with high recovery and pure fractions possible. In the following example, pure material for sequencing was obtained in a single step, despite the large number of fragments.

Bovine serum albumin was pyridylethylated to label Cys residues as described in (25) and digested with Lys-C endopeptidase. Pyridylethylated BSA digest corresponding to 70 pmol was separated. Manual peak fractionation was performed using the manual FEED TUBE command of SMART Manager to collect material eluted with the peaks (Fig. 10.69). Material corresponding to peak 23 and peak 54 was chosen for amino acid sequence analysis.

The amino acid sequence of the material eluted with peak 23 was determined to be Ala-Phe-Asp-Glu-Lys, a sequence explaining the high A254/A280 absorbance ratio of the fragment. Furthermore, the analysis of the material corresponding to peak 54 confirmed its Tyr and pyridylethylated Cys content (as suggested by the relatively high absorbance at A254nm and the A254/A280 ratio of 2.5). The amino acid sequences of both fragments are found within the BSA molecule (23).

Beyond the sensitivity limits of amino acid sequence analysis

Modern protein sequencing technology permits sequence data to be obtained at 5-10 pmol levels (32). When using micropurification to prepare material for sequencing, the factor that limits lower level is no longer the chromatography equipment (see Fig. 10.50, where 34 fmole insulin has been detected) but rather the nature of the peptide itself or the amino acid sequencer.

In gas phase sequence analysis, a hydrophobic peptide tends to wash off the resin after each cycle of the analysis. Despite this difficulty, confirmation of the N-terminal sequence of 10 pmol ß-casomorphin-8, a hydrophobic octapeptide which had been purified using SMART System, was possible (Fig. 10.70). However, 4 pmol of the peptide, which also gave a significant UV signal at 214 nm, was beyond the limits of sequence analysis.

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Column:	µRPC C2/C18 SC 2.1/10
Sample:	Pyridyl-ethylated BSA digested with Lys-C endopeptidase, 70 pmol in 10 µl
Eluent A:	0.065 % TFA in water
Eluent B:	0.05 % TFA in acetonitrile
Flow rate:	100 µl/min
Gradient:	0 % B for 6 min, 1-41 % B for 160 min, 41 % B for 14 min
Detection:	µPeak Monitor, 215 nm, 254 nm, 280 nm

Result of sequence analysis:
Peak 23 = Ala-Phe-Asp-Glu-Lys
Peak 54 = Asp-Asp-Pro-His-Ala-Cys-Tyr-Ser-Thr-Val-Phe-Asp-Lys

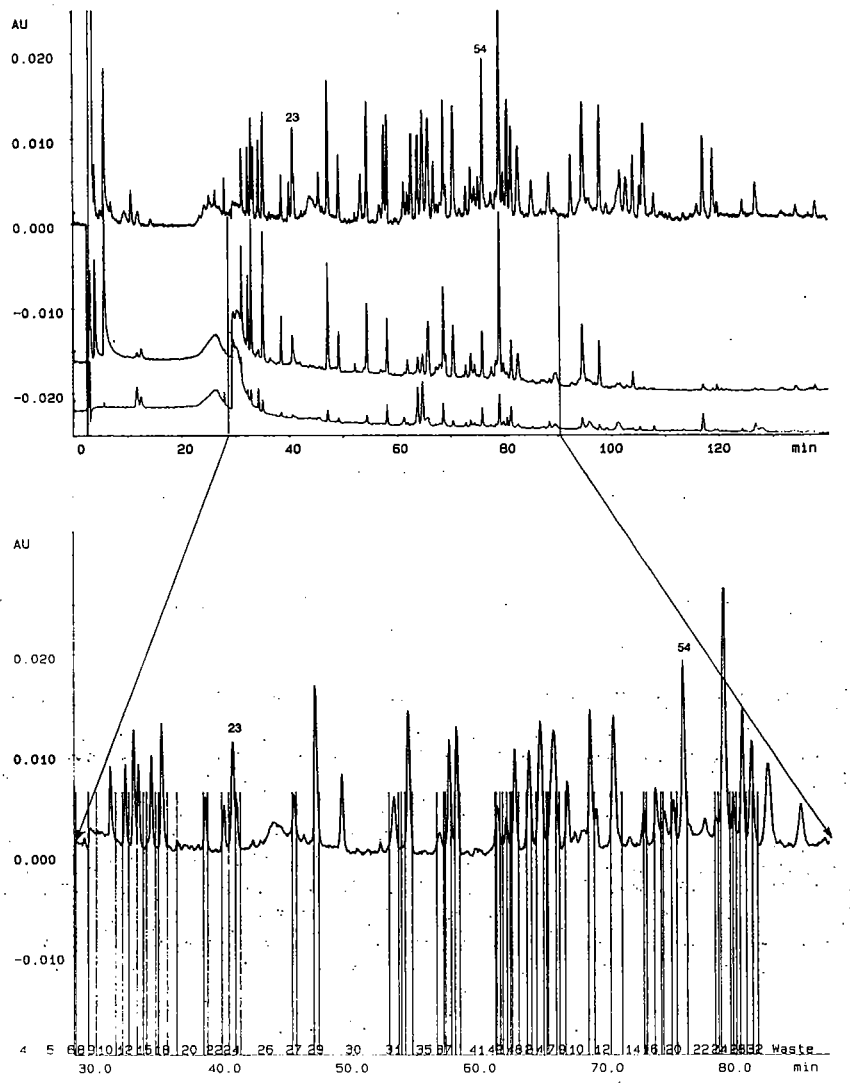


Fig. 10.69. Amino acid sequence analysis after a one-step purification of a BSA digest (70 pmol) on µRPC C2/C18 SC 2.1/10.

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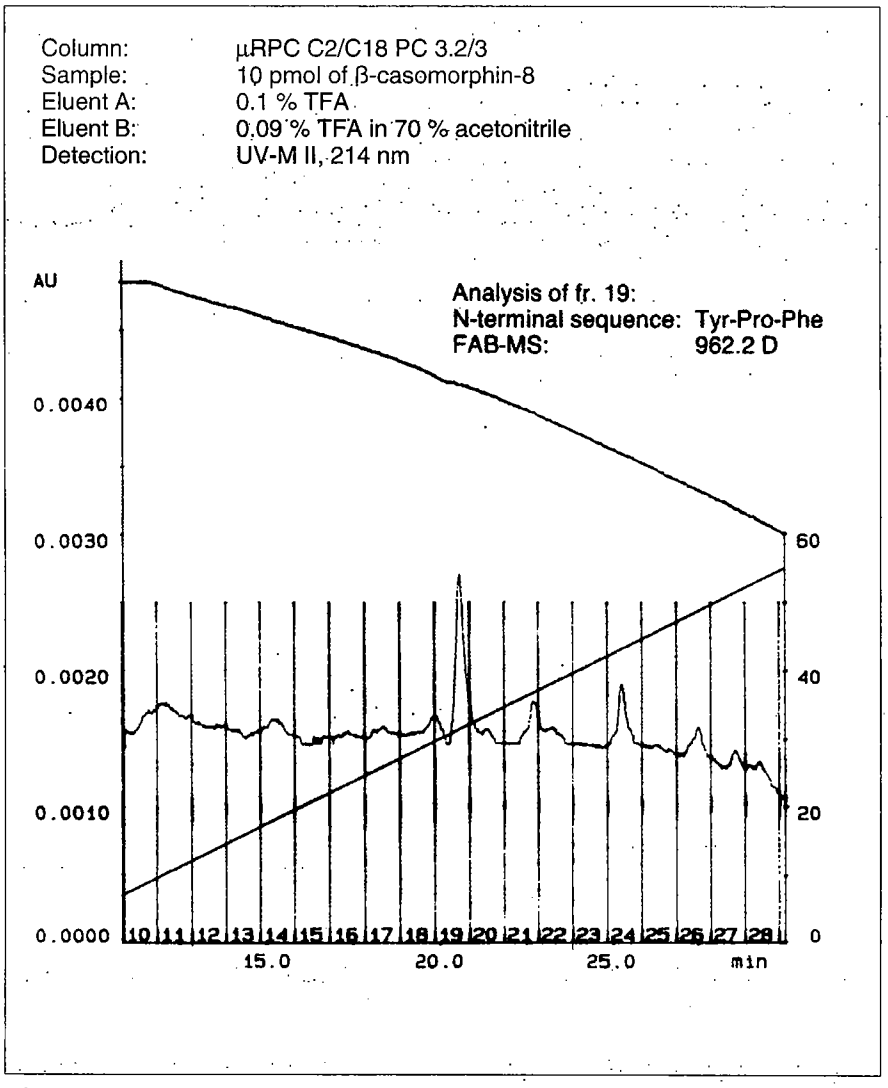


Fig. 10.70. Micropurification and desalting of ß-casomorphin on µRPC C2/C18 PC 3.2/3.

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