Applications and features of the Agilent 1100 Series micro fraction collector

Application

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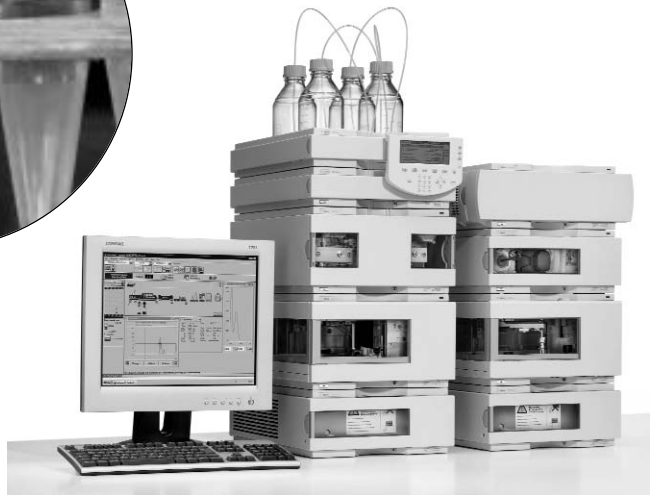

Abstract

To reduce the complexity of a sample it is often necessary to purify or fractionate samples prior to analysis. This holds especially true for applications in proteomics, metabolomics, natural product research and library generation where the sample is usually highly complex. If in addition, only a limited amount of the sample is available, the problem becomes much more challenging. For this purpose the Agilent 1100 Series micro fraction collector was developed. In combination with the Agilent 1100 Series capillary LC, the micro fraction collector offers a high performance solution for processing these special samples. This Application Note describes the field of application for the Agilent 1100 Series micro fraction collector and discusses the features and software control of this device. In addition, the main modules of the micro fraction collection system are also discussed - the Agilent 1100 Series capillary pump, the Agilent 1100 Series diode-array detector with 80-nL cell and the Agilent 1100 Series micro well-plate autosampler .

Agilent Technologies

Introduction

Life science researchers from several disciplines are often faced with the difficulty that they have samples of very high complexity. On one hand, a large number of compounds might be of investigational interest, or on the other hand a few compounds in a large background of undesired compounds might be the focus of the analysis. This is often accompanied with the problem that only a limited amount of sample is available.

These factors principally determine the strategy for the analysis of the sample. In order to process samples available in low concentrations only, the fractionation collection or purification device must be adapted. Therefore, the Agilent 1100 Series micro fraction collector[1], which is best suited to the Agilent 1100 Series capillary LC system, was developed.

Several strategies can be employed using capillary LC separation in combination with micro fractionation (figure 1). These include direct analysis after fractionation, chemical modification of compounds in the fractions with subsequent analysis of the derivatives or further fractionation in a second orthogonal separation dimension. An example of the last strategy is the analysis of proteome samples by off-line two-dimensional LC/MS. Here a digested proteome sample of high complexity is fractionated by SCX (strong cation exchange) chromatography in the first dimension with the Agilent 1100 Series micro fraction collection system. The tryptic peptides contained in the fractions are subsequently further separated with nano RP chromatography in the second dimension and analyzed by tandem mass spectrometry[2].

Further useful applications for the Agilent 1100 Series micro fraction collection system, could be the multidimensional fractionation of peptide samples in one LC system, for example, the purification of biomarkers, the isolation of metabolic compounds out of a complex biological matrix, compound isolation from combinatorial libraries and MALDI-TOF analysis[3] as well as purification of a small amount of a natural product for biological screening or fractionation of a very complex naturally occurring or synthetically gained mixture into a chemical library for screening of active compounds in drug discovery[4]. In addition, micro fraction collection allows storage of fractions for repeated analyses or running parallel analytical techniques like mass spectrometry in combination with other analytical techniques further downstream (figure 2). It is also possible to deposit small fractions on a MALDI-plate concurrent to an electrospray mass spectrometry analysis.

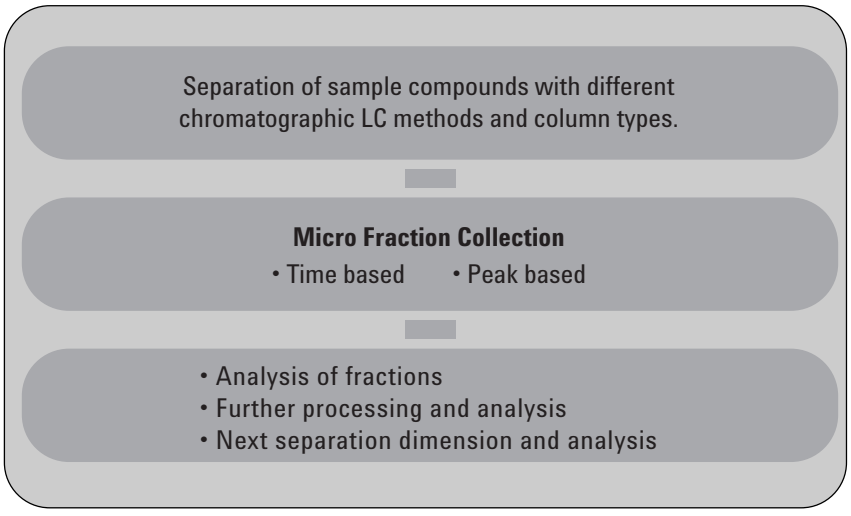

Figure 1
Fractionation strategies with the Agilent 1100 Series micro fraction collection system

Equipment

An Agilent 1100 Series micro fraction collection system[1] consisting of the following modules was used (figure 3):

- Agilent 1100 Series capillary pump with 20-µL or 100-µL flow sensor for capillary columns with 0.3–1.0 mm i.d. and micro vacuum degasser
- Agilent 1100 Series thermostatted micro well-plate autosampler
- Agilent 1100 Series diode-array detector with 500-nL cell or 80-nL cell
- Agilent 1100 Series thermostatted column compartment
- Agilent 1100 Series thermostatted micro fraction collector
- Agilent ChemStation A10.02

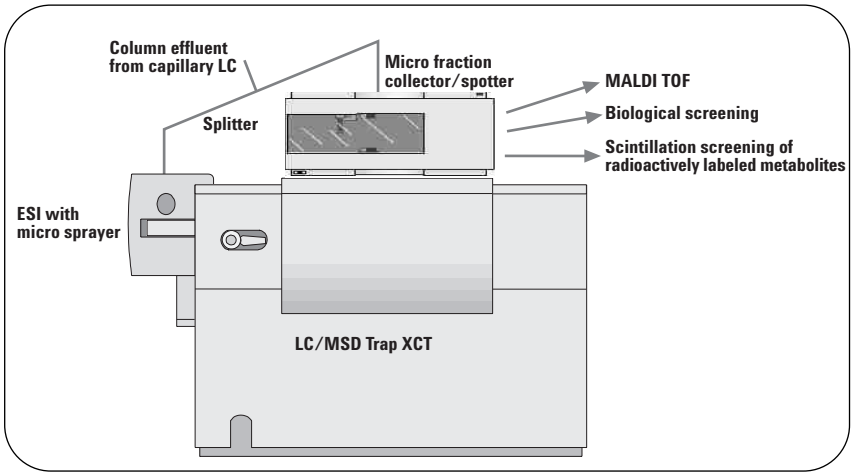

Figure 2
Parallel analysis with ESI MS and other downstream analytical methods of the collected micro fractions

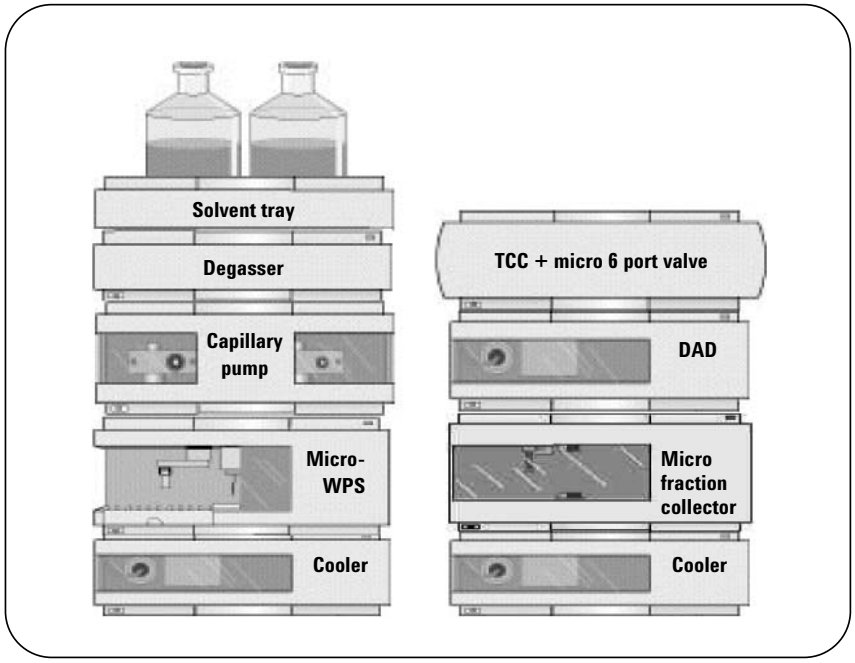

Figure 3
Components of the Agilent 1100 Series micro fraction collection system

Results and discussion

The Agilent 1100 Series micro fraction collector

The liquid contact control, a unique collection principle which is illustrated in figure 4, has been developed to reliably collect small volumes in the lower µL range. The Agilent 1100 Series micro fraction collector ensures that the droplet forming at the capillary tip is in contact with the bottom of the well at the beginning and in constant contact with the solvent during the remaining fraction collection process. At the start of fraction collection, the droplet being formed is delivered to the bottom of the well. As the well is being filled the capillary tip moves continuously upwards, keeping in constant contact with the solvent surface. At the end of fraction collection, the capillary tip moves sharply upwards, delivering the droplet currently being formed into the well. As the speed at which the well is filled is not only dependent on flow rate but also on the geometry of the well, both factors are taken into consideration during the collection process. The parameters of the liquid contact control mode, which can be controlled by the ChemStation software, are the distance between the capillary and the well bottom respective to the surface of the liquid as well as the capillary extraction speed. This method produces accurately collected fractions free from air bubbles. Cross contamination between fractions is also prevented using this approach. It is also possible to set the collection time for each fraction to a minimum of 3 seconds in order to collect very small fraction volumes using a time-based method and a given flow rate.

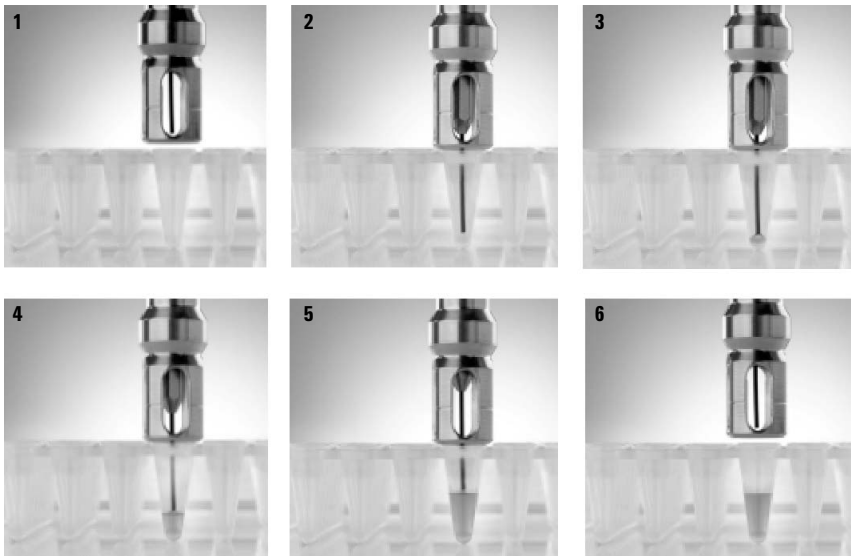

Figure 4
Liquid contact control is the basis for collecting micro fractions without cross contamination and air bubbles

Capillary i.d. [µm]	Flow rate [µL]	Delay volume [nL]
25	< 4	250
50	4 - 30	1000
100	30 -100	4000

Table 1
Delay volume and corresponding flow rate of the capillaries used in the Agilent 1100 Series micro fraction collector

Any component in the flow path which could contribute to additional delay volume, peak dispersion[5] or could be considered as a source of cross contamination is eliminated due to the low flow rates and liquid volumes. In particular a diverter valve, which switches the flow path between waste and fraction collection, if for example, a peak is detected, can be considered such an item. Therefore, there is no diverter valve in the micro fraction collector. The micro fraction collector has a complete flow-through design without any critical parts in the flow path between the detector and capillary tip. The delay volume caused by the capillary itself can be calculated approximately subject to the inner diameter of the capillary, which is used according to the flow rate (table 1). A delay calibration is recommended for a more precise determination of the delay volume, which also includes the capillary, the connection to the detector cell and the detector response time. To avoid possible cross contamination of the collected fraction

with waste compounds, the droplet formed at the capillary tip is continuously directed to the waste container by means of a special flap septum, which is in contact with the capillary when it is at the waste position.

The fractions can be collected into a variety of standard vials or well-plates of different geometry and brands with the Agilent 1100 Series micro fraction collector. In addition, Eppendorf cups (2.0 mL, 1.5 mL , 0.5 mL) can be used as collection devices. A wide variety of collection vessels are already preconfigured and can be chosen via the ChemStation software. Alternatively, user-defined collection devices may be added. To ensure that the liquid is accurately delivered into the well, the exact position and height of the capillary is automatically measured by a sensor pad during initialisation of the micro fraction collector. In addition, it is possible to calibrate the capillary position in the diagnosis mode of the ChemStation software especially for 384 well-plates.

The eluting compounds can be fractionated in a time-based or in a peak-based manner with the micro fraction collector. For instance, a complex cell lysate from yeast *(Saccharomyces cerevisiae)* was fractionated using a time-based method from an SCX chromatography in the first dimension of an offline two-dimensional LC/MS proteome analysis (figure 5). The fractions were collected every three

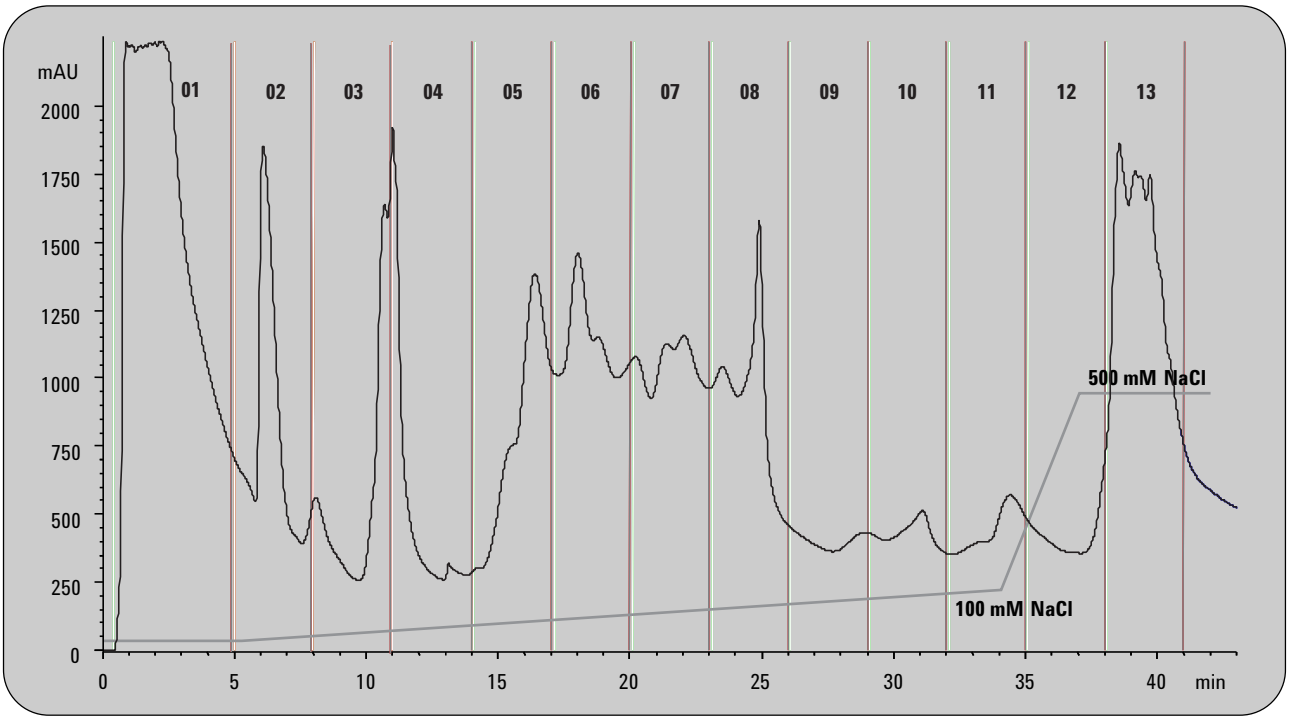

Figure 5
Time based fraction collection of a complex cell lysate from yeast

minutes at a flow rate of 5 µL/min. The tryptic peptides contained in these fractions were separated in the second dimension by nano RP chromatography and subsequently analyzed by MS/MS. After a database search with the acquired MS/MS spectra and comparison of the obtained protein hits with other methods, it was determined that the offline 2D LC/MS method using a micro fraction collection system for the first dimension separation is superior to other online 2D LC/MS methods for proteomics applications[2].

In complex compound mixtures as obtained, for example, from natural product extracts often only the single compounds are of interest for an initial biological screening accomplished with a small amount of the material. In this case, it is possible to fractionate the mixture with the micro fraction collector using a peak-based method (figures 6 and 7). Figure 6 shows the chromatogram with the peak-based collected fractions from a complex crude extract from the plant *Rheum palmatum*. These fractions were subsequently reanalysed, which proves the good separation of the compounds. This holds especially true for the peaks eluting between 16 and 17 minutes (figure 7). In this case, fractions with only a few micro liters were deposited precisely on the bottom of a conical shaped 384-well-plate.

Often it is necessary to obtain more material for screening tests. In this event the instrument allows pooling of fractions from multiple runs into the same wells of a given well-plate. In order to avoid any loss of column effluent from very valuable samples during peak-based collection, the ChemStation software "recovery on the track" tool was designed for the micro fraction collector. By using this feature, the complete column effluent in between fractions can be collected in the well-plate for

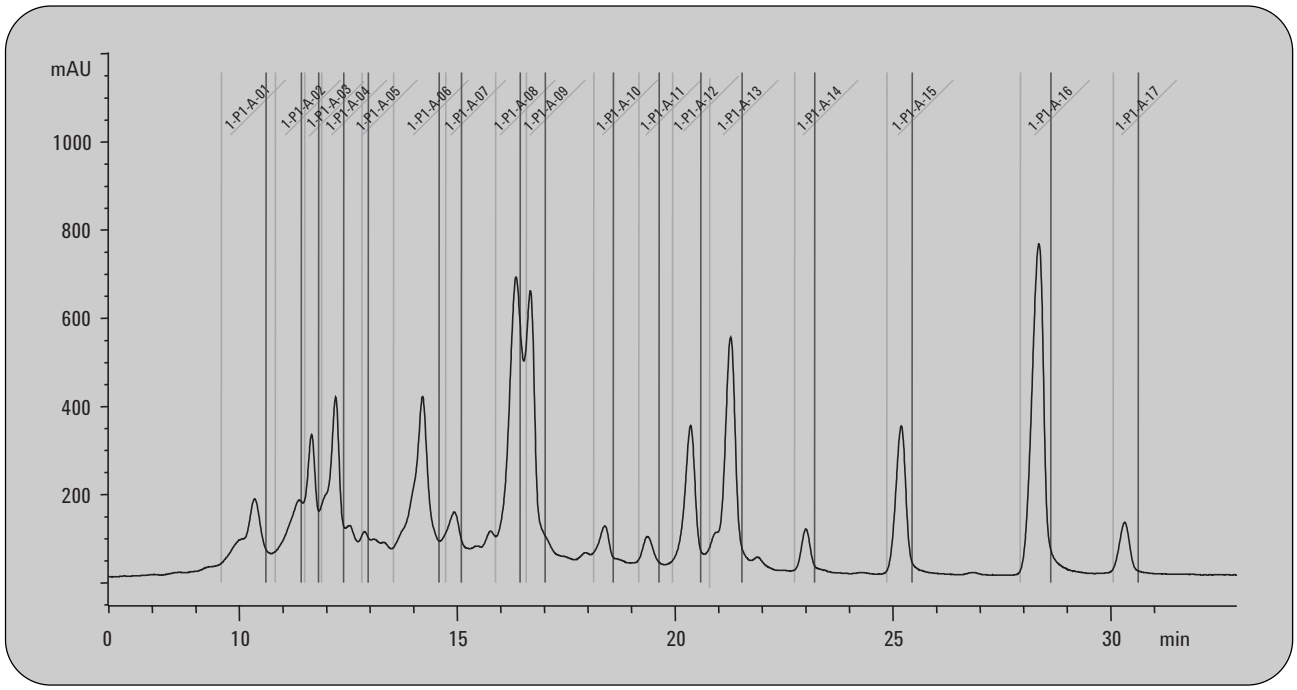

Figure 6
Peak based separation of a crude plant extract from *Rheum palmatum* with the Agilent 1100 Series micro fraction collection system

reanalysis and a new fraction is collected only when a peak is detected and matches the user defined peak-triggered fraction collection parameter. After the compound is fractionated, the micro fraction collector starts to collect the column effluent again in a new well. It is certainly possible to equip the micro fraction collector with a cooler, which prevents evaporation and decomposition of valuable samples. This thermostat is able to regulate the fractions between 4° C and 40° C.

Column:	ZORBAX SB C18, 0.3 x 150 mm, 5 µm
Capillary pump	
Solvent:	A = water + 0.1 % formic acid, B = AcN + 0.1 % formic acid.
Gradient:	0 min 5 % B, 3 min 5 % b, 33 min 65 % B, 36 min 85 % B, 45 min 85 % B.
Stop time:	45 min
Post time:	15 min
Column	
Flow rate:	10 µL/min
Micro well-plate autosampler	
Injection:	1 µL for purification and 10 µL for reanalysis (fractions were diluted to 10 µL with water).
Injector valve:	Bypass at 3 min, needle wash for 10 sec, cleaning procedure for lowest carry-over at 40 min
Diode-array detector:	80 nL flow cell, 254 nm +/- 8, ref. 360 nm.
Fraction collector:	Peak based between 8 min and 33 min, threshold = 50, up and down slope = 5

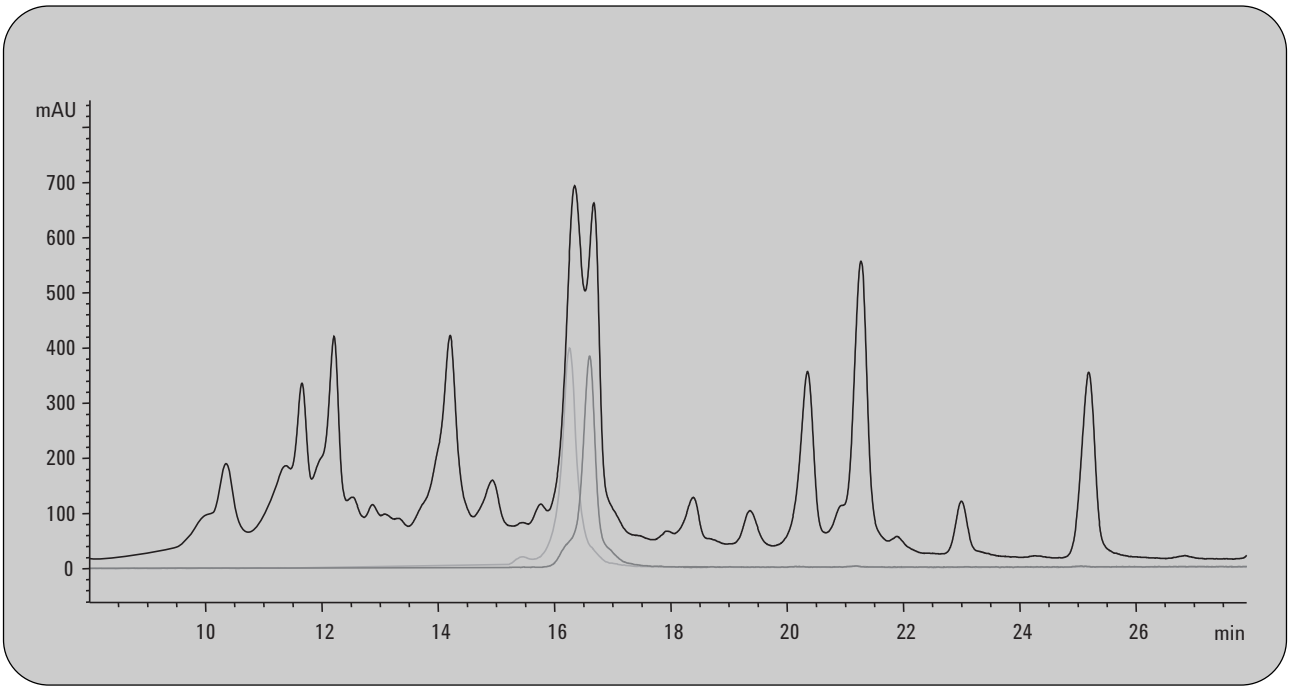

Figure 7
Reanalysis of collected fractions and separation of peaks eluting between 16 and 17 minutes

The Agilent 1100 Series capillary pump

For robust and reliable fraction collection in the lower micro liter range it is crucial that the pump delivers the flow with highest stability, thus ensuring retention time stability of the separated compounds. The Agilent 1100 Series capillary pump is equipped with a special Electronic Flow Control (EFC) which delivers a very stable and robust liquid flow in the micro liter flow rate range instead of a non-regulated passive splitter. The EFC divides the primary flow rate into the micro liter column flow and a waste flow. The micro liter column flow is monitored with a special flow sensor to control the active splitting ratio. The calibration curves for all common HPLC solvents and their mixtures are included in the ChemStation software for this sensor. The sensor signal drives the split ratio at the electromagnetic proportioning valve (EMPV) (figure 8a). The flow sensor[6] (figure 8b) consists of a stainless steel capillary, two temperature sensors and a heater around the capillary. If there is no flow through the capillary the temperature profile around the heater is symmetric. If there is a flow though the capillary the temperature profile shifts downstream. The shift in the temperature profile represents a temperature difference caused by the heat transport of the flowing fluid. The heat transport is proportional to the flow rate. Therefore, the sensor measures the flow of the fluid and delivers a feedback to the EMPV (figure 8c). This ensures an outstanding flow stability independent from system backpressure fluctuations. There are two flow sensors available, a 20-µL sensor and a 100-µL sensor for the capillary pump.

The composition accuracy and precision as well as the mixing noise was calculated with the results obtained from three runs with a tracer gradient from 0 to 10 % in increments of 1 %. For every pump this is the most critical performance range. The injection valve was switched into the bypass mode after one minute to exclude the well-plate sampler volume from the system delay volume. The primary flow rate was set to low solvent consumption and the column flow rate to
4 µL/min.

The main features of the capillary pump include:

- Accuracy of step height has an average deviation of 0.036 -0.066 %.
- The step height precision typically shows a standard deviation of 0.024 - 0.041 %.
- Mixing noise was typically < 0.027 % with the autosampler set to the mainpass position, 420-µL mixer installed and low solvent consumption.

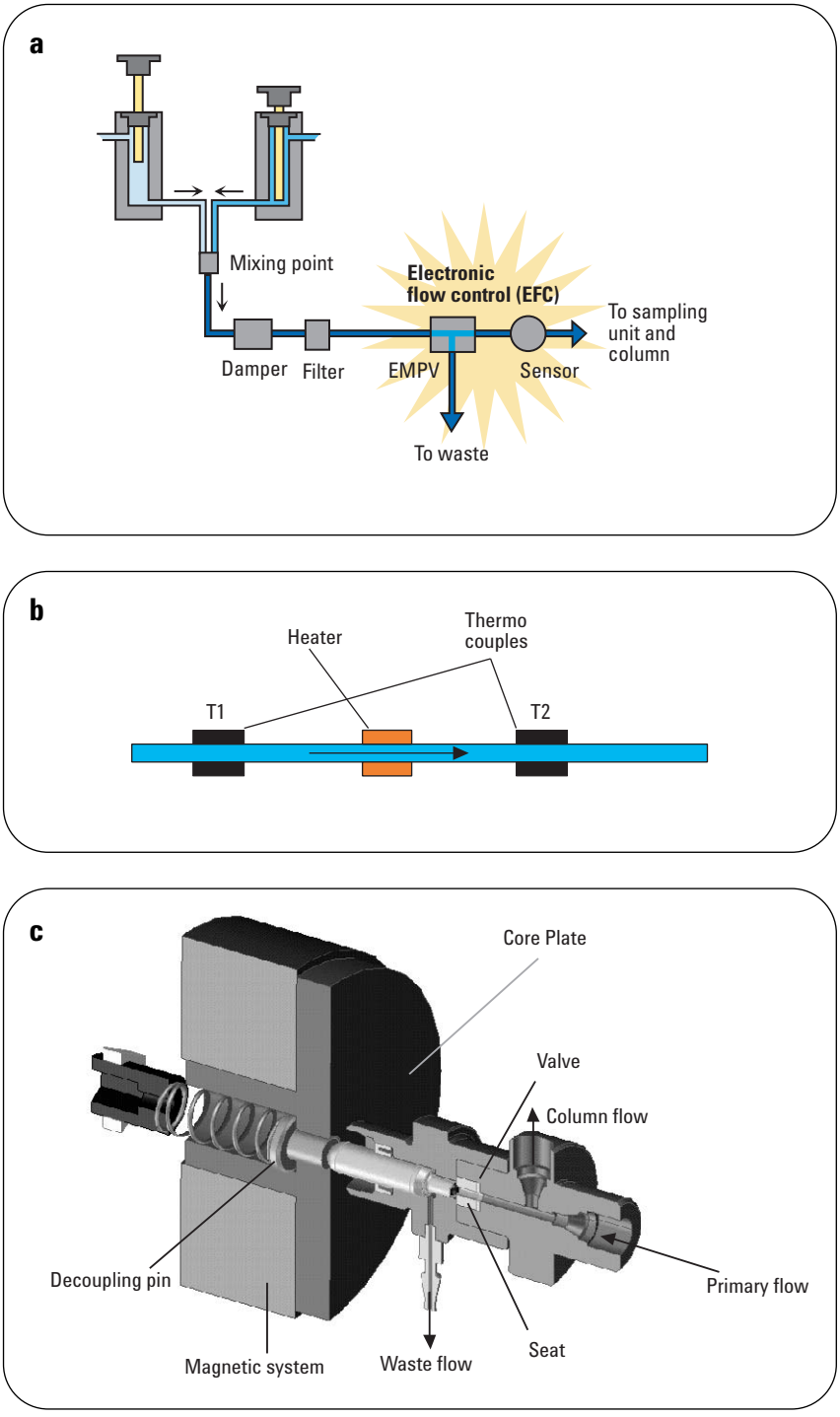

Figure 8
The electronic flow control of the Agilent 1100 Series capillary pump used in the Agilent 1100 Series micro fraction collection system.

These results demonstrate the extreme robustness of the capillary pump flow rates, which ensures the reliable and strong flow for fraction collection, and retention time stability. The measurements are described in detail in a separate Application Note about the Agilent 1100 Series capillary LC system[7].

Agilent 1100 Series micro well-plate autosampler

The application field of the Agilent 1100 Series micro fraction collection system covers a broad range of accessible sample volumes. Therefore, it is essential that the autosampler included in the micro fraction collection system is able to extract very low sample as well as larger sample volumes with high precision. The Agilent 1100 Series micro well-plate autosampler offers two different sample loops, an 8-µL and a 40-µL loop. The injection volumes are set in 10-nL steps beginning at 20 nL. The recommended minimum injection volume is 200 nL. The injection precision was determined by 1-µL injections of a 100 fmol BSA digest and measurement of detected peak areas of selected ions with ion trap MS. Under these conditions the RSD of the peak area was between 2.58 % and 3.45 %[8]. Due to the high value and limited volume of some samples it is also very important that the autosampler is able to extract as much sample volume as possible from a given volume out of the sample vial. In this case, with a vial-optimized method, the Agilent 1100 Series micro well-plate autosampler is able to extract

nearly the complete sample volume out of the vial. This can be accomplished using the "bottom sensing" feature of the autosampler. To inject low volume samples 300-µL wide open conical polypropylene vials (Agilent part number 5182-0549) and 100-µL polypropylene inserts (Agilent part number 9301-0978) are recommended. The Agilent 1100 Series micro well-plate autosampler is able to extract four full 2-µL injections out of 10-µL sample volume from these vial inserts[7] .

Carryover of compounds between different samples is always a critical issue for the autosampler. To determine the carryover for a small pharmaceutical molecule as well as for a large peptide several experiments were carried out[7,8]. Azithromycine, a macrolide antibiotic compound, which is very critical for carryover because it sticks to the valve in the autosampler, was tested to develop a special cleaning method to avoid carryover from the autosampler in general. This procedure consists of a capillary wash prior to the injection to clean the capillary exterior.

After the elution of all sample compounds the autosampler valve is switched two or three times between the bypass and mainpath position to clean the valve grooves in the high organic solvent stream. Figure 9 shows the results obtained using this cleaning method are shown in the extracted ion chromatograms. After the injection of 2-µg azithromycine water was injected to evaluate the carryover. Using the special cleaning procedure only < 0.01 % carryover of azithromycin could be detected compared to 0.95 % without this procedure[6].

Summary of the main features:
- Two different loop sizes – 200 nL minimum injection volume in 10-nL steps
- RSD of peak area is 2.58 %
- Four full 2-µL injections out of 10 µL
- Lowest carryover <0.01 % with special cleaning function

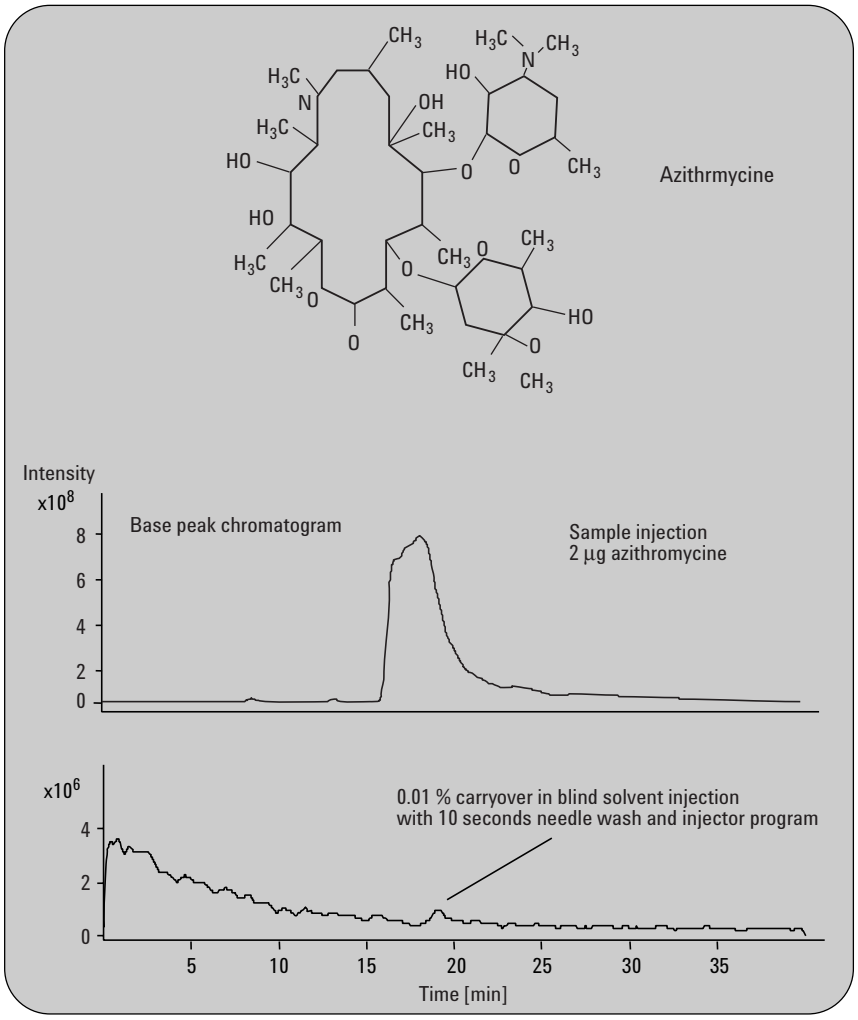

Figure 9
Analysis of azithromycine and evaluation of carryover using a special cleaning procedure

The Agilent 1100 Series diode-array detector with 500-nL and 80-nL flow cell

An Agilent 1100 Series diode-array detector equipped with an easily exchangeable 500-nL or 80-nL flow cell is integrated into the Agilent 1100 Series micro fraction collection system for peak detection and for triggering the micro fraction collector in the peak-based fraction collection mode. Both flow cells have an optimized geometry and flow path for minimized dispersion, high sensitivity and high resolution. Due to the low internal cell volume in the 80-nL flow cell, the peak width, the resolution and the chromatographic plates are improved compared to the 500-nL cell. More details about both flow cells are outlined in table 2 and described in a separate Application Note[9].

Conclusion

The Agilent 1100 Series micro fraction collection system is a tool for fractionation of samples in the lower micro liter range with lowest delay volume and peak dispersion. The fractions can be collected either in a high precision time or peak based manner using the "liquid contact control" mode, which precisely deposits small amounts of liquid with a minimum of 2 µL. To protect valuable samples from decomposition it is possible to equip the micro fraction collector with an additional cooler. Therefore, the Agilent 1100 Series micro fraction collection system can be used in proteomics, metabolomics and natural product purification. The Agilent 1100 Series micro fraction collection system includes the Agilent 1100 Series capillary pump, which delivers high precision micro liter flow rates, the Agilent 1100 Series micro well-plate autosampler, which is capable of injecting sample volumes in a broad range suitable to a large field of applications requiring highest precision and lowest carry over and the Agilent 1100 Series diode-array detector with the 500-nL or the 80-nL flow cell for highest sensitivity and resolution.

Flow cell volume [µL]	Path length [mm]	Operating range [bar]	Sensitivity of standard cell	Noise of standard cell	Recommended flow rate [µL]	Recommended column [mm i.d.]
500	10	0 - 50	1/3	3 x *	50 - 200	1 - 2
80	6	0 - 50	1/10	10 x**	0 - 50	0.3 - 0.5

* +/- 30µAU = 60µAU according to ASTM methode at 254 nm

** +/-100µAU = 200 µAU according to ASTM methode at 254 nm

Table 2
Performance of the 500-nL and 80-nL flow cells

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