

## Application Note

### ► Quantitative analysis of Caffeine using the KNAUER HPLC Educational system



<b>Category</b>	Pharmaceutical analysis
<b>Matrix</b>	Tablets
<b>Method</b>	HPLC
<b>Keywords</b>	HPLC educational system, introduction to HPLC, quality control, calibration, quantitative analysis, internal standard
<b>Analytes ID</b>	caffeine, paracetamol, theophylline (IS) VSP0009N_A_E

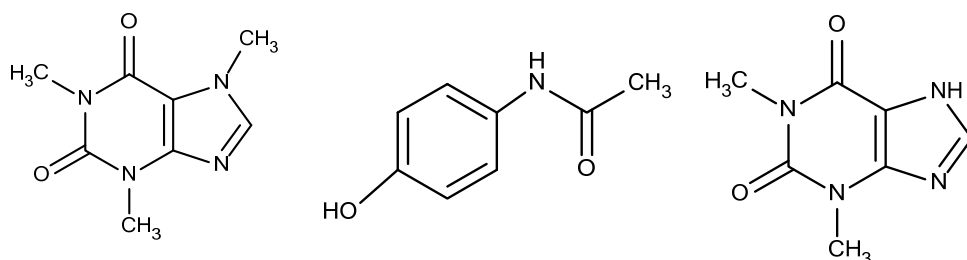
#### Summary

The KNAUER HPLC Educational system allows an easy and fast implementation of liquid chromatography (HPLC, high pressure liquid chromatography) and promotes a deeper understanding of this separation method. A simple example is given describing the determination of a sample containing caffeine and paracetamol. The separation of the components is demonstrated by HPLC and the substance peaks are assigned from the corresponding chromatogram. The concentration of caffeine is quantified using a multi-point calibration method.

#### Introduction

Caffeine and paracetamol are widely-used pharmaceutical components. Both substances are present as ingredients in many analgetics (so called "painkillers", see figure 1). Hence, they are often determined simultaneously in routine analysis. Theophylline, a substance chemically closely related to caffeine, is used to serve as an internal standard (figure 1).<sup>1</sup> To analyze the components, the KNAUER HPLC Educational system is utilized providing isocratic elution HPLC in combination with UV detection. The samples are injected via a manual injection valve. Based on the KNAUER AZURA Compact series, this system layout represents an easy and convenient solution for the current application.

The determination of a sample containing caffeine and paracetamol is a typical example from applied research for the implementation of the KNAUER HPLC Educational system. Numerous other applications are available. For additional video tutorials on software handling and data analysis please see [www.knauer.net](http://www.knauer.net).



**Figure 1**

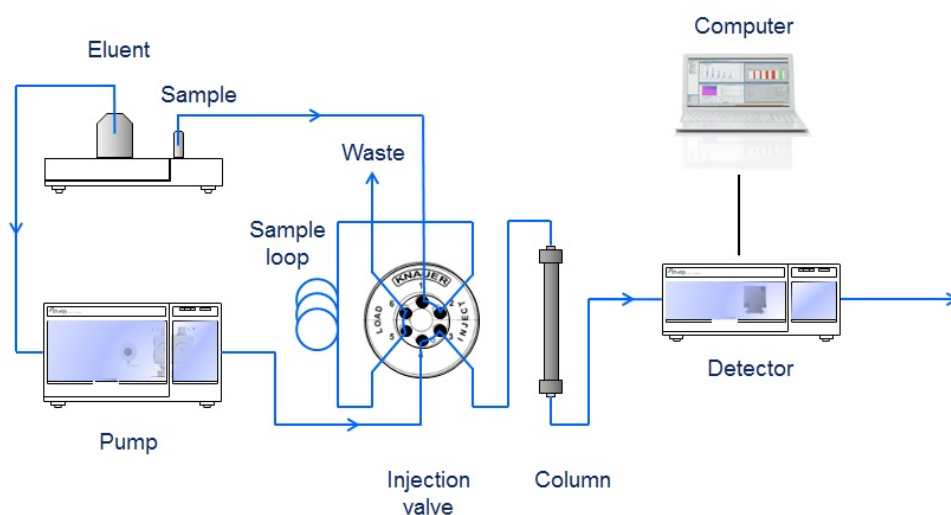
Chemical structures of the investigated compounds

## Principle of HPLC

Liquid chromatography is a well-established technique for the separation of substances. In particular, high performance liquid chromatography (HPLC) is a suitable method for the analysis of pharmaceutical samples.

The separation principle is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase. The specific intermolecular interactions between the molecules of a sample and the packing material define their time "on-column". Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample ingredients is achieved. A detection unit (e.g. UV detector) recognizes the analytes after leaving the column. The signals are converted and recorded by a data management system (computer software) and then shown in a chromatogram. After passing the detector unit, the mobile phase can be subjected to additional detector units, a fraction collection unit or to the waste.

In general, a HPLC system contains the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and a data processing unit (see figure 2).



**Figure 2**

Schematic layout of a HPLC system

The solvent (eluent) is delivered by the pump at high pressure and constant speed through the system. To keep the drift and noise of the detector signal as low as possible, a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by the injection valve.

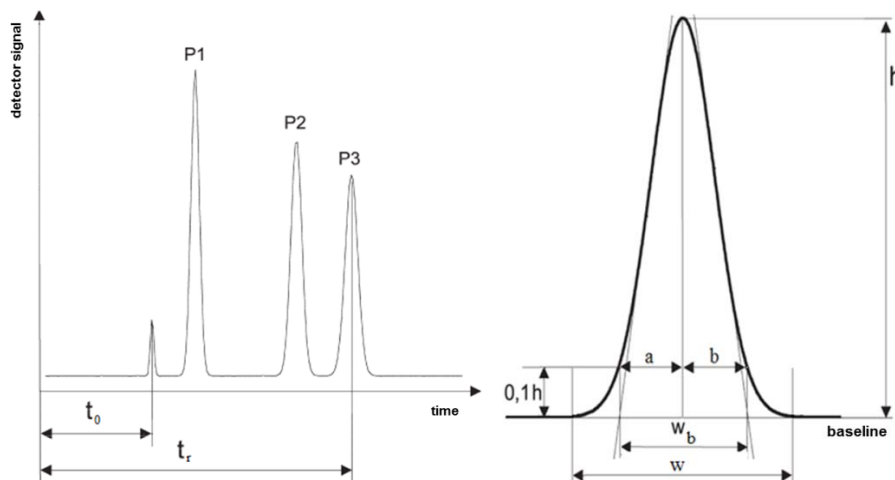
Depending on the composition of the mobile phase, two different modes are generally applicable. If the makeup of the mobile phase remains constant during the separation process, the HPLC system is defined as an isocratic elution system. When the composition of the mobile phase is changed during separation, the HPLC system is defined as a gradient elution system.<sup>2,3</sup> Using a gradient system, two different techniques are available: a low pressure gradient (LPG) and a high pressure gradient (HPG). A low pressure gradient means that the mixing of the solvents is carried out upstream of the pump (suction side). In a high pressure gradient system the different solvents are supplied by individual pumps and mixed after the pumps (discharge side).<sup>2</sup>

The column represents the heart of any HPLC system. It is responsible for the adequate separation of the sample ingredients. The separation efficiency correlates with the column inner diameter, the length of the column and the type and particle size of the column packing material. Depending on the desired application, numerous HPLC columns are commercially available. Different packing materials support different separation mechanisms – common are materials for normal-phase, reversed-phase, size exclusion, ion exchange, affinity, chiral, or hydrophilic interaction HPLC.<sup>2</sup>

The task of the detector unit is to register the time and amount of a substance which is eluted from the column. The detector perceives the change in the composition of the eluent and converts this information into an electrical signal which is evaluated by the aid of a computer.<sup>2</sup> A variety of detectors is available depending on the structural characteristics of the analyte. Common detector units are refractometric, UV/VIS, electrochemical and fluorescence detectors.

### Chromatographic parameters

The separated analytes which are transported by the mobile phase are recorded as signal peaks by the detector unit. The total amount of all peaks is called chromatogram. Each individual peak provides qualitative and quantitative information of the analyte. Qualitative information is given by the peak itself (e.g.: shape, intensity of the signal, time of appearance in the chromatogram). In addition, the area of a peak is proportional to the concentration of the substance. Hence, the chromatography data management software can calculate the concentration of the sample by integration. This provides quantitative information. Ideally the peaks are recorded as a Gaussian bell-shaped curve. A schematic example is illustrated in figure 3. The basic parameters of a chromatographic separation are discussed in the section below.



**Figure 3**  
Schematic illustration of a chromatogram<sup>4</sup>

#### Delay time $t_0$

The delay time refers to the time which is required for a non-retarded compound to be transported from the injection site to the detector unit (where the compound is recorded). During this time, all sample molecules are exclusively located in the mobile phase. In general, all sample molecules share the same delay time. The separation is caused by differing adherence of the substances with the stationary phase.

#### Retention time $t_r$

The retention time refers to the time which is required for a compound from the moment of injection until the moment of detection. Accordingly, it represents the time the analyte is in the mobile and stationary phase. The retention time is substance-specific and should always provide the same values under the same conditions.

**Peak width  $w$** 

The peak width covers the period from the beginning of the signal slope until reaching the baseline after repeated drop in the detector signal.

**Tailing factor  $T$** 

In practice, perfectly symmetric peaks are very rare. In a chromatogram they often show some degree of tailing. Peak tailing is measured by the tailing factor  $T$ . This factor describes the peak asymmetry, i.e. to which extent the shape is approximated to the perfectly symmetric Gaussian curve. The tailing factor is measured as:

$$T = \frac{b}{a}$$

$a$  represents the width of the front half of the peak,  $b$  is the width of the back half of the peak. The values are measured at 10% of the peak height from the leading or trailing edge of the peak to a line dropped perpendicularly from the peak apex (see figure 3).<sup>5</sup>

$T = 1$  represents a symmetrical peak. For  $T > 1$  the peak profile is named tailing. For  $T < 1$  the peak profile is named fronting.

**Multi-point calibration method**

In chromatography an internal standard represents a compound which is added to a sample in known concentration. It is used to facilitate the qualitative identification and/or quantitative determination of the sample components. An internal standard has to be very similar but not identical to the chemical species of the analyte. Moreover, it should not occur in the investigated sample. Therefore, theophylline is an appropriate internal standard for the quantitative analysis of caffeine (see chemical structures in figure 1).

Here, samples with a caffeine concentration in the range of 5-80 g/ml are sequentially injected into the HPLC system. Each concentration level is injected three times in the system for sufficient data analysis. A known quantity of the internal standard theophylline is added to each of the diluted samples to balance pipetting or injection errors.

All samples are analyzed via HPLC using a multi-point calibration method. For this purpose, five different concentrations of the analytes are examined. The concentration of the internal standard remains constant. For each measurement, the concentration and the peak area of the analyte are compared with those of the internal standard. Additionally, the HPLC software generates a single calibration curve by correlating the peak areas and the given concentration of caffeine. The resulting calibration curve enables the determination of the caffeine concentration from an unknown sample by measurement of the peak area.

**Preparation of standards**

At first, individual stock solutions are prepared from caffeine, paracetamol, and theophylline. The initial weight of the substances should be about 100 mg. However, it is important to note the exact sample weight in order to obtain accurate results for the quantitative analysis. The substances are dissolved in 10 ml of methanol and sonicated to yield stock solutions of approximately 10 mg/ml.

To identify the individual substances directly by HPLC, the substances are diluted 1:100 with water (cf. table 1).

**Table 1**

Initial weight and dilution of stock solutions

	initial weight [mg]	final concentration stock solution [mg/ml]	final concentration diluted solution [µg/ml]
caffeine	99.3	9.9	99.3
theophylline	113.2	11.3	113.2
paracetamol	107.7	10.7	107.7

Secondly, a single calibration solution is prepared from the caffeine and paracetamol stock solution. For this purpose, 50 µl of the caffeine stock solution and 50 µl of the paracetamol stock solution are combined and diluted with water to a final volume of 5 ml (1:100 dilution). Thus, each individual substance has a concentration of approximately 100 µg/ml.

For HPLC analysis, the dilution levels of the calibration solution should cover a range from 5-80 µg/ml. To ensure a correct measurement, at least four different dilution levels should be achieved. For the current application, five dilution levels (cf. table 2, dilution factor: 0.05, 0.2, 0.4, 0.6 and 0.8) have been prepared. The corresponding solutions are named standard 1 - 5 (volume: 1.00 ml).

An additional calibration solution of the internal standard becomes necessary for quantitative HPLC analysis. For this purpose, 100 µL of the theophylline stock solution are diluted with water to a final volume of 1.00 mL (1:10 dilution, concentration approx. 1 mg/ml). Subsequently, a volume of 20 µL (final concentration approx. 20 µg/ml) of this solution is added to standard 1 - 5.

The concentration of caffeine in the final dilution stage is calculated by addition of the internal standard, i.e. it is important to ensure the final volume to be included. Here, 20 ml of the internal standard have been added to the volume of the standard (1.00 ml). This yields a total volume of 1.02 ml. Accordingly, the concentration of caffeine relates to a volume of 1.02 ml (see table 2).

**Table 2**

Caffeine standard 1 - 5

caffeine standard	projected caffeine concentration (V = 1.00 ml) [µg/ml]	actual caffeine concentration (V = 1.02 ml) [µg/ml]
1	5	4.9
2	20	19.5
3	40	38.9
4	60	58.4
5	80	77.8

**Preparation of the sample**

The sample is solid (tablet) and contains caffeine and paracetamol of an unknown concentration. The sample is accurately weighted and completely dissolved in 10 ml of methanol. Subsequently, it is diluted 1:100 with water.

Similarly to the standard solutions, the internal standard theophylline (20 µl, final concentration approx. 20 µg/ml) is added to 1.00 mL of the sample solution.

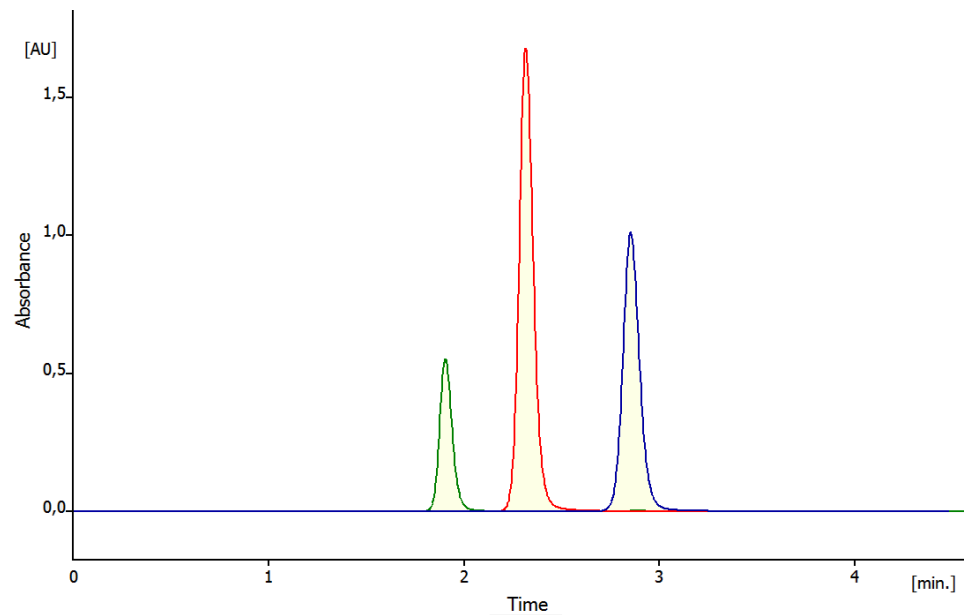
After proper mixing of the sample solution, it is ready for HPLC analysis.

**Method parameters**

column	Eurospher II 100-5 C18, 125 x 4 mm
eluent	water/methanol 60:40 (v/v)
flow rate	0.8 ml/min
injection volume	10 µl
column temperature	room temperature
system pressure	approx. 110 bar
detection	UV at 273 nm
run time	4 min

## Results

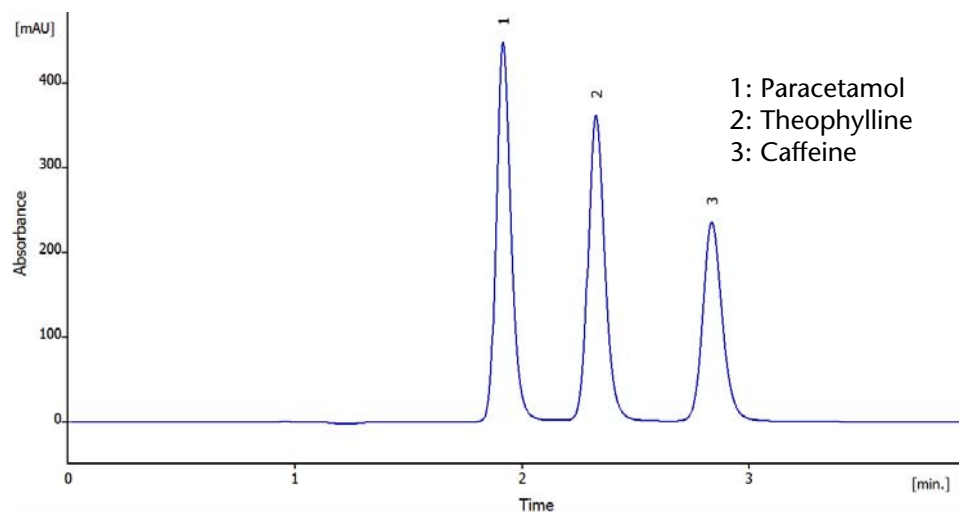
Figure 4 shows the overlay of the chromatograms of the individual stock solutions containing caffeine, paracetamol, and theophylline. The measurements indicate sufficient baseline separation. The substances can be separated and identified simultaneously under the given conditions.



**Figure 4**

Superposition of chromatograms of the individual stock solutions (green: paracetamol, red: theophylline, blue: caffeine)

Subsequently, a mixture of the three stock solutions is analyzed. The chromatogram is shown in figure 5.

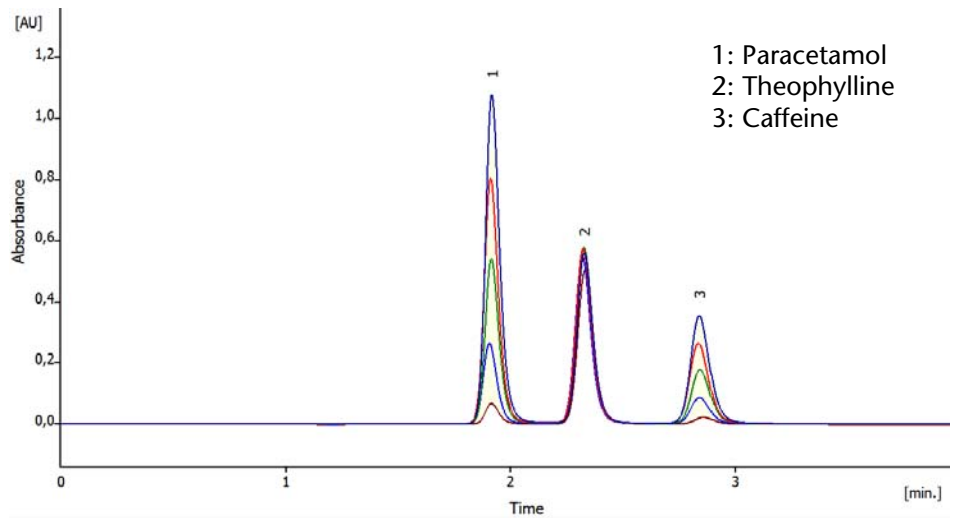


**Figure 5**

Chromatogram of mixed stock solutions

To calibrate the system, the standard solutions (see table 2) are injected into the system to analyze the peak areas. Each standard solution is injected three times to ensure sufficient data acquisition.

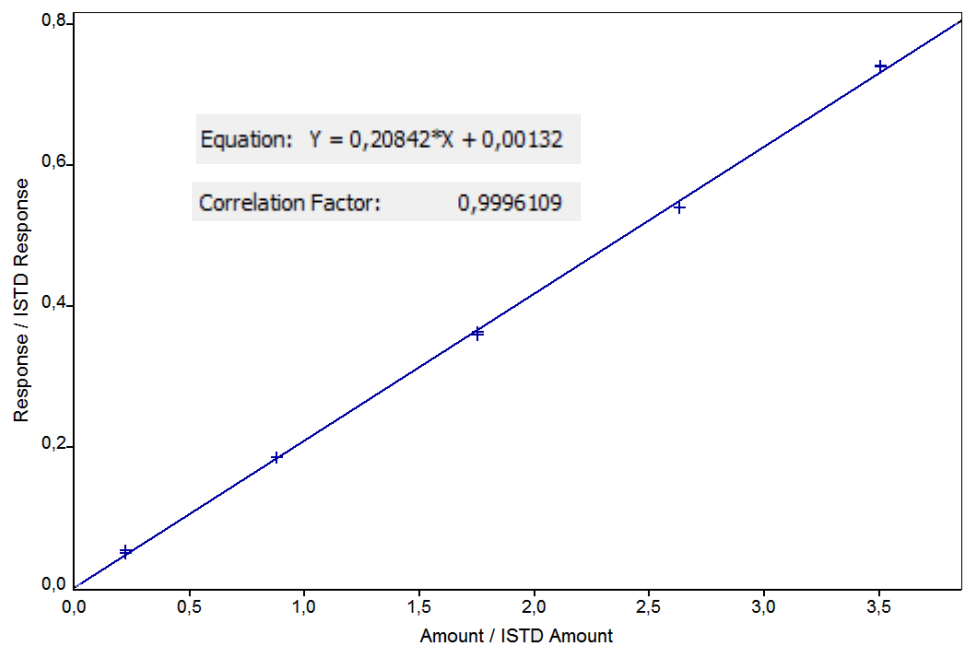
An overlay of the chromatograms corresponding to the five standards is illustrated in Figure 6. An increase in the sample ingredients (caffeine, paracetamol) results in an increase of the peak area. Note that the concentration of the internal standard remains



**Figure 6**  
Overlay of standard solution chromatograms

The HPLC software correlates the peak areas of the standard solutions and the peak area of the internal standard for each concentration (method: .ISTD calibration curve).

The obtained calibration curve for caffeine is shown in Figure 7. In the ideal case, the calculated correlation factor is 1. The present example shows that the calculated correlation factor is 0.9996109. Hence, the chosen method is suitable and reproducible for the quantitative analysis of caffeine.



**Figure 7**  
Calculated ISTD calibration curve

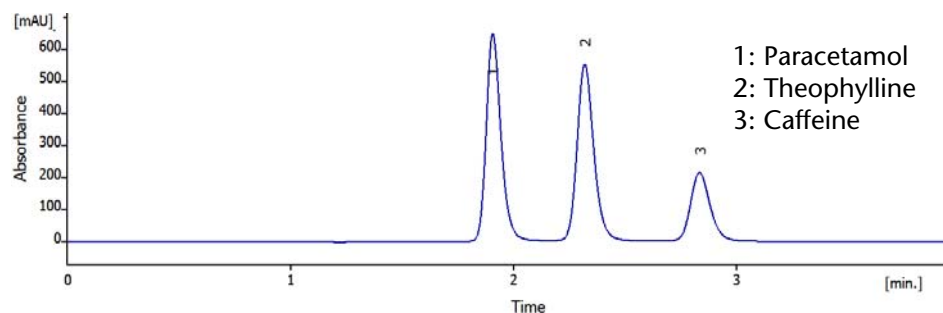
Subsequently, the unknown sample is analyzed by HPLC using the calibration curve method. Figure 8 shows the corresponding chromatogram.

The software automatically calculates the concentration of caffeine from data of the calibration method.

As a result, a caffeine concentration of 46.7 µg/ml in the sample is evaluated (see result table in Figure 8). The total caffeine content of the unknown sample is calculated from the initial weight and the dilution factor.



With the initial weight of 105.0 mg (sample) and the dilutions of 1:10 (solvent: methanol) and 1:100 (solvent: water) plus the addition of the internal standard, the total caffeine content in the solid sample is 47.6 mg. This equals a caffeine concentration of 45 percent by mass.



**Figure 8**

Quantitative analysis of the unknown sample and determination of the caffeine concentration

Result Table (ISTD - 130508\_Coffein\_Probe - ASM2.1LUV)

	Reten. Time [min]	Response	Amount [µg/ml]	Amount [%]	Peak Type	Compound Name
1	1,903	3032,161	N/A	N/A	Error	Paracetamol
2	2,317	2923,127	ISTD	ISTD	ISTD	Theophyllin
3	2,833	1327,957	46,665	100,0	Ordnr	Coffein
	Total		46,665	100,0		

## Conclusion

The KNAUER HPLC Educational System provides both, a qualitative and quantitative analysis of caffeine from different chemical probes. The system is compact, very simple to operate and can be ideally used for practical training courses.

The integrated chromatography data system ClarityChrom offers an intuitive system configuration, control and the evaluation of data.

More detailed information on hardware and software is available by videos and manuals which will be delivered with the system.

## References

- 1 Entry: *internal standard*. In: [IUPAC Compendium of Chemical Terminology \(the "Gold Book"\)](#). doi:10.1351/goldbook.i03108.
- 2 Meyer, V. R.: *Practical High Performance Liquid Chromatography*. 5. Aufl. Chichester: WILEY, 2010.
- 3 Böcker, J.: *Chromatographie*. Würzburg: Vogel Buchverlag, 1997.
- 4 Kromidas, S.; Kuss, H.-J.: *Chromatogramme richtig integrieren und bewerten: Ein Praxishandbuch für die HPLC und GC*. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA, 2008.
- 5 Dolan, J. W.: *Peak Tailing and Resolution*. LC•GC Europe, 2002.

## Authors

**Dr. Silvia Marten**, Head of Columns and Applications Department, KNAUER

**Mareike Margraf**, Columns and Applications Department, KNAUER

**Dr. Christian Textor**, Product Management, KNAUER



### Properties of the column



Eurospher II is characterized by outstanding mechanical and chemical stability and also offers excellent peak symmetry for acidic, basic, and neutral compounds.

<b>Stationary phase</b>	Eurospher II 100-5 C18
<b>USP code</b>	L1
<b>Pore size</b>	100 Å
<b>Pore volume</b>	0.8 ml/g
<b>Specific surface area</b>	320 ± 20 m <sup>2</sup> /g
<b>Particle size</b>	5 µm
<b>Shape</b>	spherical
<b>Carbon content</b>	16 % C
<b>Endcapping</b>	yes
<b>Dimension</b>	125 x 4 mm
<b>Order No.</b>	12DE181E2J

### System



With the AZURA HPLC Educational system we want to support you in applied science. For this purpose we customized the ideal AZURA-based training package for you. It contains the isocratic HPLC AZURA Compact unit including manual injection valve, pump, column, detector unit plus eluent tray and eluent supply bottles. Hence, beginners in HPLC techniques will achieve an easy follow and understanding of practical aspects, e.g. injection, pumping, separation, detection and evaluation.

To control the instrument and to illustrate the data analysis, the system is equipped with Laptop and HPLC software ClarityChrom.

Description	Order No.
Complete system	AYLXBACA-E
Components:	
AZURA Assistant for Compact HPLC isocratic, educational system; Pump P4.1S with 10 ml pump head stainless steel, UV/VIS Detector UVD 2.1S, manual injection valve	AYLXBACA
Analytical flow cell for UV detection, 10 mm path length, 10 µl volume, 1.1 mm ID, 1/16", stainless steel	A4061
AZURA Haltewinkel	A9853
Manual injection valve with 20 µl sample loop	A1357
Injection syringe (100 µl volume)	A0726
Start-up kit 1/16"	A9851
Magnetic clip for HPLC column	A9847
ClarityChrom Educational system license (2 offline licenses)	A1672-5
Laptop	A1304-1
LAN-Router (8-port)	A64808

Optional accessory:

AZURA Eluent tray E 2.1L for up to 6 x 1000 ml bottles	AZC00
Set of eluent supply bottles (2 x 1000 ml)	A5324-1
AZURA tool kit	A1033
Cutter for PEEK tubing	A0851

### Contact information

Wissenschaftliche Gerätebau  
Dr. Ing. Herbert Knauer GmbH  
Hegauer Weg 38  
14163 Berlin, Germany

Tel: +49 30 809727-0  
Fax: +49 30 8015010  
E-Mail: [info@knauer.net](mailto:info@knauer.net)  
Internet: [www.knauer.net](http://www.knauer.net)