

## Application Note

# ► UHPLC-MS method for the quantification of steroid hormones

Category	Pharmaceutical
Matrix	-
Method	UHPLC-ESI-MS
Keywords	Steroid hormones, Pharmaceutical, Forensic
Analytes	Cortisone, Corticosterone, Deoxycorticosterone, Norgestrel, Progesterone, Testosterone
ID	VPH0043N, 08/11

PLATIN blue  
by Knauer

### Summary

In this application note we present a method combining the fast UHPLC separation and the mass sensitive and exact MS detection of six steroid hormones. The developed UHPLC-ESI-MS method allows for the qualification and quantification of six steroids in only one run. A binary high pressure gradient is used at a flow rate of 0.2 ml/min. By applying a BlueOrchid 175-1.8 C18 column in the dimension 50 x 2 mm ID, analysis time could be reduced to less than 2.5 minutes and the total run time to 4 minutes when a washing step and re-equilibration of the column is included. The sensitive and exact detection of the steroid hormones is figured out using the MSQ Plus single quadrupole mass spectrometer.

### Introduction

Steroids are a unique class of compounds in which all structural variation is centered on a common conjugated ring system (see fig. 1). Double bonding and various functional groups lead to chemical diversity (see fig. 2). The three main classes of sex steroids are androgens (like testosterone), estrogens and progestagens (like progesterone). These steroid hormones are all derived from cholesterol and the most important human derivatives are testosterone and estradiol. Progesterone is the most important and only naturally-occurring human progestagen. Steroid hormones are widely used throughout the body and are involved in numerous regulatory pathways including immune response, sexual differentiation and metabolic function. This widespread activity makes steroids an important class of therapeutic compounds and often drug monitoring is required to minimize numerous side effects.<sup>1</sup> Endocrine disrupting chemicals in the environment are also a topic of growing concern. A variety of commonly used chemicals have endocrine-disrupting properties. But sex hormones have the most estrogenic potential.<sup>2</sup>

Human excretion and agricultural runoff are the primary sources of steroids. Environmental studies of wastewater have shown that compounds are generally not affected by standard wastewater treatment practices. Such systemic effects often require drug monitoring. The analytical determination of steroid hormones as micropollutants and also the clinical determination in the blood are no easy tasks, first, because of the complexity of the matrices, and second, because of the usually extremely low concentrations of the target compounds. Thus, to achieve the required sensitivity and selectivity for their analysis at physiologically active concentrations (pg-ng/L in water), time-consuming procedures are required. For many years, the environmental determination of steroid sex hormones has been dominated by the use of biological techniques, like immunoassays, and gas chromatography/mass spectrometry (GC/MS). However, recently, the application of liquid chromatography/mass spectrometry (LC/MS) has experienced rapid growth, in part due to the instrumental developments afforded in this field. GC/MS analyses are usually carried out after derivatization of the analytes with bis(trimethylsilyl)trifluoroacetamide (BSTFA) what means an additional step is needed that raises the probable errors of the method.<sup>3</sup>

Because steroids are neutral compounds, the selection of an alkyl (i.e. C18) stationary phase is the first choice. The C18 reversed phase BlueOrchid column is able to separate the analytes on the basis of overall hydrophobicity plus additional polar interactions in high speed mode. Related to the more traditional columns packed with 3 and 5  $\mu\text{m}$  particles the advantages of sub 2  $\mu\text{m}$  columns are shorter analysis times, improvements in resolving power, sensitivity and peak capacity. The present work describes a rapid gradient UHPLC method for simultaneous determination of six steroid hormones in less than 2.5 min.

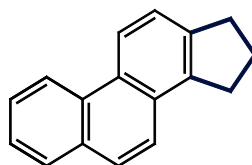
### Experimental preparation of standard solution

A 1 mg/ml steroid solution (single standard) was prepared in 50:50 (v/v) water-acetonitrile. The mixture of steroid hormone standard was prepared with concentrations of 0.05 mg/ml up to 0.17 mg/ml for every single compound in 50:50 water-acetonitrile for calibration of the system.

### Experimental sample preparation

Due to the complex sample matrix steroid hormones are often present in, a hydrolysis procedure is needed to remove proteins and peptides (acidic hydrolysis or enzymatic hydrolysis) before running the sample cleaning SPE procedure. Acid hydrolysis can be realized by adding 750  $\mu\text{l}$  hydrochloric acid to 5 ml sample, heating of sample for 60 min at 100°C (oven or water bath), adding 0.1 N sodium hydroxide to adjust the pH value (pH 7). The recommended sample cleaning for purifying human plasma or urine samples is SPE technology. The most often used SPE cartridges are based on C18 material (Thermo HyperSep C18 SPE tube; 200 mg bed weight; 3 ml column volume). The cartridge is conditioned in sequence with 2 ml of 8:2 (v/v) water-acetone, 2 ml of methanol and 2 ml of water. The hydrolyzed or unhydrolyzed sample is added to the cartridge. Wash the cartridge in sequence with 2 ml of water and 2 ml of 8:2 (v/v) water-acetone. The elution of the steroids is done with 1 ml methanol. Collect the eluent, evaporate it and reconstitute it in 100  $\mu\text{l}$  of acetonitrile, methanol or HPLC eluent starting conditions.

### Phenanthrene



Cyclopentane

Fig. 1

Base structure of steroid molecules

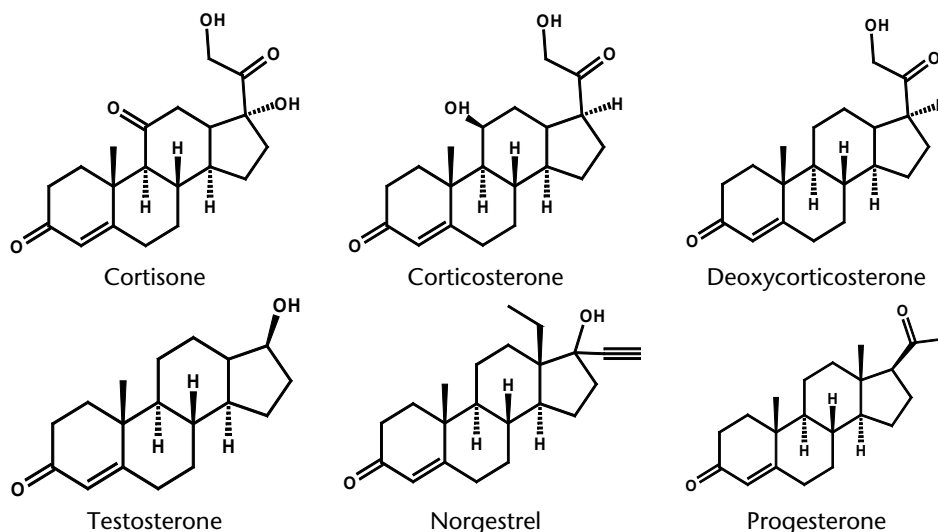


Fig. 2

Chemical structures of the analyzed Steroids

## Method parameters

<b>Column</b>	BlueOrchid 175-1.8 C18, 50 x 2 mm ID		
<b>Eluent A</b>	Water + 0.1 % Formic Acid		
<b>Eluent B</b>	Acetonitrile + 0.1 % Formic Acid		
<b>Gradient</b>	<b>Time [min]</b>	<b>% A</b>	<b>% B</b>
	0.00	60	40
	1.00	60	40
	1.50	10	90
	2.00	10	90
<b>Flow rate</b>	0.4 ml/min		
<b>Injection volume</b>	5 µl		
<b>Column temperature</b>	40 °C		
<b>Run time</b>	3.0 min		
<b>MS Detection Parameters</b>	Ionization mode	ESI, positive mode	
	Needle Voltage	3.5 kV	
	Cone Voltage	20 V	
	Probe temperature	350 °C	
	Mode	Full scan mode, m/z 270 – 370	

## Results

For MS detection, the characteristic mass spectra for every compound were figured out. The fragment with the highest intensity is chosen for quantification. Fragment ions with lower intensities can be used as qualifiers in real samples. Parameters important for MS detection are shown in table 1.

**Table 1**

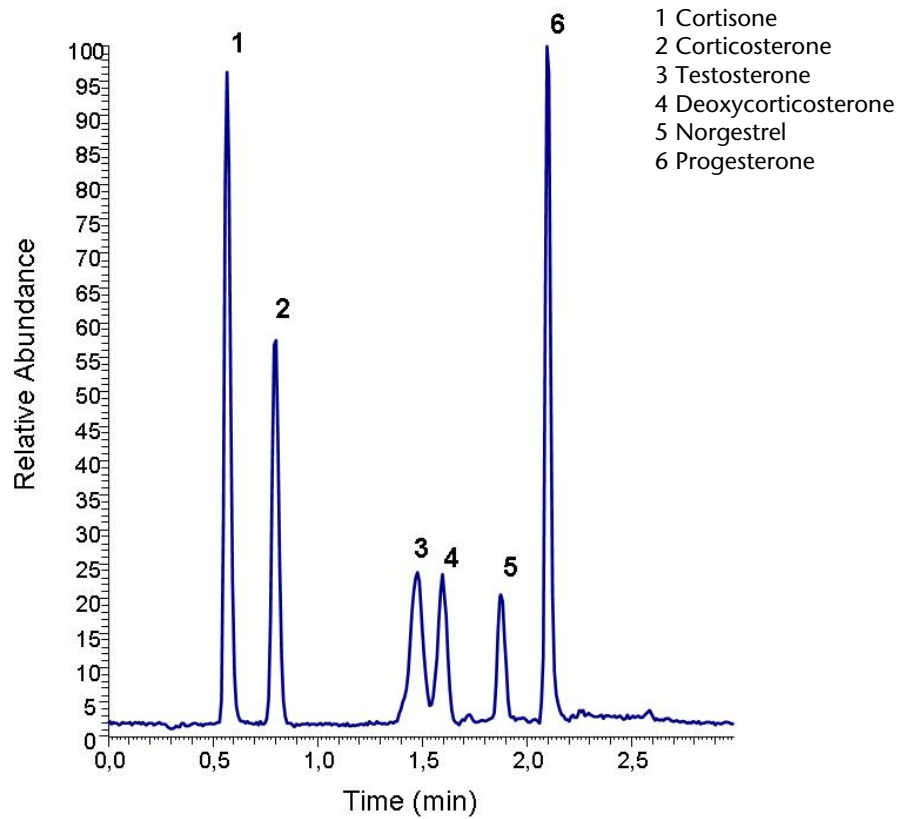
Analyzed steroids and their MS Parameters

Steroid	Ionisation	Mass [g/mol]	m/z expected [M+H] <sup>+</sup>	m/z found
Corticosterone	ESI +	346.4	347	347
Cortisone	ESI +	360.4	361	361
Deoxycorticosterone	ESI +	330.5	331	331
Norgestrel	ESI +	312.4	313	313
Progesterone	ESI +	314.5	315	315
Testosterone	ESI +	288.4	289	289

All analyzed steroid hormones were detected as expected as singly charged quasi-molecular ions [M+H]<sup>+</sup>. Therefore, this mass was at any one time chosen for quantification.

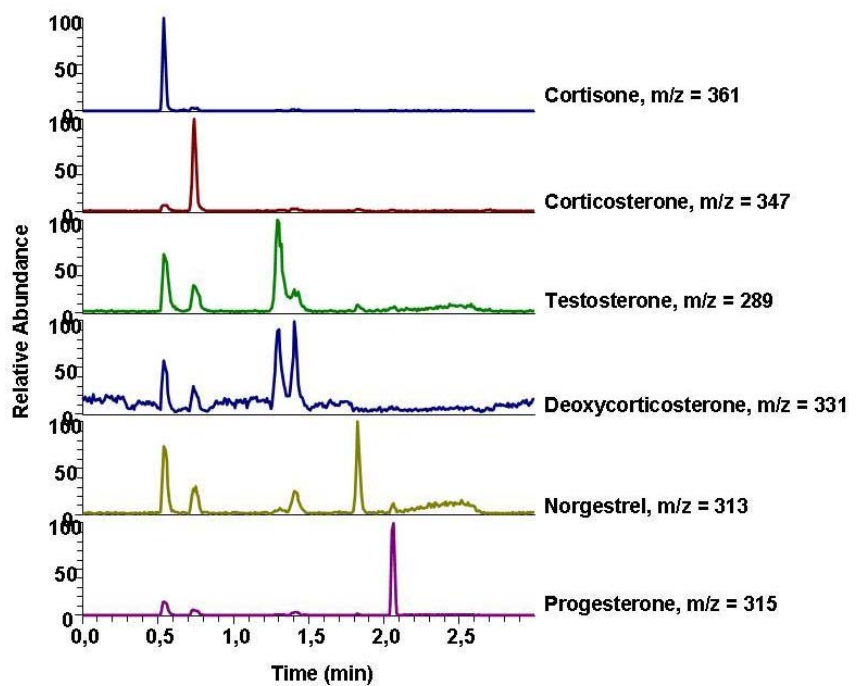
For the separation, a BlueOrchid 175-1.8 C18 50 x 2 mm ID column was chosen. The resulting total ion chromatogram (TIC) shown in figure 3 clearly shows the chromatographic separation of all Steroids in less than 2.5 minutes. The scan range of m/z = 270 – 370 was chosen as narrow as it could be.

Calibration was carried out for every steroid hormone using the method of external standards and the resulting calibration curves are shown in figures 6 - 10. The linear calibration functions and the correlation coefficients are shown above the curves. For integration, the TIC was used.

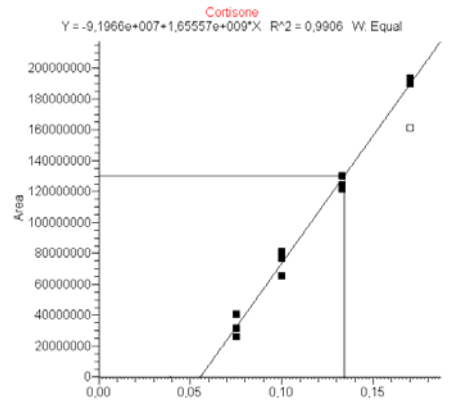
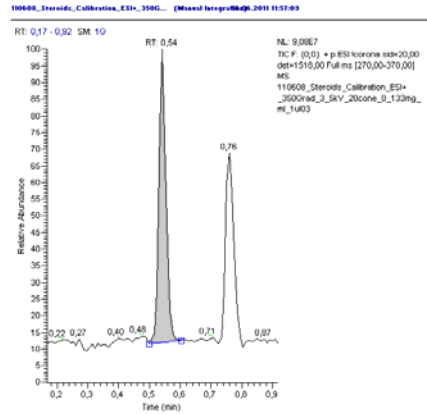


**Fig. 3**  
 Total Ion Chromatogram of the steroid standard

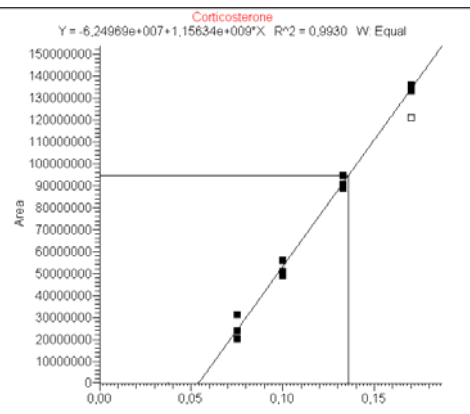
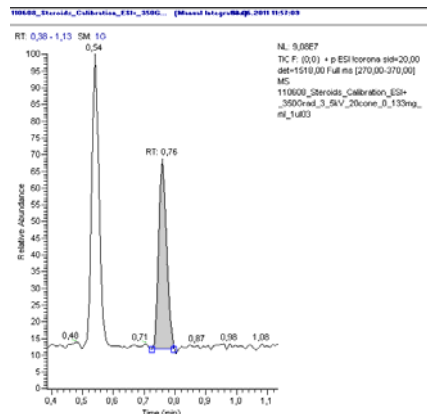
Using the Xcalibur software, the user is able to extract the single ion chromatograms (SICs) for every single compound from the TIC which gives much sharper and singly occurring peaks that are easier and more exact to quantify. The SICs for the quantified masses are shown in figure 4.



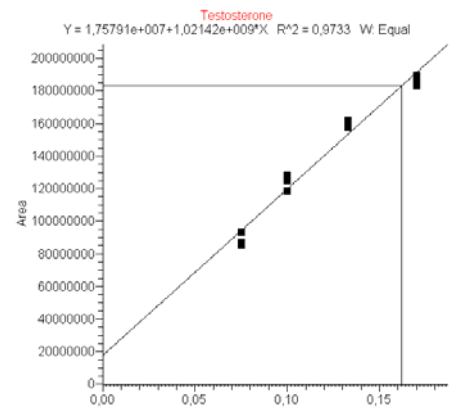
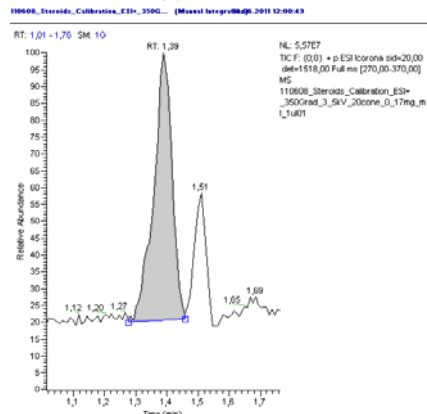
**Fig. 4**  
 SICs extracted from grouped SIM scans of the steroid hormone standard



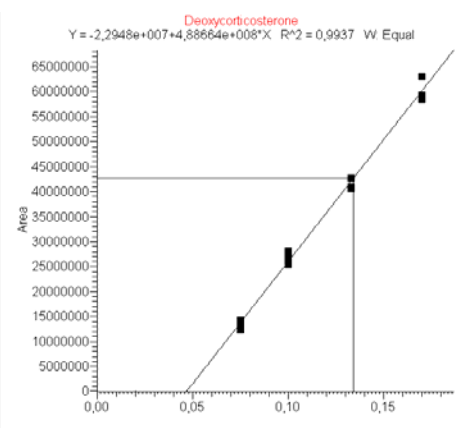
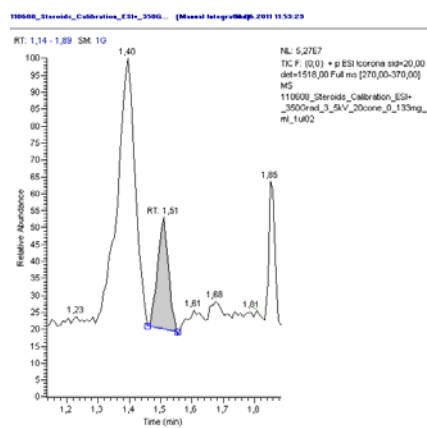
**Fig. 5**  
 Calibration curve of Cortisone (0.075 - 0.170 mg/ml)



**Fig. 6**  
 Calibration curve of Corticosterone (0.075 - 0.170 mg/ml)



**Fig. 7**  
 Calibration curve of Testosterone (0.075 - 0.170 mg/ml)



**Fig. 8**  
 Calibration curve of Deoxycorticosterone (0.075 - 0.170 mg/ml)

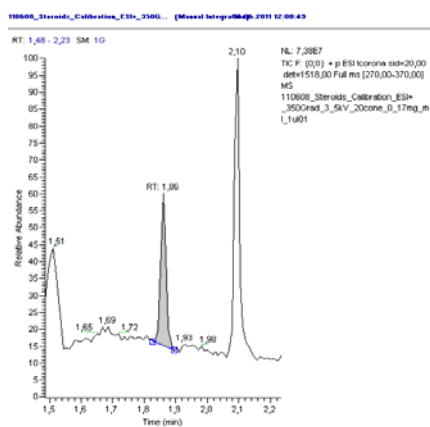


Fig. 9

Calibration curve of Norgestrel (0.075 - 0.170 mg/ml)

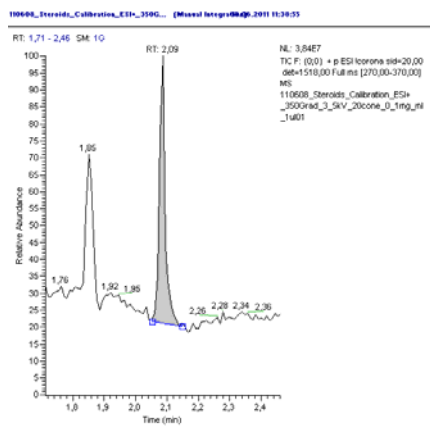
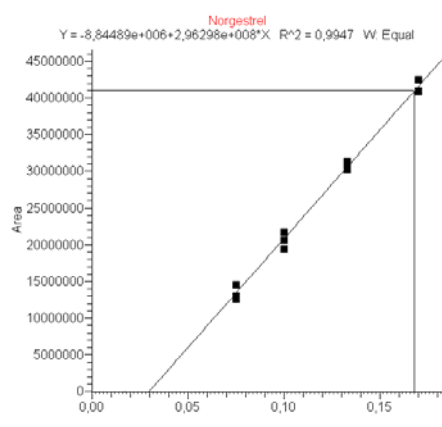
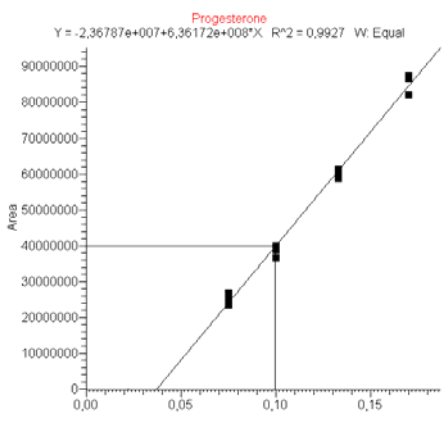


Fig. 10

Calibration curve of Progesterone (0.075 - 0.170 mg/ml)



For every compound without Testosterone the correlation coefficient is higher than 0.995. For Testosterone it lies in the range of 0.973. The limit of detection lies in the range of 0.017 mg/ml for Cortisone up to 0.063 mg/ml for Deoxycorticosterone.

Method performance

Limit of detection	0.017 – 0.063 mg/mL range (S/N = 3)
Goodness of linearity fit (r <sup>2</sup> )	> 0.995 (Testosterone > 0.973)

Conclusion

The UHPLC-ESI-MS method presented in this application note describes the fast and simultaneous separation, qualification and quantification of six steroid hormones. Including a washing step and re-equilibration of the column, only four minutes are needed for the analysis of one sample. This underlines that the method is well-suited for the use in routine analyses. Caused by the high speed and at the same time low eluent flow, only about 1.6 ml eluent are needed for one run, what means less than 1 ml of acetonitrile are used. This underlines the economy and the environmental compatibility of the method. Additionally, the method can easily be extended for the detection of a greater variety of steroid hormones, caused by the mass sensitive detection method.

## References

- 1 Peng, L., Farca, T., McGinley, M. Identification of steroids in urine and plasma, LCGC Europe, Sep. 2005
- 2 Kuster, M., Lopez, M.J., Barcelo, D. Estrogens and Progesterons in Wastewater, Sludge, Sediments and Soil, Handbook of Environmental Chemistry pp.3
- 3 Díaz-Cruz, M. S., López de Alda, M. J., López, R., Barceló, D. Determination of estrogens and progestogens by mass spectrometric techniques (GC/MS, LC/MS and LC/MS/MS), J. Mass Spectrom., 38: 917–923. 2003

## Physical properties of recommended column



BlueOrchid C18 use hydrophobic interactions for separation mechanism and offers an extended pH range for analysis of acidic, basic and neutral analytes in reversed phase mode. All BlueOrchid phases feature exceptional peak symmetry and resolution. Due to the narrow particle size distribution, the column back pressure of all BlueOrchid columns is lower than other high speed column materials on the market.

<b>Stationary phase</b>	BlueOrchid C18 1.8 µm
<b>USP code</b>	L1
<b>Pore size</b>	175 Å
<b>Pore volume</b>	0.98 ml/g
<b>Particle size</b>	1.8 µm
<b>Form</b>	spherical
<b>Surface area</b>	320 m <sup>2</sup> /g
<b>% C</b>	10
<b>Endcapping</b>	yes
<b>Dimensions</b>	50 x 2 mm
<b>Order number</b>	05BI181BOE

## Recommended Instrumentation



This application requires the PLATINblue binary high pressure gradient UHPLC system equipped with degasser, autosampler, column thermostat and MSQ PLUS MS. Other configurations are also available. Please contact KNAUER to configure a system that's perfect for your needs.

Description	Order No.
PLATINblue UHPLC-MS System	A69450
PLATINblue Pump P-1	
PLATINblue Pump P-1 with Degasser	
PLATINblue Autosampler AS-1	
PLATINblue Column Thermostat T-1 Basic	
PLATINblue modular eluent tray	
PLATINblue stainless steel capillary kit	
MSQ PLUS MS, Single Quadrupole MS with Edwards vacuum pump, PC, Xcalibur SW, ESI and APCI	

## Authors

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

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

## Contact information

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

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