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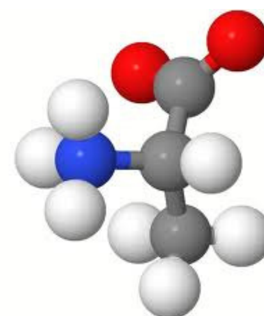


Application Note

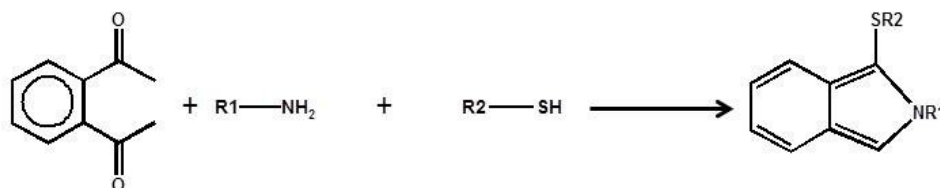
► Determination of Amino acids by UHPLC with automated OPA-Derivatization by the Autosampler

Category Bio Analysis
Matrix -
Method UHPLC
Keywords Proteinogenic Amino acids, Canonical Amino acids, *o*-Phtalaldehyde (OPA), derivatization
Analytes Alanine (Ala), Arginine (Arg), Aspartic acid (Asp), Glutamic acid (Glu), Glycine (Gly), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Serine (Ser), Threonine (Thr), Tyrosine (Tyr), Valine (Val)
ID VBS0029N, August 2012

PLATIN blue by Knauer

Summary

Amino acid analysis is a considerable application applied in research, clinical facilities and industrial processes. A rapid and sensitive UHPLC method for the determination of amino acid concentrations and compositions has been worked out in this application note. Using a KNAUER BlueShell core shell column and a high speed gradient method on the KNAUER PLATINblue UHPLC system, a complex mixture of 15 derivatized amino acids could be separated in less than 8 minutes.
The method described in this study uses *o*-Phtalaldehyde (OPA) as pre-column derivatizing reagent. OPA is a well-known and reliable derivatization reagent for amino acid analysis by UHPLC. Its significantly lower price compared to other derivatization reagents like 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) makes this reagent very attractive for the use in routine analyses.



o-Phtalaldehyde (OPA) Fluorescent derivative

Fig. 1
Scheme of OPA derivatization

Introduction

Amino acids are highly active compounds present for example in food and beverages affecting the quality of foodstuffs (taste, aroma and colour).[1] There is a continued interest in the development of a reliable, rapid and accurate method for the determination of food quality in regulatory purposes. Many analytical methods have already been proposed and ion-exchange chromatography has been the most common one.[2] Amino acid analysis by reversed-phase HPLC is also a well-established analytical technique used for quality or quantity control of industrial products as well as for diagnostic analyses and research. The amino acid composition and concentration of proteins or peptides can be determined if the protein or peptide is available in pure condition.

Also the analysis of the amount of proteins or free amino acids is possible. Two steps are necessary to analyze the amino acids of proteins and peptides. The first step is the hydrolysis to split off the amino acids. Typically acidic hydrolysis is the method of choice.[3] Second the derivatization, separation and detection of all amino acids have to be performed. For the derivatization, different reagents are commercially available.[4] The pre-column derivatization of amino acids with ortho-phthalaldehyde (OPA) and a thiol compound is one of most popular techniques today.[5] HPLC run times of about 60 minutes as well as high sensitivity are special characteristics of the classical OPA methods. It is also very important to know, that OPA reacts really fast with amino acids and forms derivatives that are only stable for several minutes. So it is inevitable to derivatize amino acids with the autosampler and inject them directly to the UHPLC system to get reproducible results.
The amino acid analysis reported in this application note allows for the further development of the already described HPLC method using OPA as precolumn derivatization reagent. The focus for the UHPLC OPA method includes simple derivatization handling by automatic derivatization by the autosampler unit as well as robustness and short analysis times. A much more sensitive detection can be realized with a fluorescence detector at excitation at 340 nm and emission at 455 nm. UV detection at 230 nm can be the second choice, but it is less sensitive compared to fluorescence detection.

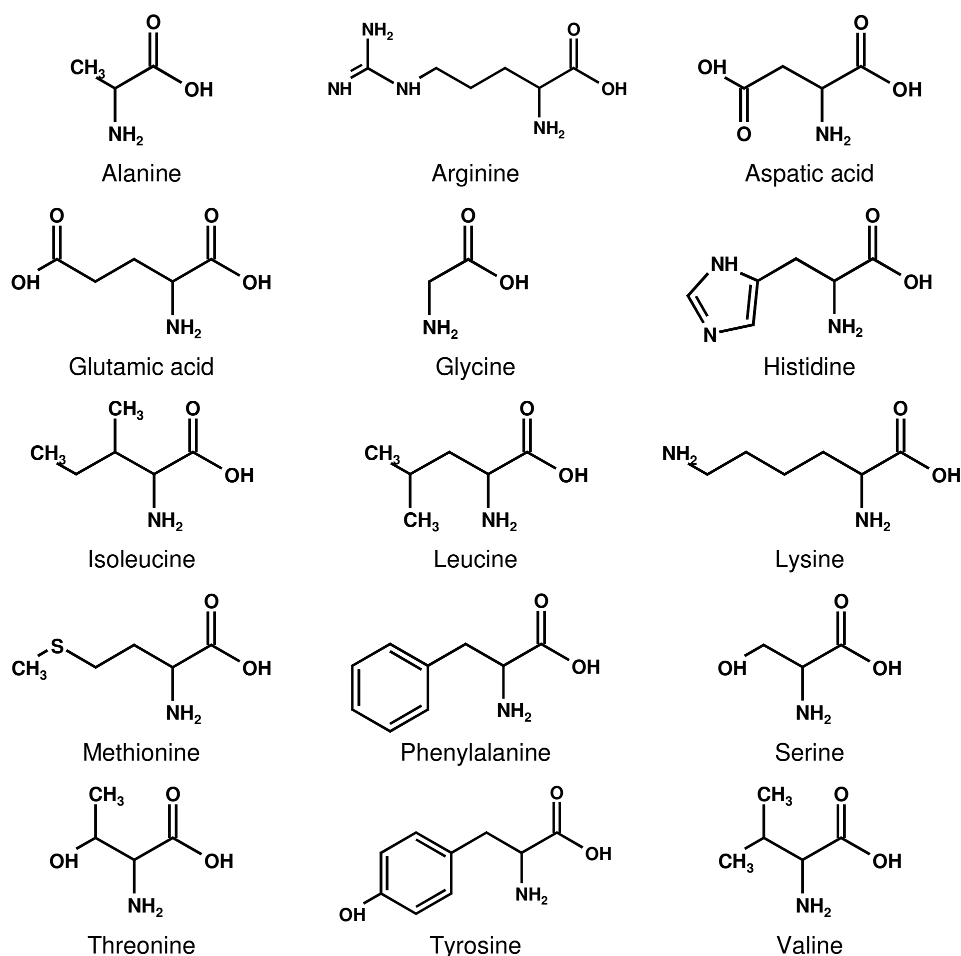


Fig. 2

Chemical structures of the analyzed Amino acids

Experimental preparation of standard solution

The stock standard mixture was received in the concentration of 2.5 µmol/mL per amino acid in 0.1 N HCl. For derivatization, it is essential that the pH value of the standard is in the range of pH 7. Therefore the standard was first diluted 1:1 with 0.1 N NaOH. Afterwards, the standard mixture was diluted using water to reach concentrations of 50 – 200 pmol/µl for calibration.

Experimental OPA-derivatization by autosampler

The OPA derivatization was automated by the PLATINblue autosampler AS-1. In the instrument configuration, two reagent vials can be defined. In our case reagent vial A was filled with borate buffer and reagent vial B with OPA reagent. Figure 3 shows the configuration in the KNAUER Chromgate software for the AS-1 equipped with two standard 48 vial trays.

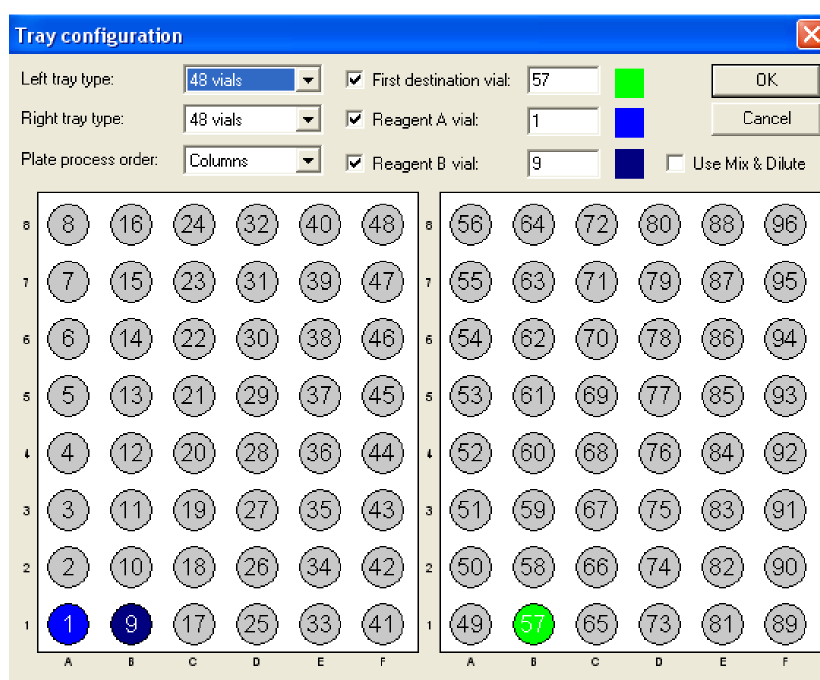


Fig. 3

Configuration of the AS-1 tray

For the reagent A vial position 1 and for the reagent B vial position 9 is chosen. For the described method, one destination vial is needed per sample. Vials 57 – 96 are configured as destination vials what means that vials 17 – 56 can be used as sample positions. At positions 57 – 96 the user has to place empty vials with sufficient volume as destination vials. They have to be closed with caps like all the other vials with septa where the autosampler needle can get through.

The borate buffer consists of 0.5 M disodium-tetraborate at pH 9.2. Because the OPA reaction is very pH sensitive, it is important to adjust the pH value of the buffer carefully. OPA reagent was created by mixing 100 mg *o*-phthaldialdehyde with 9 ml methanol, 1 ml borate buffer and 100 µl mercaptoethanol.

In a last step, the washing solvent bottle of the autosampler was filled with neutralization buffer consisting of 100 ml 0.05 M Sodium-acetate added with 25 ml methanol brought to pH 6.1 with 0.75 M HCl. The pH value is very important to neutralize the basic OPA-derivatization solution before injection to the UHPLC system. This neutralization buffer is in this case also used as the autosampler´s needle wash solvent.

In the following, a scheme is shown how the derivatization should be done. Of course the volumes of the solvents can be adjusted as long as the relations stay the same.

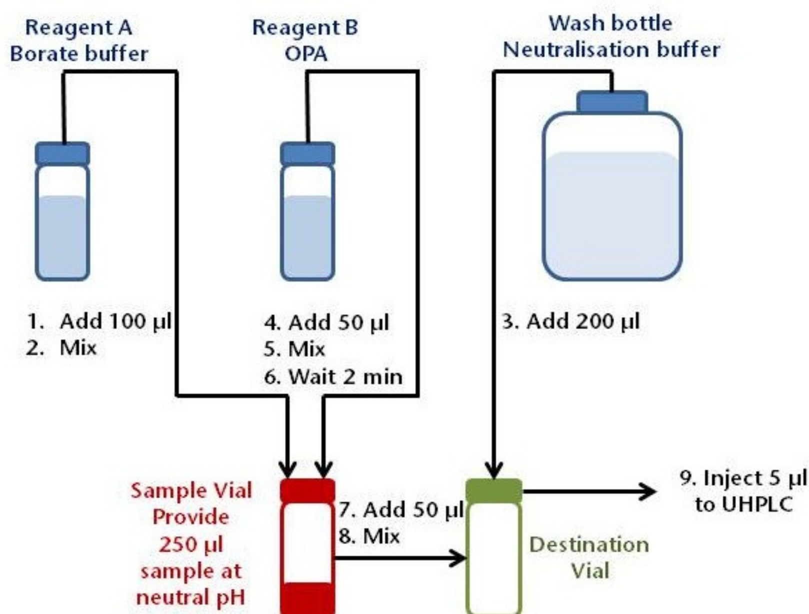


Fig. 4

Scheme of the OPA derivatization method

This mixing method was created with the software to get an automated derivatization technique. The mixing method programmed in KNAUER´s Chromgate is shown in figure 5.

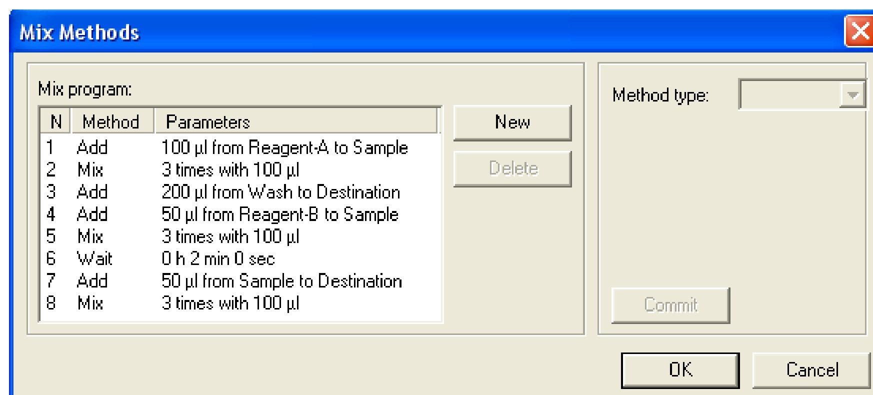


Fig. 5

Autosampler mixing method

Method parameters

Column	BlueShell 80-2.6 C18 A core shell, 150 x 2.0 mm ID		
Eluent A	50 mM Sodium acetate pH 7.2		
Eluent B	50 mM Sodium acetate pH 7.2/Methanol 25:75 (v/v)		
Gradient	**Time [min]**	**% A**	**% B**
	0.00	90	10
	1.50	90	10
	5.00	50	50
	8.00	0	100
	10.00	0	100
Flow rate	0.8 ml/min		
Sample loop	100 µl loop		
Injection mode	partial loop fill		
Injection volume	5 µl		
Column temperature	45 °C		
Run time	10.0 min		
Detection	PDA-1, 230 nm, 10mm cell, 50 Hz, 0,02 s		
Sample preparation:	automated derivatization of Amino acid standard by autosampler AS-1 method		

Results

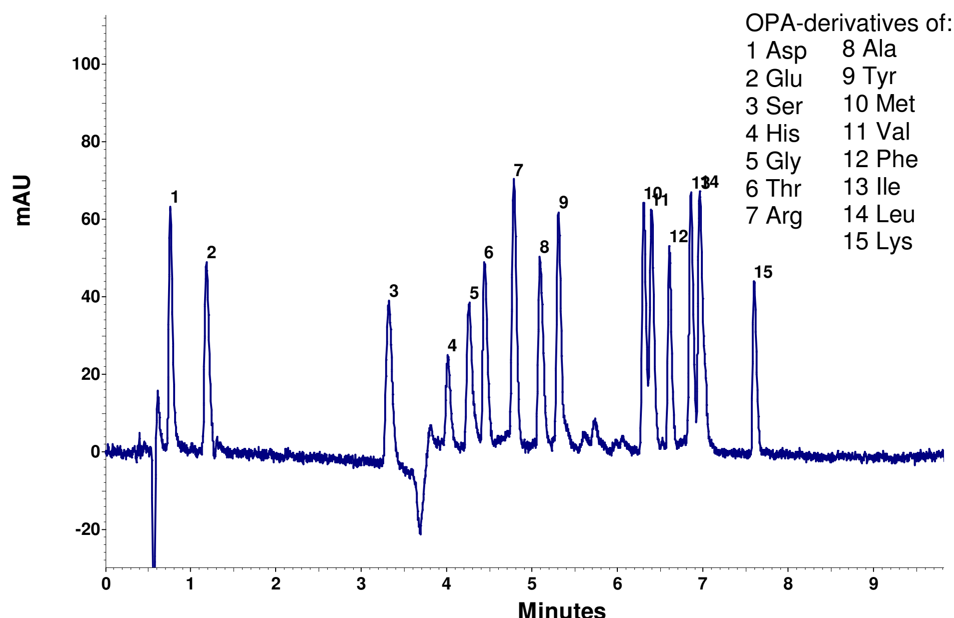


Fig. 6

Chromatogram of the analyzed Amino acid standard after OPA derivatization

To prove the reproducibility of the shown method 4 replicate runs were performed with the same parameters and the results were evaluated statistically exemplarily for 4 peaks. Figure 5 shows an overlay of the chromatograms with a mark on the peaks that were chosen for evaluation.

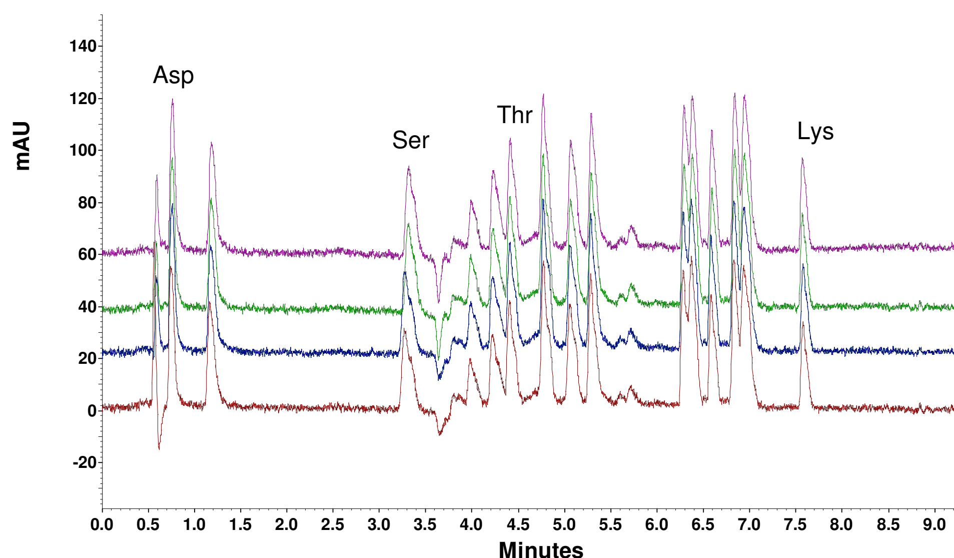


Fig. 7

Overlay of 4 chromatograms of the amino acid standard to prove the reproducibility of the method.

Table 1 shows the statistical evaluation of retention time (RT) and peak area.
From figure 5 and table 1 it becomes obvious, that the presented method works reproducible and robust. Concerning retention time the relative standard deviation is in the range of lower than 1 %. Regarding the peak area standard deviation is lower than 2.5 % when 4 replicate runs are regarded.

Table 1

Reproducibility of the described method

	Asp		Ser		Thr		Lys	
Nr.	RT [min]	Area	RT [min]	Area	RT [min]	Area	RT [min]	Area
1	0,76	207268	3,31	227463	4,41	206555	7,57	145498
2	0,76	203662	3,31	222842	4,41	203643	7,57	147362
3	0,76	201471	3,27	234330	4,41	206412	7,58	139491
4	0,76	198986	3,27	231090	4,41	204709	7,58	145521
Ave-rage	**0,76**	**202847**	**3,29**	**228931**	**4,41**	**205330**	**7,57**	**144468**
RSD	**0,0%**	**1,7%**	**0,7%**	**2,2%**	**0,0%**	**0,7%**	**0,1%**	**2,4%**

With these results the method presented in this application note was found to be well suited for the analysis of amino acids and in the following, a calibration was done. An overlay of the resulting chromatograms of amino acid standards with 3 different concentrations in the range of 50 – 200 pmol/µl can be seen in figure 6.

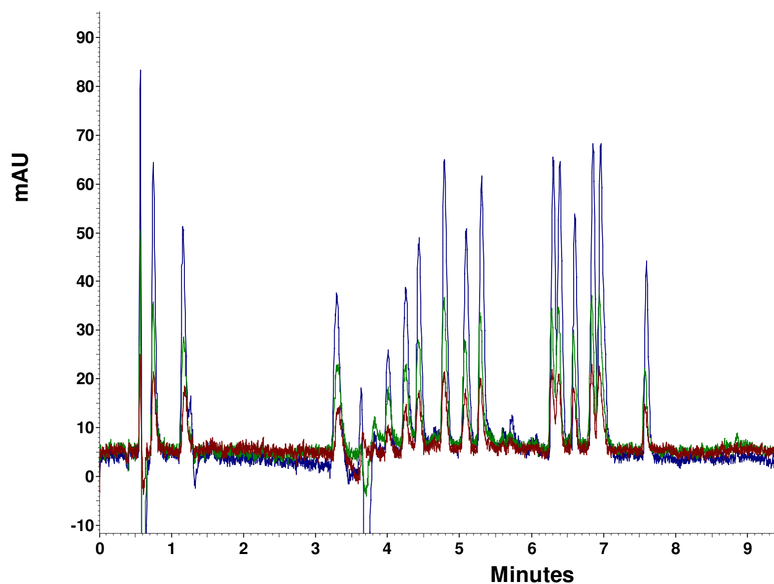


Fig. 8

Overlay of the calibration chromatograms of the OPA amino acid standard, red: 50 pmol/µl, green: 100 pmol/µl, blue: 200 pmol/µl

Calibration was done for all amino acids in the standard mix. Calibration curves are shown exemplarily for the 4 selected amino acids Aspartic acid, Serine, Threonine and Lysine.

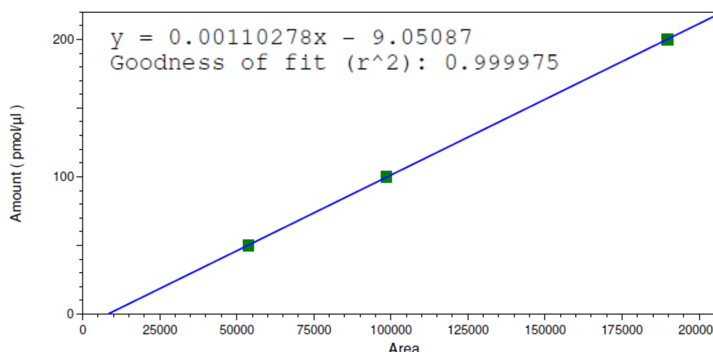


Fig. 9

Calibration for Asp

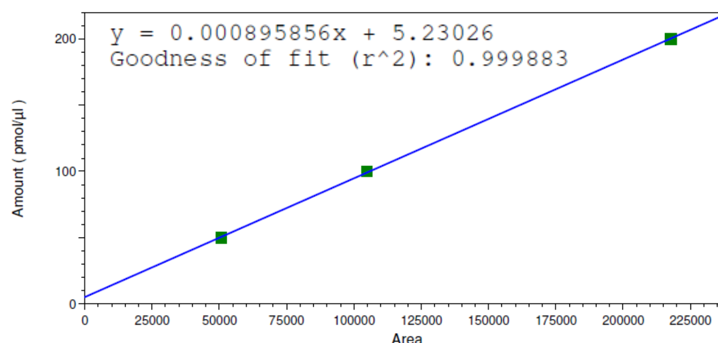


Fig. 10

Calibration for Ser

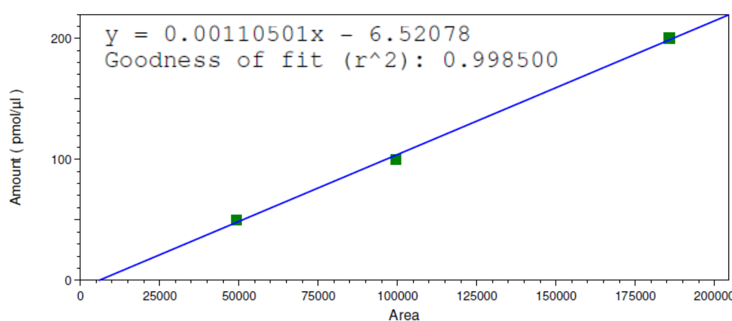


Fig. 11

Calibration for Thr

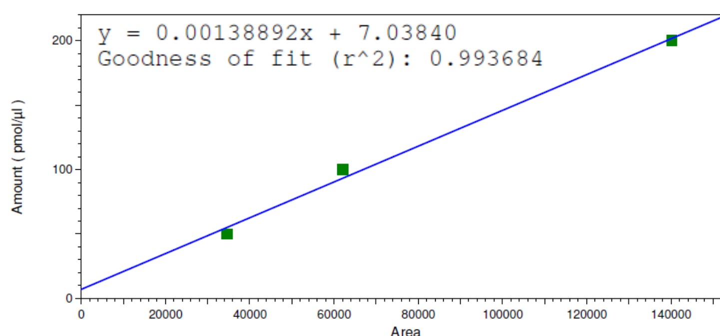


Fig. 12

Calibration for Lys

Conclusion

The UHPLC method presented in this application note describes the fast and simultaneous separation and qualification of 15 amino acids after automated precolumn derivatization with OPA. Including the derivatization, a washing step and re-equilibration of the column, only 15 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, less than 12 ml eluent are needed for one complete run. This again underlines the economy and the environmental compatibility of the method. Additionally, if higher sensitivity is needed for example for clinical analyses, detection by fluorescence can be applied what will dramatically increase sensitivity.
The automated derivatization can easily be done by the PLATINblue autosampler AS-1. This guarantees consistent results and a stable reaction time. This means that the automated derivatization by the autosampler is not only time- and manpower saving but also a need to get stable and reproducible results.

References

1. 1 P. Hernandez-Orte, J. Cacho, Ferreira, V., J. Agric. Food Chem, 50:2891 (2002).
2. S.Moore, W.H.Stein, J. Biol. Chem. 176 (1948) 367-388.
3. I. Davidson; Hydrolysis of Samples for Amino Acid Analysis in *Methods in Molecular Biology* Vol. 211 (2002) pp 111-122.
4. W.D. Hil, F.H. Walters, T.D. Wilson, J.D.Stuart, Anal. Chem. 51, 138-141 (1979).
5. S.A. Cohen, D.P. Michaud, Anal.Biochem. 211, 279-287 (1993).

Authors

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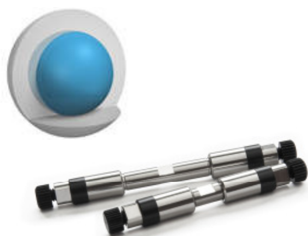
Physical properties of recommended column for UHPLC

To obtain ultra-high performance results comparable with sub-2 µm columns, without the disadvantage of high backpressure, the new BlueShell columns are your first choice. BlueShell columns are packed with special core-shell particles, developed to provide improved speed, higher resolution, and reduced eluent consumption, all while keeping moderate HPLC backpressures.

BlueShell C18 A is a polar endcapped C18 phase for alternative selectivity; designed for use with 100 % aqueous eluents for analysis of very polar compounds, basic pharmaceutical ingredients, water soluble vitamins, catecholamines as well as organic acids.



Stationary phase	BlueShell 80 - 2.6 C18 A
USP code	L1
Pore size	80 Å
Pore volume	0.8 ml/g
Particle size	2.6 µm
Form	spherical
Surface area	130 m^2/g
% C	9
Endcapping	Yes, polar
Dimensions	150 x 2 mm
Order number	15BD184SHA

Recommended Instrumentation

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

Description	**Order No.**
PLATINblue UHPLC-System	A69420
PLATINblue Pump P-1	
PLATINblue Pump P-1 with Degasser	
PLATINblue Autosampler AS-1	
PLATINblue Column Thermostat T-1	
PLATINblue Detector PDA-1	
PDA-1 flow cell (10 mm, 2 µl)	
PLATINblue modular eluent tray	
PLATINblue CG Data system	
PLATINblue CG PDA license	
PLATINblue stainless steel capillary kit	

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