

Application Note

► High speed separation and detection of 17 AQC derivatized amino acids using UHPLC-ESI-MS

Category	Bio science, food
Matrix	Baby food
Method	UHPLC-MS
Keywords	Proteinogenic amino acids, canonical amino acids, 6-aminoquinolyl- <i>N</i> -hydrosysuccinimidyl carbamate (AQC), derivatization, MS detection, MSQ Plus
Analytes	Alanine (Ala), arginine (Arg), aspartic acid (Asp), cystine (Cys), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), taurin, threonine (Thr), tryptophan (Trp), tyrosine (Tyr), valine (Val)
ID	VBS0013N, 03/11



Summary

Amino acid analysis is a considerable application applied in research, clinical facilities and industrial processes. A rapid and sensitive UHPLC method coupled to MS detection for the determination of amino acid concentrations and compositions in food samples is presented in this application note. Using a KNAUER Bluespher® 100 x 2 mm ID column and a high speed gradient method on the KNAUER PLATINblue UHPLC-MS system, a complex mixture of 18 derivatized amino acids could be separated in less than 8 minutes. The nature and the concentration of the mobile phase modifier were optimized in order to obtain sensitive MS signals. The method described in this study uses AQC as a pre-column derivatization reagent. AQC is ideal for amino acid analysis by UHPLC-MS caused by its fast and easy operability. The amino acid-AQC derivatives are substantially more stable than other commonly used reagents like o-phthalaldehyde (OPA) or 9-fluorenyl-methoxycarbonyl chloride (Fmoc).

Introduction

Amino acids are highly active compounds present for example in food and beverages affecting the quality of foodstuffs (taste, aroma and color).¹ There is a continued interest in the development of a reliable, rapid and accurate method for the determination of the quality or quantity control of industrial products as well as for diagnostic analyses and research. Many analytical methods have already been proposed and amino acid analysis by reversed-phase HPLC coupled to MS detection is still a promising technique especially for the selective measurement of the compounds at low concentrations in complex matrices.² 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. The first step is the hydrolysis to split off the amino acids and typically acidic hydrolysis is the method of choice.³ The next step is the analysis by HPLC or UHPLC methods with or without precolumn derivatization. Although, analysis of underivatized amino acids can reduce the errors introduced by side reactions and reagent interferences, there are still some notable disadvantages that can not be overcome at present. Firstly, the molecular mass of most amino acids is below 200 what leads to interferences of the mobile phase and sample matrix for the fragmentation acquirement of

the individual amino acids. Secondly, the detection limits of most amino acids are in MS detection much higher than with UV or fluorescence detection. In this context, the precolumn derivatization method is more promising for the analysis of amino acids by UHPLC-MS.² Electrospray-Ionisation (ESI) was chosen as it is ideal for the relatively small and polar molecules of derivatized amino acids.

In this application note, the already described HPLC method using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) as the precolumn derivatization reagent is advanced. This highly reactive amine derivatization reagent can be used in an easy one step procedure. The compound reacts with amines through nucleophilic attack on the carbonyl carbon of AQC. This reaction results in the loss of N-hydroxysuccinimide (NHS) and CO₂ (Fig. 1). Excess AQC is rapidly hydrolyzed in water to 6-aminoquinoline (AMQ), CO₂ and NHS (half-life = 15 s).⁴ The resulting stable derivatives are readily amenable to the analysis by reversed phase HPLC. Primary and secondary amino acids are derivatized quickly and they are stable for more than 7 days at room temperature.⁵ In contrast, by using other techniques such as OPA derivatization, some amino acid derivatives are stable only for a few minutes.

Theoretically, a baseline separation of the amino acids by UHPLC is unnecessary in MS detection, but there are some cases where limitations can occur. The isomers (in this application Ile and Leu) can not be separated by mass, the chemical suppression phenomenon can occur if several molecules are co-eluted and ionized together and the presence of isotopes can interfere with other molecules, especially if their mass difference is only 1 or 2. Therefore, a good chromatographic separation is still essential for sensitive MS analysis.

In spite of all the advantages, the identification and quantification of AQC-amino acid derivatives with MS has only been reported a little with analysis times of up to 45 minutes.² This application note describes a very fast UHPLC-MS method for the determination of 18 amino acids and taurin (a derivative of the sulfur-containing amino acid cysteine that is often found in hydrolyzed food samples) in hydrolyzed baby food samples.

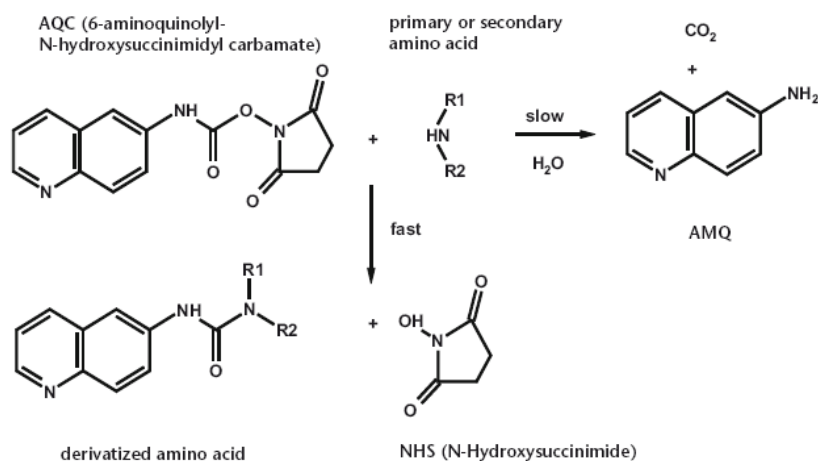


Fig. 1

Reaction scheme of AQC with primary or secondary amino acids

Experimental preparation of standard solution

The amino acid standard solution used in this work contains all canonical amino acids except asparagine and glutamine. Because its thiol group is highly susceptible to oxidation, cysteine is present in the dimeric form cystine. Additionally, taurin is added as a derivative of cysteine commonly found in hydrolyzed food samples. Because it lacks a carboxyl group, it is not an amino acid. The concentration of every amino acid is 2.5 μmol except 1.25 μmol for cystine. In a first step, the standard amino acid mix is diluted to 100 pmol/μl for every amino acid except cystine (50 pmol/μl) with deionized water. According to the care and use manual of the AccQ Fluor reagent Kit (Waters), 10 μl of this standard were mixed with 70 μl buffer solution (0.2 M borate buffer) and afterwards 20 μl derivatization reagent (2 mg/ml AQC) were added. A few minutes at 50 °C are recommendable to build stable derivates. Afterwards, the derivatized standard solution was directly injected to separate the amino acids by UHPLC. For calibration, the standard solution was diluted in the desired factors with the mobile phase A.

Experimental sample preparation

Additionally, different samples of acidic hydrolyzed baby food were derivatized and analyzed. For the derivatization according to the AccQ Fluor reagent Kit (Waters), 20 µl of the hydrolyzed sample were mixed with 60 µl buffer solution (0.2 M borate buffer) and afterwards 20 µl derivatization reagent (2 mg/ml AQC) were added. After a few minutes at 50 °C, the solution was directly injected into the UHPLC system.

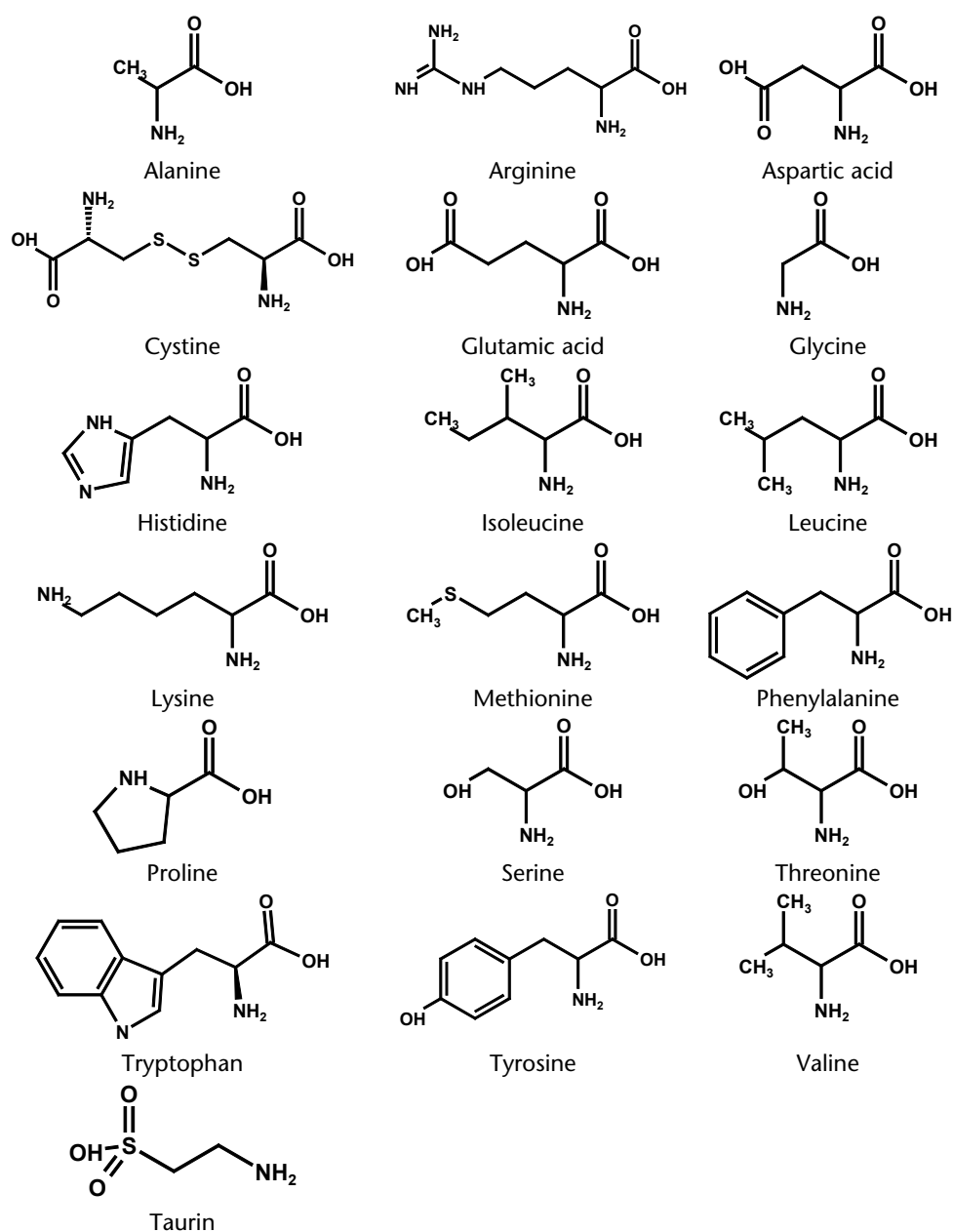


Fig. 2

Chemical structures of the analyzed amino acids and the sulfonic acid taurin

Method parameters

Column	Bluespher® 100-2 C18, 100 x 2 mm ID		
Eluent A	2.5 mM ammonium acetate, pH 5.75		
Eluent B	2.5 mM ammonium acetate, pH 6 / acetonitrile 30:70 v/v		
Gradient	Time [min]	% A	% B
	0.00	95	5
	3.00	90	10
	4.75	75	25
	6.50	68	32
	7.50	68	32
Flow rate	0.8 ml/min		
Injection volume	10 µl		
Column temperature	45 °C		
System pressure	approx. 660 bar		
Run time	7.5 min		
MS Detection Parameters	Ionization mode	ESI, positive and negative	
	Needle Voltage	3 kV	
	Cone Voltage	75 V	
	Probe temperature	350 °C	
	Mode	SIM scans	

Amino acids were detected as their AQC-derivatives. From figure 1 it becomes obvious that the mass of one AMQ molecule is added to every individual amino acid. Lysine has two derivatization sites (see figure 2) and therefore the mono- and di-derivatized form could be detected by the MS. Cystine is the dimeric form of cysteine and has also two derivatization sites, but the di-derivatized form could not be detected in this case.

Typically, amino acid masses + 170 g/mol for one AMQ molecule were detected by the MS what leads to the following table:

Table 1

Detected amino acids and their masses

Amino acid	Molecular mass [g/mol]	Derivatized		Ionization mode
		Molecular mass [g/mol]	m/z detected	
Asp	133	303	304	ESI +
Glu	147	317	318	ESI +
Ser	105	275	276	ESI +
Gly	75	245	246	ESI +
Taurin	125	295	294	ESI -
Lys	146	316/486	317/487	ESI +
Thr	119	289	290	ESI +
His	155	325	326	ESI +
Ala	89	259	260	ESI +
Arg	174	344	345	ESI +
Pro	115	285	286	ESI +
(Cys) ₂	240	410	411	ESI +
Tyr	181	351	352	ESI +
Val	117	287	288	ESI +
Met	149	319	320	ESI +
Ile	131	301	302	ESI +
Leu	131	301	302	ESI +
Phe	165	335	336	ESI +
Trp	204	374	373	ESI -

Results

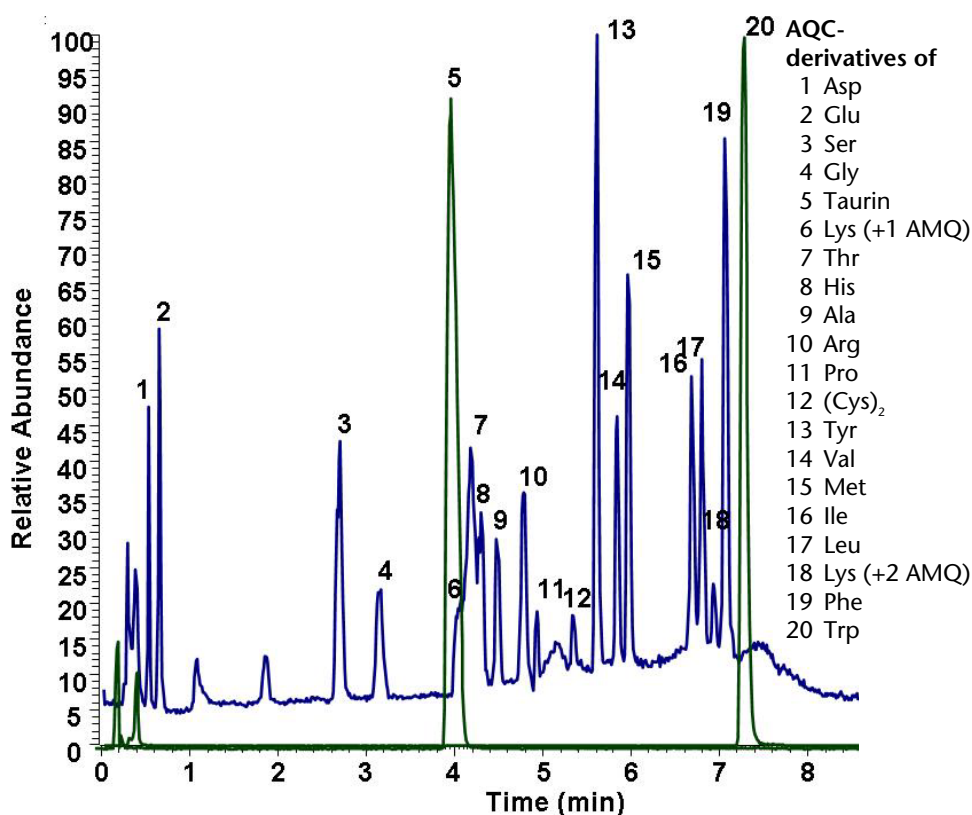


Fig. 3

TIC from grouped SIM scans of the AQC-derivatized amino acid standard solution
 ESI positive ion mode (blue)
 ESI negative ion mode (green)

The total ion chromatogram (TIC) shown in figure 3 clearly shows the chromatographic separation of all derivatized amino acids in less than 8 minutes. Peaks 6 and 18 both belong to the lysine derivative and could be identified as the mono- and di- derivatized amino acid by their m/z values.

During method development, different buffer types and concentrations were tested. Sodium acetate in the concentration of 50 mM with acetic acid worked well for the amino acid separation and the detection using the UV- or fluorescence detector. For MS detection, a volatile buffer is needed. After switching to ammonium acetate and formic acid, MS signals became much higher. Further improvement was gained by reducing the buffer concentration to 2.5 mM as described before.^[2] Excess inorganic ion can deteriorate the MS response severely even if a volatile substance is chosen. Although it has a negative effect on the chromatographic separation of the amino acids, the modifier concentration was lowered in order to gain higher sensitivity in MS detection. From the SIM scans in figure 4 it becomes obvious, that every amino acid could be quantified using their single ion chromatogram (SIC), because the isomers isoleucin and leucin are chromatographically separated.

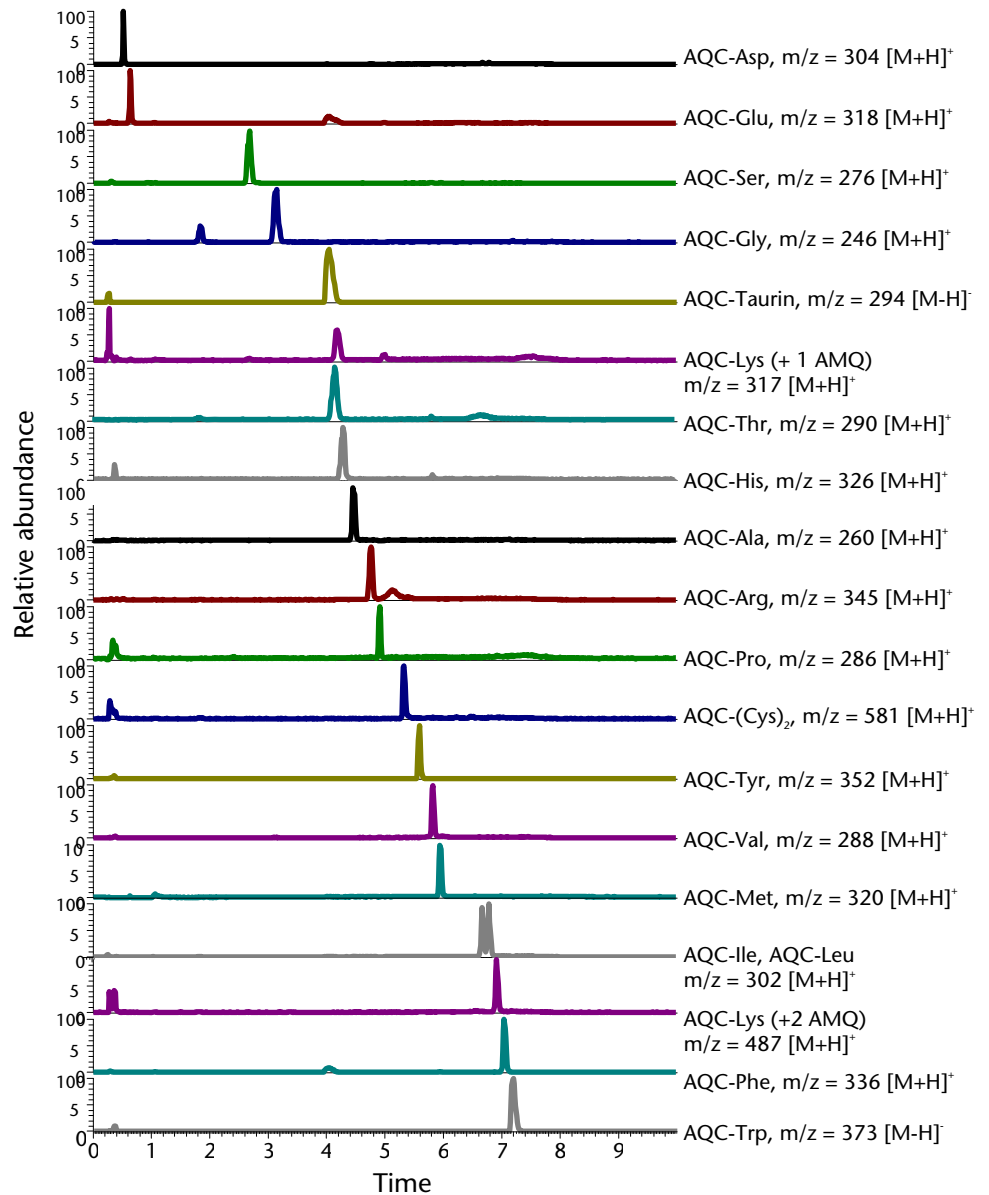


Fig. 4
SICs extracted from grouped SIM scans of the AQC-derivatized amino acid standard

A statistical evaluation was carried out for all amino acids over four replicate runs and is shown in table 2 for the peaks of asparagine, threonine, tyrosine and phenylalanine exemplarily that are randomly distributed over the chromatogram.

Table 2
Statistical evaluation

File Nr.	Asp		Thr		Tyr		Phe	
	Area	RT	Area	RT	Area	RT	Area	RT
1	428064	0.52	750655	4.19	1619025	5.60	1525830	7.07
2	459530	0.52	730120	4.17	1667579	5.57	1662220	7.04
3	436888	0.51	740870	4.15	1674333	5.56	1767494	7.03
4	438766	0.51	750319	4.16	1706136	5.59	1754782	7.04
Average	440812	0.52	742991	4.17	1666768	5.58	1677581	7.04
StDev	13322	0.01	9706	0.02	35996	0.02	111518	0.02
StDev [%]	3.02%	1.46%	1.31%	0.40%	2.16%	0.30%	6.65%	0.23%

Retention time stability is in the range of < 2.2 % RSD and peak area precision < 7 % RSD. The detection limits (S/N=3) lie in the range of 0.03 -0.1 pmol/μl. Detection limits were typically lower for the later eluting amino acids, what is caused by the mobile phase containing higher amounts of organic solvent leading to a better spray in the ESI mode.

Calibration was carried out for every detected amino acid. As an example, the calibration curve of asparagin is shown in figure 6. It has to be noted that the dilution factors resulting from the derivatization step are not incorporated in the calibration (see X-axis in figure 5).

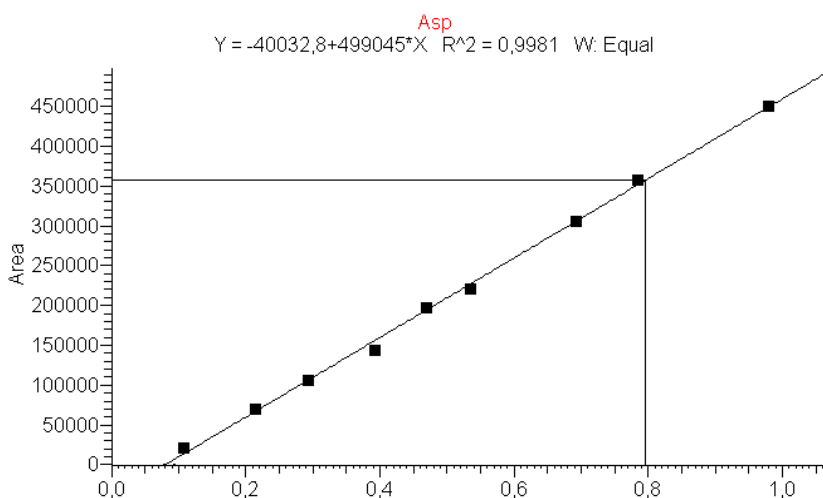


Fig. 5
Calibration curve of asparagine
(0.1 - 0.98 pmol/μl)

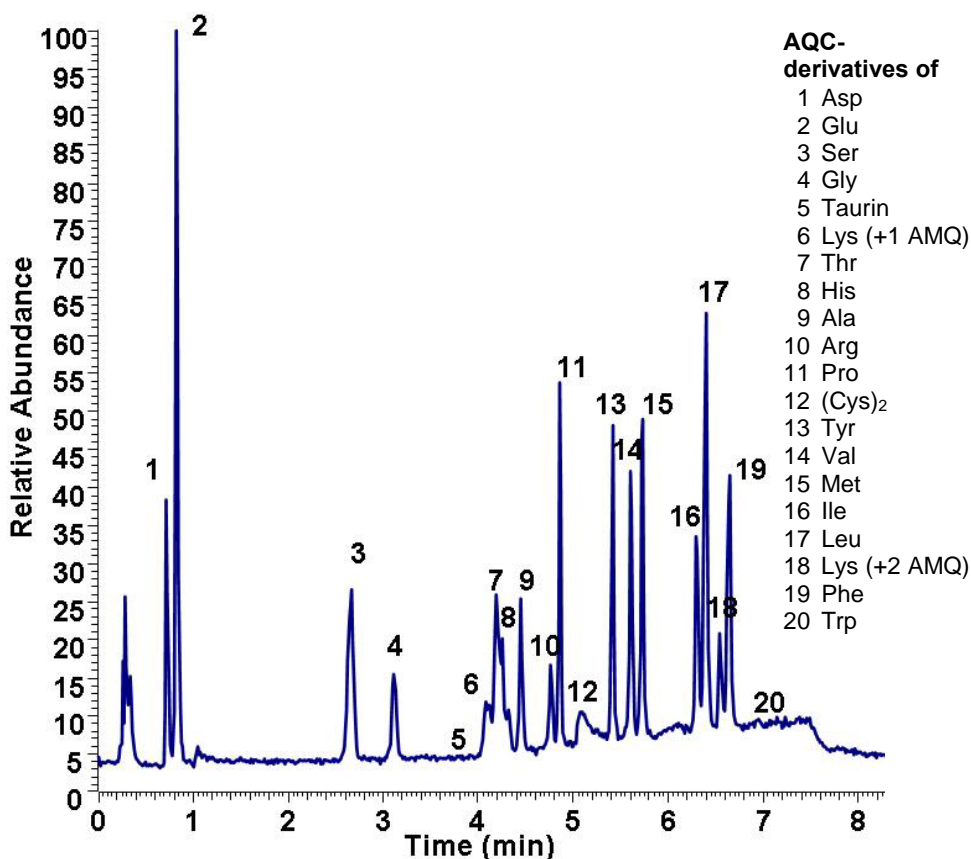


Fig. 6
TIC from grouped SIM scans of
the AQC-derivatized hydrolyzed
baby food sample

Food samples like acidic hydrolyzed baby food show high values of proteinogenic amino acids (see fig. 6). All compounds of the standard solution except cystine, taurine and tryptophan could be detected with good resolution values what allows for the quantification after calibration is done using the presented method. Peak heights show clearly that MS detection sensitive enough for the determination of amino acid concentrations as they appear in food samples. All detected amino acids could be analyzed using their individual SIM scans what allows for the integration of every single peak without doubt.

Method performance

Limit of detection	0.03 – 0.1 pmol/μl range (S/N = 3)
Goodness of linearity fit (r^2)	> 0.990
Retention time precision*	< 2.2 % RSD*
Peak area precision*	< 7.0 % RSD*

*repeatability calculated over 4 replicate runs

Conclusion

The developed method shows the very fast and simultaneous determination of 18 AQC derivatized amino acids in less than 8 minutes. The pre-column AQC derivatization results in stable derivatives of primary and secondary amino acids and can be figured out in just one simple step. This step can also be automatized using the autosampler unit at ambient temperature. But caused by the excellent stability of the derivatives shown by peak areas for derivatized amino acids staying essentially unchanged for at least 7 days, derivatization by hand and storage of the samples is also feasible. The resulting AQC-derivatized amino acids are separated in less than 8 minutes using the KNAUER PLATINblue UHPLC-MS system and a Bluespher® C18 column. With the demonstrated method, the LOD lies in the range of 0.03 – 0.1 pmol/μl applying ESI-MS detection in the SIM mode. The SIM scans for all analyzed substances were grouped by the software and the settings for the analyzer quad for a given mass were held to a narrow window width for about 20 milliseconds in order to maximize data acquisition. Grouped SIM scans have the advantage that one total ion chromatogram (TIC) is acquired for each polarity, but also the selected ion chromatograms (SIC) can easily be viewed (see figures 3 and 4). With this method, also peaks that are barely visible in the TIC can easily be quantified using the SIC. Applying UHPLC-MS and its advantages, long equilibration and analysis times can be avoided and a detection of amino acid concentrations down to 0.03 pmol/μl can be realized. The separation of hydrolyzed baby food demonstrates the potential of this method for several application areas. Applying the MSQ Plus™ mass detector, all amino acids could easily be determined and also quantified even in a very complex food matrix.

References

1. P. Hernandez-Orte, J. Cacho, Ferreira, V., J. Agric. Food Chem, 50:2891 (2002).
2. S. Hou et al., Talanta (2009), doi: 10.1016/J.talanta.2009.07.013
3. I. Davidson; Hydrolysis of Samples for Amino Acid Analysis in *Methods in Molecular Biology* Vol. 211 (2002) pp 111-122.
4. S.A. Cohen, D.P. Michaud, Anal.Biochem. 211, 279-287 (1993).
5. M. P. Bartolomeo and F. Maisano; J. Biomol Tech. 2006 April; 17(2): 131–137.

Physical properties of recommended column



Bluespher® columns are packed with ultra pure silica stationary phase to provide excellent separation performance and are well-suited for either routine analysis or ambitious chromatography in high speed mode where resolution, sensitivity and sample throughput are critical. These columns are your first choice for high-throughput-screening, quality control, and method development.

Stationary phase	Bluespher® 100-2 C18
USP code	L1
Pore size	100 Å
Pore volume	0.8 ml/g
Specific surface area	320 m ² /g
Particle size	2 µm
Form	spherical
% C	16
Endcapping	yes
Dimensions	100 x 2 mm
Order number	10BE181BSF

Recommended instrumentation



This application was carried out on a PLATINblue binary high pressure gradient UHPLC system equipped with degasser, autosampler, column thermostat and an 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 ESI and APCI ion sources, Xcalibur™ data system including PC	

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
Internet: www.knauer.net