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Application Note

► Determination of 17 AQC derivatized Amino acids in baby food samples

Category	Bio science, food
Matrix	Baby food
Method	UHPLC
Keywords	Proteinogenic amino acids, canonical amino acids, 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate (AQC), derivatization
Analytes	Alanine (Ala), arginine (Arg), aspartic acid (Asp), cystine $(Cys)_2$, glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), valine (Val)
ID	**VBS0011N**, 05/11

PLATIN blue

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 in food samples has been worked out in this application note. Detection by UV and also fluorescence can be used to underline the variability of the presented method. Using a KNAUER Bluespher® 100 x 2 mm ID column and a high speed gradient method on the KNAUER PLATINblue UHPLC system, a complex mixture of 17 derivatized amino acids could be separated in less than 8 minutes. Short Bluespher® columns filled with small particles are the most suitable way for avoiding long equilibration and analysis times. The method described in this study uses AQC as pre-column derivatizing reagent. AQC is an excellent derivatization reagent for amino acid analysis by UHPLC caused by its fast and easy operability. The amino acid-AQC derivatives are substantially more stable than other commonly used reagents like ortho-phthaldialdehyde (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 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 of 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 and high sensitivity are characteristics of the

OPA method. The amino acid analysis reported in this application note allows further development of an already described HPLC method using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) as the precolumn derivatization reagent. This highly reactive amine derivatization reagent can be used in an easy one step procedure.[6] The compound reacts with amino acids to form stable urea derivatives which are readily amenable for 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. In contrast, by using other techniques such as OPA derivatization, some amino acids are stable only for a few minutes. The second advantage of this method is that UV detection can be applied what allows for the quantification of tryptophan with high sensitivity which is not detected in fluorescence detection of OPA adducts.

The focus for the UHPLC AQC method includes simple derivatization handling by either using manual or automatic derivatization by the autosampler unit as well as robustness and short analysis times. A sensitive detection can be realized with a fluorescence detector at excitation at 250 nm and emission at 395 nm. UV detection at 254 nm can be the second choice, but it is less sensitive compared to fluorescence detection. Both detection methods are described in this application note pointing out their advantages and disadvantages.

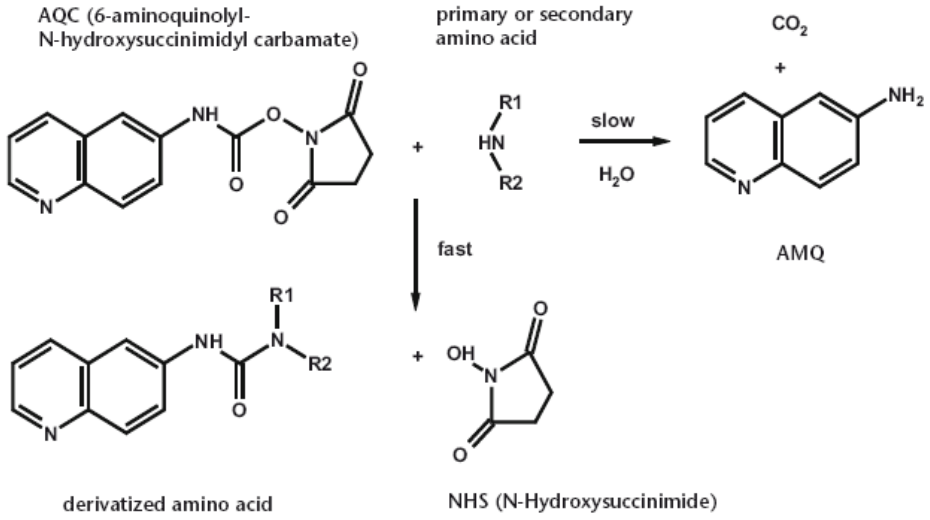

Fig. 1

Reaction scheme of AQC with primary or secondary amino acids

Experimental

preparation of standard solution

The amino acid standard solution (Sigma Aldrich) used in this work contains all canonical amino acids except tryptophan, asparagine and glutamine. Because its thiol group is highly susceptible to oxidation, cysteine is present in the dimeric form cystine. 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.
For the identification of the individual amino acids, single standards solutions were made by weighing out approximately 5 mg of the pure substance and dilution with water and acetonitrile to 10 ml. Afterwards, the derivatization was carried out as described before.

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.

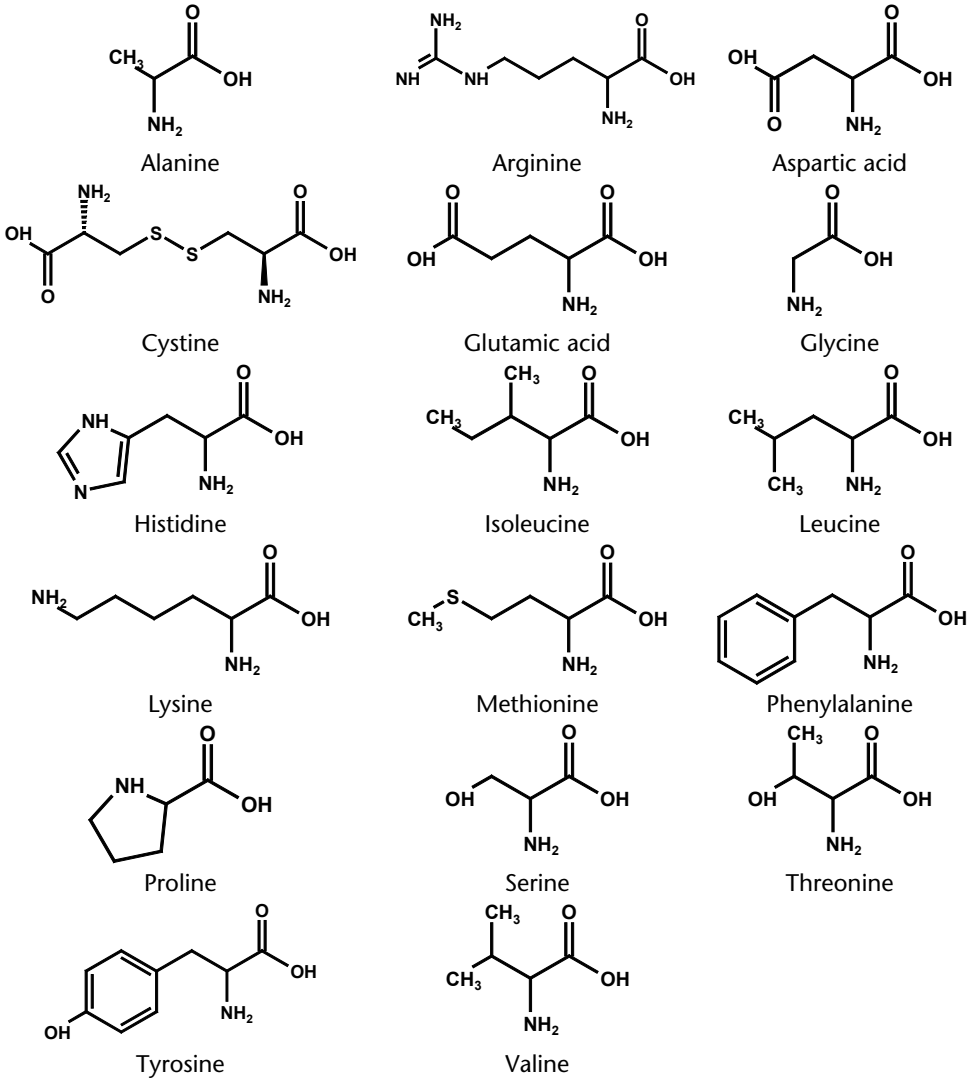

Fig. 2

Chemical structures of the analyzed Amino acids

Method parameters

Column	Bluespher® 100-2 C18, 100 x 2 mm ID		
Eluent A	50 mM Sodium acetate, pH 5.75		
Eluent B	50 mM Sodium 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	1 - 10 µl		
Column temperature	45 °C		
System pressure	approx. 660 bar		
Detection	UV at 245 nm (50 Hz, 0.02 s), 2 µl flow cell Fluorescence RF 20A-xs (Ex 250 nm, Em 395 nm)		
Run time	7.5 min		

Results

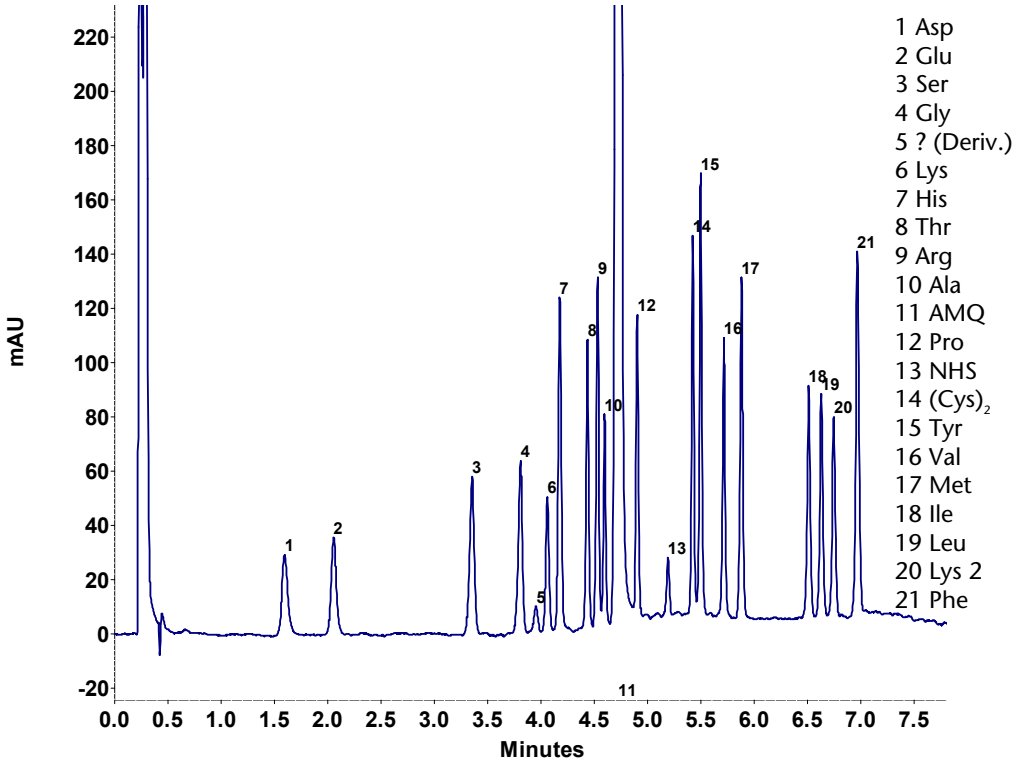

Fig. 3

Chromatogram of the AQC-derivatized amino acid standard with UV detection

The chromatograms clearly show the baseline separation of all derivatized amino acids in less than 8 minutes. The AMQ peak in the middle of the chromatogram is also separated from the target compounds. Peak 5 could not be identified but it seems to be a by-product of the derivatization reaction. Peaks 6 and 20 both belong to the lysine derivative as verified by the measurement of the pure lysine standard. A possible explication for this phenomenon is, that lysine has two derivatization sites (see figure 2) and that in the chromatogram the mono- and di-derivatized forms of lysine are detected. This is only an acceptance and can for example be proven by means of MS detection.

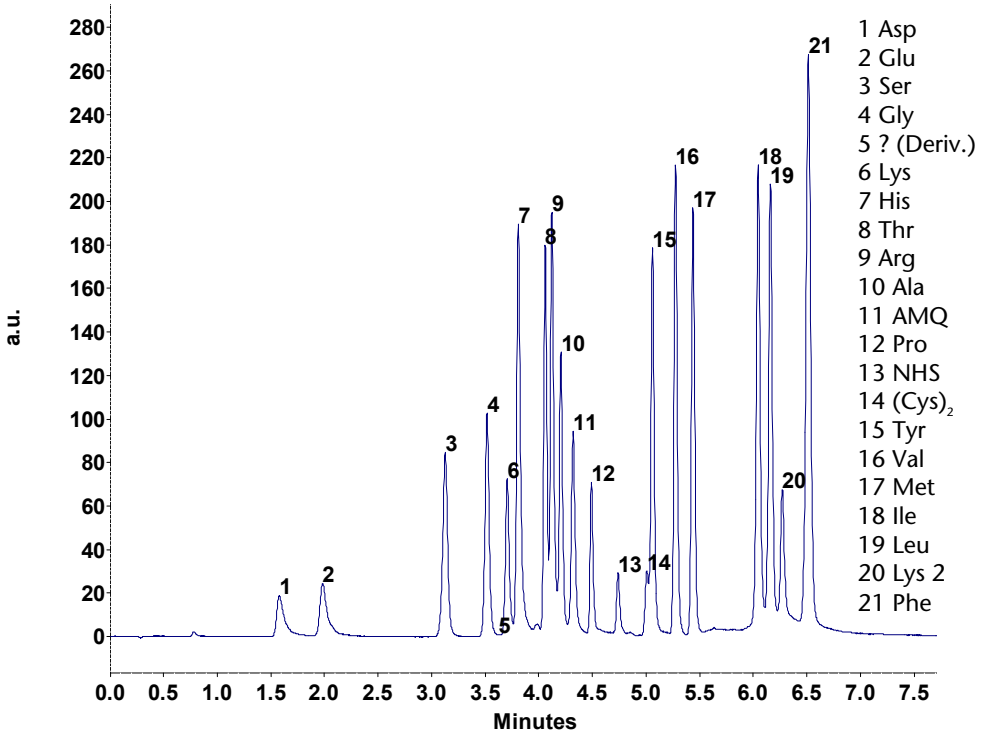

Fig. 4

Chromatogram of the AQC-derivatized amino acid standard with fluorescence-detection (baseline subtract)

Comparing figure 3 and 4 it can also be seen that a loss of resolution is noted by switching from UV to fluorescence detection. This is caused by the higher volume of the fluorescence detector´s flow cell. The very small volume of 2 µl for the flow cell of the PLATINblue PDA-1 minimizes the dwell volume of the system. Applying the fluorescence detector, still all peaks are resolved but they already become a little broader what is caused by the higher volume of its flow cell.

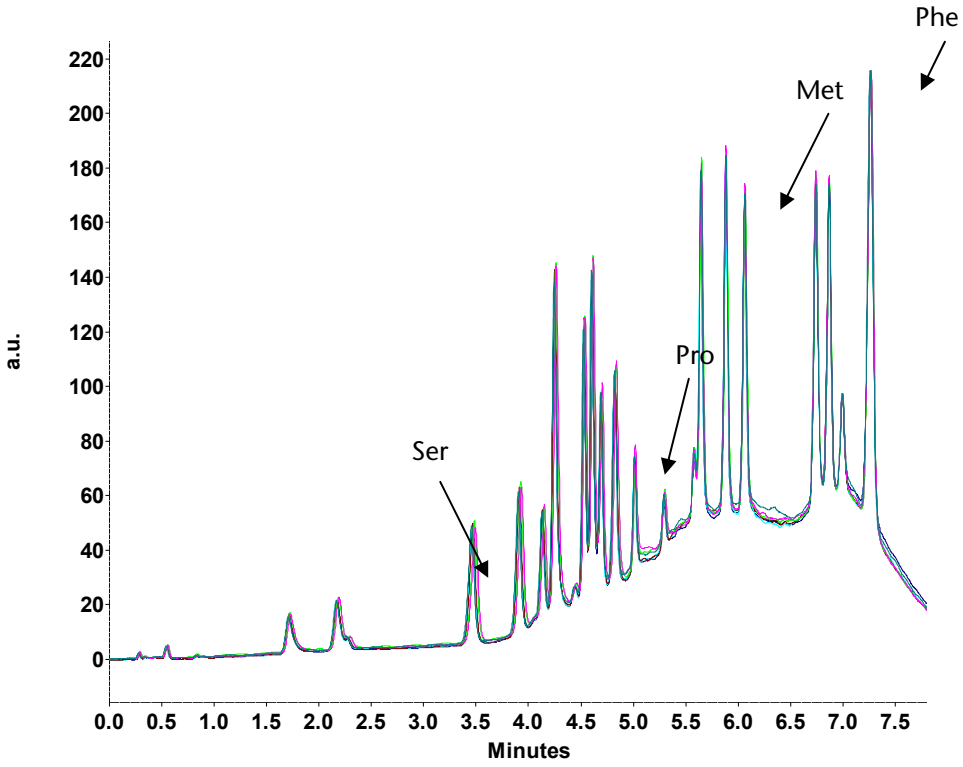

Fig. 5

Overlay of 5 replicate runs of the AQC-derivatized amino acid standard measured with fluorescence-detection

The overlay of five chromatograms in figure 5 shows the excellent reproducibility and robustness of the method. For a statistical evaluation, four peaks randomly distributed over the whole chromatogram were chosen as pointed out with arrows in figure 5. The statistical evaluation over the five replicate runs can be found in table 1.

Table 1

Statistical evaluation

RF-10AxI Data Filename	Ser Retention Time	Pro Retention Time	Met Retention Time	Phe Retention Time
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_1zu10_002.dat	2.24	3.23	3.79	4.68
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_1zu10_003.dat	2.24	3.24	3.79	4.69
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_1zu10_004.dat	2.25	3.24	3.80	4.69
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_AS1zu10_002.dat	2.23	3.23	3.79	4.68
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_AS1zu10_003.dat	2.23	3.23	3.79	4.68
Min:	2.23	3.23	3.79	4.68
Max:	2.25	3.24	3.80	4.69
Mean:	2.24	3.23	3.80	4.68
Std Dev:	0.01	0.00	0.00	0.00
%RSD:	0.31	0.09	0.05	0.03

RF-10AxI Data Filename	Ser Area	Pro Area	Met Area	Phe Area
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_1zu10_002.dat	112827.00	60792.00	219607.00	401894.00
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_1zu10_003.dat	114191.00	62204.00	220734.00	400554.00
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_1zu10_004.dat	113000.00	60080.00	215430.00	401853.00
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_AS1zu10_002.dat	111524.00	62800.00	209898.00	399107.00
110112_BSC18_1101_02_M21_RF20AXS_Gain16_45Grad_1ul_AS1zu10_003.dat	110080.00	60389.00	210224.00	403953.00
Min:	110080.00	60080.00	209898.00	399107.00
Max:	114191.00	62800.00	220734.00	403953.00
Mean:	112324.40	61253.00	215178.60	401472.20
Std Dev:	1570.92	1186.65	5073.69	1796.84
%RSD:	1.40	1.94	2.36	0.45

Relative standard deviation of retention time and peak area were calculated for four representative peaks in the chromatogram (see table 1). Retention time stability is in the range of < 0.5 % RSD and peak area precision < 2.5 % RSD. The detection limits (S/N=3) lie in the lower pmol range for UV detection and even lower for fluorescence detection.

Calibration was carried out for every detected amino acid. As an example, the calibration curves of prolin are shown in figure 6 for the UV detector and in figure 7 for the fluorescence detector. In figure 6 it becomes obvious that an additional concentration of about 1.2 pmol/µl was added that does not lie in the range of the other calibrated concentrations. This was carried out because the measured samples contained a significantly higher amino acid concentration than expected. It has to be noted that the dilution factors resulting from the derivatization step are already incorporated in the calibration (see Y-axis in figure 6 and 7).

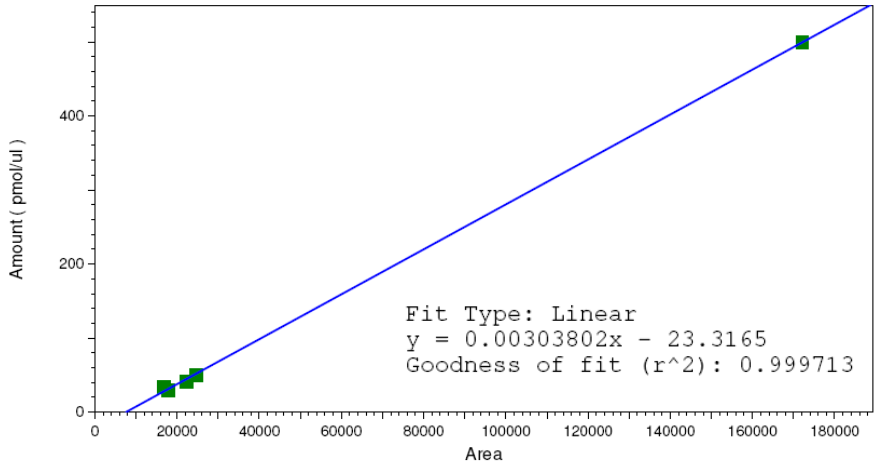

Fig. 6

Calibration of prolin with UV detection (0.7 – 1.2 pmol/µl, 10 µl injection volume)

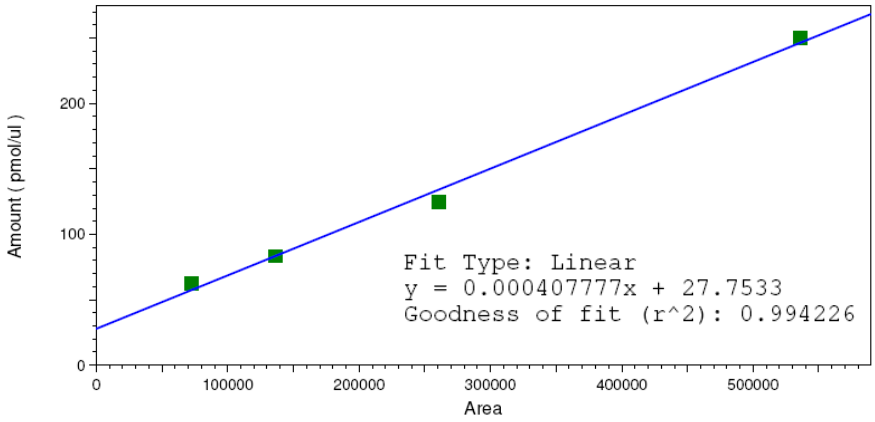

Fig. 7

Calibration of prolin with fluorescence detection (0.3 – 1.2 pmol/µl, 1 µl injection volume)

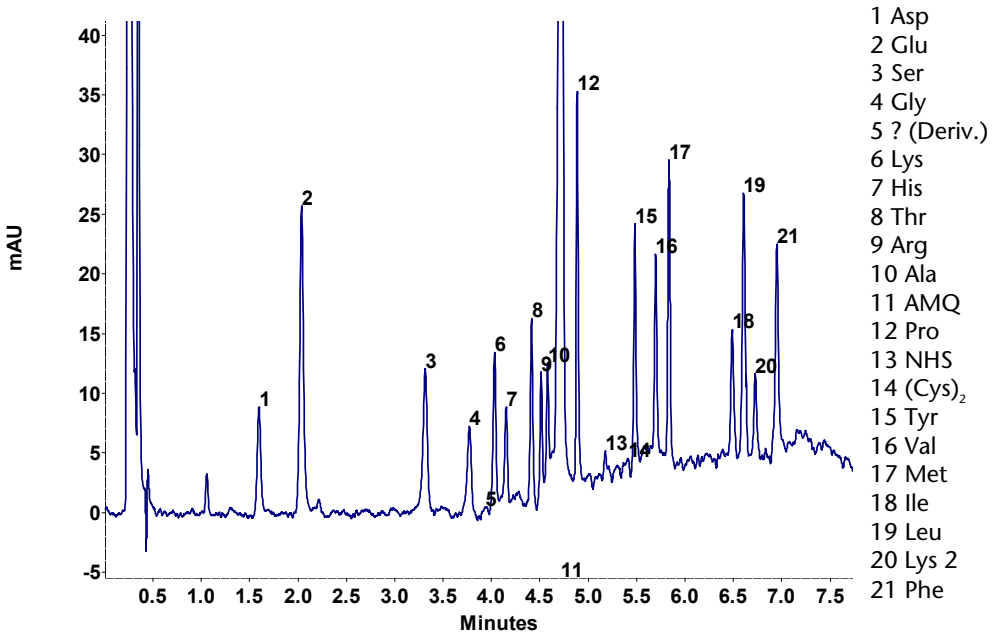

Fig. 8

Chromatogram of an AQC-derivatized hydrolyzed baby food sample measured with UV detection

Food samples like acidic hydrolyzed baby food are showing high values of proteinogenic amino acids (see fig.8). In such a sample, all amino acids except cystine could be detected with baseline separation what allows for the quantification after calibration is done using the presented method. Table 2 shows the evaluation of an AQC-derivatized hydrolyzed baby food sample measured with UV detection. It shows clearly that the detection by the PDA-1 is completely sufficient for amino acid concentrations as they appear in food samples and there is no need for a fluorescence detector in this case. After calibration for every compound, the concentrations of amino acids in baby food samples could be figured out easily and automatically by the software.

Table 2

Evaluation and quantification of an AQC-derivatized hydrolyzed baby food sample measured with UV detection

PDA-1 [Channel 1] Results Pk #	Retention Time	Name	Area	ESTD concentration	Units
1	1.60	Asp	22824	82.796	pmol/ul
2	2.04	Glu	65914	279.742	pmol/ul
3	3.31	Ser	37333	87.850	pmol/ul
4	3.77	Gly	23194	47.455	pmol/ul
5	3.94	? (Derivatization)	2851	0.000	
6	4.03	Lys	28707	61.814	pmol/ul
7	4.15	His	18265	26.108	pmol/ul
8	4.42	Thr	29156	66.745	pmol/ul
9	4.51	Arg	20283	29.105	pmol/ul
10	4.58	Ala	22489	77.880	pmol/ul
11	4.72	AMQ	407823	0.000	
12	4.89	Pro	50032	128.682	pmol/ul
13	5.18	NHS	3537	0.000	
14	5.41	CysCys	2187	0.000	pmol/ul
15	5.48	Tyr	33935	51.885	pmol/ul
16	5.70	Val	28360	68.860	pmol/ul
17	5.83	Met	44852	86.327	pmol/ul
18	6.49	Ile	21335	44.289	pmol/ul
19	6.61	Leu	49110	124.288	pmol/ul
20	6.73	Lys 2	15877	17.061	pmol/ul
21	6.95	Phe	36106	57.656	pmol/ul

Method performance

Limit of detection	UV detection: 0.4 pmol range (S/N = 3) Fluorescence detection: 0.004 pmol range (S/N = 3)
Goodness of linearity fit (r^2)	> 0.900
Retention time precision*	< 0.5 % RSD
Peak area precision*	< 2.5 % RSD

*repeatability calculated over 5 replicate runs

Conclusion

The developed method shows the very fast and simultaneous determination of 17 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 can be separated in less than 8 minutes using the KNAUER PLATINblue UHPLC system and a Bluespher® C18 column. With the demonstrated method, the LOD lies for UV detection in the range of 0.4 pmol and with fluorescence detection even by a factor 100 lower in the range of 0.004 pmol. For the amino acid quantification in the analyzed baby food samples, UV detection was absolutely sufficient. The recommendation is for samples in the food area with relatively high amounts of amino acids UV detection can be used. In the clinical sector where very small amounts of amino acids have to be determined, fluorescence detection is recommended because it is much more sensitive.
Applying UHPLC and its advantages, long equilibration and analysis times can be avoided and a fluorescence-detection of amino acid concentrations in the range of down to 0.004 pmol can be realized. The separation of hydrolyzed baby food demonstrates the potential of this method for several application areas. Investigations of hydrolyzed protein samples like baby food demonstrate accurate compositional analysis in the submicrogram level.

References

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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 requires the PLATINblue binary high pressure gradient UHPLC system equipped with degasser, autosampler, column thermostat, and fluorescence- and / or 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 Basic	
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	
Fluorescence detector RF-20A xs	A59201

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