

Application Note

▶ UHPLC separation and determination of 17 proteinogenic amino acids in baby food

Category	Food
Matrix	Baby food
Method	UHPLC
Keywords	proteinogenic amino acids; AQC derivatization
Analytes	Alanine (Ala), arginine (Arg), aspartic acid (Asp), cysteine (Cys), 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	VBS0001N, 10/09, updated 05/11



Summary

The amino acid analysis is applied in research, clinical facilities, and industry. A rapid and sensitive UHPLC method, coupled with precolumn derivatization and UV detection, has been worked out to determine the amino acid concentrations and composition of baby food. Short columns with small particles are the most suitable way to prevent long equilibration and analysis times. This application uses a 100 mm column with a high speed gradient UHPLC method for the separation of a complex mixture of derivatized amino acids in less than 12 minutes. The method described in this note operates with AQC as precolumn derivatizing reagent. AQC is an excellent derivatization reagent for amino acid analysis. The amino acid-AQC derivatives are substantially more stable than other reagents like o-phthalaldehyde (OPA) or 9-fluorenyl-methoxycarbonyl chloride (Fmoc).

Introduction

Amino acids are active biomolecules and often present in food and beverages. They affect the quality of foodstuffs (taste, aroma and color).¹ There is a continued interest in the development of a reliable, rapid and accurate method of analysis for assessing the quality of foods for regulatory purposes. Many analytical methods have been proposed for the analysis of amino acids. Until a few decades ago analysis of amino acids via ion-exchange chromatography was by far the most common method for quantification of these biological compounds.² Amino acid analysis by reversed-phase HPLC is a well established analytical technique and used for quality or quantity control of e. g. industrial products, diagnostic analysis as well as applies in 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 acid is possible. Two steps are necessary to analyse the amino acids of proteins and peptides. The first step is the hydrolysis to split of the amino acids. Typically the acidic hydrolysis is the main method.³ Secondary the derivatization, separation and detection of all amino acids have to be performed. For derivatization different reagents are available.⁴ The precolumn derivatization of amino acids with ortho-phthalaldehyde (OPA) and a thiol is one of most common technique today.⁵ HPLC method times of mostly 60 min. as well as high sensitivity are special characteristics of the OPA method. The amino acid analysis reported in this application note is considered to enhance the already described HPLC method with AQC.⁶ The highly reactive amine derivatization reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate can be used in a one step procedure.⁶ The compound reacts with amino acids to form stable unsymmetrical urea derivatives which are readily

Introduction (continued)

amenable for analysis by reversed phase HPLC. Primary and secondary amino acids were derivatized quickly and they are stable for more than 7 days at room temperature. With other techniques such as OPA derivatization, some amino acids are stable only for minutes. The second advantage of the AQC method is the possibility of UV detection in contrast to fluorescence detection of OPA adducts. With UV detection tryptophan can also be quantified with high sensitivity. The focus for the UHPLC AQC method includes simple derivatization handling by using the autosampler unit as well as robustness and short analysis time. A sensitive detection can be realized with fluorescence detector at excitation at 250 nm and emission at 395 nm. UV detection at 254 nm can be the second choice but is less sensitive comparing to fluorescence.

 Experimental
 Preparation of
 standard solution

A standard amino acid solution (Sigma Aldrich) contains all proteinogenic amino acids which codes for genetic information except for tryptophan, asparagine and glutamine. The concentration of every amino acid was 2.5 μmol and 1.25 μmol for cysteine. 10 μl of hydrolysis standard as well as the hydrolyzed baby food samples were mixed with 70 μl buffer solution (0.2 M borate buffer) and afterwards 20 μl derivatization reagent (2 mg/ml AQC) was added. A few minutes at 50 °C are recommendable to build the stable derivatives. The solution was directly injected to separate the amino acids by UHPLC. Additionally acidic hydrolyzed baby food was derivatized and analyzed in the same way.

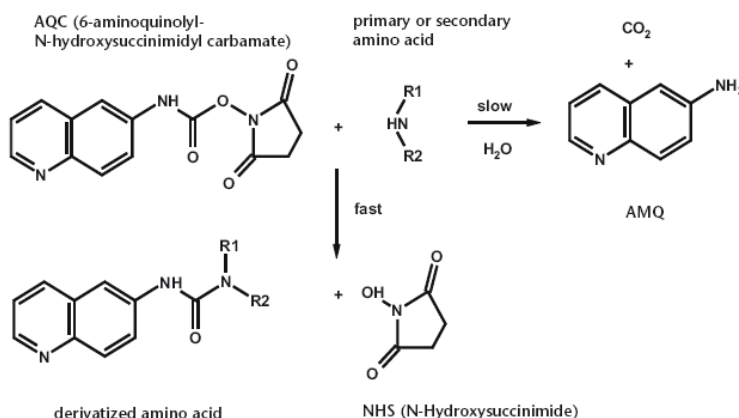
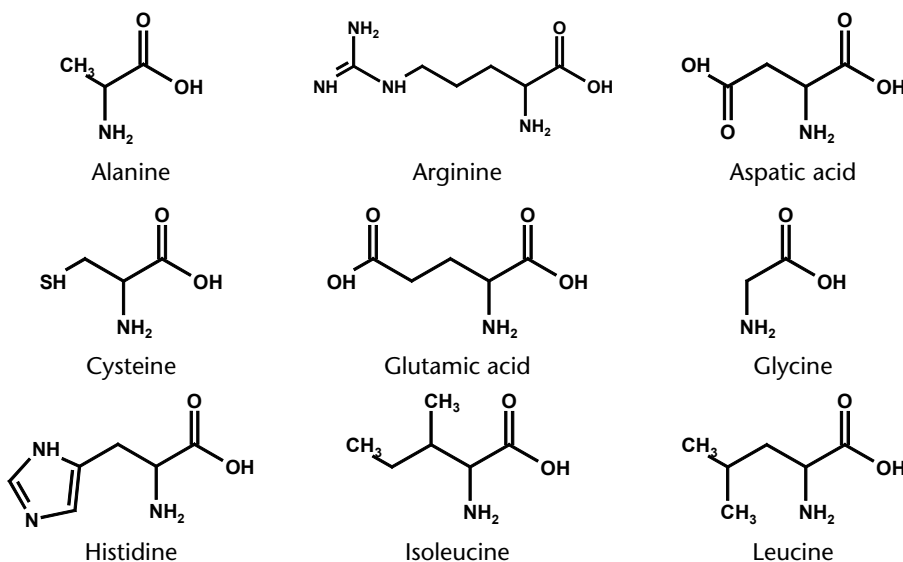


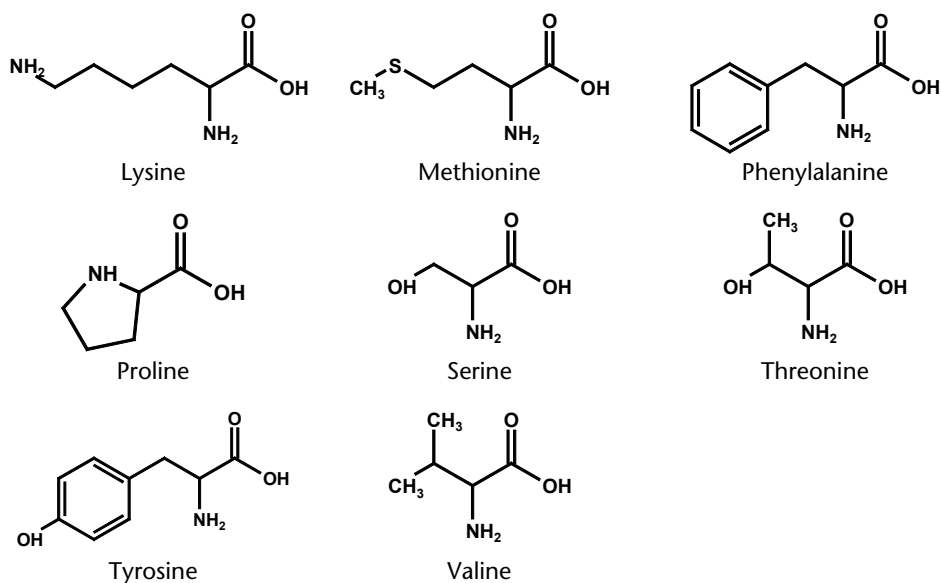
Fig. 1

Reaction scheme of AQC with Amino acids

Chemical structures

Amino acids are organic compounds with at least one carboxyl- and one amino group. The analysed amino acids belong to the alpha amino acids and L-amino acids and are only different in side chain (see chemical structures).





Method parameters

Column	BlueOrchid 1.8 C18 100 x 2 mm		
Eluent A	50 mM Na acetate (pH 6)		
Eluent B	Eluent A / ACN 40:60		
Gradient	Time (min)	% A	% B
	0.00	95	5
	5.50	90	5
	12.00	65	35
	13.00	65	35
	13.50	95	5
	15.00	95	5
Flow rate	0.8 ml/min		
Injection volume	1 µl		
Column temperature	40 °C		
System pressure	approx. 750 bar		
Detection	UV at 254 nm		
Run time	12 min (15 min incl. regeneration)		

Results

The one step AQC derivatization processes by adding 70 µl of 0.2 M borate buffer (pH 8.8) and 20 µl derivatization reagent AQC (2 mg/ml) to 10 µl amino acid sample can be automatized by using the autosampler unit at ambient temperature. Peak areas for derivatized amino acids were essentially the same for at least 7 days. Complete chromatographic resolution of the derivatized amino acid can be realized at slightly acidified mobile phase where AMQ is eluting in front of the chromatogram (recommended cut off at 0.75 min). The robustness of the gradient method is demonstrated in fig. 2 by overlaying five runs. Relative standard deviation of retention time was calculated for three representative peaks in the chromatogram. Retention time stability for peak 3 (serine 0.72%), peak 13 (proline 0.24%) and peak 21 (phenylalanine 0.19%) is calculated. Calibration standard concentrations were in the range of 25 - 500 pmol with detection limits (S/N=3) in the lower pmol range (5 pmol). Food samples like acidic hydrolysed baby food are showing high values of proteinogenic amino acids (see fig.3).

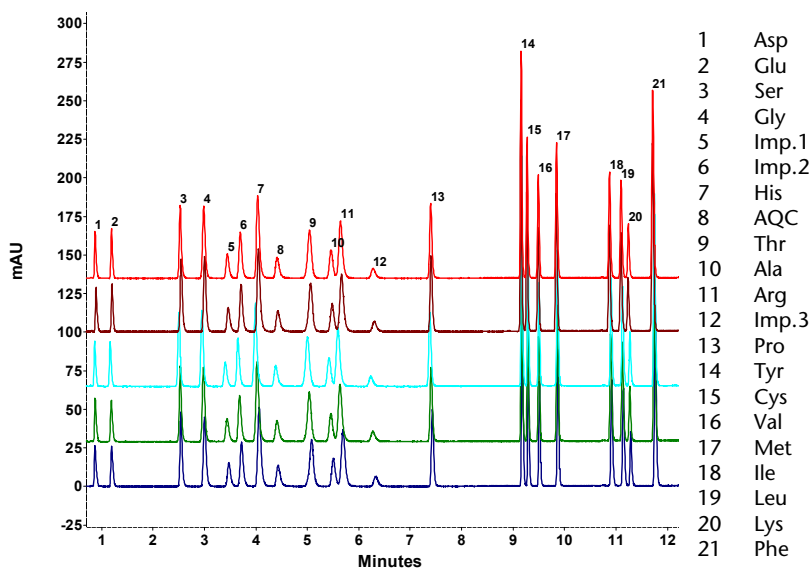


Fig. 2
Separation of AQC derivatized SIGMA amino acid standard (overlay of five runs)

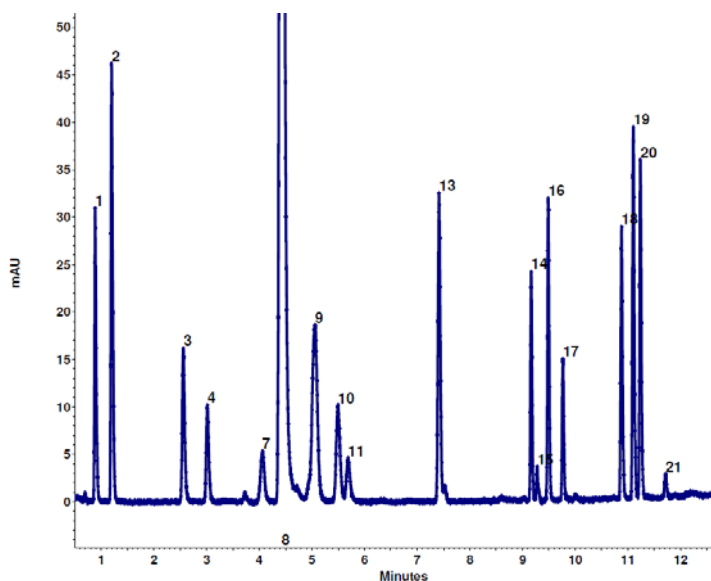


Fig. 3
Separation of AQC derivatized baby food sample

Method performance

Limit of detection	5 pg/ml range (S/N = 3)
Linearity range	25 to 500 pmol
Retention time precision*	Serine < 0.75 % RSD Proline < 0.25 % RSD Phenylalanine < 0.20 % RSD

*repeatability calculated over 5 replicate runs

Conclusion

The developed method shows a fast and simultaneous determination of AQC derivatized amino acids in less than 12 minutes. The precolumn AQC derivatization results in stable derivates of primary and secondary amino acid and is done in only one simple step. By using UHPLC, long equilibration and analysis times can be avoided. Furthermore a UV detection of amino acid concentrations in the range of 5 pmol to 500 pmol can be realized. The separation of hydrolyzed baby food demonstrates the potential of this analysis for several application areas. Investigations of hydrolyzed protein samples like baby food demonstrate accurate compositional analysis in the submicrogram level. Sensitive fluorescence detection can improve the detection limit if necessary. The one step derivatization of the quantitative AQC reaction with primary and secondary amino acids can be easily and successfully realized without additional heating steps. The resulted AQC derivatized amino acids are extremely stable and can be separated in less than 12 minutes

by using UHPLC technology. With the demonstrated method linearity amino acid concentrations in the range of 5 pmol up to 500 pmol can be realized by using UV-detection. Perspectives investigations of AQC adducts promise detection limit reduction by using capillary columns (300 µm ID) or nanobore columns (< 100 µm ID).

References

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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.

Stationary phase	BlueOrchid 1.8 C18
USP code	L1
Particle size	1.8 µm
Form	spherical
pH range	1-10
% C	10
Endcapping	yes
Dimensions	100 x 2 mm
Order number	10BI181BOE

Recommended instrumentation



This application was realized using the PLATINblue binary gradient UHPLC system equipped with degasser, autosampler, column oven, and multi-wavelength UV 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	A69410
PLATINblue Pump P-1	
PLATINblue Pump P-1 with Degasser	
PLATINblue Autosampler AS-1	
PLATINblue Column Thermostat T-1 Basic	
PLATINblue Detector MW-1	
PDA-1 flow cell (10 mm, 2 µl)	
PLATINblue CG Data system	
PLATINblue CG spectra license	
PLATINblue stainless steel capillary kit	

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