CUSTOMER APPLICATION NOTE 73761

Impurity analysis of L-aspartic acid and glycine by HPLC-UV-CAD

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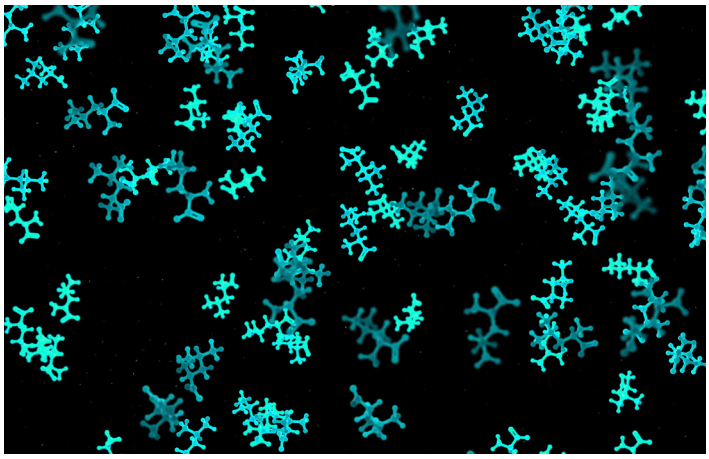
Keywords: Amino acids, impurity analysis, ion pair chromatography, Vanquish Horizon Charged Aerosol Detector, Variable Wavelength Detector



Application benefits

- Replacement of error-prone derivatization methods by more robust and reliable UV-CAD methods
- Simultaneous detection of amino acids and organic acids by a single method instead of two separate methods offering reduced analysis time and costs
- Improved sensitivity for weak-chromophoric amino acids by hyphenated detection technologies

Goal

This application aims to offer a more reliable alternative method to the compendial methods for the impurity analysis of L-aspartic acid and glycine by high performance liquid chromatography with ultraviolet and charged aerosol detection (HPLC-UV-CAD) methods. The used detection technique enables the quantitation of polar amino acids and related organic acids in a single chromatographic run. With this approach the currently required two compendial methods for the respective impurity classes could be substituted by a single analysis. In addition, the selectivity and the limits of quantification (LOQs) of the new methods are comparable with the established methods.

Introduction

Amino acids are among the most versatile biomolecules, serving as building blocks for proteins, nutritional supplements, or even as a medication. Due to their widespread use, the quality of the raw amino acids needs to be controlled, which is performed according to the respective compendial monographs. The polar nature of amino acids and their lack of a suitable chromophore make their analysis challenging. As a standard technique for amino acid analysis, the European Pharmacopoeia

(Ph. Eur.) employs several derivatization techniques. One of those is the Amino-Acid-Analyser (AAA) using ninhydrin as reagent;[1] the impurities detected by this procedure are called ninhydrin-positive substances. The AAA, however, can only detect amine-containing structures, thus other related impurities, like organic acids, must be determined separately. The objective of this application work was to develop methods for simultaneous detection and quantitation of amino acids and their possible impurities, in particular organic acids, in a single chromatographic run. The hyphenation of a CAD and a UV detector can help to overcome the aforementioned issues. L-aspartic acid (Asp) and glycine (Gly) were chosen as test substances, since their impurity analysis in the Ph. Eur. is currently accomplished by two different HPLC methods.[2,3] The individual impurity profiles of the two test substances are depicted in Figure 1. Volatile ion-pairing reagents were used to facilitate the separation of the amino acids and their putative impurities on a C18 Thermo Scientific™ Acclaim™ Polar Advantage II column. A Thermo Scientific™ Vanquish™ Charged Aerosol Detector H was used for the detection of the weak-chromophoric amino acids, demonstrating superior sensitivity with LOQs of 5–50 ng on column compared to what is achievable with low wavelength (<210 nm) UV detection (LOQs >75 ng).

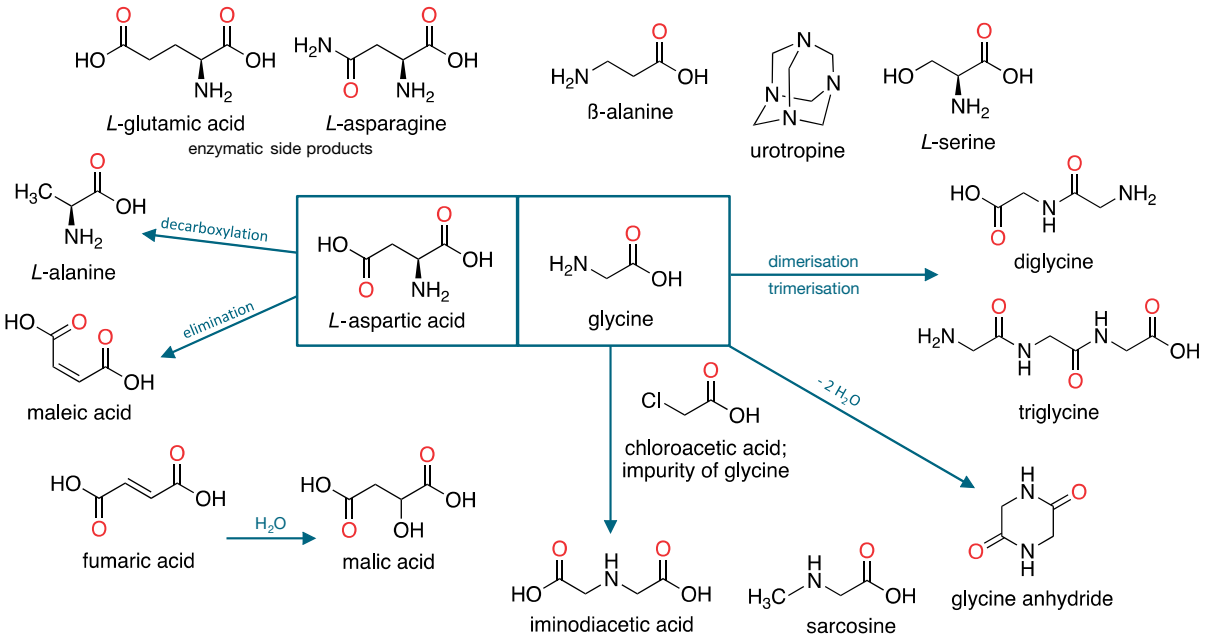


Figure 1. Impurity profile of Asp and Gly with respect to the Ph. Eur. 10.2[2,3]

Experimental

Chemical name	Part number
Deionized water, 18.2 MΩ/cm resistivity or higher	N/A
Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (ACN)	A955-212
Fisher Scientific™ Trifluoroacetic acid, Optima™ LC/MS grade (TFA)	A116-05AMP
Alfa Aesar™ Perfluoropentanoic acid, 97% (NFPA)	AAB2156706
Alfa Aesar™ Perfluoroheptanoic acid, 98+% (TDFHA)	AAA1209206
Fisher Scientific™ analytical grade amino acids, organic acids, glycine anhydride, and urotropine	
Acclaim Polar Advantage II (150 × 4.6 mm, 3 µm)	063191

Sample handling	Part number
Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer	14-955-152
Vials (amber, 2 mL), Fisher Scientific	03-391-6
Cap with Septum (Silicone/PTFE), Fisher Scientific	13-622-292

Sample preparation

Stock solutions of each impurity substance were prepared by accurately weighing 10.0 mg of the impurity and dissolving it in 10.0 mL deionized water. The stock solutions were used as calibration or external standards and for spiking of the sample solutions by appropriate dilution with mobile phase. The stock solutions were stored at 8 °C and were stable for at least one week.

The sample solutions were freshly prepared daily by weighing 50 mg of Asp or 100 mg of Gly and dissolving in 10.0 mL mobile phase. Due to its low solubility, the sample solution of Asp had to be stirred and heated at 50 °C for about 10 min to achieve complete dissolution. No visible precipitation occurred after cooling to room temperature. The sample solutions were stable for at least one day at room temperature.

Instrumentation

Module	Part number
Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of:	
System Base Vanquish Horizon	VH-S01-A
Vanquish Binary Pump H	VH-P10-A
Vanquish Split Sampler HT	VH-A10-A
Vanquish Column Compartment H	VH-C10-A-02
Vanquish Variable Wavelength Detector F	VF-D40-A
Standard Flow Cell, biocompatible, 11 µL	6077.0200
Vanquish Charged Aerosol Detector H	VH-D20-A

Table 1. Chromatographic conditions

Aspartic acid method	
Column	Acclaim Polar Advantage II, 150 × 4.6 mm, 3 µm
Mobile phase	7 mM NFPA, 4 mM TFA in water
Run time	10 min isocratic
Flow rate	0.8 mL/min
Column temperature	25 °C
Autosampler temperature	8 °C
Autosampler wash solvent	Methanol
Injection volume	20 µL
Detector settings (CAD)	Evaporation temperature: 50 °C; Power function value: 1.0; Filter constant: 5 s; Data collection rate: 10 Hz
Detector settings (VWD)	Detection wavelength: 210 nm; Data collection rate: 20 Hz; Response time: 0.20 s

Glycine method		
Column	Acclaim Polar Advantage II, 150 × 4.6 mm, 3 µm	
Mobile phase	A: 1.25 mM TDFHA, 6.5 mM TFA in water B: 1.25 mM TDFHA in ACN	
Gradient	Time (min)	%B
	0.0	1
	8.0	1
	10.0	10
	18.0	10
	20.0	1
	35.0	1
Flow rate	0.8 mL/min	
Column temperature	25 °C	
Autosampler temperature	8 °C	
Autosampler wash solvent	Methanol	
Injection volume	10 µL	
Detector settings (CAD)	Evaporation temperature: 70 °C; Power function value: 1.0; Filter constant: 10 s; Data collection rate: 10 Hz	
Detector settings (VWD)	Detection wavelength: 210 nm; Data collection rate: 20 Hz; Response time: 0.20 s	

Chromatography data system

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.6 was used for data acquisition and analysis.

Results and discussion

Method development and retention time reproducibility

Ion pair chromatography has already been successfully applied to the separation of closely related amino acids.[4-7] Thus, volatile ion-pairing reagents were used as mobile phase additives on a C18 reversed phase column to enhance the selectivity of the methods. A C18 Acclaim Polar Advantage II column was chosen as stationary phase since it provided excellent resolution of the test substances and their impurities. In addition, the embedded amide moieties of the column caused stability of the chromatographic system under the fully aqueous conditions of the Asp method.

The complete chromatographic separation of Asp from its impurities was achieved isocratically using a mixture of 7 mM perfluoropentanoic acid (NFPA) and 4 mM trifluoroacetic acid (TFA) in deionized water as mobile phase. For the Gly method, the selectivity had to be increased by switching from NFPA to perfluoroheptanoic acid (TDFHA). The final method mobile phase consisted of A: 1.25 mM TDFHA and 6.5 mM TFA in deionized water and B: 1.25 mM TDFHA in ACN. Gradient elution was employed in case of the Gly method to shorten the run time and to improve the peak shape of the late eluting impurities.

Since the extent of adsorption of the ion-pairing reagent on the surface of the stationary phase is likely to increase when switching from NFPA to TDFHA, a column washing procedure[4] was applied to ensure reproducible retention times of the analytes when switching between methods. The column was flushed with 25 column volumes of ACN and 15 column volumes of methanol as a regeneration procedure before changing the ion-pairing reagent. Prior to the Gly testing, the column had to be equilibrated at the final gradient conditions of 10% mobile phase B to minimize retention time shifts. The retention time reproducibility was satisfactory after injection of three blank runs (%RSD RT < 0.5), which corresponds to 34 column volumes. As a consequence of the greater adsorption of TDFHA on the surface of the stationary phase, the column equilibration time (~90 min) was significantly increased compared to NFPA (~25 min).

UV-CAD detection

A variable wavelength detector (VWD) and a CAD were connected inline to facilitate the simultaneous detection of the weak-chromophoric amino acids and their volatile impurities, in particular organic acids. Among the adjustable CAD settings, the evaporation temperature may be considered as the most relevant setting to adjust the CAD's sensitivity towards a specific analyte, since it influences the analyte's response as well as the background noise.[8] The hyphenation of detectors proved very beneficial, since the background noise of the CAD could be effectively reduced at elevated evaporation temperatures regarding the non-volatile impurities, while the decreased response of the more volatile impurities was tolerable due to the inline UV detection. The optimized evaporation temperature was higher (70 °C) for the Gly method compared to the Asp method (50 °C), since Asp contained the rather volatile impurity malic acid that was also preferentially detected by CAD. The weak-chromophoric impurities of Asp, namely asparagine, alanine, and glutamic acid were detected by CAD, whereas the more volatile organic acids maleic acid and fumaric acid were determined by UV detection (Figure 2). The impurities of Gly were, except for the UV-detectable chloroacetic acid, non-volatile, thus CAD alone was used for their detection (Figure 3). Besides the evaporation temperature, the filter constant setting of the CAD was also adjusted to optimize the signal-to-noise ratio (S/N). A higher filter constant (10 s) was applied to the Gly method compared to the Asp method (5 s) due to the higher background noise of the method caused by the relatively low volatility of the additive TDFHA.

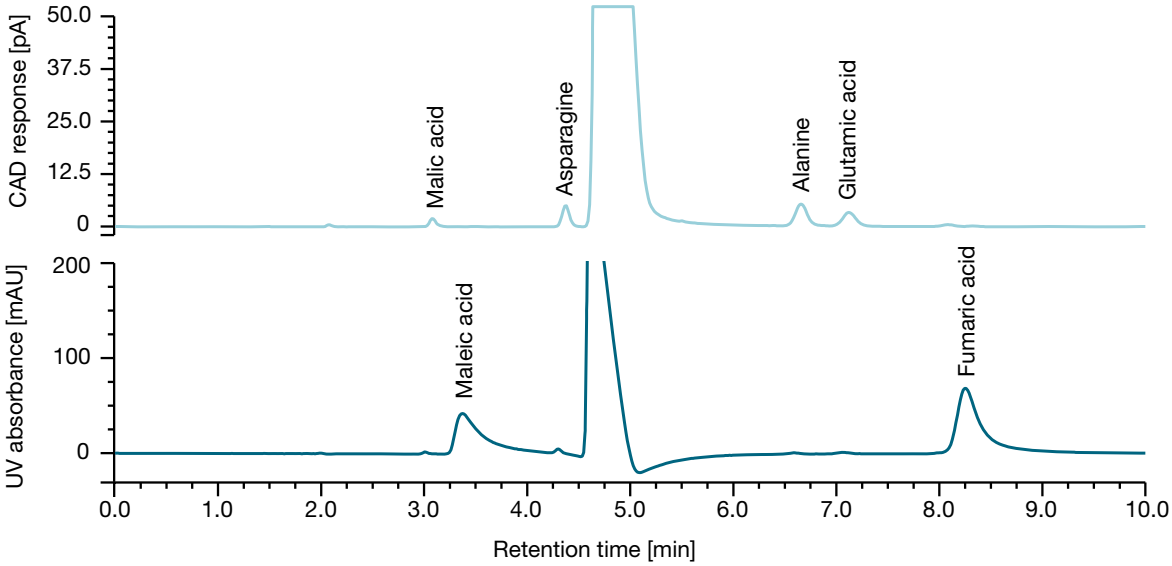


Figure 2. Chromatogram of an Asp sample solution (5 mg/mL) spiked with 0.15% of each impurity

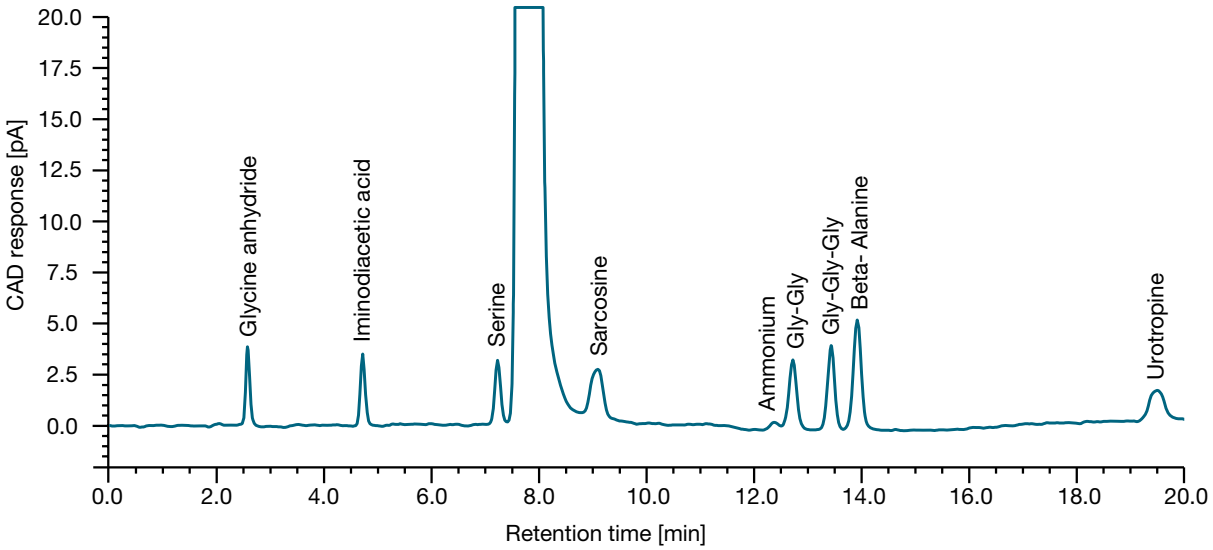


Figure 3. Chromatogram of a Gly sample solution (10 mg/mL) spiked with 0.15% of each impurity. The UV signal is not displayed as chloroacetic acid, the only UV-absorbing compound, was not found in any of the investigated batches.

Validation and batch analysis

Both methods were validated according to the international conference on harmonisation (ICH) guideline Q2(R1).[9] Therefore, the specificity, linearity, accuracy, precision, LOQs, and the robustness of the methods were assessed. Some key validation data are illustrated in Tables 2 and 3.

The methods were then applied to batch analysis. Four batches from various manufacturers were tested for each impurity. With exception of maleic acid, all specified impurities (alanine, asparagine, fumaric acid, glutamic acid, maleic acid, and malic acid) were found in the examined Asp samples at relevant levels. The only impurity found at relevant level in the Gly samples was sarcosine, besides small quantities of iminodiacetic acid below the reporting threshold and traces of diglycine and ß-alanine. Since no chloroacetic acid was detected in the investigated batches, it was possible to cover all occurring impurities by CAD only.

UV-CAD detection vs. compendial procedures

The impurity analysis of Asp according to the Ph. Eur. comprises a post-column ninhydrin derivatization procedure for the amino acid impurities, and one UV detection method to address the organic acid impurities.[2] The run time of the UV method is 30 min, whereas the derivatization method is a generic amino acid analysis method with a run time of at least 80 min. Thus, the total run time can be reduced by more than 90% when using the UV-CAD method with a run time of 10 min. The reporting threshold of the two compendial methods is set to a concentration of 0.05% with respect to the concentration of the sample solution, although the ICH guidelines outline requirements as a reporting threshold of 0.03% for drugs with an average daily intake of >2 g,[10] which holds true for Asp. The UV-CAD method, however, provides a reporting threshold of 0.03% due to its lower LOQs.

In case of Gly, the amino acid impurities are detected by the same method that is applied to Asp. The remaining impurities are detected by an UV method with a run time of 22 min.[3] Consequently, the total run time can be reduced by more than 65% when using the UV-CAD method with a run time of 35 min. The compendial reporting thresholds for Gly correspond to the Asp values (0.05%). Except for the unspecified impurities chloroacetic acid and urotropine that were not found in any of the investigated batches, the reporting threshold could be lowered to 0.03% when using the UV-CAD method.

Table 2. Linearity and LOQ results of the Asp and Gly methods (with respect to CAD unless otherwise stated)

Asp method			Gly method		
Compound	R^2 (linear regression)	LOQ (ng on column)	Compound	R^2 (linear regression)	LOQ (ng on column)
Asp	0.9995	8	Gly	0.9997	30
Alanine	0.9987	8	ß-Alanine	0.9983	30
Asparagine	0.9982	8	Chloroacetic acid	0.9992 (UV)	50 (UV)
Fumaric acid	0.9997 (UV)	5 (UV)	Glycine anhydride	0.9984	20
Glutamic acid	0.9991	10	Diglycine	0.9975	30
Maleic acid	0.9998 (UV)	10 (UV)	Triglycine	0.9962	30
Malic acid	0.9991	20	Iminodiacetic acid	0.9987	25
			Sarcosine	0.9972	30
			Serine	0.9978	30
			Urotropine	0.9967	50

Table 3. Recovery rate in % obtained by linear regression and %RSD of replicate injections (n=6) for three concentration levels (0.05% instead of 0.03% for the lowest level in case of chloroacetic acid and urotropine) with respect to the concentration of the sample solution

	Asp method					
	Spiked concentration [%]					
	0.03		0.15		0.24	
Compound	Recovery [%]	%RSD	Recovery [%]	%RSD	Recovery [%]	%RSD
Alanine	103	2.6	104	3.3	98	3.0
Asparagine	92	3.2	101	1.6	94	2.6
Fumaric acid	100	3.5	100	0.7	100	0.1
Glutamic acid	111	3.4	101	3.1	98	2.4
Maleic acid	104	2.7	100	2.3	101	0.9
Malic acid	104	4.0	100	3.7	104	1.0

	Gly method					
	Spiked concentration [%]					
	0.03		0.15		0.24	
Compound	Recovery [%]	%RSD	Recovery [%]	%RSD	Recovery [%]	%RSD
ß-Alanine	112	4.7	97	1.5	100	1.1
Chloroacetic acid	90 (0.05%)	3.9 (0.05%)	96	4.0	97	2.3
Glycine anhydride	98	5.5	104	1.5	100	2.8
Diglycine	111	6.6	100	2.5	100	1.5
Triglycine	112	4.9	97	1.8	100	0.9
Iminodiacetic acid	107	5.2	103	1.5	97	3.3
Sarcosine	75	7.3	101	3.4	91	4.3
Serine	111	3.9	98	3.4	100	2.4
Urotropine	97 (0.05%)	4.9 (0.05%)	102	2.6	88	1.7

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Conclusion

- To enable the simultaneous detection of volatile and weak-chromophoric impurities in amino acids, HPLC methods using UV-CAD detection were evaluated as an alternative to the compendial methods that employ UV and post-column derivatization procedures.
- The inline coupling of both detectors was successfully applied to the impurity profiling of the polar amino acids Asp and Gly.
- Two separate methods were developed for Asp and Gly and, subsequently, validated.
- Both methods were applied to batch analysis, demonstrating superior selectivity and time savings (65% and 90%, respectively) compared to the compendial procedures.

Acknowledgements

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