Improved and Simplified Liquid Chromatography/Atmospheric Pressure Chemical Ionization Mass Spectrometry Method for the Analysis of Underivatized Free Amino Acids in Various Foods

Application

Food

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Abstract

An improved analytical method that offers rapid, accurate determination and identification of 22 amino acids in a variety of matrixes is reported. The amino acids were extracted from the matrixes using acidified water. Simultaneous determination of 22 underivatized amino acids was carried out by liquid chromatography–mass spectrometry (LC/MS). A narrow-bore column allowed rapid screening and quantitative analysis by LC/atmospheric pressure chemical ionization (APCI) MS in positive ion mode.

Introduction

There are a total of 22 amino acids classified by their functional group, or their "R" group. Of those 22 amino acids, eight are histidine, lysine, phenylalanine, methionine, leucine, isoleucine, valine, and threonine considered essential, which means that humans need to get a certain amount of them in their diet to function properly. The determination of amino acids is of great importance to the food industry because of nutritional labeling requirements.

There are several approaches to amino acids analysis based on the pre- or post-column derivatization techniques, which have demonstrated good results but have drawbacks of very long analysis time or poor repeatability. Most of the other methods used for amino acid analysis involve a chromatographic separation following or preceding the derivatization of amino acids with UV absorptive or fluorescent functional group detection. Although these methods are often faster than the classical ion-exchange method, which involves quantification after ninhydrin and OPA derivatization and offers lower detection limits, these methods are still very time consuming, taking 20 to 75 min, are not repeatable, and are difficult to undertake.

The objectives of the present study were 1) to develop an analytical method for the simple, rapid, accurate, and repeatable determination of 22 free amino acids in various foods using an improved LC/APCI-MS method, 2) to develop an easy aqueous extraction of amino acids without clean-up, and 3) to perform screening and both qualitative and quantitative analysis on a narrow-bore column with a short run time.

Method

LC Conditions

Column:	ZORBAX Bonus-RP, narrow-bore (100 mm × 2.1 mm, 3.5 µm)
Flow rate:	0.2 mL/min
Mobile phase:	0.01 mM acetic acid in 0.2% aqueous solution of formic acid
Injection:	20 µL out of 1000 µL

MS Conditions

Ionization mode:	Positive APCI
Nebulizer pressure:	55 psi
Drying gas flow:	4 L/min
Drying gas temperature:	320 °C
Vaporizer temperature:	425 °C
Skimmer:	20 V
Capillary voltage:	3 kV
Fragmentor voltage:	55 V
Dwell time:	27 ms

Experimental

LC/MS experiments were performed using an Agilent 1100 Series HPLC system consisting of a binary pump, an autosampler, and a temperature-controlled column oven, coupled to an Agilent 1100 MS detector equipped with an atmospheric pressure chemical ionization (APCI) interface.

Data acquisition was performed in SIM mode using the interface parameters: drying gas (N_2, 100 psig) flow of 4 L/min, nebulizer pressure of 55 psig, drying gas temperature of 320 °C, vaporizer temperature of 425 °C, capillary voltage of 3 kV, corona current of 8 µA, fragmentor voltage of 55 V, and dwell time of 27 msec. Ions monitored for 22 underivatized amino acids are given in Table1.

The chromatographic separations were performed on a ZORBAX Bonus-RP, narrow-bore (100 mm × 2.1 mm, 3.5 µm) using the isocratic mixture of 0.01 mM acetic acid in a 0.2% aqueous solution of formic acid at a flow rate of 0.2 mL/min at 400 °C.

Sample preparation

According to the sample matrix, the sample was ground (by using a blender, mesh size 2 mm) or mixed (by Ultra Turrax). Dry samples like baby foods, breakfast cereal, and cookies were milled, and soft samples, like tomato, and liquid samples, like juices, were mixed with an Ultra Turrax blender. The pH of each homogenized sample was measured before sample preparation. Subsamples of the homogenate were stored at –20 °C in high-density polyethylene bottles with plastic screw-cap lids. Finely ground or homogenized sample (1 g) was weighed (fresh weight [FW]) into a 10 mL glass centrifuge tube with cap. After mixing in a vortex mixer for 2 min, the mixture was centrifuged at 5,000 rpm for 10 min at –5 °C. The clear supernatant was quantitatively transferred into a vial, avoiding the top oil layer if present. The supernatant was filtered through 0.45-µm nylon syringe filter prior to LC/MS analysis.

Table 1. Characteristic Fragments of 22 Amino Acids and Ions Used in SIM Mode for Quantification

ID	Fragment ions *m/z*	Selected ion *m/z*
Alanine (ala)	90, 73	90
Arginine (arg)	175, 129	175
Asparagine (asn)	133, 116, 87, 74	133
Aspartic acid (asp)	134, 116, 88	134
Cysteine (cys)	122, 105, 87, 73	122
Cystine(cys-cys)	241, 122	241
Glutamic acid (glu)	148, 130, 102	148
Glutamine (gln)	147, 130, 101	147
Glycine (gly)	76, 59	76
Histidine (his)	156, 110, 96, 73, 59	156
Hydroxyproline (hyp)	132, 86	132
Leucine-isoleucine (leu-ile)	132, 86	132
Lysine (lys)	147, 130, 84	147
Methionine (met)	150, 133, 104	150
Phenylalanine (phe)	166, 149, 120	166
Proline (pro)	116, 70	116
Serine (ser)	106, 88, 60	106
Threonine (thr)	120, 102, 74	120
Tyrosine (tyr)	182, 165, 136, 123	182
Tryptophan (trp)	205, 188, 130	188
Valine (val)	118, 72	118

Results and Discussion

Optimization of MS fragmentation and chromatographic conditions

Fragmentation patterns were reproducible in both modes, and quantification could be performed on single *m/z* fragments. In-source collision induced dissociation at 55, 70, and 100 V was applied for all amino acids, and optimum conditions were found to be 55 V and positive polarity. All the most abundant fragmentations were present, with optimization of the MS parameters. Under the positive APCI conditions applied here, SIM-MS analysis showed excellent identification and quantification conditions within the 7.5 min total run time.

Specificity

MS detection has the advantage of providing structural information about the eluted compounds. Moreover, resolution of co-eluting compounds can be achieved by selecting different *m/z* ions for monitoring. In this study, a simple, rapid, and economic sample preparation method was used.

During the extraction with a 0.2 mM acetic acid solution, colloids soluble in water (starch) were simultaneously precipitated by centrifugation. Fat was separated by cold centrifugation, if present in the food sample. Figure 1 shows the MS signals for specific *m/z* ions of 22 amino acids in green peas using a single injection.

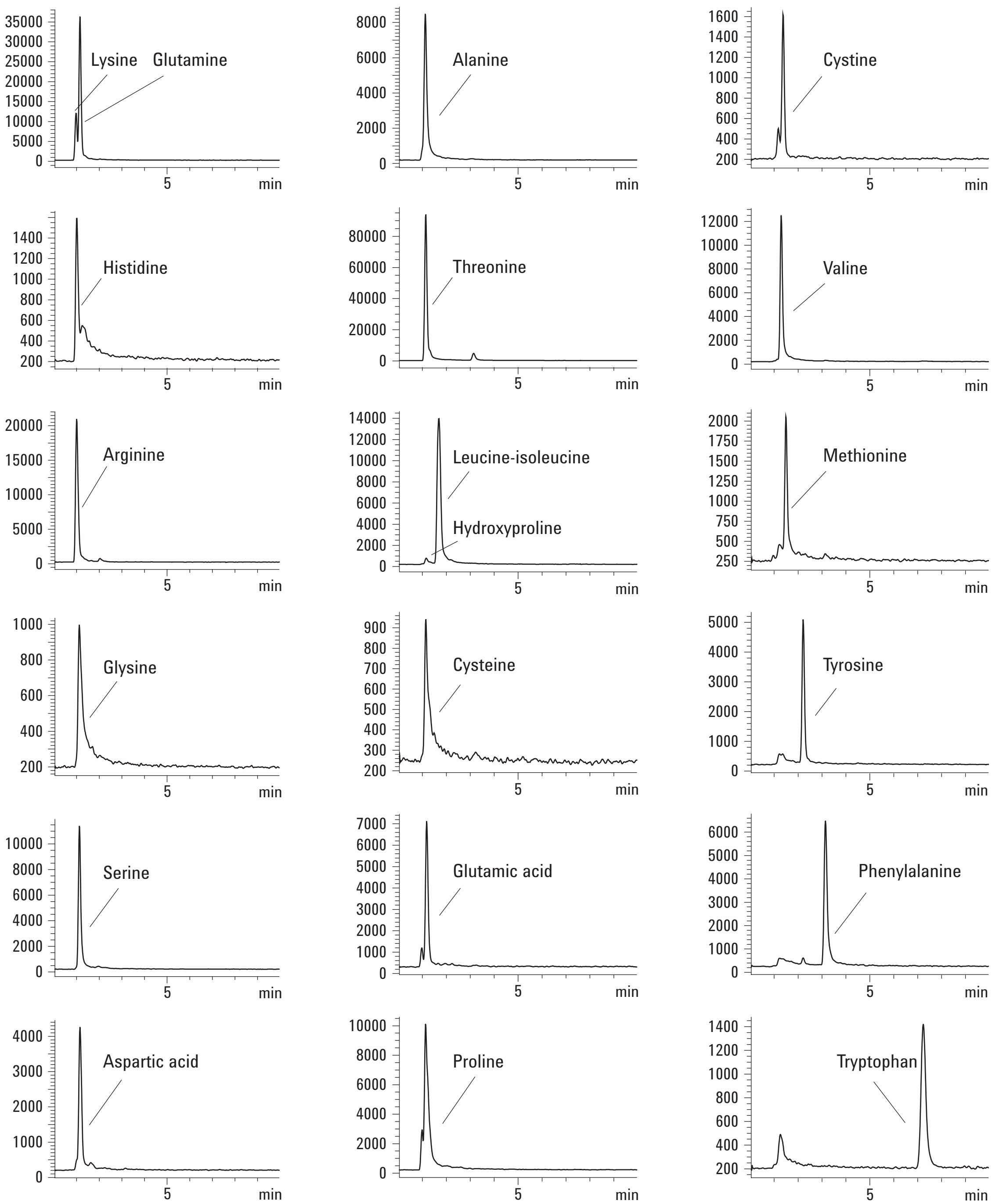

Figure 1. **Determination of underivatized free amino acids using LC/APCI-MS. LC conditions: column, ZORBAX Bonus RP (2.1 mm × 100 mm, 3.5 µm); flow rate, 0.2 mL min^{-1}; mobile phase composition, 0.01 mM acetic acid in a 0.2% aqueous solution of formic acid; and MS signals for specific *m/z* ions of 22 amino acids in green pea.**

Food samples

In this study, 22 food samples were analyzed for their amino acid content. The food groups are baby foods, vegetables, fruits, juices, nuts, wine, beer, milk, chicken, and honey. The mean concentrations of amino acids in each food are given in Table 2.

Table 2. The Results of 22 Free Amino Acids in Various Food, as mg/100 g FW

Food Types	Ala	Arg	Asn	Asp	Cys	Cys-Cys	Glu	Gln	Gly	His	Hyp
Baby food	3.59	1.25	1.07	0.50	0.17	9.77	0.37	1.06	0.52	0.30	1.42
Tomato	10.07	3.94	11.02	36.86	1.31	1.52	85.94	49.64	2.03	0.34	0.73
Pea	69.84	37.26	112.10	30.22	6.96	3.62	15.78	57.49	7.92	3.27	1.17
Pear	< LOD	1.81	45.27	17.01	0.60	13.58	8.19	86.33	1.14	< LOD	< LOD
Apple paste	< LOD	1.40	3.80	3.56	0.30	16.04	2.18	23.46	< LOD	< LOD	< LOD
Apple juice	2.03	1.79	12.80	10.58	0.98	12.52	4.82	39.89	< LOD	0.13	0.43
Sour cherry juice	1.53	1.62	24.10	7.30	0.38	10.95	7.11	52.45	< LOD	1.01	0.32
Orange juice	3.95	8.30	8.08	15.15	0.70	9.99	5.10	3.36	1.38	1.11	0.53
Pomegranate juice	15.66	1.99	13.58	17.33	0.27	16.53	17.23	42.35	< LOD	0.17	0.44
Peach juice	3.38	1.64	78.57	21.52	0.84	12.26	3.38	10.05	1.44	0.14	0.35
White grape juice	6.54	2.18	10.50	16.08	0.50	16.06	8.34	41.50	1.26	0.11	0.43
Red grape juice	8.58	0.60	5.32	5.54	0.74	17.60	7.98	4.17	1.52	1.46	0.52
Beer	4.00	0.88	2.98	1.15	< LOD	5.33	1.89	1.54	1.48	0.26	8.59
Milk	7.02	16.50	8.99	4.00	0.36	16.12	9.06	4.85	1.38	0.75	0.36
Wine	4.49	1.35	0.94	0.55	0.66	0.92	0.73	0.42	1.69	0.46	0.24
Honey	12.23	< LOD	4.16	8.43	< LOD	203.22	7.93	18.90	2.93	1.26	0.76
Green coffee	41.06	2.38	31.36	70.26	43.19	14.82	57.18	3.89	3.43	3.27	< LOD
Hazelnut	12.98	1.28	2.21	13.05	< LOD	6.02	21.01	0.51	2.57	< LOD	< LOD
Walnut	6.56	2.71	0.71	7.42	< LOD	4.90	46.80	3.55	1.72	0.38	0.74
Almond	11.39	4.14	57.51	44.92	0.73	4.50	3100	6.31	3.76	0.34	0.46
Pistachio	25.15	7.58	14.45	27.40	1.36	5.82	35.64	39.28	2.88	< LOD	7.26

Food Types	Leu-ile	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Trp	Val
Baby food	0.30	< LOD	0.20	0.26	0.78	0.40	1.66	0.50	0.12	1.21
Tomato	0.96	< LOD	0.63	3.25	6.30	3.38	2.70	2.05	0.74	2.43
Pea	26.84	17.41	4.44	13.52	21.50	55.52	0.03	15.45	3.31	38.60
Pear	1.38	< LOD	< LOD	0.44	17.92	3.86	1.23	0.68	0.48	5.25
Apple paste	< LOD	< LOD	< LOD	< LOD	< LOD	0.57	< LOD	< LOD	< LOD	< LOD
Apple juice	0.32	< LOD	< LOD	< LOD	4.48	0.78	0.33	< LOD	< LOD	1.70
Sour cherry juice	0.23	< LOD	4.52	0.14	6.52	1.29	0.53	0.25	< LOD	2.62
Orange juice	0.37	1.76	4.72	0.99	30.55	6.07	1.13	0.99	0.58	3.64
Pomegranate juice	1.37	< LOD	0.53	0.94	8.52	3.01	1.94	1.76	0.64	4.62
Peach juice	< LOD	< LOD	< LOD	0.45	12.89	2.48	1.17	0.33	< LOD	2.18
White grape juice	1.54	1.21	0.89	0.81	8.94	2.80	1.75	1.80	0.64	5.10
Red grape juice	1.80	1.39	0.64	1.88	14.05	4.30	5.33	3.90	0.75	5.03
Beer	< LOD	1.17	0.30	0.15	0.84	< LOD	0.32	0.27	< LOD	3.84
Milk	2.04	1.43	1.80	0.61	11.61	3.14	3.12	2.33	0.65	5.40
Wine	0.23	1.28	0.25	0.25	30.50	0.36	0.31	2.37	1.86	0.88
Honey	2.06	4.80	< LOD	11.18	81.32	6.31	2.65	8.63	< LOD	6.34
Green coffee	6.37	4.35	1.66	14.31	32.30	20.48	3.63	13.58	7.66	8.61
Hazelnut	3.24	2.06	< LOD	2.41	5.38	3.84	2.38	3.69	2.16	4.08
Walnut	1.81	1.64	0.52	1.09	5.50	1.44	1.12	1.71	1.75	3.01
Almond	7.21	4.93	1.93	6.08	33.65	7.82	3.34	4.07	2.28	10.33
Pistachio	2.75	3.64	1.43	381	55.98	3.80	3.71	6.00	1.51	5.73

There was a great variation in the amino acid levels in each food. The variations in amino acid concentrations might be due to the differences in the composition of formulas and/or the differences in processing conditions. Also the protein composition can give rise to slight differences in the free amino acid profiles of foods. The pH of the samples was measured before extraction and found to range from 2 to 3 for juices, beer, and wine; about pH 4 for tomato; pH 6 for peas; and pH 7 for milk. It is very well known that the measurement of amino acids is very important in various areas, such as:

1) The determination of the geographical sources of honey from the ratios between concentrations of amino acids

2) Nutritional analysis as the amino acid supply in an infant's first month of life must be sufficient in quantity and quality to fulfill the needs of this critical period.

3) Some of the important free amino acids, such as asparagine, glutamine, aspartic acid, and glutamic acid, are important precursors for acrylamide in various products.

Conclusion

A simple, reliable, and rapid LC/MS method for the determination of 22 free amino acids in foods was developed in this study. The method was found to be applicable for a wide variety of foods. Our goal was to obtain a single-run LC/APCI-MS analysis of underivatized amino acids exhibiting sensitivity in positive mode, and this was improved by the use of further acidic LC/MS mobile phase to achieve a very short run time, ca. 7.5 min. Sample preparation and the subsequent chromatographic run took < 25 min to complete. Previous studies, which used conventional LC columns, long extractions with clean-up, and subsequent derivatization were expected to take one day to analyze five samples, whereas with this new, simplified, faster method, a batch of 20 samples can be completed in the same time.

Reference

S. Özcan, H. Şenyuva, Improved and simplified liquid chromatography/atmospheric pressure chemical ionization mass spectrometry method for the analysis of underivatized free amino acids in various foods, *J. Chromatogr. A* (2006), Doi:10.1016/j.chroma.2006.09.039

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