High-Performance Liquid Chromatographic Analysis of Free Amino Acids in Fruit Juices Using Derivatization with 9-Fluorenylmethyl-Chloroformate

A. Fabiani, A. Versari*, G.P. Parpinello, M. Castellari, and **S. Galassi**
Corso di Laurea in Scienze e Tecnologie Alimentari, Università di Bologna, Via Ravennate, 1020 – 47023 Cesena (FC), Italy

Abstract

A simple, rapid, and reliable reversed-phase high-performance liquid chromatographic method for the analysis of 16 amino acids of main interest in commercial fruit juices (pear, orange, grapefruit, pineapple, peach, and apricot) is described. No sample cleanup is required. The pH of the fruit juices is adjusted to alkaline value (8.5) using 200mM borate buffer, then amino acid is converted to stable derivatives using 9-fluorenylmethyl-chloroformate. The excess of derivatization reagent is removed by a hydrophobic amine, 1-amino-adamantane hydrochloride. The derivatization procedure is simple, fast, and described in detail. Amino acids are detected at 263 nm and eluted within 35 min. The calibration, precision (≤ 6.1%), and recovery (102% ± 4%) of the method are reported. The conditions of separation are optimized; however, serine partially overlapped with aspartic acid. The amino acid profile of fruit juices is consistent with data from the literature.

Introduction

Amino acids, a class of biologically active compounds present in food and beverages, are important for human nutrition (1) and affect the quality of foods including taste, aroma, and color (2–4). Amino acids are useful markers to define fruit juice genuineness; however, their use is complicated by the natural variability of fruit compositions (5,6). Proline (PRO) has already been included within the European regulatory values for testing the authenticity of fruit juices and nectars (7).

There is an ongoing interest in the development of a reliable, rapid, and accurate method of analysis to assess the quality of foods for nutritional and regulatory purposes. Many analytical methods have been proposed for the analysis of amino acids, including gas chromatography (8,9), high-performance liquid chromatography (HPLC) (10), and capillary electrophoresis (11,12). Amino acids present in foods are usually analyzed after their derivatization. Reversed-phase HPLC with precolumn derivatization is preferred because of the short time, simple instrumentation, and low cost required. Typical reagents for precolumn derivatization are phenylisothiocyanate (PITC); *o*-phthalaldehyde (OPA); 9-fluorenylmethyl-chloroformate (FMOC-Cl); 1-fluoro-2,4-dinitrobenzene; 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; and dansyl-chloride (10). Each of these reagents have particular advantages and limitations. Among them, only PITC and OPA are widely used for the analysis of amino acids. FMOC-Cl has been used in combination with 1-amino-adamantane hydrochloride (ADAM) for the analysis of FMOC–amino acids (FMOC–AA) in protein hydrolysates, coffee beans, and algae (13–17). In fact, ADAM is a hydrophobic amine that reacts with FMOC-Cl in excess only to form a complex (FMOC-ADAM), thus allowing for the reduction of the chromatographic interference of FMOC-OH formed in ambient alkaline.

In this study, an HPLC method for the analysis of selected amino acids in fruit juices without sample cleanup was developed and validated. The use of FMOC-Cl (in combination with ADAM) for the analysis of amino acids as FMOC derivatives in fruit juices is reported for the first time.

Experimental

Chemicals

Hydrochloridic acid, (Carlo Erba, Milano, Italy), sodium hydroxide, sodium acetate, sodium borate, HPLC-grade acetonitrile, chlorure and carbonate buffers (Merck, Darmstad, Germany), FMOC-Cl, and ADAM (Sigma, Milano, Italy) were obtained from commercial sources.

Standards and sample preparation

Standards of amino acids (Sigma) were dissolved in 0.01M hydrochloridic acid, then derivatized (as will be described) and filtered through a 0.45-µm polytetrafluorethylene (PTFE) membrane (Gyrodisc, Orange Scientific, Waterloo, Belgium) prior to HPLC analysis. The molar absorptivity (ε) of each derivatized amino acid (FMOC–AA, Sigma) was determined according to the Lambert-Beer law (A = εbc). The absorbance at 263 nm was measured within the range of linearity using a Uvikon XS spec-

* Author to whom correspondence should be addressed: email versari@foodsci.unibo.it.

trophotometer (Uvikon, Milano, Italy), and each amino acid was dissolved in a mixture of acetonitrile–acetate buffer whose composition resembled the mobile phase that occurred at the time of peak (amino acid) elution during the HPLC run.

Commercial fruit juices (pear, orange, grapefruit, pineapple, peach, and apricot) obtained from the local markets were centrifuged at 1500 g for 15 min at 4°C (ALC4239R, ALC International, Milano, Italy). The supernatant was derivatized and then filtered using a 0.45-µm PTFE membrane before injection in HPLC. The composition of the amino acids was referred to as fruit juices with 11.5° Brix.

Derivatization procedure

Amino acids were derivatized (FMOC–AA) at room temperature using a precolumn procedure. An aliquot of 300 µL of fruit juice (or a standard solution of amino acids) was added with 600 µL of a 200mM borate buffer (pH 10.0). Then, 600 µL of 15mM FMOC-Cl (in acetonitrile) was added to the fruit juice and derivatization occurred. After 5 min, the reaction was stopped by the addition of 600 µL of 300mM ADAM (water–acetonitrile, 1:1, v/v), and the reaction lasted for 1 min to form the FMOC–ADAM complex (Figure 1). Then, the sample was filtered and analyzed by HPLC. The total time required for the derivatization procedure was 6 min.

HPLC analysis

An LC-1500 HPLC system (Jasco, Tokyo, Japan) was equipped with an MD-1510 diode-array detector set at 263 nm (λ_{max}). Data were acquired and processed using Borwin-PDA Version 1.50 software (JMBS Developments, Grenoble, France). Samples were injected with a 20-µL loop using a 7125 valve (Rheodyne, Cotati, CA) onto a Purospher RP-18 column (250- × 4-mm, 5-µm i.d.) protected with a guard column of the same material (Merck). The column operated at 25°C (Jones Chromatography, Mid Glamorgan, U.K.) with a flow rate of 1.0 mL/min using 50mM acetate buffer (pH 4.2) as eluent A and acetonitrile as eluent B. Amino acids were separated with the following linear gradient elution conditions (min/A%): 0/72, 3/72, 27/55, 32/0, 37/0, 39/72, and 47/72.

Peak identification and quantitation

Identification was based on the comparison between the retention time of the standards of the amino acids and those in fruit juices and was confirmed by a fortification technique (spiking). Quantitation was based on the external standard method using calibration curves fitted by linear regression analysis (Statistica 5.1, StatSoft, Tulsa, OK).

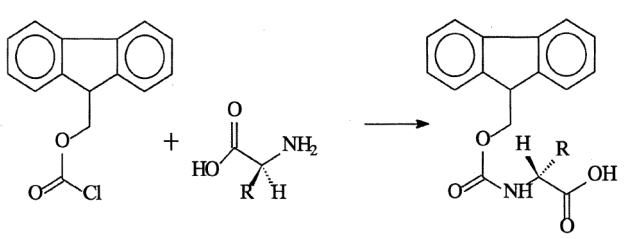

Figure 1. Chemical reaction between FMOC-Cl and ADAM.

Method validation

In order to assess the linearity of the relationship between the concentration of the amino acids and the peak area, five amino acid standard solutions (ranging from 0.25 to 1.25mM) were prepared in 0.01M hydrochloridic acid and analyzed in duplicate. The calibration curves, relating the signal (peak area) to the analyte concentration, yielded the linear equation:

$$y = a + bx \qquad \text{Eq. 1}$$

with y being the signal (µAU), a the intercept (signal), b the slope (signal/concentration), and x the amino acid concentration (mM). The precision of the method (repeatability) was determined by measuring the peak area of a single fruit juice injected six times. In order to assess the accuracy of the method, a recovery study was carried out by adding each amino acid at three concentration levels (0.04, 0.06, and 0.08mM) to a fruit juice that was previously analyzed. The resulting fortified samples were analyzed in duplicate. The limit of detection (LOD) was evaluated as three times the signal-to-noise ratio. The stability of the derivatives at 20°C was studied at 0, 50, and 100 min.

Results and Discussion

Optimization of the reaction conditions

The derivatization of amino acids with FMOC-Cl requires a buffered alkaline pH (≥ 8.0). Because fruit juices have acidic pH values and a natural buffering capacity, the preliminary optimization of sample alkalinization was studied. Sodium hydroxide (5%) and several buffers (including borate, chloride, and carbonate) were tested (Table I). The alkalinization with sodium hydroxide and carbonate buffer led to the formation of an insoluble deposit during the derivatization procedure. Instead, best results were achieved by using a 0.2M borate buffer at pH 10 with a 2:1 buffer–juice ratio.

Precolumn derivatization with FMOC-Cl was fast (5 min), and the amino acid derivatives were stable up to 100 min. The subsequent reaction of FMOC-Cl with ADAM allowed for the reduction of the FMOC-OH in excess, thus avoiding the presence of interferences during the chromatographic analysis. The derivatization yield was dependent on both the reagent concentration and the reaction time. An excess of reagents was required to provide effective derivatization. In this study, the ratios of 5 for FMOC-Cl–amino acids and 20 for ADAM–FMOC-Cl were found to be appropriate. Most of the amino acids were fully derivatized after 30 s, whereas aspartic acid (ASP) and glutamic acid (GLU)

Table I. Effect of Buffer Type and Concentration on the pH Value of Fruit Juices

Buffer (M)	pH	Buffer–juice ratio (v/v)	pH value of juice		
			Orange	Pineapple	Grapefruit
Borate (0.20)	8.2	1		4.1	4.8
3.3					
Carbonate (0.25)	10.7	1		10.0	10.5
9.1					

required a longer time of reaction (14).

Separation and identification

The proposed HPLC method allowed for the simultaneous analysis of 16 amino acids of interest in fruit juices within 35 min (Figure 2). Phenylalanine (PHE), isoleucine (ILE), hystidine (HYS), and lysine (LYS) were detected in standard solutions only, with their content in the analyzed fruit juices being too low or absent. Therefore, the chromatograms of fruit juices were cut at 30 min in order to improve the quality of presentation (Figures 3–6). Fruit juices showed a complex amino acid profile. The major peaks were identified and quantitated, whereas additional peaks (tentatively attributed to secondary amino acids) were only detected. Serine (SER) (t_R = 11.7) partially overlapped with ASP (t_R = 12.0), and the attempt to improve their separation using different C-18 columns (5-µm Lichrospher and Purospher (Merck), 5-µm Adsorbosphere (Alltech), and 3-µm Luna (Phenomenex)) and several elution and flow-rate conditions were thus far only partially successful. In particular, the analysis with a 3-µm C-18 column reduced the peak width and allowed for a slight improvement in the separation of SER (t_R = 13.8) and ASP (t_R = 14.2); however, these two amino acids were still not resolved at baseline. Additional information on the effect of the mobile phase composition, its flow rate, column temperature, and type of column would require a chemometric approach, such as a central composite or a modified Box & Behnken experimental design. Information from the literature (16,17) confirms the problematic separation between SER and ASP when these amino acids are analyzed as FMOC derivatives. Péter et al. (17) also showed a satisfactory HPLC separation of SER–FMOC and ASP–FMOC by using a Vydac 218TP54 C-18; however, a coelution between ASP

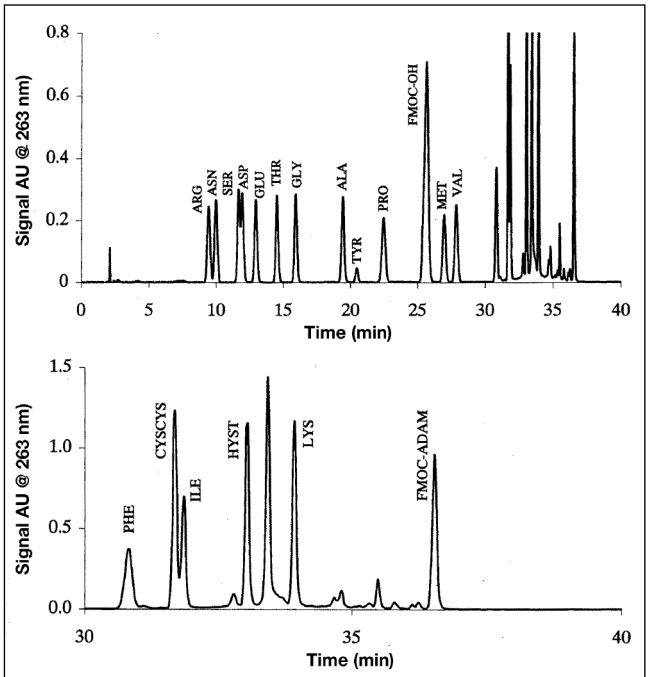

Figure 2. Chromatograms of a standard mixture: threonine, THR; alanine, ALA; and cystine, CYSCYS.

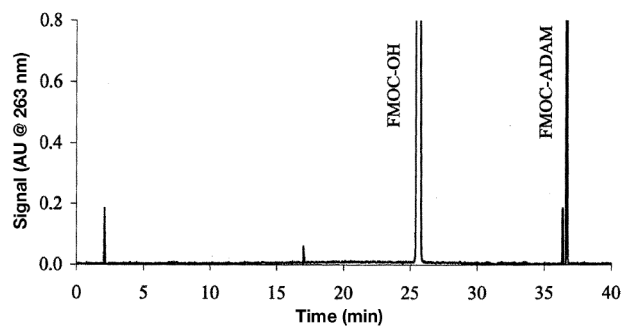

Figure 3. Chromatogram of a blank (derivatization reagent).

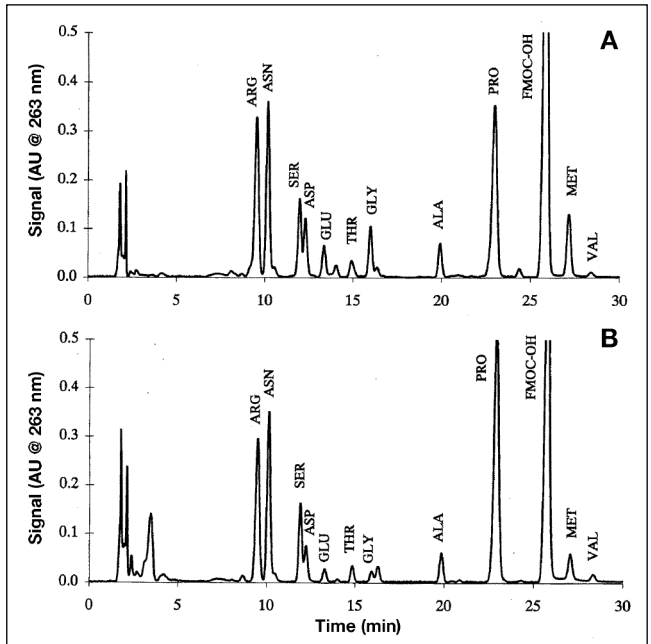

Figure 4. Chromatograms of (A) orange and (B) grapefruit juices: threonine, THR, and alanine, ALA.

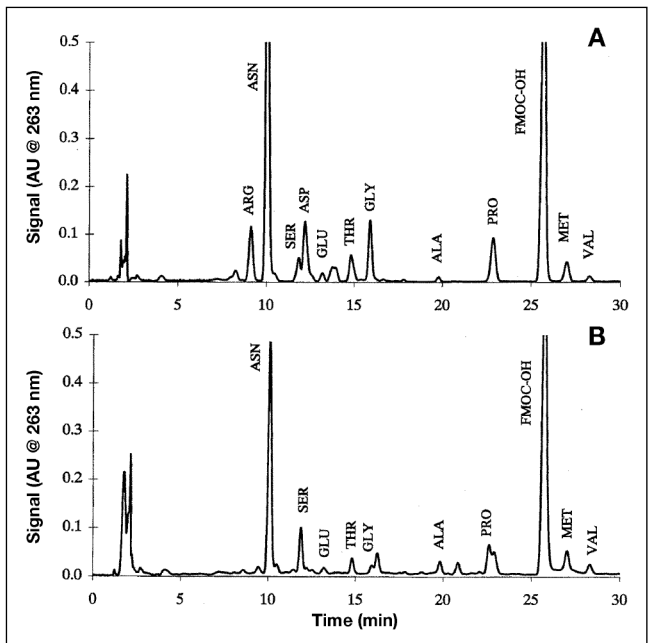

Figure 5. Chromatograms of (A) pear and (B) pineapple juices: threonine, THR, and alanine, ALA.

and GLU occurred.

Asparagine (ASN) was found in all the fruit juices (its content being highest in apricot and peach), and PRO was abundant in orange, pineapple, and grapefruit. Arginine (ARG) was typical in orange and grapefruit, whereas glycine (GLY) was high in pear and orange juices and methionine (MET) was high in grapefruit. The amino acid profile found in the fruit juices was consistent with data from the literature (5,6,18–22), with the secondary peaks detected in the fruit juices being probably minor amino acids such as glutamine, leucine, ornithine, or γ-aminobutyric acid.

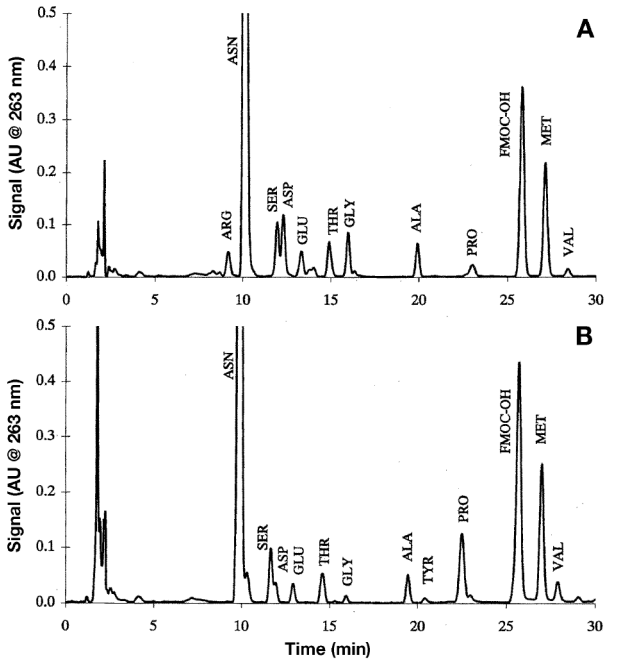

Figure 6. Chromatograms of (A) peach and (B) apricot juices: threonine, THR, and alanine, ALA.

Method validation

The amino acids showed similar molar absorptivity and sensitivity (slope) as a consequence of their derivatization (Table II). HYS and LYS showed the highest value of molar absorptivity because of the presence of two chromophores in their molecules. The linearity of the method was satisfactory, which implies the reliable quantitation of amino acids. Furthermore, precision showed a range between 2.4 and 6.1%, with ASN and valine (VAL) having the extreme values (Table III). A recovery test was performed to verify the yield of derivatization and the lack of interference effects resulting from the matrix composition. Table III shows the recovery of amino acids. ASP was only partially derivatized by FMOC-Cl, probably because of the low pH value of the reaction. However, tyrosine (TYR) (a basic amino acid) probably formed an unstable derivative, TYR–FMOC, which decomposed because of the high pH of reaction. In fact, the recovery of TYR was improved at a pH of 8.2. However, by lowering the pH value the solubility of ASP and GLU derivatives decreased. According to Einarsson et al. (13), the derivatization of HYS with FMOC-Cl is problematic because it produces multiple peaks, which implies the poor quantitation of this amino acid. The LOD showed a range of 3 to 6µM. Arnold et al. (16) reported an improved sensitivity in the femtomole range by using fluorescence detection.

The precolumn derivatization for the HPLC analysis of amino acids has advantages and drawbacks depending on the method used (10,23–26). For example, OPA does not react with secondary amino acids such as PRO and hydroxyproline. Derivatization with dansyl-chloride is slow, derivatives have poor stability, and interfering side-products usually occur. However, PITC requires a long time for derivatization and the removal of reagents, and it is not suitable for an automatic procedure.

Table II. Molar Absorptivities, Retention Times, Calibration Parameters, and General Composition Ranges of Juices

Amino acid	Molar absorptivity	t_R (min)	Calibration parameters (n = 10)					Juice composition range (mM)
			Intercept ± SE*	Slope ± SE*	r^2	Curve SE*	p-value†	
ARG	17600	9.4	0.38 ± 0.26	2.97 ± 0.43	0.998	0.04	—	0.1–2.3
ASN	19100	9.9	–0.79 ± 1.77	3.97 ± 0.32	0.987	1.44	—	1.7–18.6
SER	19300	11.7	0.00 ± 0.07	3.04 ± 0.09	0.997	0.06	—	0.1–1.4
ASP	18400	12.0	–0.02 ± 0.04	3.16 ± 0.06	0.997	0.06	—	0.1–2.4
GLU	18900	12.9	0.01 ± 0.02	2.95 ± 0.04	0.999	0.03	—	0.1–0.7
Threonine	18600	14.5	0.01 ± 0.02	3.03 ± 0.03	0.999	0.03	—	0.2–0.3
GLY	19600	15.9	0.02 ± 0.03	3.15 ± 0.06	0.997	0.05	—	0.1–0.8
Alanine	19500	19.4	0.00 ± 0.03	3.19 ± 0.04	0.998	0.04	—	0.1–0.8
TYR	19000	20.5	n.d.‡	n.d.	n.d.	n.d.	n.d.	n.d.
PRO	18200	22.4	0.00 ± 0.08	3.14 ± 0.01	0.996	0.06	—	0.1–5.0
MET	19000	26.9	0.51 ± 0.08	2.59 ± 0.13	0.990	0.11	—	0.1–3.1
VAL	19500	27.8	0.00 ± 0.03	3.20 ± 0.04	0.998	0.04	—	0.1–0.2
PHE	19300	30.7	–0.07 ± 0.07	4.71 ± 0.10	0.998	0.08	—	n.d.
ILE	18800	31.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HYS	33800	32.9	–0.12 ± 0.12	5.64 ± 0.22	0.991	0.14	—	n.d.
LYS	37000	33.8	0.07 ± 0.11	6.06 ± 0.19	0.992	0.15	—	n.d.

* SE, standard error (× 10^{-6}).
† Significant at a p-level ≤ 0.01.
‡ n.d., not determined.

Table III. Precision, Stability, Recovery, and LOD for Selected Amino Acids Found in Fruit Juices

Amino acid	Precision CV*,†	Stability CV‡	Recovery mean ± standard deviation†	LOD (µM)
ARG	5.1	0.1	100.7 ± 2.5	4
ASN	2.4	0.5	104.9 ± 2.1	4
SER	3.8	0.6	103.9 ± 2.7	5
ASP	4.6	0.4	93.6 ± 10.7	5
GLU	3.0	0.2	106.7 ± 5.4	3
Threonine	5.3	0.6	97.0 ± 6.1	3
GLY	5.7	0.2	105.6 ± 7.7	5
Alanine	2.8	0.3	100.3 ± 4.4	4
TYR	n.d.§	n.d.	n.d.	n.d.
PRO	3.2	0.6	104.3 ± 1.2	6
MET	4.0	n.d.	n.d.	n.d.
VAL	6.1	0.6	99.1 ± 4.3	4

* CV, coefficient of varience.
† $n = 6$.
‡ $n = 3$.
§ n.d., not determined.

Conclusion

A simple and rapid HPLC method for the simultaneous analysis of 16 amino acids is presented. Precolumn derivatization with FMOC and ADAM was fast, the derivatives were stable, and both primary and secondary amines could be detected. This method of analysis was applied to the characterization of some fruit juices without sample cleanup. The method validation was satisfactory, which implies a great potential for the research and routine analyses of selected amino acids in food science and biotechnology.

Acknowledgments

The authors gratefully acknowledge M.U.R.S.T. (Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Italy) for the financial support of this research (Project 40%, No. 9807031463) and M. Marabini for technical support. G.P. Parpinello is a recipient of a research contract from the Centro di Gestione della Spesa (CGS, sede di Cesena, Università di Bologna).

References

1. K.A. Massey, C.H. Blakeslee, and H.S. Pitkow. A review of phisiological and metabolic effects of essential amino acids. *Amino Acids* **14:** 271–300 (1998).
2. J. Kirimura, A. Shimizu, A. Kimizuka, T. Ninomiya, and N. Katsuya. The contribution of peptides and amino acids to the taste of foodstuffs. *J. Agric. Food Chem.* **17:** 689–95 (1969).
3. R.J. Haefeli and D. Glaser. Taste responses and threshold obtained with the primary amino acids in humans. *Lebensm.-Wiss. U.-Technol.* **23:** 523–27 (1990).
4. J.M. Ames. Application of the Maillard reaction in the food industry. *Food Chem.* **62:** 431–39 (1998).
5. H.F. Linskens, J.F. Jackson, and T.T. Fang. *Modern Methods of Plant Analysis*. Springer-Verlag, Berlin, Germany, 1988, pp. 51–68.
6. S. Nagy, J.A. Arraway, and M.E. Rhodes. *Adulteration of Fruit Juice Beverages*. Marcel Dekker, New York, NY, 1988, pp. 21–48.
7. *RSK values. The complete manual, Verlag Flüssiges OBST, Association of the German Fruit Juice Industry*. Schönborn, Germany, 1987.
8. A.M.P. Vasconcelos and H.J. Chaves das Neves. HRGC of wine free amino acids as tools for elementary wine characterization. *J. High Resolut. Chromatogr.* **13:** 494–98 (1990).
9. P. Husék. Rapid derivatization and gas chromatographic determination of amino acids. *J. Chromatogr.* **552:** 289–99 (1991).
10. G. Sarwar and H.G. Botting. Evaluation of liquid chromatographic analysis of nutritionally important amino acids in foods and physiological samples. *J. Chromatogr.* **615:** 1–22 (1993).
11. H.J. Issaq and K.C. Chan. Separation and detection of amino acids and their enantiomers by capillary electrophoresis: a review. *Electrophoresis* **16:** 467–80 (1995).
12. C. Corradini and A. Cavazza. Application of capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MECK) in food analysis. *Ital. J. Food Sci.* **4:** 299–316 (1998).
13. S. Einarsson, B. Josefsson, and S. Lagerkvist. Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **282:** 609–18 (1983).
14. B. Gustavsson and I. Betnér. Fully automated amino acid analysis for protein and peptide hydrolydates by precolumn derivatization with 9-fluorenylmethylchloroformate and 1-aminoadamantane. *J. Chromatogr.* **507:** 67–77 (1990).
15. P.A. Haynes, D. Shemack, J. Kibbi, and J.W. Redmond. Amino acids analysis using derivatization with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **540:** 177–85 (1991).
16. U. Arnold, E. Ludwig, R. Kühn, and U. Möschwitzer. Analysis of free amino acids in green coffee beans. I. Determination of amino acids after precolumn derivatization using 9-fluorenylmethylchloroformate. *Z. Lebensm-Unters. Forsch.* **199:** 22–25 (1994).
17. A. Péter, D. Tourwé, M.E.M. Baumann, M. Elskens, and L. Goeyens. High performance liquid chromatographic determination of free amino acid in algae. *J. Liq. Chrom. & Rel. Technol.* **22:** 1077–93 (1999).
18. J.P. Chaytor. The analysis of amino acids in fruit juices by high-performance liquid chromatography. *J. Sci. Food Agric.* **37:** 1019–26 (1986).
19. D.W. Kuneman, J.K. Braddock, and L.L. McChesney. HPLC profile of amino acids in fruit juices as their (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide derivatives. *J. Agric. Food Chem.* **36:** 6–9 (1988).
20. M. Dizy, P.J. Martín-Alvarez, M.D. Cabezudo, and M.C. Polo. Grape, apple and pineapple juice characterisation and detection of mixtures. *J. Sci. Food Agric.* **60:** 47–53 (1992).
21. H. van Gorsel, C. Li, E.L. Kerbel, M. Smits, and A.A. Kader. Compositional characterization of prune juice. *J. Agric. Food Chem.* **40:** 784–89 (1992).
22. M.D. del Castillo, G. Santa-María, E. Pueyo, N. Corzo, and A. Olano. Differences in amino acid composition in commercial orange juices. *J. Agric. Food Chem.* **46:** 2329–31 (1998).
23. S.A. Cohen and D.J. Strydom. Amino acids analysis utilizing phenylisothiocyanate derivatives. *Anal. Biochem.* **174:** 1–16 (1988).
24. J.F. Davey and R.S. Ersser. Amino acid analysis of physiological fluids by high-performance liquid chromatography with phenylisothiocyanate derivatization and comparison with ion-exchange chromatography. *J. Chromatogr.* **528:** 9–23 (1990).
25. B.N. Jones and J.P. Gilligan. *o*-Phthaldiadehyde precolumn derivatization and reversed-phase high-performance liquid chromatography of polypeptides hydrolysates and physiological fluids. *J. Chromatogr.* **266:** 471–82 (1983).
26. P. Lehtonen. Determination of amines and amino acids in wine—a review. *Am. J. Enol. Vitic.* **47:** 127–33 (1996).

Manuscript accepted July 27, 2001.