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Fully automated precolumn derivatization, on-line dialysis and high-performance liquid chromatographic analysis of amino acids in food, beverages and feedstuff

Dany Heems, Genevieve Luck, Christophe Fraudeau, Eric Vérette*

Gilson S.A., 72 Rue Gambetta, BP 45, 95400 Villiers-le-Bel, France

Abstract

A reliable, high throughput method is described for the routine analysis of amino acids in food, beverages and feedstuff. A fully automated sample processor performs precolumn derivatization of both primary and secondary amino acids with *o*-phthalaldehyde–3-mercaptopropionic acid and 9-fluorenylmethyl chloroformate (FMOC), respectively. Following dilution and derivatization, an on-line dialysis step is performed to remove macromolecular, microparticulate and insoluble interferents resulting from complex matrices and the FMOC derivatization. The optimization strategies for sample preparation and HPLC are discussed in this work. Twenty-five amino acids (including cystine) present in food products are separated in 24 min using a simple one-step binary gradient, with an excellent resolution and outstanding reproducibility. Analytical data are provided both for standard solutions and real samples. © 1998 Elsevier Science B.V.

Keywords: Food analysis; Wine; Derivatization, LC; Fruit juices; Dialysis; Amino acids

1. Introduction

The determination of amino acids is of great importance in the food industry [1]. It plays an essential role in assessing the nutritional value of food and feedstuff [2–4], and in the control of food products fortified with proteins. Amino acid analysis finds other applications in the origin identification or possible adulteration of foods and beverages, for example Pro, γ-aminobutyric acid (GABA) and Arg are used as indices to show significant differences among grape varieties [5–7]. Amino acid determination also gives an indication of the eventual transformations occurring during food-processing techniques including fermentation [8–10]. More specific analyses of particular amino acids are also done in certain areas, examples are the determination of monosodium glutamate which enhances food flavour, or taurine (Tau) which is an important amino acid in baby milk products and in cat food preparations [11].

The traditional approach to amino acid analysis is based on the technology developed by Moore and Stein [12], in which amino acids with free amino groups are separated by cation-exchange chromatography followed by a post-column derivatization (ninhydrin or *o*-phtalaldehyde). This technique which has been largely applied during the last 40 years, has demonstrated good results; however analysis times are very long (around 2 h and more), peak broadening with occasional overlapping can occur, and post-column derivatization systems are difficult to operate and maintain in a routine basis, and are cost-effective in terms of equipment and reagents [13].

More recently, interest has been focused on pre-column derivatization followed by reversed-phase

*Corresponding author.

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HPLC which constitutes a much faster and more efficient and sensitive alternative to former amino acid analyzers [13–17]. For amino acid analysis on a routine basis, the ideal precolumn derivatizing agent must satisfy several requirements such as to react rapidly under mild conditions, to provide both primary and secondary stable derivatives, to be easily automated; also there should not be any interference from the reagent, breakdown products or side reactions, and the response should be linear over the concentration ranges typical of most applications; moreover the reversed-phase HPLC procedure should be simple, robust and easily reproducible in other laboratories. Among the numerous compounds used in pre-column derivatization of amino acids, two reagents seem to be favoured: *o*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC). Still, none of the solutions is individually satisfactory; the major disadvantage of the OPA procedure is the lack of reaction with secondary amino acids, and the main drawback of FMOC is its insolubility and the interferences created by this compound in excess and by its hydrolysis products. More recently, these limitations have been minimized with automated systems in using the two derivatization agents, respectively, OPA and FMOC, in conjunction [18–20]. Still, most of the proposed reversed-phase HPLC procedures following derivatization are particularly difficult to reproduce from one laboratory to another, mainly due to complicated solvent composition and multi-step binary or even ternary gradients; and this is a major limitation in the routine amino acid analysis of food samples.

Moreover, samples containing complex matrices necessitate at least a filtration step, if not a more elaborated sample clean-up such as precipitation/centrifugation, to eliminate macromolecules such as proteins, microparticulates and insoluble interferents coming from FMOC in excess. Such procedures are indispensable to obtain correct chromatographic results, to avoid any contamination in the system and to preserve the column efficiency; but most of the time, when they are considered, they remain manual and off-line [1]. To overcome this problem, the ASTED sample processor, with its on-line dialysis step, enables an efficient removal of these interferents. ASTED technology has been widely and successfully applied in many fields including food analysis for the determination of numerous small analytes [19,21–23]. Among these, an application has been developed for the amino acid analysis in raw biological fluids, which also utilizes a two-step precolumn derivatization (OPA/FMOC) for primary and secondary amino acids [19].

Regarding the general requirements for a reliable precolumn derivatization/reversed-phase HPLC procedure to analyze amino acids, the present study describes a fully automated method based on the previous technique [19], but which has been optimized in replacing 2-mercaptoethanol by 3-mercaptopropionic acid (3-MPA) to provide more stable OPA derivatives, and with a much simpler and more robust reversed-phase HPLC technique. The method is specially dedicated to food applications for numerous analyses on a routine basis, and is employed here to determine a feedstuff protein hydrolysate, an orange juice and a red wine.

2. Experimental

2.1. Chemicals and samples

Chemicals were of analytical or Suprapur grade, and solvents were of HPLC grade. The amino acid standards and internal standards (norvaline and thioproline) and the following reagents: OPA, 3-MPA, FMOC, iodoacetic acid (IDA), sodium tetraphenyl borate and sodium azide were obtained from Sigma (St. Quentin Fallavier, France). Other reagents: 85% phosphoric acid, dibasic sodium phosphate anhydrous, 30% hydrochloric acid, 30% sodium hydroxide, boric acid and dry acetone were obtained from Merck–Clevenot (Nogent-sur-Marne, France). Water, acetonitrile and tetrahydrofuran (THF) were obtained from J.T. Baker (Noisy-le-sec, France), and methanol from Carlo Erba (Nanterre, France). Real samples were respectively a soya-bean cattle-cake obtained from Laboratoires Wolff (Paris, France), a commercial orange juice and a red wine, Pinot noir 1995, obtained from Domaine Chevrot (Cheilly-les-Maranges, France).

2.2. Preparation of reagents and solvents

100 m*M* Borate buffer: boric acid (1.24 g) was dissolved in 200 ml of water and adjusted to pH 10.4

with sodium hydroxide. This buffer can be stored at room temperature for two weeks.

Internal standards–3-MPA solution (reagent A): 25 μl of norvaline (I.S.1) and 50 μl of thioproline (I.S.2), both solutions at 2 mg/ml in water, were added to 9.875 ml of borate buffer, then 50 μl of 3-MPA were added. This reagent must be prepared weekly and kept at 4°C.

100 m*M* IDA solution (reagent B): IDA (0.208 g) was dissolved in 10 ml borate buffer. This reagent can be kept at room temperature for two weeks.

37.2 m*M* OPA–100 m*M* 3-MPA (reagent C): OPA (50 mg) was dissolved in 1 ml of methanol, then borate buffer (8.9 ml) was added, followed by 3-MPA (100 μl). This reagent must be stored in amber glass vials at 4°C, and freshly prepared every week.

5 m*M* FMOC solution (reagent D): FMOC (13 mg) was dissolved in 10 ml of dry acetone. This reagent must be freshly prepared every week and kept at 4°C.

HPLC mobile phase A: 10 m*M* phosphate buffer was adjusted to pH 7.5 with phosphoric acid and completed with 0.8% of THF.

HPLC mobile phase B: mobile phase A–methanol–acetonitrile (20:50:30, v/v).

HPLC mobile phase A and B lines were respectively equipped with aqueous and organic solvent in-line filters/degassers of 0.2 μm, produced by Whatman and obtained from Prolabo (Fontenay-sous-Bois, France), to improve reproducibility and preserve the analytical column.

2.3. Preparation of protein hydrolysates

The soya-bean cattle-cake was first ground. Then hydrolysis was performed according to the procedure described in the literature [1,2]. Ground sample (100 mg) was accurately weighed into a screw-capped test tube and 6 *M* hydrochloric acid (2 ml) was added. Tubes were capped and hydrolysed for 24 h at 110°C. After hydrolysis, the mixture was evaporated to dryness under vacuum. Hydrolysates were reconstituted in 2 ml of 0.1 *M* hydrochloric acid.

2.4. Instrumentation

2.4.1. ASTED sample processor (Fig. 1)

Dilution, derivatization and sample clean-up were performed using the Gilson ASTED sample processor (Gilson, Villiers-le-Bel, France). The ASTED consists of a large XYZ autosampler, a Model 402 dilutor (equipped with two 1-ml syringes), a flat-bed dialyser (cellulose acetate membrane with 15 000 molecular mass cut-off; 100 μl donor channel; 175 μl recipient channel) and two six-port Model 7010 valves (Rheodyne, Berkeley, CA, USA), one of which was fitted with a 20 μl injection loop. Samples and reagents racks were thermostated at 4°C using the Model 832 temperature regulator. Control was from the 722 keypad (version 2.0) software.

2.4.2. HPLC

The ASTED was interfaced to a HPLC system (Gilson) consisting of two Model 306 pumps fitted with 5SC pump heads, a Model 805 manometric module, a Model 811C mixer module, a Model 831 column oven, a Model 122 fluorometer, and Unipoint (version 1.4) System Software for HPLC control and data handling. A Hypersil BDS C_{18} column (3 μm; 150×4.6 mm) from Life Sciences International (Eragny-sur-Oise, France) was used.

2.5. Methodology

2.5.1. Derivatization and sample clean-up conditions

After performing an automatic dilution step (when required), ASTED automated the derivatization as follows: sample (20 μl) was added to 50 μl of reagent A, then reagent B (50 μl) was added to the sample-reagent A mixture, followed by 20 μl of reagent C (OPA–3-MPA), and finally 10 μl of reagent D (FMOC). After each reagent addition, two mixing cycles were performed. The reaction mixture was then cleaned up by on-line dialysis over 1.5 min. Both the donor and recipient channels of the dialyser, containing aqueous 0.05% sodium azide solution to avoid contamination resulting from bacterial growth, were held in the static mode. The dialysate was then injected onto the HPLC system, and the donor channel was regenerated with 1 ml of acetonitrile, followed by 4 ml of water, and the recipient channel was regenerated with 5 ml of water.

2.5.2. HPLC conditions

A simple binary gradient elution was performed from 0 to 70% of mobile phase B over 24 min, at 1.2

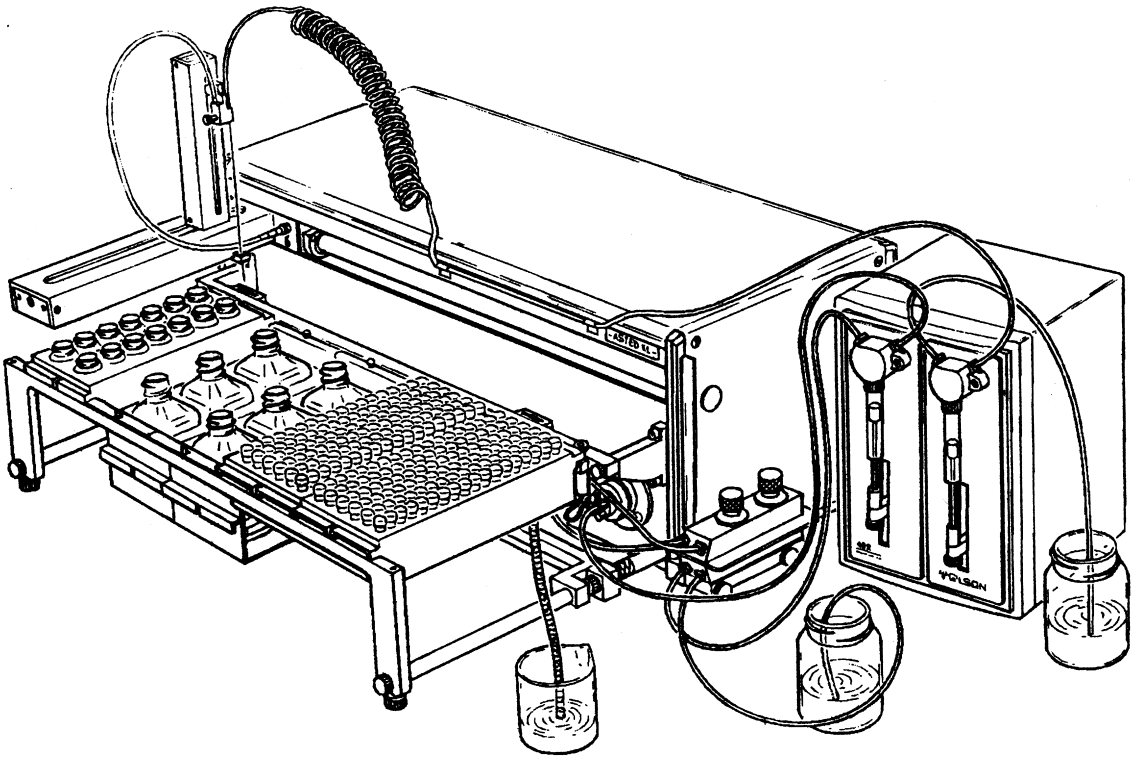

Fig. 1. ASTED sample processor.

ml/min, the column was then regenerated with 100% mobile phase B during 5 min and before returning to initial conditions. The column was thermostated at 40°C. OPA–3-MPA derivatives were detected by the programmable fluorometer with excitation (λ_{ex}) and emission (λ_{em}) wavelengths set at 335 and 440 nm, respectively; the FMOC derivatives were detected at λ_{ex} 260 nm and λ_{em} 315 nm; the wavelength change occured at 18.50 min. ASTED processed samples simultaneously with HPLC. The analysis time between injections was 38 min including the preparation time (15 min) and the reconditioning of the column.

3. Results and discussion

3.1. Optimization of sample preparation and HPLC

Sample preparation and chromatographic conditions were optimized using analytical standards diluted with 0.1 *M* hydrochloric acid.

3.1.1. Derivatization

Derivatization was performed by ASTED. 3-MPA was chosen in preference to other mercapto reagents such as 2-mercaptoethanol [19], since it led to increased sensitivities and a better stability [16], as well as more polar OPA derivatives [18,20] for a complete HPLC separation between primary and secondary amino acids. With OPA, cystine does not directly result in a fluorescent complex, and the cysteine derivative has only minimal fluorescence [20,24]. To overcome this problem, cystine (when present) was reduced to cysteine using 3-MPA (reagent A), then alkylated with IDA (reagent B) to form a fluorescent complex with OPA–3-MPA (reagent C). OPA–3-MPA reacts with ammonia, but the fluorescence response of this derivative is approximately 100-fold less intense than with amino acids [25]. When ammonia is present in high concentrations, it can interfer with amino acid derivatives.

Sodium tetraphenyl borate was therefore added to reagent A (200 mg in 10 ml) to form an insoluble precipitate, which was removed by the on-line dialysis step. In a previous investigation, the best stability of amino acid derivatives with OPA–3-MPA was achieved after an incubation period of 400 s with no variation over 1 h [16]. In the present study, the time taken between the addition of reagent C to the sample and injection was 9 min without any additional incubation period, allowing sufficient time for the optimum stability to be achieved.

Imino acids were then derivatized with FMOC (reagent D). Excess FMOC and its hydrolysis products were removed by on-line dialysis.

3.1.2. Sample clean-up by on-line dialysis

Following derivatization of primary and secondary amino acids, the mixture was loaded into the donor channel of the dialyser. Among the dialysis modes available (static, pulsed or continuous), the best option was to hold both the donor and recipient streams static for a period of 1.5 min for an optimum diffusion. The dialysate in the recipient channel was then transferred to the injection loop for HPLC.

3.1.3. Separation of amino acid derivatives

OPA derivatives of primary amino acids were best separated at a pH greater than 6.5. Therefore, a phosphate buffer was preferred to acetate, because of its superior buffering stability, which is optimum at pH=p$K\pm1$ [26]. Therefore, an acetate buffer at pH over 5.8, although previously used in other methods, should not be employed; indeed, experiments with an acetate buffer at pH 6.8 resulted in a poor day-to-day reproducibility due to a constant decrease in pH. For the same reason, a more resistant type of column, such as Hypersil BDS with its special ''end-capping'' treatment process was selected and preferred to more conventional ODS columns, not only for its excellent resolution and peak symmetry, but also for its longer lifetime at pH 7.5 and reproducibility from one batch of columns to another.

All other HPLC parameters were optimized to get the best separation of 25 amino acids present in food products, using the simplest and most robust conditions, within a reasonable time (Fig. 2).

Robustness was studied using small modifications in pH (±0.1 unit), % THF (±0.1%), column temperature (±1°C) and methanol–acetonitrile composition (55:25 and 45:35, v/v). Separations were essentially unchanged with only slight modifications in the separation of Tau/GABA (peak 13/peak 14) and Val/Met (peak 16/peak 17), except when 55% methanol was used in solvent B, which resulted in a significant decrease in the resolution of Val/Met.

3.2. Analytical data

Table 1 shows a summary of the repeatability and the reliability of this method. The R.S.D.$_{RT}$ values of

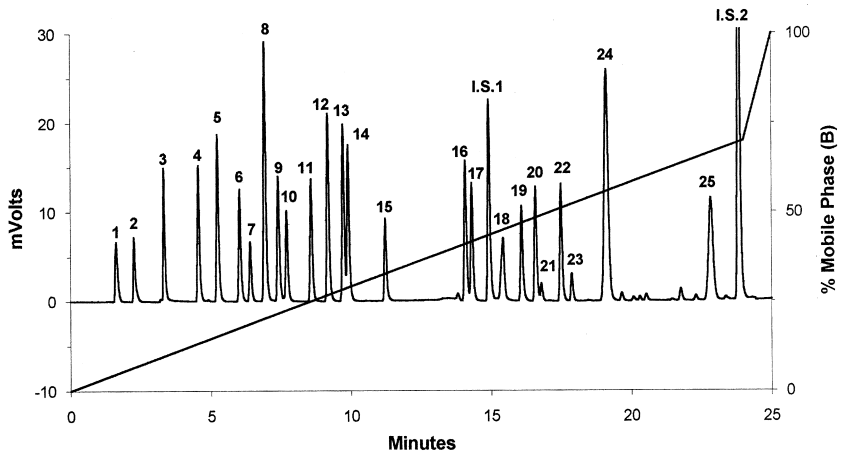

Fig. 2. HPLC analysis of standard OPA/FMOC amino acid derivatives (10 mg/l of each amino acid), after derivatization and clean-up using ASTED. 1: Asp, 2: Glu, 3: Cys, 4: Asn, 5: Ser, 6: Gln, 7: His, 8: Gly, 9: Thr, 10: Cit, 11: Arg, 12: Ala, 13: Tau, 14: GABA, 15: Tyr, 16: Val, 17: Met, 18: Trp, 19: Phe, 20: Ile, 21: Orn, 22: Leu, 23: Lys, 24: Hyp, 25: Pro.

Table 1
Analytical data from the HPLC analysis of 25 OPA/FMOC amino acid derivatives, using ASTED

Peak No.	Amino acid	Retention time (min)	R.S.D.$_{RT}$ (%; n=20)	R.S.D.$_{A}$ (%; n=20)[a]	L.O.D. (μg/l)	Linearity (mg/l)
1	Asp	1.62	0.3	3.3	112	0.6–60
2	Glu	2.28	0.3	1.7	89	0.5–50
3	Cys	3.32	0.3	4.3	152	0.5–50
4	Asn	4.56	0.3	1.0	41	0.5–100
5	Ser	5.24	0.3	4.6	37	0.5–100
6	Gln	6.04	0.3	1.4	51	0.6–110
7	His	6.42	0.2	2.5	89	0.4–80
8	Gly	6.93	0.3	2.3	23	0.6–60
9	Thr	7.41	0.2	1.9	48	0.5–50
10	Cit	7.72	0.2	1.6	65	0.5–100
11	Arg	8.60	0.2	2.2	50	0.4–90
12	Ala	9.17	0.2	1.6	31	0.5–100
13	Tau	9.72	0.2	1.6	32	0.7–130
14	GABA	9.89	0.2	1.9	35	0.5–100
15	Tyr	11.21	0.2	1.9	71	0.6–110
16	Val	14.04	0.1	1.6	55	0.5–50
17	Met	14.28	0.1	1.5	51	0.5–110
18	Trp	15.40	0.1	1.9	57	2.5–100
19	Phe	16.04	0.1	1.5	59	0.5–110
20	Ile	16.53	0.1	1.2	51	0.5–50
21	Orn	16.74	0.1	3.7	278	0.5–100
22	Leu	17.42	0.1	1.4	51	0.5–100
23	Lys	17.80	0.1	3.0	200	0.4–80
24	Hyp	19.06	0.1	4.3	46	0.5–50
25	Pro	22.76	0.1	4.7	49	0.6–60

[a] For an amino acid concentration of 10 mg/l.

retention time (n=20) were less than 0.3% for all analytes; and the R.S.D.$_{A}$ values of peak areas were between 1% for Asn and 4.7% for Pro; and for 18 amino acids, R.S.D.$_{A}$ values were less than 2.5%. Limits of detection are given for a ratio signal-to-noise ratio of 3, and were less than 100 μg/l for most of the amino acids. Limits of detection can be lowered further, using a 100 μl injection loop instead of a 20 μl loop, in this case it is necessary to replace the water in the recipient channel with 10 m*M* phosphate buffer at pH 7.5 to improve peak shape of the more polar derivatives (i.e. Asp, Glu and Cys). Other solutions are to use a larger dialysis block and/or to replace the injection loop by a trace enrichment cartridge [19,21]. For most amino acids, linearities range from 0.5 to 100 mg/l, with coefficients of correlation between 0.997 and 1.0; these linearities are perfectly suited to the amino acid concentrations normally found in food products. Recoveries were determined from a direct injection of a 0.2 mg/l amino acid standard solution in a 20 μl injection loop, and an injection of a 10 mg/l amino acid standard solution after dialysis; they ranged from 1.3% for Lys to 3.2% for Tau, and varied between 2 and 3% for 14 amino acids. An additional study on 40 consecutive injections gave a very good repeatability from the first to the last chromatogram.

3.3. Amino acid analysis of food sample

All food samples were automatically diluted before derivatization by ASTED to be in accordance with the amino acid linearity ranges, and analysed using the internal standard method. Table 2 summarises the results obtained from three real samples, with their amino acid concentrations and respective R.S.D.$_{A}$ values (n=10).

3.3.1. Soya-bean cattle-cake hydrolysate (Fig. 3)

Sample was hydrolysed according to the procedure

Table 2
Amino acid content of a soya-bean cattle-cake hydrolysate, an orange juice, and a red wine (Pinot noir), with corresponding $R.S.D._A$

Peak No.	Amino acid	Cattle-cake hydrolysate		Orange juice		Red wine Pinot noir	
		Conc. (g/100 g)	$R.S.D._A$ (%; $n=10$)	Conc. (mg/l)	$R.S.D._A$ (%; $n=10$)	Conc. (mg/l)	$R.S.D._A$ (%; $n=10$)
1	Asp	4.8	1.5	332.2	2.2	10.3	2.4
2	Glu	8.0	4.2	125.2	2.6	24.5	1.3
3	Cys	0.2	n.d.	–	–	3.8	5.5
4	Asn	–	–	371.5	1.5	7.0	0.9
5	Ser	2.0	1.4	157.0	1.9	7.8	1.3
6	Gln	–	–	30.0	2.5	–	–
7	His	1.4	1.9	11.5	n.d.	2.6	7.6
8	Gly	2.0	1.8	17.1	1.8	11.3	0.8
9	Thr	1.6	2.1	15.7	n.d.	4.4	1.5
10	Cit	–	–	–	–	1.3	6.6
11	Arg	3.4	1.3	795.5	1.4	6.9	1.9
12	Ala	2.0	1.0	97.7	2.2	24.1	0.7
13	Tau	–	–	–	–	–	–
14	GABA	–	–	295.7	1.9	12.7	0.9
15	Tyr	1.4	1.0	11.5	n.d.	5.0	1.8
16	Val	2.4	1.2	16.5	n.d.	–	–
17	Met	0.5	1.5	–	–	–	–
18	Trp	–	–	–	–	–	–
19	Phe	2.5	1.1	–	–	5.9	3.1
20	Ile	2.4	1.1	11.0	n.d.	2.1	3.6
21	Orn	–	–	–	–	5.1	7.5
22	Leu	3.6	1.0	6.2	n.d.	5.9	2.1
23	Lys	2.9	1.5	34.7	6.9	12.1	4.0
24	Hyp	–	–	–	–	3.8	1.8
25	Pro	2.0	3.0	1787.5	1.7	552.3	2.7

n.d.: not determined.

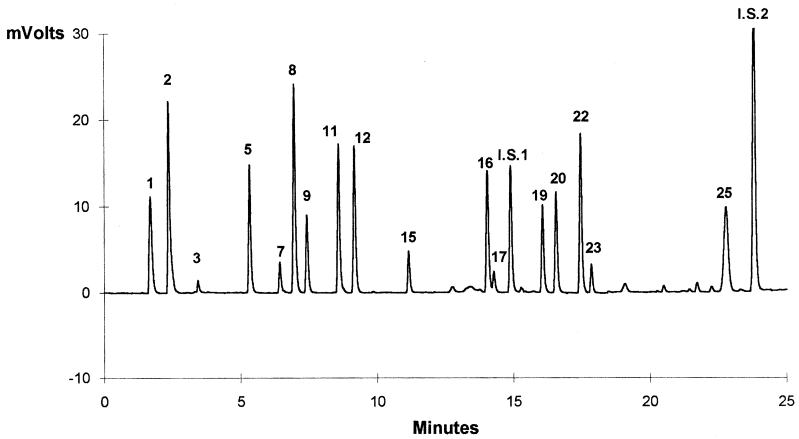

Fig. 3. HPLC analysis of OPA/FMOC amino acid derivatives of a soya-bean cattle-cake hydrolysate, after derivatization and clean-up using ASTED.

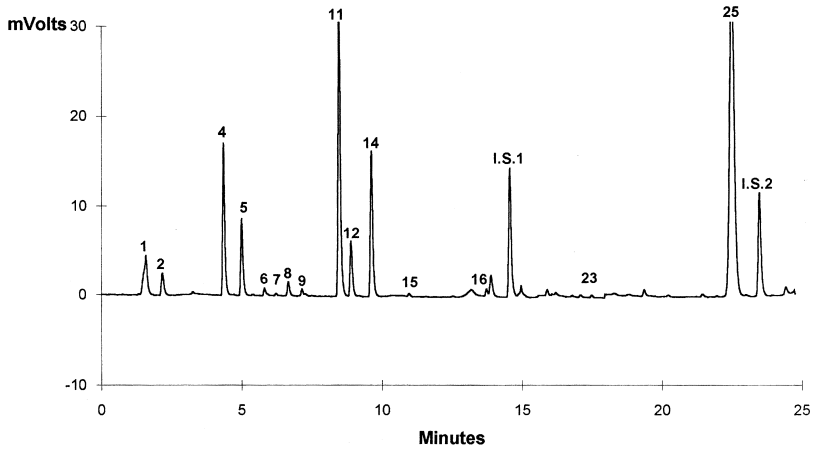

Fig. 4. HPLC analysis of OPA/FMOC amino acid derivatives of an orange juice, after derivatization and clean-up using ASTED.

described in Section 2.3. As expected, Trp could not be analysed since this amino acid undergoes degradation during acid hydrolysis [1]. R.S.D.$_A$ values of amino acids were mostly in the range of 1.0% for Ala, Tyr and Leu to 2.1% for Thr, except for Pro (3.0%) and Glu (4.2%). These results were in good agreement with the amino acid concentrations reported in a compilation of worldwide data sources [27].

3.3.2. Free amino acids from an orange juice (Fig. 4)

The main amino acid found in this sample was Pro with a rather high concentration (1787.5 mg/l) which represented 42% of total amino acid content; others were Arg (19%), Asn (9%), Asp (8%), GABA (7%), Ser (4%) and Glu (3%). The results were in a good agreement with the ranges determined in a previous work using another method, and realised on 64 orange juices [6]. Most R.S.D.$_A$ values were between 1.4 and 2.6%.

3.3.3. Free amino acids from a red wine (Fig. 5)

Proline is also the most abundant amino acid in wine. In this sample, the proline concentration was 552.3 mg/l, representing 78% of the total amino acid content. The next most abundant amino acids were Glu (4%), Ala (4%) and GABA (2%). Most amino

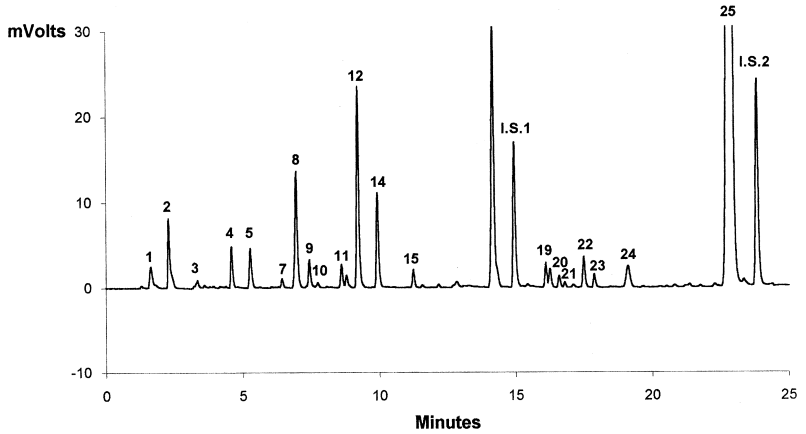

Fig. 5. HPLC analysis of OPA/FMOC amino acid derivatives of a red wine (Pinot noir), after derivatization and clean-up using ASTED.

acid concentrations were in good agreement with those obtained from seven French red wines [10], except for Arg which was present at a smaller concentration (6.9 mg/l instead of 23–64 mg/l), this is probably due to varietal differences [7]. The major peak before I.S.1 was an OPA derivative, but did not correspond to Val or Met, this has been confirmed with spiked samples. Most $R.S.D._A$ values were between 0.7 and 4.0%.

4. Conclusion

The work described here was directed to develop a reliable and high throughput method for separating and quantifying 25 amino acids in the routine analysis of food products. The method is based on the automated precolumn derivatization of liquid samples using a combined OPA/FMOC reaction, followed by an on-line dialysis procedure to remove macromolecular interferents, microparticulates contained in the sample matrix and insoluble compounds resulting from derivatization. The derivatized complexes were consecutively separated by a simple and robust reversed-phase HPLC technique using a single-step gradient, to give excellent results within a reasonable analysis time. The method applied here to a protein hydrolysate, a fruit juice and a red wine, was also used for other types of samples such as baby milk and cat food preparations, which demonstrates its wide applicability. All samples determined in this work had to be automatically diluted before analysis, which means that no sensitivity limitation was encountered. However, if such limitation occurs, further developments are available using a larger injection loop, a larger dialysis block and/or a trace enrichment cartridge to increase recovery and sensitivity.

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