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Rapid automated high performance liquid chromatography method for simultaneous determination of amino acids and biogenic amines in wine, fruit and honey

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ABSTRACT

This paper reports a new, simple, rapid and economical method for routine determination of 24 amino acids and biogenic amines in grapes and wine. No sample clean-up is required and total run time including column re-equilibration is less than 40 min. Following automated in-loop automated pre-column derivatisation with an *o*-phthaldialdehyde, N-acetyl-L-cysteine reagent, compounds were separated on a 3 mm × 25 cm C_{18} column using a binary mobile phase. The method was validated in the range 0.25–10 mg/l; repeatability was less than 3% RSD and the intermediate precision ranged from 2 to 7% RSD. The method was shown to be linear by the 'lack of fit' test and the accuracy was between 97 and 101%. The LLOQ varied between 10 μg/l for aspartic and glutamic acids, ethanolamine and GABA, and 100 μg/l for tyrosine, phenylalanine, putrescine and cadaverine. The method was applied to grapes, white wine, red wine, honey and three species of *physalis* fruit. Grapes and *physalis* fruit were crushed, sieved, centrifuged and diluted 1/20 and 1/100, respectively, for analysis; wines and honeys were simply diluted 10-fold. It was shown using this method that the amino acid content of grapes was strongly correlated with berry volume, moderately correlated with sugar concentration and inversely correlated with total acidity.

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1. Introduction

Amino acids contribute to the nutritional value of several fresh foods and their importance in grape juice is that primary amino acids are a significant source of yeast nutrition. Microbial and enzymatic conversion of amino acids produces key aroma and flavour compounds in foods such as cheese, wine, honey and other fermented foodstuffs [1], and decarboxylation of amino acids by bacteria leads to the formation of physiologically active biogenic amines.

There is growing interest in the analysis of individual amino acids in grape juice due their pivotal role as precursors to aromas released during fermentation or ageing. For example, isoamyl, isobutyl and phenylethyl alcohols are derived from respectively leucine, isoleucine and valine [2]. The same authors also showed that threonine, phenylalanine and aspartic acid are the amino acids which most influence the fermentation process. A relationship was demonstrated between the amino acid profile in grape juice and the concentration of some important volatile compounds in wine [3]. It has also been shown [4] that the amino acids remaining in wine after fermentation have an influence on aromas during the maturing process.

It has recently been shown that adding ammonium salts to grape juice (to increase its fermentability) can reduce by up 30% the production of aromatic thiols such as 4-methyl-4-mercaptopentan-2-one (4MMP) from their precursors through the phenomenon of NCR—nitrogen catabolic repression [5]. For this reason, new research is beginning to focus on adapting vineyard practice (for example the judicious application of nitrogen fertilisation, irrigation or fertigation) in order to increase the concentration of amino acids in the grapes at harvest.

The demand by consumers for better and healthier foods has led to renewed interest in biogenic amines, given their importance for human health and food safety. The aliphatic polyamines, putrescine, cadaverine, spermine and spermidine, are pharmacologically active and reportedly toxic [6,7]. Putrescine and cadaverine play an important role in food poisoning as they can enhance the toxicity of histamine [8]. They play an essential part in tissue growth, and because of this, it has been suggested that they may be involved in the development of tumours [9,10]. Furthermore, putrescine and cadaverine can react with nitrite to form

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heterocyclic nitrosamines which are carcinogenic [11]. Thus, the determination of biogenic amines in foods is of interest not only due to their possible toxicity, but also due to their role as potential indicators to determine the quality of freshness or spoilage of food products.

Current analytical methods for the determination of biogenic amines in foods have recently been reviewed [12]. High performance liquid chromatography is by far the most widely used technique for the determination of amino acids and biogenic amines in a diversity of matrices. More recently, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has been shown to be a very specific and sensitive technique for the determination of underivatized amino acids [1,13], but reported applications of these methods to food measurements are limited as LC–MS/MS instrumentation is expensive, requires a higher level of technical skill than GC–MS and is not available in many research laboratories.

In recent years, the original HPLC technique of ion-exchange chromatography followed post-column derivatisation with ninhydrin on a dedicated amino acid analyser has been largely supplanted by pre-column derivatisation, due to the flexibility of the technique and relative simplicity of the apparatus without the requirement for a dedicated instrument. A variety of derivatising reagents are available for pre-column derivatisation followed by HPLC with fluorescence detection, each with their advantages and drawbacks. For example, the advantage of fluorenylmethyl chloroformate (FMOC-Cl) [14], the use of which has been recently reviewed [15], is that it reacts with both primary and secondary amines, but it is troublesome in that requires quenching and even then produces a large reagent peak in the chromatogram. Dabsyl chloride also reacts with both primary and secondary amines, produces stable derivatives and allows for sensitive detection, however, the methods described for its application in the analysis of biogenic amines are rather complex, involving heating at 70 °C, intermediate mixing and cooling in an ice bath [16,17]. A relatively recent fluorescent derivatising reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was first reported in 1993 [18] is available commercially from the Waters Corporation. It provides selective fluorescence detection of stable derivatives with no significant interference from the only major fluorescent reagent by-product, 6-aminoquinoline. This technique is growing in popularity, though it does suffer the drawback of requiring heating, and a reaction time of 15 min is essential for optimum results.

Since it was first described as a fluorigenic reagent by Roth in 1971 [19] OPA (*o*-phthaldialdehyde) has become arguably the most widely used derivatising agent in the chromatographic determination of primary amino acids and biogenic amines. The reaction takes place almost instantaneously at room temperature at alkaline pH in the presence of a thiol-containing reducing agent, the most commonly used of which is as 2-mercaptoethanol (MCE) [20]. However, the isoindole derivatives produced by OPA–MCE are unstable, and more bulky thiols such as N-acetyl-L-cysteine (NAC) or 3-mercaptopropionic acid (MPA), provide more stable derivatives [21]. The characteristics and stability of OPA–NAC-amine derivatives have been intensively investigated [21–26].

There are several publications on the simultaneous analysis of amino acids and biogenic amines, using OPA either alone [13,21–26] or combined with FMOC [27–30]. These methods provide varying degrees of sensitivity, selectivity and ease of execution, however, in general they involve long analysis times (>60 min) with flow-rates as high as 1.8 ml/min [28] resulting in high solvent consumption. Therefore, given growing awareness of environmental issues, the rising costs of organic solvents (especially acetonitrile) and of their disposal, the objective of this study was to develop and validate a rapid and economical method for routine analysis of several amino acids and biogenic amines common to wines, grapes and other food matrices without compromising on selectivity or sensitivity.

2. Materials and methods

2.1. Reagents

All chemicals and reagents were of analytical or HPLC grade or equivalent. Methanol, acetonitrile, acetic acid, sodium acetate, potassium chloride, boric acid, hydrochloric acid 0.1 M and sodium hydroxide 1 M were obtained from Carlo Erba (Carlo Erba Réactifs, BP 615, Val de Reuil, France). The 16 amino acids as their hydrochloride salts and the seven biogenic amines in addition to *o*-phthaldialdehyde (OPA) and N-acetyl-L-cysteine (NAC) were purchased from Sigma (Sigma–Aldrich Chimie, Lyon, France). Doubly distilled water was used to prepare solutions and for washing all consumable materials.

2.2. Analyte solutions

Stock solutions (approximately 4 g/l accurately weighed) of the analytes were made with 0.1 M HCl, except for tyrosine, which was prepared in 0.1 M NaOH. These solutions stored at −20 °C and were stable for several months. A stock mixture containing approximately 40 mg/l of the analytes was prepared in freshly distilled water on a weekly basis, however, it was stable at −20 °C for several weeks. Calibration standards of 0.25–0.5–1–2–5–10 mg/l were prepared on a daily basis by serial dilution of the stock mixture in freshly distilled water.

2.3. Samples

The method was applied to the determination of amino acids and biogenic amines in grape juice, wine, honey and physalis fruit. Frozen grapes were thawed at room temperature, crushed in a mortar and pestle, sieved to remove solid matter and then centrifuged at 7000 × *g*. Physalis fruit was homogenised in a domestic blender and then filtered to remove solid matter. The grape juice supernatant was diluted 20-fold with freshly distilled water and filtered using a 0.45 μm membrane. The physalis fruit filtrate was diluted 100-fold and also filtered. Wine and honey were diluted 1 in 10 (v/v) and (w/v), respectively with distilled water and filtered using a 0.45 μm membrane. Prepared samples were placed in the autosampler for in-loop derivatisation.

2.4. Derivatisation reagent

Fifty mg of *o*-phthaldialdehyde (OPA) were dissolved in 10 ml methanol and 400 mg N-acetyl-L-cysteine (NAC) were dissolved in 50 ml of a 0.2 M borate buffer adjusted to pH 9.5 with sodium hydroxide. The derivatisation reagent consisting of 2 ml NAC solution and 0.5 ml OPA solution was prepared on a daily basis and allowed to stabilise at room temperature for 90 min before use. The OPA solution was stable for >10 days but was prepared weekly and the NAC solution was prepared every 14 days.

2.5. Instrumentation and operating conditions

A Hewlett-Packard (Agilent Technologies Massy, France) 1100 series HPLC instrument was used, consisting of a model G1322A degasser, a G1312A binary pump, a model G1313A autosampler and a G1321A fluorescence detector set at excitation and emission wavelengths of 330 nm and 440 nm, respectively. Separations were carried out on a 250 mm × 3 mm Equisil® column (CIL, Bordeaux, France), protected by a 1 mm C18 SecurityGuard® cartridge supplied by Phnomenx (France). Mobile phase A consisted of 95%

Table 1
Gradient programme.

Time (min)	% B
0	3
4.5	5
10	19
16	27
20	42
25	48
32	60
35	3

0.05 M sodium acetate buffer, pH 6.5 and 5% methanol, filtered under vacuum using a 0.22 μm nylon membrane. Mobile phase B consisted of methanol–acetonitrile 70–30. Separations were carried out at 25 °C with a flow rate of 0.5 ml/min. The total run time (including re-equilibration of the column), was 39 min and the gradient programme is shown in Table 1.

2.5.1. Derivatisation

The in-loop derivatisation method was as follows: draw 2 μl from OPA–NAC reagent, draw 2 μl sample (or standard). Mix 15 times in seat. Wait 2 min. Draw 3 μl of distilled water. Mix three times in seat. Inject. The injection volume was thus 7 μl.

2.6. Validation

Calibration standards at six concentration points (0.25–0.5–1–2–5 and 10 mg/l) were prepared in doubly distilled water spiked with the analyte mixture. Standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data. The slope and intercept of the calibration graphs were determined through linear regression of the peak areas versus concentration plot. Individual peak areas were then interpolated on the calibration graphs to determine the found (back calculated) concentrations. The quality of fit was determined using back-calculated-to-nominal concentrations and the 'lack of fit' test was used to confirm the linearity of the method.

Within-day and between-day precision and accuracy of the method were determined by carrying out replicate analyses of the calibration standards. Repeatability was determined by preparing and analysing each calibration standard five times within a single day (i.e. 30 standards in total) under the same operating conditions (e.g. same operator, same mobile phase). The intermediate precision was determined by carrying out the same operations over 5 days under different operating conditions. The precision was given by mean relative standard deviation of the back-calculated (found) concentrations, and the accuracy of the method was evaluated as 100 × [mean found concentration/nominal concentration].

Recovery in the matrix was determined by spiking wine and grape juice with the amino acid–biogenic amine mixture (20 mg/l for arginine and 10 mg/l all other compounds). It was determined by comparing the back-calculated (found) concentrations and the nominal concentrations using the standard additions method; it was expressed as 100 × [mean found concentration/nominal concentration].

3. Results and discussion

3.1. Chromatographic separation

Based on a literature survey the selected starting point was a method published in 2003 for the simultaneous determination of amino acids and biogenic amines in wine, beer and vinegar [24]. The column used in that method was a Hypersil ODS 5 μm dp 200 mm × 4 mm with a 20 mm × 4 mm guard column. In this study the a more cost-effective 'generic' version was chosen with a column diameter of 3 mm, which enabled a flow rate of 0.5 ml/min to be used (as opposed to 1.3–1.7 ml/min in the above reference), thus ensuring further savings on solvents. The guard column was an economical, compact 4 mm × 2 mm cartridge system, which produced virtually no change in peak shape or retention time. Initially a ternary gradient program similar to that described in the above reference was used, however, the program was entirely revised to reduce the amount of acetonitrile in the organic modifier and to enable the method to be executed on a less costly binary pump system (see experimental section). A small proportion of methanol (5%) was included in mobile phase A to prolong its stability vis à vis to microbial growth. The further advantage of the optimised gradient program is that complete elution of all 24 analytes plus re-equilibration of the column is achieved in less than 40 min.

3.2. Derivatisation

N-acety-L-cystine was selected as the source of thiol in the derivatising reagent as it does not need any particular handling requirements, and as has been reported [21] and references contained therein, the isoindoles formed with OPA–NAC are more stable than those obtained with the more commonly used OPA–mercaptethanol reagent. However, in the literature, the reported relative molar proportions of OPA to NAC varied from 25:1 in a method applied to milk and oyster samples [31] to 1:50 in the method described in Ref. [21]. Since the latter research team stated in an earlier paper that optimum analytical conditions are obtained using a mole ratio of OPA/SH group additive of 1:3 [23] three different molar ratios of OPA/NAC were investigated. To simplify the procedure, solutions of OPA (50 mg/10 ml methanol) and NAC (400 mg/50 ml borate buffer pH 9.5) were mixed in three different proportions to produce different molar ratios (OPA/NAC 5:1, 1:1 and 1:5). Once prepared, the mixtures were used after 90 min to allow time for the self-fluorescence of the reagent to disappear [21]. Derivatisation was then carried out by adding 150 μl of 0.2 M borate buffer to 100 μl of the 20 mg/l amino acid and amine mixture followed by 100 μl OPA/NAC mixture—a sample-reagent ratio previously described in amino acid and amine analysis of wine [13]. A 20 μl aliquot was manually injected after 3 min reaction time. The OPA/NAC 1:5 reagent, prepared by adding 0.5 ml of the OPA methanolic solution to 2.5 ml of the NAC solution, gave the most satisfactory results in terms of peak height and shape and was therefore was used for all subsequent analyses. The reagent was freshly prepared on a weekly basis and stored at 4 °C. The derivatisation procedure was then transferred to the automatic injector of the HPLC instrument. Initially the injector program was:

1. Draw 1 μl borate 0.2 M, pH 9.5.
2. Draw 2 μl from sample.
3. Mix five times.
4. Needle wash three times (10% MeOH).
5. Draw 2 μl reagent.
6. Mix 15 times.
7. Wait 3 min.
8. Draw 3 μl water.
9. Mix five times.
10. Inject.

It was found that the addition of water in step 8 improved peak shape, presumably by reducing the ionic strength of the injected aliquot. Later on in the study, when it emerged that the sensitivity of the method vis à vis to the studied matrices was such that it was necessary to dilute grape juice 20-fold, and wine 10-fold to avoid saturation of the detector, steps 1 and 3 in the above program were deleted with no compromise in sensitivity. This had the added

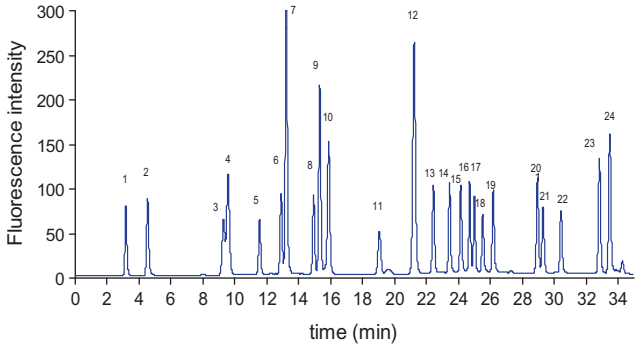

Fig. 1. Chromatogram 2 mg/l standard solution. 1 – Aspartic acid, 2 – glutamic acid, 3 – serine, 4 – asparagine 5 – glutamine, 6 – threonine, 7 – glycine, 8 – arginine, 9 – alanine, 10 – GABA, 11 – tryosine, 12 – ethanolamine, 13 – valine, 14 – methionine, 15 – phenylalanine, 16 – histamine, 17 – isoleucine, 18 – lysine, 19 – leucine, 20 – tyramine, 21 – putrescine, 22 – cadaverine, 23 – isoamylamine, 24 – phenylethylamine, and R = reagent.

advantages of enabling steps 2 and 5 to be inversed and step 3 to be deleted, thus reducing the injector program by approximately 90 s.

3.3. Validation

In preliminary studies, it emerged that other than dilution, no other sample preparation steps were required. In addition to obvious advantages of reducing the introduction of errors and obviating the need for an internal standard, this also justifies the decision to obtain some of the validation data using authentic standards.

The results of the validation study are summarised in Table 2A (repeatability or intra-day data) and Table 2B (intermediate precision or inter-day data). For each point of the calibration standards, the concentrations were back-calculated from the equation of the linear regression curves, and the precision was given by the mean relative standard deviation of the these values.

As may be seen in Table 2, the intra-day precision varies from less than 0.2% RSD at the 10 mg/l calibration point to 5–7% at 0.25 mg/l, and the intermediate precision varies from less than 0.4% at 10 mg/l to up to 10% at 0.25 mg/l. Amino acids and amines with a low yield under the reaction conditions (e.g. serine, tyrosine phenylalanine, putrescine—Fig. 1) tended to have higher % CVs at all concentrations and in fact 0.25 mg/l was the lower limit of quantification (LLOQ) for these compounds. The LLOQ was determined as being the concentration which gave an intermediate precision coefficient of variation of 20% or greater, in accordance with the US FDA guidelines for the validation of bioanalytical methods [32]. The LLOQ for compounds with higher yields—aspartic and glutamic acids, glutamine and ethanolamine was 0.05 mg/l, and for those of intermediate yield, the limit of quantification was 0.1 mg/l.

The precision within the different matrices was determined by six replicate analyses of grape juice, wine and honey carried out over 3 days. It varied from 1.07% for leucine in white wine to 8.33% for glycine in honey. Table A in the supplementary material—"precision in the matrices" presents the mean peak area, standard deviation and coefficient of variation for each individual compound in grape juice, wine and honey.

The "lack of fit" test showed no significant deviation from linearity; the bias, calculated as the sum of the residuals (nominal–back-calculated concentration) was non-significant. Linear regression of the back-calculated-to-nominal concentrations provided slopes of generally 0.999–1.000 and intercepts equal to 0 (Student's *t*-test). The accuracy of the method was evaluated as 100 × (mean found concentration/nominal concentration) and as may be seen from Table 3, the mean values for both the intra- and inter-day data are approximately 100%.

Table 2A
Repeatability (intra-day precision).

Nominal amount	0.25 mg/l			0.5 mg/l			1 mg/l			2 mg/l			5 mg/l			10 mg/l			Mean CV
	Mean AF*	SD**	CV %	Mean AF	SD	CV %	Mean AF	SD	CV %	Mean AF	SD	CV %	Mean AF	SD	CV %	Mean AF	SD	CV %	
Aspartic acid	0.245	0.017	6.90	0.503	0.011	2.28	1.01	0.034	3.34	1.99	0.014	0.680	4.99	0.032	0.631	10.0	0.013	0.131	2.33
Glutamic acid	0.248	0.009	3.49	0.511	0.017	3.28	0.993	0.019	1.89	1.99	0.009	0.460	4.99	0.042	0.842	10.0	0.018	0.182	1.69
Asparagine	0.252	0.014	5.60	0.488	0.025	5.11	0.988	0.039	3.95	1.98	0.067	3.37	4.98	0.058	1.16	9.99	0.033	0.333	2.35
Serine	0.252	0.017	6.90	0.501	0.012	2.03	0.987	0.013	1.32	2.01	0.011	0.530	5.00	0.019	0.371	9.99	0.009	0.093	1.88
Glutamine	0.250	0.015	5.91	0.493	0.013	2.12	0.997	0.009	0.92	2.01	0.013	0.650	5.00	0.047	0.942	9.99	0.022	0.222	1.80
Threonine	0.247	0.018	7.32	0.489	0.018	3.71	1.00	0.013	1.32	1.99	0.008	0.420	5.02	0.037	0.745	9.99	0.018	0.181	2.28
Glycine	0.247	0.013	5.34	0.505	0.017	3.28	0.983	0.024	2.48	2.01	0.038	1.90	5.02	0.062	1.24	9.99	0.023	0.232	2.40
Arginine	0.240	0.018	7.42	0.491	0.016	3.27	1.00	0.018	1.76	2.00	0.015	0.730	5.03	0.034	0.692	9.98	0.018	0.184	2.34
Alanine	0.234	0.015	6.51	0.500	0.009	1.80	1.01	0.025	2.49	2.01	0.020	0.991	4.996	0.024	0.481	9.99	0.011	0.115	2.06
GABA	0.238	0.014	6.04	0.485	0.013	2.62	1.02	0.019	1.87	2.00	0.018	0.911	5.02	0.028	0.563	9.99	0.011	0.112	2.01
Tyrosine	0.242	0.013	5.32	0.502	0.008	1.69	1.02	0.039	3.84	2.00	0.018	0.892	4.98	0.058	1.17	10.0	0.027	0.277	2.19
Ethanolamine	0.244	0.012	4.72	0.495	0.012	1.93	1.01	0.019	1.84	1.99	0.020	1.02	5.01	0.027	0.545	9.99	0.011	0.113	1.71
Valine	0.246	0.017	6.81	0.485	0.013	2.05	1.02	0.029	2.85	2.01	0.020	0.993	4.99	0.036	0.716	10.0	0.014	0.142	2.26
Methionine	0.253	0.007	2.70	0.487	0.011	2.25	0.999	0.013	1.28	2.00	0.022	1.09	5.02	0.045	0.89	9.99	0.020	0.202	1.40
Phenylalanine	0.246	0.028	11.1	0.489	0.015	3.04	1.01	0.021	2.06	2.00	0.025	1.27	5.00	0.043	0.856	9.99	0.023	0.233	3.13
Histamine	0.250	0.017	6.64	0.491	0.016	1.97	1.00	0.023	2.27	1.99	0.009	0.445	5.01	0.039	0.778	9.99	0.019	0.194	2.04
Isoleucine	0.246	0.021	8.63	0.489	0.012	2.43	1.01	0.039	3.87	2.00	0.046	2.29	5.00	0.044	0.882	9.99	0.021	0.215	3.05
Lysine	0.246	0.019	7.82	0.487	0.012	2.56	1.01	0.018	1.79	1.99	0.024	1.18	5.03	0.051	1.00	9.98	0.024	0.243	2.44
Leucine	0.251	0.014	5.51	0.492	0.017	1.95	1.01	0.016	1.54	1.99	0.019	0.942	5.02	0.056	1.11	9.99	0.023	0.232	1.88
Tyramine	0.244	0.012	4.90	0.490	0.021	4.32	1.01	0.021	2.05	2.01	0.014	0.691	4.99	0.034	0.686	10.0	0.018	0.183	2.14
Putrescine	0.246	0.015	6.22	0.515	0.034	5.79	1.01	0.022	2.19	1.99	0.027	1.373	4.99	0.022	0.457	10.0	0.014	0.141	2.69
Cadaverine	0.244	0.019	7.64	0.499	0.007	1.46	1.01	0.021	2.12	2.00	0.008	0.401	4.99	0.024	0.473	10.0	0.012	0.122	2.03
Isoamylamine	0.240	0.013	5.61	0.491	0.013	2.73	1.01	0.007	0.70	2.00	0.016	0.821	5.02	0.028	0.562	9.98	0.014	0.143	1.76
Phenylethylamine	0.244	0.008	3.53	0.494	0.012	2.42	1.00	0.009	0.91	2.00	0.019	0.932	5.02	0.043	0.871	9.99	0.019	0.195	1.46

*, **: Mean AF: mean amount found ($n = 5$); SD: standard deviation.

Table 2B
Intermediate precision.

Nominal amount	0.25 mg/l			0.5 mg/l			1 mg/l			2 mg/l			5 mg/l			10 mg/l			Mean CV
	Mean AF*	SD**	CV%	Mean AF	SD	CV%	Mean AF	SD	CV%	Mean AF	SD	CV%	Mean AF	SD	CV%	Mean AF	SD	CV%	
Aspartic acid	0.232	0.022	9.31	0.493	0.014	2.89	1.01	0.025	2.45	2.01	0.038	1.91	5.02	0.026	0.513	9.99	0.028	0.287	2.89
Glutamic acid	0.251	0.017	6.83	0.499	0.014	2.82	1.01	0.015	1.51	1.98	0.094	4.74	5.02	0.081	1.62	9.99	0.033	0.3341	2.98
Asparagine	0.251	0.021	8.45	0.510	0.025	4.90	0.985	0.032	3.25	1.97	0.054	2.73	4.98	0.106	2.13	9.98	0.084	0.84	4.02
Serine	0.230	0.036	15.0	0.485	0.038	7.82	0.975	0.094	9.64	2.06	0.141	6.82	5.02	0.085	1.68	9.98	0.044	0.445	7.02
Glutamine	0.241	0.025	10.1	0.485	0.046	9.63	1.01	0.012	1.20	2.00	0.031	1.56	5.03	0.068	1.35	9.99	0.045	0.457	4.06
Threonine	0.233	0.024	10.2	0.494	0.029	5.94	0.988	0.013	1.26	2.02	0.034	1.67	5.02	0.057	1.14	9.99	0.043	0.438	3.44
Glycine	0.248	0.022	8.93	0.505	0.017	3.35	1.01	0.023	2.30	1.99	0.046	2.32	5.00	0.055	1.10	10.0	0.038	0.382	3.05
Arginine	0.258	0.016	6.45	0.492	0.018	3.72	1.03	0.029	2.80	1.98	0.022	1.10	4.98	0.046	0.933	10.0	0.023	0.23	2.53
Alanine	0.241	0.019	7.87	0.493	0.020	3.96	1.00	0.012	1.21	2.01	0.016	0.80	5.02	0.039	0.781	9.99	0.031	0.319	2.48
GABA	0.254	0.010	3.81	0.508	0.015	3.01	0.990	0.030	3.01	2.02	0.039	1.95	4.98	0.067	1.35	10.0	0.022	0.223	2.22
Tyrosine	0.252	0.026	10.0	0.508	0.019	3.81	1.02	0.025	2.43	1.99	0.018	0.902	4.97	0.037	0.754	10.0	0.025	0.254	3.04
Ethanolamine	0.244	0.010	4.07	0.503	0.018	3.53	1.02	0.026	2.55	1.98	0.017	0.843	5.00	0.038	0.773	10.0	0.012	0.125	1.98
Valine	0.259	0.022	8.66	0.487	0.024	4.90	1.02	0.024	2.32	2.01	0.021	1.06	4.97	0.079	1.58	10.0	0.030	0.307	3.14
Methionine	0.242	0.009	3.81	0.495	0.017	3.38	1.02	0.025	2.46	2.03	0.054	2.65	4.95	0.053	1.08	10.0	0.022	0.223	2.27
Phenylalanine	0.231	0.027	11.1	0.490	0.014	2.92	1.04	0.037	3.57	1.97	0.037	1.90	5.03	0.099	1.97	9.99	0.029	0.294	3.73
Histamine	0.245	0.022	9.12	0.506	0.012	2.44	0.995	0.019	1.86	1.99	0.025	1.26	5.03	0.076	1.51	9.99	0.042	0.422	2.76
Isoleucine	0.257	0.022	8.74	0.515	0.027	5.32	1.00	0.017	1.71	1.99	0.018	0.906	4.98	0.055	1.09	10.0	0.024	0.242	2.99
Lysine	0.235	0.023	9.81	0.487	0.017	3.55	1.02	0.028	2.70	2.02	0.021	1.02	4.99	0.013	0.255	10.0	0.016	0.161	2.91
Leucine	0.238	0.024	10.3	0.511	0.020	3.87	1.00	0.015	1.48	2.03	0.023	1.13	4.97	0.057	1.16	10.0	0.023	0.232	2.97
Tyramine	0.237	0.023	9.66	0.494	0.010	2.01	1.00	0.010	1.01	2.02	0.019	0.92	5.00	0.037	0.737	10.0	0.027	0.271	2.42
Putrescine	0.240	0.019	7.84	0.487	0.030	6.23	0.987	0.054	5.44	2.01	0.025	1.26	5.06	0.157	3.10	9.99	0.079	0.793	4.10
Cadaverine	0.239	0.022	9.42	0.491	0.012	2.46	0.998	0.030	2.98	2.03	0.054	2.64	5.01	0.024	0.489	9.99	0.026	0.265	3.03
Isoamylamine	0.237	0.017	7.44	0.496	0.017	3.40	0.998	0.022	2.20	2.01	0.068	3.36	5.04	0.069	1.37	9.98	0.038	0.381	3.01
Phenylethylamine	0.243	0.017	6.92	0.509	0.022	4.34	1.010	0.018	1.80	1.99	0.044	2.22	5.00	0.114	2.27	10.0	0.049	0.494	3.00

*, **: Mean AF: mean amount found (back-calculated concentration) ($n = 5$); SD: standard deviation.

Table 3
Accuracy.

	Accuracy $n = 5$				
	Slope	Intercept	Mean FC/NC* (%)	SD	RSD (%)
Aspartic acid	1115	74.8	98.9	3.05	3.09
Glutamic acid	587	43.4	100	0.711	0.709
Asparagine	233	35	98.9	3.90	3.96
Serine	228.	62.5	99.3	1.96	1.97
Glutamine	439	46.6	99.0	2.92	2.95
Threonine	614	64.6	100	0.802	0.802
Glycine	29	95.1	101	2.00	1.99
Arginine	453	31.2	99.4	1.64	1.65
Alanine	1052	27.8	100.5	1.02	1.02
GABA	1049	85.2	100.6	1.06	1.05
Tyrosine	466	5.78	100	1.40	1.40
Ethanolamine	1663	22.2	100	2.00	1.99
Valine	873	110	99.8	1.87	1.87
Methionine	589	62.9	99.0	3.98	4.01
Phenylalanine	398	100	99.8	1.14	1.14
Histamine	646	68.6	100	1.63	1.61
Isoleucine	517	81.6	99.1	2.90	2.92
Lysine	358	60.1	99.8	2.40	2.41
Leucine	555	125	99.2	2.39	2.40
Tyramine	522	84.3	99.0	1.94	1.96
Putrescine	378	67.9	99.2	1.97	1.99
Cadaverine	445	72.0	99.1	2.23	2.25
Isoamylamine	708	99.9	100	1.49	1.49
Phenylethylamine	892	102	97.7	1.30	1.34

*, **: AF/NC = 100 × (found concentration/nominal concentration).

Recovery was given by the formula: Recovery (%) = 100 × (amount found/amount added). The "amount found" was obtained by subtracting the peak area of the unspiked sample from the peak area of the spiked sample and then by interpolating the difference on the standard curve. As may be seen from Table 4, recovery was generally greater than 80% and frequently in excess of 90%. Chromatograms of white wine, red wine and grape juice, both unspiked and spiked are presented in Fig. 2.

3.4. Applications

3.4.1. Analysis of grape juice

The method was used to determine the effect of different levels of sunlight in interaction fruit yield on the amino acid and biogenic

Table 4
Recovery (%).

	Grape juice	White wine	Red wine
Aspartic acid	98.0	96.1	94.2
Glutamic acid	104	103	104
Asparagine	94.0	93.0	92.5
Serine	78.3	81.2	79.1
Glutamine	87.2	87.8	91.2
Threonine	82.9	81.1	82.7
Glycine	95.5	93.3	96.2
Arginine	94.2	94.11	95.3
Alanine	90.9	91.3	90.4
GABA	96.9	95.5	94.7
Tyrosine	90.3	90.6	89.5
Ethanolamine	98.0	90.1	97.5
Valine	85.6	85.4	84.4
Methionine	93.7	85.33	91.4
Phenylalanine	88.5	91.5	88.7
Histamine	91.1	88.5	91.3
Isoleucine	92.5	89.4	91.9
Lysine	87.0	87.3	85.9
Leucine	86.4	84.8	82.0
Tyramine	88.6	85.0	87.4
Putrescine	88.9	89.3	86.6
Cadaverine	79.1	77.4	78.1
Isoamylamine	92.8	93.1	94.4
Phenylethylamine	91.5	92.4	90.9

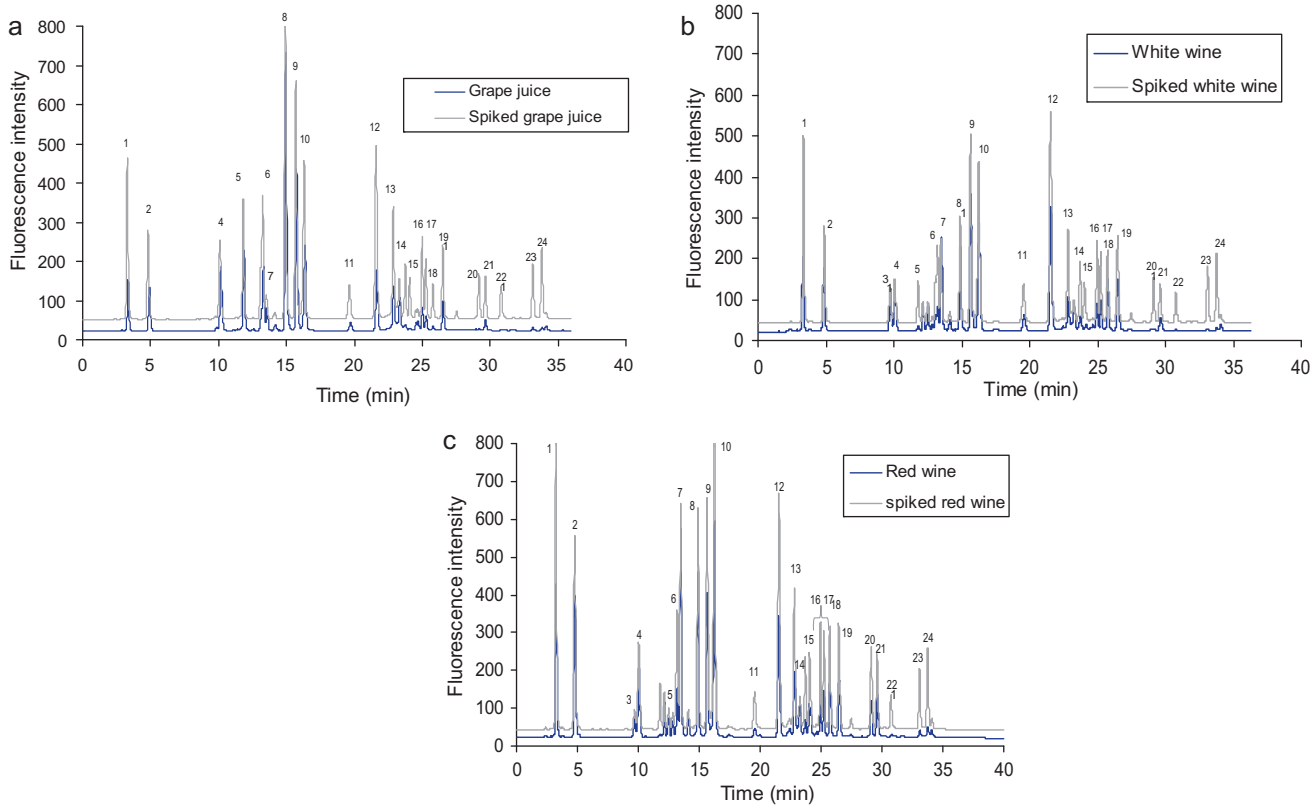

Fig. 2. Chromatograms of grape juice (a), white wine (b), red wine (c) unspiked and spiked with 10 mg/l standard mixture. Peak numbers as per Fig. 1.

amine content of Syrah grapes. The grapes received two (high and low) levels of sunlight. Natural sunlight was reduced by 17% using a black canopy, and was increased by 28% using a reflecting surface. For each level of sunlight, three yields were studied: 14 tonnes/ha (the natural yield of the parcel) and 9 and 6 t/ha obtained by removing green bunches before veraison. As may be observed in Fig. 3 in the case of both the 6 tonne and 14 tonne yields (only the high and low yields are presented for ease of interpretation), grapes that received more intense levels of sunlight were richer in all compounds though this effect is less intense (but still present) if the sugar concentrations are taken into account; there was a moderate correlation between sugar concentration and total amino acid concentration (0.76 and 0.66, for the 6 tonne and 14 tonne yields, respectively). As may also be observed in Fig. 3, yield had a more pronounced impact on amino acid and amine levels, and although the differences were not uniform across the range of analytes, the fact that this observation may due to a concentration phenomenon could not be excluded.

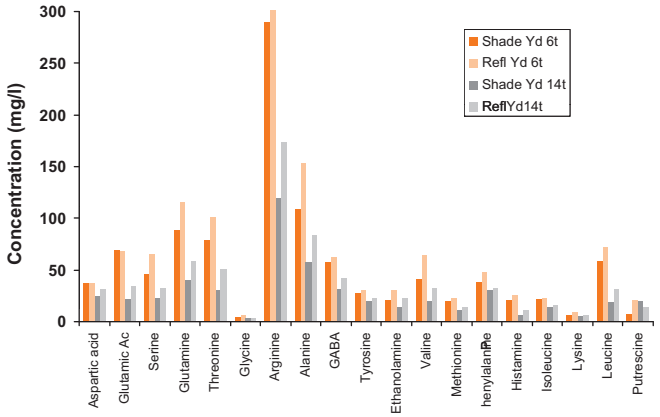

Fig. 3. Histogram showing the effect of shade and yield on amino acid and amine concentration of Syrah grapes.

3.4.2. *Analysis of wine*

In the first of two different wine studies, a comparison was made between wines produced at one site in France and one site Romania, given that the winemaking was supervised by the same consultant at both sites. In total, 11 wines were analysed: four Cabernet sauvignons and three Merlots from Romania, and two Merlots and two Syrahs from France. The predominant amino acids were aspartic and glutamic acids, glycine, threonine, alanine and GABA. Putrescine was the principal biogenic amine (and the only biogenic amine in the Merlots) with small quantities of cadaverine in the other wines. Within a given variety, the chromatographic profiles were very similar with differences observed only in concentrations (Fig. A supplementary material), though as indicated in the figure, the French Merlot contained residual arginine and a higher concentration of putrescine. The total concentration of amino acids and biogenic amines varied from approximately 300 mg/l to over 1 g/l in the case of one of the Cabernet sauvignon wines. The Romanian Merlot wines generally contained lower concentrations of amino acids and amines than the French Merlots or the Syrah wines, though this was in no way related to overall wine concentration, as all wines contained between 13.5 and 14% alcohol.

In the second study, the aim was a preliminary observation of the impact of sulphur dioxide (used as an antiseptic) on the amino acid and amine concentrations in wines made from the same Syrah grapes. Both wines were entirely fermented and aged in stainless steel tanks. Although both wines contained exactly the same compounds, the wine without the sulphur dioxide was slightly richer in all compounds (Fig. B supplementary material). However, due to the fact that replicate samples were not available no firm conclusions could be drawn in respect to the impact of SO_2 on wine amino acid and biogenic amine content.

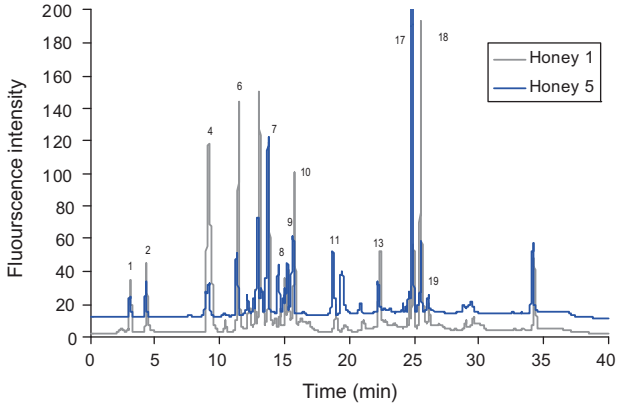

Fig. 4. Chromatogram of oak honey (1) and Eyne valley honey (5). Oak honey was distinctive from the other honeys in that it contained substantially more lysine than isoleucine.

3.4.3. Analysis of honey

Among other parameters, such as the concentration of mineral elements, the amino acid content has been proposed as a method of determining the botanical and/or geographical provenance of food products, and honey is has been frequently been targeted in this approach [33–35]. However, in this study, the method was applied to the analysis of five honey samples in the context of a larger multi-disciplinary study into the pharmacological properties of honey. The honey samples originated from the same producer from the Roussillon region of the south of France, but were from different sources (oak, chestnut, Mediterranean scrubland, Eyne valley and rhododendron). The oak honey was the deepest colour and was richest in total and individual amino acids (Table A supplementary material). Oak honey was also the richest in % saccharose (10.62%), whereas the honey with the lowest levels of amino acids (chestnut) also contained the lowest % saccharose. Oak honey was also distinctive in that it was particularly rich in lysine—77 mg/kg (Fig. 4) and contained less isoleucine (8.2 mg/kg) than scrub and Eyne valley honeys, which were the second and third richest in total amino acids, respectively (Table B supplementary material). The concentrations of aspartic and glutamic acids were similar, or at least of the same order of magnitude (2–10 mg/kg) as those previously reported. However, in other studies, the most abundant amino acid after proline was GABA, but in the honeys investigated in this study, the most abundant compounds were isoleucine and lysine and glutamine. Curiously, although the abundance of phenylalanine has been widely reported in nectar honeys has been reported at length [33–37], no phenylalanine was found in any of the honeys analysed in the present study. Clearly, however, it would be necessary to analyse a far greater number of samples and apply principal component analysis techniques to explore the possibilities of applying this methodology for discriminatory purposes.

3.4.4. Analysis of Physalis fruit

As part of a study on the nutra-ceutical and economic potential of different *Physalis* species, a preliminary analysis of the amino acid content was determined in various species of *Physalis*—*Physalis peruviana* L., otherwise known as goldenberry or cape gooseberry, *Physalis pruinosa* L. also known as ground cherry or husk tomato and *Physalis ixocarpa* Brot., commonly known as the tomatillo. Physalis fruit and juice are nutritious, containing particularly high levels of niacin, carotenoids and minerals [38]. The lipid [39] and aroma [40,41] fractions of *P. peruviana* have been investigated, and there is one report on biochemical composition and sensory properties of *P. pruinosa* [42] and the volatile composition of *P. ixocarpa* has also been reported [43].

All three species were extremely rich in amino acids (containing in excess of 2 g/l amino acids) to the degree that it was necessary to dilute the juice 100-fold before filtration and analysis. As may be observed in Fig. C (supplementary material), the amino acid distribution of all three species are similar; *P. peruviana* is considerably richer in the more abundant amino acids (particularly aspartate, glutamate, glutamine and GABA) than *P. pruinosa* or *P. ixocarpa*, with a total amino acid content of 6.3 g/l as opposed to 4 and 2.3 g/l for the other two species, respectively. The fact that all three species contain similar amounts of the less abundant amino acids (threonine, glycine, arginine, ethanolamine, methionine, and leucine) would indicate that this is not merely a concentration/dilution phenomenon. These findings concur with a previous study on the chemical composition of *Physalis pubescens* [38] where the total essential amino acid content was in excess of 3 g/l thus adding to the growing evidence of the nutritional benefits of this plant.

4. Conclusion

A new rapid and economical method has been presented using a fully automated in-loop derivatisation procedure for the simultaneous determination of 24 amino acids and biogenic amines. The method was statistically validated and applied to the determination of these compounds in grapes, wines, honey and *physalis* species. Due to the high sensitivity of the method no sample preparation, other than a simple dilution is required before derivatisation, which obviates the need for an internal standard. This considerably reduces the complexity of the method because, due to the widely varying of polarities among the different compounds, two internal standards are usually required to adequately compensate for variations in extraction procedures. The method has proved suitable for the analysis of these compounds in a variety of matrices and it is expected that it would be applicable to an even more diverse number of biological media; further studies on its application for the analysis of different plant parts are envisaged.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.09.047.

References

[1] S. Özcan, H. Senyuva, J. Chromatogr. A 1135 (2006) 179.
[2] P. Hernandez-Orte, M.J. Ibarz, J. Cacho, V. Ferreira, Food Chem. 98 (2006) 300.
[3] P. Hernandez Orte, J. Cacho, V. Ferreira, J. Agric. Food Chem. 50 (2002) 2891.
[4] A. Escudero, P. Hernandez-Orte, J. Cacho, V. Ferreira, J. Agric. Food Chem. 48 (2000) 4268.
[5] M. Subileau, J.-M. Salmon, R. Schneider, E. Degryse, FEMS Yeast Res. 8 (2008) 771.
[6] H. Tabor, C.W. Tabor, Pharmacol. Rev. 16 (1964) 245.
[7] H.P. Til, H.E. Falke, M.K. Prinsen, M.I. Willems, Food Chem. Toxicol. 35 (1997) 337.
[8] A.L. Cinquina, A. Calì, F. Longo, L. De Santis, A. Severoni, F. Abballe, J. Chromatogr. A 1032 (2004) 73.
[9] N. Seiler, Digestion 46 (1990) 319.
[10] C.W. Tabor, H. Tabor, Ann. Rev. Biochem. 53 (1984) 749.
[11] J.H. Hotchkiss, Cancer Surv. 8 (1989) 295.
[12] A. Önal, Food Chem. 103 (2007) 1475.
[13] V. Pereira, M. Pontes, J.S. Camara, J.C. Marques, J. Chromatogr. A 1189 (2008) 435.
[14] T. Bauza, M.T. Kelly, A. Blaise, Food Chem. 105 (2007) 405.
[15] A. Jámbor, I. Molnár-Perl, J. Chromatogr. A 1216 (2009) 3064.
[16] I. Krause, A. Bockhardt, H. Neckermann, T. Henle, H. Klostermeyer, J. Chromatogr. A 715 (1995) 67.

[17] O. Pinho, I. Ferreira, E. Mendes, B.M. Oliveira, M. Ferreira, Food Chem. 75 (2001) 287.
[18] S.A. Cohen, D.P. Michaud, Anal. Biochem. 211 (1993) 279.
[19] M. Roth, Anal. Chem. 43 (1971) 880.
[20] R. Hanczkó, A. Jámbor, A. Perl, I. Molnár-Perl, J. Chromatogr. A 1163 (2007) 25.
[21] I. Molnár-Perl, I. Bozor, J. Chromatogr. A 798 (1998) 37.
[22] D. Kutlán, P. Presits, I. Molnár-Perl, J. Chromatogr. A 949 (2002) 235.
[23] Y. Mengerink, D. Kutlán, F. Tóth, A. Csámpai, I. Molnár-Perl, J. Chromatogr. A 949 (2002) 99.
[24] D. Kutlán, I. Molnár-Perl, J. Chromatogr. A 987 (2003) 311.
[25] A. Vasanits, D. Kutlán, P. Sass, I. Molnár-Perl, J. Chromatogr. A 870 (2000) 271.
[26] I. Molnár-Perl, A. Vasanits, J. Chromatogr. A 835 (1999) 73.
[27] P. Herbert, M.J. Cabrita, N. Ratola, O. Laureano, A. Alves, J. Food Eng. 66 (2005) 322.
[28] P. Herbert, L. Santos, A. Alves, J. Food Sci. 66 (2001) 1319.
[29] Á. Kőrös, Zs. Varga, I. Molnár-Perl, J. Chromatogr. A 1203 (2008) 146.
[30] R. Hanczkó, Á. Kőrös, F. Tóth, I. Molnár-Perl, J. Chromatogr. A 1087 (2005) 210.
[31] S. Rubio-Barroso, M.J. Santos-Delgado, C. Martín-Olivar, L.M. Polo-Díez, J. Dairy Sci. 89 (2006) 82.
[32] Food Drug Administration, Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Rockville, MD, 2001.
[33] P.M. Cometto, P.F. Faye, R. Di Paola Naranjo, M.A. Rubio, M.A.J. Aldao, J. Agric. Food Chem. 51 (2003) 5079.
[34] M.T. Iglesias, C. De Lorenzo, M.C. Polo, P.J. Martiín-Álvarez, E. Pueyo, J. Agric. Food Chem. 52 (2004) 84.
[35] A.M. González Paramás, J.A. Gómez Báreza, C. Cordón Marcosa, R.J. García-Villanova, F. Sánchez Sánchez, J. Food Chem. 95 (2006) 148.
[36] G. Bosi, M. Battaglini, J. Appl. Res. 17 (1978) 152.
[37] L.S. Conte, M. Miorini, A. Giomo, G. Bertacco, R. Zironi, J. Agric. Food Chem. 46 (1998) 1844.
[38] A.F. El Sheikha, M.S. Zaki, A.A. Bakr, M.M. El Habashy, D. Montet, FOOD, vol. 2, Global Science Books Ltd., UK, 2008, p. 124.
[39] M.F. Ramadan, J.T. Mörsel, J. Agric. Food Chem. 51 (2003) 969.
[40] H. Mayorga, C. Duque, H. Knapp, P. Winterhalter, Phytochemistry 59 (2002) 439.
[41] H. Mayorga, H. Knapp, P. Winterhalter, C. Duque, J. Agric. Food Chem. 49 (2001) 1904.
[42] A.F. El Sheikha, M.S. Zaki, A.A. Bakr, M.M. El Habashy, M. Magida, D. Montet, J. Food Proc. Pres. 34 (2010) 541.
[43] Y. Xu, S. Barringer, J. Food Sci. 75 (2010) C268.