

Simultaneous analysis of free amino acids and biogenic amines in honey and wine samples using in loop orthophthalaldehyde derivatization procedure

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Abstract

This work presents a RP-HPLC method for the simultaneous quantification of free amino acids and biogenic amines in liquid food matrices and the results of the application to honey and wine samples obtained from different production processes and geographic origins. The developed methodology is based on a pre-column derivatization with *o*-phthalaldehyde carried out in the sample injection loop. The compounds were separated in a Nova-Pack RP-C₁₈ column (150 mm × 3.9 mm, 4 μm) at 35 °C. The mobile phase used was a mixture of phase A: 10 mM sodium phosphate buffer (pH 7.3), methanol and tetrahydrofuran (91:8:1); and phase B: methanol and phosphate buffer (80:20), with a flow rate of 1.0 ml/min. Fluorescence detection was used at an excitation wavelength of 335 nm and an emission wavelength of 440 nm. The separation and quantification of 19 amino acids and 6 amines was carried out in a single run as their OPA/MCE derivatives elute within 80 min, ensuring a reproducible quantification. The method showed to be adequate for the purpose, with an average RSD of 2% for the different amino acids; detection limits varying between 0.71 mg/l (Asn) and 8.26 mg/l (Lys) and recovery rates between 63.0% (Cad) and 98.0% (Asp). The amino acids present at the highest concentration in honey and wine samples were phenylalanine and arginine, respectively. Only residual levels of biogenic amines were detected in the analysed samples.

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1. Introduction

Amino acids and biogenic amines co-exist in biological and food matrices and participate in several transformation processes [1]. Amino acids play a central role as building blocks of proteins and as intermediates in the metabolism. They represent an important part of the human body as proteins are found in muscles, tendons, organs, glands, nails and hair, and promote growth, repair and maintenance of the cells. Amino acids microbial catabolism produces key flavour compounds in foods such as cheese, wine, honey and other fermented foodstuff [2]. From their enzymatic decarboxylation results the formation of biogenic amines [3], undesirable compounds when in higher levels due to the physiological effects in the human organism, consequence of their toxicology. Biogenic amines are a group of organic bases, namely aliphatic (putrescine and cadaverine),

aromatic (tyramine) and heterocyclic amines (histamine) with low-molecular weight [4]. Consumption of high amounts of these amines, namely histamine, can result in intoxication symptoms such as headache, nausea, rushes, hypo- or hypertension and digestive problems, whereas tyramine and phenylethylamine have been associated with migraines and hypertension [5]. The study of biogenic amines can also be used as an indicator of food quality since their occurrence is normally associated with inadequate sanitary conditions during the production procedures. The determination of the amino acids and biogenic amines is of great importance in food industry due to nutritional labelling requirements, control of process operating conditions and, eventually, in the determination of origin, as used for honey [6] in substitution of mellissopalynology. This method, recommended for the analysis and identification of pollens contained in honey, is extremely tedious, high time-consuming and requires a very skilful analyst for data interpretation [7]. Considering that about 1% of the non sugar/water fraction of honey is due to amino acids related to animal and vegetal sources, mainly bees and pollen [8], the amino acids profile can be advantageously used for the

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characterization. In a similar way, they can be used for the characterization of wines. Amino acids act as a source of nitrogen (30–40%) for yeast during alcoholic fermentation, interacting in the aromatic composition of wine and, according to several authors [9–12], their composition may be a suitable method for the classification of wines according to variety, geographical origin, wine-making technologies and vintage.

Biogenic amines, naturally present in grapes and in wines, derive mostly from malolactic fermentation [13,14] from the decarboxylation of free amino acids by the action of lactic acid bacteria. In recent years, new trends in food safety, together with the consumers' demand for quality and healthier products, have encouraged several authors to study these compounds in several wines [4,15–17] while in honeys, and as far as we know, no studies have been published. The most common biogenic amines found in wines are histamine, tyramine, putrescine and phenylethylamine [18].

Diverse analytical methods have been proposed for the analysis of amino acids and biogenic amines including gas chromatography (GC) [19–21], high-performance liquid chromatography (HPLC) [22,23] and capillary electrophoresis (EC) [24,25]. 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 [2,26–31], but reported applications of these methods to biological measurements are limited. Besides involving shorter analysis times the LC–MS/MS technique is expensive and is not available in many research laboratories. There are some methods used for the simultaneous determination of amino acids and biogenic amines by HPLC [32–35], micellar electrokinetic capillary chromatography (MECC) [36] and micellar liquid chromatography (MLC) [37]. Traditionally, the determination of amino acids has been conducted by ion-exchange chromatography, followed by post-column derivatization with ninhydrin. So far, the analysis of these compounds using pre-column derivatization and reversed-phase HPLC separation of the derivatives has become widely accepted and usually shows great sensitivity. Typical derivatization reagents include 9-fluorenylmethyl chloroformate (FMOC-Cl) [38,39], *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (FMOC-OSu) [35], carbazole-9-yl-acetyl chloride (CRA-Cl) [40], orthophthalaldehyde (OPA) [41,42], phenyl isothiocyanate (PITC) [41,43], 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide [44] and dansyl-chloride [45,46].

This study intended to develop a simple RP-HPLC methodology for the simultaneous identification and quantification of amino acids and biogenic amines in liquid food matrices, based on a pre-column OPA derivatization carried out in the chromatograph injection loop. The OPA/MCE reagent was selected due to its high sensitivity and response to minor amino acids. OPA in the presence of 2-mercaptoethanol (MCE) reacts with amino acids and biogenic amines and proceeds to isoindolic derivatives, at room temperature, in a quick and simple reaction. The secondary amino acids, proline and hydroxyproline, are not determined as they do not react. Some derivatives are unstable making crucial an appropriate control of the reaction and injection

time [47]. Furthermore, this derivatization reagent allows the simultaneous analysis of these compounds without extraction and purification processes preceding the derivatization with fluorescent functional group detection [1,24]. In order to simplify the derivatization procedure and the reproducibility of the results, this work was focused in the derivatization operating conditions to be accomplished in the sample injection loop. This methodology was applied to honey and wine samples obtained from different production processes and geographic origins.

2. Experimental

2.1. Standards and reagents

Ultra-pure water was obtained from a Milli Q-System (Millipore, Milford, MA, USA) while HPLC-grade methanol was obtained from Sigma–Aldrich (St. Louis, MO, USA). Tetrahydrofuran (99.5%), ethanol (99.9%), sodium hydroxide (98%), sodium phosphate monobasic monohydrate (98%) are from Panreac Quimica SA (Barcelona, Spain). *o*-Phthaldialdehyde (p.a.), 2-mercaptoethanol (99%) were supplied by Acros Organics (Geel, Belgium), hydrochloric acid (p.a.) by Riedel-de Haën (Seelze, Germany) and boric acid (99.5%) by Merck Co. (Darmstadt, Germany).

A kit of high purity L-amino acids (>98%) was supplied by Sigma–Aldrich (St. Louis, MO, USA) and consisted of 1 g of each of the following standards: aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), methionine (Met), tryptophan (Trp), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu) and lysine (Lys).

The following standards were supplied by Fluka BioChemika AG (Buchs, Switzerland): γ -aminobutyric acid (GABA) and the biogenic amines: histamine (Him), tyramine (Tym), phenylethylamine (Phm) isopentylamine (Ism), and cadaverine (Cad). Tryptamine (Trm) was purchased from Acros Organics (Geel, Belgium).

A concentrated 10 g/l stock solution of each amino acid and biogenic amine was prepared in 0.1 M HCl. Calibration standards (ranging from 0.5 to 60.0 mg/l) were prepared in 0.1 M HCl from the concentrated standard solution. Finally, they were filtered through a 0.45 μ m filter (Acrodisc[®] CR-PTFE, Ann Arbor, SOM, USA), stored in a refrigerator and protected from light.

2.2. Equipment

Amino acids and biogenic amines were simultaneously separated in a HPLC system using a Waters (Milford, MA, USA) liquid chromatograph controlled by the Empower Pro software and equipped with an auto-injector (Waters 2695, separations module) and a Multi λ Fluorescence detector (Waters 2475). Chromatographic analysis were performed using an analytical scale (3.9 mm \times 150 mm) Nova-Pack RP-C₁₈ column, with a particle size of 4 μ m, purchased from Waters (Milford, MA, USA).

Table 1
Gradient program employed for the separation of amino acids and biogenic amines

Time (min)	Flow (ml/min)	Eluent A (%)	Curve
0	1.00	100	6
6	1.00	100	6
17	1.00	85	6
25	1.00	80	6
33	1.00	70	6
45	1.00	60	6
61	1.00	20	6
67	1.00	0	6
70	1.00	0	6
71	1.00	100	1
80	1.00	100	6

2.3. Chromatographic conditions

HPLC conditions were as follow: mobile phase A: 1% of tetrahydrofuran, 8% methanol and 91% phosphate buffer (10 mM). Mobile phase B: 80% methanol and 20% phosphate buffer (10 mM).

The flow rate was set at 1 ml/min and the column maintained at 35 °C. The eluted OPA derivatives were detected by monitoring their fluorescence at 335 and 440 nm as excitation and emission wavelengths, respectively. The injections were performed in less than 80 min, including column regeneration and stabilization during the last 13 min. The gradient program used is shown in Table 1.

2.4. Samples

A total of 21 samples were analyzed with the developed method (12 honeys and 9 wines—see Table 2). The honey samples include nine multifloral and three monofloral, from different origins namely Madeira islands (H1–H5), Portugal mainland (H6–H8) and Canary islands (H9–H12), purchased in local stores. The wine samples include four Madeira fortified wines (W1–W4), three Madeira table wines (W5–W7) and two Canarian table wines (W8 and W9) from the following grape varieties: Malvasia, Tinta Negra Mole and Sercial.

Before the derivatization procedure, 200 µl of the sample were added to 1.5 ml of a 0.4 M borate buffer solution (pH 10.5), homogenized in a vortex agitator and then filtered through 0.45 µm PTFE filter. In case of honey samples, 5 g were diluted with ultrapure water into a 10 ml volumetric flask and filtered.

2.5. Derivatization

OPA derivatization solution was prepared in a 10 ml volume flask by dissolving 250 mg of reagent in 1.5 ml of ethanol and making up the volume with 0.4 M borate buffer (pH 10.5). Finally 200 µl of 2-mercaptoethanol was added. At last, the reagent solution was left to settle for 90 min, stored in dark glass vials at 4 °C and freshly prepared every 9 days.

The derivatization procedure was performed in the sample injection loop according to the following sequence: 10 µl of

Table 2
Samples analyzed by the developed HPLC method

Samples	Characteristics	Origin
Honey		
H1	Multifloral	Madeira islands
H2	Multifloral	
H3	Multifloral	
H4	Multifloral	
H5	Multifloral	
H6	Monofloral	Portugal mainland
H7	Multifloral	
H8	Monofloral	
H9	Multifloral	Canary islands
H10	Monofloral	
H11	Multifloral	
H12	Multifloral	
Wine		
Fortified		
W1	Malvasia (sweet)	Madeira islands
W2	Sercial (dry)	
W3	Tinta Negra Mole (sweet)	
W4	Tinta Negra Mole (dry)	
Table		
W5	Malvasia	Madeira islands
W6	Tinta Negra Mole (rosé)	
W7	Tinta Negra Mole (red)	
Table		
W8	Malvasia	Canary islands
W9	Malvasia	

buffered sample mixture were aspirated to the injection loop followed by 10 µl of OPA solution and maintained for 3 min to promote the derivatization reaction. During this period, the flow is maintenance at 0 ml/min to keep the reagent into the loop. Then, the loop content (20 µl) was forced to enter into the column by changing the mobile phase flow to 1 ml/min.

2.6. Calculations

The concentration of each analyte was obtained by direct interpolation of the peak area in the correspondent linear calibration curve (peak area vs. concentration, ranging from 0.5 to 60.0 mg/l). Samples were diluted when needed to comply with the working range.

3. Results and discussion

3.1. Derivatization procedure

Derivatization of the standard amino acid and biogenic amines mixture was performed by OPA/MCE in boric buffer (0.4 M sodium borate, pH 10.5). The first experiments were carried out using an injection volume of 50 µl (25 µl of buffered sample mixture and 25 µl of OPA/MCE reagent) but the volume was reduced in order to extend column life without compromising the good response.

Table 3
Retention times, calibration curves ($y = ax + b$), correlation coefficient (r^2), limits of detection (LOD) in mg/l, repeatability (RSD%) and recovery (Rec%) of amino acids and biogenic amines

	Retention times (min)	$a (\times 10^5)$	$b (\times 10^5)$	r^2	SD ($\times 10^5$)	LOD	Rep (RSD%)	Rec (%)
Amino acids								
Aspartic acid	1.33 ± 0.28	23.30	27.40	0.998	14.68	0.71	0.60	98
Glutamic acid	2.52 ± 0.30	21.20	13.40	0.999	14.45	1.41	1.65	91
Asparagine	8.60 ± 0.52	19.10	6.94	0.999	11.89	1.50	1.49	90
Serine	11.10 ± 0.35	32.00	12.30	0.998	28.69	2.30	0.14	85
Glutamine	13.74 ± 0.25	2.29	0.58	0.994	4.07	5.08	4.14	90
Histidine	14.25 ± 0.45	9.54	-3.67	0.998	8.57	2.73	1.92	90
Glycine	17.25 ± 0.38	45.50	-11.70	0.997	52.92	3.75	1.09	87
Threonine	19.15 ± 0.30	24.10	8.92	0.999	18.73	1.96	2.55	88
Arginine	21.89 ± 0.65	17.40	1.72	0.998	15.79	2.62	0.58	85
Alanine	26.15 ± 0.25	36.20	-0.14	0.998	36.15	3.00	0.72	85
GABA	28.50 ± 0.25	30.50	-22.40	0.996	42.26	4.89	0.89	83
Tyrosine	32.50 ± 0.40	17.60	4.87	0.999	12.14	1.79	0.51	91
Methionine	45.20 ± 0.25	21.40	-0.89	0.991	45.14	6.37	7.92	98
Tryptophan	46.25 ± 0.35	31.20	16.70	0.999	16.98	1.10	0.39	91
Valine	47.50 ± 0.20	13.60	-1.56	0.998	12.49	2.87	0.56	82
Phenylalanine	50.20 ± 0.30	19.10	2.21	0.999	15.97	2.39	0.63	83
Isoleucine	52.50 ± 0.45	29.90	10.30	0.999	19.27	1.59	0.88	91
Leucine	53.90 ± 0.25	27.30	3.42	0.999	21.30	2.21	0.67	88
Lysine	59.50 ± 0.40	6.78	-8.75	0.990	15.74	8.26	5.41	82
Biogenic amines								
Histamine	49.10 ± 0.45	29.50	-25.30	0.996	4,209,260	5.14	0.40	87
Tyramine	58.90 ± 0.25	28.90	-24.30	0.994	4,972,310	6.00	6.04	82
Tryptamine	63.50 ± 0.30	21.90	-25.10	0.994	3,815,051	6.37	0.66	81
Phenylethylamine	64.75 ± 35	24.20	-18.10	0.995	3,963,053	5.66	0.24	78
Isopentylamine	65.80 ± 0.20	35.00	1.75	0.994	4,184,279	3.54	5.14	91
Cadaverine	67.00 ± 0.20	20.50	-19.80	0.992	4,182,516	7.09	1.09	63

Most part of published methods requires sample pre-treatment before derivatization [6,48,49]. Paramás et al. [48] developed an OPA/MCE derivatization method for the determination of amino acids in honey that includes a clean-up step and an extraction procedure before derivatization. The developed method has the advantage of a simpler methodology, not requiring any complex pre-treatment for liquid food matrices and only a dilution is carried out, if necessary. OPA-derivatization times are short (3 min) when compared when other derivatization reagents are used for the simultaneous determination of these compounds [32,33,35]. The proposed method by Bauza et al. [33] using FMOC as derivatization reagent needs 6 min for reaction development, while Krause et al. [32] used a dabsyl method and the derivatization time was 20 min. Lately, Lozanov et al. [35] proposed the use of FMOC-OSu reacting during 20 min. OPA derivatization does not show the presence of excess reagent, interfering with the analytes resolution, as detected when using FMOC derivatization methods [35]. Furthermore, the derivatization reaction is automatic, occurs in the injection loop and shows sensitive and consistent results.

3.2. Method validation

The sample analytes were identified by comparison with the retention times of amino acid standard solutions. For the determination of retention times, the reference standards were injected both individually and as a mixture. Quantification was

performed by the external standard method based on peak areas of the eluted amino acid and biogenic amines derivatives.

The linearity was evaluated by the construction of calibration curves, using the chromatographic peaks areas of the fluorescence response from triplicate injections of standards, at six increasing concentrations in the 0.5–60.0 mg/l range for all amino acids and biogenic amines. The linear relationship between concentrations and peak area is given by a , b and r^2 —see Table 3, where a and b are the coefficients of the regression equation $y = ax + b$, x being the concentration of the analyte, y the peak area and r^2 the coefficient of determination. For this calculation, all obtained values were used instead the average of the three injections. In all cases, the relationship between concentrations and peak areas were linear over the tested range, with coefficients of determination greater than 0.990. The repeatability of the method was evaluated by nine consecutive injections of the same sample during a working day. Detection limits (defined as three times the signal-to-noise ratio) ranged from 0.71 mg/l (Asp) to 8.26 mg/l (Lys)—Table 3.

To determine intra-sample and inter-day precisions for the analyses of amino acids and biogenic amines in honeys and wines, three identical samples were run on three separate days. Intra-samples precisions for individual measurements of amino acids range from 1.9 to 4.8% and the inter-day precisions range from 4.2 to 9.4% (RSD). Biogenic amines were not considered as only vestigial quantities were found. The calculated concentrations of individual amino acids showed residual standard

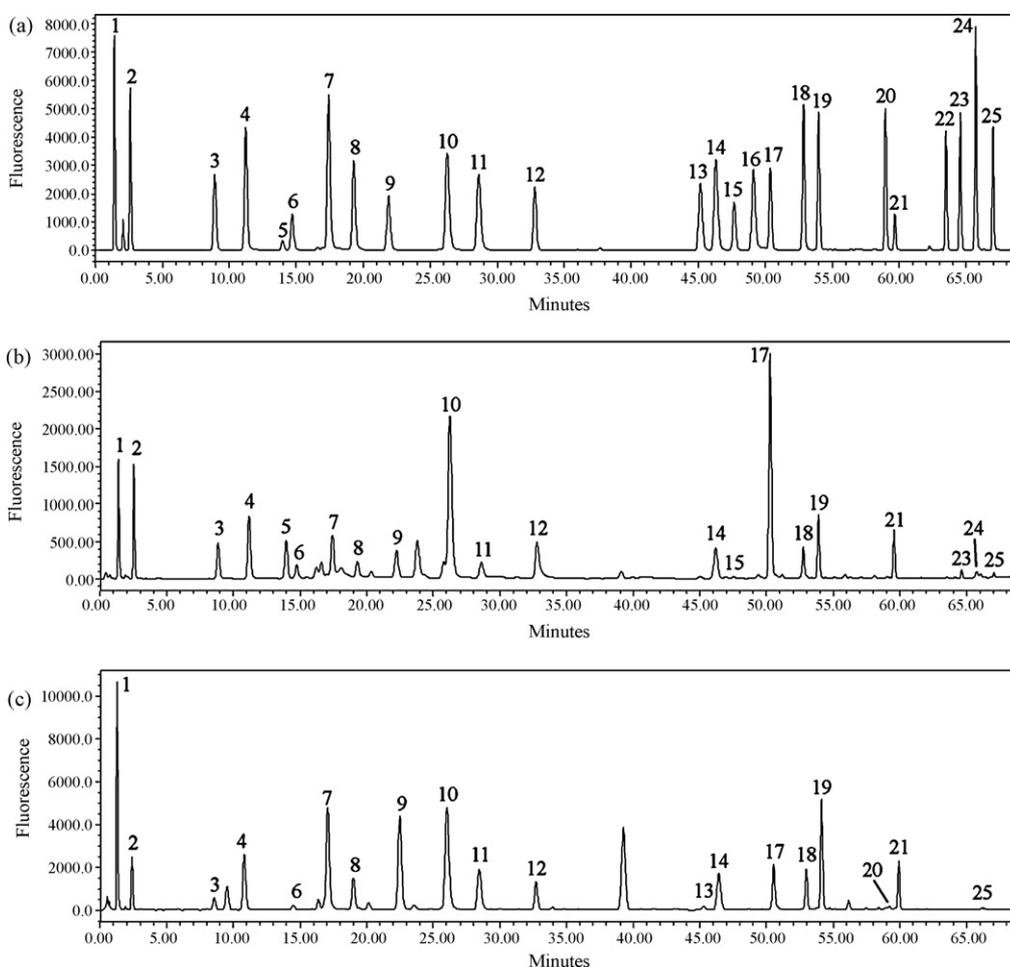


Fig. 1. Typical chromatogram profile of amino acids and biogenic amines in: (a) 20 mg/l standard mixture, (b) H5 honey and (c) W4 wine. Peak identification: (1) aspartic acid, (2) glutamic acid, (3) asparagine, (4) serine, (5) glutamine, (6) histidine, (7) glycine, (8) threonine, (9) arginine, (10) alanine, (11) GABA, (12) tyrosine, (13) methionine, (14) tryptophan, (15) valine, (16) histamine, (17) phenylalanine, (18) isoleucine, (19) leucine, (20) tyramine, (21) lysine, (22) tryptamine, (23) phenylethylamine, (24) isopentylamine and (25) cadaverine.

deviations (RSD) of about 2% in the analyses of wine and honey samples.

The accuracy was estimated by means of the recovery tests. For the evaluation of the recovery rate, H3 honey and W7 wine ($n=5$) were spiked with 10 mg/l standard solution, derivatized and quantified. The recovery rate averages obtained were acceptable, with values ranging from 82% (Lys) to 98% (Asp) for amino acids, and 63% (Cad) to 91% (Ism) for biogenic amines—Table 3.

3.3. Chromatographic analysis

The proposed HPLC method allows the simultaneous determination of 19 amino acids and 6 biogenic amines in 83 min, including the column regeneration (9 min) and derivatization time (3 min), slightly higher than the methodology suggested by Alberto et al. [34], 63 min, but ensuring better separation. The applied methodology allows the total separation of all amino acids and biogenic amines in the standards solutions and analysed matrices, overcoming some peak overlay obtained by several authors [8,50–52] namely Asn + Ser [50,8], Gln + Thr [50], Asp + Asn [51], Glu + Gln [51] and Thr + Ala [52]. Fig. 1

shows the separation obtained for the amino acids and biogenic amines present in a 20 mg/l standard solution, together with typical chromatograms obtained for honey (H5) and wine (W4).

More than the determination of amino acid composition, this methodology is a useful tool for the control of biogenic amines with known toxic activity, like phenylethylamine, histamine (maximum recommended levels of 5–6 mg/l in Belgium

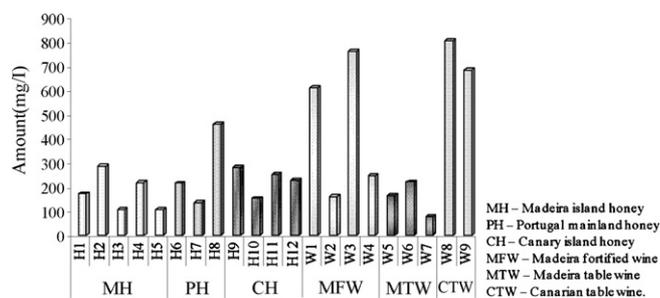


Fig. 2. Amount of amino acid in the liquid food matrices studied. MH, Madeira islands honey; PH, Portugal mainland honey; CH, Canary islands honey; MFW, Madeira fortified wine; MTW, Madeira table wine; CTW, Canarian table wine.

Table 4
Levels (mg/l) of free amino acids in studied honey and wine samples with the respective standard deviation of the mean value ($n = 3$ for each data point)

	Asp	Glu	Asn	Ser	Gln	His	Gly	Thr	Arg	Ala	GABA	Tyr	Met	Trp	Val	Phe	Ile	Leu	Lys
Honeys																			
Madeira island																			
H1	3.73 ± 0.080	4.65 ± 0.11	2.83 ± 0.01	<LOD	39.99 ± 0.30	3.20 ± 0.20	<LOD	<LOD	3.36 ± 0.03	3.69 ± 0.04	<LOD	4.77 ± 0.35	n.d.	3.31 ± 0.00	<LOD	76.38 ± 0.72	2.24 ± 0.02	10.74 ± 0.12	12.58 ± 0.83
H2	3.89 ± 0.01	5.64 ± 0.01	2.85 ± 0.03	2.67 ± 0.00	55.19 ± 0.48	4.71 ± 0.00	<LOD	<LOD	2.99 ± 0.00	7.77 ± 0.01	<LOD	8.44 ± 0.02	n.d.	5.56 ± 0.00	<LOD	146.65 ± 1.65	4.07 ± 0.01	23.09 ± 0.02	12.48 ± 0.12
H3	3.33 ± 0.04	4.94 ± 0.04	3.01 ± 0.00	3.44 ± 0.03	32.71 ± 0.37	2.73 ± 0.18	<LOD	<LOD	3.61 ± 0.03	12.41 ± 0.05	<LOD	5.47 ± 0.01	n.d.	2.03 ± 0.01	n.d.	19.99 ± 0.02	<LOD	3.24 ± 0.00	9.75 ± 0.08
H4	6.26 ± 0.27	11.77 ± 0.27	3.29 ± 0.00	4.77 ± 0.11	34.06 ± 0.86	6.73 ± 0.21	<LOD	3.21 ± 0.04	<LOD	6.41 ± 0.05	<LOD	20.65 ± 0.28	n.d.	3.37 ± 0.00	6.45 ± 0.00	97.76 ± 0.08	2.36 ± 0.01	1.79 ± 0.01	9.43 ± 0.06
H5	3.54 ± 0.04	6.36 ± 0.01	5.51 ± 0.05	2.73 ± 0.00	32.56 ± 0.22	5.07 ± 0.00	<LOD	<LOD	3.67 ± 0.00	4.45 ± 0.09	<LOD	6.49 ± 0.02	n.d.	2.45 ± 0.01	<LOD	20.37 ± 0.06	<LOD	<LOD	12.63 ± 0.16
Portugaal mainland																			
H6	3.82 ± 0.04	2.84 ± 0.06	4.95 ± 0.05	3.56 ± 0.01	20.08 ± 0.11	4.77 ± 0.05	<LOD	<LOD	4.27 ± 0.15	3.72 ± 0.08	<LOD	33.84 ± 0.35	n.d.	1.65 ± 0.05	<LOD	109.61 ± 0.29	1.62 ± 0.02	11.11 ± 0.12	8.71 ± 0.12
H7	5.49 ± 0.13	6.58 ± 0.14	3.81 ± 0.10	3.33 ± 0.09	26.93 ± 1.19	4.92 ± 0.03	<LOD	<LOD	4.00 ± 0.03	8.02 ± 0.01	<LOD	8.91 ± 0.14	n.d.	2.37 ± 0.00	<LOD	47.69 ± 0.39	<LOD	2.32 ± 0.02	9.49 ± 0.03
H8	23.65 ± 0.28	39.23 ± 0.45	15.17 ± 0.89	7.76 ± 0.53	154.42 ± 0.91	4.07 ± 1.36	4.15 ± 0.18	3.26 ± 0.16	9.98 ± 0.26	11.36 ± 0.29	8.70 ± 0.17	9.07 ± 0.06	n.d.	6.79 ± 0.07	<LOD	145.28 ± 1.53	3.70 ± 0.02	3.02 ± 0.00	8.42 ± 0.09
Canary island																			
H9	2.29 ± 0.10	4.74 ± 0.04	2.66 ± 0.04	3.19 ± 0.03	31.34 ± 0.06	6.21 ± 0.07	<LOD	<LOD	3.43 ± 0.05	3.58 ± 0.00	<LOD	40.67 ± 0.01	n.d.	2.06 ± 0.02	<LOD	159.68 ± 0.17	<LOD	3.85 ± 0.01	15.62 ± 0.00
H10	17.04 ± 0.39	9.28 ± 0.27	17.93 ± 0.36	3.85 ± 0.03	57.08 ± 1.26	5.77 ± 0.05	<LOD	<LOD	4.58 ± 0.03	5.24 ± 0.02	<LOD	6.10 ± 0.05	n.d.	3.44 ± 0.04	n.d.	10.44 ± 0.05	1.85 ± 0.02	<LOD	8.85 ± 0.12
H11	43.85 ± 0.91	45.58 ± 0.47	4.42 ± 0.26	7.36 ± 0.82	91.68 ± 2.74	3.05 ± 0.20	8.42 ± 0.03	<LOD	<LOD	19.99 ± 0.06	<LOD	2.64 ± 0.18	n.d.	5.21 ± 0.09	n.d.	15.74 ± 0.36	<LOD	3.89 ± 0.00	<LOD
H12	6.84 ± 0.06	7.48 ± 0.06	7.63 ± 0.54	3.83 ± 0.09	61.35 ± 1.41	9.64 ± 0.38	<LOD	2.62 ± 0.07	<LOD	5.29 ± 0.15	<LOD	19.66 ± 0.13	n.d.	3.58 ± 0.04	<LOD	74.62 ± 1.84	2.09 ± 0.08	<LOD	21.11 ± 0.24
Wines																			
Madeira fortified																			
W1	14.62 ± 0.08	12.16 ± 0.10	2.32 ± 0.04	21.23 ± 0.09	<LOD	4.38 ± 0.09	14.03 ± 0.10	19.39 ± 0.60	313.46 ± 1.62	85.74 ± 0.22	95.60 ± 1.16	5.69 ± 0.04	<LOD	10.61 ± 0.00	n.d.	8.51 ± 0.02	3.49 ± 0.12	8.33 ± 0.02	<LOD
W2	20.90 ± 0.01	6.46 ± 0.00	3.03 ± 0.02	6.98 ± 0.02	1.17 ± 0.05	3.49 ± 0.13	10.54 ± 0.02	6.88 ± 0.10	15.46 ± 0.05	16.51 ± 0.20	9.41 ± 0.07	6.37 ± 0.05	1.39 ± 0.00	6.52 ± 0.09	n.d.	9.83 ± 0.19	4.81 ± 0.09	13.56 ± 0.32	15.43 ± 0.45
W3	30.44 ± 0.41	11.47 ± 0.15	4.89 ± 0.04	16.77 ± 0.04	<LOD	4.73 ± 0.09	11.85 ± 0.07	21.96 ± 0.40	459.56 ± 6.29	87.23 ± 0.56	33.57 ± 0.21	18.17 ± 0.09	n.d.	11.40 ± 0.07	n.d.	11.33 ± 0.07	6.72 ± 0.02	12.01 ± 0.03	14.96 ± 0.15
W4	29.52 ± 0.19	8.45 ± 0.08	3.88 ± 0.12	12.20 ± 0.01	<LOD	3.69 ± 0.10	18.84 ± 0.52	9.10 ± 0.08	47.42 ± 0.32	28.67 ± 0.15	13.91 ± 0.34	11.78 ± 0.07	<LOD	10.58 ± 0.07	n.d.	15.40 ± 0.03	6.72 ± 0.05	20.61 ± 0.05	33.40 ± 1.49
Madeira table																			
W5	10.67 ± 0.06	26.48 ± 0.11	11.03 ± 0.29	4.86 ± 0.01	<LOD	5.29 ± 0.21	5.61 ± 0.12	3.47 ± 0.00	19.43 ± 0.50	17.58 ± 0.03	5.18 ± 0.01	4.70 ± 0.03	<LOD	4.16 ± 0.01	n.d.	6.64 ± 0.03	3.17 ± 0.01	11.00 ± 0.02	19.86 ± 0.09
W6	10.35 ± 0.02	29.64 ± 0.01	5.39 ± 0.36	4.35 ± 0.02	28.37 ± 0.05	6.78 ± 0.28	8.41 ± 0.21	2.47 ± 0.18	24.73 ± 0.15	14.90 ± 0.02	31.27 ± 0.11	8.61 ± 0.02	<LOD	3.74 ± 0.15	n.d.	7.13 ± 0.05	2.35 ± 0.03	9.13 ± 0.01	20.11 ± 0.14
W7	1.55 ± 0.01	9.49 ± 0.04	1.39 ± 0.01	3.60 ± 0.06	21.36 ± 0.17	2.03 ± 0.07	5.43 ± 0.05	0.85 ± 0.02	5.58 ± 0.08	8.31 ± 0.09	6.27 ± 0.11	2.05 ± 0.04	0.15 ± 0.01	1.50 ± 0.11	n.d.	1.93 ± 0.06	0.64 ± 0.06	2.01 ± 0.04	2.75 ± 0.05
Canarian table																			
W8	30.13 ± 0.62	46.52 ± 0.62	3.20 ± 0.10	12.09 ± 0.14	<LOD	84.26 ± 0.71	25.14 ± 0.32	11.69 ± 0.02	278.09 ± 6.29	66.12 ± 0.67	101.65 ± 3.55	29.83 ± 0.20	6.51 ± 0.04	23.45 ± 0.07	n.d.	17.90 ± 0.62	7.92 ± 0.02	22.18 ± 0.09	34.38 ± 0.29
W9	37.17 ± 0.56	53.21 ± 1.01	4.42 ± 0.30	12.65 ± 0.06	6.18 ± 0.56	71.18 ± 0.44	25.80 ± 2.06	10.24 ± 0.61	181.55 ± 1.16	48.28 ± 1.08	98.85 ± 1.26	25.92 ± 0.38	6.99 ± 0.00	13.44 ± 0.26	<LOD	17.51 ± 0.43	6.44 ± 0.03	21.50 ± 0.00	37.38 ± 0.47

and 10 mg/l in Switzerland [53]) and tyramine, considered toxic to human health (25–40 mg/l) [54].

The results obtained for the honey and wine samples are summarized in Table 4, where only amino acid concentrations are shown as biogenic amines were found only in vestigial quantities, usually below the LOD. The main amino acids found in honey samples were phenylalanine, glutamine and lysine and in wines were arginine, alanine and GABA. Methionine showed very low level in wines and was not detected in honeys.

The total amount of the primary amino acids found is described in Fig. 2. The values range from 76.89 mg/l (W7) to

802.40 mg/l (W8). From the analyzed honeys, Madeira multifloral honey (H2) presents the highest amount of amino acid found, 286.00 mg/l. Analysing Madeira wines from similar grape varieties, it was observed that fermented ones show lower values (about 172.94 mg/l) than the wines submitted to partial fermentation, like sweet wines (684.73 mg/l in average for W1 and W3), as expected since amino acids are catabolised in several reactions during this step. Comparing the results obtained for the same variety submitted to a different fermentation process (W3 and W4), the content of the following acids decrease at least 50% during fermentation: arginine, alanine, γ -aminobutyric acid and

Table 5
Relative abundance (%) of each amino acid in honeys and wines under study

Amino acids (%)	Madeira island honeys					Portugal mainland honeys			Canary island honeys			
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Asp	9	9	8	14	8	9	13	54	22	39	100	16
Glu	10	12	11	26	14	6	14	86	10	20	100	16
Asn	16	16	17	18	31	28	21	85	15	100	25	43
Ser	–	34	44	61	35	46	43	100	41	50	95	49
Gln	26	36	21	22	21	13	17	100	20	37	59	40
His	33	49	28	70	53	49	51	42	64	60	32	100
Gly	–	–	–	–	–	–	–	49	–	–	100	–
Thr	–	–	–	98	–	–	–	100	–	–	–	80
Arg	34	30	36	–	37	43	40	100	34	46	–	–
Ala	18	39	62	32	22	19	40	57	18	26	100	26
GABA	–	–	–	–	–	–	–	100	–	–	–	–
Tyr	12	21	13	51	16	83	22	22	100	15	6	48
Met	–	–	–	–	–	–	–	–	–	–	–	–
Trp	49	82	30	50	36	24	35	100	30	51	77	53
Val	–	–	–	100	–	–	–	–	–	–	–	–
Phe	48	92	13	61	13	69	30	91	100	7	10	47
Ile	55	100	–	58	–	40	–	91	–	45	–	51
Leu	47	100	14	8	–	48	10	13	17	–	17	–
Lys	60	59	46	45	60	41	45	40	74	42	–	100
Total compounds	13	14	13	16	12	14	13	17	13	13	12	13

Amino Acids (%)	Madeira island wines				Table			Canary island wines	
	Fortified				Table			Table	
	W1	W2	W3	W4	W5	W6	W7	W8	W9
Asp	39	56	81	79	28	28	4	80	100
Glu	23	12	21	16	49	55	18	86	100
Asn	21	27	44	35	100	49	13	29	42
Ser	100	33	79	57	23	20	17	57	60
Glu	2	4	8	1	7	100	75	5	20
His	5	4	6	4	6	8	2	100	85
Gly	56	42	47	75	22	33	22	100	97
Thr	88	31	100	41	16	11	4	53	45
Arg	68	3	100	10	4	5	1	61	39
Ala	98	19	100	33	20	17	10	76	54
GABA	94	9	33	14	5	31	6	100	96
Tyr	19	21	61	39	16	29	7	100	88
Met	9	20	–	22	29	22	2	93	100
Trp	45	28	49	45	18	16	6	100	58
Val	–	–	–	–	–	–	–	–	100
Phe	48	55	63	86	37	40	11	100	96
Ile	44	61	85	85	40	30	8	100	81
Leu	38	61	54	93	50	41	9	100	97
Lys	10	42	40	90	54	54	7	93	100
Total compounds	18	18	17	18	18	18	18	18	19

threonine, showing the importance of these amino acids in the formation of typical aromas present in Madeira wines (together with cysteine, not determined with this method, due to low sensitivity). The total amino acid content in Madeira fortified dry wines is similar to Madeira table wines, since the fermentation is almost complete. Canarian table wines present high levels of these compounds (741.64 mg/l in average) close to Madeira submitted to partial fermentation, explained by the fact that those wines were produced from over-matured Malvasia grapes.

The percentage of the amino acid relative abundance is exposed in Table 5. The samples with the highest relative abundance of amino acids were H8 (honey) due to serine, glutamine, threonine, arginine, GABA and tryptophan and W8 (wine) due to histidine, glycine, GABA, tyrosine, tryptophan, phenylalanine, isoleucine and leucine. Wines besides having higher amounts of amino acids also possess the richest amino acids profile.

The applicability of the reported procedure for simultaneously analysis of amino acids and biogenic amines has been demonstrated for the analysis of honey and wine samples. The reported method is routinely used in our laboratory.

4. Conclusions

A simple RP-HPLC analytical method for the simultaneous analysis of amino acids and biogenic amines in liquid food matrices is proposed based on a pre-column derivatization with OPA, performed in the sample injection loop, and fluorescence detection. The separation and quantification of 19 amino acids and nine amines was carried out in a single run as their OPA/MCE derivatives elute within 80 min, ensuring a reproducible quantification. The practical utility of the proposed chromatographic procedure was shown by the analysis of the amino acid and biogenic amine content in honey and wine samples without any preliminary separation or clean-up steps. The method showed high sensitivity and response to minor compounds with the exception to proline, cysteine and hydroxyproline. Future trends pass through the use of shorter columns, to reduce analysis and the application of this procedure to others food matrices.

Relatively to the analysed samples, the amino acid present at the highest concentration in honeys was phenylalanine and in wines was arginine. The biogenic amines suspected to cause toxicological effects (histamine, tyramine and phenylethylamine) were no cause for concern in the analysed honey and wine samples since they are present in vestigial quantities.

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