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Simultaneous HPLC Analysis of Biogenic Amines, Amino Acids, and Ammonium Ion as Aminoenone Derivatives in Wine and Beer Samples

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A method has been developed for the simultaneous analysis of biogenic amines, amino acids, and the ammonium ion in wine and beer. Aminoenones formed by the reaction of amino acids, biogenic amines, and the ammonium ion with the derivatization reagent diethyl ethoxymethylenemalonate are separated by HPLC. Reaction takes place in methanolic alkaline medium for 30 min in an ultrasonic bath. Further heating at 70 °C for 2 h produces complete degradation of excess derivatization reagent and byproducts. Comparison of the results of ammonium analysis and enzymatic analysis showed a good correlation ($r = 0.953$). The proposed analytical method has the following advantages: easy derivatization of wines and beers; quantification of 24 amino acids, nine biogenic amines, and the ammonium ion in a single injection; use of the photodiode array detector; complete degradation of excess derivatization reagent during sample preparation; and detection limits below 0.40 mg/L for amino acids and below 0.06 mg/L for biogenic amines.

KEYWORDS: Biogenic amines; amino acids; ammonium ion; diethyl ethoxymethylenemalonate; wine; beer; HPLC

INTRODUCTION

Amino acids and the ammonium ion are essential growth factors for proper implementation and growth of yeasts and lactic acid bacteria during the course of alcoholic and malolactic fermentation, respectively (*1*, *2*). Some biogenic amines form in variable amounts in wines by decarboxylation of their precursor amino acids due to the action of yeasts during alcoholic fermentation, lactic acid bacteria during malolactic fermentation, or other contaminating microorganisms (*3*). As in wine, biogenic amines are also present in other fermented foods or beverages such as cheese and beer. Some of these amines, such as histamine, tyramine, and phenylethylamine, are harmful to one's health, causing sensitive individuals to suffer nausea, headaches, and respiratory disorders, particularly when accompanied by alcohol and acetaldehyde (*4*). For this reason, despite the fact that no official method has been developed yet for its analysis, some countries have established legal or recommended limits for histamine concentrations in wine.

The final concentration of the previous compounds in wine varies depending on a number of factors, including grape variety and root stock, nitrogenated fertilizer, season, ripeness, and oenological practices. For instance, some authors have used amino acid and biogenic amine composition or the combination of both with the composition of polyphenols, organic acids, or volatile compounds as a method for differentiating the grape varieties used to produce the wine (*5*−*9*), their geographical origin (*7*−*9*), the vintage (*7*−*8*), or the genuineness of some liqueur wines prepared from botrytized grapes (*6*, *10*).

The analysis of these two important families of compounds of the nitrogenated fraction is difficult due to their different structures and the absence of a specific chromophore, and hence, they are normally derivatized using different chemical compounds to improve detection limits and to avoid matrix interferences. It is usual to analyze biogenic amines and amino acids separately by HPLC after pre- or post-column derivatization: as fluorescent derivatives with *o*-phthaldialdehyde (OPA), although the main amino acid in wine, proline, does not react in this case (*6*); or with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (*11*); or by forming strong UV−vis absorbing derivatives with ninhydrin (*8*) or dansyl chloride (*12*).

Some recent methods have been published that allow the joint analysis of amino acids and biogenic amines in wine. However, despite their expected advantages over separate analysis of these two families of compounds, they still have some limitations. For instance, Lozanov et al. (*13*) developed an HPLC method able to separate and quantify 20 amino acids and four

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polyamines after derivatization with *N*-(9-fluorenylmethoxycarbonyloxy)-succinimide (FMOC-Su); however, the excess reagent and its hydrolysis product (FMOC-OH) are still present as majority peaks in the chromatogram, and the method does not determine the aromatic biogenic amines histamine and tyramine, which are the most important given their effects for consumer health, or γ-aminobutyric acid (GABA), a characteristic amino acid in grapes. Another simultaneous analysis based on OPA derivatization quantifies 20 amino acids and 17 amines, but the reagent does not serve to determine proline, quantitatively the most important amino acid in grape and wine (*14*). A third simultaneous method requires dual OPA/FMOC derivatization and also uses a fluorescence detector (*15*).

The present paper reports the development of a new method for simultaneous analysis of biogenic amines, amino acids, and ammonium ion in wines and beers. It is based on a method that was initially developed for analysis of protein hydrolysates (*16*, *17*) and other biological samples (*18*) and subsequently used to analyze free amino acids in foods (*19*, *20*) but had never hitherto been used to analyze biogenic amines. It consists of reversed-phase separation by HPLC and UV−vis detection of the aminoenones formed by the reaction of amino acids, biogenic amines, and ammonium ion with the derivatization reagent diethyl ethoxymethylenemalonate (DEEMM).

MATERIALS AND METHODS

Reagents. Super-gradient HPLC grade acetonitrile and methanol were obtained from Labscan (Dublin, Ireland), and ultrapure water generated by the Milli-Q system Millipore (Bedford, MA) was used. L-Cysteine, L-leucine, L-phenylalanine, L-lysine, ammonium chloride, L-histidine, agmatine sulfate, cadaverine, L-arginine, histamine, L-proline, L-α-alanine, spermidine, glycine, β-alanine, L-aspartic acid, L-glutamic acid, L-tyrosine, L-valine, and L-serine were from Fluka Chemie (Buchs, Switzerland); isoamylamine, diethylethoxymethylenemalonate (DEEMM), putrescine, L-glutamine, tyramine, and *trans*-4-hydroxy-L-proline were from Aldrich Chemie (Steinhein, Germany); L-2-aminoadipic acid, L-ornithine monohydrochloride, L-tryptophan, L-asparagine, L-threonine, γ-aminobutyric acid (GABA), L-isoleucine, L-methionine, phenylethylamine, and sodium azide were from Sigma Chemie (Steinhein, Germany). Solutions of amino acids and biogenic amines were prepared with HCl 0.1 N.

Materials. Twenty-eight red wines and 14 white wines from the 2005 harvest were kindly supplied by 10 different wineries from the Spanish region of Castilla-La Mancha. Beer was purchased in a retail store.

Enzymatic Determination of Ammonium Ion. Ammonia was determined with an enzymatic kit for the determination of urea and ammonium ion in foodstuffs from Boehringer Mannhein/R-Biopharm (Darmstadt, Germany). Wine samples were previously treated with polyvinylpolypyrrolidone (PVPP) to avoid the interference of tannins as specified by the manufacturer.

Reaction of Derivatization. Aminoenone derivatives were obtained by reaction of 1.75 mL of borate buffer 1 M (pH = 9), 750 μL of methanol, 1 mL of target sample without any pretreatment, 20 μL of internal standard (L-2-aminoadipic acid, 1 g/L), and 30 μL of DEEMM in a screw-cap test tube over 30 min in an ultrasound bath. The sample was then heated at 70 °C for 2 h to allow complete degradation of excess DEEMM and reagent byproducts.

HPLC Analysis. The analyses were performed on a Varian ProStar HPLC (Varian Inc., Walnut Creek, CA) comprising a ProStar 240 ternary pump, a ProStar 410 autosampler, and a ProStar 330 array photodiode detector.

Chromatographic separation was performed in an ACE HPLC column (5 C18-HL) particle size 5 μm (250 mm × 4.6 mm) thermostatized at 16 °C in an MFE-01 oven (Análisis Vínicos, Tomelloso, Spain) through the binary gradient shown in **Table 1** (phase A, 25 mM acetate buffer pH = 5.8 with 0.02% sodium azide; phase B, 80:20 mixture of acetonitrile and methanol) and a flow rate 0.9 mL/min. For detection, a photodiode array detector monitored at 280, 269, and 300 nm was used. In the proposed conditions, 34 compounds were separated, identified, and quantified in a single injection: 24 amino acids (plus the internal standard), the ammonium ion, and nine biogenic amines.

Table 1. Eluent Gradient for HPLC Determination of Aminoenone Derivatives of Amino Acids, Biogenic Amines, and Ammonium Ion

time (min)	0.0	20.0	30.5	33.5	65.0	73.0	78.0	82.0	85.0
eluent A (%)	90	90	83	83	60	28	18	0	0
eluent B (%)	10	10	17	17	40	72	82	100	100

The target compounds were identified according to the retention times and UV−vis spectral characteristics of the derivatives of the corresponding standards and were quantified using the internal standard method. Detection limits were calculated according to the OIV Oeno 7/2000 (*21*) method as 3 times the baseline noise.

Statistical Analysis. Statistical analysis was performed using SPSS 12 statistical software (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Method Development. On the basis of an original method designed for the analysis of only free amino acids (*19*, *20*), we developed a new method that improved amino acid separation and allowed us to extend the analysis to other nitrogenated compounds. The amino acids asparagine and serine, which coeluted in the original method, were successfully separated, and a new compound, hydroxyproline, was also introduced with the amino acids analyzed. At the same time, further changes were necessary to separate the aminoenone derivatives of nine biogenic amines in the same chromatogram. To do this, it was necessary to extend the chromatographic running time from 40 to 85 min and also to change the composition of the mobile phase B, which acts as an organic modifier, from 100% acetonitrile to a mixture of 80% acetonitrile and 20% methanol.

With the analytical method developed, correct chromatographic separation of 24 amino acids, nine biogenic amines, and ammonium ion was successfully achieved (**Figure 1**). The maximum absorption wavelengths in the UV of the aminoenone derivatives of amino acids, biogenic amines, and ammonium ion were found between 269 nm (aminoenone of the ammonium ion) and 292 nm (aminoenones of proline and hydroxyproline), and the intermediate maximum absorption wavelengths were 279−284 nm (aminoenones of the primary amino acids), 277−278 nm (aminoenones of lysine and ornithine), and 278−280 nm (aminoenones of the polyamines or biogenic amines). On the basis of these data, we selected 280 nm as the wavelength for quantifying all the biogenic amines and most of the amino acids, with the exception of asparagine, serine, and hydroxyproline (peaks 3, 4, and 5); as shown in **Figure 1**, these displayed better separation at 300 nm since hydroxyproline only appeared as a shoulder of serine in the chromatogram recorded at 280 nm. The ammonium ion could also have been quantified at 280 nm, but it was decided to use the response at 269 nm to increase the intensity of its signal.

This analytical procedure has various advantages over the methods reported in the literature for simultaneous analysis of amino acids and biogenic amines (*13*−*15*). An important feature common to all these other methods is the use of fluorescent derivatizing agents, which requires HPLC equipment with this type of detector. However, the aminoenone derivatives formed by reaction with DEEMM can be detected with a photodiode array detector and also with an ultraviolet detector, the two most common detectors in HPLC equipment. Some ultraviolet detectors can be set at several simultaneous detection wave-

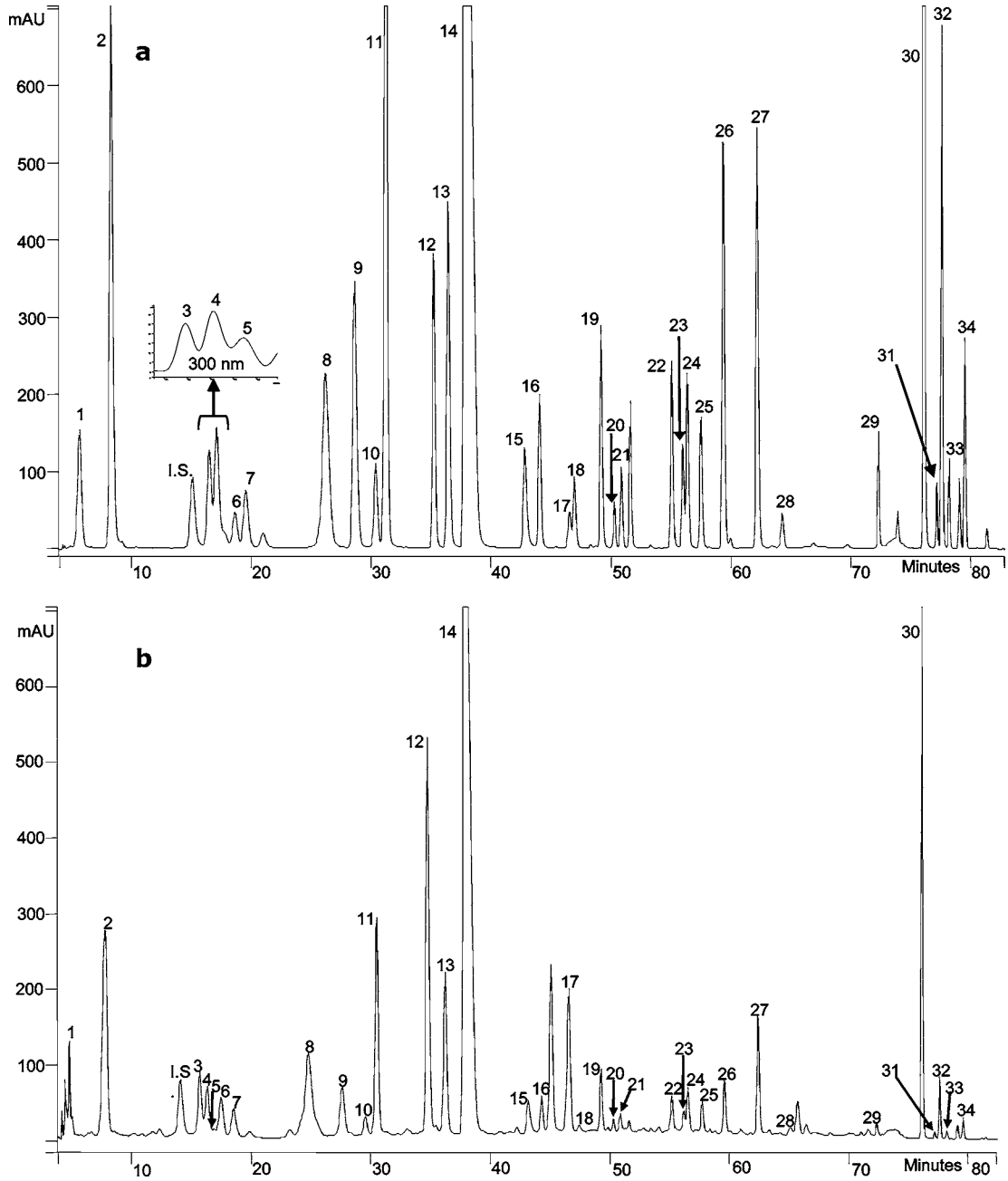

Figure 1. HPLC chromatogram of aminoenone derivatives of amino acids, ammonium ion, and biogenic amines at 280 nm. (**a**) Standard solution and (**b**) red wine. Peak assignments: 1, aspartic acid; 2, glutamic acid; I.S., internal standard (L-2-aminoadipic acid); 3, asparagine; 4, serine; 5, HO-proline; 6, glutamine; 7, histidine; 8, glycine; 9, threonine; 10, β-alanine; 11, arginine; 12, α-alanine; 13, GABA; 14, proline; 15, histamine; 16, tyrosine; 17, ammonium ion; 18, agmatine; 19, valine; 20, methionine; 21, cysteine; 22, isoleucine; 23, tryptophan; 24, leucine; 25, phenylalanine; 26, ornithine; 27, lysine; 28, spermidine; 29, tyramine; 30, putrescine; 31, tryptamine; 32, cadaverine; 33, phenylethylamine; and 34, isoamylamine.

lengths, but, if this is not possible, the quantification can be performed at 280 nm as well, although in that case, there is a probability of considerable error in the quantification of hydroxyproline. Compared with the analytical procedure developed by Herbert et al. (*15*), our method resolved the same number of compounds in a much shorter analysis time (85 min instead of 135 min) and was also more reproducible and accurate. As regards the method proposed by Kutlán and Molnár-Perl (*14*), our method enabled us to quantify proline, the most abundant free amino acid in grape and wine, and also hydroxyproline, which did not react with the derivatizing agent used by them. When compared with the method developed by Lozanov et al. (*13*), the advantage of our method was that it enabled us to determine histamine and tyramine, the most important aromatic biogenic amines given their effects on consumer health, and γ-aminobutyric acid, an amino acid characteristic of grapes.

Table 2 shows the quantification wavelengths, linear calibration interval, r^2 coefficients, variation coefficients obtained in the consecutive analysis of 10 samples, and the detection limits.

The calibration curves with the commercial standards were plotted by covering the range of concentrations normally present in oenological products. These yielded regression coefficients (r^2) above 0.995 in almost all cases, with the exception of hydroxyproline, proline, methionine, and cadaverine, indicating excellent linearity of the response of the derivatives.

The test of repeatability of the method consists of consecutive analysis of 10 replicates of a standard mixture and 10 replicates

Table 2. Measured Wavelengths, Calibration Parameters, Repeatability, and Detection Limit for Aminoenone Derivatives of Amino Acids, Biogenic Amines, and Ammonium Ion

		calibration		repeatability (CV %)		DL
compound	λ (nm)	range (mg/L)	r^2	standard	wine	(mg/L)
aspartic acid	280	2.28–91.00	0.9996	3.53	2.96	0.11
glutamic acid	280	9.98–399.2	0.9991	1.44	1.53	0.05
asparagine	300	2.01–80.20	0.9962	1.64	3.87	0.40
serine	300	2.05–81.80	0.9996	1.04	1.70	0.32
HO-proline	300	0.53–21.00	0.9941	2.94	4.07	0.14
glutamine	280	1.00–39.80	0.9997	3.82	3.96	0.10
histidine	280	1.73–69.20	0.9997	2.04	4.62	0.13
glycine	280	2.79–111.4	0.9991	1.83	3.21	0.06
threonine	280	4.87–186.6	0.9995	2.20	3.05	0.05
β-alanine	280	1.00–40.20	0.9998	4.31	3.99	0.03
arginine	280	18.00–360.5	0.9975	1.86	2.90	0.05
α-alanine	280	2.48–99.20	0.9993	1.06	1.91	0.02
GABA	280	4.02–160.6	0.9986	1.02	1.99	0.03
proline	280	124.20–2484	0.9949	2.88	2.85	0.25
histamine	280	1.67–66.80	0.9993	1.28	2.49	0.04
tyrosine	280	2.88–115.0	0.9997	1.71	4.96	0.04
ammonium ion	269	2.30–37.8	0.9996	2.52	3.46	0.01
agmatine	280	1.80–72.00	0.9992	2.24	n.d.[a]	0.06
valine	280	2.20–88.00	0.9995	1.18	2.70	0.01
methionine	280	1.07–42.60	0.9672	4.06	4.96	0.04
cystenine	280	1.70–68.00	0.9997	1.69	4.18	0.03
isoleucine	280	2.02–80.80	0.9994	1.01	4.55	0.01
tryptophan	280	1.91–76.20	0.9991	2.66	3.36	0.02
leucine	280	2.01–80.40	0.9997	1.21	3.14	0.01
phenylalanine	280	2.07–82.80	0.9994	1.09	3.51	0.02
ornithine	280	3.61–144.20	0.9989	1.15	4.28	0.01
lysine	280	3.16–126.40	0.9988	1.09	1.80	0.01
spermidine	280	0.62–24.60	0.9963	4.35	4.06	0.06
tyramine	280	0.99–39.60	0.9992	1.37	2.72	0.02
putrescine	280	4.90–73.4	0.9975	1.07	2.42	0.01
tryptamine	280	0.57–22.8	0.9991	0.83	n.d.	0.04
cadaverine	280	1.96–78.20	0.9870	1.12	3.42	0.01
phenylethylamine	280	0.62–24.8	0.9993	1.07	n.d.	0.02
isoamylamine	280	1.24–49.60	0.9988	1.16	1.65	0.01

[a] n.d.: nondetectable.

Table 3. HPLC Recovery for Determination of Aminoenone Derivatives of Amino Acids and Biogenic Amines in Wine and Beer

	initial concentration (mg/L)		added concentration (mg/L)		recovery wine (%)		recovery beer (%)	
compound	wine	beer	min	max	mean	SD	mean	SD
aspartic acid	7.43	5.56	3.19	13.18	97.2	4.7	96.4	1.7
glutamic acid	51.99	6.29	12.64	57.10	96.1	2.7	101.7	4.6
asparagine	11.96	1.97	2.71	11.97	98.1	2.6	103.5	1.1
serine	6.02	1.22	2.33	11.06	102.9	4.8	103.9	3.6
HO-proline	1.86	0.41	0.72	2.96	102.3	2.6	101.5	3.1
glutamine	11.84	3.13	0.37	1.55	100.2	3.4	100.3	3.9
histidine	6.90	8.57	1.87	8.69	97.7	1.1	96.5	1.6
glycine	16.42	11.81	3.37	15.61	95.7	2.1	102.1	3.5
threonine	7.72	1.53	5.51	25.96	96.4	1.1	102.1	4.4
β-alanine	1.58	0.71	0.98	4.63	99.7	2.2	100.8	0.9
arginine	34.45	6.43	20.49	97.00	93.5	1.1	104.1	2.2
α-alanine	47.09	25.33	2.96	14.13	95.7	3.3	95.6	1.6
GABA	21.30	32.42	4.96	22.83	99.0	3.6	97.9	3.0
proline	853.56	219.27	155.89	726.66	100.6	3.0	101.5	5.0
histamine	5.79	0.34	2.09	9.50	101.1	1.6	95.5	3.1
tyrosine	4.44	1.19	3.20	15.28	95.5	1.6	104.7	0.3
agmatine	0.00	6.96	1.95	9.17	95.7	4.0	98.8	2.3
valine	5.06	11.45	2.31	12.63	99.0	2.0	97.9	1.2
methionine	2.81	0.61	1.87	7.59	97.7	1.5	95.3	3.4
cystenine	2.60	0.63	1.82	8.01	96.4	2.6	100.5	5.1
isoleucine	2.95	2.45	2.40	11.27	95.3	1.8	99.1	4.9
tryptophan	3.81	12.53	2.78	11.33	100.8	2.7	96.6	2.5
leucine	3.85	4.13	2.25	10.81	95.6	2.2	101.9	5.7
phenylalanine	3.46	11.30	2.26	10.80	98.5	2.7	100.3	3.6
ornithine	2.24	2.33	4.43	20.97	97.0	2.5	102.6	2.2
lysine	7.58	1.78	3.90	18.38	95.4	1.6	104.9	4.8
spermidine	0.64	0.14	0.52	2.62	100.4	4.2	95.5	0.9
tyramine	0.51	18.51	1.08	5.36	101.5	3.4	100.5	2.2
putrescine	14.30	1.68	5.98	26.98	90.4	1.7	104.3	3.1
tryptamine	n.d.[a]	0.10	0.67	3.10	99.1	1.7	99.3	0.3
cadaverine	0.37	0.20	2.68	12.61	98.2	1.6	102.8	2.8
phenylethylamine	n.d.	0.10	0.73	3.43	96.6	1.8	102.2	4.5
isoamylamine	n.d.	n.d.	1.53	7.17	100.0	1.2	104.2	0.9

[a] n.d.: nondetectable.

of a red wine produced with Cencibel grapes. All the resulting variation coefficients were below 5% (i.e. within normally acceptable limits).

The detection limits for the amino acids were below 0.1 mg/L, with the exception of aspartic acid, asparagine, serine, hydroxyproline, histidine, and proline, for which the limits were below 0.4 mg/L. In the case of the biogenic amines analyzed, the detection limits were below 0.06 mg/L in all cases. These limits were lower than required for the proposed applications and at the same time similar to, or below, those reported in the literature (*13*−*15*).

Recovery in the analytical method was also studied by adding three increasing amounts of each target compound to a red wine to cover the expected range of concentrations and then analyzing each one in triplicate. The results are shown in **Table 3**; the mean recoveries were between 95 and 103%, except in the cases of arginine and putrescine, which were 94 and 90%, respectively.

To determine whether the method developed in this study could be applied to other fermented foods, we decided to perform the recovery study on beer. The data in **Table 3** show that the analytical procedure can be applied to beer since the results were as good as or better than those achieved in wine. The recoveries in the case of beer ranged from 95% of methionine to 105% of lysine.

In the case of the ammonium ion, results of the HPLC analysis of its aminoenone derivatives were also compared with those achieved with an enzymatic kit. For this purpose, ammonium content in 28 red wines produced with Cencibel grapes was analyzed using both methods. **Figure 4** shows the high correlation ($r = 0.9529$; $p < 0.0001$) between the results achieved with both methods.

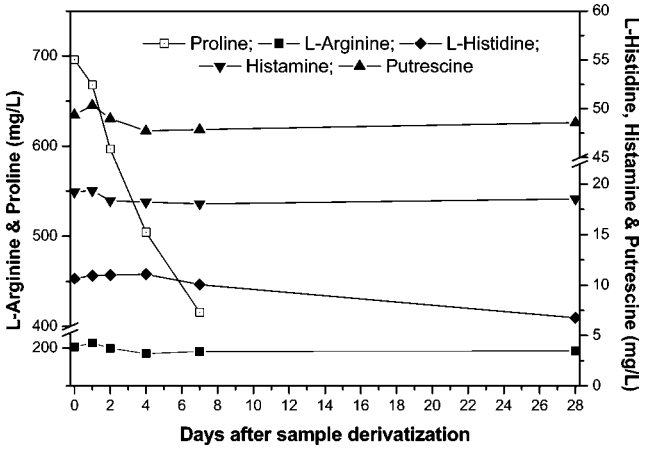

Figure 2. Evolution of the aminoenone derivatives of amino acids and biogenic amines after the derivatization reaction.

Study of Stability of Aminoenone Derivatives. To determine the stability of the derivatives, a same sample was analyzed in triplicate at the time of preparation, after 1, 2, and 4 days and after 1 and 4 weeks. **Figure 2** shows the variations in the concentrations of three amino acids and two amines over time, by way of example. As can be seen, in most cases, the

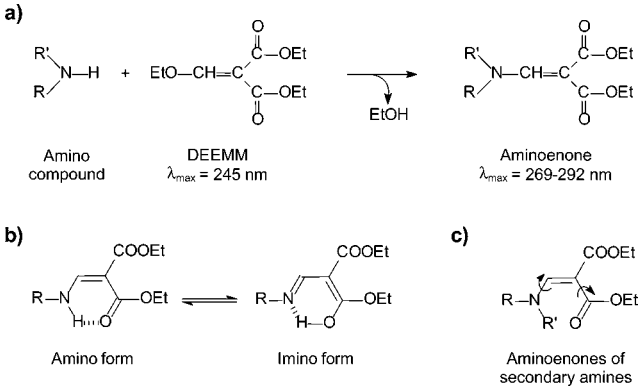

Figure 3. Structure of aminoenone derivatives: (**a**) derivatization reaction; (**b**) primary amino acid derivative; and (**c**) secondary amino acid derivative.

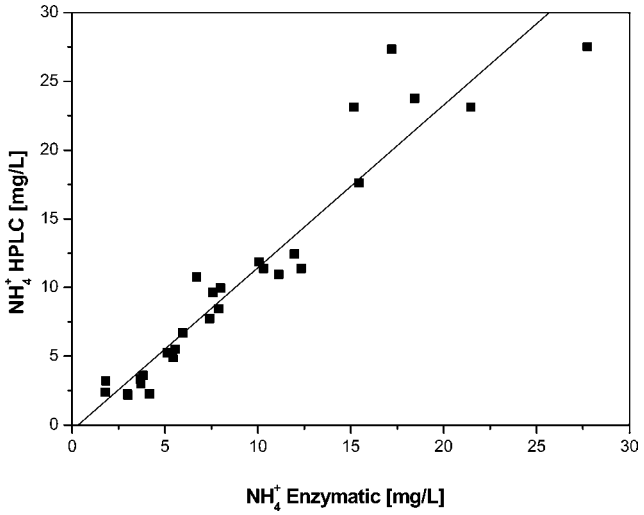

Figure 4. Ammonium content determined by HPLC vs ammonium content as determined using an enzymatic kit, in Cencibel red wines ($n = 28$).

compounds produced by the derivatization reaction were perfectly stable, at least over the first week. The observed differences between the first and the last analyses did not normally exceed 0.1 mg/L and were below 0.2 mg/L in the case of histidine, glycine, arginine, and putrescine. The only compounds that were not stable over time in the reaction conditions were proline and hydroxyproline; hence, if these are to be quantified, the analysis would have to be performed within 24 h of the derivatization reaction.

The aminoenone derivatives of proline and hydroxyproline were less stable due to the nature of the secondary amino group of these two compounds. Formation of the aminoenone derivative entails substitution of the ethoxy group in DEEMM by the amino group (**Figure 3a**). If the amino group involved in the reaction is primary (R-NH_2), as occurs in most amino acids and biogenic amines, the resulting aminoenone is stabilized particularly by the formation of a hydrogen bridge between the free amino hydrogen and the oxygen of one of the carbonyl groups of the derivatizing reagent (**Figure 3b**). This hydrogen bridge enables the aminoenone group to adopt a planar arrangement that facilitates the balance of amino-imino tautomerism, resulting in a stronger N−C bond in the aminoenone since there is a certain degree of double bonding. In the case of proline and hydroxyproline, the hydrogen bridge cannot be formed in the resulting aminoenone because there is no free hydrogen bonded to the nitrogen. Consequently, the single bonds (N−C and C−C) of the aminoenone system, lacking the characteristics of double bonds, can rotate freely (**Figure 3c**).

Table 4. Amino Acid, Biogenic Amine, and Ammonium Ion Content of Cencibel Red Wines and Airén White Wines from Castilla-La Mancha

	red wine[a] ($n = 18$)				white wine[a] ($n = 14$)			
compound	mean	SD	min	max	mean	SD	min	max
aspartic acid	12.45	5.03	6.09	19.94	14.48	6.43	2.95	24.58
glutamic acid	51.16	25.52	23.51	79.94	27.22	10.92	9.79	49.96
asparagine	19.76	8.21	10.59	32.65	15.13	7.25	3.40	28.49
serine	9.65	4.95	3.25	16.19	10.31	7.39	3.46	33.41
HO-proline	4.33	0.56	3.60	5.41	12.56	1.66	8.96	15.31
glutamine	16.96	9.29	4.84	32.68	9.63	7.95	3.16	31.05
histidine	17.69	17.78	2.85	61.12	9.12	2.90	5.57	14.79
glycine	22.09	9.08	11.94	36.31	7.89	3.08	4.64	15.46
threonine	12.80	8.14	4.54	30.35	8.46	3.81	2.85	16.61
β-alanine	2.92	0.98	1.28	4.11	2.37	0.61	1.57	3.54
arginine	48.22	33.22	13.21	105.80	25.95	5.51	17.89	36.75
α-alanine	53.65	27.30	23.91	95.68	26.95	13.16	11.84	53.60
GABA	43.77	34.30	10.82	115.80	22.33	19.05	7.55	67.42
proline	1511	217	1131	1798	904	105	592	1013
histamine	2.74	3.61	0.55	11.95	0.30	0.20	n.d.[a]	0.67
tyrosine	7.67	3.05	4.58	11.55	8.89	3.44	3.52	16.21
ammonium ion	10.29	3.89	6.66	16.95	4.36	2.79	1.29	12.81
agmatine	0.50	0.37	n.d.	1.07	0.70	0.20	0.44	1.07
valine	7.93	4.51	2.33	15.24	8.53	3.12	2.68	13.12
methionine	4.86	2.80	2.07	9.57	3.65	1.18	2.24	6.60
cystenine	5.48	2.41	1.95	9.16	7.94	4.26	2.33	15.89
isoleucine	6.12	2.64	3.21	10.74	5.84	2.45	1.81	9.64
tryptophan	7.22	2.63	3.47	11.12	2.79	2.24	0.37	7.60
leucine	9.62	5.75	4.16	21.53	15.27	7.66	4.06	29.42
phenylalanine	6.07	3.31	2.85	13.13	11.04	4.89	3.87	20.59
ornithine	24.20	33.54	1.04	109.14	4.60	2.96	1.18	11.09
lysine	11.27	5.07	4.39	19.77	22.96	11.74	8.99	47.94
spermidine	2.67	0.67	1.91	3.84	0.93	0.58	0.27	2.35
tyramine	1.09	0.68	0.47	2.63	0.91	1.38	n.d.	5.48
putrescine	8.16	4.37	3.92	15.35	6.16	3.68	3.88	18.43
tryptamine	0.06	0.02	0.03	0.07	0.10	0.10	n.d.	0.27
cadaverine	0.65	0.14	0.35	0.80	0.23	0.05	0.15	0.33
phenylethylamine	0.04	0.01	n.d.	0.06	0.04	0.02	n.d.	0.09
isoamylamine	0.01	0.00	n.d.	0.02	0.07	0.05	n.d.	0.15
total amino acids	1917	413	1301	2431	1187	238	707	1583
total amines	15.92	7.95	10.39	33.50	9.97	5.03	5.41	26.71

[a] Concentration expressed as mg/L. [b] n.d.: nondetectable.

Since the aminoenone system in the cases of proline and hydroxyproline was unable to adopt a preferential planar arrangement, the N−C bond was weaker. The lack of planarity also meant that the molar absorption coefficients of the proline and hydroxyproline aminoenones were substantially lower than those corresponding to aminoenones derived from primary amines; it also accounts for the fact that their chromatographic peaks were proportionally less intense than expected from their concentration.

Analysis of Wine Samples. As a specific application of the proposed method, 18 red wines produced with Cencibel grapes were analyzed upon conclusion of malolactic fermentation, and 14 white wines produced with Airén grapes were analyzed as well. All the wines were of the 2005 vintage and were obtained from wineries in the region of Castilla-La Mancha (Spain). **Table 4** shows the mean results for red and white wines.

The first noteworthy aspect was the enormous variation among the different red and white wines, as revealed by the standard deviation values of amino acids and biogenic amines. The most abundant amino acid in both types of wine was proline, with concentrations ranging from 1100 to 1800 mg/L and from 590 to 1010 mg/L in red and white wines, respectively. The next amino acids in order of abundance were α-alanine, arginine, GABA, and glutamic acid, together with ornithine in red wines and lysine in white wines. All other amino acids were present in mean amounts that did not exceed 20 mg/L in the case of the red wines and 15 mg/L in the white wines. It is also interesting to note that the red wines displayed higher mean amino acid contents. This difference was probably due to

maceration with grape skins during alcoholic fermentation in the case of red wines to extract their characteristic color (*22*).

Also, concentrations of biogenic amines were higher in red wines; this difference was probably due to the malolactic fermentation of red wines following alcoholic fermentation (*3*). As reported in the literature (*9*, *23*, *24*), the main biogenic amine in both types of wine was putrescine, followed in terms of abundance by histamine, spermidine, cadaverine, tyramine, and agmatine. The minority biogenic amines, present in concentrations close to their detection limits, were tryptamine, phenylethylamine, and isoamylamine. In the case of histamine, the principal biogenic amine from the standpoint of effects on human health, the mean values in the red wines were below the threshold considered to produce adverse effects (*4*) and only exceeded 10 mg/L in one of the wines. White wines do not undergo malolactic fermentation, and therefore, the concentrations of this biogenic amine were much lower, the maximum value being 0.67 mg/L in the white wines studied here.

LITERATURE CITED

(1) Feuillat, M.; Charpentier, C.; Mauhean, A. Les composés azotés. In *Oenologie: Fondements scientifiques et technologiques*, 1st ed.; Flanzy, C., Ed.; Technique et Documentation: Paris, France, 2001; pp 94−116.

(2) Yatin, M. Polyamines in living organisms. *J. Cell Mol. Biol.* **2002**, *1*, 57−67.

(3) Lonvaud-Funel, A. Biogenic amines in wine: Role of lactic acid bacteria. *FEMS Microbiol. Lett.* **2001**, *199*, 9−13.

(4) Silla Santos, M. H. Biogenic amines: their importance in food. *Int. J. Food Microbiol.* **1996**, *29*, 213−231.

(5) Vasconcelos, A. M. P.; Neves, H. J. C. Characterization of elementary wines of *Vitis vinifera* varieties by pattern recognition of free amino acid profiles. *J. Agric. Food Chem.* **1989**, *37*, 931−937.

(6) Soufleros, E.; Barrios, M. L.; Bertrand, A. Correlation of biogenic amines and other wine compounds. *Am. J. Enol. Vitic.* **1998**, *49*, 266−278.

(7) Soufleros, E. H.; Bouloumpasi, E.; Tsarchopoulos, C.; Biliaderis, C. G. Primary amino acid profiles of Greek white wines and their use in classification according to variety, origin, and vintage. *Food Chem.* **2003**, *80*, 261−273.

(8) Hérberger, K.; Cosmos, E.; Simon-Sarkadi, L. Principal component and linear discriminant analyses of free amino acids and biogenic amines in Hungarian wines. *J. Agric. Food Chem.* **2003**, *51*, 8055−8060.

(9) Landete, J. M.; Ferrer, S.; Polo, L.; Pardo, I. Biogenic amines in wines from three Spanish regions. *J. Agric. Food Chem.* **2005**, *53*, 1119−1124.

(10) Kiss, J.; Sass-Kiss, A. Protection of originality of Tokaji Aszú: Amines and organic acids in botrytized wines by high-performance liquid chromatography. *J. Agric. Food Chem.* **2005**, *53*, 10042−10050.

(11) Torrea, D.; Ancín, C. Influence of yeast strain on biogenic amines content in wines: relationship with the utilization of amino acids during fermentation. *Am. J. Enol. Vitic.* **2001**, *52*, 185−190.

(12) Galgano, F.; Caruso, M.; Favati, F.; Romano, P.; Caruso, M.; HPLC determination of agmatine and other amines in wine. *J. Int. Sci. Vigne Vin* **2003**, *37*, 237−242.

(13) Lozanov, V.; Petrov, S.; Mitev, V. Simultaneous analysis of amino acid and biogenic polyamines by HPLC after pre-column derivatization with *N*-(9-fluorenylmethoxy-carbonyloxy)succinimide. *J. Chromatogr., A* **2004**, *1025*, 201−208.

(14) Kutlán, D.; Molnár-Perl, I. New aspects of the simultaneous analysis of amino acids and amines as their *o*-phthaldialdehyde derivatives by high-performance liquid chromatography. *J. Chromatogr., A* **2003**, *987*, 311−322.

(15) Herbert, P.; Santos, L.; Alves, A. Simultaneous quantification of primary and secondary amino acids and biogenic amines in musts and wines using OPA/3-MPA/FMOC-Cl fluorescent derivatives. *J. Food Sci.* **2001**, *66*, 1319−1325.

(16) Alaiz, M.; Girón, J.; Hidalgo, F. J.; de la Maza, M. P.; Zamora, R.; Vioque, E. Esterification of amino acids as their 2,2-bis-(ethoxycarbonyl)vinyl derivatives. *Synthesis* **1989**, *7*, 544−547.

(17) Alaiz, M.; Navarro, J. L.; Girón, J.; Vioque, E. Amino acid analysis by HPLC after derivatization with diethyl ethoxymethylenemalonate. *J. Chromatogr.* **1992**, *591*, 181−186.

(18) Girón, J.; Alaiz, M.; Vioque, E. HPLC determination of *N*-ϵ-(2-propenal)-lysine in biological samples after derivatization with diethyl ethoxymethylenemalonate. *Anal. Biochem.* **1992**, *206*, 155−160.

(19) Chicón, R.; Hermosín, I.; Cabezudo, M. D. Método de análisis de los aminoácidos libres y del ión amonio en vinos y mostos, por HPLC tras derivatización con etoximetilénmalonato de dietilo (EMMDE). *Tecnol. Vin.* **2001**, *1*, 95−100.

(20) Hermosín, I.; Chicón, R. M.; Cabezudo, M. D. Free amino acid composition and botanical origin of honey. *Food Chem.* **2003**, *83*, 263−268.

(21) O.I.V. 2000. *Résolution Oeno 7/2000*; 80 Assemblée Générale: Paris, June, 23 2000.

(22) Dubourdieu, D.; Ollivier, C.; Boidron, J. N. Incidence des opérations préfermentaires sur la composition chimique et les qualités organoleptiques des vins blancs secs. *Conn. Vigne Vin.* **1986**, *20*, 53−76.

(23) Jimenez, M. N.; Torrea Goñi, D.; Ancín Azpilicueta, C. Changes in amine concentrations during aging of red wine in oak barrels. *J. Agric. Food Chem.* **2003**, *51*, 5732−5737.

(24) Marcobal, A.; Polo, M. C.; Martín-Álvarez, P. J.; Moreno-Arribas, M. V. Biogenic amine content of red Spanish wines: Comparison of a direct ELISA and an HPLC method for the determination of histamine in wine. *Food Res. Int.* **2005**, *38*, 387−394.

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