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High-Performance Liquid Chromatography−Ultraviolet Detection Method for the Simultaneous Determination of Typical Biogenic Amines and Precursor Amino Acids. Applications in Food Chemistry

Eleonora Mazzucco, Fabio Gosetti,* Marco Bobba, Emilio Marengo, Elisa Robotti, and Maria Carla Gennaro

DISAV Dipartimento di Scienze dell'Ambiente e della Vita, University of Piemonte Orientale "Amedeo Avogadro", Viale Teresa Michel 11, 15121 Alessandria, Italy

A reversed-phase high-performance liquid chromatography (HPLC) method was developed for the simultaneous determination in food of biogenic amines and their precursor amino acids after a precolumn derivatization with dansyl chloride. The chromatographic conditions, selected to be suitable for mass spectrometry detection, were optimized through experimental design and artificial neural networks. The HPLC-UV method was validated by comparing the separation results with those obtained through a HPLC method, working under the same chromatographic conditions but employing mass spectrometry detection. The HPLC-UV method was then applied to the analysis of different food samples, namely, cheese, clams, salami, and beer. For all of the matrices, recoveries (relative standard deviation always <5%) always >92% were obtained. The results are discussed as a function of the total biogenic amine content and of the concentration ratio between amines and precursor amino acids.

KEYWORDS: Biogenic amines; amino acids; HPLC-UV; experimental design; artificial neural networks; HPLC-MS

INTRODUCTION

Biogenic amines (BA) can naturally form in food from decarboxylation of amino acids. They are induced by vasoactive and psychoactive effects, depending on the toxicity level of individual metabolism. Legal threshold concentrations allowed in food are available only for histamine and tyramine. The maximum concentration admitted for histamine is 2.0 mg L^{-1} in alcoholic beverages and 100−200 mg kg^{-1} in fish (fish subjected to enzyme ripening treatment in brine may contain up to double amounts), whereas the potentially toxic concentration of tyramine ranges between 100 and 800 mg kg^{-1} (*1*, *2*).

Because the content of BA is influenced by ripening and quality of storage conditions (*3*−*7*), interest in the presence and amount of BA in food is always increasing, not only because of the potential toxicity but also because the relative content of BA and of their precursor amino acids is a useful parameter to evaluate the extent of ripening and the quality of storage.

Liquid chromatographic methods with fluorescence or UV detection are reported in the literature for the determination of biogenic amines, after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (*8*), benzoyl chloride (*9*), dabsyl chloride (*10*, *11*), dansyl chloride (*6*, *12*, *13*), and *o*-phthaldialdehyde (*12*, *14*). Also, amino acids were determined by liquid chromatography methods, after derivatization with dansyl chloride (*15*) and *o*-phthalaldehyde (*13*).

Few studies deal with the simultaneous chromatographic separation of amines and amino acids and are based on precolumn derivatization with dabsyl chloride (*16*), fluorenylmethylchloroformate (*17*), *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (*18*), and *o*-phthalaldehyde (*19*, *20*) with fluorometric or UV detection.

In our laboratory, two high-performance liquid chromatography methods, hyphenated with mass spectrometry detection (HPLC-MS/MS), have been previously developed to quantify BA in cheese (*21*, *22*). When the reversed-phase HPLC-MS/MS method with electrospray ionization (ESI) was used, the matrix effect led to a signal suppression, which was overcome by applying the standard addition method for quantification of each amine in cheese samples (*21*). Also, when in the same analysis the hydrophilic interaction liquid chromatography (HILIC) HPLC-MS/MS method with atmospheric pressure chemical ionization (APCI) was applied, a matrix effect was observed, but it led to a signal enhancement. The standard addition method was again used for suitable quantitation, but it could here be employed for mixtures of more analytes (*22*).

The aim of this work was the development of a HPLC-UV method for the simultaneous separation in food of the most representative BA (cadaverine, histamine, tyramine, and tryptamine) and their respective precursor amino acids (lysine, histidine, tyrosine, and tryptophan). The eight analytes are characterized by different acidic properties, as well as by aromatic

*Corresponding author (telephone +39 0131 360280; fax +39 0131 360365; e-mail fabio.gosetti@mfn.unipmn.it).

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and aliphatic structures that largely affect the choice of both chromatographic and detection conditions. Taking into account the drawback of the relevant matrix effect expected on the basis of our previous results (*21*, *22*), a reversed-phase HPLC method was developed with a precolumn derivatization step with dansyl chloride, selective for the amino groups. The chromatographic conditions, chosen to be suitable for the transfer of the HPLC-UV method to a HPLC-MS method, were optimized by multivariate analysis techniques. The results obtained with the HPLC-MS method permitted validation of the HPLC-UV developed.

MATERIALS AND METHODS

Reagents and Standard Solutions. Methanol (Chromasolv, >99.9%), hydrochloric acid (37%), ammonium formate (99.995%), and acetone (Chromasolv, 99.8%) were acquired from Sigma-Aldrich (Milwaukee, WI). Cadaverine dihydrochloride (>99%), histamine dihydrochloride (>99%), tryptamine hydrochloride (99%), tyramine hydrochloride (99%), L-lysine (98%), L-histidine (≥99.5%), L-tyrosine (≥99.0%), L-tryptophan (≥99.5%), dansyl chloride (≥99.0%), formic acid (98%), and sodium hydrogen carbonate (≥99%) were acquired from Fluka (Buchs, Switzerland). Ammonia (30%) was purchased from Carlo Erba (Milan, Italy). HPLC gradient grade acetonitrile (99.9%) and buffer solutions at pH values of 4.00, 7.00, and 10.00 (at 20 °C) were acquired from Merck (Darmstadt, Germany). Ultrapure water was obtained through a Millipore Milli-Q system (Milford, MA).

The 1000.00 mg L^{-1} standard solutions of BA and amino acids were prepared in 0.10 M HCl and kept in dark plastic bottles at −20 °C. All of the stock solutions were stable for about 3 months, and the solutions at the desired concentrations were prepared every day in 0.10 M HCl.

Instrumentation. The chromatographic analyses were carried out by a Thermo Finnigan (San Jose, CA) HPLC system equipped with a degasser SCM1000, a gradient pump Spectra System P4000, and an autosampler Spectra System AS3000, interfaced by the module SN4000 to a diode array detector Spectra System UV6000LP. HPLC-MS analyses were carried out by another Thermo Finnigan Mat Spectra System with mass spectrometry detection. Mass spectrometry experiments were conducted by means of the Thermoquest LCQ Duo ion trap mass spectrometer from Finnigan (San Jose, CA) equipped with ESI and APCI sources.

A Symphony SB70P pH-meter (VWR, Darmstadt, Germany), equipped with a combined glass Ag/AgCl electrode, was employed for the pH measurements.

A homogenizer Ultra-Turrax T25 (IKA-Werke, Staufen, Germany), a Stomacher Circulator (PBI International, Milan, Italy), an IEC CL31R multispeed centrifuge (Thermo Electron Corp., Milford, MA), and an ultrasonic cleaner (Emmegi, Milan, Italy) were employed in the treatment of the samples. The cleanup procedure was conducted using solid-phase extraction (SPE) C18 cartridges (Phenomenex, Bologna, Italy). The software Statistica 7.1 (StatSoft, Tulsa, OK) was used for ordinary least-squares (OLS) models, whereas the software applications Matlab 7.6 (The MathWorks, Natick, MA) and neural network toolbox 6.0 (The MathWorks) were used for grid search algorithm and for artificial neural network (ANN) models, respectively.

Real Sample Preparation. *Cheeses and Clams*. A sample of 5.0 g of cheese or clams, accurately weighed and cut into small pieces, was homogenized with 25.0 mL of 0.10 M HCl solution at 13500 rpm for 10 min in a 50.0 mL Falcon tube and then centrifuged at 4000 rpm at 4 °C for 20 min. The liquid fraction was collected in a plastic tube and subjected to the dansylation reaction.

Salami. A volume of 100.0 mL of 0.10 M HCl was added to 10.0 g of salami accurately weighed, cut into small pieces, homogenized by the Stomacher at 270 rpm for 5 min, and centrifuged at 8000 rpm for 10 min. The liquid fraction was collected in a plastic tube and subjected to dansylation reaction.

Beer and Clam Broth. A volume of 10.0 mL of beer or clam broth was sonicated for 30 min, double-filtered on a 0.2 μm PTFE membrane (VWR), and then subjected to dansylation reaction.

Derivatization Procedure with Dansyl Chloride. A volume of 1200.0 μL of a mixture of standard amines and amino acids (100.00 mg L^{-1} each) or of the food sample extracts was treated with 1200.0 μL of 0.020 M dansyl chloride in acetone and 1200.0 μL of 0.500 M $NaHCO_3$. The solution was protected from light by aluminum foil and kept at 65 °C for 40 min. The solution was centrifuged at 10000 rpm for 2 min, and the supernatant was submitted to SPE.

SPE Cleanup Procedure. Different eluent compositions, solvents (methanol, acetonitrile, mixture) and sorbents were tried and compared in the extraction process. Due to the complexity of the food samples investigated, the matrix effect was always relevant. Although the extraction process was not efficacious enough, the matrix effect could be greatly reduced by the use of the dansylation reaction, which is selective toward the amino group.

The SPE sorbent was conditioned with 2.0 mL of methanol and 2.0 mL of a 50:50 (v/v) acetone/ultrapure water mixture and then loaded with 2.0 mL of the dansylated standard solution or the dansylated sample extract. After a washing with 3.0 mL of ultrapure water, the sorbent was dried with nitrogen gas for 5 min and the analytes were eluted with 2.0 mL of a acetonitrile/methanol 50:50 (v/v) mixture. The eluate, after proper dilution in the mobile phase, was injected in the HPLC-UV or in the HPLC-MS system.

Chromatographic Conditions. The stationary phase was a LiChrospher C18e (250 mm × 4 mm, 5 μm) column equipped with a C18e precolumn (15 mm × 4 mm, 5 μm) from Merck. The mobile phase was a mixture of acetonitrile (A) and a solution (B) of ammonium formate (9.0 mM, brought to pH 3.40 with concentrated formic acid at 98%), flowing under gradient elution. The gradient program consisted of 0−20 min, 59% A; 20−21 min, 70% A; 21−40 min, 70% A; and 40−50 min, 59% A, to equilibrate the system at the initial mobile phase composition. The flow rate was 1.00 mL min^{-1} and the injection volume 100.0 μL. The UV detector was set at 254 nm.

Mass Spectrometry Conditions. In the ESI source, high-purity nitrogen was employed as the nebulizer (sheath and auxiliary gas pressures set at 40 and 10 of the arbitrary 0−100 scale of the instrument, respectively), and helium (>99.999%) was the quenching agent. The ESI probe tip and capillary potential were set at 5.00 kV and 3.00 V, respectively. The heated capillary was set at 270.00 °C, and the ion optics optimized parameters were as follows: tube lens offset, −17.00 V; first octapole voltage, −3.50 V; interoctapole lens voltage, −16.00 V; second octapole voltage, −5.00 V. Positive ion mode full scan data acquisition was made over the m/z 250−1200 range. The maximum injection time and the number of microscans per second were set at 50 ms and 3, respectively.

In the APCI source, high-purity nitrogen was the nebulizer (sheath and auxiliary gas pressures both set at 30 of the arbitrary 0−100 scale of the instrument), and helium (>99.999%) served as the quenching agent. The APCI spray current and capillary potential were set at 5.00 μA and 3.00 V, respectively. The heated nebulizer and heated capillary were set at 450.00 and 220.00 °C, respectively, and the optimized ion optics parameters were as follows: tube lens offset, −17.00 V; first octapole voltage, −3.50 V; interoctapole lens voltage, −16.00 V; second octapole voltage, −5.00 V. Positive ion mode full scan data acquisition was made over the m/z 250−1200 range. The maximum injection time and the number of microscans per second were set at 50 ms and 3, respectively.

Experimental Design. The behavior of the experimental system was investigated by means of a Hoke design (*23*), namely, a 2^3 full factorial design (FFD) augmented with a star design, with all of the external experiments laying on the surface of the cube defined by the FFD experiments. This experimental design permits the calculation of regression models containing all factor interactions and their square terms. It was used as the basis for calculating either traditional ordinary least square (OLS) models or, when necessary, ANN models.

ANNs. ANNs are mathematical algorithms that simulate the functions of the human brain and are used to solve complex problems. They usually provide better results than OLS, especially when nonlinear relationships are present (*24*, *25*).

ANNs were used to model the analyte retention times instead of OLS, when the latter did not provide satisfactory results. The back-propagation algorithm (BP) was used to optimize the ANNs (*26*). To avoid overfitting, the regularization method (*27*), implemented in Matlab, was used.

Grid Search Optimization. The OLS or BP-ANN models were used to simulate the retention times of the eight analytes. The grid search algorithm employed consists in calculating a simulated chromatogram on

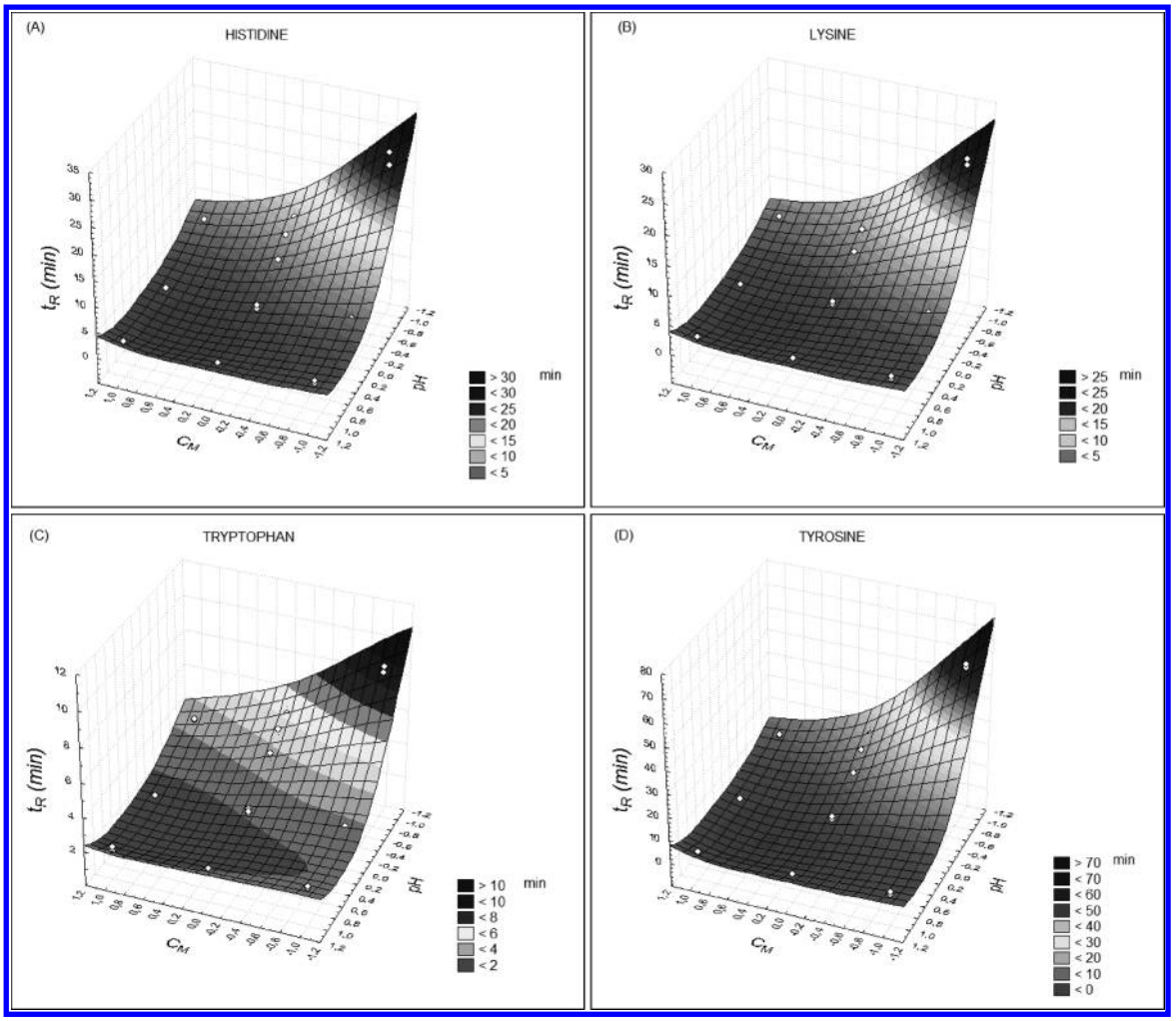

Figure 1. Response surfaces for retention times of histidine, lysine, tryptophan, and tyrosine as a function of the most relevant factors: C_M and pH.

Table 1. Retention Times Obtained by the Grid Search Optimization Performed with Constrained Maximum Analysis Time

	analysis time constraint				
analyte	20 min	30 min	40 min	50 min	60 min
cadaverine	10.75	14.27	16.02	18.00	22.75
histamine	11.64	15.41	17.29	19.34	25.88
histidine	5.69	6.78	7.24	7.34	9.34
lysine	4.63	5.38	5.29	5.94	7.15
tryptamine	6.87	8.37	9.07	9.83	11.62
tryptophan	3.42	3.73	3.69	3.91	4.59
tyramine	18.21	29.64	35.91	42.83	59.58
tyrosine	8.01	10.03	10.45	11.35	14.32

a grid of points that spans the experimental domain with a preselected step. The step can be iteratively reduced as a function of the desired accuracy. The optimal conditions consist in the values of the experimental factors that provide the maximum value of the *target function*, here defined as the difference to the nearest chromatographic peak. This guarantees the maximum resolution. The target function can be modified to constrain the maximum analysis time to be smaller than a desired value. This can be easily obtained by not considering, along the grid search optimization, those conditions that lead to too long total analysis time.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Separation. Taking into account that the analytes under study were characterized by different chemical structures (aliphatic, aromatic, heterocyclic) and by different acidic properties, three variables with potential effect on the chromatographic retention were considered: the concentration of the organic modifier (acetonitrile concentration, C_M), the concentration of the ion pair reagent (ammonium formate concentration, C_{IP}), and the pH value of the ammonium formate aqueous solution.

Preliminary experiments permitted us to evaluate the experimental domain range of the experimental factors as follows: C_M varied between 50.0 and 70.0%, C_{IP} between 5.0 and 10.0 mM, and pH between 3.00 and 7.50.

A two-level FFD was initially used, considering the retention times of the analytes as the experimental responses. The experiment in the central point was repeated three times to estimate the pure experimental error and to check the stability of the chromatographic performance (memory effects).

The possibility of significant quadratic effects was tested by calculation of an F value by the equation (*28*)

$$F_{(1,\nu,\alpha)} = \frac{(\bar{y}_0 - \bar{y}_F)^2}{s_{pe}^2 \times \left(\frac{1}{n_0} + \frac{1}{n_F}\right)}$$

where $\bar{y}_0$ was the average response of the central experiments, $\bar{y}_F$ was the average response from the factorial design experiments, which represents the estimated value in the center; s_{pe}^2 was the pure experimental error (estimated from the repeated experiments), n_0 was the number of the central experiments, and n_F was the number of FFD experiments. Because the results of the F test suggested for all of the analytes the presence of quadratic effects,

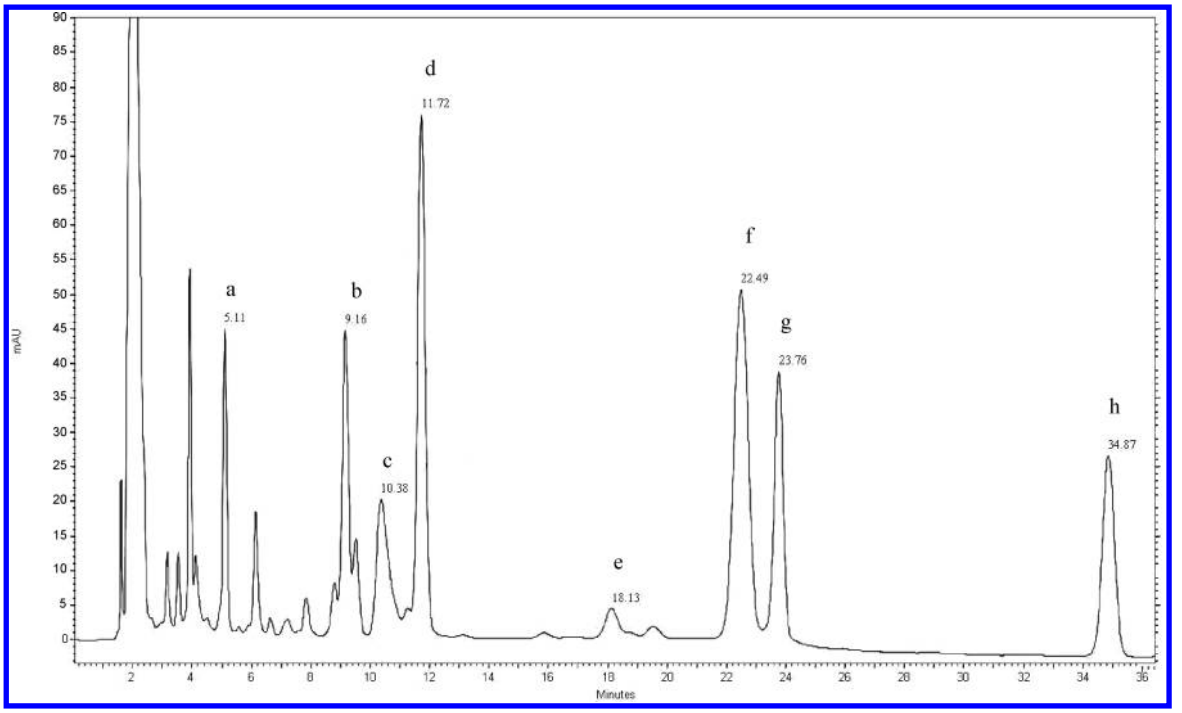

Figure 2. Typical chromatogram of the separation of a standard mixture of tryptophan (a), lysine (b), histidine (c), tryptamine (d), tyrosine (e), cadaverine (f), histamine (g), and tyramine (h) (1.50 mg L^{-1} each). Chromatographic conditions: stationary phase, LiChrospher C18e column (250 mm × 4 mm, 5 μm) equipped with a C18e precolumn (15 mm × 4 mm, 5 μm); mobile phase, mixture of acetonitrile (A) and a solution (B) of ammonium formate (9.0 mM, brought to pH 3.40 with concentrated formic acid), flowing under gradient elution; gradient program, 0−20 min, 59% A, 20−21 min, 70% A, 21−40 min, 70% A, and 40−50 min, 59% A, to equilibrate the system at the initial mobile phase composition; flow rate, 1.00 mL min^{-1}; injection volume, 100.0 μL. The UV detector was set at 254 nm.

six experiments of a star design were added to the FFD experiments, to provide a central composite design (CCD).

The regression models from the experimental data obtained showed that the retention times (the responses) of all analytes were mostly affected by the variables C_M and pH.

For all amines good linear relationships with R^2 always >0.995 were obtained, whereas for the four amino acids the OLS regression models were not so satisfactory. To improve the regression models, BP-ANNs were employed.

BP-ANN Models. The BP-ANNs consist of one input layer with three neurons corresponding to the three variables of the FFD (C_M, C_{IP}, and pH), one hidden layer with a variable number of neurons, and one output layer, with four neurons associated with the responses (the retention times of the four amino acids). Different architectures were tried, changing the number of the neurons in the hidden layer. The best results were obtained by selection of one hidden layer containing one neuron. Two parameters, called the learning rate (set at 0.3) and the momentum (set at 0.3), were used for controlling the size of weight adjustment along the descending direction and for the dampening oscillations of the iterations. The transfer function was a sigmoid.

Figure 1 shows the response surfaces drawn by reporting for the four amino acids the retention times as a function of C_M and pH: a nonlinear behavior was found for all amino acids, in particular in the region that corresponds to the lower values of pH. Additional experiments were therefore performed in the pH range between 5.25 and 3.00.

The statistical features were all satisfactory, with R^2 values always >0.98.

Search for the Optimal Conditions. To optimize the separation of the eight analytes, a grid search algorithm was employed, to search for the set of experimental factors that provided the best resolution either without constraint on the total analysis time or with different constraints (**Table 1**). The best unconstrained chromatographic conditions were found by iterating the optimization procedure with progressively shorter search steps. Because under the best conditions identified ($C_M = 59.0\%$, $C_{IP} = 9.0$ mM, and pH 3.40) the retention time of the last analyte that exited the column (tyramine) was around 57 min, a gradient elution was introduced to reduce the total analysis times. The conditions were therefore changed at time = 20 min, by increasing the acetonitrile percentage from 59 to 70%, whicht led to a total analysis time of 36 min. No further gradient optimization was possible, because increasing percentages of acetonitrile led to coelution of the analytes corresponding to peaks f and g (**Figure 2**) in the chromatogram obtained for the standard mixture of the eight analytes (1.50 mg L^{-1} each).

Table 2. LOD and LOQ Values of the Analytes in HPLC-UV and HPLC-MS Analysis

	HPLC-UV		HPLC-APCI MS		HPLC-ESI MS	
analyte	LOD (μg L^{-1})	LOQ (μg L^{-1})	LOD (μg L^{-1})	LOQ (μg L^{-1})	LOD (μg L^{-1})	LOQ (μg L^{-1})
cadaverine	17	52	8	23	10	29
histamine	34	104	12	47	16	62
histidine	51	159	34	94	28	77
lysine	22	69	18	67	14	52
tryptamine	14	45	7	28	10	39
tryptophan	51	163	38	110	30	87
tyramine	21	62	10	36	12	43
tyrosine	67	229	58	197	54	183

Calibration Curves. A calibration curve for each analyte was constructed by plotting peak area (y) versus concentration (x) for five concentration levels. Three replicates gave an intraday precision always $<5.0\%$ for peak area and $<1.0\%$ for retention time. For each analyte, in the concentration range between the

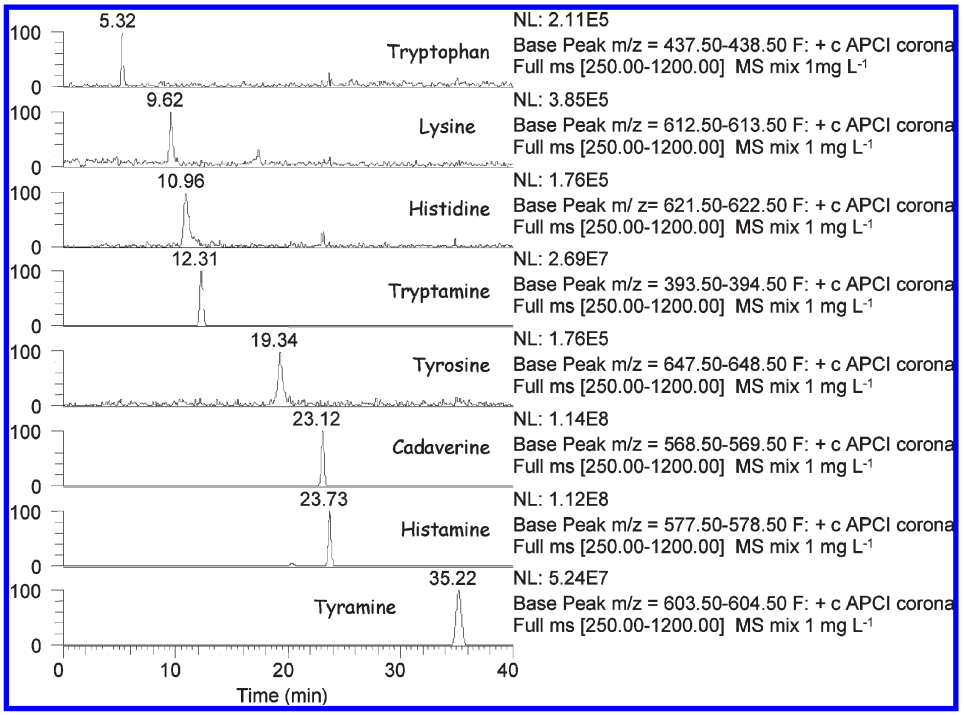

Figure 3. HPLC-APCI MS separation of a standard mixture of tryptophan, lysine, histidine, tryptamine, tyrosine, cadaverine, histamine, and tyramine (1.00 mg L^{-1} each) extracting the characteristic *m*/*z* signal of the dansylated analytes. Chromatographic conditions were as in **Figure 2**.

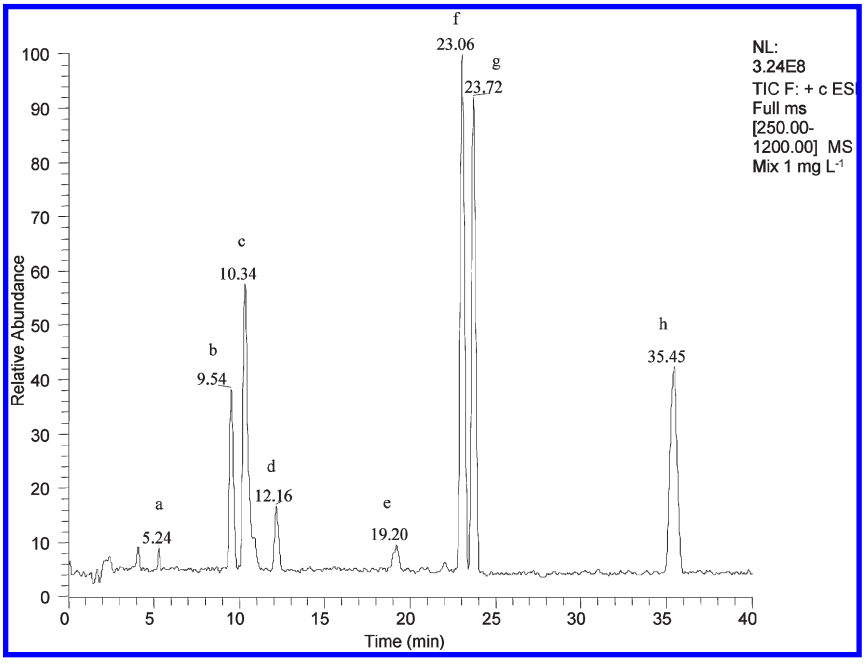

Figure 4. TIC HPLC-ESI MS separation of a standard mixture of tryptophan (a), lysine (b), histidine (c), tryptamine (d), tyrosine (e), cadaverine (f), histamine (g), and tyramine (h) (1.00 mg L^{-1} each). Chromatographic conditions were as in **Figure 2**. Split flow rate ratio is 1:5.

limit of quantitation value and 3500 μg L^{-1}, good linearity was obtained, with R^2 values always >0.99.

The limit of detection (LOD), expressed as the concentration of the analyte that gives a signal equal to the average background (S_{blank}) plus 3 times the standard deviation (s_{blank}) of the blank (LOD = $S_{blank} + 3s_{blank}$) and the limit of quantitation (LOQ), given as LOQ = $S_{blank} + 10s_{blank}$ (*29*), are reported in **Table 2** for all the analytes.

Transfer of the HPLC-UV Method to Mass Spectrometry Detection. Due to the relatively large amount of BA in food, the sensitivity offered by the HPLC-UV method is generally suitable in food analysis. The method can find large application, because UV detection is more often possible in food analysis laboratories than in mass spectrometry detection. Nevertheless, to validate the method developed, to offer wider applications, and for pursuing greater sensitivities, we explored the transfer of the method to a mass spectrometric detection with both APCI and ESI sources.

Figure 3 shows the good results obtained in the analysis of a standard mixture of the eight analytes (1.00 mg L^{-1} each) in HPLC-APCI MS by extracting the characteristic *m*/*z* signal of each dansylated analyte; all of the analytes were well resolved.

Table 3. Analytes Quantified in Food Samples[a]

			Muletta Monferrina salami (mg kg^{-1})					
	Toma Piemontese D.O.P. cheese (mg kg^{-1})	Pecorino Sardo cheese (mg kg^{-1})	t_0	t_1	t_2	clams (mg kg^{-1})	clam broth (mg L^{-1})	beer (mg L^{-1})
cadaverine	19.5 (±0.9)	23 (±1)	40 (±2)	56 (±3)	146 (±7)	5.2 (±0.2)	1.83 (±0.08)	<LOD
histamine	36 (±2)	27 (±2)	160 (±9)	237 (±13)	292 (±16)	34 (±2)	7.2 (±0.4)	<LOD
histidine	304 (±20)	351 (±21)	196 (±12)	124 (±7)	80 (±5)	21 (±1)	11.2 (±0.7)	11.4 (±0.7)
lysine	457 (±24)	<LOD	<LOD	<LOD	<LOD	57 (±3)	<LOD	29 (±2)
tryptamine	8.0 (±0.3)	3.1 (±0.1)	26 (±1)	35 (±1)	49 (±2)	<LOD	<LOD	11.5 (±0.5)
tryptophan	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
tyramine	40 (±2)	15.7 (±0.8)	130 (±7)	104 (±6)	138 (±7)	<LOD	<LOD	2.1 (±0.1)
tyrosine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
total BA	104 (±3)	69 (±2)	356 (±12)	432 (±15)	625 (±19)			
histamine/histidine concentration ratio	0.12 (±0.01)	0.08 (±0.01)	0.82 (±0.08)	1.9 (±0.2)	3.6 (±0.3)			

[a] In samples of salami, the amounts at three different times of aging (t_0, not aged; t_1, after 3 months; and t_2, after 6 months) are reported. The standard error is reported in parentheses. The total amount of BA and the histamine/histidine concentration ratio were evaluated.

The ionization process provided for the dansylated amines a greater efficiency and consequently greater intensity of the m/z signals than for the dansylated amino acids.

ESI (mobile phase eluting at a flow rate of 0.2 mL min^{-1}, after 1:5 splitting) led to comparable efficiencies for amines and amino acids. **Figure 4** presents the total ion current (TIC) separation of the standard mixture of the eight dansylated analytes (1.00 mg L^{-1} each).

The results obtained in mass spectrometry detection represented a validation of the chromatographic separation. As can be seen in the chromatograms of **Figures 2**−**4** the retention time reproducibility was always within 7%. For each analyte, standard calibration curves using both ESI and APCI sources were plotted, and LOD and LOQ values were calculated as reported under Calibration Curves (linearity ranges between LOQ value and 1.50 mg L^{-1} and R^2 values always >0.99). LODs and LOQs obtained in mass spectrometry detection with both APCI and ESI sources were for all of analytes lower than those obtained for UV detection (**Table 2**). Cmparison with the results of the previous works, concerning the analysis of the same matrix without derivatization reaction (*21*, *22*), showed that the precolumn selective dansylation process is able to lower in MS detection the matrix effect.

Recovery Evaluation. To apply the optimized HPLC-UV method in the analysis of food samples, the recovery yield of each analyte was evaluated by spiking the food matrices with knownamounts of standard analytes and quantifying them. Because recovery depends on the concentration level of the analyte in the matrix (*30*), in representative samples (Toma cheese, unripened salami, preserved clams, and beer), a previous qualitative and quantitative evaluation of amine and amino acid content was performed: the native amounts (already contained in the matrix) were so evaluated. Standard analytes at concentrations comparable with those quantified were added (surrogates) and the samples subjected to the whole treatment of dansylation, SPE, and HPLC-UV analysis. The recovery was evaluated for each analyte by comparing the amount found after spiking (with respect to that initially estimated) and the amount added (surrogate). Recovery percentages were always >92% (relative standard deviation (RSD) < 5%).

HPLC-UV Analysis of Real Samples. The optimized HPLC-UV method was applied to the analysis of typical foods. For the quantitative analysis both external calibration plots (peak area versus concentration of standard solutions) and standard addition method plots (peak area versus concentration of standard solutions added to the sample) were built and compared. The slopes of the two plots are not significantly different, indicating no significant matrix effect in the determination. In a semihard cow's milk cheese, namely, Toma Piemontese D.O.P. produced in Piedmont (Italy), four amines (cadaverine, histamine, tryptamine, and tyramine) were identified and quantified. For tyramine and tryptamine, typically present in cheese, we did not detect their precursor amino acids, whereas the presence of cadaverine and histamine was accompanied by the precursor amino acids lysine and histidine. The quantitative data are given in **Table 3**.

For comparison, a Pecorino Sardo cheese produced in another Italian region (Sardinia) with ewe's milk and characterized by a very different taste was also analyzed. The results (**Table 3**) unexpectedly showed, apart from the absence of lysine, a BA composition similar to that of Toma Piemontese D.O.P. On the basis of the higher total amount of BA and the higher histamine/histidine concentration ratio, Toma Piemontese D.O.P. seems to be more mature than Pecorino Sardo.

To check if the parameters of biogenic amine amount and of higher concentration ratios between an amine and its precursor amino acid are really useful in the evaluation of the ripening time of food products, samples of Muletta Monferrina salami (typical of Alessandria, Piedmont) of known ripening time were analyzed; samples of the fresh product (t_0) and after ripening times of 3 months (t_1) and 6 months (t_2) were available. The amounts obtained for cadaverine, histamine, histidine, tyramine, and tryptamine are shown in **Table 3**. **Figure 5** reports typical chromatograms recorded for the three ripening times. As expected, both the total biogenic amine amount and the histamine/histidine concentration ratio increased with the maturation of the salami.

Histamine typically is reported as present in preserved seafood; jarred clams acquired in a local supermarket were analyzed. Cadaverine and histamine were detected and quantified together with their precursor amino acids, lysine and histidine. Apart from the absence of lysine, a similar composition was found in the clam broth (**Table 3**).

A homemade beer aged for 4 months was analyzed. Histidine, lysine, tyramine, and tryptamine were identified and quantified (**Table 3**). Because the suggested threshold concentration for alcoholic beverages addresses only histamine (*1*), particular attention was paid to its possible presence, but no evidence was found, at least at LOD concentration levels.

In conclusion, in this paper a new HPLC-UV method was developed for the simultaneous identification and determination of four BA and their precursor amino acids. The

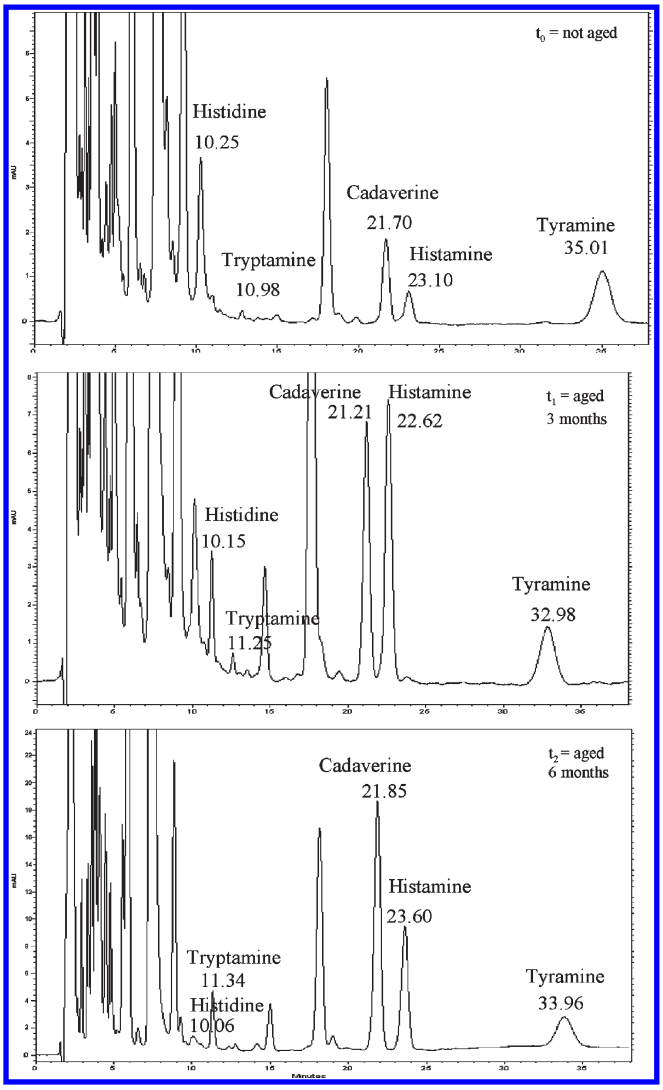

Figure 5. HPLC-UV chromatograms of samples of Muletta Monferrina salami at different aging times: t_0 (fresh product), t_1 (3 months), and t_2 (6 months). Chromatographic conditions were as in **Figure 2**.

chromatographic conditions were optimized by experimental design, OLS, and BP-ANNs. OLS and BP-ANN permitted good models relating the retention times of the analytes to the chromatographic operative conditions to be obtained. A grid search algorithm provided the best conditions for performing an optimal separation of the eight analytes. Also, the best chromatographic conditions for analysis with constrained total analysis time were searched for.

The HPLC-UV method was validated for comparison of the results with those obtained under the same conditions but in mass spectrometry detection, which also offered greater sensitivities. In addition, the robustness of the chromatographic method was proven when it was transferred to a different HPLC instrumentation and a different detector.

The whole extraction and HPLC-UV procedure was applied to the analysis of different kinds of food, and the results were discussed and employed to evaluate ripening time on the basis of biogenic amine/precursor amino acid ratio and total biogenic amine amount.

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LITERATURE CITED

(1) Silla Santos, M. H. Biogenic amines: their importance in foods. *Int. J. Food Microbiol.* **1996**, *29*, 213–231.
(2) European Commission Directive 2073/2005.
(3) Draisci, R.; Riannetti, L.; Boria, P.; Lucentini, L.; Palleschi, L.; Cavalli, S. Improved ion chromatography-integrated pulsed amperometric detection method for the evaluation of biogenic amines in food of vegetable or animal origin and in fermented foods. *J. Chromatogr., A* **1998**, *798*, 109–116.
(4) Innocente, N.; Biasutti, M.; Padovese, M.; Moret, S. Determination of biogenic amines in cheese using HPLC technique and direct derivatization of acid extract. *Food Chem.* **2007**, *101*, 1285–1289.
(5) Rabie, M.; Simon-Sarkadi, L.; Siliha, H.; El-seedy, S.; El Badawy, A.-A. Changes in free amino acids and biogenic amines of Egyptian salted-fermented fish (Feseekh) during ripening and storage. *Food Chem.* **2009**, *115*, 635–638.
(6) Mardiana, S.; Bahruddin, S.; Noor Hasani, H.; Abdussalam, S. M. A.; Muhammad, I. S. Determination of biogenic amines in selected Malaysian food. *Food Chem.* **2009**, *113*, 1356–1362.
(7) Mah, J.-H.; Hwang, H.-J. Effects of food additives on biogenic amines formation in *Myeolchi-jeot*, a salted and fermented anchovy (*Engraulis japonicus*). *Food Chem.* **2009**, *114*, 168–173.
(8) Busto, O.; Gulasch, J.; Borrull, F. Determination of biogenic amines in wine after precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. *J. Chromatogr., A* **1996**, *737*, 205–213.
(9) Hwang, D. F.; Chang, S. H.; Shiua, C. Y.; Chai, T. J. High-performance liquid chromatographic determination of biogenic amines in fish implicated in food poisoning. *J. Chromatogr., B* **1997**, *693*, 23–29.
(10) Romero, R.; Gázquez, D.; Bagur, M. G.; Sánchez-Viñas, M. Optimization of chromatographic parameters for the determination of biogenic amines in wines by reversed-phase high-performance liquid chromatography. *J. Chromatogr., A* **2000**, *871*, 75–83.
(11) Romero, R.; Sánchez-Viñas, M.; Gázquez, D.; Bagur, M. G. Characterization of selected Spanish table wine samples according to their biogenic amine content from liquid chromatographic determination. *J. Agric. Food Chem.* **2002**, *50*, 4713–4717.
(12) Moret, S.; Smela, D.; Populin, T.; Conte, L. S. A survey on free biogenic amine content of fresh and preserved vegetables. *Food Chem.* **2005**, *89*, 355–361.
(13) Soufleros, E. H.; Bouloumpasi, E.; Zotou, A.; Loukou, Z. Determination of biogenic amines in Greek wines by HPLC and ultraviolet detection after dansylation and examination of factors affecting their presence and concentration. *Food Chem.* **2007**, *101*, 704–716.
(14) Alberto, M. R.; Arena, M. E.; Manca de Nadra, M. C. A comparative survey of two analytical methods for identification and quantification of biogenic amines. *Food Control* **2002**, *13*, 125–129.
(15) Naval, M. V.; Gómez-Serranillos, M. P.; Carretero, M. E.; De Arce, C. Value of high-performance liquid chromatographic analysis of amino acids in the determination of *Panax ginseng* radix extract effect in cultured neurons. *J. Chromatogr., A* **2006**, *1121*, 242–247.
(16) Krause, I.; Bockhardt, A.; Neckermann, H.; Henle, T.; Klostermeyer, H. Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dabsyl derivatives. *J. Chromatogr., A* **1995**, *715*, 67–79.
(17) Bauza, T.; Blaise, A.; Daumas, F.; Cabanis, J. C. Determination of biogenic-amines and their precursor amino-acids in wines of the Vallée du Rhône by high-performance liquid-chromatography with precolumn derivatization and fluorometric detection. *J. Chromatogr., A* **1995**, *707*, 373–379.
(18) Lozanov, V.; Petrov, S.; Mitev, V. Simultaneous analysis of amino acid and biogenic polyamines by high-performance liquid chromatography after pre-column derivatization with *N*-(9-fluorenylmethoxycarbonyloxy)succinimide. *J. Chromatogr., A* **2004**, *1025*, 201–208.
(19) Kőrös, Á.; Varga, Z. S.; Molnár-Perl, I. Simultaneous analysis of amino acids and amines as their *o*-phthalaldehyde-ethanethiol-9-fluorenylmethyl chloroformate derivatives in cheese by high-performance liquid chromatography. *J. Chromatogr., A* **2008**, *1203*, 146–152.

(20) Pereira, V.; Pontes, M.; Câmara, J. S.; Marques, J. C. Simultaneous analysis of free amino acids and biogenic amines in honey and wine samples using in loop orthophthalaldeyde derivatization procedure. *J. Chromatogr., A* **2008**, *1189*, 435–443.
(21) Gosetti, F.; Mazzucco, E.; Gianotti, V.; Polati, S.; Gennaro, M. C. High performance liquid chromatography/tandem mass spectrometry determination of biogenic amines in typical Piedmont cheeses. *J. Chromatogr., A* **2007**, *1149*, 151–157.
(22) Gianotti, V.; Chiuminatto, U.; Mazzucco, E.; Gosetti, F.; Bottaro, M.; Frascarolo, P.; Gennaro, M. C. A new hydrophilic interaction liquid chromatography tandem mass spectrometry method for the simultaneous determination of seven biogenic amines in cheese. *J. Chromatogr., A* **2008**, *1185*, 296–300.
(23) Hoke, T. A. Economical second-order designs based on irregular fractions of the 3*n* factorial. *Technometrics* **1974**, *16*, 375–384.
(24) Zupan, J.; Gasteiger, J. In *Neural Network for Chemists: an Introduction*; Wiley: New York, 1993.
(25) Marengo, E.; Gennaro, M. C.; Angelino, S. Neural network and experimental design to investigate the effect of five factors in ion-interaction high-performance liquid chromatography. *J. Chromatogr., A* **1998**, *799*, 47–55.
(26) Wythoff, B. J. Backpropagation neural networks − a tutorial. *Chemom. Intell. Lab. Syst.* **1993**, *18*, 115–155.
(27) Foresee, F. D.; Hagan, M. T. Gauss-Newton approximation to Bayesian learning. *IEEE Trans. Neural Networks*, **1997**, *3*, 1930−1935.
(28) Deming, S. N.; Morgan, S. L. *Experimental design: a chemometric approach*, 2nd ed.; Elsevier: Amsterdam, The Netherlands, 1993; pp 109.
(29) Miller, J. N.; Miller, J. C. Calibration methods in instrumental analysis: regression and correlation. In *Statistics and Chemometrics for Analytical Chemistry*, 4th ed.; Prentice Hall: Upper Saddle River, NJ, 2000; pp 120.
(30) Polati, S.; Roz, M.; Angioi, S.; Gianotti, V.; Gosetti, F.; Marengo, E.; Rinaudo, C.; Gennaro, M. C. Statistical evaluation of recovery of 3,4-dichloroaniline in soil as function of particle size and analyte concentration. *Talanta* **2005**, *68*, 93–98.

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