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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

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Abstract

An improved method for the simultaneous determination of underivatized biogenic amines, cadaverine, putrescine, spermidine, histamine, tyramine and some amino acids precursors, histidine and tyrosine, in food products, based on ion-exchange chromatography (IC) with integrated pulsed amperometric detection (IPAD) has been developed. The method was successfully used for the analysis of biogenic amines and amino acids in food both of vegetable (kiwi, *Actinidia chinensis*) and animal origin, (fish, pilchard), as well as in fermented foods, such as cheese (Emmenthal) and dry sausages (salami). The method was also successfully used to study the changes in biogenic amines during the ripening of dry fermented sausages (salami). The analytes were extracted from foods with perchloric acid and the extracts were purified by liquid–liquid partition using *n*-hexane. Determination of biogenic amines was performed through cation-exchange chromatography with isocratic elution and IPAD. The detection limits for the analytes under investigation were found to range from 1.25 to 2.50 ng, at a signal-to-noise ratio of 3:1. Average recoveries ranged from 85.5 to 97.4% and R.S.D. values ranged from 3.4 to 8.8. The proposed method offers a number of advantages over our previous IPAD method, such as the application to a larger number of analytes and matrices, a simpler extraction procedure and clean-up, isocratic elution using low acid and base concentrations, an improved chromatographic separation and a lower detection limit. © 1998 Elsevier Science B.V.

Keywords: Food analysis; Vegetables; Fish; Cheese; Biogenic amines; Amino acids

1. Introduction

Biogenic amines are aliphatic, alicyclic and heterocyclic organic bases of low molecular mass that possess biological activity. They are widely distributed in food products of both animal [1–10] and vegetable [11,12] origin, as well as in fermented foods [13–15], originating mainly by microbial decarboxylation of amino acids [10,16–19]. The importance of estimating the levels of biogenic amines in foods and beverages is related to their impact on human health and food quality.

The first concern regards food safety, as biogenic amines are responsible for human food-borne chemical intoxications. The most frequent foodborne intoxication caused by biogenic amines involves histamine and tyramine. The first is well recognized to cause ‘scombroid fish poisoning’, an illness generally associated with the consumption of scombroid fish, such as tuna, mackerel and sardines, which contain high levels of histamine [20], while tyramine is responsible for the foodborne intoxication known

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as 'cheese reaction', which is caused by the ingestion of high tyramine doses in cheese [21]. An important role in human foodborne intoxication can also be played by secondary amines, such as putrescine and cadaverine, since they can potentiate the toxicity of histamine [22–26]. In addition, secondary amines can react with nitrite to form carcinogenic nitrosamines [27].

On the other hand, the assessment of the biogenic amine content in foods has received crucial attention in recent years due to the possibility of using amine concentrations as an index of food quality [28–30], as the original concentration levels of biogenic amines can be changed during food processing and storage and are influenced by the hygienic conditions employed.

Due to their important roles and ubiquity in foodstuffs, various high-performance liquid chromatographic (HPLC) methods have been developed for determining biogenic amines in food products. Although the methods are usually based on pre- or post-column derivatization of amines using several derivatizing agents [31–42], detection methodology that does not require derivatization is preferred for convenience and simplicity.

Integrated pulsed amperometric detection (IPAD) [43] is a new variant of pulsed amperometric detection (PAD), which is useful for amines and sulfur species, because their oxidation at metal electrodes is catalysed by the formation of metal oxide [44]. While in PAD, current is measured after a pulse and a short delay, to allow charging current to decay, in IPAD current is integrated continuously during a cycle where the electrode is oxidized and then reduced back to the original state. The advantage of IPAD is that, by cancelling the charge from oxide formation and reduction, the effect on the baseline is greatly minimized.

Previously, we employed a gradient ion-exchange chromatography (IC) separation method coupled with IPAD using a gold electrode material to determine biogenic amines in fish products [43]. In this research, the method has been improved in order to perform both the isocratic elution and the determination of other biogenic amines, such as tyramine and its amino acid precursor, tyrosine, in different food products, of vegetable (kiwi, *Actinidia chinensis*) and animal origin, such as cured meat (salami), fish (pilchard) and cheese (Emmenthal). The evolution of biogenic amines has also been evaluated during the ripening of dry fermented sausage (salami) samples through this method.

2. Experimental

2.1. Equipment

A model DX-500 liquid chromatograph (Dionex, Sunnyvale, CA, USA) was used for the analyses. IPAD was performed with a model ED40 electrochemical detector (Dionex). An electrochemical cell equipped with a gold working electrode, a stainless-steel counter-electrode and a combined pH–Ag/AgCl Na-reference electrode was used. A reagent delivery module, RDM (Dionex), was used for post-column sodium hydroxide addition. Samples were injected using a Rheodyne model 9126 injection valve with a polyether ether ketone (PEEK) sample loop of 25 μl (Cotati, CA, USA). The system was interfaced via proprietary network chromatographic software, PeakNet (Dionex), to an 80486-based personal computer, for data acquisition and instrumentation control.

An Ultra-Turrax T25 blender (Janke and Kunkel, Staufen, Germany) and a model ALC 4237 centrifuge (ALC International, Milan, Italy) were used for sample preparation.

2.2. Chemicals

All solutions were prepared from reagent grade chemicals. Sodium perchlorate and perchloric acid were from Carlo Erba (Rodano, Italy), sodium hydroxide, putrescine, cadaverine dihydrochloride and tyrosine, tyramine were from Novachimica (Milan, Italy), histamine dihydrochloride, L-histidine hydrochloride and spermidine trihydrochloride were from Aldrich (Milwaukee, WI, USA). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA).

Biogenic amine and amino acid stock solutions containing 1000 μg/ml of each compound were prepared by dissolving the pure compound in 0.375 *M* perchloric acid. The solutions were stored under refrigeration (+4°C) and were stable for at least

fifteen days. Standard calibration solutions in the range 0.2–10.0 μg/ml were prepared daily by diluting the stock solutions with 0.375 *M* perchloric acid. Standard fortification solutions were prepared in the same way as the standard curve solutions.

2.3. Samples

Samples of kiwi (*Actinidia chinensis*), cheese (Emmenthal) and fish (pilchard) were purchased from commercial suppliers in Rome, Italy. Dry fermented sausages were obtained by simulating the industrial manufacturing process for production of Italian dry sausages (salami). In order to assess the changes to biogenic amines during the seasoning of dry fermented sausages, samples were collected at the following times during the ripening process: 0, 5, 12, 19, 33 and 47 days.

2.4. Chromatographic conditions

Separations were performed on an IonPac CS10 cation-exchange analytical column (Dionex) that was coupled to an IonPac CG10 guard column (Dionex) packed with 8.5 μm solvent-compatible ethylvinylbenzene–divinylbenzene 55% XL substrate, agglomerated with 175 nm sulphonated cation-exchange latex for a cation-exchange capacity of approximately 80 μequiv./column.

Isocratic separation was performed with an eluent containing 1 *M* sodium perchlorate–0.375 *M* perchloric acid–water (81:5:14, v/v/v) at a flow-rate of 1 ml/min.

2.5. Integrated pulsed amperometric detection

Table 1 shows the optimized waveform time-potential used during the experiments. In order to achieve a basic pH for the IPAD on a Au electrode, 0.25 *M* NaOH was added at a flow-rate of 0.8 ml/min using a mixing tee to the column eluate via the RDM.

2.6. Sample preparation

A 30-ml volume of 0.375 *M* perchloric acid was added to 10 g of homogenized sample of kiwi, meat, fish or cheese. Samples were homogenized (1000 *g*, 5 min) and quantitatively transferred into a 50-ml volumetric flask for kiwi and cheese or a 100-ml flask for fish and meat. The aqueous solution was then extracted with *n*-hexane (2:1, v/v) for meat, fish and cheese and centrifuged at 1000 *g* for 10 min, and the supernatants were discarded. Finally, the aqueous phase was filtered through a 0.2-μm filter and injected for the IC–IPAD analysis.

Table 1
Waveform time-potential used for integrated pulsed amperometry

Time (s)	Potential (V)[a]	Integration
0.00	0.25	
0.20	0.25	Begin
0.30	0.65	
0.40	0.65	
0.50	0.25	
0.70	0.25	End
0.71	1.20	
0.90	1.20	
0.91	−0.60	
1.00	−0.60	

[a] Na-reference electrode.

3. Results and discussion

Previously, we investigated the suitability of using HPLC coupled to IPAD with a gold electrode material to determine cadaverine, putrescine, spermidine and histamine in fish products [43].

Keen interest in optimizing this method has recently developed in this laboratory, in order to determine different biogenic amines, such as cadaverine, putrescine, spermidine, histamine, tyramine and some precursors of these, i.e. amino acids such as histidine and tyrosine, in a number of food products, both of vegetable and animal origin.

The cation-exchange separation of amines and precursor amino acids was performed by a comparatively simple isocratic elution instead of the gradient elution used in our previous method [43]. As for the mobile phase, chromatographic separations were performed at 1 ml/min with an eluent composition of 1 *M* sodium perchlorate–0.375 *M* perchloric acid–water (81:5:14, v/v/v). The use of acetonitrile was avoided, thus ruling out the possible formation of acetonitrile by-products in basic media, which could lead to possible interferences in detection [45].

As for the IPAD, the waveform proposed is designed for oxidation of the amine on the electrode surface, followed by a cycle of oxidation/reduction of the electrode to bring the electrode surface back to the initial conditions. The IPAD program, using short positive and negative pulses following the integration cycle to coulometrically remove the oxide background signal from the electrode surface, virtually eliminates drift and changes associated with small variations in pH and composition of the mobile phase. The reference electrode was used in the sodium form in order to reduce the interference caused by perchlorate eluent with other reference electrodes [46]. The basic pH in the electrochemical cell needed for detection using the gold working electrode was achieved by the post-column addition of 0.25 *M* NaOH.

Under the new IC–IPAD conditions, very good separation of cadaverine, putrescine, spermidine, histamine, tyramine, histidine and tyrosine was achieved in 25 min with sharp and symmetrical peaks (Fig. 1). Linearity of the IPAD response to cadaverine, putrescine, spermidine, histamine, tyramine, histidine and tyrosine, separated by isocratic elution, was verified in the range 0.2–10.0 μg/ml. The data obtained from the external standard calibration curves were submitted to linear regression analysis and correlation coefficients greater than 0.997 were obtained for all curves.

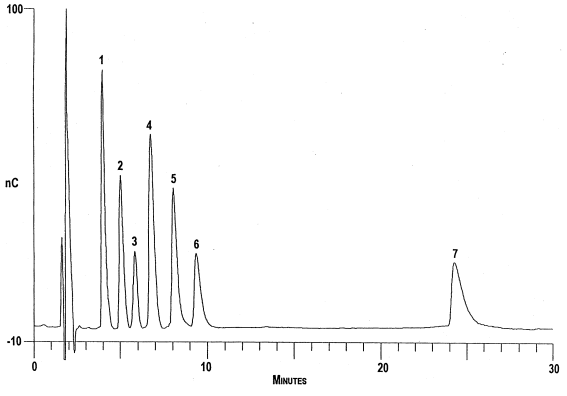

Fig. 1. Chromatogram of a standard solution containing biogenic amines and amino acids. Peaks: **1**, putrescine (2.0 μg/ml); **2**, histidine (2.0 μg/ml); **3**, tyrosine (2.0 μg/ml); **4**, cadaverine (2.0 μg/ml); **5**, histamine (2.0 μg/ml); **6**, tyramine (2.0 μg/ml) and **7**, spermidine (2.0 μg/ml). Columns, IonPac CG10+CS10; eluent, 1 *M* sodium perchlorate–0.375 *M* perchloric acid–water (81:5:14, v/v/v); flow-rate, 1 ml/min; detection, IPAD (Au working electrode), see Table 1.

The lowest detectable amounts for the analytes under investigation, based on a 25-μl injection, were found to be 1.25 ng for putrescine; 1.65 ng for cadaverine; 1.90 ng for histidine; 2.00 ng for histamine; 2.35 ng for tyrosine; 2.42 ng for tyramine and 2.50 ng for spermidine, at a signal-to-noise ratio of 3:1.

The repeatability of the retention time was less than 1% for five injections.

3.1. Determination of biogenic amines in food products

In order to simplify sample preparation, we cut out the clean-up step by solid-phase extraction (SPE) that was used previously [43]. The new protocol simply involves the extraction of the analytes using 0.375 *M* perchloric acid and washing the extract with *n*-hexane, in order to remove the lipidic fraction from the fat matrices, namely fish, meat and cheese extracts.

Accuracy and precision data for the IC–IPAD method were generated each day for two days from the analysis of duplicate control samples that were fortified with standard fortification solution containing 2, 5, 10 and 50 μg/g (kiwi and cheese) and 5, 10 and 50 μg/g (salami and pilchard) of each analyte under investigation.

The average recoveries, reported in Table 2, ranged from 85.5%, for tyramine in a fruit product, to 97.4%, for putrescine in fish. These values are satisfactory considering the complexity of the biological matrices under investigation. The precision was also satisfactory at all levels, with R.S.D. values ranging from 3.42 to 8.81.

The IC–IPAD method was then applied to the determination of biogenic amines in food products both of vegetable and animal origin. Typical chromatogram profiles of fruit products (kiwi, *Actinidia chinensis*), meat products (salami), fish (pilchard) and cheese (Emmenthal) are shown in Figs. 2–5, respectively. No interfering peaks appeared at the retention times of the analytes. However, some samples with high concentrations of biogenic amines

Table 2
Recovery of biogenic amines from fortified kiwi (*Actinidia chinensis*), meat product (salami), fish (pilchard) and cheese (Emmenthal) samples

Analyte	Amount added (μg/g)	Kiwi		Cheese		Pilchard		Salami	
		Recovery (%)[a]	R.S.D. (%)[a]	Recovery (%)[a]	R.S.D.[a]	Recovery (%)[a]	R.S.D.[a]	Recovery (%)[a]	R.S.D.[a]
Putrescine	2.0	96.8	7.37	97.3	8.33	n.p.[b]	n.p.[b]	n.p.[b]	n.p.[b]
	5.0	94.3	7.12	95.1	6.68	92.0	7.22	94.0	7.10
	10.0	95.7	5.56	97.3	4.83	98.7	4.54	95.7	4.54
	50.0	96.5	4.04	97.7	3.48	101.5	3.42	97.3	4.43
Average recovery (%)		95.8		96.9		97.4		95.7	
Cadaverine	2.0	94.8	6.58	94.7	6.60	n.p.[b]	n.p.[b]	n.p.[b]	n.p.[b]
	5.0	96.0	5.76	93.1	7.19	94.1	8.67	95.3	6.07
	10.0	97.3	4.19	96.0	4.47	95.3	6.07	97.7	4.10
	50.0	95.0	3.58	95.7	4.63	93.7	3.45	94.3	3.77
Average recovery (%)		95.8		94.9		94.4		95.7	
Histamine	2.0	98.0	8.81	90.8	6.32	n.p.[b]	n.p.[b]	n.p.[b]	n.p.[b]
	5.0	96.6	7.31	92.5	6.45	92.8	6.04	94.0	6.85
	10.0	95.0	4.01	96.3	4.01	94.1	4.33	94.7	4.89
	50.0	94.5	4.58	95.3	3.64	94.8	3.89	94.0	4.24
Average recovery (%)		96.0		93.7		93.9		94.2	
Tyramine	2.0	82.0	7.90	94.6	6.70	n.p.[b]	n.p.[b]	n.p.[b]	n.p.[b]
	5.0	85.5	4.98	90.8	5.35	90.8	6.70	94.0	6.54
	10.0	87.7	5.83	87.3	4.78	86.7	4.05	96.3	4.15
	50.0	86.8	3.96	91.6	4.72	91.5	4.16	96.0	4.46
Average recovery (%)		85.5		91.1		89.7		95.4	
Spermidine	2.0	93.3	6.57	94.6	7.26	n.p.[b]	n.p.[b]	n.p.[b]	n.p.[b]
	5.0	94.5	5.80	93.7	6.43	85.4	7.79	90.8	7.43
	10.0	97.0	4.00	97.0	4.21	97.1	4.71	97.7	4.25
	50.0	96.0	3.62	96.7	5.64	97.8	4.64	94.0	5.71
Average recovery (%)		95.2		95.5		93.4		94.2	

[a]Each value is the average of four extractions (two per day for two days);
[b]n.p.: not performed.

and/or free amino acids may have to be diluted before injection.

3.2. Evaluation of biogenic amines during the seasoning of dry fermented sausages

Fermented sausages are defined as ground meat mixed with salt and curing agents, stuffed into casings and subjected to a fermentation process in which microorganisms play a crucial role. Dry fermented sausage can usually accumulate biogenic amines during seasoning and storage, and the concentration of biogenic amines in fermented sausages tends to increase with ripening time [47,48].

The IC–IPAD method was therefore applied to investigate the formation of biogenic amines during the ripening process in the seasoning of dry fermented sausages (salami). Fig. 6 shows the levels of biogenic amines determined in dry fermented sausages as a function of ripening time. Putrescine was determined (5.1 μg/g) from the initial day of the ripening process and increased up to 46.2 μg/g after 47 days, while tyramine was detected starting at day twelve (28.2 μg/g) and showed its highest level (55.9 μg/g) after 33 days' seasoning. Increases in cadaverine concentrations started at day five and the highest content (19.0 μg/g) was recorded after 33 days. Histamine was detected at low concentrations (8.0 μg/g) only after 33 days of storage. Spermidine

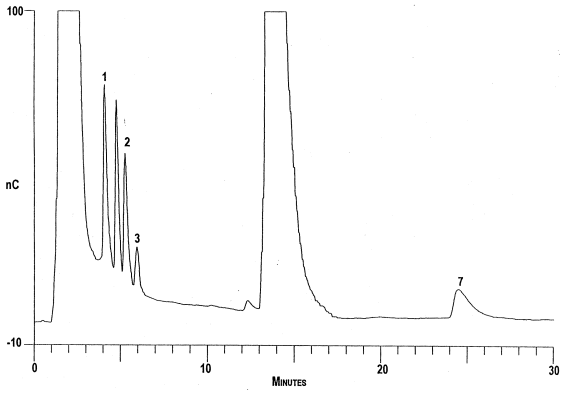

Fig. 2. Chromatogram of a kiwi (*Actinidia chinensis*) sample. Peaks: **1**, putrescine (6.7 μg/g); **2**, histidine (7.8 μg/g); **3**, tyrosine (5.5 μg/g) and **7**, spermidine (4.3 μg/g). Analytical conditions as in Fig. 1.

was not found to accumulate during the ripening time investigated.

4. Conclusions

An improved IC–IPAD method for the simultaneous determination of underivatized biogenic amines, cadaverine, putrescine, spermidine, histamine, tyramine and some amino acids precursors, histidine and tyrosine, in food products has been developed and successfully applied to the analysis of foods of vegetable (kiwi, *Actinidia chinensis*) and animal origin, such as cheese (Emmenthal), fish (pilchard) and cured meat (salami), as well as in assessing the changes that take place in the levels of biogenic amines during the ripening of dry fermented sausages (salami).

The main advantages of this method over the previous method [43] are its application to a larger number of analytes and matrices, a simpler extraction and clean-up procedure, isocratic elution using

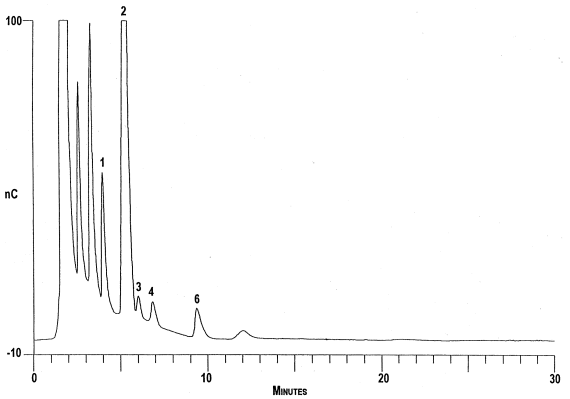

Fig. 3. Chromatogram of a meat (salami) sample. Peaks: **1**, putrescine (49.6 μg/g); **2**, histidine (1087.0 μg/g); **3**, tyrosine (18.3 μg/g); **4**, cadaverine (14.1 μg/g) and **6**, tyramine (42.5 μg/g). The extract was diluted fivefold before injection. Analytical conditions as in Fig. 1.

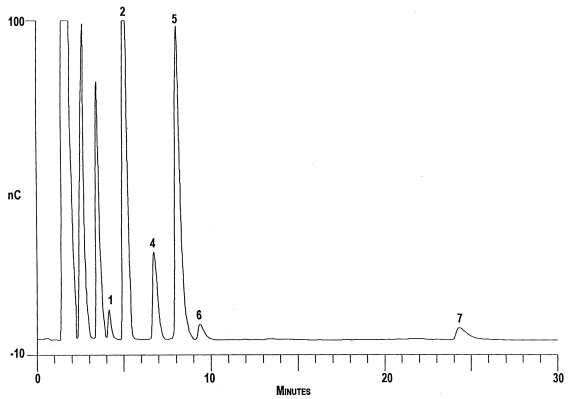

Fig. 4. Chromatogram of a fish (pilchard) sample. Peaks: **1**, putrescine (15.5 μg/g); **2**, histidine (1065.0 μg/g); **4**, cadaverine (45.0 μg/g); **5**, histamine (185.3 μg/g), **6**, tyramine (32.1 μg/g) and **7**, spermidine (28.2 μg/g). The extract was diluted fivefold before injection. Analytical conditions as in Fig. 1.

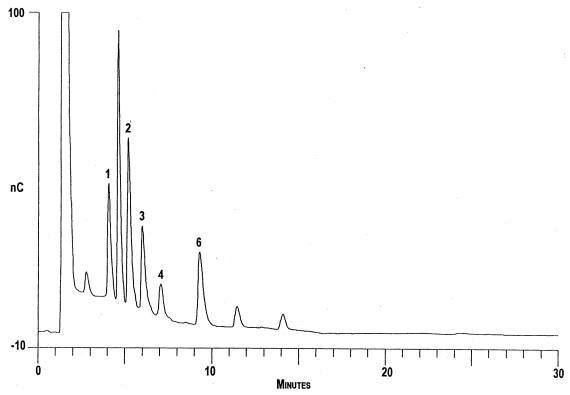

Fig. 5. Chromatograms of a cheese (Emmenthal) sample. Peaks: **1**, putrescine (4.9 μg/g); **2**, histidine (13.5 μg/g); **3**, tyrosine (12.6 μg/g); **4**, cadaverine (2.6 μg/g) and **6**, tyramine (12.7 μg/g). Analytical conditions as in Fig. 1.

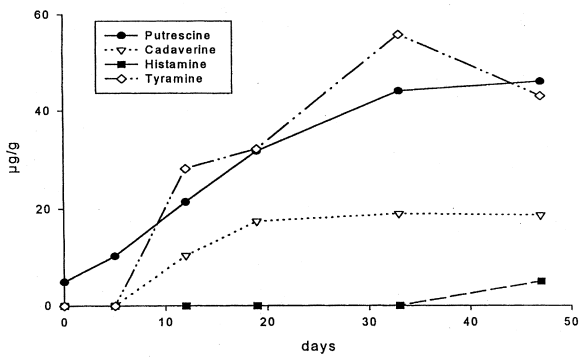

Fig. 6. Evolution of the biogenic amine content in a dry fermented sausage sample. Analytical conditions as for Fig. 1.

low acid and base concentrations, thus, obtaining an improved chromatographic separation and a lower detection limit.

This method offers a simplified, sensitive, selective and rapid alternative to spectrophotometric and fluorimetric HPLC methods, which require cumbersome derivatization reactions and purification steps, for the determination of biogenic amines in different food products.

The developed IC–IPAD method seems to be suitable for the detection of biogenic amines in a large number of samples and is particularly useful for routine checks in repetitive analyses.

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