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Journal of Chromatography A, 1098 (2005) 111–115
JOURNAL OF CHROMATOGRAPHY A
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Determination of biogenic amines in chocolate by ion chromatographic separation and pulsed integrated amperometric detection with implemented wave-form at Au disposable electrode

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Received 19 May 2005; received in revised form 9 August 2005; accepted 23 August 2005
Available online 8 September 2005

Abstract

A rapid and selective cation exchange chromatographic method coupled to integrated pulsed amperometric detection (PAD) has been developed to quantify biogenic amines in chocolate. The method is based on gradient elution of aqueous methanesulfonic acid with post column addition of strong base to obtain suitable conditions for amperometric detection. A potential waveform able to keep long time performance of the Au disposable electrode was set up. Total analysis time is less than 20 min. Concentration levels of dopamine, serotonin, tyramine, histamine and 2-phenylethylamine were measured, after extraction with perchloric acid from 2 g samples previously defatted twice with petroleum ether. The method was used to determine the analytes in chocolate real matrices and their quantification was made with standard addition method. Only dopamine, histamine and serotonin were found in the analysed real samples. Repeatabilities of their signals, computed on their amounts in the real samples, were 5% for all of them. Repeatabilities of tyramine and phenethylamine were relative to standard additions to real samples (close to 1 mg/l in the extract) and were 7 and 3%, respectively. Detection limits were computed with the 3 s of the baseline noise combined with the calibration plot regression parameters. They were satisfactorily low for all amines: 3 mg/kg for dopamine, 2 mg/kg for tyramine, 1 mg/kg for histamine, 2 mg/kg for serotonin, 3 mg/kg for 2-phenylethylamine.

Keywords: Biogenic amines; Integrated pulsed amperometric detection; Au disposable electrode; Chocolate; Ion chromatography

1. Introduction

It is well known that biogenic amines (BAs) are a group of naturally occurring amines derived from enzymatic decarboxylation of some natural amino acids and proteins. Many of them have powerful physiological effects (e.g., histamine, serotonin, dopamine, tyramine) and have an important biological activity [1]. For these reasons, it is important to monitor biogenic amine levels in foodstuffs [2,3], beverages [4,5] and plants [6,7], in view of their importance for human health and food safety as they are members of pressor amine group and tend to cause hyper or hypotension [8]. Some of them, in particular aliphatic diamines, were found in tumour cells and their detection in organ transplant recipients was the basis for monitoring the extent of tissue rejection [9]. Moreover, secondary amines such as putrescine and cadaverine play an important role in food poisoning as they can potentiate the toxicity of histamine [10]. All of them can also react with nitrites to form nitrosamines, which are carcinogenic compounds. The detection of aromatic BAs has become particularly important also in chocolate [11] as they are found at significant concentration levels. Tyramine, serotonin, 2-phenylethyl amine and dopamine content can be modified during technological processes from cocoa to the final product and is influenced by the prevailing hygienic conditions.

Determination of BAs is mainly based on gas or liquid chromatographic separation followed by different detection approaches including FID [12], UV [2,11,13], fluorescence

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doi:10.1016/j.chroma.2005.08.065

[5,14]. The main drawbacks of these methods are related to the process of pre- or post-column derivatisation leading to long analysis times, low reproducibility, interferences and problems connected to the stability of derivatisation products. Other methods [3,15,16] do not involve derivatisation and use conductometric detection after ion chromatographic separation with chemical suppression of the eluent or integrated pulsed amperometric detection (PAD) in various operative conditions [17,18]. In particular, integrated square wave detection (ISWD) was used for standard mixtures [19], and applied to determine BAs in soybean seeds [20]. Pulsed amperometric detection was used in milk samples [21] and electrochemical detection at fixed potential at carbon electrodes [22] was used for serum matrices. Moreover, carbon electrodes modified with gold nanoparticles [23] were applied to extracted sardine samples. The drawback of conductometric detection is the large presence of alkaline and alkaline-earth cations detected together with amines usually much less concentrated. Amperometric detection ensures good sensitivity and does not require derivatisation, however, the reported methods usually lack in repeatability above all when applied to real matrices owing to electrode surface poisoning. In the present work an improved method of detection of non derivatised dopamine (DOP), serotonin (SER), tyramine (TYR), histamine (HIS) and 2-phenylethylamine (PHE) in chocolate has been developed by using a suitable ion chromatographic separation coupled with an integrated pulsed amperometric detection at gold disposable electrodes able to improve signal repeatability. This detection technique resulted more selective than the potentially usable UV one.

2. Experimental

2.1. Reagents

All reagents were of analytical grade and were used as purchased: dopamine 99% (3-hydroxytyramine hydrochloride, Janssen, Geel, Belgium), serotonin hydrochloride 99% (Lancaster, Morecambe, England), tyramine >99% (Fluka, Milan, Italy), histamine base >97% (Fluka, Milan, Italy), 2-phenylethylamine >99% (Fluka, Milan, Italy), $HClO_4$ 70% RP Normapur (Prolabo, France), NaOH (Riedel de Haën-Fluka, Milan, Italy), methanesulfonic acid 99% (Sigma-Aldrich, Milan, Italy), petroleum ether 35–60 °C (Prolabo, France), CH_3CN HPLC grade 99.8% (Baker, Deventer, Holland). The solutions were prepared with milliQ water (Millipore Plus System, Milan, Italy, resistivity 18.2 Mohm·cm). Standard solutions of BAs were prepared in $HClO_4$ 0.1 M at 100 mg/l and stored at 5 °C. For the evaluation of recovery from chocolate, a more concentrated solution was used (about 4000 mg/l), to avoid the addition of large amount of solution.

2.2. Instrumentation

A Dionex GP50 gradient pump equipped with an EG40 electrochemical detector and a LC25 oven was used as chromatographic system. All devices were controlled by the Chromeleon software, version 6.20. The column used was a Dionex CS17 (2 mm × 250 mm, 5 μm) with a pre-column CG17 (2 mm × 50 mm, 5 μm) thermostated at 40 °C. The eluent was methanesulfonic acid (MSA) 6 mM for 3.5 min, then linearly to 27 mM in 7.5 min. This last concentration was maintained constant for 7 min. The chromatographic run was preceded by 7 min of equilibration at 6 mM methanesulfonic acid. The flow rate was 0.35 ml/min. A post column addition of 0.1 M NaOH was made by using a Biorad 1350T pump at 0.35 ml/min. The injected volume was 10 μl. Extracted samples were centrifuged with a Serafini centrifuge, mod. 322, and sonicated with a Branson, mod. 2200, ultrasonic bath. The flow-through electrochemical cell (Dionex) consisted of a 1.0 mm diameter Au disposable working electrode, a titanium counter electrode and a pH–Ag/AgCl combination reference electrode. The adopted waveform and the corresponding integration interval (charge is measured) is reported in Fig. 1.

2.3. Procedures

2.3.1. Extraction of amines from chocolate

Chocolate was grated and fats were twice extracted from 2 g of sample with two aliquots of 10 ml petroleum ether. Extraction were made by shaking for 10 min and then centrifuging for 10 min at 3000 rpm. Then, the petroleum ether solution was discarded and the residual ether eliminated with a nitrogen flow. Amines were then extracted with 10 ml of 0.1 M $HClO_4$ in an ultrasonic bath for 20 min and centrifuged at 4500 rpm for 45 min. Solution was filtered with a 0.2 μm PTFE filter and diluted 1:1 prior injection. A second extraction with 5 ml of $HClO_4$ was made to verify the completion of extraction. Ether used for fats extraction was tested for the presence of amines.

2.3.2. Standard additions calibration and recovery tests

Three standard additions were made to calibrate the analytical method and to evaluate the recovery of the analytes in the extraction procedure. For this purpose 2 g of grated plain chocolate were added of 10, 20, 30 μl of a standard solution containing DOP (4000 mg/l), TYR (2000 mg/l), HIS (900 mg/l), SER (4000 mg/l) and PHE (3000 mg/l). The added solution was

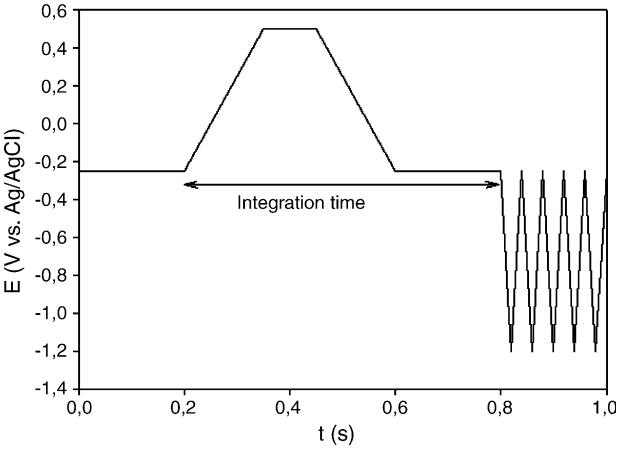

Fig. 1. Detection waveform.

immediately adsorbed by the matrix. Chocolate samples were homogenized before extraction. Amines were then extracted from chocolate as previously described. In the case of 85% cocoa chocolate the added volumes were 20, 40 and 60 μl.

3. Results and discussion

3.1. Choice of the potential waveform

As mentioned in the introduction section, a quite important problem connected to the use of gold electrodes in real matrices, is the surface poisoning that can be caused either by adsorption of oxidation products or by Au oxidation for the presence of ligands in the matrix or for too oxidative applied potentials. In one of their papers, Hoekstra and Johnson [15] showed the way to obtain an optimal waveform for the biogenic amine detection but, unfortunately, its use in real matrices usually can lead to erosion and recession of the electrode surface. We tried to overcome this problem by setting up suitable reductive steps in order to preserve or to recover the Au metal surface. The potential waveform proposed in this paper (Fig. 1) is a modified version of the Hoekstra and Johnson's one. On the basis of cyclic voltammograms in alkaline media (pH close to 13) of the different amines under study, the waveform begins at a rest potential of −0.25 V were gold is at its metal state. Potential was kept at that value for 0.2 s and then was positively scanned up to 0.50 V in 0.15 s. During this period electrode is covered with an oxide layer and, in the presence of amines, their oxidation does occur. Potential remains at 0.5 V for 0.1 s (this step was included also to stabilise the base-line signal) and then returns back to −0.25 V with a reverse scan opposite to the rising one remaining at that value up to 0.8 s. The linear scans (positive and negative) placed in this first part of the waveform, minimize any contribution to the net analytical signal from the formation and reduction of surface oxide because the measured charges are of opposite polarity. The period of time at 0.5 V increases the sensitivity towards amines and the extra amount of oxide formed is recovered by the following steady potential step at −0.25 V. This trapezoidal part of the waveform is essentially the combination of the IVD and ISWD modes reported in Ref. [15]. In the last part of the wave-form, five rapid cyclic scans from −0.25 V to −1.2 V are applied up to a 1 s time length. This cyclic sequence allowed the electrode surface to stabilise at metallic state and probably to condition at negative potential before performing a new charge sampling period. Charge is measured from 0.2 to 0.8 s. We empirically verified that the chosen number of triangular cycles ensures the minimum base-line noise and short surface stabilisation time. Inside each cycle, very important parameters were, obviously, the step time of each potential level and the potential levels themselves. Slight variations of time length of the individual steps inside the cycle did not affect very much the overall performance of the detection. On the other hand, potential levels were quite critical. In this context a helpful check for the optimal performance of the waveform is the base-line signal level. The best detection performance was obtained when residual charge was as close as possible to 0 nC at least at the beginning of the chromatographic gradient. In other words, the potential levels proposed in this paper might need a slight correction to compensate little differences of the reference electrode potential and to compensate the potential shift due to differences of the pH value of solution.

3.2. Post-column reagent

The necessity to detect amines at alkaline pH range compelled the use of a post-column addition of a strong base. Hoekstra and Johnson [19] suggested the use of a cationic micromembrane suppressor to change the elution pH from acidic to alkaline. The idea was based on the usual ion exchange of the methyl sulfonate ion with hydroxide, giving a neutral pH, and to a concomitant slight NaOH permeation from the suppression membrane, producing an alkaline pH depending on the NaOH concentration of the regenerating solution. Although a cation self regenerating suppressor (CSRS-II), tested by us, would have the great advantage of minimizing dilution and producing a very stable base-line, we verified that: (1) it eliminates tyramine and decrease partially also the response of other amines; (2) the amount of permeated NaOH did not allow a strict control of the detection pH; (3) the pH is usually much lower than 13 (NaOH 0.1 M) and in this condition, alkaline and alkaline-earth cations (usually present in real matrices even at high concentrations) can produce a signal due to the substitution of their counter ions with OH^- responsible of the signal change. For all these reasons the suppression would be avoided in the determination of amines in real matrices. We therefore decided to pump and mix NaOH directly into the elution stream by a "tee" junction so that the operative measured pH was always close to 12.7. The added NaOH concentration delivered was 0.1 M in order to limit the dilution effect. Higher concentrations, coupled to slower flow rates did not produce better results owing to the increased difficulty of controlling low flow-rates and for problems of effective solutions mixing. The experimental conditions chosen allowed the use of Au disposable electrodes that remained stable for almost two weeks of continuous work without any decrease of the response.

3.3. Calibration and analysis of real matrices

The proposed detection wave-form was applied to the determination of BAs in various chocolate matrices. From preliminary experiments, we verified the absence of BAs in white chocolate and presence of trace amounts in milk chocolate. We therefore chose to optimise the analytical method with plain and 85% cocoa chocolate. The chromatograms of Fig. 2b and 3 show the separation obtained for two real samples with the chosen elution gradient compared to the five standard analytes (Fig. 2a) all at the concentration of 2.0 mg/l. In particular, the reported chromatograms refer to a plain chocolate sample containing a declared cocoa amount of 50% (Fig. 2b) and a chromatogram relative to a sample containing 85% of cocoa (Fig. 3). A sinusoidal base-line noise, produced by the post-column pump used for the NaOH delivering, becomes evident at trace level analysis while it has no effect when detecting the amine contents usually present in chocolate. This noise can be further reduced by using a pressurized post-column device. In this case, also

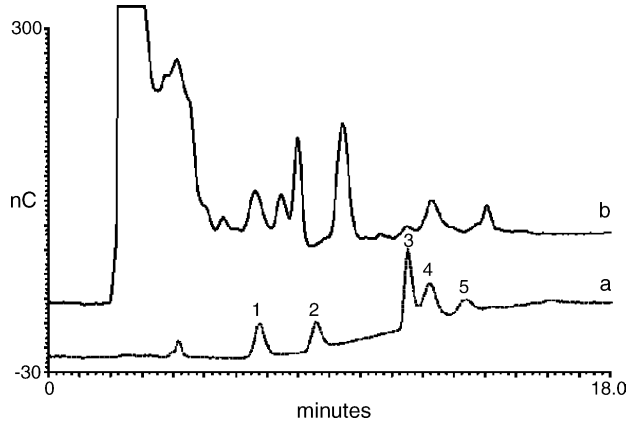

Fig. 2. Chromatograms relative to (a) standards at 2 mg/l concentration level and (b) plain chocolate sample. Experimental conditions: column Dionex CS17 with precolumn; methanesulfonic acid gradient; flow rate 0.35 ml/min; post column NaOH at 0.35 ml/min; injection volume 10 μl; $T = 40$ C. For the electrochemical detection conditions, see Fig. 1: (1) dopamine; (2) tyramine; (3) histamine; (4) serotonin; (5) 2-phenylethylamine.

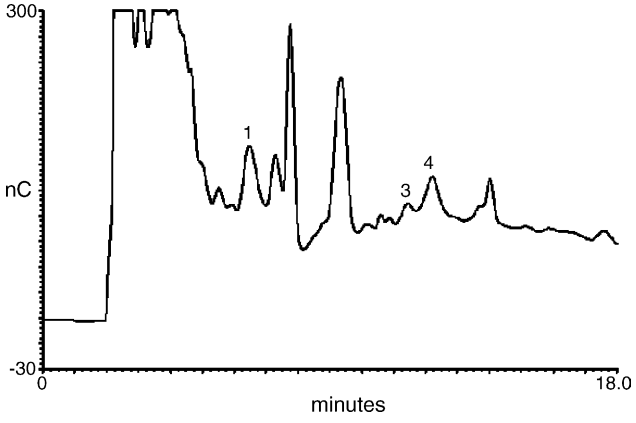

Fig. 3. Chromatogram relative to a 85% cocoa chocolate sample. Experimental conditions: column Dionex CS17 with precolumn; methanesulfonic acid gradient; flow rate 0.35 ml/min; post column NaOH at 0.35 ml/min; injection volume 10 μl; $T = 40$ C. For the electrochemical detection conditions, see Fig. 1: (1) dopamine; (3) histamine; (4) serotonin.

the detection limits may be lowered. The base-line drift evidenced in the standard run corresponds to the variation of eluent concentration but it does not interfere with the determination of the BAs in real samples as demonstrated by the real sample chromatogram. This fact points out the great sensitivity of the electrode to minimal variation of the pH value. Anyhow, the effect is hidden by the head components of the matrix likely responsible of a modification of the electrode response. Column temperature plays a significant role in peak separation. Higher temperature produced sharper peaks improving both efficiency and selectivity so that the chosen temperature was 40 °C, the highest possible to keep the column safety unchanged. It must be noted that all the tested amines have UV active groups and therefore they could be potentially detected also by UV technique. Anyhow, we verified that this detection technique cannot be used owing to the presence of a huge signal of absorbing components present in the cocoa real matrices that hid all the amine peaks. Therefore, a relevant improvement in terms of selectivity has been obtained with the present IPAD technique. Quantification has been made by standard addition method as reported in the procedure section. We chose this calibration method on the basis of the following results: (1) the sensitivities of the external calibration are different from those of the standard addition method as a result of the influence of the matrix on the electrode surface performance; (2) the chocolate matrices are usually different from one another and therefore it seems reasonable to use standard additions; (3) if the injected sample is a real matrix the analytes retention times are slightly different from those of the external standards (compare the two chromatograms of Fig. 2) so that the presence of close interfering peaks can give some interpretation problems. Anyway, the amount of amines added in the standard addition method has been chosen on the basis of an estimate of the analyte concentration in the real matrices with external standards. Table 1 reports the regression parameters, intercept (b_0), slope (b_1), their standard deviations (s_{b_0} and s_{b_1}, respectively), standard deviation of residuals ($s_{y/x}$, for a confidence level of 95%) and correlation coefficient (R), relative to the used standard addition method at the temperature of 40 °C.

The recovery of the analytes was checked by making a second extraction on the same chocolate sample and also by extracting the petroleum ether solution used to eliminate fats. Both these two second extractions did not show any peak of the analytes so that we assumed our extraction procedure as quantitative. The reported chromatograms point out the absence of TYR and PHE in the sample extract while the found amount of DOP, HIS and SER in the extract of the 85% cocoa sample were 9.6, 1.0, 6.1 mg/l, respectively. In the case of the plain chocolate, the found amounts were 8.2, 0.46, 4.8 mg/l, respectively. The concentration in mg/kg can be obtained by multiplying the given values by 10. In our opinion, the determination of the limit of detection (LOD) would be made on the basis of the calibration plot statistics as both blank and analytes are accounted for and this method would be applied to a low standard concentration range [24]. We tried this approach but the external calibration plots gave slopes statistically different from those obtained by

Table 1
Regression parameters of the calibration plots (standard addition method) of the five analytes in the case of 85% cocoa sample

	b_0 (nC min)	b_1 (nC min l mg^{-1})	s_{b_0} (nC min)	s_{b_1} (nC min l mg^{-1})	$s_{y/x}$ (nC min)	R
DOP	35.5	3.71	0.3	0.04	0.5	0.9996
TYR	0.9	3.5	0.4	0.1	1.2	0.998
HIS	8.5	8.2	0.5	0.3	0.9	0.997
SER	27.4	4.5	1.0	0.1	1.8	0.997
PHE	2.5	3.6	1.2	0.2	1.0	0.994

Linear model: $y = b_0 + b_1 x$.

Table 2
LOD and repeatabilities of the different amines computed from the 85% cocoa chocolate sample

	LOD (mg/l)	Concentration (mg/l)	RSD (%)[a]
DOP	0.3	9.6[b]	5
TYR	0.2	0.95[c]	5
HIS	0.1	1.0[b]	7
SER	0.2	6.1[b]	5
PHE	0.3	1.3[c]	3

[a] $N = 5$.
[b] Found concentration.
[c] Added concentration.

standard additions so that this approach is meaningless if a "blank" real matrix is not available, as in the present situation. The Eurachem alternative method [25] is equally unsuitable because, also in this case, the real matrix is not accounted for. Our choice was therefore the use of "3 s" approach in conjunction with the slope of the standard addition calibration plot. The second column of Table 2 reports the computed LODs for each amine. These concentration values in mg/l correspond to 3 mg/kg for DOP, 2 mg/kg for TYR, 1 mg/kg for HIS, 2 mg/kg for SER, 3 mg/kg for PHE. The repeatability (fourth column of Table 2) was computed from five repeated injection of the real samples. As only DOP, HIS and SER were present in the analysed real samples, the concentration levels chosen for the calculation were those effectively present in the sample for DOP, HIS, SER and one addition close to 1.0 mg/l for TYR and PHE (third column of Table 2).

4. Conclusions

The use of a reductive multiple step potential wave-form after the current integration step applied to Au disposable electrodes, together with a gradient separation by cation exchange, allows a selective and sensitive determination of five BAs usually present in chocolate (dopamine, serotonin, tyramine, histamine and 2-phenylethylamine). The sample preparation is very simple consisting in fats elimination with petroleum ether followed by an extraction of the analytes with perchloric acid. The quantification of the analytes in real samples by standard addition method is suitable for the determination of the BAs in complex matrices such as chocolate. Detection limits and repeatabilities of the measurements are good enough to give robust results and to propose the analytical approach for the determination of the studied BAs also in different real matrices.

Acknowledgements

The financial support of the Italian Ministry of University and Research (MIUR) is gratefully acknowledged.

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