Determination of serotonin and its precursors in chocolate samples by capillary liquid chromatography with mass spectrometry detection

V. Guillén-Casla*, N. Rosales-Conrado, M.E. León-González, L.V. Pérez-Arribas, L.M. Polo-Díez

Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, E-28040 Madrid, Spain

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ABSTRACT

A method for the analysis of serotonin (5-HT) and its precursors, 5-hydroxytryptophan (5-HTP) and L-tryptophan (TP) in chocolate samples by capillary liquid chromatography–mass spectrometry (cLC–MS) has been developed. Optimum chromatographic conditions were established by using a personalized multifactorial experimental design. Finally the cLC separation was achieved through a mixture of acetonitrile and 5 mM ammonium formate at pH 4 (3:97, v/v) as mobile phase in gradient elution, setting the injection volume at $10\,\mu L$ and using pure water as injection solvent for focusing purposes on the head of the capillary column. For extraction of targets in chocolate samples a new, fast and simple procedure based on the use of acidic extraction medium and sonication was developed. Working in selected ion mode (m/z 177 for 5-HT, m/z 205 for L-tryptophan and m/z 221 for 5-HTP) detection limits were between 0.01 and $0.11\,\mu g\,g^{-1}$ and linearity was in the concentration range of $0.5–25\,\mu g\,g^{-1}$. Recoveries higher than 76% with RSDs lower than 8% were obtained from spiked samples for all analytes, showing the effectiveness of the proposed method. Serotonin and its precursors were determined in 5 kinds of commonly consumed chocolates with different cocoa contents (70–100%). The highest serotonin content was found in chocolate with a cocoa content of 85% ($2.93\,\mu g\,g^{-1}$). Regarding L-tryptophan, the highest content of this amino acid ($13.27–13.34\,\mu g\,g^{-1}$) was found in chocolate samples with the lowest cocoa content (70–85%). 5-Hydroxytryptophan was not detected in any chocolate samples.

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1. Introduction

Chocolate is a highly demanded food considered as "food of happiness" that contains cocoa. The cocoa is produced through a process of fermenting the seeds from the pods of the cocoa tree (*Theobroma cacao* L.). The beans are processed resulting an intermediate product which is homogenized with sugar, cocoa butter and sometimes with milk to produce commercial chocolate [1].

Although chocolate consumption is often associated with situations related to anxiety, stress or worries due to the stimulating effect of caffeine and other bioactive amines [2], recent studies have attributed beneficial health effects to its consumption such as protective effect against cardiovascular diseases or inhibition of fluid secretion in the small intestine relieving diarrhea [3].

The bioactive indolamine serotonin (5-hydroxytryptamine, 5-HT) is an established neurotransmitter and vasoconstrictor in the central nervous system. Serotonin has a broad activity in human brain, playing an important role in the modulation of anger, aggression, mood and sexuality, appetite, as well in muscle contraction or blood pressure regulation [4–6]. According to de Jong et al. [7], this neurotransmitter is synthesized only from the L-enantiomer of the essential amino acid tryptophan through a short metabolic pathway consisting of two enzymes: tryptophan hydroxylase and amino acid decarboxylase with 5-hydroxytryptophan (5-HTP) as an intermediate [8,9]. On the other hand, interest in the analysis of tryptophan is relevant because it is nutritionally essential for humans and it also takes part in several biological processes [10]. 5-Hydroxytryptophan results due to decarboxylation of serotonin in the presence of vitamin B6 and magnesium. 5-HTP is not found in significant quantities in the diet but it can be obtained from foods with high tryptophan content [11–13].

Pastore et al. [14] and Herraiz [15] detected the presence of serotonin in chocolate at low $\mu g\,g^{-1}$ level. Chocolate contains serotonin, at concentrations which depend on the chocolate type and its cocoa content, dark chocolate containing more than 60% of cocoa is a good source of serotonin and a concentration of $1.4–5\,\mu g\,g^{-1}$ has been reported in it [16]. However, information is scarce about the levels of serotonin and its precursors in chocolate and also, how they are affected during production and processing. Although serotonin

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* Corresponding author at: Department of Analytical Chemistry, Faculty of Chemistry, UCM, Avda. Complutense s/n, E-28040 Madrid, Spain. Tel.: +34 91 394 41 96; fax: +34 91 394 43 29.

E-mail addresses: vguillen@quim.ucm.es, vanesaguillencasla@gmail.com (V. Guillén-Casla).

could be found only in small amounts in chocolate, once tryptophan is within the central nervous system, it is readily converted into serotonin. Moreover, sugar content of chocolate facilitates the absorption and transport of this amino acid on the brain [16].

Several analytical methods including capillary electrophoresis, spectrophotometry, immunoassay, amperometric sensors or thin layer chromatography have been used to determine serotonin [4,17–21]. The major difficulty in using these methods in biological samples is that many substances can interfere with the analytes, making the assay almost undependable [13]. Although the use of gas chromatography coupled to mass spectrometry (GC–MS) has been reported [22,23] and has shown good sensitivity and selectivity, derivatization is required prior to analysis; therefore, high-performance liquid chromatography (HPLC) is the technique most extensively used. HPLC has been coupled to different detection techniques such as electrochemical, fluorescence, UV pulsed amperometry or fluorimetric detection [24–28]. Main drawbacks are related to the derivatization process and low reproducibility owing to the possibility of interferences. On the other hand, HPLC–MS is a powerful technique to determine serotonin and its precursors in complex samples such as chocolate [15], platelet depleted plasma [5], *Griffonia* seed [13], fruits, urine, gut lavage fluid, rat brain, and plants [29–34] by identifying the analytes based on their mass-to-charge ratios thus providing a detection specificity that most other types of detection methods lack.

Nowadays, capillary liquid chromatography (cLC) results particularly advantageous for coupling with MS detection due to the use of more compatible low flow rates [35]. Moreover, cLC is environmentally friendly as well as cost saving. The main drawback of cLC is the loss of sensitivity due to the small volumes or masses injected. In some cases, sensitivity can be increased by the use of the so-called on-column focusing techniques with large injection volumes in which the sample solvent has significantly lower elution strength compared to the mobile phase at the beginning of the chromatographic run [36,37].

Optimization of the chromatographic conditions requires taking into account several adjustable parameters as a whole, and therefore, multivariate approaches and experimental design based on response surface methodologies (RSA) are useful with the aim to model analytical responses and to find optimal factors combination [38]. This process involves finding the values of the experimental factors that maximize peak area and minimize peak width or achieve a balance between resolution and analysis time [37,39,40].

In this paper, a method based on capillary liquid chromatography with MS detection (cLC–MS) for the determination of serotonin and its precursors is presented. Experimental design methodologies based on response surface analysis and multiple response analyses (MRAs) have been employed to achieve optimum chromatographic conditions. Special attention has been paid to the analyte extraction procedure based on two steps, the first one involving fat extraction and the other one analyte extraction. The optimized cLC–MS method and the sample preparation procedure were applied to the analysis of target analytes in chocolate samples with different cocoa contents (70–100%).

2. Experimental

2.1. Chemicals and standards

All chemicals and solvents were of analytical grade and purified water from a Milli-Q system was used in all procedures (Millipore, Bedford, MA, USA). Methanol, acetonitrile (LC–MS quality) and hexane (gradient-HPLC quality) were supplied by Scharlab (Barcelona, Spain). Reagents, including petroleum ether (bp 40–60 °C) and formic acid (99.8%) were purchased from Panreac (Barcelona,

Spain). Glacial acetic acid (99.5%) was from Carlo Erba (Milan, Italy) and ammonium hydroxide (33%) was supplied by Riedel de Häen (Seelze, Germany). L-Ascorbic acid was from Sigma–Aldrich (St. Louis, MO, USA).

The studied analytes L-tryptophan, L-5-OH-tryptophan and serotonin hydrochloride were also provided by Sigma–Aldrich. According to the supplier, chemical purity of all compounds was higher than 98%.

Mobile phases were filtered through Teknokroma nylon membrane filters (Barcelona, Spain) with 0.20 μm pore size in a Visiprep vacuum manifold system (Supelco, Bellfonte, PA, USA), and sample extracts were filtered through PTEF and nylon membrane filters (0.20 μm pore size) from Scharlab.

Analyte standard stock solutions (200 mg L^{-1}) were prepared in water and stored in the dark at 4 °C. Fresh working standard solutions were daily prepared by suitable dilution of the stock solutions.

2.2. Chocolate samples

Chocolate samples with different cocoa contents (70–100%) were purchased from a local supermarket of Madrid. They were stored at room temperature.

2.3. Equipment

Chromatographic analysis by cLC was performed by an Agilent cLC instrument Mod. 1100 Series (Agilent Technologies, Madrid, Spain) which was equipped with a G1376A binary capillary pump, a G1379A degasser and a G1315B diode array detector (500 nL, 10 mm path length). Mass spectrometry detection was carried out by an Agilent 6120 Quadrupole LC/MS. The instrument was operated using an ESI source equipped with a micro electrospray nebulizer in SIM positive mode. Nitrogen was used both as drying and nebulizer gas. The drying gas temperature was set to 325 °C and a flow-rate of 8.0 L min^{-1}. The nebulizer pressure was fixed at 17 psi. The ESI capillary voltage was fixed at 3.5 kV. Data acquisition and processing were made by using the Agilent Chemstation software package for Microsoft Windows.

An external stainless steel sample loop with a volume of 10 μL was placed into a Rheodyne® injection valve. A Zorbax SB-C18 column (150 mm × 0.5 mm I.D., 5 μm) supplied by Agilent (Agilent Technologies, Madrid, Spain) was used, which was thermostatized at 25 °C during the chromatographic run by a MISTRAL programmable oven (Spark Holland, Emmem, The Netherlands).

Extraction of serotonin and its precursors from chocolate samples was carried out in an ultrasound bath provided by P-Selecta; a vortex mixer from VELP Scientifica (Usmate, Italy) and a mechanical shaker model Vibromatic from P-Selecta were also used. A Unicen centrifuge model 21 supplied by Ortoalresa (Madrid, Spain) was used for centrifugation of chocolate extracts. Solvent evaporation under nitrogen stream was carried out by means of a VacElut 20-place vacuum manifold supplied by Varian (Harbor City, CA, USA).

The software package Statgraphics Plus version 5.0 (Statistical Graphics Corp., Rockville, MD, USA), running under Windows XP, was used for application of chemometric tools.

3. Procedures

3.1. Chromatographic determination of serotonin, 5-hydroxytryptophan and L-tryptophan by cLC–MS

A linear gradient elution program was applied at a flow rate of 20 μL min^{-1}. The mobile phase consisted of ammonium formate 5 mM at pH 4 (solvent A) and acetonitrile (solvent B). Initial solvent B percentage was 3% (v/v) which was held for 2 min, increasing

linearly to 7% in 1 min and held for 5 min, returning to initial conditions in 2 min and maintained for 12 min. Run analysis took place in 12 min.

MS full-scan spectra were acquired for each analyte with identification purposes in automatic mode. The ESI source was operated in positive ionization mode. [M+H]+ adducts were monitored working in selected ion monitoring (SIM) for quantitative analyses using m/z ions of 177, 205 and 221 for serotonin, L-tryptophan and 5-hydroxytryptophan, respectively.

To improve sensitivity large injection sample volumes (10 μL) and pure water as injection solution were employed for on-column focusing.

3.2. Extraction of serotonin, 5-hydroxytrytophan and L-tryptophan from chocolate

Sample treatment according to the method proposed by Pastore et al. [14] was slightly modified for analysis by cLC–MS. Briefly, a portion of about 10 g of chocolate was finely crushed by using a knife. Intermixing was accomplished by the sampling tabling method [41]; 1 g was exactly weighted and fats were twice extracted with two aliquots of 5 mL petroleum ether by shaking for 10 min in a mechanical shaker and then centrifuging for 10 min at 4200 rpm. The petroleum ether extracts were combined and discarded and the residual ether was eliminated under nitrogen stream. Analytes were then extracted from the chocolate residue with 5 mL of 0.2 M formic acid in the ultrasonic bath for 20 min and centrifuged at 4200 rpm for 45 min. A volume of 50 μL of the final extract was filtered through a 0.2 μm PTEF membrane and diluted with pure water up to 1 mL for focusing purposes. A total of 10 μL of the resulting solution was injected in the cLC system and analyzed under conditions described in Section 3.1.

3.3. Determination of serotonin, 5-hydroxytrytophan and L-tryptophan in chocolate samples

The above cLC–MS method and extraction procedure were applied to chocolate samples with different cocoa contents (70–100%). For recovery studies, chocolate samples were spiked with the targets at levels between 2 and 9 μg g^{-1}. Their concentrations were determined by the standard addition method.

4. Results and discussion

4.1. Optimization of the cLC–MS method by experimental design

Initial experiments showed that to achieve a reasonable analytes peak separation, a mobile phase with a high aqueous content was required. Due to this reason, different ratios of acetonitrile/ammonium formate buffer and acetonitrile/ammonium acetate were tested in the range of 3–8% for the modifier. From this study, the best conditions were achieved by using slight linear elution gradients using a mobile phase consisting of ammonium

formate (solvent A) and acetonitrile (solvent B). The elution gradient program was applied at a flow rate of 20 μL min^{-1} as follows: initial conditions of 3% (v/v) of solvent B were held for 2 min, increasing linearly to 7% in 1 min. This composition was held for 5 min, returned to initial conditions in 2 min and finally maintained for 12 min. This gradient allowed a complete separation between 5-hydroxytrytophan and the injection peak, avoiding the instability in the base line on the first minutes of analysis and, at the same time, an excessive L-tryptophan's peak broadening and therefore, a loss of sensitivity for this amino acid.

When cLC is applied for trace analysis and high injection volumes are used; achieving the optimum on-column focusing conditions and working in the most appropriate pH and buffer concentration in the mobile phase are critical factors to reach a good analyte ionization efficiency in ESI-MS [42] and therefore considerable values on peak area. Although peak width in linear gradient elution gets a quite constant value for high retention, potentially, it may depend on other variables such as elution strength of the mobile phase or volume and nature of the injection solution. Moreover, the dispersion caused by injection of high sample volumes can be expected to be dependent on other variables, including composition of injection solution, pH and nature of the analytes injected [36]. Consequently, mobile phase pH, buffer concentration, injection volume and sample focusing conditions were assumed to be the most important factors to optimize in order to get the experimental conditions that provide better sensitivity in MS detection.

The optimization study of the chromatographic conditions was done by using personalized multifactorial experimental design. To obtain the response function, four factors including pH (4–6) and buffer concentration of the mobile phase (5–20 mM), injection volume (5–20 μL) and focusing conditions (with or without buffer solution) were considered, and two (pH and focusing conditions) or three levels (buffer concentration and injection volume) were studied for each selected factor. All the experiments were carried out at 25 °C and 20 μL min^{-1} flow rate, by using standard solutions of 50 μg L^{-1}, prepared with the convenient mobile phase. A total of 48 experiments (36 runs and 12 replicates corresponding to the central zone of the studied domain) for each analyte were carried out (Table 1, Supplementary data).

To evaluate the sensitivity in MS detection, peak area and peak width were selected as experimental responses. Responses (y) were correlated with experimental factors x_1, buffer concentration; x_2, injection volume; x_3, buffer pH; x_4, focusing conditions with or without buffer by means of a first-order general polynomial equation:

$$y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_1x_2 + a_6x_1x_3 + a_7x_1x_4$$
$$+ a_8x_2x_3 + a_9x_2x_4 + a_{10}x_3x_4 \tag{1}$$

Coefficients a_0–a_{10} represent the weight of each studied factor (x_1–x_4) and their interactions on area and peak width (Table 2, Supplementary data).

Mathematical models with acceptable correlation coefficients (R^2), higher than 0.781 and good standard estimation errors (SEE)

Table 1
Analytical characteristics obtained from standard solutions.

Analyte	Linearity		LOD[a] (μg L^{-1})	LOQ[b] (μg L^{-1})	Intra-day precision (RSD, %)[c], n = 5			Inter-day precision (RSD, %)[c], n = 15		
	Concentration range (μg L^{-1})	R^2			Peak area	Peak width	t_r (min)	Peak area	Peak width	t_r (min)
5-Hydroxytrytophan		0.9995	0.44	1.46	4.7	1.7	0.3	5.3	1.1	0.4
Serotonin	3–500	0.9991	0.50	1.65	2.4	1.8	0.6	3.3	2.8	0.8
L-Tryptophan	0.25–500	0.9982	0.04	0.13	2.3	2.1	0.1	4.5	1.9	0.4

[a] Detection limit (signal/noise ratio 3:1).
[b] Quantitation limit (signal/noise ratio 10:1).
[c] Coefficient of variation for serotonin and its precursors standard solutions at a concentration level of 50 μg L^{-1}.

Table 2
Analytical characteristics and recovery studies from serotonin and its precursors in chocolate samples.

Cocoa content (%)	LOD[a] (µg g⁻¹)			LOQ[b] (µg g⁻¹)			Linearity range[c] (µg g⁻¹)			Added (µg g⁻¹)	Recovery (%)[d] (mean ± RSD)		
	5-HTP	5-HT	TP	5-HTP	5-HT	TP	5-HTP	5-HT	TP		5-HTP	5-HT	TP
100	0.10	0.09	0.01	0.33	0.32	0.06	0.5–25	6.0		2.5	76 ± 1	84 ± 5	86 ± 3
											85 ± 3	86 ± 1	85 ± 3
										9.0	94 ± 3	97 ± 2	95 ± 2
100 ecological	0.12	0.10	0.02	0.41	0.34	0.07	0.5–25	6.0		2.5	80 ± 4	82 ± 6	90 ± 5
											94 ± 2	89 ± 7	95 ± 1
										9.0	95 ± 2	97 ± 1	97 ± 1
99	0.06	0.11	0.01	0.22	0.37	0.09	0.5–25	6.0		2.5	81 ± 5	86 ± 6	86 ± 3
											97 ± 2	86 ± 3	93 ± 4
										9.0	98 ± 2	97 ± 2	97 ± 2
85	0.08	0.12	0.01	0.26	0.40	0.04	0.5–25	6.0		2.5	85 ± 4	91 ± 2	89 ± 8
											90 ± 5	88 ± 1	94 ± 3
										9.0	96 ± 3	97 ± 2	97 ± 4
70	0.07	0.11	0.01	0.25	0.37	0.04	0.5–25	6.0		2.5	81 ± 7	89 ± 7	86 ± 3
											96 ± 2	87 ± 2	94 ± 3
										9.0	96 ± 3	95 ± 2	93 ± 2

[a] Detection limit (signal/noise ratio 3:1).
[b] Quantitation limit (signal/noise ratio 10:1).
[c] $n = 7$, $R^2 \geq 0.972$.
[d] $n = 3$.

in the range 3258–7476 and 0.017–0.023 for peak area and peak width were achieved, showing the validity of these equations. The coefficients which showed higher weight on both responses studied were a_1–a_3 corresponding to buffer concentration, injection volume and buffer pH, respectively. Regarding interaction terms, the coefficients of the interaction between buffer concentration and injection volume (a_5) or injection volume and buffer pH (a_8) showed an important weight in peak area.

In the studied domain and for all targets, both peak area and width were significantly affected by injection volume, which increased with the increase of the injected volume.

In general, buffer concentration and pH mainly affected peak area, which decreased when these two factors increased. Only in the case of serotonin's peak width, buffer concentration had a significant positive effect, showing the highest value of this response at the high concentration value. Regarding focusing conditions, in general, they affected peak width of serotonin; the use of buffered injection solutions produced an increase in this response. Moreover, pH showed a strong effect on peak width of serotonin and L-tryptophan. While in the case of serotonin an increase in pH produced a decrease in peak width, in tryptophan and 5-hydroxytryptophan this fact produced the opposite effect. The main effect of pH on peak width is probably related to the targets pK_a. In the case of L-tryptophan (pI 5.93) and 5-hydroxytryptophan (pI 5.76) which have two pK_a values, the isoelectric points of both were close to 6 so, net charge of these analytes is zero around this pH. Consequently, maximum hydrophobic interactions between stationary phase and these amino acids occurred, and wider peaks are obtained [43]. In the case of serotonin (pK_a = 10.2) this effect was not found. The nature of the injection solutions has a remarkable effect on serotonin's peak width; when the injection solution is buffered, wider peaks are obtained. The opposite effect is found in the other targets; a peak width increase is obtained when the injection solution does not contain buffer. This effect could be related to acid–base properties of L-tryptophan and 5-hydroxytryptophan in which pH effect was stronger.

The interaction among factors was significant. Regarding peak area, the interaction between buffer concentration and injection volume had a negative effect in the case of 5-hydroxytrytophan and tryptophan whereas the interaction term between injection volume and pH only has a negative effect for serotonin and tryptophan. On the other hand, interactions both between injection volume and pH and between injection volume and sample composition only had a remarkable effect in peak width of L-tryptophan and serotonin, respectively.

In order to visualize the most important effects on peak area and width, estimated response surfaces were obtained for each analyte through their respective equations. These three-dimensional graphs showed distorted planes due to the importance of interaction factors. As an example, response surfaces obtained for serotonin adjusting the two main variables at a buffer concentration of 5 mM and using pure water as injection solution can be observed in Fig. 1.

To get optimum chromatographic conditions for separation of serotonin and its precursors and taking into account the obtained experimental responses, multiple response analysis (MRA) was tried. The optimization criterion involved both maximizing peak area and minimizing peak width. This chemometric procedure allowed determining the combination of experimental factors which simultaneously optimize the two studied responses and therefore, that maximize the desirability function over the selected region. A mixture of ACN/ammonium formate 5 mM pH 4 (3:97, v/v) as mobile phase in the specified elution gradient mode, setting injection volume at 10 µL and using pure water as injection solution were selected as a compromise to get optimum conditions (maximum value of desirability function = 0.7). Fig. 2 shows
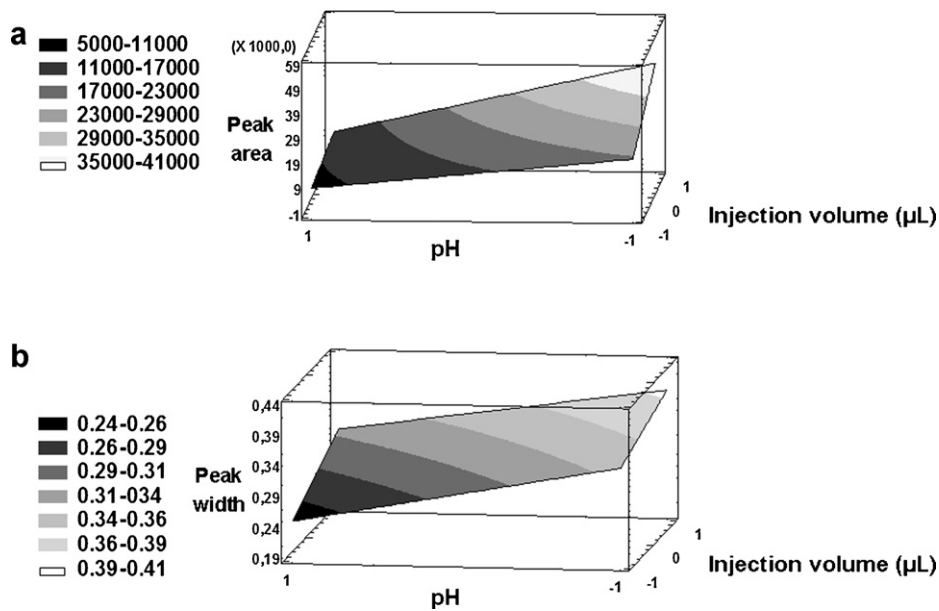
Fig. 1. Estimated response surfaces (normalized) of serotonin obtained for peak area (a) and peak width (b), using the variables with higher weight in the model at a buffer concentration of 5 mM and using pure water as injection solution.

a representative cLC–MS chromatogram obtained under these conditions.

Under these conditions, the estimated response surfaces predicted peak area values of 21,653, 31,497 and 64,881 for 5-hydroxytrytophan, serotonin and L-tryptophan, and peak widths of 0.28 (L-tryptophan), 0.29 (5-hydroxytrytophan) and 0.36 (serotonin). These expected values were experimentally assessed and results were in agreement with those predicted, achieving RSDs from the predicted values in the range 1.7–7% for peak area and 0.20–2.4% for peak width. In addition, variance analysis of one factor showed that there were no significant differences between experimental results and those predicted by Eq. (1) with a confidence level of 95%.

Regarding mass spectrometry analysis, results were in agreement which those obtained by Peterson et al. [4] and Numan and Danielson [44]. Essentially two types of ions were observed: $[M+1]^+$ molecular ions and intense adduct ions that resulted from $[M+1-17]^+$ corresponding to the lost either of an −OH or an −NH$_3$ group; both are possible in case of serotonin (m/z 160) and 5-hydroxytrytophan (m/z 204). However, L-tryptophan, which does
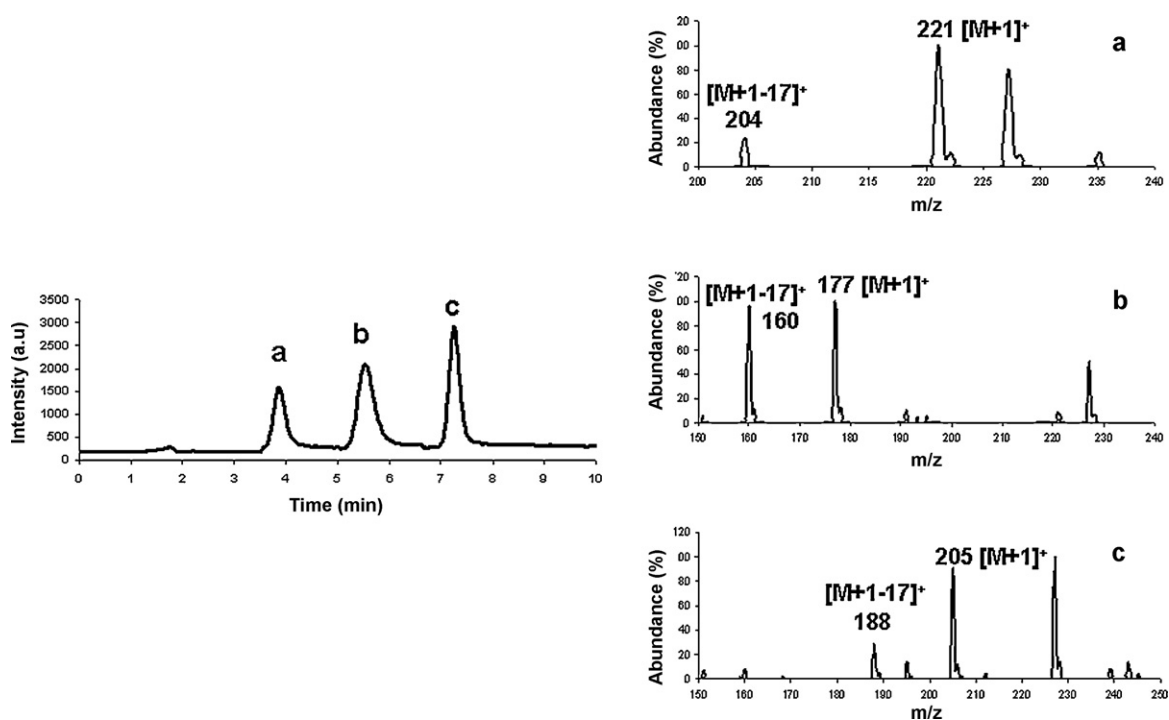
Fig. 2. cLC–MS chromatogram (SIM mode) from a 50 μg L^{-1} standard solution and representative mass spectra from serotonin and its precursors obtained under selected conditions specified in Section 3.1, (a) 5-hydroxytrytophan (m/z 221), (b) serotonin (m/z 177) and (c) L-tryptophan (m/z 205).

Table 3
Concentration of serotonin and its precursors in chocolate samples, and ANOVA test table for concentration of analytes among cocoa content.

Cocoa content (%)	5-Hydroxytrytophan ($\mu g\,g^{-1}$)	Serotonin ($\mu g\,g^{-1}$)	L-Tryptophan ($\mu g\,g^{-1}$)	*F*-ratio		*P*-value[*]	
				Serotonin	L-Tryptophan	Serotonin	L-Tryptophan
Chocolate 100% cocoa content[a]	n.d.	1.30 ± 0.06	4.06 ± 0.04				
Chocolate 100% cocoa content (ecological product)[a]	n.d.	1.58 ± 0.07	6.60 ± 0.04	260.78	901.18	0.0001	0.0001
Chocolate 99% cocoa content[a]	n.d.	1.38 ± 0.03	4.19 ± 0.04				
Chocolate 85% cocoa content[a]	n.d.	2.93 ± 0.01	13.27 ± 0.02				
Chocolate 70% cocoa content[a]	n.d.	1.53 ± 0.02	13.34 ± 0.02				

n.d. = not detected.

[a] $n=3$, mean ± RSD, referred to g of chocolate.

[*] Since the *P*-value of the *F*-test is less than 0.05, there is a statistically significant difference between the mean concentrations from one level of doses to another at the 95.0% confidence level.

not contain a hydroxyl, still formed this type of ions (m/z 188) suggesting that the NH_3 group was lost. Fig. 2 shows mass spectra of the targets obtained under selected chromatographic conditions.

4.2. Analytical characteristics from standard solutions

LODs and LOQs were estimated at 3 and 10 times the signal-to-noise ratio (S/N), respectively [45]. These parameters were calculated experimentally by analysis of a series of decreasing concentrations of analyte solutions. The obtained values for each analyte are shown in Table 1, in which it can be observed that LODs and LOQs were lower or equal than 0.5 and 1.65 $\mu g\,L^{-1}$, respectively.

Evaluation of linearity was done based on analyses of standard solutions prepared in pure water. Linearity was in the range of 3–500 $\mu g\,L^{-1}$ ($n=9$) for 5-hydroxytrytophan and serotonin and 0.25–500 $\mu g\,L^{-1}$ ($n=12$) for L-tryptophan. When regression analysis was performed high correlation coefficients (R^2) for all peak areas were obtained (between 0.9982 and 0.9995).

The intra-day precision was evaluated by injecting standard solutions at 50 $\mu g\,L^{-1}$, five consecutive times on the same day ($n=5$). The inter-day variation was similarly conducted in three successive days ($N=15$). The intra-day and inter-day variability, expressed as RSD (%), was evaluated for peak area, peak width and retention time (t_r). Results are summarized in Table 3. It can be seen, the intra-day variation was lower than or equal than 4.7%, 2.1% for peak area and peak width and 0.6% for t_r. Similar RSD values were obtained for inter-day precision ($\leq 5.3\%$, $\leq 2.8\%$ or $\leq 0.8\%$ for peak area, peak width and t_r), showing the suitability of the proposed chromatographic separation.

4.3. Determination of serotonin and its precursors in chocolate samples

The usefulness of the developed cLC–MS method was evaluated by analyzing chocolate samples with different cocoa contents. Firstly an extraction procedure was individually optimized and validated for each analyte.

4.3.1. Optimization of the extraction method

Sample treatment according to the method proposed by Pastore et al. [14] was slightly modified for analysis by cLC–MS. Extraction efficiency studies were carried out from chocolate aliquots of 1 g spiked with 25 $\mu g\,g^{-1}$ of each target.

In order to achieve the maximum analyte recovery, several variables such as nature of the cleaning extraction organic solvent as well as acidic extraction medium, ultrasonication time and nature of the filter were evaluated.

The first sets of experiments were carried out to select hexane, or petroleum ether as cleaning extraction organic solvent. When chocolate was twice extracted with two petroleum ether aliquots

of 5 mL cleaner extracts were obtained avoiding the presence of fats that could interfere the determination of analytes. Thus, petroleum ether was selected as the most appropriate cleaning extraction organic solvent.

Extraction was carried out with 5 mL from different acidic media (formic acid 0.2 M, acetic acid 0.2 M and ascorbic acid 1 mM). As reported in the literature for the extraction of different biogenic amines in foods [46], a strong acidic medium is required. Among all, best recoveries for serotonin were obtained with formic acid as extraction medium. Therefore, it seems clear that the use of an acidic medium contributes to desorb biogenic amines and it promotes their extraction from the chocolate matrix.

Ultrasonication time was tested at 10, 20 and 30 min. When the extraction was made during 10 min, considerable recoveries decrease was observed for all the analytes. On the other hand, an extraction time of 30 min promoted a large co-extraction of endogenous matter causing obstruction of the filter used. Consequently, 20 min was selected as the most appropriate ultrasonication time. Finally, the nature of the filter was studied; PTEF and nylon filters were evaluated. When nylon filters were used, a significant decrease of serotonin recovery was observed. It could be probably due to the analyte retention in the filter. Consequently, PTEF filters were selected as the most convenient.

4.3.2. Matrix effect study

The potential matrix effect in the suppression/enhancement of the analytes ESI/MS signal was studied. Zrostlíková et al. [47] suggest that organic compounds present in the sample with concentrations exceeding 10^{-5} M may compete with the analyte for access to the droplet surface for gas phase emission; or if the sample contains non-volatile matrix components, droplets are prevented from reaching their critical radius and surface field, hence a reduction of the ion signal for an analyte occurs. As a result of these matrix effects the response of a standard analyte in pure solvent can differ significantly from that in matrix sample.

To evaluate this matrix effect, some chocolate samples were spiked with increasing and known amounts of target standard solutions. The initial concentrations of serotonin and its precursors in chocolate were obtained from the intercept of the linear regression with the y-axis. At the same time these concentrations were determined by using external calibration. The results obtained by the two calibration methods (direct determination and standard addition) were compared, showing that the concentrations determined with standard addition were slightly different than those calculated with the external calibration; so, matrix effect was investigated by comparing the slopes of the direct calibration curve for each analyte with the one obtained by the standard addition method in each chocolate sample. Therefore, an *F* test was applied to compare the standard deviations of the slopes of both calibration methods. In most cases significant differences among them at a 95% confidence level were found. Then, a Student's *t*-test comparison was applied.

This test showed that there was a statistically significant difference between the slopes at the 95.0% confidence level. It can be concluded therefore that there is a slight matrix effect at the concentration levels of this study. In order to eliminate or compensate this possible matrix effect, standard addition method was selected as the most appropriate method to apply in chocolate samples.

4.3.3. Analytical characteristics and recovery study in chocolate samples

Performance characteristics of the optimized extraction method were established from samples of different cocoa contents (70–100%), studying linearity, LODs, LOQs and accuracy (expressed as recovery).

Linearity and correlation coefficients of the calibration graphs ($n = 7$) for each compound were obtained in the range 0.5–25 µg g^{-1} for serotonin and its precursors. Good linearity of the calibration curves was observed for the targets at concentrations within the tested intervals, with R^2 higher than 0.972 in all cases.

In the case of serotonin and L-tryptophan, LODs and LOQs were estimated by analyzing chocolate samples at a concentration close to 3 and 10 times the signal-to-noise ratio (S/N) by the standard addition method, for 5-hydroxytrytophan in the same way as explained in Section 4.2 by using matrix matched calibration. Resulting values for serotonin and its precursors are shown in Table 2. Good results were achieved for all compounds with LOD and LOQ values lower than or equal to 0.12 and 0.40 µg g^{-1} chocolate, respectively.

To evaluate the efficiency and applicability of the proposed extraction methodology, a recovery study was carried out. Several amounts of serotonin and its precursors in the range of 2.5–9.0 µg g^{-1} (Table 2) were added to aliquots of 1 g chocolate to obtain a final concentration of tryptophan around the maximum levels found in some varieties of pure cocoa beans [48]. Recoveries were determined by analysis of spiked chocolate samples with target compounds at the three selected concentration levels; after spiking, samples were left to stabilize for 30 min in dark, to allow sample equilibration. Recoveries were calculated by using the standard addition method for serotonin and tryptophan determining the analyte initially present in the sample and subtracting their contribution to the respective value obtained from the spiked sample; the matrix-matched calibration was used for 5-hydroxytrytophan. Repeatability, expressed as RSD (%), was evaluated by performing three replicates at each spiked level. It can be observed in Table 2; best recoveries were obtained at the highest spiked levels for all targets.

4.3.4. Application of the proposed cLC–MS to the analysis of serotonin and its precursors in commercial chocolate samples

The proposed extraction method combined with the cLC–MS allowed establishing the total content of the analytes in chocolate samples. Table 3 summarizes the obtained results. It can be seen, 5-hydroxytrytophan was not detected in any samples. These results are in agreement with those authors who suggest that 5-HTP is not found in significant quantities in the diet [12,13,17]. On the other hand, serotonin and L-tryptophan were found in chocolate of any cocoa content in significant amounts. In order to detect possible significant differences between mean concentrations of each compound from one type of chocolate to another they were compared by means of ANOVA test; Table 3 shows the results of F-ratios and P-values obtained. As both serotonin and L-tryptophan showed significant differences among cocoa contents at the 95% confidence level, multiple range tests allowed determining among which percentages of cocoa significant differences occurred.

Regarding L-tryptophan, significant differences among samples were observed for a cocoa content of 70%. The highest content (13.27–13.34 µg g^{-1}) was found in chocolate samples with the lowest cocoa content (70–85%). The results obtained are in agreement with some authors reporting that the addition of milk to chocolate (decrease in cocoa content) increases the protein content of food due to the presence of milk proteins [49].

Serotonin synthesis requires an adequate amount of vitamin B6 and magnesium to transform tryptophan into serotonin [50]. Dark chocolate usually contains higher amounts of magnesium and vitamin B6 than chocolate with lower cocoa content or milk chocolates [49]. This could be the reason of the decrease of L-tryptophan concentration in chocolate with the highest cocoa content.

The content of tryptophan found in the selected commercial chocolates is remarkably lower than the concentrations found in pure cocoa beans [50], suggesting the amounts of this amino acid could be affected during production and processing.

Regarding serotonin, significant differences between samples were observed from a chocolate with a cocoa content of 85%, being this chocolate the one which showed the highest content (2.93 µg g^{-1}). The obtained results were in agreement with those referred by some authors [2,9,10] reporting the presence of serotonin in chocolate samples in a concentration range of 0.10–2.9 µg g^{-1} suggesting that it could be the reason of chocolate potential craving sensation or the reason of abnormal behavior and psychological sensation similar to those of other addictive substances.
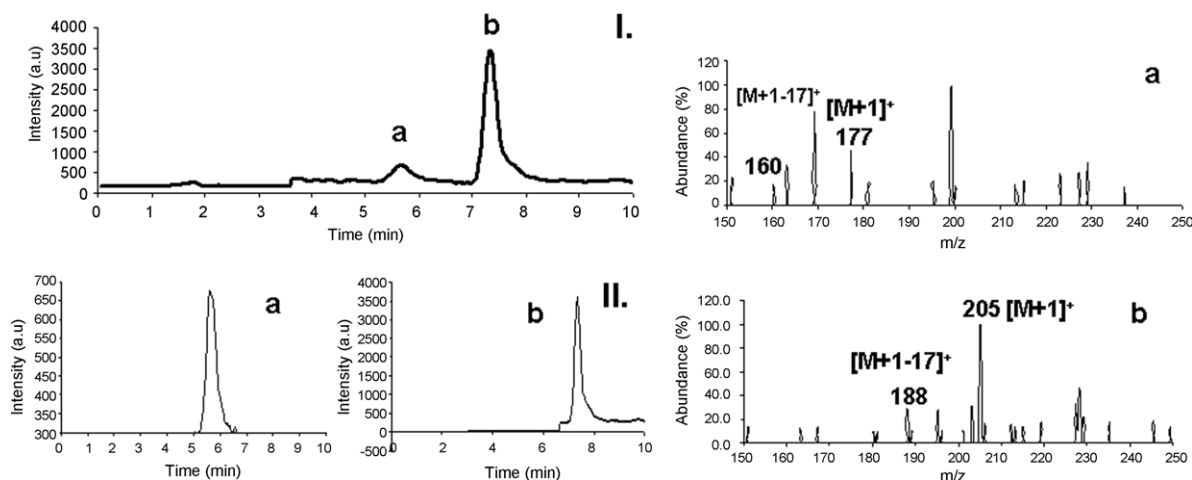

Fig. 3. cLC–MS chromatogram (I. SIM mode, II: extracted ion mode) from a chocolate sample with 99% cocoa content and representative mass spectra obtained under selected conditions specified in Section 3.1, (a) serotonin (m/z 177) and (b) L-tryptophan (m/z 205).

Fig. 3 shows an experimental chromatogram and representative mass spectra of targets in a chocolate sample with a cocoa content of 99% obtained by using the proposed methods.

5. Conclusions

The developed cLC–MS method combined with the optimized sample extraction procedure was found to be suitable to determine serotonin and its precursor's (5-hydroxytrytophan and L-tryptophan) at low $\mu g\,g^{-1}$ levels in chocolate samples having different cocoa contents (70–100%). The proposed rapid, easy and simple sample extraction procedure has minimized common problems of sample clean-up when complex matrices are analyzed, being suitable for capillary liquid chromatography and providing acceptable and constant target recoveries. Thus, serotonin and L-tryptophan could be detected in all chocolate samples in the concentration range of low $\mu g\,g^{-1}$, while 5-hydroxytrytophan was not found in any studied chocolate samples.

In addition, this work has also demonstrated the effectiveness of chemometric tools to achieve the best chromatographic conditions in complex separations.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.11.037.

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