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High-performance Liquid Chromatographic Determination of Four Biogenic Amines in Chocolate

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Some biogenic amines occur in a wide variety of foods including cheese, fish, bakery products, milk products and chocolate. This study was undertaken to analyse and quantify four of the biogenic amines thought to occur in chocolate. Tyramine, tryptamine, 2-phenylethylamine and serotonin (5-hydroxytryptamine) were chosen as the amines of interest. Two high-performance liquid chromatographic (HPLC) systems were used for the final analysis of amine extracts. Both systems employed dual detection, with the first using ultraviolet absorbance at 254 nm and the formation of a post-column *o*-phthaldehyde derivative. The second method used ultraviolet absorbance at 254 nm and the natural fluorescence of the four amines. Thin-layer chromatography (TLC) was performed on all of the extracts to provide further confirmation. All four amines were detected and quantified at varying levels in extracts of several kinds of chocolate and chocolate liquors.

Keywords: *High-performance liquid chromatography*; *biogenic amines*; *chocolate*; *food*

Tyramine, tryptamine, 2-phenylethylamine and serotonin are members of the pressor amine group and tend to cause a rise in blood pressure.[1,2] Many foods contain amines that are members of this pressor amine group including tomatoes[3] and other fruits, as well as fish, meat and poultry products.[4,5,6,7] As early as 1919 Pognic *et al.* suggested that allergies could cause migraines and especially implicated chocolate and the amines found in chocolate.[7] Recent literature contains conflicting information about the presence or absence of these various amines in chocolate.[9–15]

This study was undertaken to determine the levels of four of these amines in various chocolate products and cocoa liquors. Samples were de-fatted and extracted using recognised procedures for biogenic amines.[14,16,17]

The final determination was carried out on one of two HPLC systems. Both used reversed-phase HPLC with dual detection and ultraviolet absorbance at 254 nm as one of their detection modes. The systems differed in that one used the formation of a post-column *o*-phthaldehyde derivative[18] of the biogenic amines while the other used natural fluorescence. These two analytical systems gave comparable results; recovery and precision studies of samples and standards showed the methods to be satisfactory. Results using both HPLC systems are presented for several chocolate and cocoa liquor samples.

Experimental

HPLC System 1

This was a modular system consisting of an M6000A solvent delivery system, Model 440 absorbance detector at 254 nm and U6K universal injector (Waters Associates). The column was Bondapak C_{18} Reversed Phase (Waters Associates). The flow-rate was 1.0 ml min^{-1}. The fluorescence detector was a Gilson, Spectra Glo Filter Fluorimeter equipped with excitation filters (340 nm) and emission filters (418 nm) (Gilson Medical Electronics).

The post-column reaction apparatus consisted of a Milton Roy Mini-Pump (Laboratory Data Control) used to pump the *o*-phthaldehyde solution into a mixing chamber. The pump was equipped with a home-made pulse damper in the form of a standing air column. The pump effluent was passed into one port of a three-port mixing chamber at a flow-rate of 1.0 ml min^{-1}. After mixing, the *o*-phthaldehyde - amine complex was carried into a reaction coil consisting of 10 m of 0.009-in i.d. stainless-steel tubing kept at a constant temperature of 40 °C by immersion in a circulating water-bath.

HPLC System 2

This was a modular system using the same solvent delivery system, ultraviolet detector, injector and HPLC column as the HPLC System 1. The flow-rate was 1.3 ml min^{-1}. The fluorescence detector used was a Varian SF-330 spectrofluorimeter equipped with an HPLC flow cell [excitation wavelength = 285 nm, emission wavelength = 320 nm (cut-off filter)].

Reagents

HPLC mobile phase 1. Acetic acid (0.2 M) at pH 2.8 in HPLC water, the latter was prepared by passing distilled water through ion-exchange and organic absorber cartridges, and finally through a 0.1-μm filter.

HPLC mobile phase 2. An 80 + 20 (V/V) mixture of 0.2 M potassium dihydrogen phosphate solution adjusted to pH 3.7 with orthophosphoric acid and methanol. This mixture was prepared, de-gassed and passed through a 0.1-μm final system filter.

TLC developing solvent. The solvent system was chloroform - methanol - concentrated ammonia solution (28% m/V), 12 + 7 + 1.

Boric acid buffer. Boric acid solution (0.4 M) adjusted to pH 10.3 $\pm$ 0.2 with solid potassium hydroxide.

o-Phthaldehyde reagent. This reagent was prepared by dissolving 0.32 g of *o*-phthaldehyde in 100 ml of ethanol and diluting to 1 l with boric acid buffer.

Ninhydrin solution. A mixture of 0.300 g of ninhydrin, 100 ml of butan-1-ol and 3 ml of acetic acid.

Standard solutions. Tyramine and tryptamine (Calbiochem-Behring); 2-phenylethylamine and serotonin (Sigma Chemical Co.). The 2-phenylethylamine was re-distilled prior to use. All standards were prepared in HPLC mobile phase to a final concentration of 0.1 μg μl^{-1}.

Samples. Chocolate samples were of nationally distributed types while chocolate liquor samples were obtained from the Hershey Chocolate Company.

Apparatus

Centrifuge. Capable of 2000 rev min^{-1}.
Oven. Thelco Blue M.
Sorvall Omni-Mixer. DuPont Instruments.
Dual pen recorder.
TLC developing tank.
Silica gel plates. Si-60 (0.25 mm) from EM Labs.

Extraction

The procedure of Kissinger[16] was modified for the extraction of the four compounds. All samples were de-fatted with petroleum spirit (boiling range 36–60 °C) prior to extraction. One gram of de-fatted chocolate or cocoa liquor is mixed with 20 ml of 0.1 N perchloric acid using a Sorvall Omni-Mixer at setting 7 to 10 min. Alternatively this extraction could be carried out by using a wrist action shaker for 45 min. The homogenate is transferred into a centrifuge tube and centrifuged at 2000 rev min^{-1} for 10 min. The supernatant liquid is adjusted to pH 10.3 $\pm$ 0.1 with concentrated ammonia solution and then stored overnight in a refrigerator at −4 °C. The resulting solution is filtered through a Whatman No. 41 filter-paper or its equivalent. The filtrate is saturated with solid sodium chloride and then extracted four times with 5 ml of an ethyl acetate - acetone (2 + 1) mixture. After each extraction the mixture is briefly centrifuged to help separate the two layers. The first three extractions are transferred into a clean test-tube using a Pasteur pipette. The fourth extraction is filtered through Whatman IPS phase-separating paper. The organic extracts are combined and dried with anhydrous sodium sulphate. After decanting, the sodium sulphate is washed with an additional 2 ml of the ethyl acetate - acetone (2 + 1) mixture. The water-free extracts are evaporated to dryness under nitrogen at 20 °C and then dissolved in 1 ml of the HPLC mobile phase.

Analysis

The use of two HPLC systems was a result of the arrival of new equipment that would allow direct measurement of the amines by natural fluorescence[19]; earlier studies used the post-column *o*-phthaldehyde derivative formation HPLC method. Inject 20 μl of the extract and compare with injections of standards. Calculate the concentration in the extract by comparison of the peak heights of the sample and standards. Figs. 1–3 show chromatograms of standards using ultraviolet, post-column derivatisation and natural fluorescence detection, respectively. Figs. 4–6 depict chromatograms of extract using the same methods of detection.

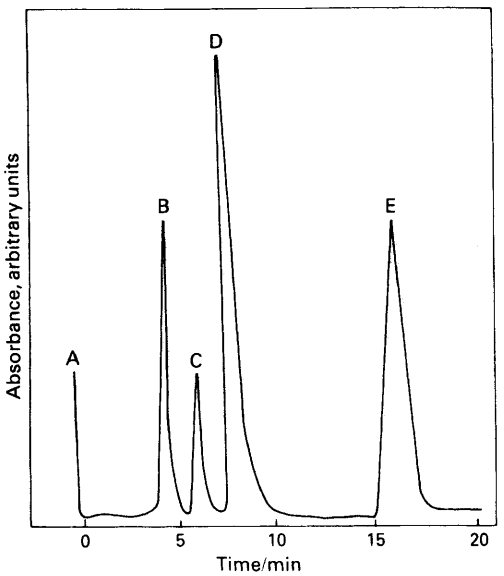

Fig. 1. Chromatogram of standards using ultraviolet detection. A is the injection point. Sample contained 20 μg each of: B, tyramine; C, serotonin; D, 2-phenylethylamine; E, tryptamine. Column, μBondapak C_{18}. Mobile phase, 0.2 M acetic acid. Detector, Waters Associates, Model 440, at 254 nm, 0.02 a.u.f.s.

Thin-layer chromatography. Spot 10 μl of each of the extracts and standards on to Si-60 TLC plates. Develop to 12–14 cm with chloroform - methanol - concentrated ammonia solution (28%) (12 + 7 + 1). Air dry the plates, then spray with ninhydrin solution and heat at 110 °C for 10 min to reveal the spots. Compare the R_F values obtained for the standards with those of the extracts.

Results

Recovery studies were conducted on the matrices of cocoa liquor and milk using the *o*-phthaldehyde post-column reaction and subsequent determination. Spiking for all recoveries was performed by the addition of standard to the perchloric acid extract. Additional recovery studies were carried out on the milk chocolate matrix using native fluorescence detection. The four amines were added at four different levels to the cocoa liquor, three different levels to the whole milk and three different levels to the milk chocolate. Tables I–III show the averages of duplicate determinations.

Tables I–III show good method accuracy using either the *o*-phthaldehyde derivative or natural fluorescence detection methods. Table IV shows the results for five 1-g samples of de-fatted milk chocolate assayed in duplicate using natural fluorescence detection.

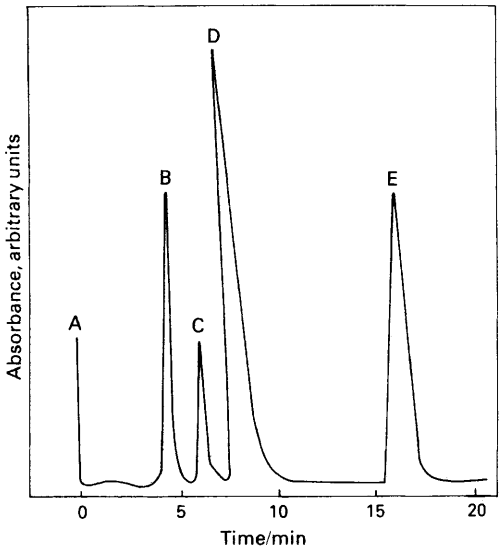

Fig. 2. Chromatogram of standards after post-column formation of *o*-phthaldehyde derivatives using fluorimetric detection. A is the injection point. Sample contained 2 μg each of: B, tyramine; C, serotonin; D, 2-phenylethylamine; E, tryptamine. Column, μBondapak C_{18}. Mobile phase 0.2 M acetic acid. Detector, Gilson Spectra-glo fluorimeter.

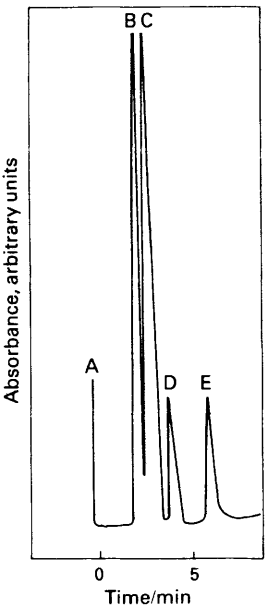

Fig. 3. Chromatogram of standards with detection by means of natural fluorescence. A is the injection point. Sample contained: 0.5 μg tyramine (B); 0.5 μg serotonin (C); 14 μg 2-phenylethylamine (D); 0.54 μg tryptamine (E). Column, μBondapak C_{18}. Mobile phase, 0.2 M potassium dihydrogen phosphate solution, pH 3.7 containing 20% of methanol by volume. Detector, Varian SF-300 with HPLC flow cell.

Tables V and VI give information pertaining to standard and sample precision studies. The ultraviolet data was included only for completeness as the ultraviolet mode was used as an additional confirmatory method and not for quantitative purposes. The lower limits of detection of four of the amines are shown in Table VII.

The average biogenic amine contents of eight selected chocolate liquors are given in Table VIII and the average amine contents of five chocolate products are given in Table IX. These tables show results obtained using both the *o*-phthaldehyde derivative and natural fluorescence methods.

Discussion

The results described indicate the presence of the biogenic amines of interest in chocolate liquor and chocolate products. As shown in Tables VIII and IX, these amines occur at varying levels and in varying ratios. It was not possible to gain information about fermentation patterns from the amine concentrations in the various liquor types, these levels vary as would be expected in natural products. One of the factors that complicates this problem is the tryptamine level. Tryptamine is a likely precursor of the plant growth hormone indole acetic acid.[19] In an attempt to gain further information about the fermentation patterns, similar samples should be analysed during all stages of growth and fermentation. From this information it might be possible to arrive at some meaningful conclusions.

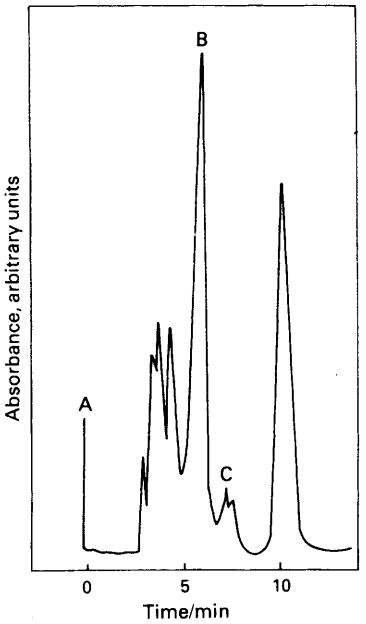

Fig. 4. Chromatogram of cocoa extract using ultraviolet detection. A is the injection point; B, tyramine; and C, serotonin. Column, μBondapak C_{18}. Mobile phase, 0.2 M acetic acid. Detector, Waters Associates, Model 440, at 254 nm, 0.02 a.u.f.s.

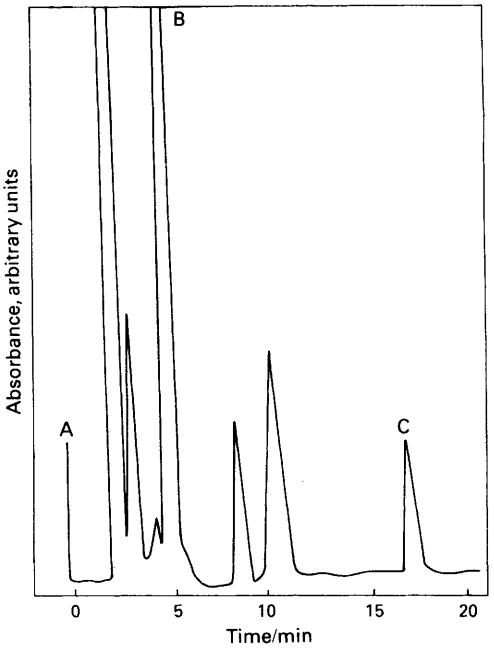

Fig. 5. Chromatogram of cocoa extract after post-column formation of *o*-phthaldehyde derivatives using fluorimetric detection. A is the injection point; B, tyramine; and C, tryptamine. Column, μBondapak C_{18}. Mobile phase, 0·2 M acetic acid. Detector, Gilson Spectra-glo fluorimeter.

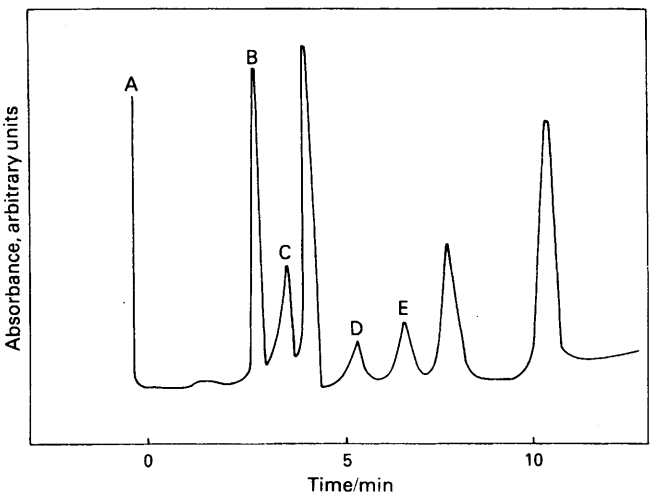

Fig. 6. Chromatogram of cocoa extract with detection by means of natural fluorescence. A is the injection point; B, tyramine; C, serotonin; D, 2-phenylethylamine; and E, tryptamine. Column, μBondapak C_{18}. Mobile phase, 0.2 M potassium dihydrogen phosphate solution, pH 3.7, containing 20% of methanol by volume. Detector, Varian SF 330 with HPLC flow cell.

TABLE I

AVERAGES OF DUPLICATE RECOVERIES FROM COCOA LIQUOR USING *o*-PHTHALDEHYDE DERIVATISATION DETECTION

Sample size = 1 g of de-fatted material in each instance, $n = 2$.

Amine	Amount added/ $\mu g\ g^{-1}$	Amount recovered/ $\mu g\ g^{-1}$	Recovery, %
Tyramine	10	9.56	95.6
	20	18.90	94.5
	50	46.80	93.6
	100	93.60	93.6
			Average = 94.3
Tryptamine	10	9.49	94.9
	20	18.40	92.0
	50	45.50	91.0
	100	88.60	88.6
			Average = 91.6
Serotonin	10	8.96	89.6
	20	19.20	96.0
	50	48.70	97.4
	100	95.80	95.8
			Average = 94.7
2-Phenylethylamine ..	10	8.45	84.5
	20	17.80	89.0
	50	44.50	88.9
	100	92.30	92.3
			Average = 88.7

The analytical data presented in this work are in agreement with those obtained by Kenyhercz and Kissinger who found tyramine levels of 8–11 $\mu g\ g^{-1}$ in cocoa.[14] The data also seem satisfactory when compared with that of Ingles *et al.*[14] where tyramine was determined in one sample at the 5 $\mu g\ g^{-1}$ level by HPLC. However, other reports have suggested that tyramine is absent from cocoa[1–3] so that the literature is confusing concerning even the presence or absence of these compounds in cocoa.

Examination of whole milk extracts indicates the presence of compounds with a primary amino group. This work did not indicate the presence of the four biogenic amines in whole milk extracts.

TABLE II

RECOVERY FROM WHOLE MILK USING *o*-PHTHALDEHYDE DERIVATISATION DETECTION

Amine	Amount added/ $\mu g\ g^{-1}$	Amount recovered/ $\mu g\ g^{-1}$	Recovery, %
Tyramine	20	18.40	92.0
	50	44.90	89.8
	100	96.80	96.8
			Average = 92.9
Tryptamine	20	17.60	88.0
	50	46.80	93.6
	100	91.80	91.8
			Average = 91.1
Serotonin	20	16.30	81.5
	50	44.70	89.4
	100	94.20	94.2
			Average = 87.8
2-Phenylethylamine ..	20	17.80	89.0
	50	42.40	84.8
	100	89.40	89.4

TABLE III

AVERAGE OF DUPLICATE RECOVERIES FROM MILK CHOCOLATE USING NATURAL FLUORESCENCE DETECTION

Sample size = 1 g.

Amine	Amount added/ $\mu g\ g^{-1}$	Amount recovered/ $\mu g\ g^{-1}$	Recovery, %
Tyramine	10	9.78	97.8
	25	23.40	93.6
	50	47.10	95.6
			Average = 95.7
Tryptamine	10	9.61	96.1
	25	24.30	97.2
	50	47.80	95.6
			Average = 96.3
Serotonin	10	9.48	94.8
	25	23.60	94.4
	50	46.55	93.1
			Average = 94.1
2-Phenylethylamine	10	9.34	93.4
	25	24.75	99.0
	50	49.65	99.3
			Average = 97.2

TABLE IV

MULTIPLE MILK CHOCOLATE ANALYSES

Tryptamine could not be detected in any of the samples.

	Amine content/$\mu g\ g^{-1}$		
Sample number	Tyramine	2-Phenylethylamine	Serotonin
1	12.02,12.02	0.42,0.46	27.10,27.30
2	11.97,12.07	0.44,0.44	27.30,27.10
3	12.03,11.99	0.41,0.47	26.80,27.60
4	12.06,11.98	0.43,0.45	26.92,27.48
5	11.95,12.09	0.40,0.48	27.03,27.37
Mean	12.03	0.44	27.2
Standard deviation	0.045	0.025	0.25
Coefficient of variation, %	0.38	5.68	0.92

TABLE V

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PRECISION STUDIES ON STANDARDS

Amine	Amount injected/ μg	n	Detection mode	Coefficient of variation, %
Tyramine	20	9	Ultraviolet	0.78
	5	5	Post-column derivatisation	1.44
	5	5	Fluorescence	1.52
Tryptamine	20	9	Ultraviolet	2.27
	5	5	Post-column derivatisation	4.63
	5	5	Fluorescence	3.91
2-Phenylethylamine	20	9	Ultraviolet	7.44
	15	5	Post-column derivatisation	8.74
	5	5	Fluorescence	6.02
Serotonin	20	9	Ultraviolet	2.01
	5	5	Post-column derivatisation	2.97
	5	5	Fluorescence	2.23

TABLE VI

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PRECISION STUDIES ON SAMPLES

Amine	Amount injected/ μg	*n*	Detection mode	Coefficient of variation, %
Tyramine	20	9	Ultraviolet	2.19
	5	5	Post-column derivatisation	1.53
	5	5	Fluorescence	0.93
Tryptamine	20	9	Ultraviolet	3.17
	5	5	Post-column derivatisation	5.03
	5	5	Fluorescence	4.46
2-Phenylethylamine ..	20	9	Ultraviolet	6.79
	15	5	Post-column derivatisation	6.08
	7	5	Fluorescence	5.58
Serotonin	20	9	Ultraviolet	1.98
	5	5	Post-column derivatisation	2.36
	5	5	Fluorescence	2.92

This study is not yet complete as other amines have been found to occur in chocolate. Kenyhercz and Kissinger,[16] using HPLC with an electrochemical detector, found octopamine, metanephrine and synephrine in chocolate. These amines need to be investigated further and quantified as they are conversion products of tyramine. The other conversion products of tyramine[6,20,21] are methyltyramine, dopamine, hordiene, methyloctopamine and noradrenaline.

These and other amines can be formed by several biochemical pathways, including amino acid decarboxylation, aldehyde amination, phospholipid decomposition and thermal amino acid composition.[6] Other amines found in chocolate are[6] methylamine, butylamine, dimethylamine, isobutylamine, ethylamine, isoamylamine, trimethylamine and triethylamine.

TABLE VII

DETECTION LIMITS FOR FOUR OF THE AMINES

Amine	Detection mode	Lower limit/μg g^{-1}
Tyramine	Post-column derivatisation	1.0
	Natural fluorescence	0.5
Tryptamine	Post-column derivatisation	0.5
	Natural fluorescence	0.5
Serotonin	Post-column derivatisation	2.0
	Natural fluorescence	0.25
2-Phenylethylamine ..	Post-column derivatisation	9.0
	Natural fluorescence	0.25

TABLE VIII

AVERAGE BIOGENIC AMINE CONTENTS OF CHOCOLATE LIQUORS

In each instance the sample size was 1 g and the mean of 2 determinations is shown.

	Amine content/μg g^{-1}							
	Tyramine		Serotonin		Tryptamine		2-Phenylethylamine	
Type	Post-column derivatisation	Natural fluorescence	Post-column derivatisation	Natural fluorescence	Post-column derivatisation	Natural fluorescence	Post-column derivatisation	Natural fluorescence
Costa Rica	7.96	7.96	15.80	16.10	3.54	3.52	N.D.*	4.34
New Guinea ..	14.70	14.70	9.19	9.23	2.88	2.93	N.D.	6.56
Lagos	2.69	2.73	3.96	3.85	0.99	1.04	N.D.	4.38
Equador	8.37	8.42	12.40	12.21	2.41	2.38	N.D.	5.13
Light Lagos ..		2.06		0.92		0.52	N.D.	2.19
Malaysian		2.74		0.18		1.07	N.D.	3.28
Sanchez		0.73		0.79		0.71	N.D.	2.55
Caracas		1.09		3.82		2.04	N.D.	8.02

* N.D. = not detected.

Table IX

Average biogenic amine content of some chocolate products

In each instance sample size = 1 g and the result given is the mean of 2 determinations.

	Amine content/$\mu g\ g^{-1}$							
	Tyramine		Serotonin		Tryptamine		2-Pheylethylamine	
Product type	Post-column derivatisation	Natural fluorescence	Post-column derivatisation	Natural fluorescence	Post-column derivatisation	Natural fluorescence	Post-column derivatisation	Natural fluorescence
Milk chocolate A ..	11.90	12.02	26.60	27.20	N.D.*	N.D.	N.D.	0.44
Milk chocolate B ..	5.96	6.04	8.51	8.33	N.D.	N.D.	N.D.	2.13
Milk chocolate C ..	3.83	3.76	5.32	5.25	N.D.	N.D.	N.D.	6.60
Milk chocolate D ..	4.27	4.41	1.04	1.02	N.D.	N.D.	N.D.	4.40
Dark chocolate ..	11.90	12.02	8.46	8.64	N.D.	N.D.	N.D.	3.84

* N.D. = Not detected.

The levels in chocolate are low when compared with levels found in other foods. Cheese has been reported as having tryptamine and 2-phenylethylamine levels ranging from below the limits of detection to over 400 $\mu g\ g^{-1}$. Sausage has been reported to contain levels of tyramine ranging from below the limit of detection to over 350 $\mu g\ g^{-1}$, 2-phenylethylamine levels from below the limit of detection to almost 700 $\mu g\ g^{-1}$ and tryptamine levels from below the limit of detection to over 50 $\mu g\ g^{-1}$. Meat and cheese are not the only foods that contain amines.

This work is not a comprehensive study of the amines that occur in chocolate and more investigations need to be made; however, it provides a method for the traction, detection and quantitation of the four biogenic amines at trace levels in chocolate products.

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