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SIMULTANEOUS EXTRACTION AND QUANTITATION OF SEVERAL BIOACTIVE AMINES IN CHEESE AND CHOCOLATE

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SUMMARY

A method is described for simultaneous extraction and quantitation of the amines 2-phenylethylamine, *tele*-methylhistamine, histamine, tryptamine, *m*- and *p*-tyramine, 3-methoxytyramine, 5-hydroxytryptamine, cadaverine, putrescine, spermidine and spermine. This method is based on extractive derivatization of the amines with a perfluoroacylating agent, pentafluorobenzoyl chloride, under basic aqueous conditions. Analysis was done on a gas chromatograph equipped with an electron-capture detector and a capillary column system. The procedure is relatively rapid and provides derivatives with good chromatographic properties. Its application to analysis of the above amines in cheese and chocolate products is described.

INTRODUCTION

The investigation of the presence of bioactive amines in foodstuffs is becoming an increasingly active area of research. It is well known that patients taking monoamine oxidase (MAO) inhibitors must avoid certain foodstuffs since these foods may cause a number of adverse effects, including hypertensive crises, in such individuals[1,2] when taken concomitantly with these drugs. It has been proposed that this is due to the presence of *p*-tyramine in these foods; this amine, which is normally metabolized by intestinal MAO, is taken up into adrenergic nerves and causes extensive release of the catecholamine noradrenaline. However, it is also possible that other structurally related amines such as 2-phenylethylamine or *m*-tyramine, may contribute to the effect; these amines are known to have strong releasing effects on catecholamines from nerve terminals *in vitro*[3,4]. It is also conceivable that histamine may be responsible for some of the effects seen when patients on MAO inhibitors ingest certain foodstuffs. Absorption of histamine from the gut is reported to be facilitated by hydrazine-type MAO inhibitors (such as phenelzine), but not by MAO inhibitors of the phenylcyclopropylamine type (*e.g.* tranylcypromine). It has been reported that the former compounds, but not the latter, inhibit diamine oxidase[5,6]. Intraduodenal administration of the histamine-rich yeast product Marmite to cats results in marked electromyographic changes and potentiation of spinal cord reflexes; these changes

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can be mimicked by administering histamine concomitantly with an MAO inhibitor[7].

Consumption of foods rich in amines may produce problems other than those mentioned above. In about 30% of patients with classical migraine, injection of *p*-tyramine or eating *p*-tyramine-containing foods can result in the production of headaches. In these patients, there seems to be a deficiency of the enzyme responsible for the formation of the sulfate conjugate of *p*-tyramine[8,9]. It has also been reported that foodstuffs with a high 2-phenylethylamine content, such as chocolate, can bring on attacks in certain migraine sufferers[9,10]. Sandler *et al.*[9] reported that migrainous patients have significantly lower 2-phenylethylamine- and tyramine-oxidizing ability than normal subjects. Histamine is of further interest since this amine is thought to be responsible for many of the symptoms observed in scombroid poisoning from consumption of fish[11–13].

Little information is available on measurement of the amount of tryptamine in foods, but this indolealkylamine may be of interest since it has been reported to have rather strong effects on the release of catecholamines and the vasoconstrictor substance 5-hydroxytryptamine from nerve terminals[3,14]. 5-Hydroxytryptamine is present in relatively large quantities in many tissues of the body and there is now a large body of evidence indicating that it may be a neurotransmitter, particularly in the central nervous system[15]. Introduction of tryptamine or 5-hydroxytryptamine into the afferent circulation is reported to cause a release of prostaglandins and other vasoactive substances into the systemic circulation[16]. In this regard, it is noteworthy that intravenous administration of prostaglandin E_1 can lead to headaches similar to those observed in spontaneous migraine[17].

2-Phenylethylamine and tryptamine, lipophilic substances which can cross the blood-brain-barrier with ease, are endogenous constituents of many tissues, including brain. Since they are excellent substrates for MAO, it is conceivable that inhibition of this enzyme may result in large amounts of 2-phenylethylamine and tryptamine from foodstuffs entering the circulation. Marked behavioural syndromes can be produced in laboratory animals[18–20] by injection of these amines, or their α-methyl derivatives (amphetamine and α-methyl-tryptamine respectively), which are protected from MAO by their α-methyl group. Chronic abuse of amphetamine in humans can result in a condition which is virtually indistinguishable clinically from paranoid schizophrenia[21], and it has been proposed that 2-phenylethylamine may be involved in the etiology of certain types of schizophrenia[22,23]. LSD-like physiological effects in dogs have been reported after injection of tryptamine[24].

At physiological pH, polyamines (putrescine, cadaverine, spermidine, spermine) are known to be protonated at all nitrogen sites; that is, they are polycations at physiological pH. This makes it possible for the polyamines to react with a number of negatively charged molecules, such as nucleic acids, proteins and anionic sites of membranes, and it has been proposed that polyamines are involved in cell growth and replication[25,26]. A major stimulus of the present interest in polyamine research evolved from the fact that enhanced polyamine metabolism was found in fast growing tumours[27,28]. The possibility that the growth rate of tumours may be hindered by inhibiting polyamine synthesis[29] has further heightened research interest in these amines.

Administration of polyamines to laboratory animals can produce a wide spectrum of physiological responses, such as curare-like paralysis of the musculature, sedation and hypothermia, hyperglycemia and convulsions[30].

Polyamines are distributed widely in nature and their presence in foodstuffs has recently attracted considerable attention in the field of food chemistry. In the presence of heat (*e.g.* in cooking), putrescine and cadaverine may be converted to pyrrolidine and piperidine, respectively[31]. If these amines are formed they may in turn be nitrosated, in the presence of nitrite, to form carcinogenic nitrosamines[32]. It is well-known that nitrite is a normal constituent of human saliva, and it has been suggested that nitrosamines can be formed in the gastrointestinal tract[33,34]. Bjeldanes *et al.*[35] suggested that there may be a synergistic relationship between histamine and the diamines, indicating that scombroid poisoning may be the consequence of ingesting a combination of histamine and diamines.

Much still remains unknown about concentrations of the amines in food products which would be required to cause possible adverse effects, and this is obviously an area deserving of further detailed investigation. Many of the studies cited above investigated only one class of amine or a small number of amines. Obviously there are several types of bioactive amines present in food products, and a comprehensive study of levels of all the amines mentioned above would be useful. We report here a rapid method, utilizing gas chromatography with electron-capture detection (GC–ECD), for simultaneous analysis of several bioactive amines and describe its application to cheese and chocolate.

The presence of a number of amines in a variety of foodstuffs has now been well established[36–51]. Several techniques have been utilized for such analysis, and these include thin-layer, paper and ion-exchange chromatographic[52–60] procedures, colorimetry[61,62], spectrophotometry[38,63], fluorometry[64–68], radioenzymic procedures[50,69], high-performance liquid chromatography (HPLC)[70–80], gas chromatography[39,81–87] and combined gas chromatography–mass spectrometry (GC–MS)[88,89].

Fluorometric methods have been reported as being laborious[83] and may present specificity problems, particularly if not used in combination with separation techniques such as HPLC[39,86,90]. Paper chromatographic techniques are semi-quantitative at best and often imprecise at low levels[39], while colorimetric and spectrophotometric procedures are often non-specific and tedious. Radioenzymic procedures have been developed for analysis of a number of bioactive amines and provide high sensitivity (see ref. 91 for review); these assays have been used more frequently for analysis in tissues and body fluids than in food products[50]. Disadvantages of this type of assay include the necessity of handling and disposing of radiochemicals and difficulties in separating some structural isomers; in addition only one or a small number of amines can be assayed practically in each sample. HPLC techniques have become very popular in recent years, as evidenced by the surge in publications utilizing this methodology. Certainly HPLC often provides a convenient means of analysis and has the advantage over GC and GC–MS of being useful for heat-sensitive compounds. However, MS provides identification of the compound of interest (or a derivative of it) and when combined with GC provides a high degree of selectivity and sensitivity; HPLC–MS is also available but still in its infancy. A major disadvantage of GC–MS is the large financial commitment required for initial purchase, operation and maintenance. It has been our experience that GC combined with a capillary column and an electron-capture detector provides a specific, sensitive and relatively inexpensive alternative; confirmation of structures of derivatives can

be carried out by interfacing the GC column with MS, and subsequent routine analysis can be done by capillary GC–ECD alone.

EXPERIMENTAL

Chemicals and reagents

The following chemicals, unless specified, were obtained from Sigma (St. Louis, MO, U.S.A.): *tele*-methylhistamine dihydrochloride, tryptamine hydrochloride, 2-phenylethylamine hydrochloride, *p*-tyramine hydrochloride, cadaverine dihydrochloride, 2-(4-chlorophenyl)ethylamine, putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, di-2-(ethylhexyl)phosphoric acid, histamine dihydrochloride (Calbiochem, Los Angeles, CA, U.S.A.), *m*-tyramine hydrochloride (Vega Biochemicals, Tucson, AZ, U.S.A.), *o*-tyramine (Dr. B. A. Davis, Saskatoon, Canada), pentafluorobenzoyl chloride (Aldrich, Milwaukee, WI, U.S.A.) potassium bicarbonate (analytical reagent grade; Mallinkrodt, Paris, KY, U.S.A.), sodium carbonate (Fisher Scientific, Fair Lawn, NJ, U.S.A.), ethyl acetate and toluene (each glass distilled) (Caledon Labs., Georgetown, Canada), ammonium hydroxide (Anachem, Portland, OR, U.S.A.), benzene (pesticide grade, Fisher Scientific), acetonitrile (ACS, Fisher Scientific), perchloric acid (Fisher Scientific), hydrochloric acid (ACS grade, The McArthur Chemical Company, Montreal, Canada). 2-(4-Chlorophenyl)ethylamine was purchased as the free base and converted to the hydrochloride salt by Dr. F. M. Pasutto.

Procedures for cleaning glassware

All glassware was rinsed with tap water before immersing in biodegradable Sparkleen (Fisher Scientific) solution. Subsequent thorough washing (and rinsing with distilled water) of beakers, flasks and measuring cylinders was done in a dishwasher (Miele Electronic G715). The glass tubes in which the extractions and analyses were conducted were immersed in Sparkleen solution, placed in an ultrasonic cleaner (Mettler Electronics) containing a solution of Decon 75 concentrate (BDH Chemicals), 20 ml to 1 l, and ultrasonicated for a minimum of 1 h. The glass tubes were then stacked in stainless-steel wire mesh baskets and rinsed with hot distilled water in the dishwasher. All glassware, after rinsing, was air-dried in a mechanical convection oven (Model 29, Precision Scientific Group).

Gas–liquid chromatography

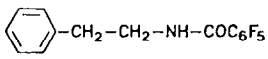
A Hewlett-Packard 5880A gas chromatograph equipped with a 15 mCi ^{63}Ni source electron-capture detector was used. The capillary column used was an SE-54 fused-silica column, 15 m × 0.25 mm I.D. Helium at 2 ml/min was used as carrier gas and argon–methane (95:5) at 35 ml/min as make-up gas to the detector. The following oven programme was employed: initial temperature of 105°C (maintained for 0.5 min), increasing at a rate of 25°C per min to 240°C. After maintaining it at this level for 3.5 min, the temperature was increased again at the same rate to 330°C. The temperature of the injection port was 250°C and that of the detector was 345°C. Injection volumes used were 1 μl or less.

Extraction and derivatization

Approximately 1 g of cheese or chocolate was cut into tiny pieces before homogenization in 10 volumes of ice-cold 0.4 *M* perchloric acid containing 10 mg% EDTA. The suspension was centrifuged for 15 min at 12 000 *g* at 0° to precipitate protein and an aliquot of the clear supernatant (1–2 ml) was used for analysis. For quantitation purposes, 2-(4-chlorophenyl)ethylamine was added to the 0.4 *M* perchloric acid during its preparation to serve as internal standard (final concentration: 1 μg/ml). After refrigerated centrifugation, a portion (2 ml) of the clear supernatant was basified by the addition of solid potassium bicarbonate. The potassium perchlorate precipitate was removed by brief centrifugation. Extraction of amines was performed by shaking the supernatant with 3 ml of the liquid ion-pairing compound di-2-(ethylhexyl)phosphoric acid (2.5% v/v in chloroform) for 10 min. After centrifugation at 1000 *g* for 5 min to separate the layers, the aqueous phase was aspirated off and discarded. The amines were back-extracted by shaking with 2.5 ml 0.5 *M* hydrochloric acid for 5 min. After centrifugation at 1000 *g* for 5 min, the hydrochloric acid layer was retained and made alkaline using a small excess of solid sodium carbonate. This was followed by the addition of 3 ml of extracting-derivatizing mixture made up of benzene–acetonitrile–pentafluorobenzoyl chloride (PFBC) in the ratio 9:1:0.01 (v/v/v). The mixture was shaken on a vortex mixer for 15 min at room temperature. The two phases were separated by centrifugation (5 min) at 1000 *g*. The organic phase was retained and taken to dryness under a stream of nitrogen. The dry residue was reconstituted with 300 μl toluene. This toluene layer was washed by shaking with 1 ml 1 *M* ammonium hydroxide for 15 s. After a brief centrifugation, the toluene layer was retained and 1 μl was injected on the SE-54 fused silica (15 m) capillary column for GC–ECD analysis.

Amine concentrations were measured using the ratio of peak heights of compounds of interest to the peak height of the internal standard. These peak height ratios were compared to those in a standard (calibration) curve, which was prepared for each batch of analyses. Construction of a calibration curve was done by adding known, varying amounts of authentic standards and a constant amount of internal standard to a series of tubes and carrying these tubes through the assay procedure in parallel with the sample tubes. Calculations were performed using a Hewlett-Packard (HP) 86 microcomputer with an HP 9130A flexible disc drive coupled to an HP 82905B printer and an HP 7475 plotter.

Although GC–ECD was used for the routine analysis, structures of the final derivatives were confirmed initially by GC–MS. The mass spectrometer was a Hewlett Packard (HP) 5985A operated in the electron-impact (EI) mode. The GC–MS system also consisted of an HP 5840A gas chromatograph, an HP 2648A graphics terminal, an HP 9876A printer, an HP 7920 disc drive (software), and an HP 21MX series E computer (Hardware). Operation conditions were as follows: ion source temperature, 200°C; interface temperature, 275°C; column pressure, 10 p.s.i.; accelerating voltage, 2200 eV; ionization voltage, 70 eV; scan speed, 100 a.m.u./s; and dwell time, 200 ms. The capillary column and oven programme were the same as those described above in the text.



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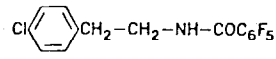

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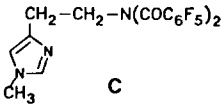

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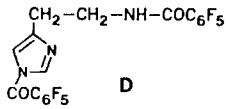

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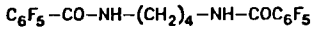

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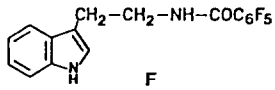

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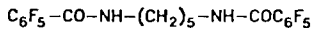

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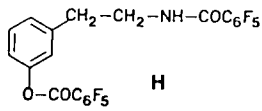

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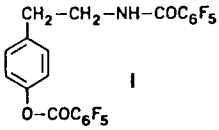

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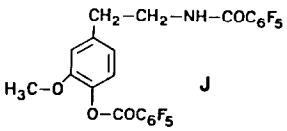

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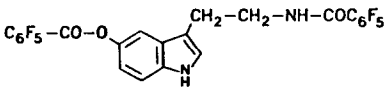

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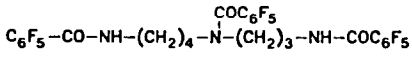

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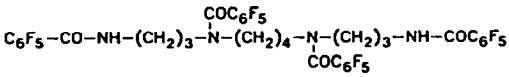

M

Fig. 1. Structures of the derivatized amines formed during the assay procedure. Derivatives of 2-phenylethylamine (A); 2-(4-chlorophenyl)ethylamine, internal standard (B); *tele*-methylhistamine (C); histamine (D); putrescine (E); tryptamine (F); cadaverine (G); *m*-tyramine (H); *p*-tyramine (I); 3-methoxytyramine (J); 5-hydroxytryptamine (K); spermidine (L); and spermine (M).

RESULTS AND DISCUSSION

Several authors have reported the use of PFBC as a derivatizing reagent for amines and phenolic amines[92–100]. This reagent frequently produces derivatives with very high sensitivity on the electron-capture detector and can react under anhydrous or aqueous conditions. The procedure described in the present paper provides a rapid and sensitive procedure for simultaneous extraction and derivatization of a number of amines with biological activity. The "on-column" sensitivites (ECD responses at least twice the blank response) were demonstrated to range from 5 pg (2-phenylethylamine) to 20 pg (tryptamine). The derivatives have excellent chromatographic properties, with little peak tailing evident, and are stable at −20°C for long periods of time. Recoveries of the amines were high in most cases, with those for the polyamines being virtually quantitative; recoveries for the other amines were as follows (means of six experiments): 2-phenylethylamine, 87%; *m*-tyramine, 82%; *p*-tyramine, 80%; *tele*-methylhistamine, 53%; histamine, 66%; tryptamine, 80%; 5-hydroxytryptamine, 42%; and 3-methoxytyramine, 79%. Calibration curves prepared by using 5–6 concentrations of the standard were utilized in each assay run, and were shown to be linear (correlation coefficient >0.99) over a 100-fold range of concentrations.

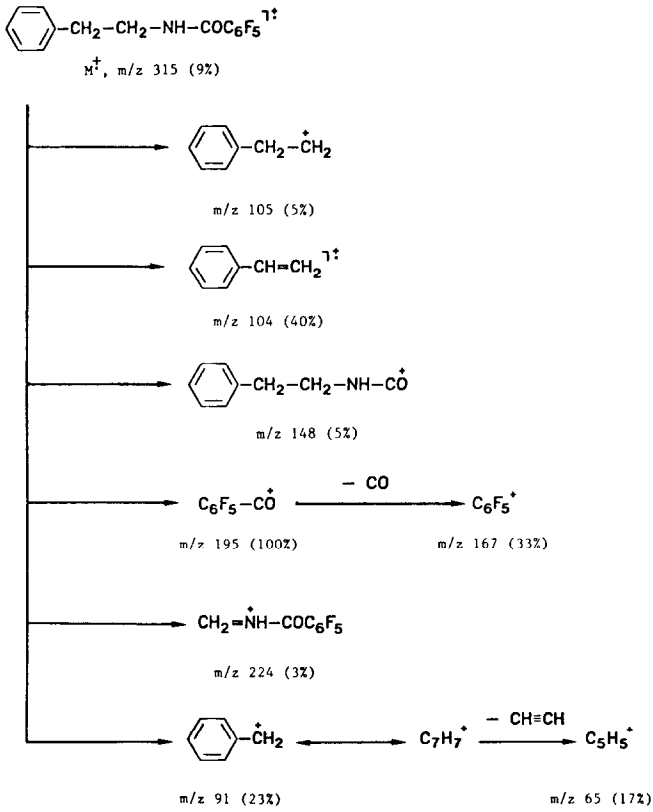

Fig. 2. Proposed mass spectral fragmentation of the pentafluorobenzoyl (PFB) derivative of 2-phenylethylamine.

The structures of the derivatives were confirmed by combined GC–MS and were consistent with the structures shown in Fig. 1. Proposed fragmentation patterns for pentafluorobenzoyl derivatives of representative amines are shown in Figs. 2–7. Only the mass spectrum of *tele*-methylhistamine was inexplicable. Unlike histamine and other amines investigated, *tele*-methylhistamine appears to be doubly derivatized on the same aliphatic nitrogen atom. This possibility is now being studied using other physico-chemical techniques.

A number of compounds [toluene, benzene, ethyl acetate, cyclohexane, toluene–acetonitrile (9:1)] were tested for suitability as a solvent for PFBC, but the cleanest GC baselines were obtained when benzene was used in the procedure.

The procedure was applied to cheese and chocolate samples, and results from four brands of each are shown in Table I. The results are the means (standard error of the mean, S.E.M.) from at least 3 samples of each, and at least 2 portions of each sample were analyzed. Examples of typical GC traces are given in Fig. 8. Preliminary findings from the application of the technique to other foodstuffs such as beer, wine, canned tuna and salmon, and soya sauce appear promising.

Thus, a rapid, sensitive procedure has been developed which utilizes extractive derivatization with PFBC and subsequent separation utilizing a gas chromatograph

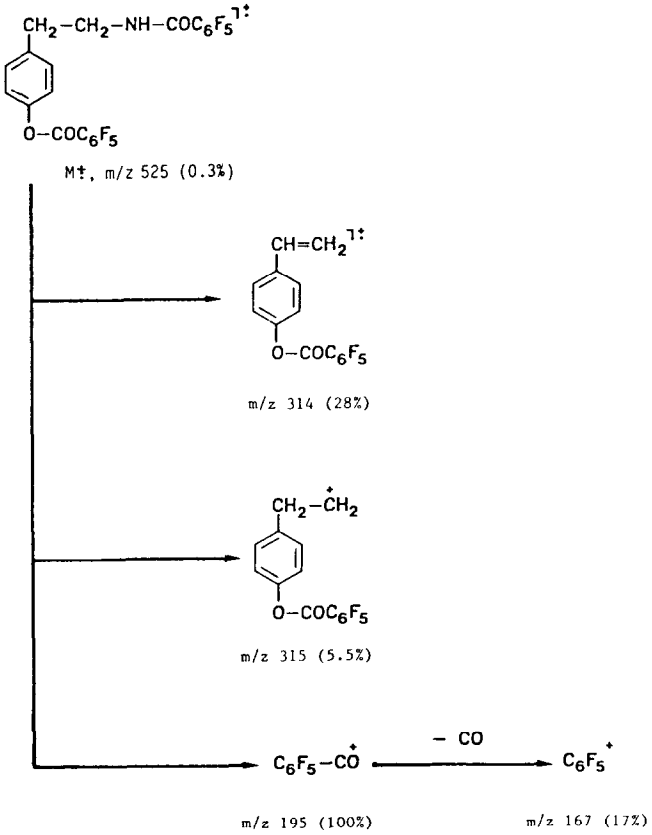

Fig. 3. Proposed mass spectral fragmentation of the PFB derivative of *p*-tyramine.

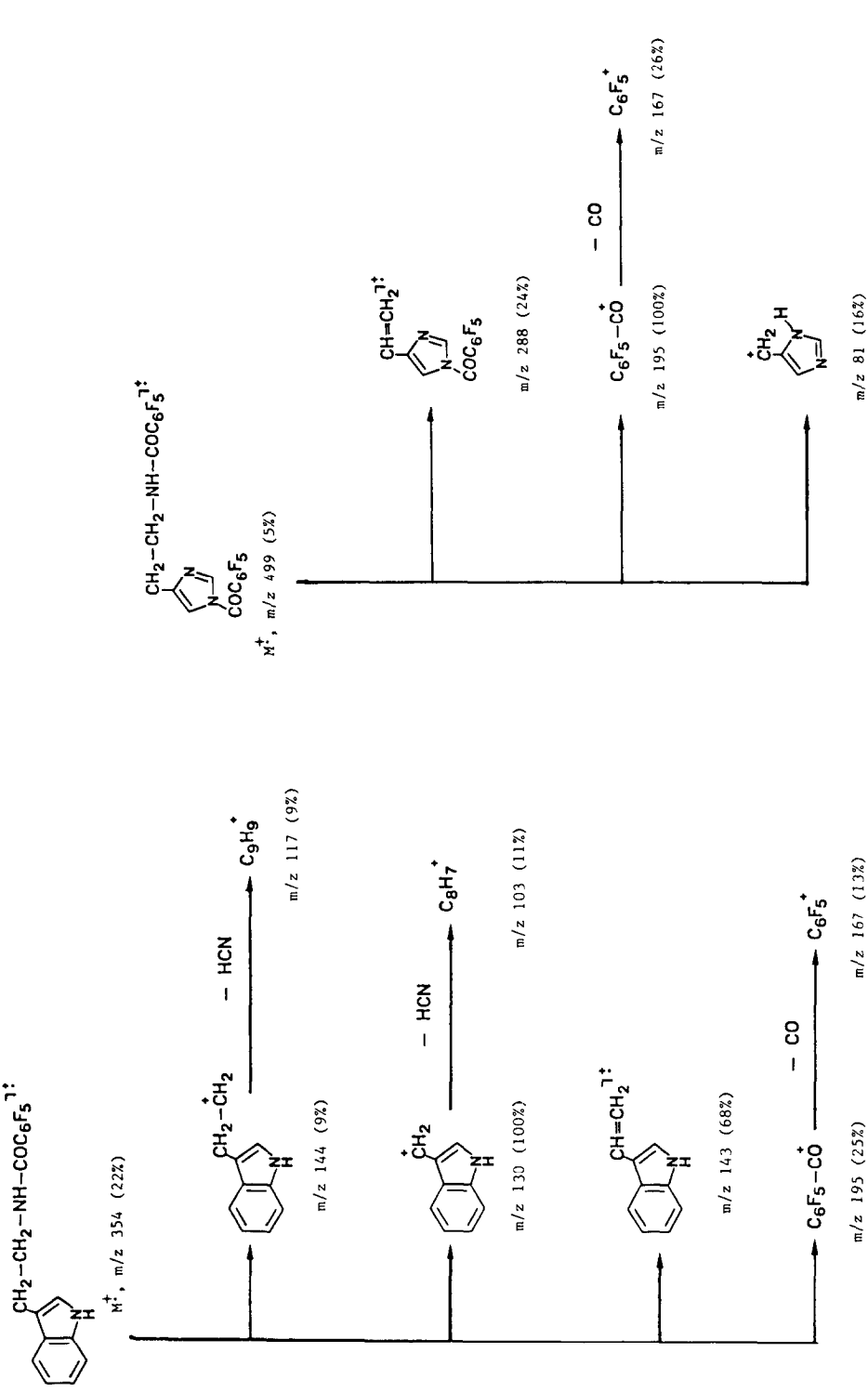

Fig. 4. Proposed mass spectral fragmentation of the PFB derivative of tryptamine.

Fig. 5. Proposed mass spectral fragmentation of the PFB derivative of histamine.

TABLE I

CONCENTRATIONS OF BIOACTIVE AMINES IN SOME CHEESE AND CHOCOLATE PRODUCTS

Results are expressed as μg/g of sample and represent means ± S.E.M. of values from 3–6 batches of each sample. At least two portions were taken for analysis from each batch. Abbreviations: PEA = 2-phenylethylamine; MeHA = *tele*-methylhistamine, a metabolite of histamine; HA = histamine; Put = putrescine; T = tryptamine; Cad = cadaverine; *m*-TA = *m*-tyramine; *p*-TA = *p*-tyramine; 3-MTA = 3-methoxytyramine, a metabolite of dopamine; 5-HT = 5-hydroxytryptamine; Spd = spermidine; Spm = spermine; and N.D. = not detectable.

Sample	*PEA*	*MeHA*	*HA*	*Put*	*T*	*Cad*	*m-TA*	*p-TA*	*3-MTA*	*5-HT*	*Spd*	*Spm*
Kraft Cheddar Cheese (old)	6.8±1.9	48.5±6.4	44.4±18.6	1.4±0.72	0.27±0.01	4.1±2.0	0.80±0.22	62.2±27.7	3.0±0.32	0.43±0.04	N.D.	N.D.
Black Diamond Hickory Smoked Cheese	9.9±2.6	4.7±1.1	5.5±2.3	49.2±19.4	0.52±0.21	25.9±6.8	N.D.	26.0±6.6	N.D.	N.D.	0.67±0.17	1.0±0.32
Woodward's Stilton Cheese	11.3±3.5	109 ±13.0	39±35	126±105	N.D.	11.6±5.9	6.1±2.8	115±42.0	5.1±0.24	0.43±0.20	5.8±1.4	1.2±0.64
Woodward's Cheshire Cheese	6.1±1.4	14.0±3.6	209±63	84±63	N.D.	80.7±69.6	5.1±0.38	144±17.0	0.86±0.28	N.D.	N.D.	N.D.
Cadbury's Hot Chocolate	1.8±0.07	0.34±0.07	0.41±0.04	N.D.	0.26±0.01	0.15±0.02	0.09±0.01	0.64±0.04	0.20±0.01	10.0±0.60	0.74±0.16	N.D.
Baker's Semi-Sweet Chocolate	6.4±0.09	1.0±0.12	0.43±0.11	N.D.	0.56±0.06	0.76±0.04	0.24±0.03	0.75±0.13	0.16±0.01	16.4±1.6	1.7±0.32	1.1±0.30
Fry's Cocoa	22.0±0.70	15.4±1.8	1.3±0.04	0.95±0.20	1.8±0.32	3.3±0.50	0.15±0.04	3.5±1.1	3.3±0.01	58.2±9.5	11.5±1.3	1.3±0.15
Rowntree's Aero Bar	0.22±0.01	0.09±0.04	0.55±0.01	N.D.	0.35±0.05	N.D.	0.07±0.02	0.22±0.03	1.5±0.02	6.2±0.2	0.53±0.09	0.46±0.05

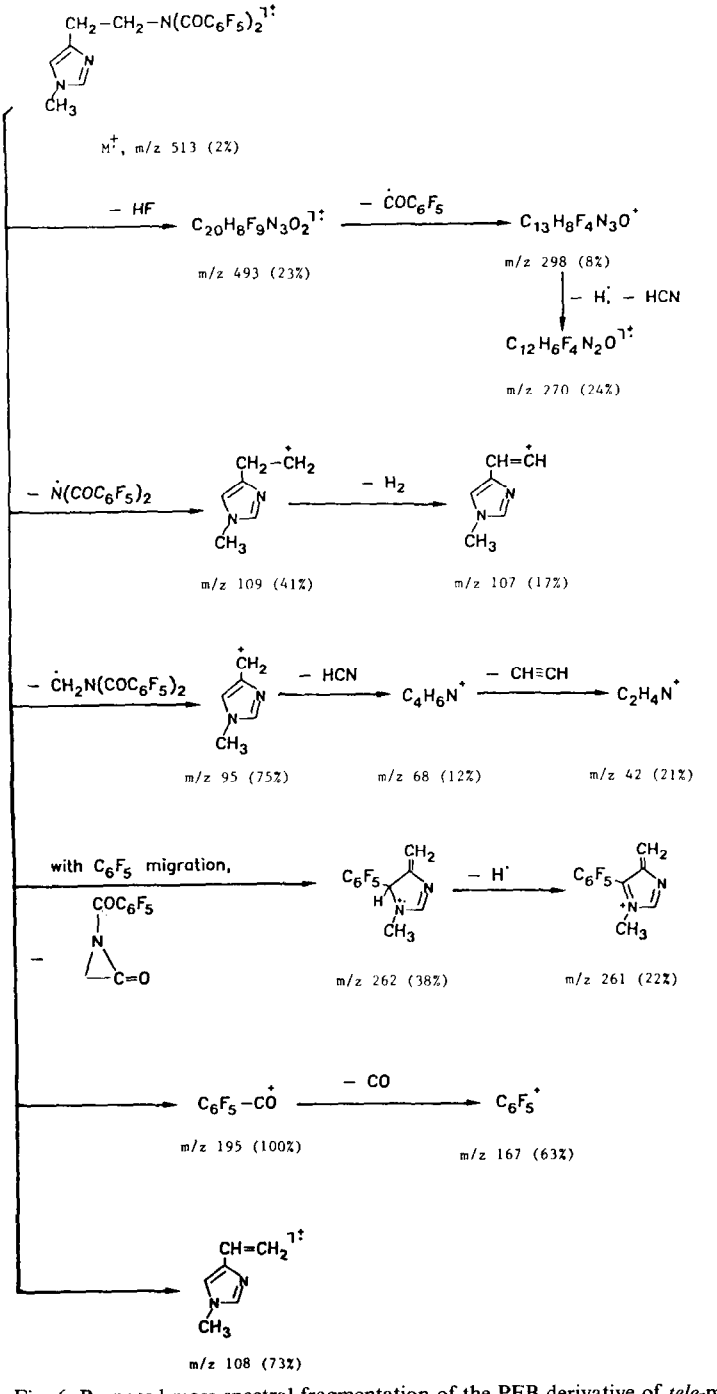

Fig. 6. Proposed mass spectral fragmentation of the PFB derivative of *tele*-methylhistamine.

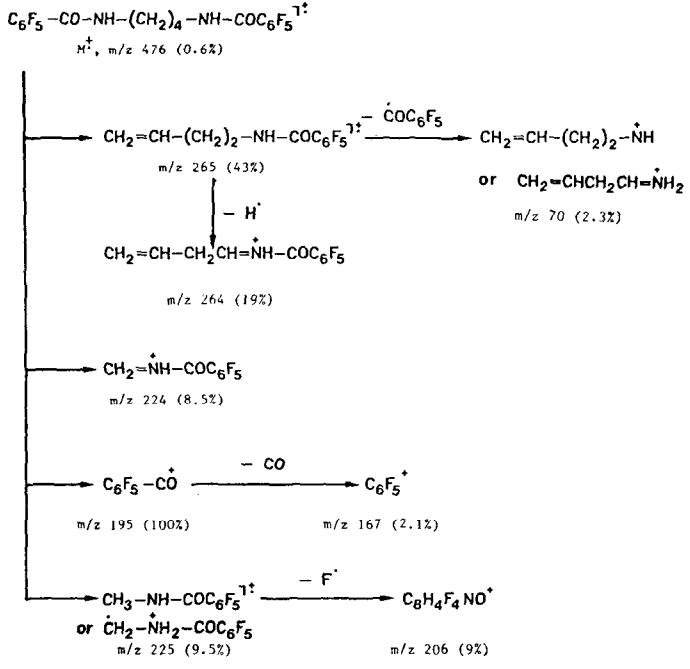

Fig. 7. Proposed mass spectral fragmentation of the PFB derivative of putrescine.

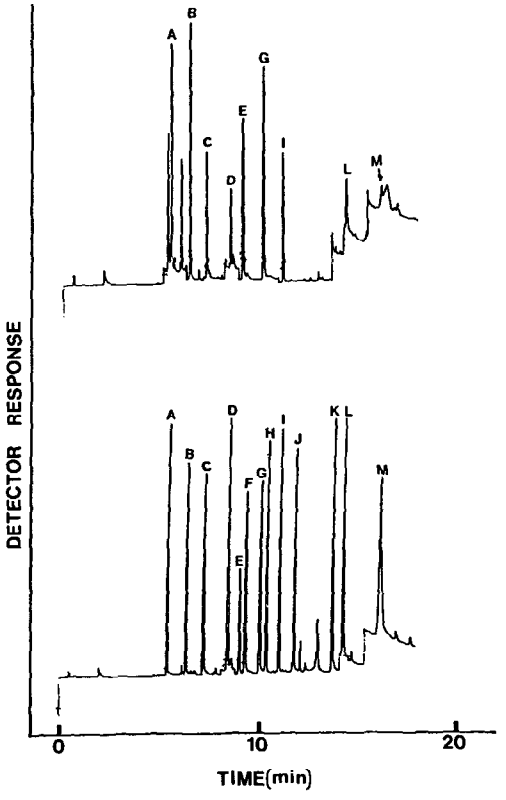

Fig. 8. Gas chromatogram of extracts of bioactive amines from (top) cheese sample and (bottom) a solution of authentic standards. Extraction and derivatization were as described in the text. Derivatives of 2-phenylethylamine (A); 2-(4-chlorophenyl)ethylamine, internal standard (B); *tele*-methylhistamine (C); histamine (D); putrescine (E); tryptamine (F); cadaverine (G); *m*-tyramine (H); *p*-tyramine (I); 3-methoxytyramine (J); 5-hydroxytryptamine (K); spermidine (L); and spermine (M). Attenuation changes were programmed in to ensure that all peaks remained on scale for the purpose of illustration.

equipped with a fused-silica capillary column and an electron-capture detector. The method provides simultaneous analysis of twelve bioactive amines and is readily applicable to the study of these amines in foodstuffs. Although we used GC–MS for initial confirmation of structures of the derivatives, GC–ECD was employed thereafter for routine analysis. Thus the assay should be a particularly attractive alternate analytical procedure to those laboratories which are interested in performing comprehensive studies of amines in food products but do not have sufficient funds for the equipment and maintenance costs involved with mass spectrometric procedures.

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