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GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF BIOACTIVE AMINES IN FOOD PRODUCTS

by

JAMES TSUN FAT WONG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF BIOACTIVE AMINES IN FOOD PRODUCTS by JAMES TSUN FAT WONG in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in Pharmaceutical Sciences (Neurochemistry).

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Date October 7, 1985

ABSTRACT

A gas-liquid chromatographic method with high specificity and sensitivity has been developed for the rapid, simultaneous analysis of a number of bioactive amines. Pentafluorobenzoylation in an aqeous environment was used successfully to derivatize the bioactive amines for their detection and quantitation on a gas chromatograph equipped with an electron-capture detection system and a high resolution capillary column. The bioactive amines of interest were β-phenylethylamine, tele-methylhistamine, histamine, tryptamine, 5-hydroxytryptamine, meta-tyramine, para-tyramine, 3-methoxytyramine and the polyamines putrescine, cadaverine, spermidine and spermine. Structures of the prepared derivatives were confirmed by mass spectrometry, and proposed mass fragmentation patterns are given. The presence of these amines in cheese and chocolate samples was determined and their levels were measured using the method developed in this study. Samples from different locations on the same block of cheese were analyzed. No significant differences in levels of amines were found between samples from the side and samples from the core of the block. Considerable variation in the concentration of amines was observed among batches of the same brand of cheese. Higher levels of 5-hydroxytryptamine than of any other of the amines were noted in the dark chocolate samples investigated; the white chocolate included for analysis in this study, however, displayed much lower levels of 5-hydroxytryptamine. The developed method can be used to detect and quantitate meta-tyramine and 3-methoxytyramine (a metabolite of dopamine). This may represent an advancement in food research since there has not been a report in the literature which analyzes these

two compounds in food products. It is anticipated that this developed method will be applicable to analyzing food products other than cheese and chocolate and also to analyzing biological samples such as body fluids.

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I. INTRODUCTION

There is a large body of supportive evidence which indicates that a number of bioactive amines may play an important role in transmitting nerve impulses and may also be etiologically involved in certain psychiatric disorders (Faurbye, 1968; Boulton, 1974, 1976, 1979; Axelrod et al., 1978). These bioactive amines include the catecholamines (dopamine, noradrenaline), 5-hydroxytryptamine (serotonin), m- and p-tyramine, β-phenylethylamine, tryptamine, histamine, tele-methylhistamine, and the polyamines (putrescine, cadaverine, spermidine, spermine). The amine theories of depression (review: Baker and Dewhurst, 1985), which state that there is a functional deficiency of one or more amines at central synapses in depressive disorders, were based in part on observations that monoamine oxidase (MAO) inhibitors were effective antidepressants. Monoamine oxidase is the major enzyme responsible for the oxidative metabolism of several biogenic monoamines, and concentrations of these amines are elevated in tissues and body fluids after administration of MAO-inhibiting drugs (Squires, 1978; Philips and Boulton, 1979). Unfortunately, it also became apparent that these MAO inhibitors could cause a number of side effects when patients taking them also ingested a number of food products (Horwitz et al., 1964; Sjogvist, 1965; Blackwell et al., 1967; Marley, 1977; Baldessarini, 1980). The most serious (and potentially fatal) of these side effects was hypertensive crisis. These adverse effects observed after co-ingestion of MAO inhibitors and certain foods were included in the term "cheese reaction" because they were first noted to be associated with the ingestion of cheese in patients receiving MAO inhibitors. Tyramine

was implicated as the pressor amine responsible for the adverse effects of the "cheese reaction" because this amine is found to exist in high concentration in cheese (Asatoor et al., 1963; Horwitz et al., 1964; Blackwell and Mabbit, 1965; Sew, 1969; Voigt et al., 1974). Since tyramine and a number of other biologically active amines are normal constituents of many foods, the analysis of these amines in foodstuffs thus became pertinent from the standpoint of health hazards to people who are taking MAO inhibitors.

A. Bioactive Amines

A.1 Tyramine (TA)

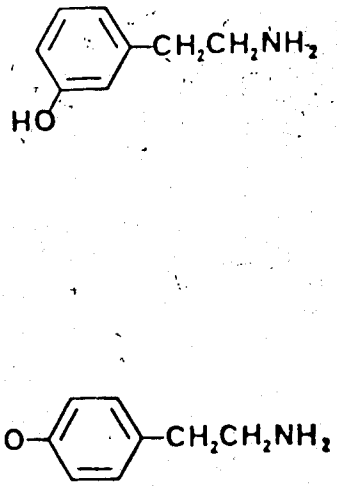


Figure 1. Structures of m-tyramine (top) and p-tyramine (bottom).

p-Tyramine (*p*-TA) is a sympathomimetic amine which acts indirectly to release noradrenaline (NA) from sympathetic nerve endings. Under normal circumstances, this amine is oxidized by MAO to the phenolic acid *p*-hydroxyphenylacetic acid (Dale *et al.*, 1909). When this metabolic oxidation is inhibited because of MAO inhibitors, ingested *p*-TA may be absorbed from the gut and enter the circulation, releasing NA from adrenergic sites. This consequently may produce a number of adverse effects, including hypertensive crisis. Depending on the amount of *p*-TA ingested and the extent which MAO is inhibited, side effects can range from mild headache, shortness of breath, and palpitation of the heart to myocardial infarction, subarachnoid hemorrhage and even death (Baldessarini, 1980). Horwitz *et al.* (1964) reported that as little as 20 g of certain cheeses or 6 mg of *p*-TA given orally would produce obvious pressor effects in human subjects receiving an MAO inhibitor. Other complications have also been reported in people ingesting foods rich in *p*-TA. About one-third of patients with classical migraine experience headaches after injection of *p*-TA and/or ingesting *p*-TA-containing foods. It is possible that these patients may have a deficiency of the enzyme forming the sulfate conjugate of *p*-TA (Hanington, 1967; Smith *et al.*, 1971; Youdim *et al.*, 1971). The *meta* isomer of tyramine (*m*-TA) has been shown to have an appreciable effect on release of the putative classical neurotransmitter amines dopamine (DA), NA and 5-hydroxytryptamine (5-HT, serotonin) from nerve terminals (Raiteri *et al.*, 1977). Thus in the quantitative analysis of TA in foodstuffs, it is important to include the *meta* isomer as well, although this has not been done in previous studies.

A.2 β-Phenylethylamine (PEA)

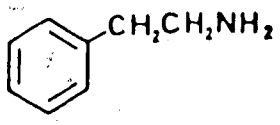


Figure 2. Structure of β-phenylethylamine.

This amine has been implicated in the onset of migraine attacks in certain migraine sufferers (Sandler et al., 1974, 1976). It is not surprising because structurally PEA is quite similar to p-TA; and, as mentioned previously, p-TA injection and/or ingestion of p-TA-containing food can induce headaches in migrainous patients. Significantly lower than normal MAO activity in migrainous individuals has been demonstrated (Sandler et al., 1974). This leads to reduced capability of the individuals to break down pressor monoamines such as PEA and p-TA. This may well explain why certain individuals suffer from dietary migraine.

β-Phenylethylamine has been termed 'the endogenous amphetamine' because it is an endogenous constituent of many tissues, including brain, and is structurally similar to amphetamine (α-methyl-β-phenylethylamine). Individuals who chronically abuse amphetamine often develop a clinical picture which is indistinguishable from paranoid schizophrenia (Ellinwood, 1967). β-Phenylethylamine has been proposed to be involved in the etiology of certain types of schizophrenia (Fischer and Heller, 1972; Sandler and Reynolds, 1976; Potkin et al., 1979), and chronic administration of PEA to laboratory rats has reported to be a good animal

model for schizophrenia (Diamond et al., 1984). Sudden development of schizophrenic symptoms has been reported as a side effect during treatment of patients with MAO inhibitors (Facts and Comparisons, 1979). Of course, this may be the result of effects on any of a number of amines, but it is of interest that in laboratory animals MAO inhibitors usually cause much larger increases in brain levels of PEA and tryptamine (T) than of the catecholamines or 5-HT (Philips and Boulton, 1979; McKim et al., 1980; Dewhurst, 1984; Baker et al., 1986).

β-Phenylethylamine has been reported to inhibit reuptake and/or release of catecholamines from heart and brain tissue (Horn, 1973; Baker et al., 1976; Raiteri et al., 1977), to alter the responses of central neurones to catecholamines (Jones, 1984), and to alter binding of 5-HT to its receptor sites in human and rat brain (Reynolds, 1984; Locock et al., 1984). Hauger et al. (1982) also reported the presence of specific binding sites for PEA in brain tissue.

A.3 Tryptamine (T)

Tryptamine (T), an indolealkylamine, is, like PEA, a structurally simple arylethylamine. β-Phenylethylamine and T are lipophilic substances and can cross the blood-brain barrier with ease (Oldendorf, 1971). Since they are excellent substrates for MAO, it is conceivable that during the inhibition of MAO, large amounts of PEA and T from foodstuffs may enter the central nervous system, causing effects resembling psychiatric disturbances. The administration of α-methyl analogues of PEA or T (amphetamine and α-methyltryptamine respectively) to laboratory animals can produce distinct behavioral syndromes (Dewhurst and Marley, 1965; Philips, 1978; Sabelli et al., 1978; Marsden, 1978; Squires,

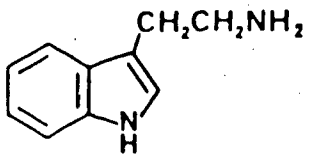


Figure 3. Structure of tryptamine.

1978; Moore, 1978). The analogues are protected from MAO-catalzyed degradation by their α-methyl group. Administration of T to dogs has been reported to produce LSD-like physiological effects (Martin et al., 1976). Tryptamine has been reported to have strong effects on inhibition of reuptake and stimulation of release of 5-HT in brain tissue (Heikkila and Cohen, 1974; Baker et al., 1977, 1980) and on release of NA in heart tissue (Paton, 1976). Receptor binding studies in vitro (Locock et al., 1984) and electrophysiological investigations in vivo (Jones, 1984) suggest that T has rather marked effects on 5-HT receptors. In addition, the presence of receptors for T itself in the central nervous system has been claimed (Dewhurst, 1968; Kellar and Cascio, 1982; Wood et al., 1985).

A.4 Histamine (HA) and tele-Methylhistamine (MeHA)

Histamine (HA) is the suggested culprit amine in many cases of food poisoning from fish products. This type of poisoning is frequently referred to as scombroid poisoning (Arnold and Brown, 1978; Gilbert et al., 1980) because in most cases the kinds of fish involved belong to

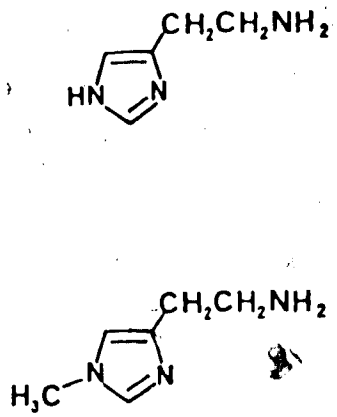


Figure 4: Structures of histamine (top) and tele-methylhistamine (bottom).

the suborder Scombroidea (e.g. tuna, mackeral, skipjack, sardines and bonito). Symptoms of scombroid poisoning include nausea, vomiting, facial flushing, headache, thirst, burning sensation in the throat and gastric pain (Edmunds and Eitenmiller, 1975). Spoilage of fish has been associated with high levels of HA in the fish. These fish usually contain no HA in their muscle tissue; but free histidine (the amino acid precursor of HA) is present in substantial amounts. Upon improper handling and poor storage of these fish, and under the right conditions, free histidine can be decarboxylated by appropriate bacteria to HA (Kimata, 1961; Arnold and Brown, 1978; Omura el al., 1978; Taylor et al., 1979).

Recently the suggested possibility that HA is a neurotransmitter has attracted considerable attention (Schwartz et al., 1980). However, not much is known about its effect in MAO inhibitor-foodstuff interactions. Intraduodenal injection of Marmite (a histamine-rich food product) in cats produces marked electromyographic changes and augmented spinal cord reflexes. These systemic responses can be reproduced by the administration of HA during MAO inhibition (Blackwell and Marley, 1966). However, HA itself is a substrate for diamine oxidase (Cooper et al., 1978). It is of interest here that phenelzine, a commonly used MAO inhibitor, also inhibits diamine oxidase (Shore and Cohn, 1960; Burkaard et al., 1962). Overdose of antihistamine drugs in humans has been reported to produce schizophreniform psychoses (Roman, 1972), and several antidepressant and antipsychotic drugs are known to be potent blockers of HA receptors in the CNS (Green and Mayaani, 1977; Kanof and Greengard, 1978; Snyder, 1978).

Although HA is not a substrate for MAO, its metabolite tele-methylhistamine (MeHA) is. This methylated compound is a major metabolite of HA in brain (Cooper et al., 1978). Little work has been done on the presence of this amine in food products or on its involvement in psychiatric disorders, although Gagne et al., (1983) have reported lower than normal urinary excretion of MeHA in depressed patients.

A.5 5-Hydroxytryptamine (5-HT, serotonin)

This amine is present endogenously in human tissues in relatively large quantities and is believed to be involved in regulation of numerous bodily activities such as hunger, thirst, sleep, mood, and sexual activities (Barchas and Usdin, 1973; Costa et al., 1974a,b; Iversen et

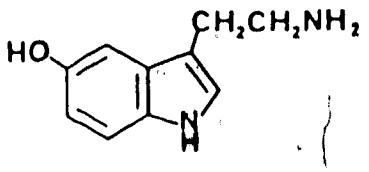
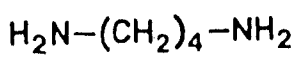


Figure 5. Structure of 5-hydroxytryptamine.

al., 1977). There is voluminous evidence supporting its role as a neurotransmitter substance in the central nervous system (Phillis, 1974; Gershon, 1977; Cooper et al., 1978; Snyder, 1980). This amine has also been suggested to play a role in the precipitation of a number of nervous disorders such as migraine, epilepsy, depression and schizophrenia (Curzon, 1968; Bruyn, 1976; Lipton et al., 1978; Legg, 1978; Essman, 1978-1979; Ban, 1981; De Lisi et al., 1981). Introduction of T or 5-HT into the afferent circulation induces release of postaglandins and other vasoactive substances into the systemic circulation (Alabaster and Bakhle, 1970). An interesting related finding is that headaches, resembling those in spontaneous migraine, can be induced by intravenous administration of prostaglandins E_1 (Carlson, 1967).

A.6 Polyamines [putrescine (PU), cadaverine (CA), spermidine (SPD), spermine (SPM)]

At physiological pH, polyamines are known to be protonated at all nitrogen sites; that is to say polyamines are polycations at physiological pH. This makes it possible for the polyamines to react with a num-



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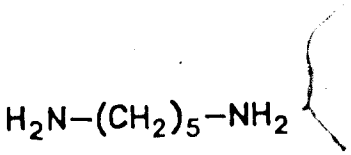

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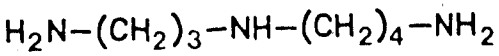

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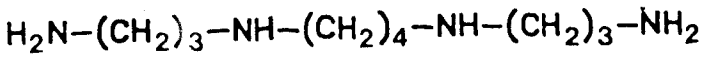

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Figure 6. Structures of the polyamines putrescine (a), cadaverine (b), spermidine (c), and spermine (d).

ber of negatively charged molecules, such as nucleic acids, proteins and anionic sites of membranes (Seiler, 1981). The suggestion that polyamines are involved in cell growth and replication is supported by numerous reports which state that polyamines affect, *in vitro*, virtually all reactions where nucleic acids are involved (Cohen, 1971; Bachrach, 1973; Janne *et al.*, 1978). A major stimulus of the present interest in polyamine research evolved from the fact that enhanced metabolism of these substances was found in fast growing tumours (Russell, 1973; Raina *et al.*, 1980). The possibility that the growth rate of tumours may be hindered by inhibiting polyamine synthesis (Seiler *et al.*, 1978) has further heightened researchers' interest in these amines.

Quantitative studies have shown that brain levels of some of the polyamines are higher than those of biogenic amines such as DA, NA and 5-HT (Seiler, 1982). Their role in central nervous system is unclear, but they are known to modulate acetylcholinesterase activity (Kossorotow, 1974; Anand *et al.*, 1976; Heinrich-Hirsch *et al.*, 1977). 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 (Shaw, 1972; Anderson and Shaw, 1974; Anderson *et al.*, 1975; Seiler, 1982).

Polyamines are distributed widely in nature and their presence in foodstuffs has recently attracted considerable attention in the field of food chemistry. These amines may present potential dangers to public health since some of these amines may be nitrosated or act as precursors for other amines capable of forming nitrosamines (Figure 7), which are highly carcinogenic. In the presence of heat (e.g. in cooking), putrescine and cadaverine may be converted to pyrrolidine and piperidine,

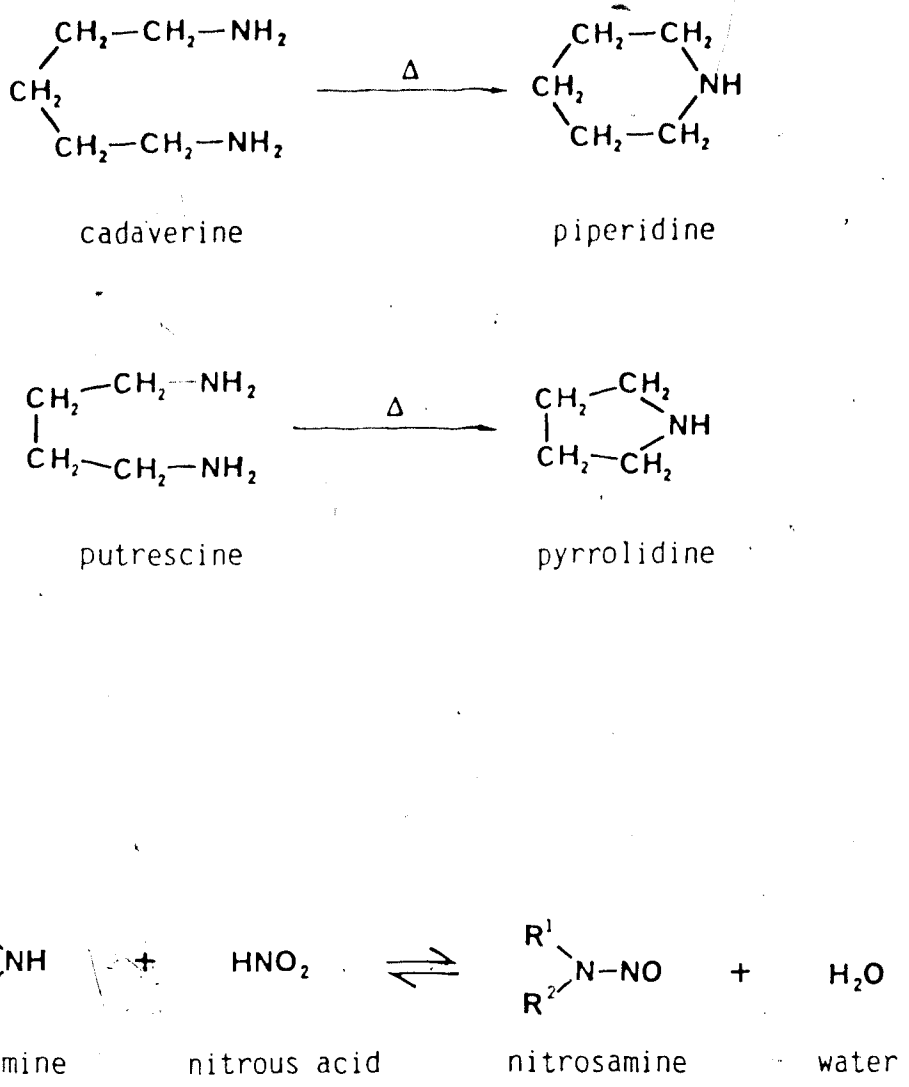


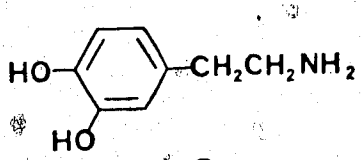
Figure 7. Formation of nitrosamines from polyamines.

respectively (Lijinsky and Epstein, 1970). These amines formed may in turn be nitrosated, in the presence of nitrite, to form the carcinogenic nitrosamines (Bills et al., 1973). It is well known that nitrite is widely used as a preservative in foods such as bacon, smoked ham and sausage. In addition nitrite has been reported as a normal constituent of human saliva, and it has been proposed that nitrosamines can be formed in the gastrointestinal tract (Tannenbaum et al., 1974; Spiegelhalder et al., 1976).

Some workers related high levels of amines, especially those of histamine, putrescine and cadaverine, in foodstuffs to the degree of food spoilage (Staruszkiewicz and Bond, 1981; Sayem-el-Daher et al., 1983) because it was thought that during microbial spoilage in food there would be increased bacterial activity, and hence increased activity of decarboxylases, enzymes which can convert amino acids to their corresponding amines. Bjeldanes et al. (1978) 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.

A.7 Catecholamines [noradrenaline (NA), dopamine (DA), adrenaline (A)]

Catecholamines are arylalkyamines with two adjacent hydroxyl groups attached to the aromatic ring. The term 'catechol' is used to indicate this dihydroxylated aromatic moiety. The catecholamines are putative neurotransmitters in the peripheral and central nervous systems (Iversen, 1973). They have been implicated in a number of psychiatric disorders, including depression and schizophrenia (Crow et al., 1976;



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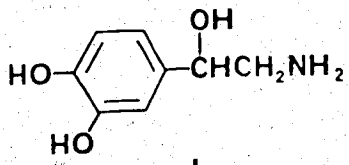

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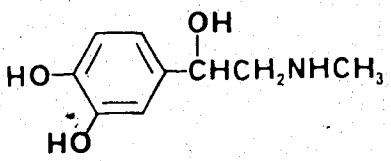

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Figure 8. Structures of the catecholamines dopamine (a), noradrenaline (b), and adrenaline (c).

Schildkraut et al., 1983). These compounds are polar and they do not cross the blood-brain barrier easily (Dewhurst, 1968; Oldendorf, 1971); presumably absorption of these amines from foodstuffs would not cause central effects even in the presence of MAO inhibitors. However, it is certainly possible that they could have dramatic actions on the cardiovascular and other peripheral systems, particularly in cases where their catabolism is hindered by the inhibition of MAO.

B. Presence of Bioactive Amines in Food Products

There is a voluminous literature on the presence of amines in food products. Much of this work has been reviewed in recent articles (Maga, 1978; Smith, 1980-81; Coutts et al., 1986) and I will not give a comprehensive overview here, but only present some of the highlights.

Examples of food products that are reported to contain substantial amounts of TA include cheese, fermented sausages, pickled herring, beef/chicken liver and yeast extract (Hughes, 1958; Hedberg et al., 1966; Blackwell et al., 1969; Sen, 1969; Boulton et al., 1970; Voigt et al., 1974; Koehler and Eitenmiller, 1978; Bacus, 1984). β-Phenylethylamine is known to exist in appreciable levels in chocolate (Saxby et al., 1980-81), and its presence has also been reported in fermented meat products such as pepperoni, smoked and cooked salami (Koehler and Eitenmiller, 1978). Tryptamine levels have been determined in numerous studies on different types of food products such as cheese, fruit, vegetables, meat products; with the exception of some aged cheeses low levels have been found (Udenfriend et al., 1959; Voigt et al., 1974; Koehler and Eitenmiller, 1978). Histamine has frequently been reported

to be present in substantial amounts in fish products. A review of the relevant literature indicates that HA is present in high concentrations in many foodstuffs (e.g. meat, fish, cheese) that are not refrigerated and stored properly (Merson et al., 1974; Foo, 1976; Taylor et al., 1978; Kim and Bjeldanes, 1979). Foodstuffs that contain considerable amounts of 5-HT include bananas, pineapples, tomatoes and plantain (Udenfriend et al., 1959; Foy and Parratt, 1961; Garcia-Moreno et al., 1980). Polyamines are found in a variety of foods including seafoods and meat, especially when these items are putrefied (Hatano et al., 1970; Mietz and Karmas, 1978; Staruszkiewicz and Bond, 1981). Catecholamines (NA, DA) have been reported mainly in fruits such as bananas and avocado (Udenfriend et al., 1959; Riggin et al., 1976); their presence in foodstuffs is not as well documented as that of other amines such as TA and HA. In general, most vegetables contain lower amounts of many of the bioactive amines than do the foods mentioned above.

Numerous articles have thus been published on the topic of amines in food products, but, much of this work suffers from the application of relatively non-specific techniques (e.g. use of paper chromatography combined with spectrophotometry, fluorescence techniques, etc.). In addition, many of the studies deal only with one or a small number of amines. Thus there was a need for a specific, sensitive analytical technique which would provide simultaneous analysis of a number of these bioactive amines.

C. Objective

As can be seen from the descriptions given above, the presence of a number of bioactive amines in foodstuffs may have very important con-

sequences for the general health of individuals. Despite this, there is very little quantitative data available on the levels of these amines in food products. Many of the techniques used for analysis in the past have low sensitivity and poor selectivity. Much of the data reported is given as less than a particular sensitivity level (usually high) or is expressed as low, medium or high (e.g. Horwitz et al., 1964, Abramowicz, 1980). It was the object of the project described in this thesis to develop a sensitive, selective, quantitative gas chromatographic procedure to provide for the simultaneous analysis in food products of as many as possible of the bioactive amines mentioned in the preceding sections.

D. Analytical Instrumentation

Because of the importance of bioactive amines in neurochemistry, numerous methods have been developed for their analysis. Among all the analytical techniques developed, the most widely used procedures include spectrophotofluorometry, radioenzymic methods, mass spectrometry (MS), high-pressure liquid chromatography (HPLC) and gas-liquid chromatography (GLC) assays. Although the mass spectrometer provides virtually unambiguous confirmation of the structures of derivatives formed, routine analysis using combined GLC-MS is expensive and requires highly trained personnel. The protocol adopted in our laboratories is to confirm structures of derivatives using the combined GLC-MS system and then use the high-resolution (capillary column) GLC with electron-capture detector for routine analysis.

Spectrophotofluorometric procedures are relatively inexpensive, but have been criticized on the grounds of lack of specificity in many cases (review: Baker and Dewhurst, 1982). Radioenzymic assays, although they have not been widely applied to analysis of amines in foodstuffs, do provide a highly sensitive means of analyzing large numbers of samples. However, they do require preparation or purchase of appropriate enzymes and purchase of expensive radiolabelled S-adenosylmethionine, complicated and/or prolonged separations of radiolabelled products in some cases, and disposal of radioactive waste. In addition, the radioenzymic procedure for TA (Tallman et al., 1976) does not distinguish between the two isomers of TA. High-pressure liquid chromatography (HPLC), especially when used with fluorometric or electrochemical detectors, has proved increasingly popular for analysis of amines in tissues and body fluids (Kissinger et al., 1981; Warsh et al., 1982; Downer et al., 1984; Anderson, 1985), and the upcoming years will probably see increased applications to analysis of these substances in food products. However, a major drawback with HPLC is the lack of certainty about the identification of the peaks measured. This problem may be overcome to a large extent by the coupling of HPLC to MS; at present such technology is still in its infancy.

After taking the above factors into consideration, it was decided to employ GLC in the present study to provide simultaneous analysis in foodstuffs of as many as possible of the important bioactive amines mentioned previously in this thesis.

D. 1 Gas liquid chromatography

Chromatography is a process of separation of components in a mixture by repetitive equilibration between two phases, a stationary phase

and a mobile phase. A GLC system consists of an injection port connected to a column which is housed in an oven where the temperature can be set at the desired level or range. At the other end of the column is situated the detector for detecting column effluent. In GLC, the stationary phase is a liquid of high boiling point housed inside a column through which the mobile phase, a carrier gas, constantly sweeps. When a sample mixture is introduced onto the column via the heated injection port, the solvent in which the sample mixture is dissolved and the components in the mixture are carried into and through the column by the carrier gas. Separation occurs inside the column according to individual components' partition coefficients between the stationary liquid phase and the gaseous mobile phase. To avoid shortening of column life and column bleed, oven temperatures should not exceed the maximum recommended by the column manufacturers. Column bleed is the eluting of stationary phase into the detector chamber; this may contaminate the detector, resulting in diminished sensitivity and poorer resolution of emerging solute peaks. Depending on the range of volatilities of compounds being analysed, the chromatographer can use either isothermal operation or temperature programming when performing analyses. The detector detects individual components in the column eluate, and this response is converted into an electrical signal and recorded as a peak on a chart recorder. The retention time of a peak is the length of time (usually in minutes) a particular component is repetitively retained in the stationary phase before eluting from the column and being detected. This is measured from the point of injection to the apex of the peak, and is characteristic of that component under those particular experimental conditions.

D.2 Columns for gas-liquid chromatography

The column in a chromatographic system is the component in which separation of mixtures takes place. Glass is usually the material of choice for making GLC columns because of its inertness in reacting with substances being chromatographed. In addition, column defects are more apparent and can be easily diagnosed. Columns made of other materials such as stainless steel, nickel, copper and aluminum are also available, but these metal columns, when heated, tend to catalyze degradation of organic compounds (Baker et al., 1982). Fused silica columns, however, are now gaining preference over glass columns because of their inertness, and high degree of flexibility and material strength, which render them almost indestructible.

If high resolution chromatography is desired, one would choose to use capillary columns over packed columns although the latter may also be useful. The dimensions of columns can vary greatly. Fused silica or glass capillary columns are usually 10-100 m in length and 0.25-0.5 mm in internal diameter (Baker et al., 1982). There are two types of columns which are differentiated by the way the stationary liquid phase is contained in the columns. Wall-coated open tubular (WCOT) columns have a thin film of liquid phase deposited directly on the inner surface of the capillary. In support-coated open tubular (SCOT) columns, the liquid phase is coated on some solid support which, in turn, is coated on the inner surface of the capillary. Generally these columns are purchased from commerical sources.

Typical dimensions of packed columns are 1 to 2 m in length and 2 to 4 mm in internal diameter. Packed columns are relatively inexpensive and the use of them allows the chromatographer to choose from numerous

commercially available stationary phases on various solid supports. Different support materials are used depending on the stationary liquid phase loadings. Low density supports such as Chromosorb® W (White) can be loaded with as much as 30% w/w stationary phase; whereas Chromosorb® P (Pink), which is mechanically stronger and has higher density, can retain as little as a monomolecular layer coating (McNair and Bonelli, 1969). In practice, the usual concentration of liquid phase on a solid support is 1-5% w/w (Moffat, 1975; Coutts and Baker, 1982). Different polymers are used in preparing different liquid phases, each having different polarity characteristics. Polar polymers such as polyethylene glycol (carbowax), non-polar polymers of dimethyl silicone (SE-30, OV-101), and semi-polar phenylmethyl silicone polymers (OV-17) are several of the many liquid phases in common use. Since 'like dissolves like', polar liquid phases retain polar solutes and vice versa (Mitchard, 1978); separation may best be achieved by matching solute and liquid types. Solutes with different polarity have varying degrees of affinity towards the type of liquid phase in use, hence making chromatographic separation possible.

D.3 Detectors in gas-liquid chromatography

The detector system of a gas chromatograph is situated at one end of the column for detecting and, when combined with an integrator, quantitating sample components emerging from the column. With appropriate electronic amplification, the detector response is converted into electrical signals and recorded on a recorder (or integrator). The detectors used most commonly in GLC work are: the thermal conductivity detector (TCD), the flame ionization detector (FID), the nitrogen-

phosphorous detector (NPD) and the electron-capture detector (ECD). The mass spectrometer can also serve as a sophisticated detector in GLC analysis.

D.3.1 Thermal conductivity detector

The TCD consists of a filament constantly heated by a steady electric current with a well-maintained constant carrier gas flow rate through the detector. A detector response corresponds to a drop in filament temperature when a gas (vaporized component) emerges from the column to cool the filament. The TCD is capable of detecting a wide range of organic compounds because of its lack of selectivity. Although it has a wide linear dynamic range, its sensitivity limit is about 1 g. The TCD is therefore not popular in analyses where specificity and sensitivity are the main themes.

D.3.2 Flame ionization detector

In an FID, an electrical field is established between a negatively charged hydrogen burner jet and a positively charged electrode. Column effluent is ionized while being combusted in the flame, and the negative ions are collected at the positive electrode. The amount of ions generated (i.e. the strength of the electric current) is proportional to the amount of compound combusted. This change (increase) in electric current is recorded as the FID response, which can be generated by most organic compounds. To ensure total ionization of the effluent, air is introduced into the FID chamber to supply an excess of oxygen for combustion. The sensitivity of this detector is approximately proportional to the number of carbon atoms in the molecule of the column effluent and is

linear over a wide range. Quantities of samples in the nanogram range can easily be detected.

D.3.3 Nitrogen-phosphorous detector

Nitrogen and/or phosphorous-containing compounds are selectively detected by the NPD. The principle for operation is similar to that of FID, the major difference being the addition of a quantity of an alkali (usually rubidium) salt near the tip of the flame. While there is not a definitely clear theoretical explanation for the operation of the NPD at present, this detector can increase response to phosphorous compounds by 5,000 to 1 and the response of nitrogen compounds by 25 to 1 over the response to pure hydrocarbons. The NPD has been used for detection of picogram quantities of nitrogen- and phosphorous-containing compounds.

D.3.4 Electron-capture detector

The electron-capture detector (ECD) is one of the most selective and sensitive GLC detection devices. Since its introduction by Lovecock and Lipsky (1960), a series of modifications have been made successfully to enhance its sensitivity and stability, which has led to its widespread use for routine analytical work. The ECD operates by sensing a decrease in the number of free electrons in the detector chamber due to their capture by electrophoric sample molecules. A suitable radioactive isotope with continuous beta decay provides the electron supply in the detector cavity. The isotope is usually nickel-63 with a half-life of 92 years. It decays by emitting beta particles (high energy electrons) which undergo repeated collisions with carrier gas molecules, producing a multitude of secondary electrons for each initial beta particle, which

is analogous to the ionization process in the ionization chamber of the Geiger-Mueller counter in radiation measurement (Shapiro, 1972). After further collisions the energy of these secondary electrons has been reduced to the thermal level and they can be captured by suitable sample molecules. When the detector is undisturbed, with only carrier gas flowing through, a beam of thermal electrons is collected at the anode and provides a baseline current. When an electrophoric component elutes from the chromatographic column and enters the detector cavity, it causes a disturbance in the baseline current by capturing some of the thermal electrons. This disturbance (i.e. reduction in current), after electronic amplification, is recorded as a peak on the chart recorder.

In practice there are several methods used for collecting electrons and measuring the resulting current. The simplest method is the constant voltage mode. A fixed, steady voltage is applied to the anode, which continuously collects electrons. This mode suffers from non-linearity in detector response and poor sensitivity. This is because as more electrophoric compounds enter the detector, a depletion of free electrons occurs; also, after the electron-capturing process, electrophoric compounds become ionized and negatively charged and compete with the free electrons for the anode. These factors render the detector non-linear and diminish sensitivity. To alleviate this problem, the pulsed voltage method was introduced. Voltage in pulses of short duration (1 microsecond) is applied to the anode at short intervals (5-150 microseconds). By careful adjustment of pulse duration and time between intervals, one may extend the linear dynamic range from 200 (of constant voltage method) to about 1,000 (Baker et al., 1981). A modified version of the pulsed voltage method, introduced by Maggs et al. (1971) probably

marks the most significant advance in ECD technology. This is the constant current, pulsed voltage method. The electron population in the ECD cell is collected periodically by applying a short voltage pulse to the cell electrodes. The cell current is constantly compared to a reference current in the ECD electronics. When there is a depletion of electrons in the cell due to the electron-capturing molecules from the column evaporates, the pulse interval is automatically adjusted to keep the cell current constant. This rise in pulse rate is recorded as a detector response to electrophoric compounds. The detector is known to detect as little as one picogram (10^{-9} g) of some compounds in a 1 µl injection. The linear dynamic range of this type of detector may reach 10,000 or more.

D.3.5 Mass spectrometer detector

To a mass spectrometrist, the GLC is an inlet for sample introduction to the mass spectrometer. By the same token, the mass spectrometer is a sophisticated and expensive detector to the chromatographer performing GLC analysis. For the mass spectrometer to function properly, carrier gas from the GLC is removed before the column effluent is passed into the ionization chamber of the mass spectrometer, where ionization of the compound occurs as a result of bombardment of high energy electrons with the molecule under investigation. To gain stability, the ionized molecule undergoes fragmentation into positive, negative and neutral fragments. Usually the positively charged fragments are collected and passed into an electron multiplier that generates an electric current which is amplified and recorded. The mass spectrometer can be programmed to amplify the current produced by an ion of a particular

mass-to-charge ratio; this is called the single ion monitoring mode of operation in mass spectrometry and is a powerful tool for selective detection.

D.3.6 Choice of detector for this project

Because of its high sensitivity, low routine cost relative to combined GLC-MS, and the relative success of its application to analysis of minute quantities of amines in biological samples such as tissues and body fluids (reviews: Baker et al., 1981; Baker and Coutts, 1982), the ECD was chosen for the study described in this thesis. In addition, we have found in our laboratories that the ECD is a more rigorous detector than the NPD for routine analytical work; the bead of the NPD requires frequent changing and varying sensitivites provided by different beads is a problem. During the course of development of the procedures described here, structures of the derivatives formed were comfirmed by combined GLC-MS. Following this confirmation and establishment of optimum conditions for separation of the derivatives of the amines, GLC-ECD was used alone for routine analysis of the food samples.

E. Chemical Derivatization for Gas-Liquid Chromatography

Prior to GLC analysis, a mixture of compounds may have to be chemically derivatized to improve their 'on-column' separation. Low volatility of some compounds renders them unsuitable for GLC analysis. This low volatility may be due to molecular complexity or the presence of certain functional groups. Compounds that contain active hydrogen atoms (such as in amine, hydroxyl, or carboxylic groups) may interact with the

liquid phase or adsorb to the support material and column surface, or form strong intermolecular hydrogen bonding, making separation (or even elution) of components impossible. Less seriously, these properties may cause excessively long retention times, drastic peak tailing, and/or diminished detector response. Generally speaking these undesirable effects are due to the presence of polar functional groups in the compounds.

Hence chemical derivatization usually involves the replacement of active hydrogen atoms of polar compounds. Procedures commonly used are acylation, alkylation, silylation and condensation (Blau and King, 1978; Knapp, 1979). General reactions for each type of derivatization are depicted in Figure 9. Some commonly used derivatizing reagents are shown in Figure 10.

In some instances, the reverse situation may also occur in that the compounds of interest are too volatile to be quantitatively handled throughout the analytical procedure. Preliminary treatment such as extraction and subsequent removal of extractant may cause substantial losses of the components being determined. The preparation of suitable derivatives usually obviates the aforementioned difficulties. In this case, chemical derivatization is adjusting the degree of volatility to suit the analytical instrumentation and experimental conditions.

Direct application of thermally unstable compounds to the heated GLC injection port may result in decomposition of compounds before they enter the column, giving rise to more than one chromatographic peak for a single component. Chemical derivatization can also be used to overcome this difficulty.

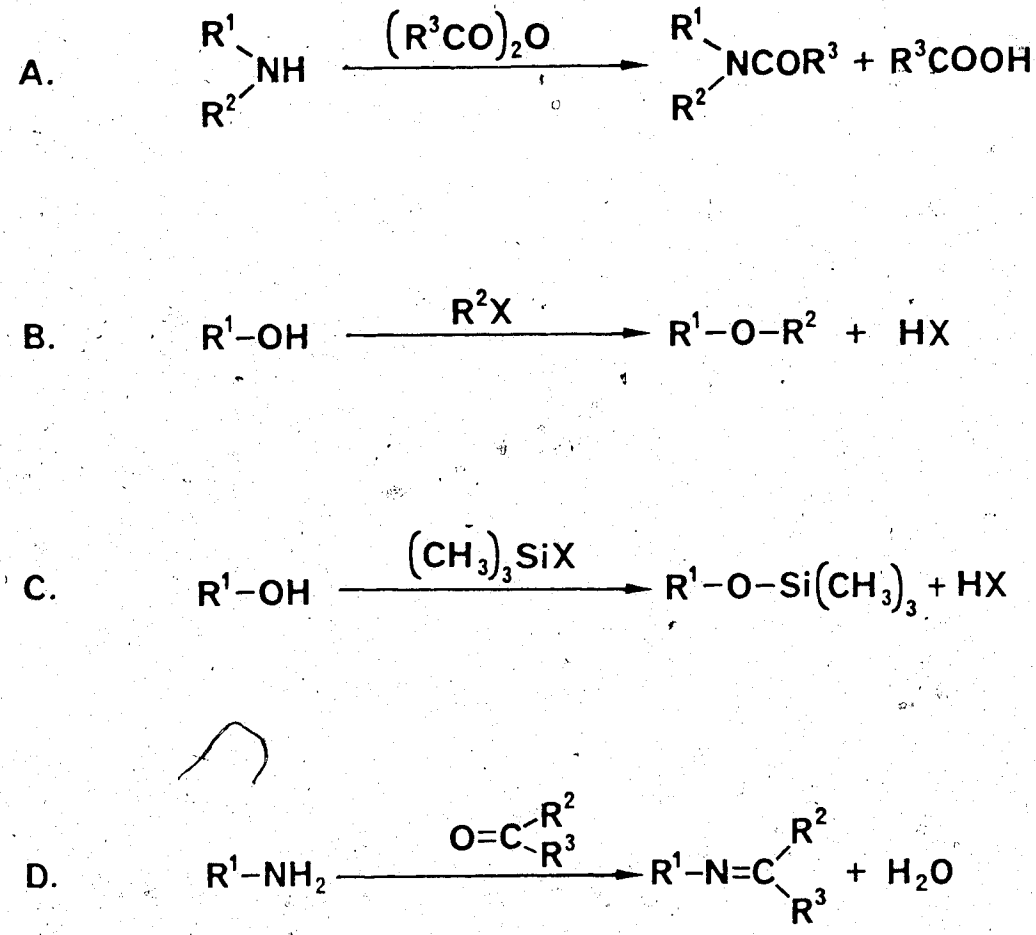
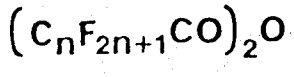
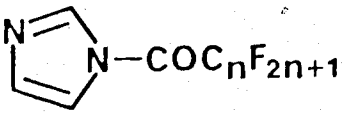


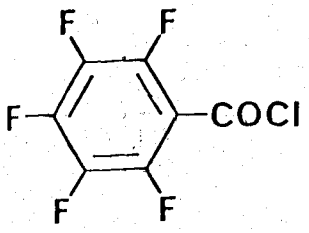
Figure 9. Derivatization reactions commonly used in gas-liquid chromatography: A, acylation; B, alkylation; C, silylation and D, condensation.



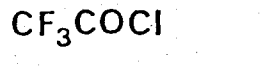
TFAA, n=1
PFPA, n=2
HFBA, n=3



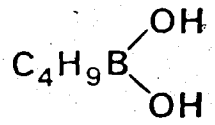
TFAI, n=1
PFPI, n=2
HFBI, n=3



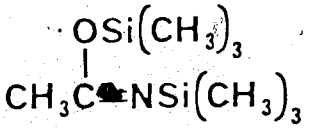
PFBC



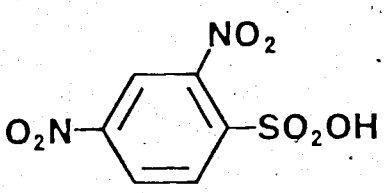
TFAC



n-Butylboronic acid



N,O-Bis(trimethylsilyl)acetamide



2,4-dinitrobenzenesulfonic acid

Figure 10. Structures of some derivatizing reagents commonly used in gas-liquid chromatography.

Another major application of chemical derivatization is the augmentation of sensitivity in selective detection systems (such as electron-capture or nitrogen-phosphorous detectors). A typical example is the preparation of derivatives possessing electronegative moieties for electron-capture detection. The ECD gives exceptionally high responses to chlorinated and fluorinated derivatives, and this accounts for the frequent use of these derivatives in trace analysis.

In the analysis of bioactive amines, a commonly used technique is the acylation of the compounds using perfluoroacylating reagents. Amino groups do not produce sufficient responses on the ECD and their presence often raises the polarity of the compounds. Amphoteric compounds (e.g. p-TA) are difficult to chromatograph because of the polarity contributed by the amino group and the phenolic group. Among the numerous commercially available halogenated acylating reagents, the most commonly and extensively used are trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA) (Bertani et al., 1970; Martin and Ansell, 1973; Belvedere et al.,1973; Wong et al., 1973; Baker et al., 1982b; Fujihara et al., 1983; Sioufi et al., 1983a,b; Jeanniot et al., 1983). These substances can acylate phenolic, alcoholic and primary and secondary amino groups relatively easily under anhydrous conditions. The use of these reagents is convenient for analysis of bioactive amines in the central nervous system (CNS) since some of these amines are multifunctional (e.g. NA, DA, 5-HT, p-TA). Typical derivatization using these reagents involves heating, usually under anhydrous conditions and often in the presence of an organic solvent. It is important to work under anhydrous conditions because the reagents (and sometimes the derivatives) are sensitive to hydrolysis in the pres-

ence of water. Products formed by reaction between TFAA and NA and 5-HT are illustrated in Figure 11. Excess reagents and the by-products (acids) may be removed by evaporation under a stream of nitrogen or helium; alternatively, especially in cases where volatile derivatives are involved, removal of unwanted products may be accomplished by partitioning the resultant reaction mixture between a hydrocarbon solvent (e.g. toluene, cyclohexane, decane) and an aqueous buffer such as saturated sodium bicarbonate or sodium borate buffer. It is important to remove the excess reagent after the reaction because sensitivity of the detector may be grossly suppressed otherwise. Further problems such as irreversible changes in the GLC columns or corrosion within the GLC or GLC-MS system may also occur (Blau and King, 1978).

The use of perfluoroacyl imidazoles as alternative acylating reagents is not uncommon (Vessman et al., 1969; Degen et al., 1972; Benington et al., 1975; Christian et al., 1975). Trifluoroacetylimidazole (TFAI), pentafluoropropionylimidazole (PFPI), and heptafluorobutyrylimidazole (HFBI) are most suitably applicable to analysis of acid-sensitive compounds which are prone to dehydration in an acid medium; the by-product imidazoles are basic and inert and do not cause decomposition of the derivatives (Suguira and Hirano, 1974). Conditions for derivatization are generally the same as those with perfluoroacyl anhydrides; that is, excess reagent, heat, reaction time, anhydrous environment, etc. The principal inconvenience in using perfluoracylated imidazoles is their high viscosity. It is difficult to draw them up into a syringe and the excess reagent is not easy to remove at the end of the reaction (Baker et al., 1981) by evaporation under a stream of nitrogen.

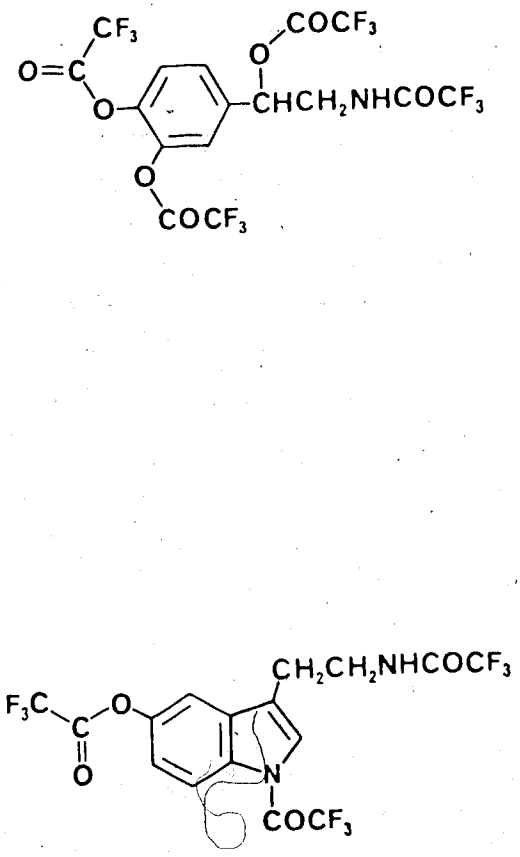


Figure 11. Structures of derivatives formed by reaction of noradrenaline (top) and 5-hydroxytryptamine (bottom) with trifluoroacetic anhydride under anhydrous conditions (from Martin and Ansell, 1973).

Another important class of acylating reagent is the perfluoroacyl halides. Pentafluorobenzoyl and trifluoroacetyl chlorides (PFBC, TFAC) are the two compounds of this class most frequently encountered in the literature. Pentafluorobenzoyl chloride has been used extensively (Anggard and Hankey, 1969; Wilkinson, 1970; Ahuja, 1976; Midha et al., 1979b) and is still gaining in popularity over many other acylating reagents because of its relatively high reactivity and the high degree of sensitivity and stability it imparts. Table I compares the relative ECD sensitivity of PFB derivatives of some amines to derivatives formed by reaction with HFBA. The PFB/HFB sensitivity ratios were 67, 500, 6.1 and 134 for the derivatives of cyclopropylamine, cyclohexylamine, benzylamine and heptylamine, respectively. Anggard and Hankey (1969) compared a number of derivatives of amphetamine with regard to ECD sensitivity and reported that the PFB derivative was 8.6, 19.3 and > 770 times more sensitive than the HFB, PFP and TFA derivatives respectively. A similar investigation by McCallum and Armstrong (1973) on the phenol thymol demonstrated that the PFB derivative was 6.9 and 5.3 times more sensitive than the HFB and PFP derivatives, respectively.

The increasing popularity of the use of PFBC can be seen from the numerous recent publications in amine analysis with ECD-GLC. This reagent has been used for analysis of amphetamine and PEA in post-mortem Parkinsonian brain after (-)-deprenyl administration (Reynolds et al., 1978). Midha et al. (1979b) conducted simultaneous analysis of norfenfluramine and fenfluramine in human plasma and urine after preparing their PFB derivatives. Bock and Waser (1981) prepared PFB derivatives of the catecholamines NA and A for ECD-GLC analysis. Pentafluorobenzoyl chloride has been shown to derivatize amphetamine and analogues in an

Table I. A comparison of the relative ECD sensitivities of HFB and PFB derivatives of amines (Cummins, 1971). Sensitivities are expressed relative to the HFB derivative of cyclohexylamine, which is given a value of 1. (Adapted from Blau and King, 1978).

Amine	Heptafluorobutyryl Derivative	Pentafluorobenzoyl Derivative
Cyclohexylamine	1	500
Cyclopropylamine	15	1,000
Benzylamine	25	133
Heptylamine	5	670

aqueous environment at room temperature in contrast to the usual anhydrous conditions and heat required with many other perfluoroacylating reagents (Cristofoli et al., 1982; Nazarali et al., 1984).

There are numerous other commercially available halogenated derivatizing reagents which have not been used as frequently as the aforementioned ones. Donike (1973) reported that N-methyl-bis(trifluoracetamide) can be used to acylate primary and secondary amines, and, in a less reactive manner, hydroxyl groups. Baker et al. (1981) reported that chlorodifluoroacetic anhydride (CDFAA) is capable of forming ECD-sensitive derivatives of catecholamines under anhydrous conditions. Aqueous derivatization of PEA and tranylcypromine (TCP) using trichloroacetic anhydride has been reported to show adequate ECD sensitivity for analysis in rat brain tissue (Baker et al., 1985). A number of derivatizing reagents containing a flophemesyl (pentafluorophenyldimethylsilyl) group have been used to derivatize alcohols, phenols, carboxylic acids and amines (Francis et al., 1978). The derivatives were reported to possess good chromatographic properties, thermal stability and high ECD sensitivity. However it is unfortunate that these derivatives are prone to hydrolysis. Because of the high molecular weight of flophemesyl group ($C_8H_6F_5Si$, mol. wt. 225), the use of these reagents is suitable for derivatizing small molecules but may be cumbersome for molecules with more than two functional groups.

The versatility and applicability of the derivatizing reagents have been compared and evaluated frequently (Clarke et al., 1966; Anggard and Sedrall, 1969; Cummings, 1971; Matin and Rowland, 1972; Moffat et al., 1972; Ko et al., 1974; Arnold and Ford, 1973; Midha et al., 1979a). However, many of these publications deal only with standards or with

samples other than food products. In cases where extracts of biological samples were analyzed, the researchers did not report a simultaneous analysis of several amines as comprehensive as that reported in this thesis.

F. Solvent Extraction and Derivatization of Amines in Aqueous Medium

Solvent extraction is a means of performing initial cleanup procedures on complex mixtures such as biological samples and food extracts. It usually involves partitioning of compounds of interest between two immiscible phases, aqueous and organic. There are certain parameters that have to be considered in order to perform an efficient extraction. One of them is the choice of solvents. Very often the starting sample is an aqueous solution. The choice of the second solvent is important because the value of the solute's partition coefficient is governed by this choice. There are situations where more than one extracting solvent appears to be satisfactory; the choice is then based on their densities, that is, whether they are heavier or lighter than water.

Another parameter that can be adjusted to achieve efficient extraction is the ionic strength of the aqueous phase. If the salt concentration of an aqueous solution is made very high, the solubility of a non-electrolyte will usually be decreased. This reduction of solubility in the aqueous phase induced by increased ionic strength is termed the 'salting-out effect'. This is usually done by incorporating a large amount of sodium chloride or similiar salt in the aqueous phase; the solute is then salted out into the organic extracting phase. Further-

more, the salt also helps to break emulsions that may form when shaking the two phases together.

Another maneuver in this context is the control of pH. It is not unusual to encounter weak acids or bases in analyses. The solubility characteristics of these substances depend upon their ionic forms, with the ionic species usually being soluble in polar (especially aqueous) solvents. By merely altering the pH of the medium, these forms can be interconverted at will, rendering pH control the most powerful means for influencing the value of the partition coefficient.

In a basic medium, amines exist in a non-ionized form and are readily extractable into an organic solvent. However, efficient extraction may be difficult if other functional groups such as phenolic hydroxyl and/or alcoholic hydroxyl groups are present as well. For example, because of its amphoteric nature, a phenolic amine may exist as the highly polar zwitterion. This would prevent the molecule from being extracted efficiently into the organic solvent. Acylation is commonly used to deal effectively with this problem. By choosing an appropriate reagent, derivatization can occur at phenolic and amino sites under aqueous conditions with ease, resulting in lowered polarity of the molecule and improved extractability into the organic solvent. Such derivatization is also useful for facilitating separation of mixtures of structurally similar molecules. Halogenated derivatizating reagents can also be used to augment sensitivity in GLC-ECD systems. Pentafluorobenzylation has been used successfully to derivatize phenolic hydroxyl groups. Ehrsson (1971) reported that pentafluorobenzyl bromide (PFBB) can be used to derivatize carboxylic acids and phenols in aqueous alkaline medium. Brotell *et al.* (1973) used PFBB to derivatize pentazocine to determine

its concentration in human plasma. The versatility of the use of PFBB was further extended by Cole et al. (1977), who used the same reagent for the quantitation of plasma morphine levels. It was found in the same study that PFBB can also be used to derivatize morphine-related phenolic alkaloids. Makita et al. (1976) reported that both phenolic hydroxyl and amino groups can be readily alkyloxycarbonylated with alkyl chloroformate in aqueous alkaline medium. This led to the development of an assay procedure using ethyl chloroformate in the gas-chromatographic determination of TA in fermented food products (Yamamoto et al., 1980) and the analysis of di- and polyamines in foods (Yamamoto et al., 1982). In case of p-TA, alkyloxycarbonylation occurred at both the amino and phenolic sites as depicted in Figure 12. This reaction can be conveniently performed in aqueous medium at room temperature. Early work indicated that phenolic and primary aliphatic amines can readily be acetylated directly using acetic anhydride in slightly alkaline aqueous solution (Chattaway, 1931; Hagopian et al., 1961). Subsequent findings showed that alcoholic hydroxyl groups cannot be acetylated under these conditions (Brooks and Horning, 1964; Laverty and Sharman, 1965; Roder and Merzhauser, 1974). Other acylated amines such as propionyl-, iso-butyryl-, n-butyryl- and pivaloyl-amines have also been prepared (Hiemke et al., 1978). Figure 13 shows the derivatives formed by reaction of acetic anhydride with p-tyramine and p-octopamine under basic aqueous conditions.

Sensitivity of acetylated primary amines to ECD may be accomplished by introducing an additional functional group which is sensitive to electron capture. This is usually done by further acylating the mono-N-acylated amines using halogenated acylating reagents under anhydrous

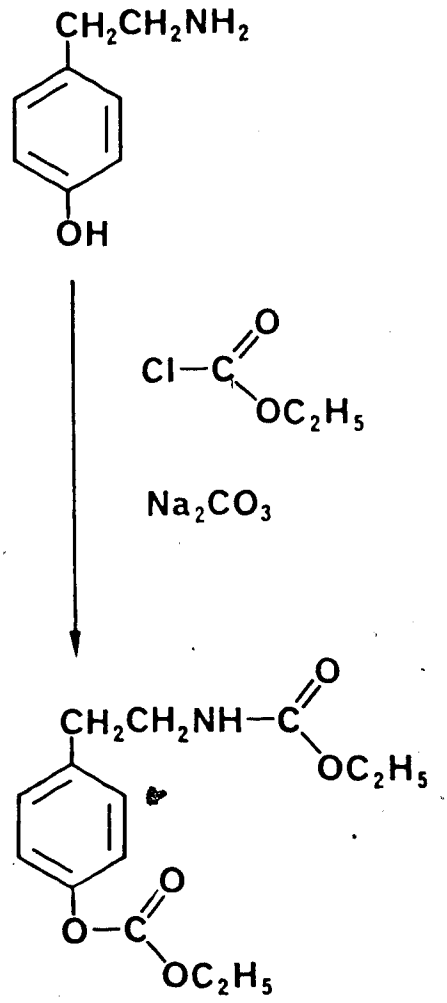


Figure 12. The product formed by reaction of p-tyramine with ethyl chloroformate (Yamamoto et al., 1980).

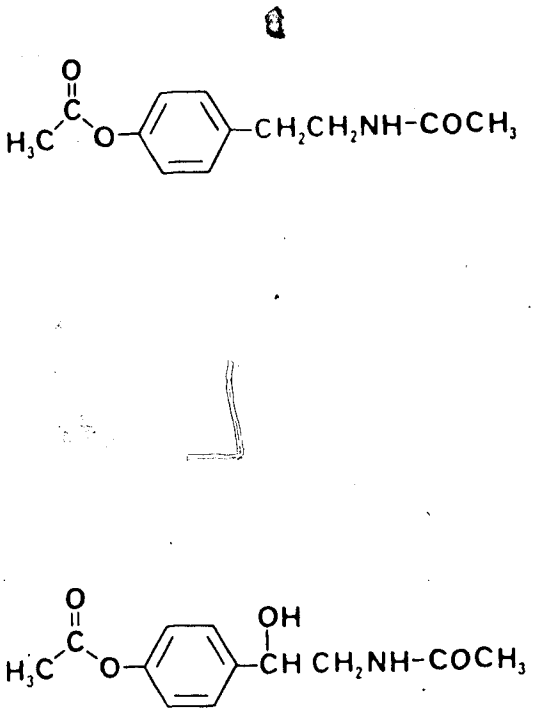
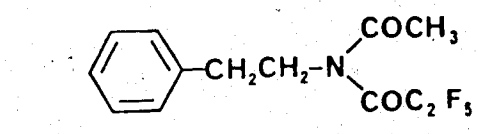


Figure 13. Products formed by reaction of p-tyramine (top) and p-octopamine (bottom) with acetic anhydride under basic aqueous conditions.

conditions. Such derivatization frequently involves the reagents TFAA, PFPA, HFBA, or PFBC. Warsh et al. (1977) developed an assay procedure for T using aqueous acetylation followed by anhydrous perfluoroacylation while Martin and Baker (1976, 1977) reported a similar procedure for PEA. Figure 14 demonstrates the derivatives formed by reaction of PEA and 5-HT with acetic anhydride under basic aqueous conditions followed by reaction with PFPA under anhydrous conditions. 5-Hydroxytryptamine forms a spirocyclic compound under these conditions. The versatility of this procedure has been demonstrated by numerous subsequent reports using similar methods to analyse amines (Baker et al., 1979, 1980; Calverley et al., 1980; Coutts et al., 1984a). The derivatizing reagent PFBC has also been used instead of PFPA or TFAA to further derivatize tranylcypromine (TCP) and PEA in rat brain extracts after initial acetylation (Hampson et al., 1984a,b). The derivatives formed are illustrated in Figure 15.

The techniques described above (i.e. acetylation followed by perfluoracylation) may also be used to derivatize phenolic amines, but the phenolic group will be acylated and not available for perfluoracylation. O-Perfluoroacyl derivatives have been reported to be more sensitive to ECD than the amino derivatives (Clarke et al., 1969), and this has initiated the development of an assay procedure which, after acetylation, incorporates selective hydrolysis of the O-acetyl groups using 10N NH_4OH solution before performing anhydrous perfluoracylation (Coutts et al., 1980b) (Figure 16). This method has been used to measure m- and p-TA levels in urine (Coutts et al., 1980a) and p-TA levels in brain (Baker et al., 1982c), to provide simultaneous extraction and quantitation of a number of biogenic amines in brain and urine (Coutts et al.,



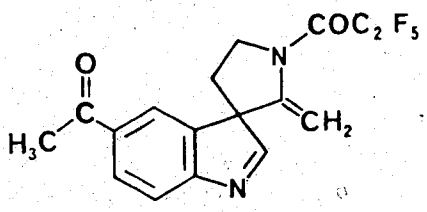
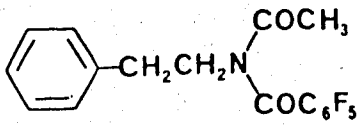
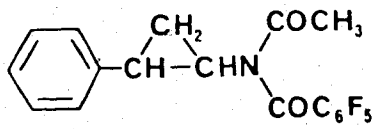


Figure 14. Derivatives formed by reaction of β-phenylethylamine (top) and 5-hydroxytryptamine (bottom) with acetic anhydride under basic aqueous conditions followed by pentafluoropropionic anhydride under anhydrous conditions. Note that 5-hydroxytryptamine forms a spirocyclic derivative under these conditions (Blau et al., 1977).



a



b

Figure 15. Products formed by reaction of β-phenylethylamine (a) and tranylcypromine (b) with acetic anhydride under aqueous conditions followed by reaction with pentafluorobenzoyl chloride under anhydrous conditions.

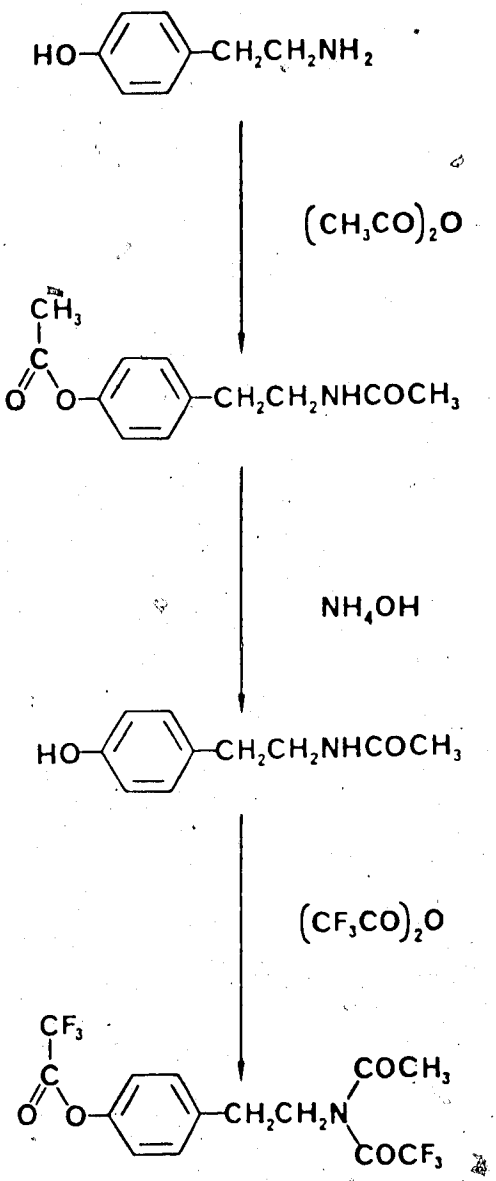


Figure 16. Derivatives formed from p-tyramine by the following sequence of reactions: acetylation under aqueous conditions, basic hydrolysis of the acetylated phenol, and reaction with TFAA under anhydrous conditions (from Coutts et al., 1980).

1980c; LeGatt et al., 1981; Baker et al., 1981; Baker et al., 1984b; Coutts et al., 1984a), and to permit analysis of p-hydroxyamphetamine and p-hydroxynorephedrine in rat brain (Coutts et al., 1984b). Despite the highly sensitive derivatives that can be obtained using this procedure, considerable time is involved in preparing them. In addition to the usual clean-up and extraction steps, the procedure requires about 20 minutes for complete aqueous acetylation, 40 minutes to specifically hydrolyze the phenolic O-acetyl groups and 30 minutes for anhydrous perfluoroacylation of the functional groups.

Decriox et al. (1968) used benzoyl chloride to benzoylate amines for efficient extraction from basic aqueous solution. Pentafluorobenzoyl chloride is the perfluoro analogue of benzoyl chloride. The use of PFBC has an added advantage in that the resultant derivatives can be detected with good sensitivity in a GLC-ECD system. Makita et al. (1975) reported an assay method for di- and polyamines using aqueous pentafluorobenzoylation with PFBC. Since, as previously mentioned, PFBC can be used to derivatize the arylalkylamines (amphetamine and analogues) in an aqueous environment at room temperature (Cristofoli et al., 1982; Nazarali et al., 1984), it would seem logical to use PFBC in an attempt to accomplish simultaneous analysis of all the bioactive amines mentioned previously in this thesis. Doing so makes it possible to shorten significantly the sample "work-up" procedure since pentafluorobenzoylation produces, in a single step, derivatives which are both readily extractable and sensitive on the ECD.

Since until now much of the data on amines in foodstuffs reported in the literature has been qualitative, despite their presence in foodstuffs being an area of concern to mental health, an assay procedure

which can provide rapid and specific quantitative determination of these amines would be extremely convenient and valuable to physicians and other health professionals and to others working in allied food, agricultural and toxicological sciences.

II. MATERIALS AND METHODS

A. Chemicals and Derivatizing Reagents

All chemicals and bioactive amines (authentic standards) used in this study are listed in Table II. Two stills, a Corning AG-3 and a Corning Mega-Pure (3 Litre Automatic), were used to generate and collect double-distilled water for use in analysis. The derivatizing reagent (PFBC) was obtained from Aldrich Chemical Company (Milwaukee, WI). All procedures which involved handling of organic solvents and derivatizing reagents were performed in fume hoods. Authentic standards were freshly prepared at the beginning of each analysis.

B. Instrumentation and Apparatus

B.1 Gas-liquid chromatography (GLC)

Gas-liquid chromatography was performed on a Hewlett Packard (HP) 5890 gas chromatograph equipped with a 15 m WCOT SE-54 fused silica capillary column and an ECD with a radioactive source of 15 mCi Nickel-63. An HP 3392A integrator was used for recording/integrating chromatographic peaks. The carrier gas, helium, was set at a flow rate of 2 ml per min. Argon-methane (95%-5%) at a flow rate of 36 ml/min was used as

Table II. Chemicals used in this study.

Chemicals	Suppliers
Acetonitrile, HPLC grade	Fisher Scientific Ltd.
Ammonium hydroxide	Fisher Scientific Ltd.
Benzene, pesticide grade	Fisher Scientific Ltd.
Cadaverine diHCl	Sigma Chemical Co.
Chloroform, reagent grade	Caledon Laboratories Ltd.
2-(4-Chlorophenyl)ethylamine HCl*	Sigma, and Drs. T. W. Hall and R. G. Micetich
Cyclohexane (glass distilled)	Caledon Laboratories Ltd.
Di(2-ethylhexyl)phosphoric acid (DEHPA)	Sigma Chemical Co.
Disodium ethylenediamine tetraacetate (EDTA)	Fisher Scientific Ltd.
Histamine diHCl	Calbiochem-Behring Corp.
Hydrochloric acid, 37-38%	Fisher Scientific Ltd.
5-Hydroxytryptamine creatinine sulfate	Sigma Chemical Co.
3-Methoxytyramine HCl	Sigma Chemical Co.
1-Methylhistamine diHCl	Calbiochem-Behring Corp.
Perchloric acid, 60%	Fisher Scientific Ltd.
β-Phenylethylamine HCl	Sigma Chemical Co.
Potassium bicarbonate	Fisher Scientific Ltd.
Potassium carbonate	Fisher Scientific Ltd.
Putrescine diHCl	Sigma Chemical Co.
Sodium carbonate, anhydrous	J. T. Baker
Spermidine triHCl	Sigma Chemical Co.
Spermine tetraHCl	Sigma Chemical Co.
Toluene (glass distilled)	Caledon Laboratories Ltd.
Tryptamine HCl	Sigma Chemical Co.
m-Tyramine HCl	Vega Biochemicals
p-Tyramine HCl	Sigma Chemical Co.

*2-(4-Chlorophenyl)ethylamine was obtained as a free base from Sigma Chemical Co. It was converted to the hydrochloride salt by Drs. T. W. Hall and R. G. Micetich (Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta).

makeup gas at the detector. The injection port temperature was 200° and the detector temperature was 345°. Chromatographic separation was accomplished using a 2-level temperature programme: an initial oven temperature of 105° was maintained for 0.5 min., and this was increased to 240° at a rate of 25°/min. After remaining at 240° for 3.5 min., the oven temperature was again increased at the same rate to 330°, at which value it was held for 8 min. Amine concentrations were measured using the ratio of peak heights of compounds of interest to the peak height of an 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.

B.2 Mass spectrometry (MS)

Chemical structures of PFB derivatives of amines were confirmed by using combined GLC-MS. The mass spectrometer was a Hewlett Packard (HP) 5985A with dual EI/CI sources and HP 7920 data system. The GLC-MS system also consisted of the inlet HP 5840A GLC, HP 2648A graphics terminal, HP 9876A printer, HP 7920 disc drive (software), and HP 21MX series E computer (hardware). Operation conditions were as follows: ion source temperature, 200°; interface temperature, 275°; column pressure, 10 p.s.i.; accelerating voltage, 2200 eV; ionization voltage, 70 eV;

scan speed, 100 amu/sec; and dwell time, 200 msec. The capillary column and oven programme were the same on those described in section B.1.

B.3 Glassware - cleaning procedure

All glassware (tubes, beakers, flasks, etc.) was rinsed with tap water before immersing in biodegradable Sparkleen (Fisher Scientific Co.) solution. Subsequent thorough washing (and rinsing with distilled water) of beakers, flasks and measuring cylinders was done in a dish-washer (Miele Electronic G715). Glass tubes were treated differently; after immersing in Sparkleen solution, they were placed in an ultrasonic cleaner (Mettler Electronics) containing a solution of Decon 75 concentrate (BDH Chemicals), 20 ml to 1 litre, and ultrasonicated for a minimum of 1 hr. The glass tubes were then stacked in stainless steel wire mesh baskets and rinsed with hot distilled water in the dish-washer. All glassware after rinsing was air-dried in a mechanical convection oven (Model 29, Precision Scientific Group). It was found that the above cleaning procedures were effective in removing adsorbed organic contaminants from glass surfaces.

B.4 Weighing balances

Weighing of chemical compounds or food samples was performed with either a Sartorius 2003 MP1 or a Mettler AE160 weighing balance.

B.5 Potter-Elvejhem homogenizer

A combination of a TRI-R Model S63C variable-speed laboratory motor with a Teflon© glass pestle and a glass grinding tube (clearance: 0.1-0.15 m) was used to homogenize food samples in ice-cold 0.4 N perchlor-

ic acid containing 10 mg% EDTA. The maximum motor speed was 12,000 r.p.m., with 10 speed levels. A speed level of 8 was used for homogenizing food samples.

B.6 Centrifugation

The bench centrifuges were a Sorvall® GLC-2B and a Sorvall® GLC-1 General Laboratory Centrifuge (Dupont Instruments). After homogenization in the perchloric acid, food samples were centrifuged in a heavy duty Damon-IEC Model B-20 refrigerated high-speed centrifuge at 12,000 x g for 15 min at 0° to remove the protein precipitate. An MSE Micro-Centaur microcentrifuge was used for centrifugation of minicentrifugation tubes (400 µl and 1.5 ml sizes).

B.7 Shaker-mixer

Two types of vortex-shakers were used: Ika-Vibrax VXR2 Shaker (Jannke and Kunkel Instruments) and a Thermolyne Maxi Mix™ vortex mixer (Sybron/Thermolyne Instruments). The VXR2 shaker has continuous variable speed of 0-2200 r.p.m. Maximum speed was used during the analysis.

C. Sample Collection and Storage

Food samples were purchased at local retail markets and were stored at -20° until analysis, if they were not analysed immediately.

D. Analysis of Bioactive Amines in Food Samples

Approximately 1 g of cheese or chocolate was cut into tiny pieces before homogenization in 10 volumes of ice-cold 0.4 N perchloric acid

($HClO_4$) containing 10 mg% EDTA. The suspension was centrifuged for 15 min at 12,000 x g at 0°. An aliquot of the clear supernatant (1-2 ml) was used for analysis. For quantitation purposes, an internal standard compound, 2-(4-chlorophenyl)ethylamine (CPEA) was added to the 0.4 N $HClO_4$ during its preparation. The final concentration was 1 µg CPEA per 1 ml $HClO_4$. After the refrigerated centrifugation, 2 ml of the clear supernatant were basified by the addition of solid potassium bicarbonate ($KHCO_3$). Potassium perchlorate precipitated out from the solution and was removed by brief centrifugation. Extraction of amines was done by shaking the supernatant with 3 ml of the liquid ion-exchanger di-(2-ethylhexyl)phosphoric acid (DEHPA, 2.5% v/v chloroform) for 10 min. After 5 min centrifugation at 3,000 r.p.m., the aqueous phase was aspirated off and discarded. The amines were back-extracted from the DEHPA-chloroform layer by shaking with 2.5 ml 0.5 N hydrochloric acid (HCl) for 5 min. After centrifugation at 3,000 r.p.m. for 5 min, the HCl layer was retained and made alkaline using a small excess of solid sodium carbonate (Na_2CO_3). This was followed by 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 bioactive amines were derivatized and extracted by vortexing the aqueous and organic phases together for 15 min at room temperature. The two phases were separated by a 5 min centrifugation at 3,000 r.p.m. The organic phase, which now contained pentafluorobenzoylated amines, was retained and taken to dryness under a gentle stream of nitrogen. The dry residue was reconstituted with 300 µl toluene. This toluene layer was shaken with 1 ml 1 N ammonium hydroxide (NH_4OH) for 15 sec to remove the unreacted excess PFBC and the possible by-product pentafluorobenzoic acid. After brief

centrifugation, the toluene layer was retained and 1 µl was injected on the SE-54 fused silica capillary column for ECD-GC analysis. Structures of the final derivatives were confirmed by gas-liquid chromatography-mass spectrometry (GC-MS) and direct insertion probe high resolution mass spectrometry (DIP-HRMS).

III. RESULTS AND DISCUSSION

A method has been developed to allow for the specific, rapid and simultaneous analysis of a number of bioactive amines. Pentafluorobenzoyl chloride was used under aqueous conditions to react with the amines before analyzing them on a capillary column/ECD-GLC system. Although the acetylation-perfluoroacylation methods of Coutts *et al.* (1980b; 1981) do provide separation of PEA, *m*- and *p*-TA, and 3-MT, it is apparent that the present method has the advantages of being more rapid and also providing simultaneous analysis of HA, MeHA, T, 5-HT, and the polyamines.

The use of PFBC gives rise to derivatives with high sensitivity on the ECD and good peak shape on the capillary column. The "on-column" sensitivity of bioactive amine derivatives prepared by this method was determined to be 5 pg or better; this was determined as the ECD response which was at least twice the blank response. The derivatives showed good stability, with little deterioration being apparent one week after storage at -20°C.

Although a variety of different types of amine (arylalkyl-, phenolic arylalkyl-, indolealkyl-, hydroxyindolealkyl-, imidazolealkyl-, and aliphatic di-, tri- and tetra-amines), which undoubtedly should possess

different chemical reactivities, were included in the present study, the derivatives prepared showed good chromatographic properties. Typical chromatograms are depicted in Figures 17-19. It is interesting to note that despite its high molecular weight, the pentafluorobenzoylated aliphatic tetraamine spermine, which was derivatized at all amino sites, still exhibited reasonably good peak shape.

To ensure completion of the derivatization reaction, 15 min of vigorous mixing was used for the derivatization step. The amine derivatives were first gas chromatographed individually to examine whether or not there were any additional peaks formed and then added successively to the mixture of standard amine derivatives, thus allowing peak identification. The reaction with PFBC produced a single chromatographically pure derivative for each amine investigated; each structure was confirmed by GLC-MS. The probable mass spectral fragmentation pathways of the derivatives are illustrated in Figures 20 to 32.

Electron impact was used in mass spectral analysis to ionize molecules entering the ionization chamber from the gas chromatograph. All the PFB derivatives of amines in this study, with the exception of spermidine and spermine, showed the presence of molecular ion. The prominent presence of PFB group is evident in all compounds investi[illegible] (PFB fragment has m/z 195). The loss of fragments of formulae COC_6F_5, $H_2N\text{-}COC_6F_5$ and $CH_2\text{-}HN\text{-}COC_6F_5$ is not uncommon in most of the derivatives. It is interesting to note that derivatives formed from the aliphatic di- and polyamines gave a fragment ion of m/z 225 with the formula $CH_3\text{-}\overset{+}{N}H\text{-}COC_6F_5$ or $\dot{C}H_2\text{-}\overset{+}{N}H_2\text{-}COC_6F_5$. A common fragment of m/z 70 with molecular formula C_4H_8N was found in the spectra of the derivatives of putrescine, spermidine and spermine; this is not surprising since these

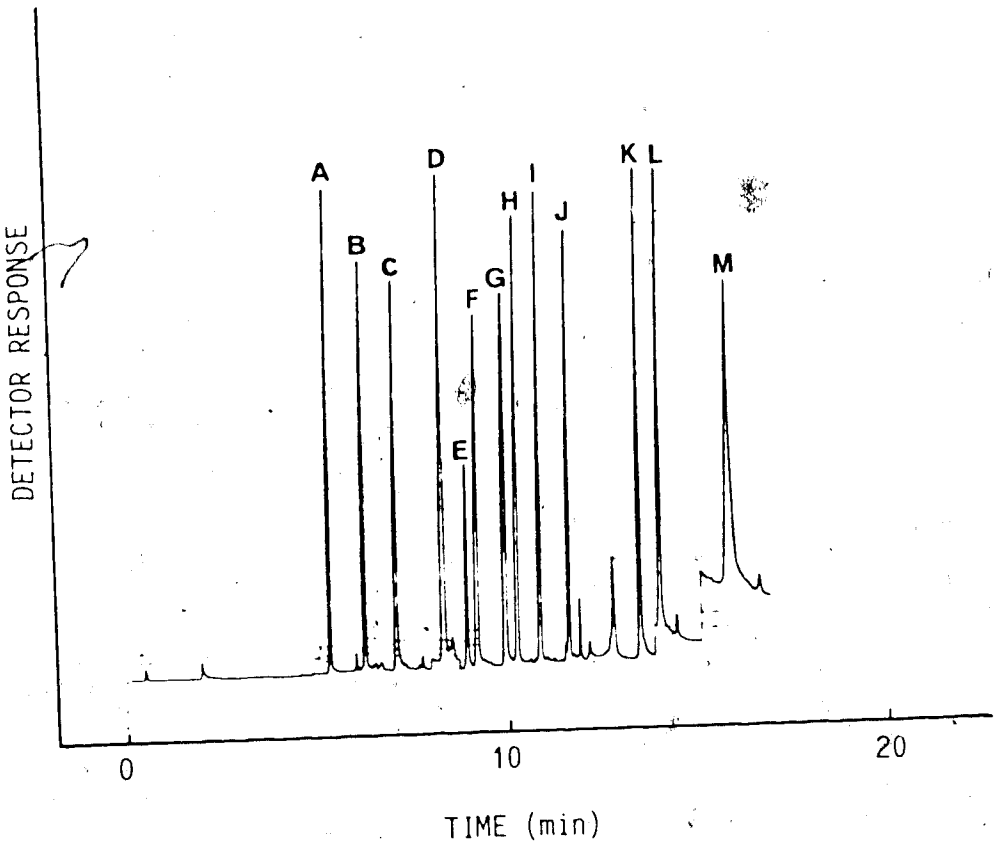


Figure 17. A typical gas chromatogram of pentafluorobenzoylated standards of β-phenylethylamine (A), 2-(4-chlorophenyl)ethylamine (B), tele-methylhistamine (C), histamine (D), putrescine (E), tryptamine (F), cadaverine (G), m-tyramine (H), p-tyramine (I), 3-methoxytyramine (J), 5-hydroxytryptamine (K), spermidine (L) and spermine (M). This represents 2-4 µg of each amine carried through the procedure.

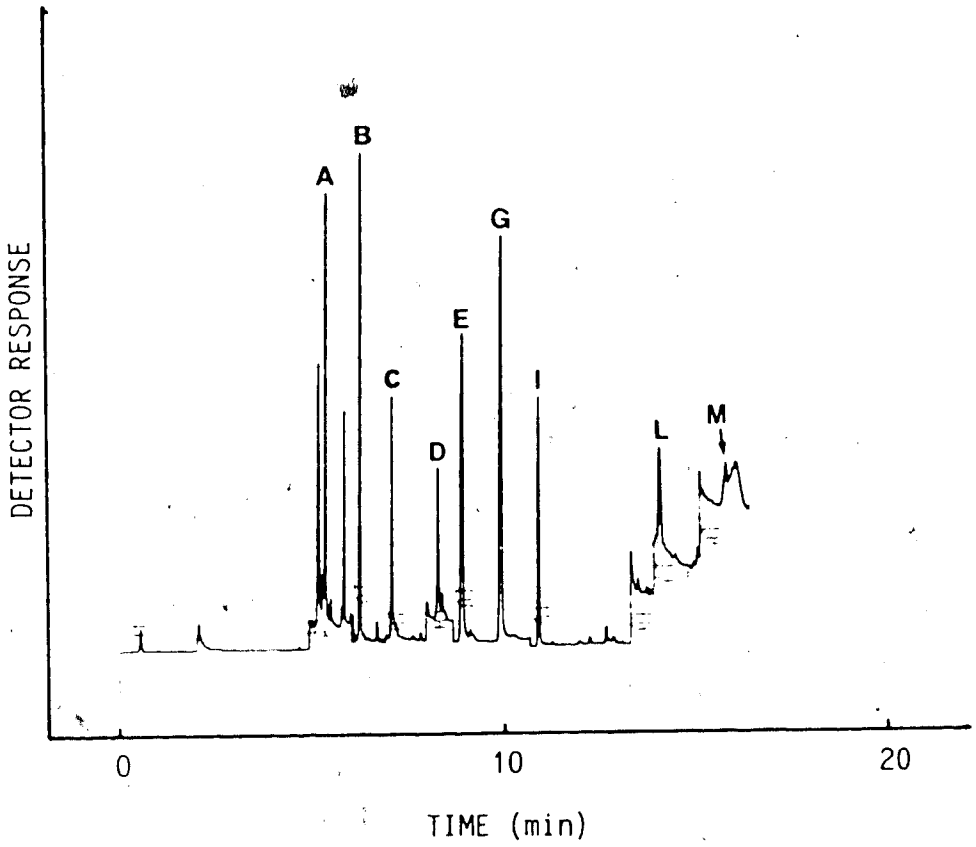


Figure 18. A typical gas chromatogram of a cheese extract carried through the pentafluorobenzoylation procedure. This extract is from a sample of hickory smoked cheese, and the peaks shown are: derivatives of β-phenylethylamine (A), 2-(4-chlorophenyl)ethylamine (B), tele-methylhistamine (C), histamine (D), putrescine (E), cadaverine (G), p-tyramine (I), spermidine (L) and spermine (M).

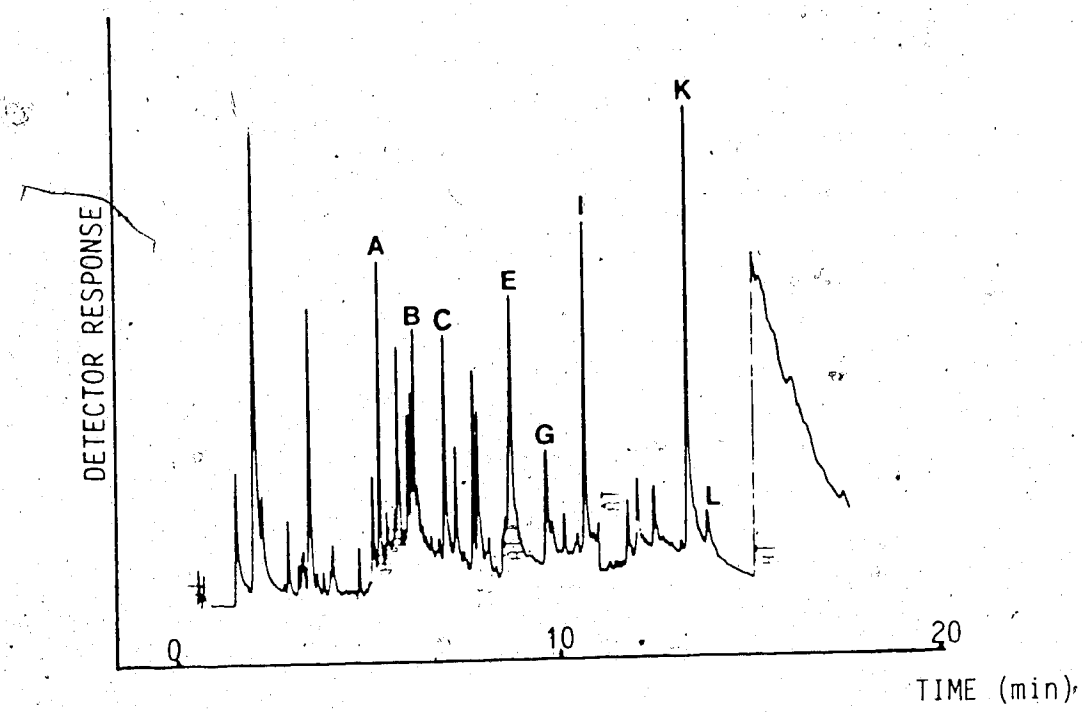


Figure 19. A typical gas chromatogram of a chocolate extract carried through the pentafluorobenzoylation procedure. This extract is from a sample of Fry's cocoa and the peaks shown are: derivatives of β-phenylethylamine (A), 2-(4-chlorophenyl)-ethylamine (B), tele-methylhistamine (C), putrescine (E), cadaverine (G), p-tyramine (I), 5-hydroxytryptamine (K) and spermidine (L).

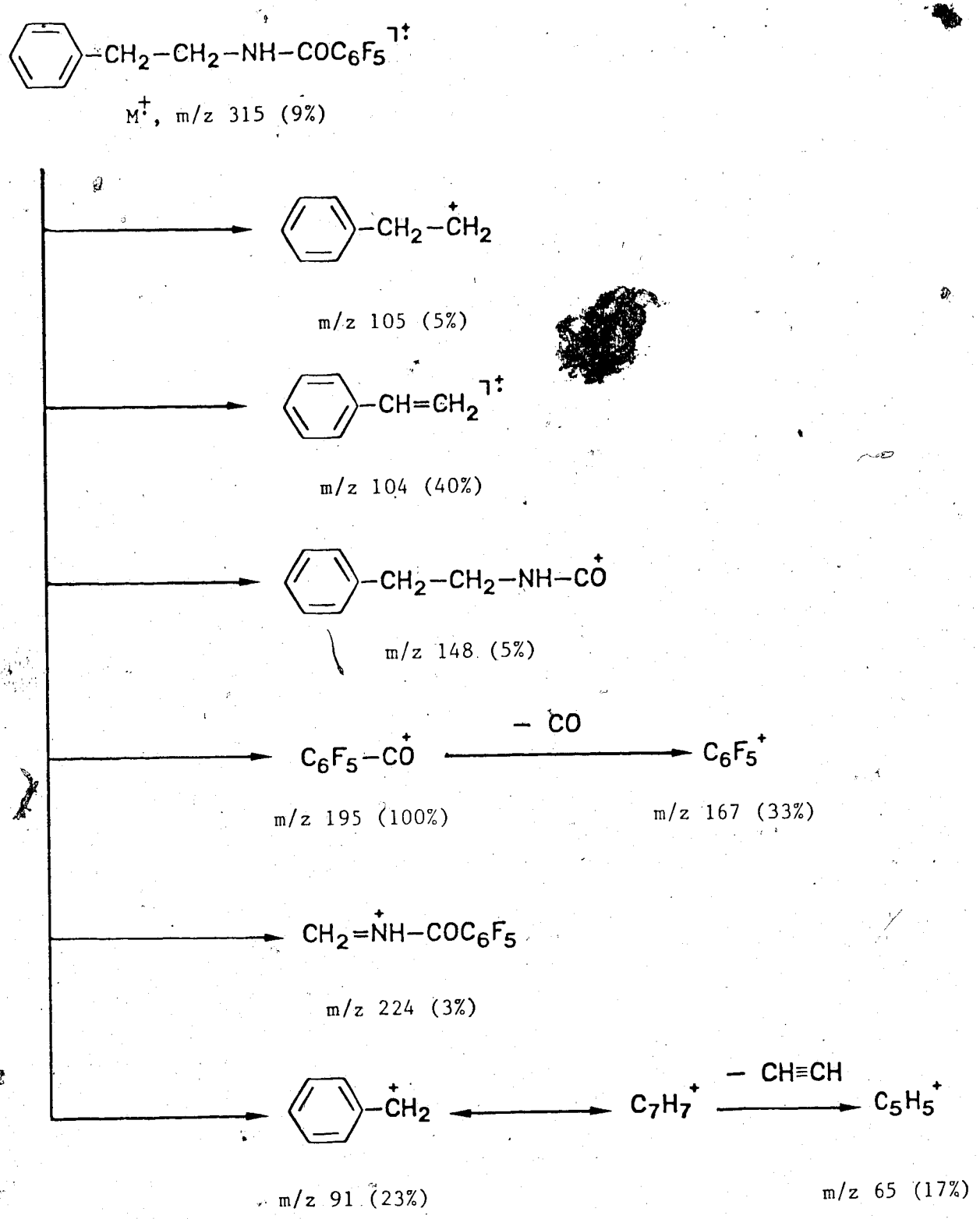


Figure 20. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of β-phenylethylamine.

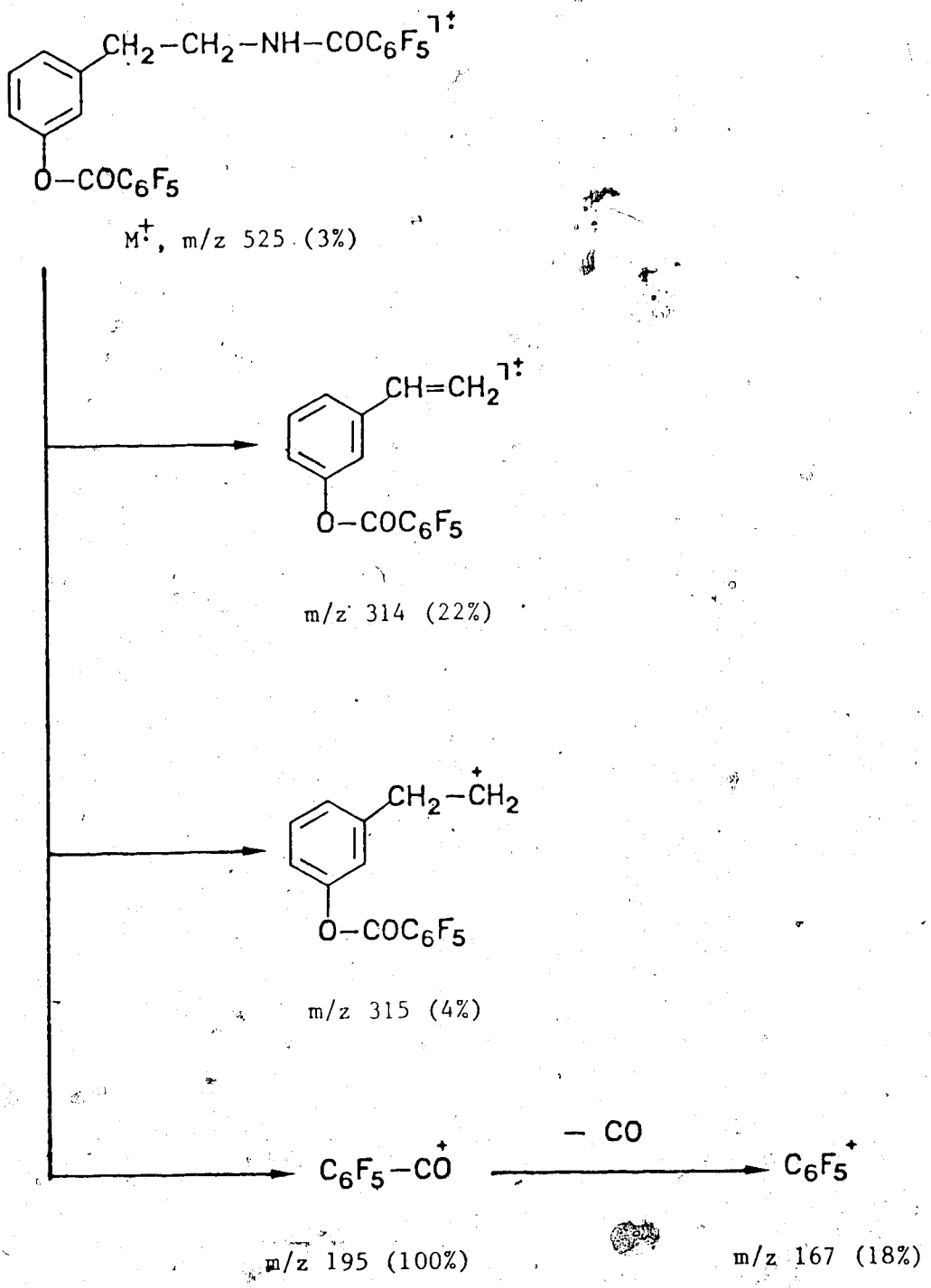


Figure 21. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of m-tyramine.

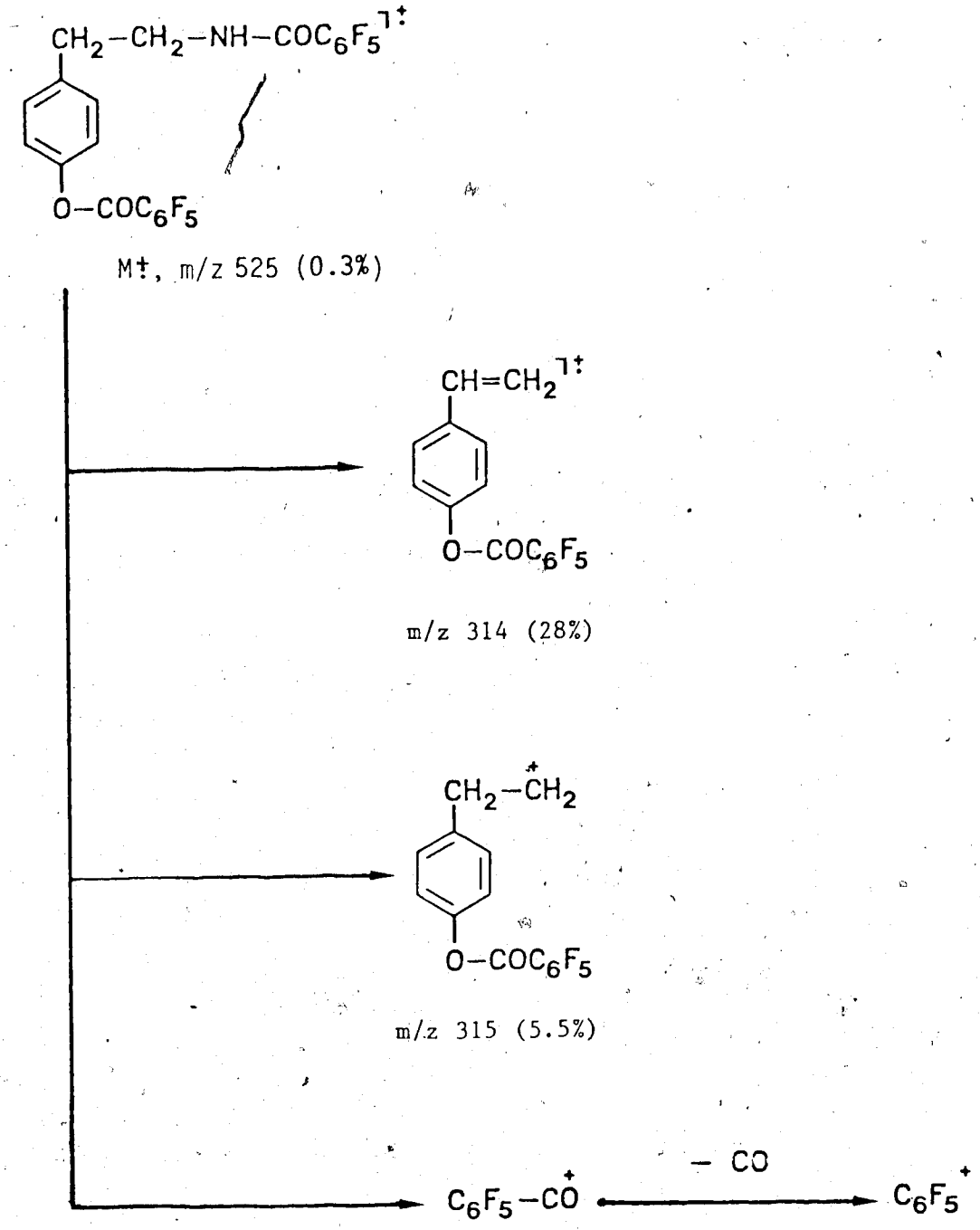


Figure 22. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of p-tyramine.

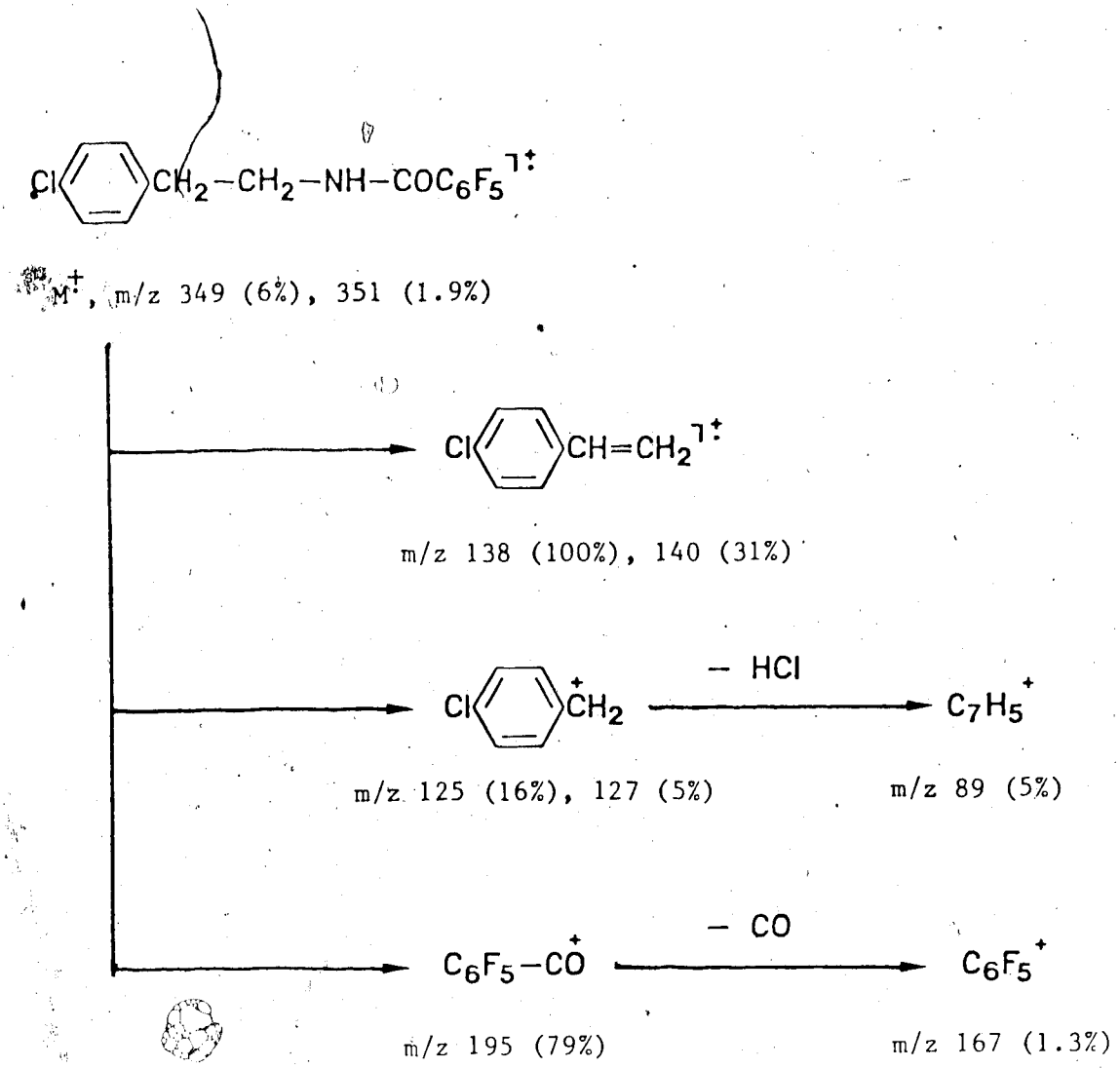


Figure 23. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of 2-(4-chlorophenyl)ethylamine.

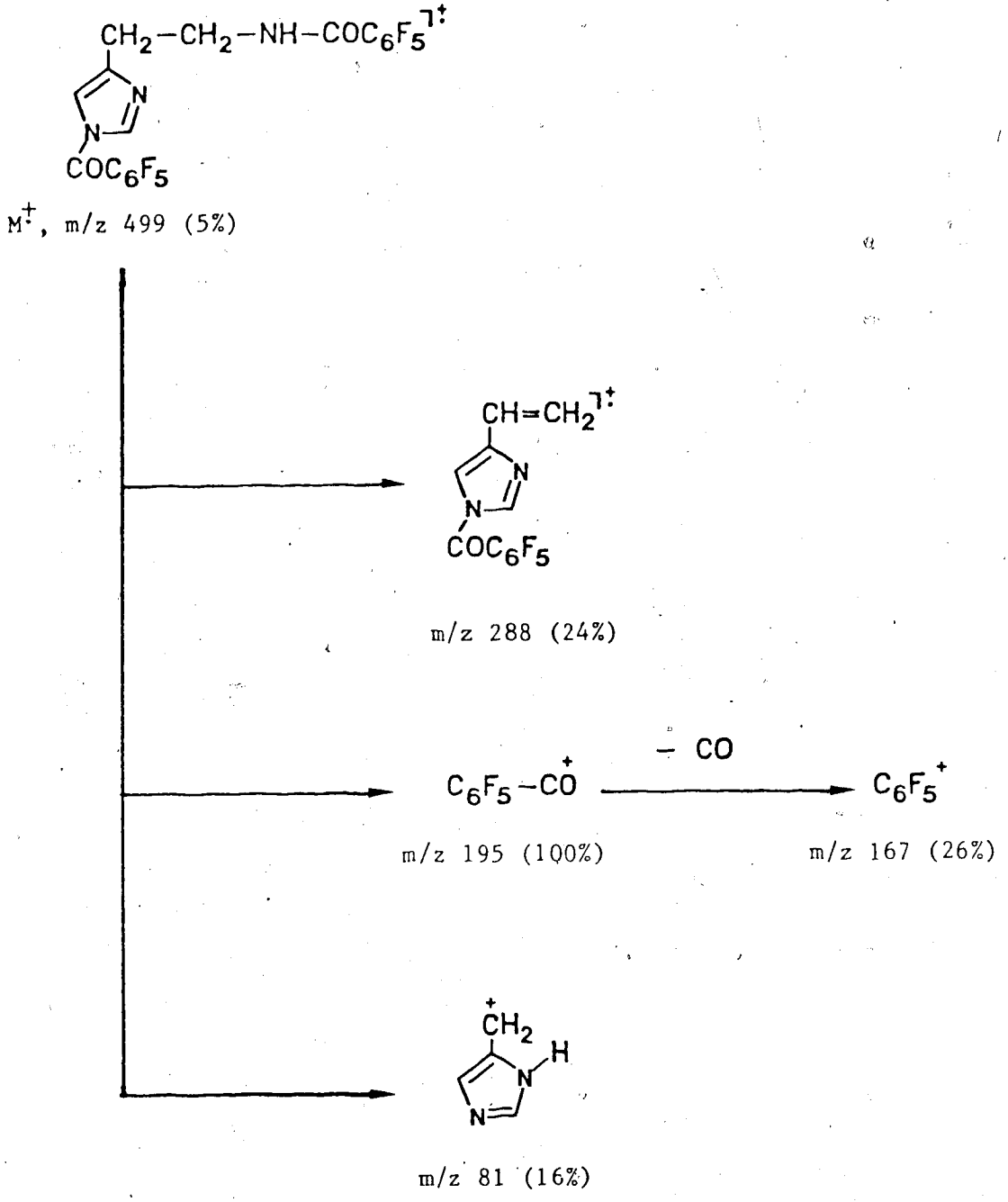


Figure 24. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of histamine.

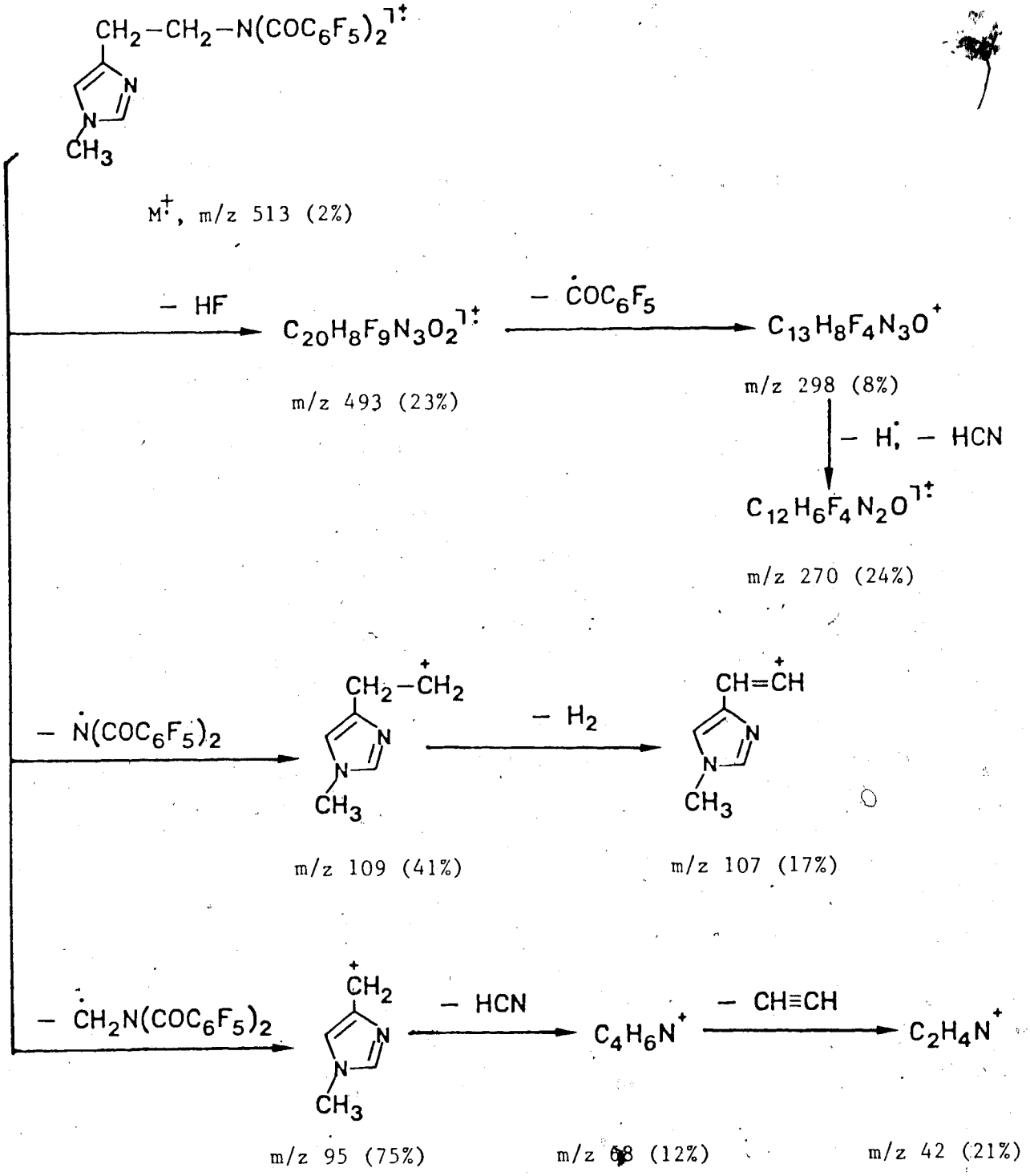


Figure 25. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of tele-methylhistamine.

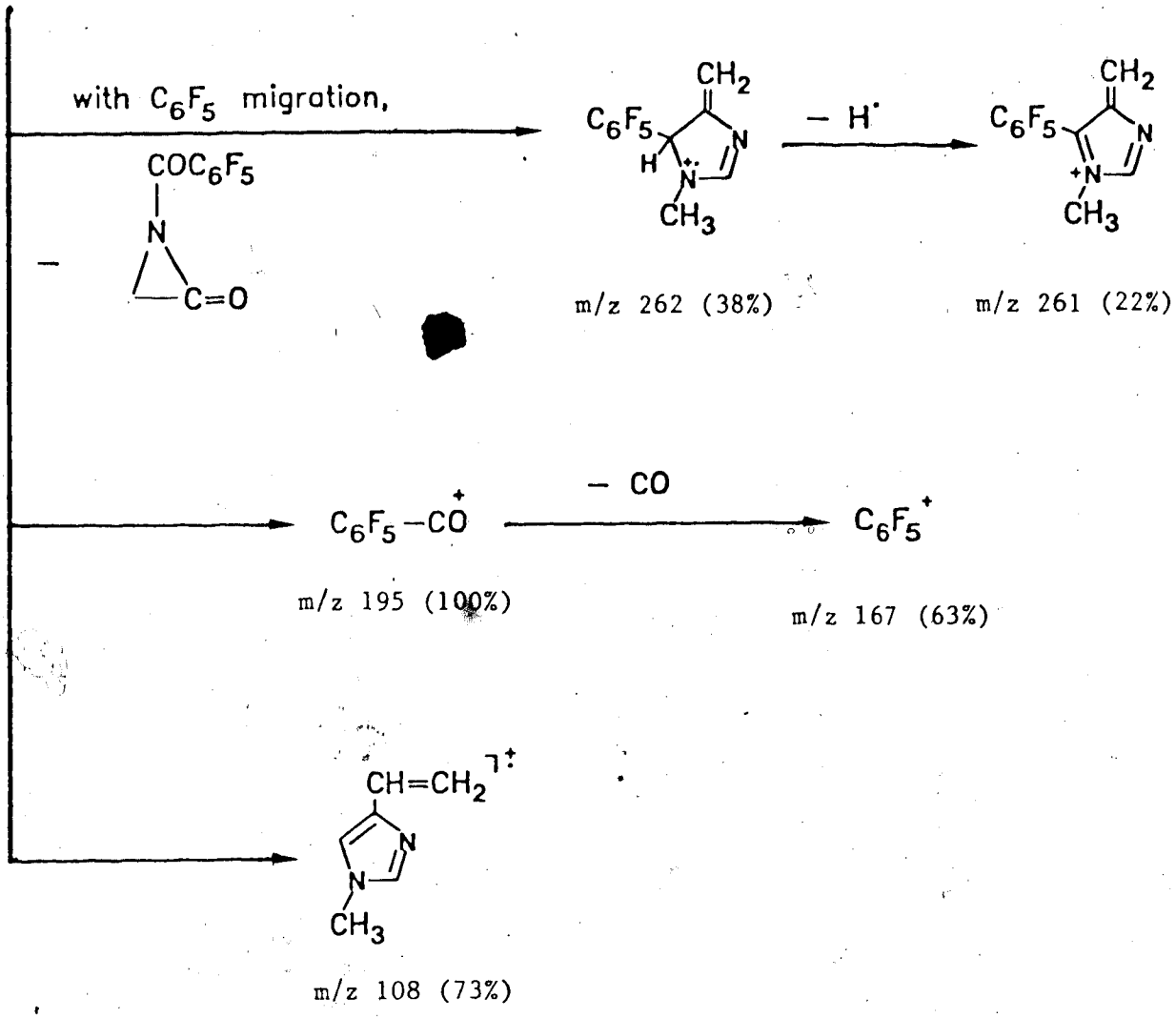


Figure 25. (cont'd)

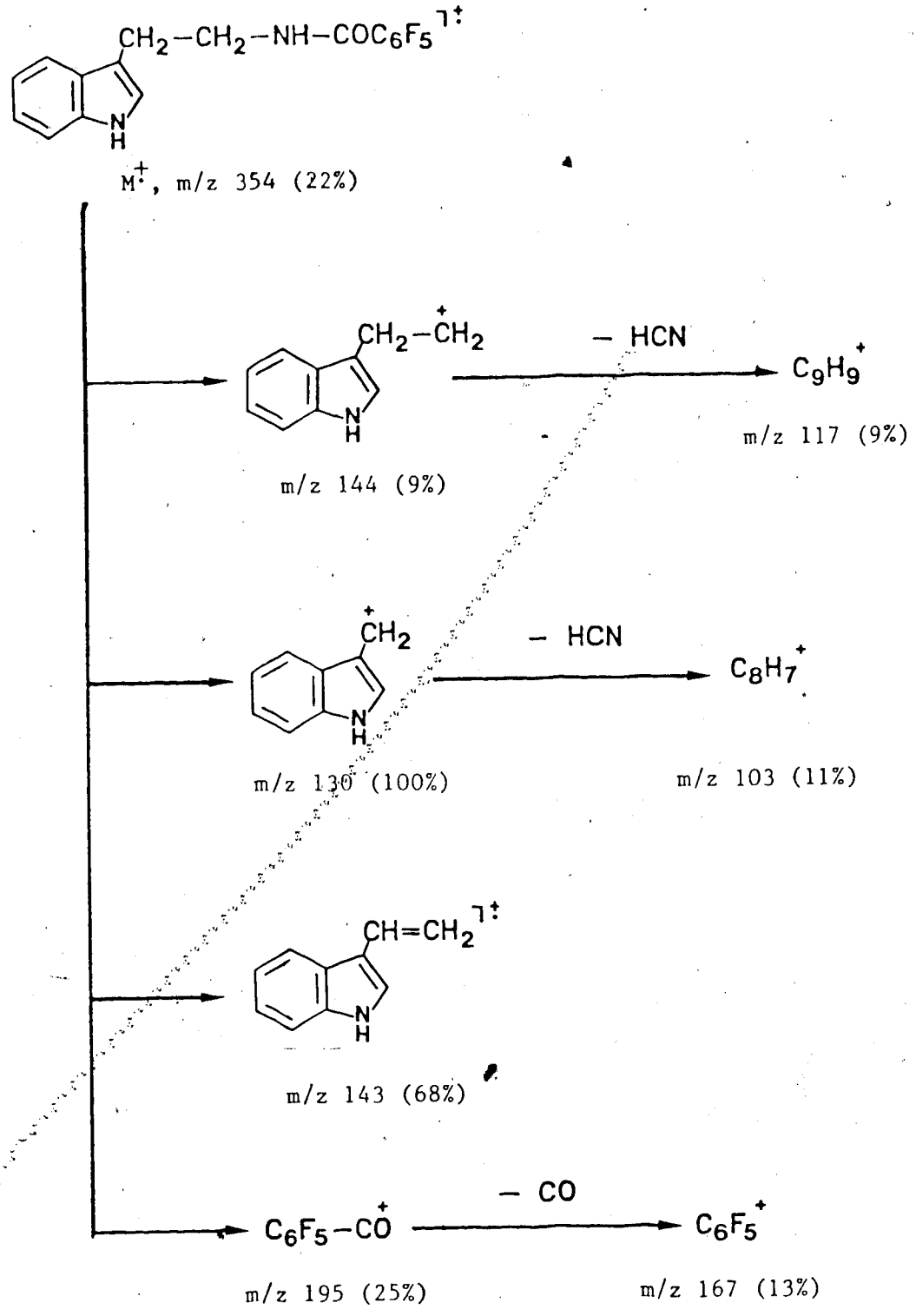


Figure 26. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of tryptamine.

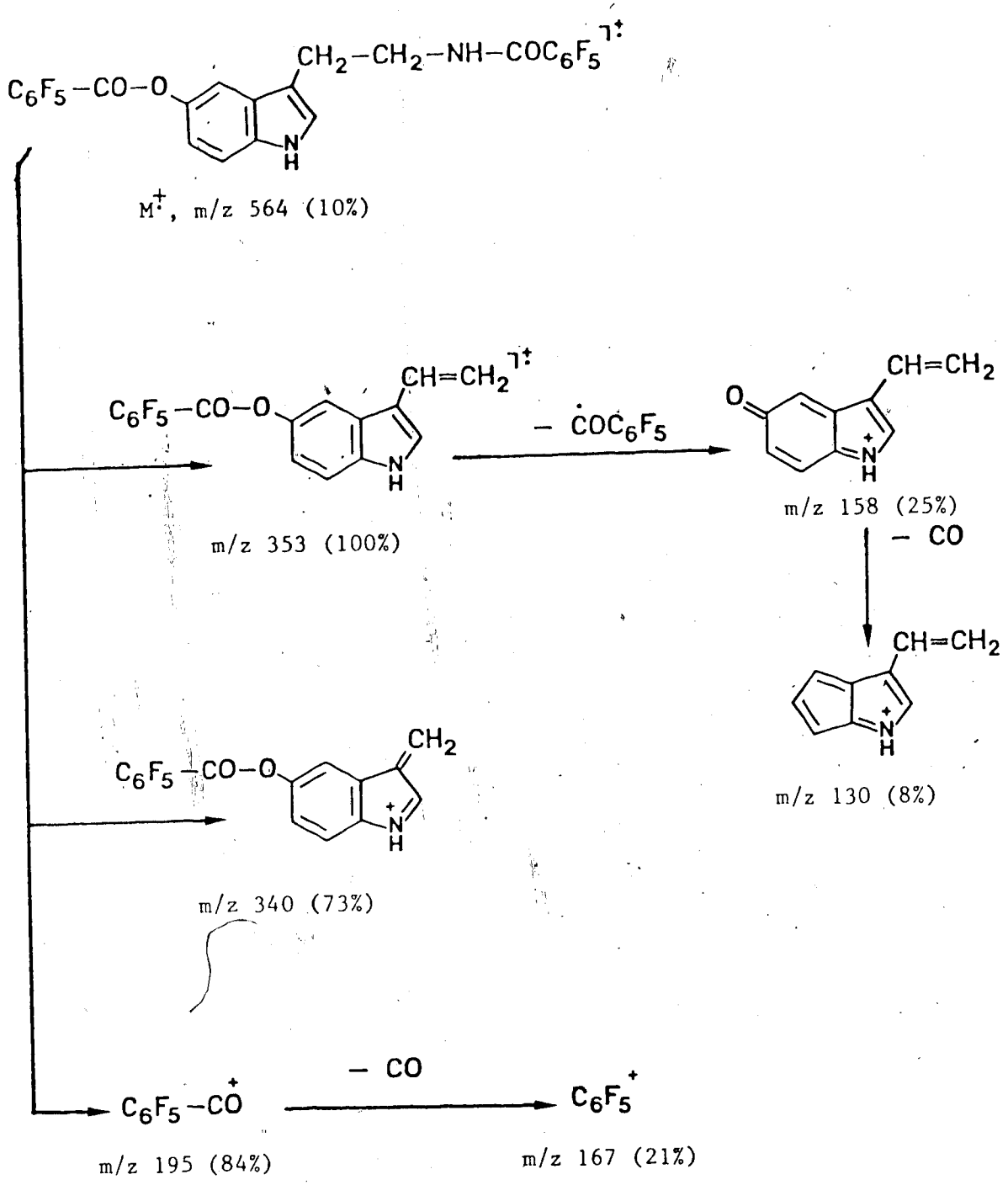


Figure 27. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of 5-hydroxytryptamine.

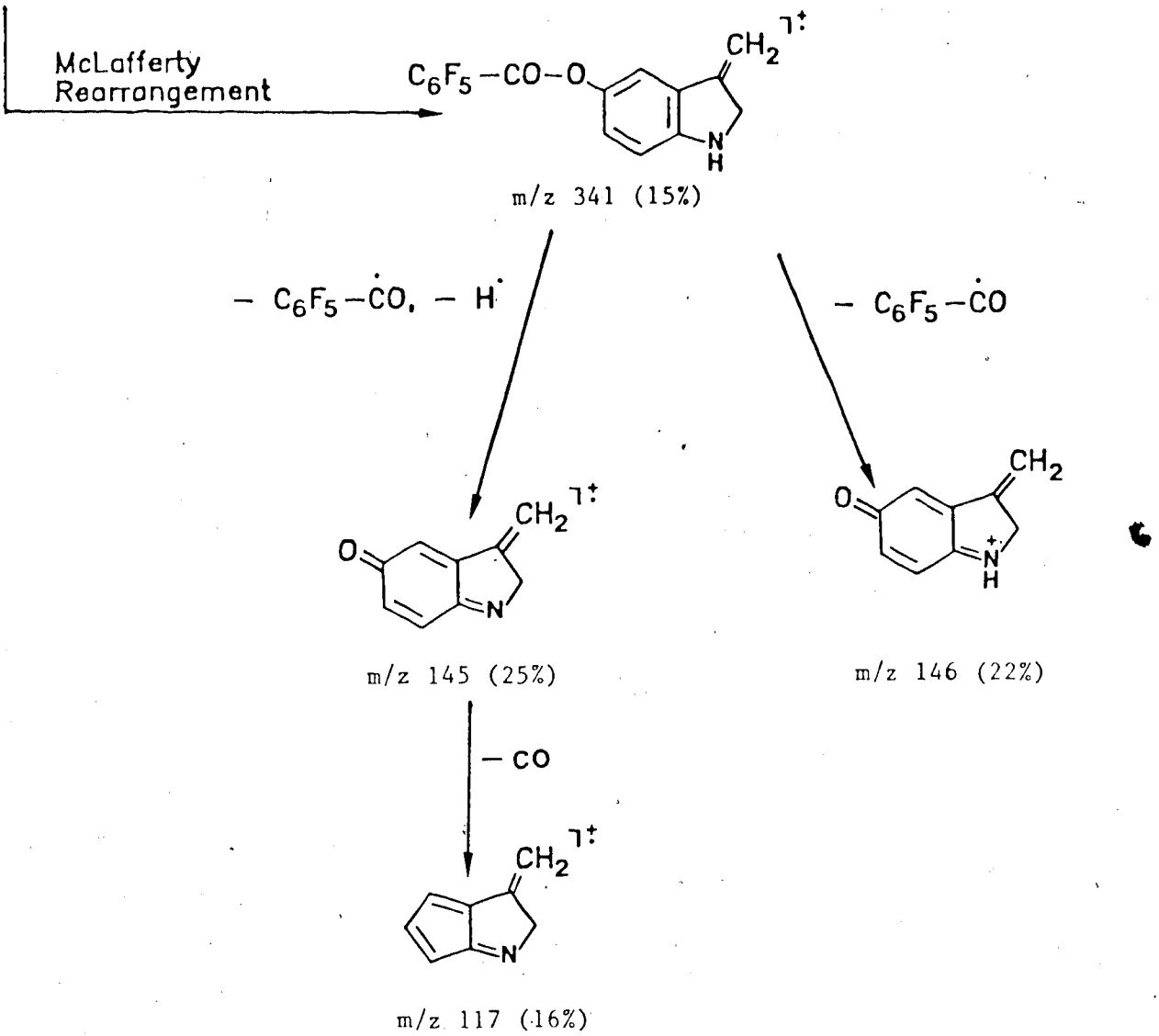


Figure 27. (cont'd)

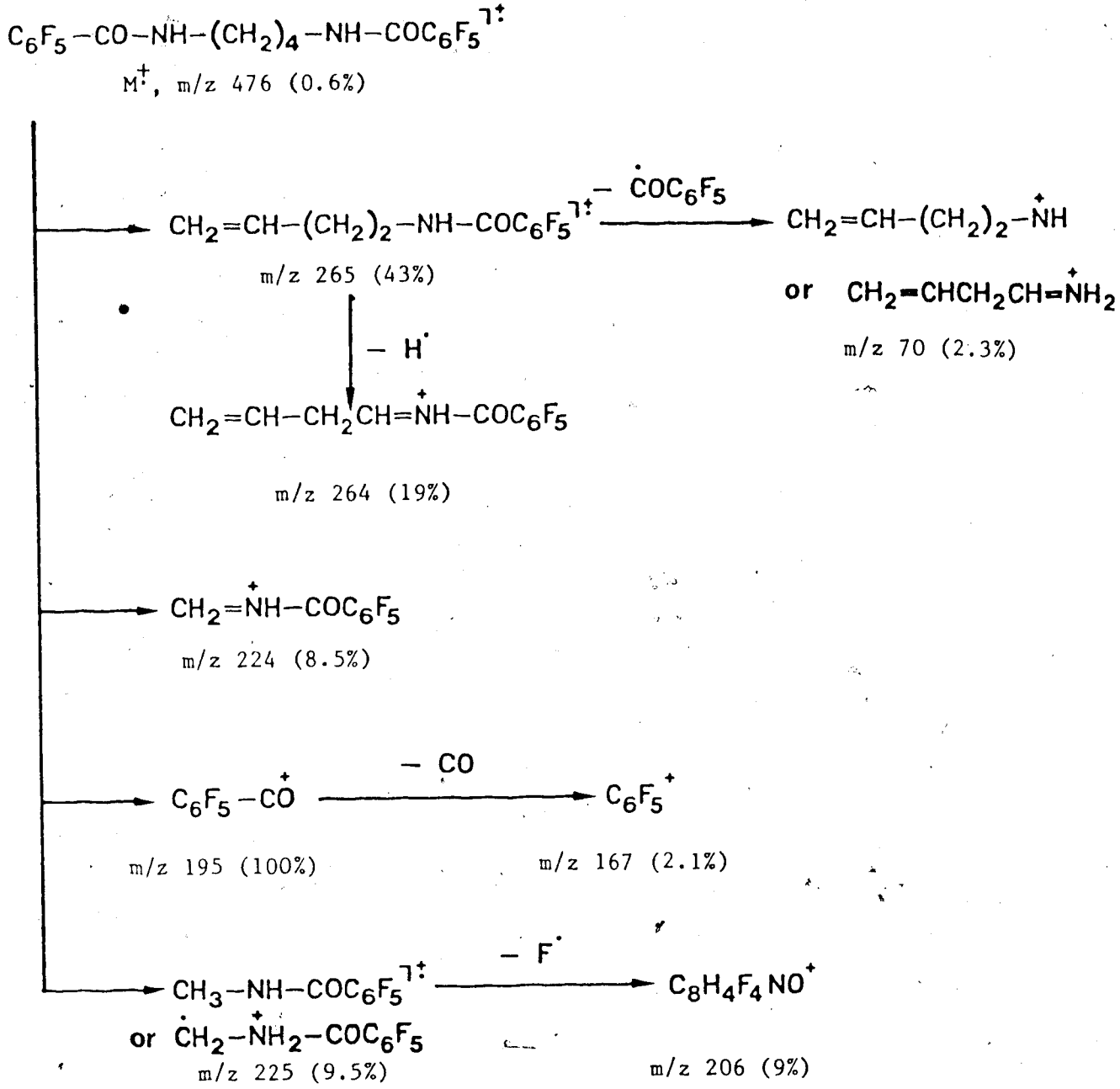


Figure 28. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of putrescine.

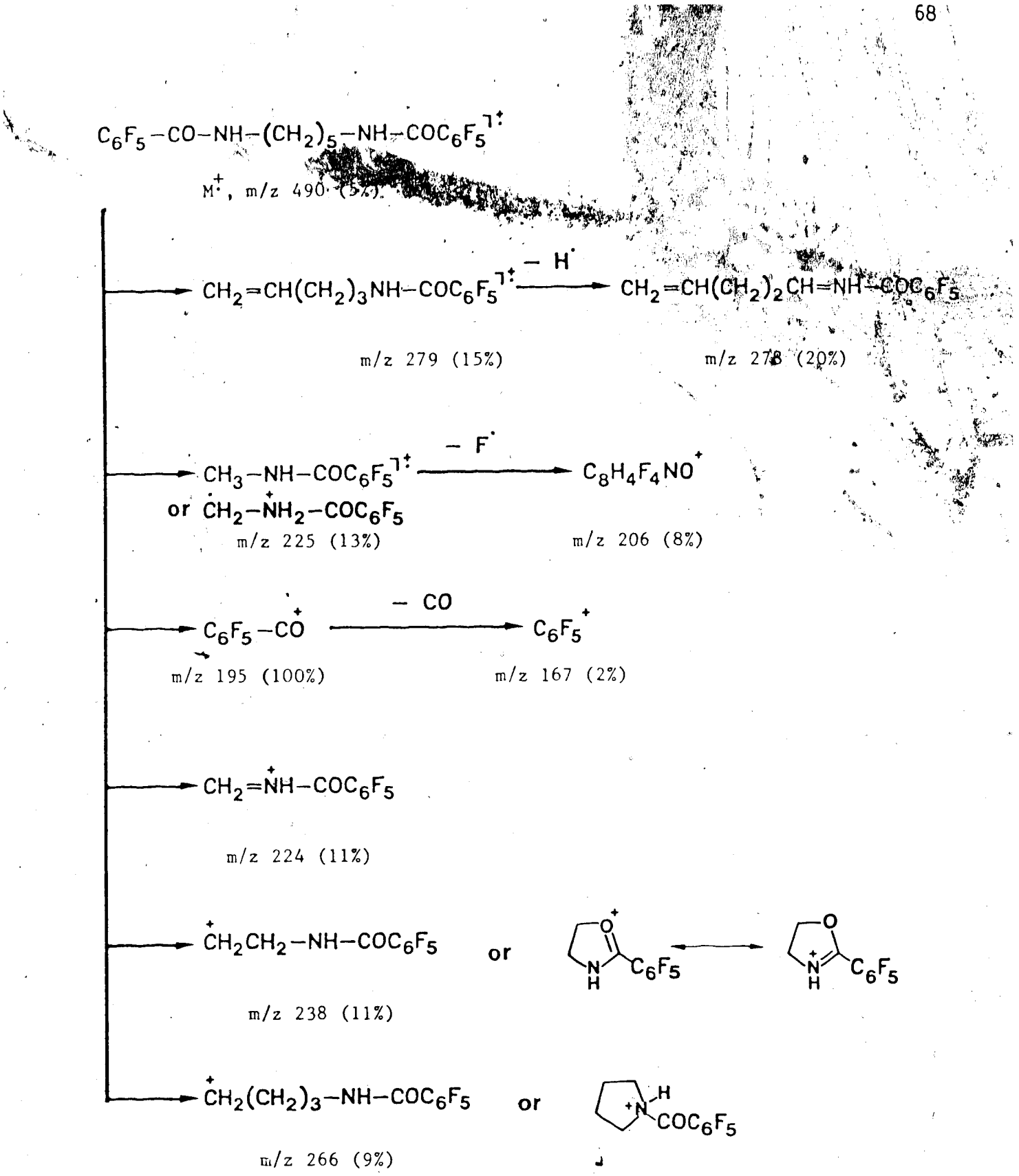


Figure 29. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of cadaverine.

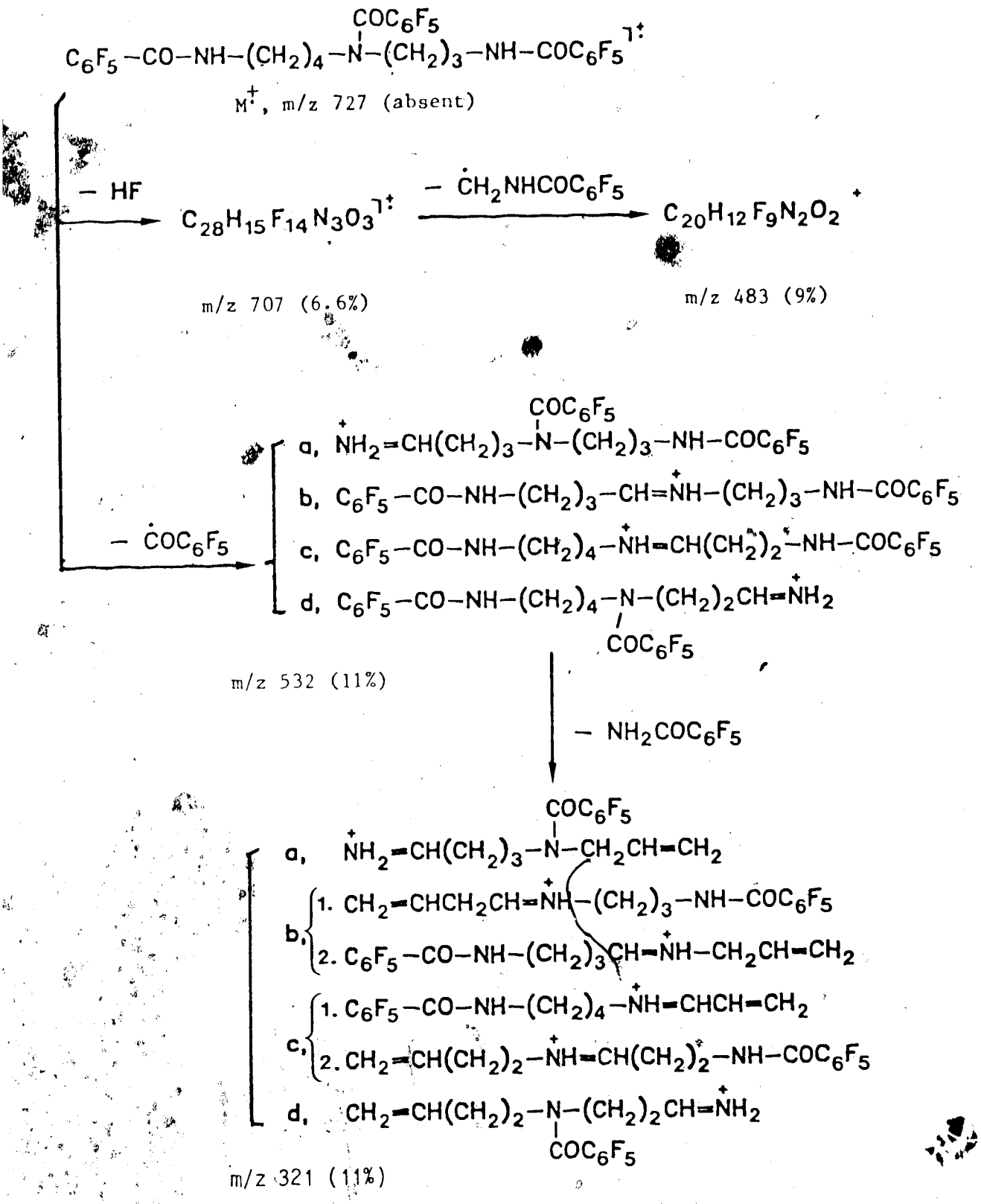
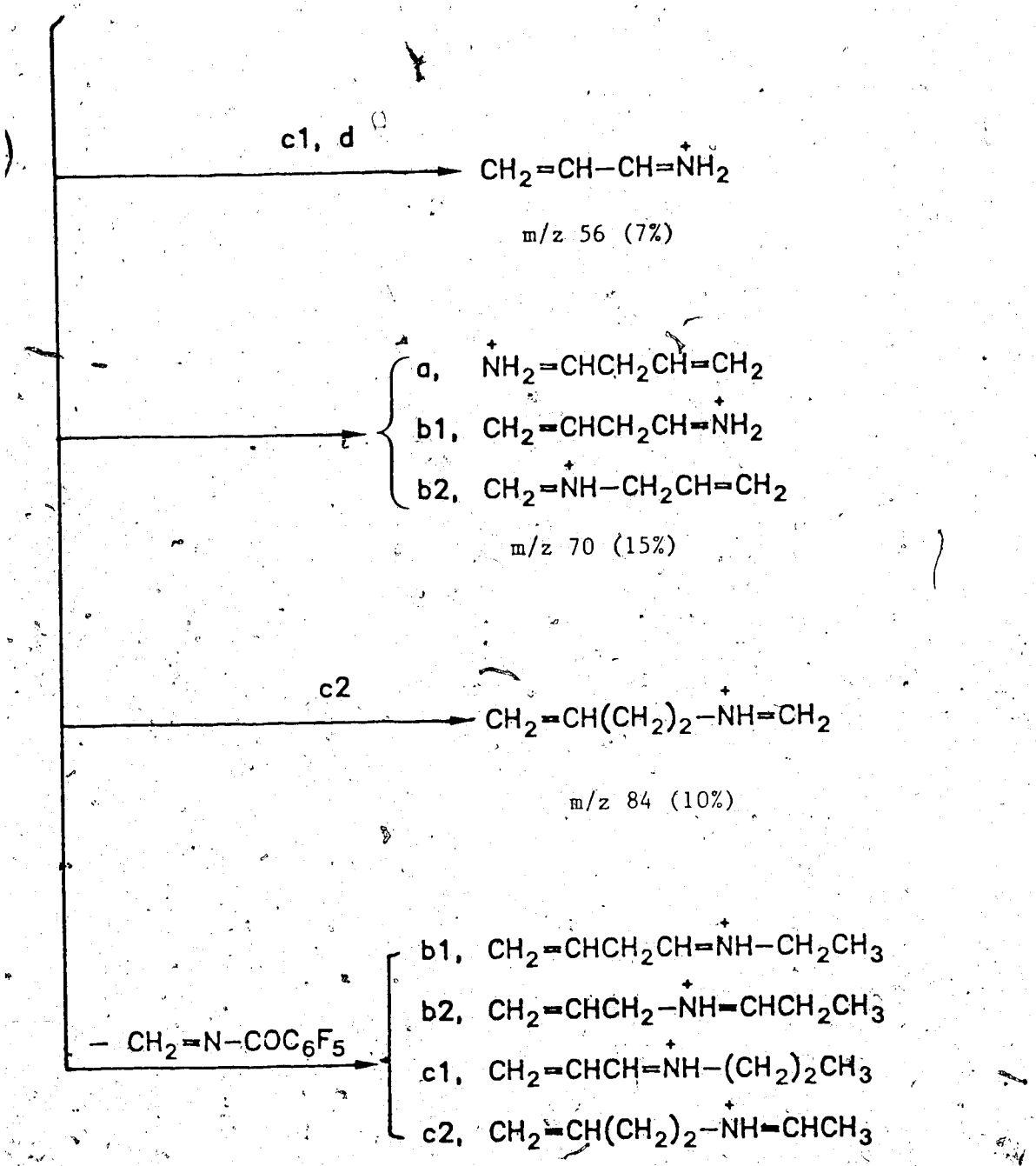


Figure 30. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of spermidine.

further fragmentation and rearrangement from (m/z 321)



from Molecular Ion,

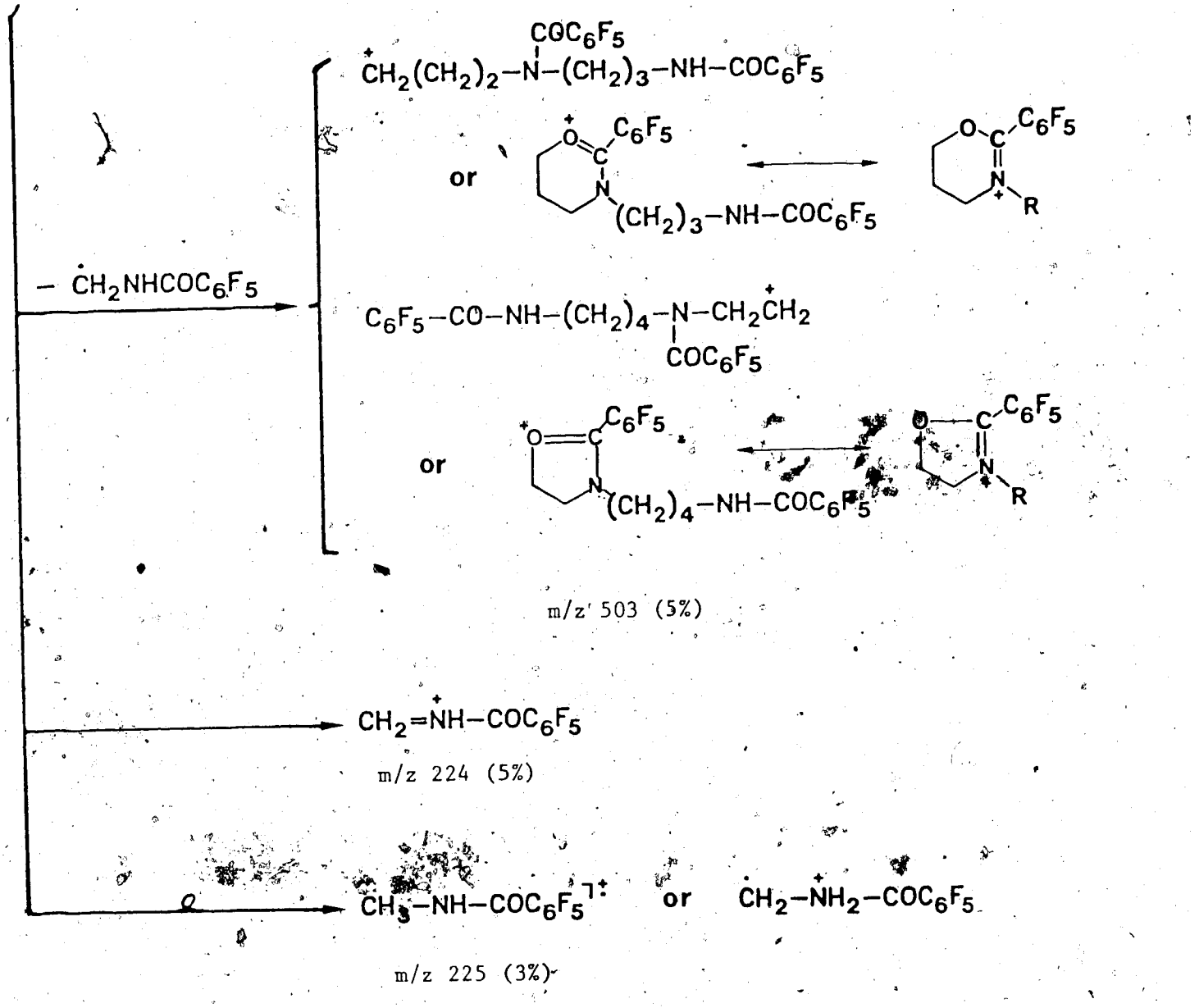


Figure 30. (cont'd)

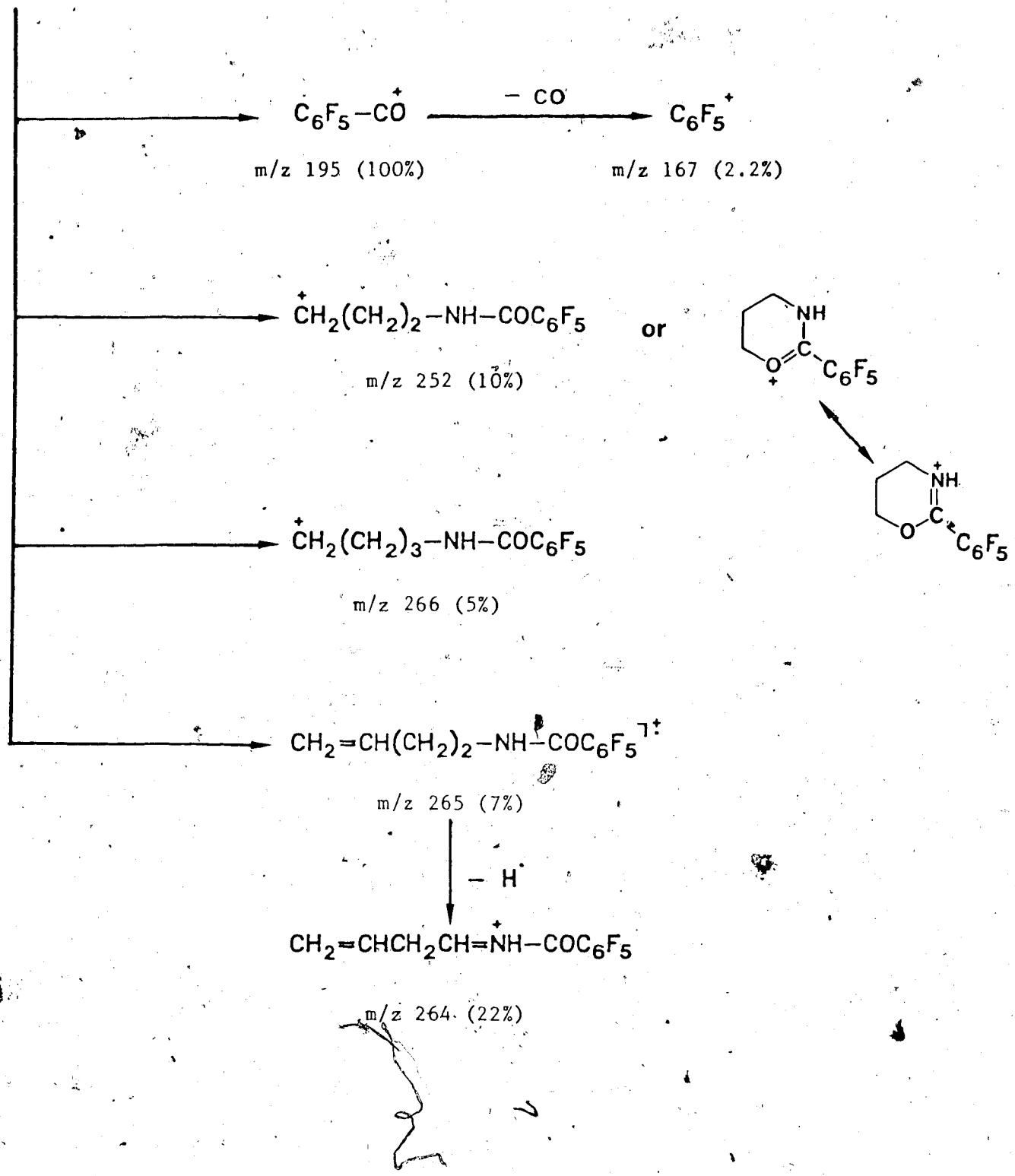


Figure 30. (cont'd)

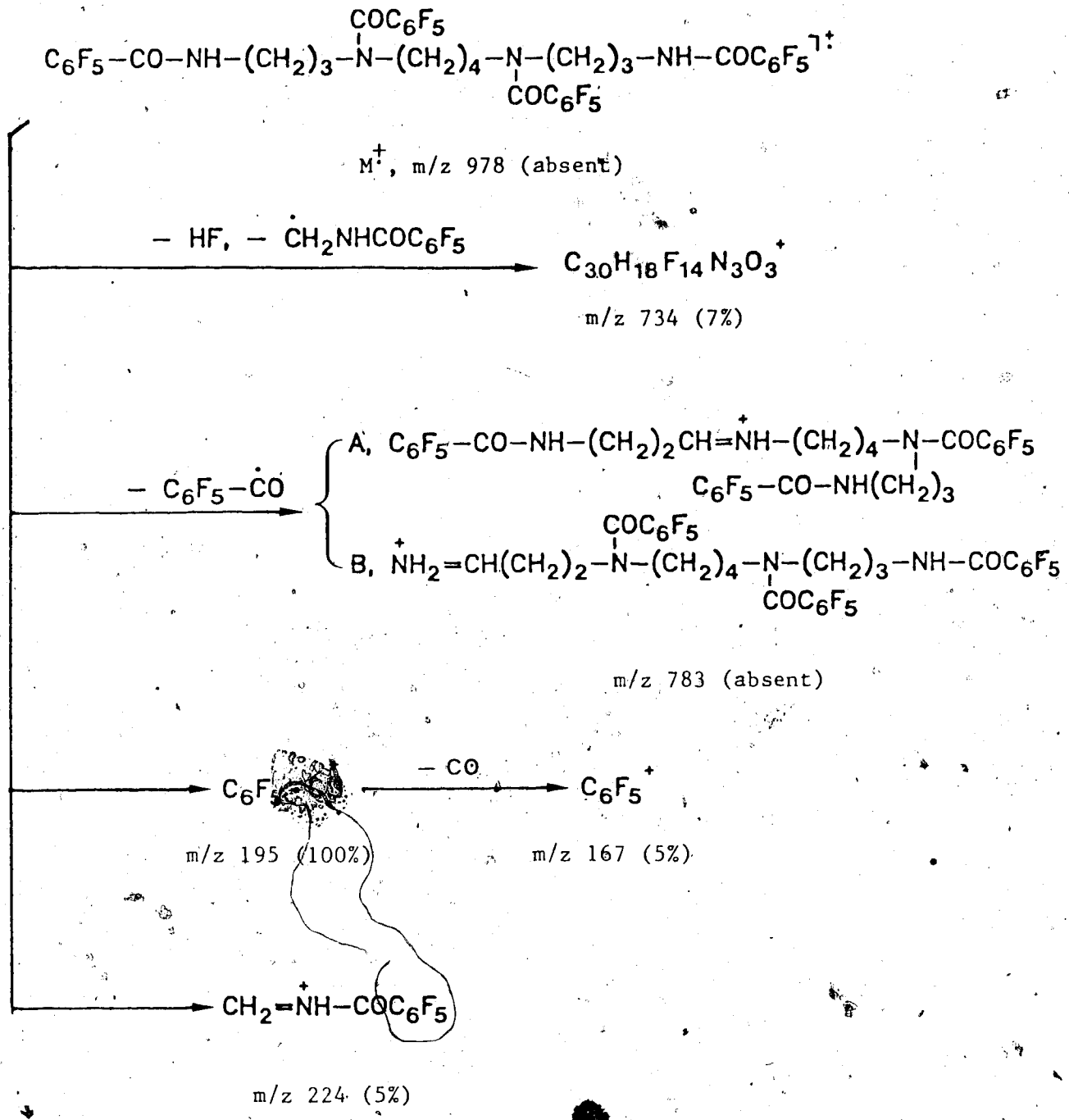


Figure 31. Proposed mass fragmentation pattern for the pentafluorobenzoyl derivative of spermine.

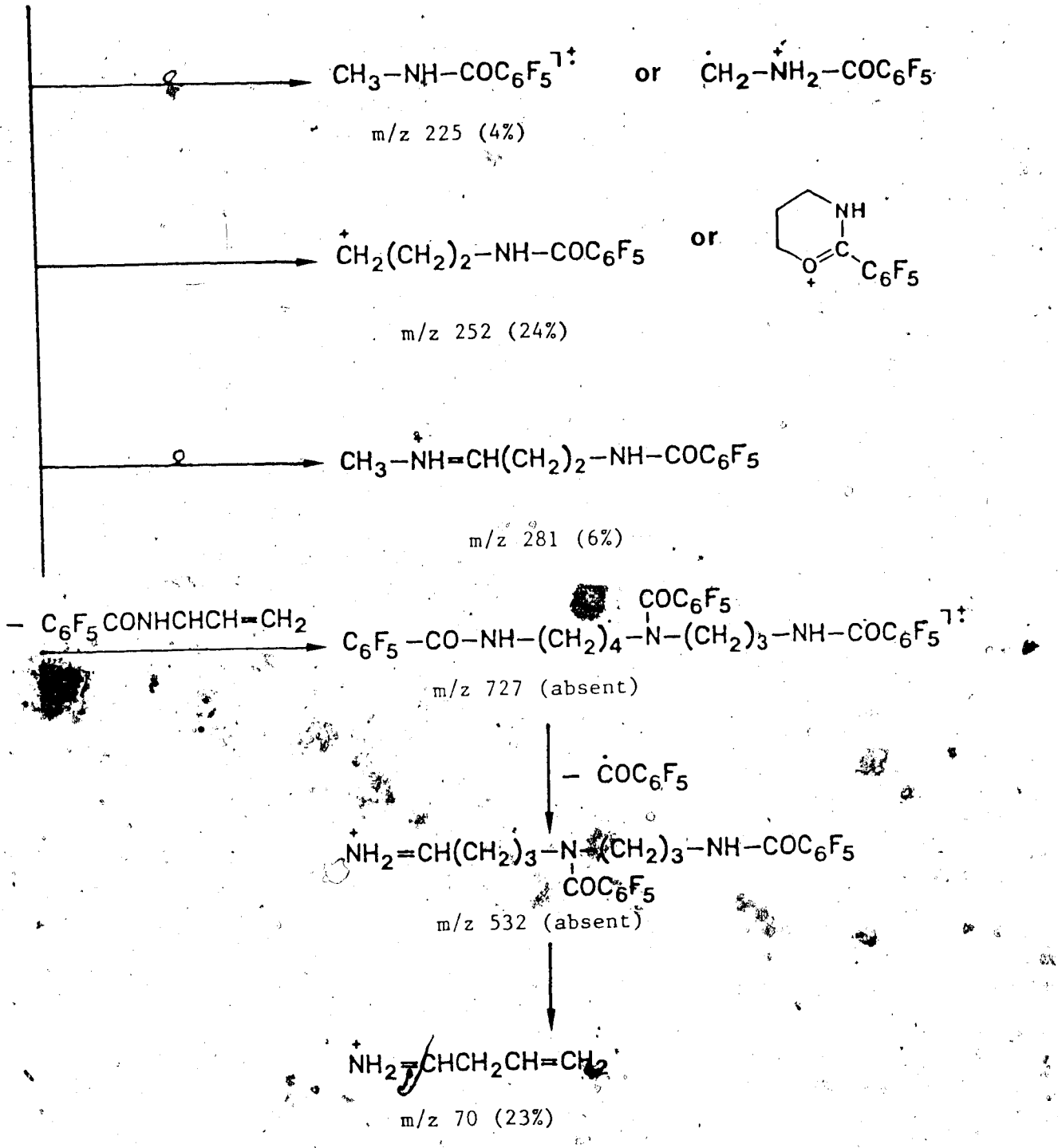


Figure 31. (cont'd)

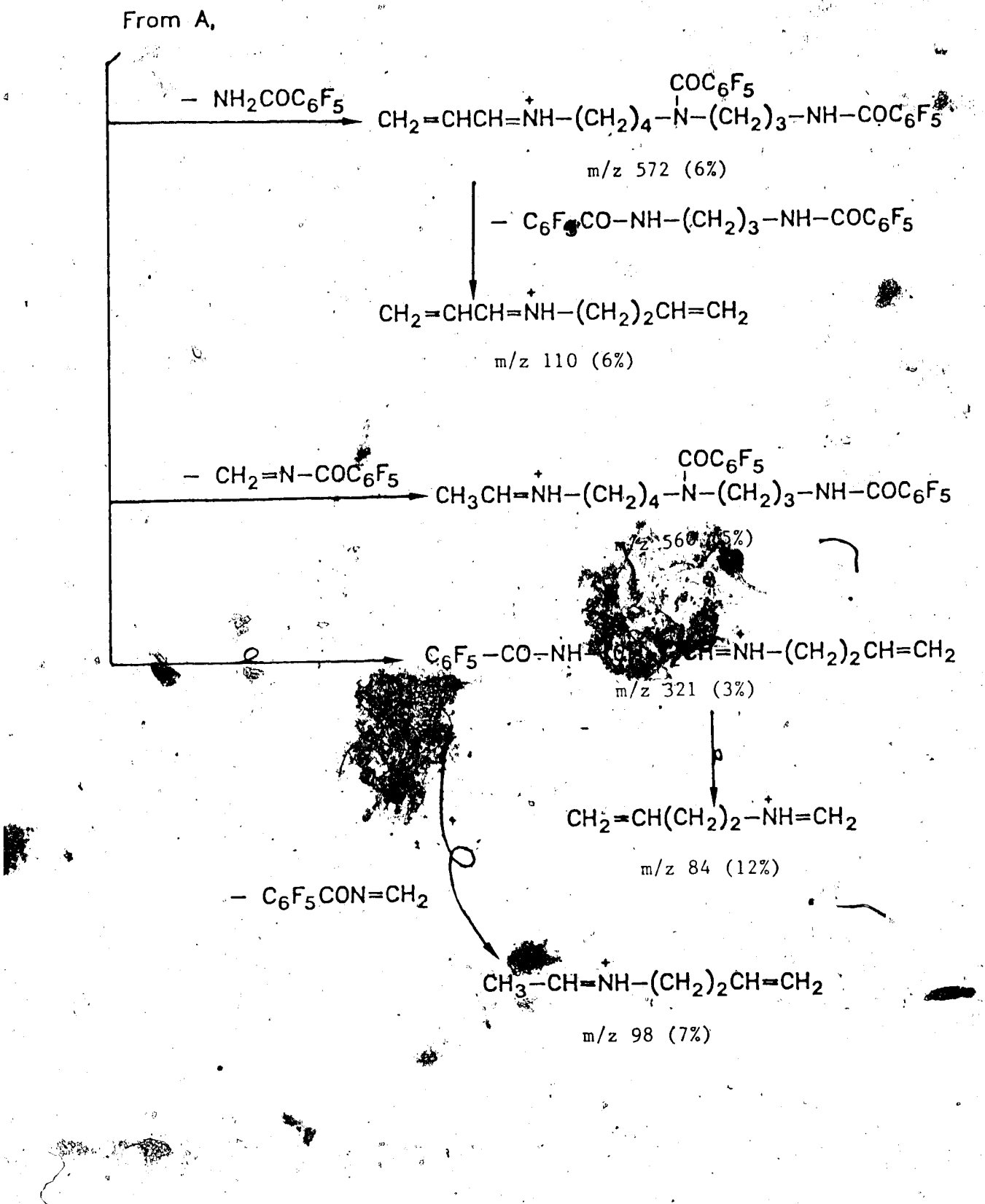
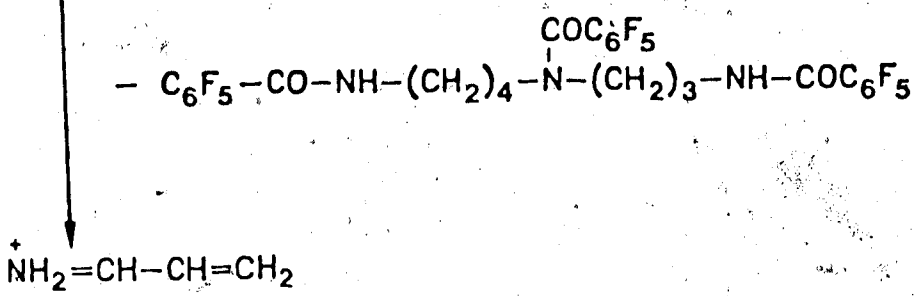


Figure 31. (cont'd)

From B,



m/z 56 (10%)

Figure 31. (cont'd)

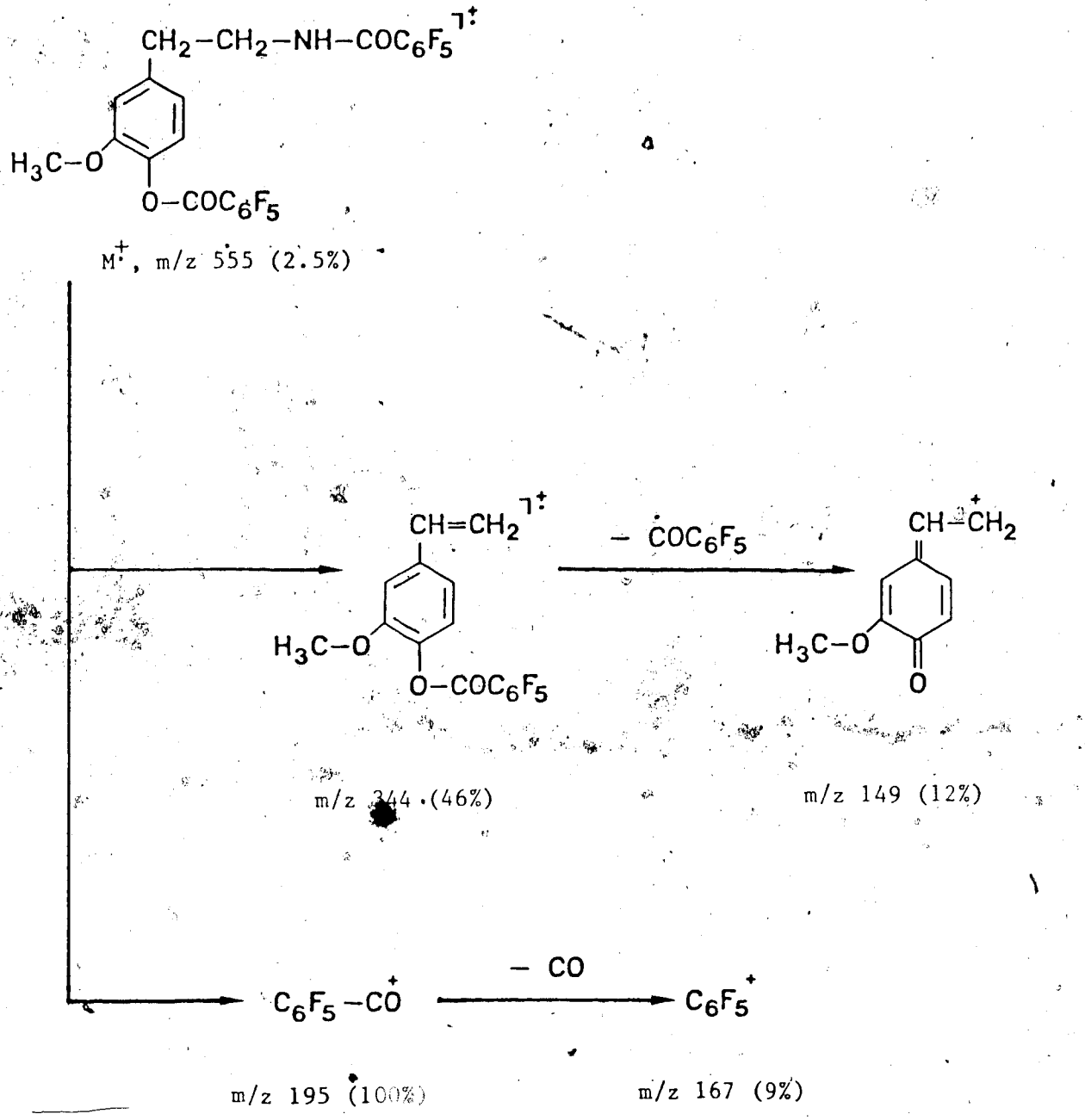


Figure 32. [redacted]ed mass fragmentation pattern for the pentafluoro-[redacted]yl derivative of 3-methoxytyramine.

amines all have the same core structure -NH-$(CH_2)_n$-NH-. Except for that of MeHA, the amino groups of all compounds reacted with PFBC to form amides of structure R-NHCOC_6F_5. It is interesting that the mass spectral fragmentation pattern of PFB-MeHA seems to suggest that the amino group of MeHA was doubly derivatized on the same aliphatic nitrogen atom. Histamine, in contrast, did not react in the same manner to give N,N-di-PFB derivative, although it contains similar amino functionality to that of MeHA. This observation may prompt a further investigation in imidazole chemistry.

In the course of method development, an effort was made to determine the most appropriate solvent to be used for the PFB-derivatization/extraction step. Benzene was chosen because enhanced extraction efficiency of the derivatives was obtained when compared to the results using other solvents such as ethyl acetate. In addition, gas chromatograms showed cleaner baselines when benzene rather than ethyl acetate, toluene or chloroform was employed as the extracting solvent.

Initially OV-1 (3%) and OV-17 (3%) packed columns were used for the analysis. It was found that although the PFB derivatives of the amines showed good chromatographic peak shapes on these columns, these columns did not provide adequate resolution. A 25 m Hewlett Packard cross-linked 5% phenylmethyl silicone fused silica capillary column was then chosen. The derivatives were all resolved and showed good gas chromatographic properties; however long analysis time was involved because of the length of column. Finally a 15 m SE-54 fused silica column was found to be most suitable for this study; it provides good separation of all amine derivatives in a relatively short period of time (17 min) (Figure 17 on page 54). In this trace, attenuation changes were programmed in

so that all peaks appeared on scale and are thus recognizable as single entities. This also allows hand integration if required.

Amines in food extracts prepared with 0.4N $HClO_4$ were isolated efficiently by the use of the liquid ion-exchanger di-(2-ethylhexyl)phosphoric acid (DEHPA). The popularity of its use in isolation of amines is well documented (Temple and Gillespie, 1966; Martin and Ansell, 1973; Nelson et al., 1979; Baker et al., 1980; Hampson et al., 1984a). Di-(2-ethylhexyl)phosporic acid is convenient to use because it is a liquid and is soluble in chloroform. During the initial cleanup stage, extraction of amines from the basified perchloric acid homogenate can be accomplished simply by liquid-liquid extraction. Since chloroform is heavier than water, subsequent separation of phases can be conveniently performed by aspiration of the top aqueous phase using a waste-trap and suction system. Interfering substances were excluded and satisfactory gas chromatograms were obtained.

In the determination of extraction efficiency of amines from basified 0.4N $HClO_4$ by 2.5% DEHPA in $CHCl_3$ and back extraction into 0.5N HCl, two salts were compared for basification. This was done by preparing three sets of six tubes containing 100 ng of amines of interest to this study. One set (set A) was analyzed using the procedure that was described in section D of Chapter II on page 50. Another set (set B) was put through the same procedure except that a different salt was used to basify the perchloric acid layer. The analysis of the third set (set C) started at the 0.5 N HCl stage, that is at the step following extraction with DEHPA, the samples were basified and derivatized with PFBC. A fixed amount of internal standard compound CPEA (2 µg) was added to all tubes of the three sets at the 0.5 N HCl stage. Per cent

recoveries were determined by dividing the quantities determined in set A or set B by the mean quantity determined in set C and multiplying by 100%. Table III shows that basification using $KHCO_3$ (vs. using K_2CO_3) generally provides better extraction efficiency. The recoveries of HA, the polyamines and the phenolic phenylethylamines were dramatically reduced when the K_2CO_3 was employed.

Calibration curves were included in each analytical run to overcome minor fluctuations in detector response. The internal standard compound, CPEA, which was included in the sample tubes and in the tubes that made up calibration curves, served as a reference to counteract losses which occur during the extraction procedure. Furthermore, the presence of an internal standard makes it possible to eliminate the necessity of having to measure an exact volume (e.g. 1.0 µl) of sample for GC injection. The calibration curves for the amines prepared from peak height ratios relative to those of the internal standard were found to be linear over a 100-fold range of concentration, and the reproducibilty of the calibration curves was found to be satisfactory for quantitative determination (see Figure 33). In initial experiments, the results determined by the electronic integrator were checked against results determined by hand measurement of peak heights using a ruler. Since there was good agreement between the results obtained using both procedures, the integrator was used for convenience in subsequent experiments.

The catecholamines were excluded from this study because the following difficulties were encountered. The PFB derivative formed from DA had relatively poor sensitivity and gave inconsistent detector response from one run to another. No peak could be found for NA or A after per-

Table III. Recoveries (mean% ± S.E.M.) of amines during the entire pentafluorobenzoylation procedure. The values of the left column represent recoveries when $KHCO_3$ was used to basify the $HClO_4$ extract and the values in the right column when K_2CO_3 was used as the basifying agent. (n=6)

	$KHCO_3$	K_2CO_3
PEA	87 ± 5	86 ± 4
MeHA	53 ± 3	54 ± 5
HA	86 ± 10	60 ± 18
PU	98 ± 14	59 ± 12
T	80 ± 2	96 ± 2
CA	107 ± 9	67 ± 5
m-TA	82 ± 4	63 ± 3
p-TA	80 ± 4	56 ± 3
3-MT	79 ± 7	58 ± 5
5-HT	74 ± 6	68 ± 7
SPD	113 ± 21	39 ± 5
SPM	112 ± 10	89 ± 3

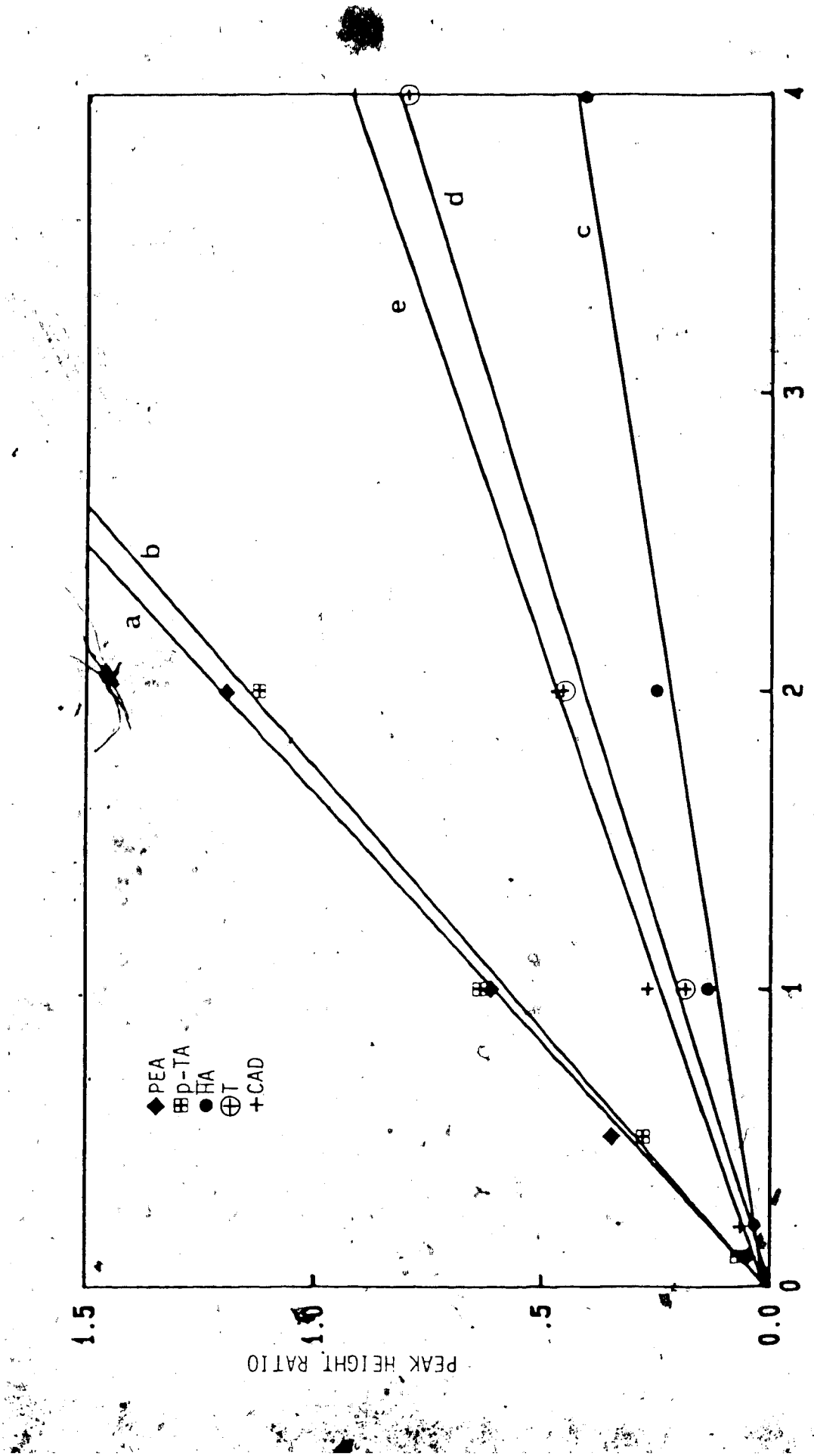


Figure 33. Typical calibration curves obtained during the pentadecafluorobenzylation procedure: β-phenylethylamine (a), p-tyramine (b), histamine (c), tryptamine (d) and cadaverine (e). These amines were chosen to represent examples of a non-phenolic phenylethylamine, a phenolic phenylethylamine, an imidazolylethylamine and an aliphatic polyamine.

forming the pentafluorobenzoylation procedure. An alternate procedure for analyzing brain catecholamines using a GLC technique has been developed (Baker et al., 1983). It consists of homogenizing samples in 0.5N HCl in ice-cold methanol. After centrifugation, the clear supernatant is taken to dryness under a gentle stream of nitrogen at room temperature in a dark room. A small amount (50 µl) of ethyl acetate is added to the dried residue followed by 75 µl PFPA. The tubes are heated to 60°C for 30 min. After cooling at room temperature for 5 min, 100 µl of hexafluoroisopropanol (HFIP) is added and the mixture is allowed to stand and react for 30 min at room temperature before taking it to dryness under a stream of nitrogen. The residue is redissolved in 400 µl cyclohexane and an aliquot is used for GLC analysis. Although this procedure is useful for analyzing catecholamines in brain tissue, it was found not applicable to analysis of food sample extracts since there were too many interfering substances in the food products.

On the basis of the results mentioned above, application of the pentafluorobenzoylation method to the determination of contents of amines (other than catecholamines) in foodstuffs was investigated. Samples to which internal standard had not been added were run to determine whether the internal standard could be confused with another peak having the same retention time and arising from a component of food. A typical gas chromatogram of a cheese sample is demonstrated in Figure 18 on page 55, showing that the internal standard region was free from interfering peaks.

The procedure was applied initially to determination of amine contents at different layers of the same block of cheese--surface, 5 mm below the surface layer, and the core. Price and Smith (1971) reported

higher amounts of p-TA in the rind of cheeses than in the core, presumably because of surface bacteria possessing decarboxylase activity, resulting in increased formation of p-TA from p-tyrosine. The present studies indicated that there was no significant difference in amine content between the surface and the layer 5 mm below the surface layer. Hence, subsequent determination of levels of amines in cheeses were done on the surface and the core layers. Levels of individual bioactive amines in some selected cheese and chocolate samples are tabulated in Tables IV and V. Results show that levels of amines in cheese vary greatly from brand to brand. These results indicate that, in general, the sharper-tasting/smelling cheeses contain higher overall levels of amines. A similar generalization may also be made about older cheeses. During the cheese-making process amines are formed by bacterial degradation of protein to amino acids and subsequent decarboxylation of the amino acids to amines. This process, the fermentation, is prolonged in the making of older cheeses, and older cheeses usually have sharper taste/smell.

Different lots of the same brand of cheese may also be substantially different in their amine contents. In most cases, amine contents of the surface layer and the core of a block of cheese are not significantly different. However, samples from both sections of the cheese were routinely analyzed and the mean value of the two areas determined for each sample.

In order to facilitate comparison of the relative amounts of amines in the various brands of cheese and chocolate, I have given, at the bottom of the appropriate table, the mean values for all the products studied. It is interesting to note that levels of p-TA are much higher

Table IV. Levels of bioactive amines in some selected cheese samples. Results are expressed as µg/g and represent the means of 3-6 batches. In all batches, samples were analyzed from both the surface and the core of the cheese sample. N.D.=not detectable.

CHEESE SAMPLES: AMINE CONCENTRATIONS IN µg/g

SAMPLE	PEA	MeHA	HA	PU	T	CA
Kraft Brick	8.6 ± 5.2	34.1 ± 2.0	.37 ± .03	2.1 ± 0.5	N.D.	.71 ± .19
Kraft Cracker Barrel (Med.)	2.1 ± .6	23.4 ± 6.2	.65 ± .36	N.D.	N.D.	.29 ± .02
Kraft Processed Slices	2.9 ± .1	16.1 ± .6	25.4 ± .7	27.9 ± 1.3	.43 ± .02	24.2 ± .7
Black Diamond Single Slices	4.6 ± 0.3	11.7 ± 6.7	5.5 ± 1.8	103 ± 22	N.D.	110 ± 27
Tiny Dane Blue	1.2 ± .3	8.1 ± 2.6	51.6 ± 9.5	6.2 ± 1.3	N.D.	3.1 ± .7
Havarti Dofino	1.0 ± .2	17.1 ± 2.2	3.0 ± 1.4	1.4 ± 0.4	N.D.	.38 ± .08
Woodward's Wensleydale	11.1 ± 1.4	40.5 ± 6.2	82.4 ± 38.2	317 ± 34	1.9 ± .4	288 ± 61
Woodward's Cheshire	6.1 ± 1.4	14.0 ± 3.6	209 ± 63	83 ± 64	N.D.	80.7 ± 70
Woodward's Stilton	11.3 ± 3.5	109 ± 13	39 ± 35	126 ± 105	N.D.	11.6 ± 5.9

Table IV. (cont'd)

SAMPLE	PEA	MeHA	HA	PU	T	CA
Armstrong Cheddar (Old)	5.9 ± 1.8	27.7 ± 1.9	.21 ± .07	4.8 ± .9	2.6 ± .5	.13 ± .03
Kraft Cheddar (Mild)	1.2 ± .4	12.5 ± 2.5	1.1 ± .4	3.7 ± .6	N.D.	N.D.
Kraft Cheddar (Old)	6.8 ± 1.9	48.5 ± 6.4	44.4 ± 18.5	1.4 ± .7	.27 ± .05	4.1 ± 2.0
Kraft Colby	14.4 ± 6.0	60 ± 21	1.3 ± .4	3.8 ± 2.2	2.3 ± .4	1.3 ± .8
Kraft Monterey Jack	.79 ± .51	5.0 ± .5	.33 ± .03	2.6 ± .4	.46 ± .12	N.D.
Safeway Edam (Denmark)	56.9 ± 13.6	17.8 ± 3.2	.79 ± .27	134 ± 23	N.D.	25.2 ± 9.2
Kraft Edam (Holland)	26.4 ± 3.3	13.3 ± 2.4	63.6 ± 25.4	40.6 ± 1.0	N.D.	7.5 ± .5
Faith Farms Cheddar (Med.)	.11 ± .02	.93 ± .10	.22 ± .01	N.D.	N.D.	N.D.
Safeway Edam (Holland)	.29 ± .21	1.4 ± .3	.23 ± .02	1.2 ± .4	N.D.	7.0 ± 2.1
Armstrong Cheddar (Med.)	N.D.	5.0 ± 1.7	1.3 ± 1.1	N.D.	N.D.	.12 ± .07

Table IV. (cont'd)

SAMPLE	PEA	MeHA	HA	PU	T	GA
Kraft Swiss	.54 ± .22	7.2 ± 1.4	4.2 ± 1.6	.39 ± .17	N.D.	2.8 ± 2.6
Black Diamond Hickory Smoked	9.9 ± 2.6	4.7 ± 1.1	5.5 ± 2.3	49.2 ± 19.4	.52 ± .21	25.9 ± 6.9
Kraft Mozarella	4.3 ± 1.7	9.9 ± 2.3	.83 ± .31	6.3 ± 5.0	.38 ± .23	.24 ± .03
Mean Value	8.0	22.2	24.6	41.6	0.4	27.0

Table IV (cont'd)

SAMPLE	m-TA	p-TA	3-MT	5-HT	SPD	SPM
Kraft Brick	.89 ± .25	45.2 ± 37.3	.66 ± .15	3.3 ± .6	.21 ± .04	N.D.
Kraft Cracker Barrell (Med.)	4.8 ± .6	.33 ± .13	.99 ± .12	.51 ± .04	.37 ± .04	.20 ± .0
Kraft Processed Slices	.50 ± .10	11.0 ± 2.2	.73 ± .15	.27 ± .01	.29 ± .01	.19 ± .08
Black Diamond Single Slices	.91 ± .2	17.3 ± 4.3	3.2 ± .8	3.7 ± 1.7	16 ± 3	16 ± 4
Tiny Dane Blue	.53 ± .05	118 ± 6	7.50 ± 3.74	N.D.	1.31 ± .11	N.D.
Havarti Dofino	.65 ± .12	4.6 ± 2.3	.61 ± .02	.72 ± .18	.45 ± .11	N.D.
Woodward's Wensleydale	.71 ± .22	159 ± 38	N.D.	.27 ± .01	.09 ± .05	N.D.
Woodward's Cheshire	5.1 ± .4	144 ± 17	.86 ± .28	N.D.	N.D.	N.D.
Woodward's Stilton	6.1 ± 2.8	115 ± 42	5.1 ± .2	.43 ± .10	5.8 ± 1.4	1.2 ± .6
Armstrong Cheddar (Old)	.50 ± .03	10.0 ± 2.1	N.D.	.26 ± .03	N.D.	N.D.
Kraft Cheddar (Mild)	.11 ± .02	.37 ± .05	.21 ± .01	.27 ± .03	.07 ± .03	.40 ± .10

Table IV. (cont'd)

SAMPLE	m-TA	p-TA	3-MT	5-HT	SPD	SPM
Kraft Cheddar (Old)	.80 ± .22	62.2 ± 27.7	3.0 ± .3	.43 ± .04	N.D.	N.D.
Kraft Colby	5.4 ± 2.3	54.2 ± 25.5	1.8 ± .2	.45 ± .14	.16 ± .05	N.D.
Kraft Monterey Jack	.02 ± .01	.04 ± .02	.17 ± .03	1.4 ± .9	N.D.	N.D.
Safeway Edam (Denmark)	.03 ± .02	61.8 ± 14.4	.05 ± .03	.44 ± .01	.53 ± .03	N.D.
Kraft Edam (Holland)	N.D.	74.1 ± 2.5	N.D.	.42 ± .02	.74 ± .04	N.D.
Faith Farms Cheddar (Med.)	N.D.	1.3 ± .2	N.D.	N.D.	N.D.	.18 ± .04
Safeway Edam (Holland)	.01 ± .01	3.9 ± 2.3	N.D.	N.D.	.16 ± .08	.16 ± .04
Armstrong Cheddar (Med.)	N.D.	.33 ± .13	.86 ± .23	.29 ± .17	.39 ± .07	N.D.
Kraft Swiss	.47 ± .20	14.1 ± 5.2	N.D.	N.D.	1.4 ± .3	.78 ± .20

Table IV. (cont'd)

SAMPLE	m-TA	p-TA	3-MT	5-HT	SPD	SPM
Black Diamond Hickory Smoked	N.D.	26.0 ± 4.5	N.D.	N.D.	.67 ± .17	1.0 ± .3
Kraft Mozarella	N.D.	14.3 ± 5.0	N.D.	.57 ± .29	1.3 ± .3	.76 ± .22
Mean Value	1.25	42.6	1.17	0.62	1.34	0.95

Table V. Levels of bioactive amines in selected chocolate samples. Results are expressed as μg/g and represent the means of 3-6 batches. Analyses on each batch were done in duplicate.

CHOCOLATE SAMPLES: AMINE CONCENTRATIONS IN μg/g

SAMPLE	PEA	MeHA	HA	PU	T	CA
Chipits	3.8 ± .3	6.3 ± 1.8	.57 ± .16	N.D.	.74 ± .30	.54 ± .23
Cadbury's Hot Chocolate	1.8 ± .1	.34 ± .07	.41 ± .09	N.D.	.26 ± .01	.15 ± .02
Nestles Quik	1.8 ± .1	.71 ± .06	.070 ± .001	N.D.	.31 ± .01	.09 ± .01
Baker's Semi-Sweet Chocolate	6.4 ± .1	1.0 ± .1	.43 ± .11	N.D.	.56 ± .06	.76 ± .16
Rowntree's Burnt Almond	2.0 ± .1	1.7 ± .2	.21 ± .02	.44 ± .25	.52 ± .03	.33 ± .04
Hershey's Special Dark	2.8 ± .1	2.4 ± .1	.25 ± .02	N.D.	.50 ± .01	.38 ± .01
~~Fry's~~ Cocoa	22.0 ± .7	15.4 ± 1.8	1.3 ± .1	.95 ± .2	1.8 ± .3	3.3 ± .04
Cadbury's Dairy Milk	.37 ± .02	N.D.	.50 ± 0	.61 ± .22	.57 ± .04	N.D.
Neilson's Jersey Milk	.92 ± .05	.18 ± .02	[illegible]	N.D.	.42 ± .05	N.D

Table V. (cont'd)

SAMPLE	PEA	MeHA	HA	PU	T	CA
Mirage	.050 ± .002	N.D.	.170 ± .002	.07 ± .01	.22 ± .02	N.D.
Zero	2.0 ± .3	2.1 ± .2	.31 ± .05	.36 ± .12	N.D.	2.2 ± .3
Hershey Big Block Milk Chocolate	.05 ± .01	N.D.	N.D.	.13 ± .02	N.D.	N.D.
Rowntree Yorkie	.31 ± .16	N.D.	N.D.	.15 ± .01	N.D.	N.D.
Nestle Gala (white chocolate)	.05 ± .01	N.D.	N.D.	.13 ± .03	N.D.	N.D.
Mean Value	3.0	2.0	0.35	0.19	0.42	0.52

Table V. (cont'd)

SAMPLE	m-TA	p-TA	3-MT	5-HT	SPD	SPM
Chipits	N.D.	.77 ± .41	.42 ± .18	14.4 ± .8	3.0 ± .6	1.1 ± .2
Cadbury's Hot Chocolate	.09 ± .01	.64 ± .04	.20 ± .01	10.0 ± .6	.74 ± .16	.72 ± .14
Nestles Quik	.06 ± .01	.34 ± .09	.210 ± .003	8.4 ± 1.1	.58 ± .16	.51 ± .03
Baker's Semi-Sweet Chocolate	.24 ± .03	.75 ± .13	.16 ± .01	16.4 ± 1.6	1.7 ± .3	1.1 ± .3
Rowntree's Burnt Almond	N.D.	.53 ± .04	.26 ± .01	12.6 ± 1.2	5.5 ± 1.4	.90 ± .15
Hershey's Special Dark	.07 ± .01	.55 ± .07	.23 ± .01	15.9 ± 1.8	2.7 ± .5	.48 ± .19
Fry's Cocoa	.15 ± .04	3.5 ± 1.1	.33 ± 0	58.2 ± 9.5	11.5 ± 1.3	1.3 ± .1
Cadbury's Dairy Milk	.09 ± .02	.13 ± .05	N.D.	3.9 ± .3	1.1 ± .1	.59 ± .02
Neilson's Jersey Milk	N.D.	N.D.	.13 ± .02	2.3 ± .8	1.3 ± .2	.20 ± .1
Mirage	.010 ± .002	.20 ± .01	N.D.	19.8 ± 1.6	.37 ± .04	.30 ± .04

Table V. (cont'd)

SAMPLE	m-TA	p-TA	3-MT	5-HT	SPD	SPM
Zero	.02 ± .01	1.2 ± .3	N.D.	9.3 ± .4	1.9 ± .3	.17 ± .07
Hershey Big Block Milk Chocolate	N.D.	.13 ± .03	N.D.	5.2 ± .7	.52 ± .05	.13 ± .06
Rowntree Yorkie	N.D.	.36 ± .05	N.D.	8.8 ± 1.2	1.6 ± .3	.29 ± .14
Nestle Gala (white chocolate)	N.D.	N.D.	N.D.	.42 ± .03	N.D.	N.D.
Mean Value	0.05	0.65	0.14	13.3	2.32	.56

than mean values in Tiny Dane Blue, Woodward's Wensleydale, Woodward's Cheshire and Woodward's Stilton cheeses. Levels of PEA are considerably higher than mean values in Safeway Edam (both Holland and Denmark brand).

If literature reports of the amounts of p-TA and PEA which cause side effects in patients on MAO inhibitors are correct (6 mg and 3 mg, respectively), then the following cheese products (based on a proposed ingestion of 100 g) should be avoided or used with caution in such patients: Safeway Edam (Denmark) (PEA, 56.9 µg/g), Tiny Dane Blue, Woodward's Wensleydale, Woodward's Cheshire, Woodward's Stilton, Old Kraft Cheddar, Safeway Edam (Denmark) (p-TA level in g/g: 118, 159, 144, 115, 62.2, 61.8, 74.1, respectively). The ingestion of 100 g of any of the chocolate samples analyzed in this study would not result in an intake of 3 mg or more of PEA. Since detailed dose studies on the physiological effects of the other amines have not been reported, to my knowledge, it is not possible at this time to elaborate on the significance of high levels of these other compounds in foodstuffs.

Several cheeses had considerably higher levels of PU than the mean value. These included Woodward's Wensleydale, Safeway Edam (Denmark), Woodward's Stilton and Black Diamond Single Slices (processed cheddar), which contained levels 7.6, 3.2, 3.0 and 2.5 times higher than the mean respectively. Three cheeses had levels of CA several times higher than the mean. These were Woodward's Wensleydale, Black Diamond Single Slices (processed cheddar) and Woodward's Cheshire (10.7, 4.1 and 3.0 times higher respectively). With regard to HA content, Woodward's Cheshire was the most remarkable (8.5 times the mean value). No consis-

tent relationship between levels of HA and MeHA was evident in the cheese.

It is interesting to note that with chocolate (Table V) 5-HT was the most prominent amine in all cases. This amine is thought to be involved in the etiology of migraine (Hunter, 1980), so perhaps it may also contribute to the migraine produced in some migraine sufferers who consume chocolate. However, unlike PEA, another amine in chocolate proposed to be a causative agent (Sandler et al., 1974, 1976) in migraine, 5-HT does not cross the blood-brain barrier readily.

It is also of interest that the sample of white chocolate tested had much lower levels of 5-HT than did any of the other chocolate samples. This observation is now being investigated further.

This is the first time, to the knowledge of the author, that m-TA has been identified in food samples. Although very low levels were found in most of the cheese and chocolate samples tested, reasonably high concentrations were present in Kraft Medium Cracker Barrel, Woodward's Cheshire, Woodward's Stilton, and Kraft Colby cheeses. As mentioned in the introduction, m-TA, like p-TA, has rather strong effects on uptake and release of catecholamines and 5-HT in nerve terminals. It is therefore conceivable that this isomer may be contributing to the adverse effects seen in MAO inhibitor-food product interactions and that it may also play a role in TA-sensitive migraine.

As well as providing analysis of PEA, the TAs, HA, MeHA, T, 5-HT and the polyamines, the pentafluorobenzoylation procedure described here also permits simultaneous quantification of the DA metabolite 3-methoxytyramine (3-MT). This represents the first time a detailed study of this amine in food products has been conducted. Levels were low in all

cheese and chocolate samples investigated, except for Black Diamond Single-Slices, Woodward's Stilton, Old Kraft Cheddar, and Kraft Colby cheeses.

IV. CONCLUSIONS

A novel GLC procedure has been developed which provides for the simultaneous analysis of the following amines: β-phenylethylamine, m-tyramine, p-tyramine, histamine, tele-methylhistamine, tryptamine, 5-hydroxytryptamine, 3-methoxytyramine, cadaverine, putrescine, spermine and spermidine.

The method, which employed GLC with electron-capture detection and a capillary column, has the significant advantages of high sensitivity, specificity and simplicity. The derivatives formed also have high stability. The versatility of the method was supported by the successful isolation of amines from the complex matrix of food samples using the liquid ion exchanger DEHPA and, following this, back-extraction with HCl, basification and aqueous derivatization using PFBC. The derivatization is rapid and can be performed under aqueous conditions; this is in contrast to many published GLC procedures for amines which require derivatization under anhydrous conditions after complicated procedures for isolation of the amines from aqueous homogenates of food samples. The method has been applied here to perform analysis on cheese and chocolate.

The procedure, as well as providing for the first time such a comprehensive quantitative simultaneous analysis of amines in food products, has yielded some other novel findings: (1) Relatively high levels

of 5-HT were found in all dark chocolate samples. The level of this amine was much lower in the one brand of white chocolate analyzed. (2) For the first time, to the knowledge of the author, the meta isomer of tyramine has been detected and quantitated in food products. (3) 3-Methoxytyramine, a metabolite of dopamine was shown to be present in relatively large amounts in several types of cheese.

Preliminary findings from the application of the new analytical procedure to analysis of other foodstuffs such as beer, wine, canned tuna fish and soya sauce are promising. The present procedure is also anticipated to be applicable to analyzing biological samples such as brain tissues, plasma, urine and cerebrospinal fluid. This may be an important asset for neurochemical research since many of the amines investigated in this study are putative neurotransmitters or neuromodulators. Furthermore, this method may be applicable to cancer research because di- and polyamines are known to be closely related to neoplastic growth; and abnormally high levels of these amines have been reported in the body fluids of cancer patients. Thus, the novel GLC procedure developed, as well as permitting comprehensive, quantitative studies of bioactive amines in food products, has the potential to be applicable to a wide variety of studies on these substances in the health-related sciences.

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