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Gas Chromatographic Analysis of Histamine in Mahi-mahi (*Coryphaena hippurus*)

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Several authors have studied histamine using gas chromatography (GC) as a tool for quantitation, but the methods used were not always suitable depending on the kind of food. Problems frequently cited include incomplete histamine elution from the columns and peak tailing. Histamine is of interest because it is the factor common to all cases of scombroid poisoning, it has physiological and biological activity, and it is a chemical indicator of fish quality. In this study a modified GC method was used to quantify histamine in mahi-mahi (*Coryphaena hippurus)*. Mean recovery was 67% for the GC method, compared with 90% for the AOAC fluorometric method. There was a 0.96 correlation of the GC histamine values with those of the AOAC fluorometric method. A temperature program, splitless/split injection, and analyte cleanup were essential for GC properties. Histamine retention time was 8.2 min. The method allowed peak height to be used for quantitation and simultaneous analysis of cadaverine and putrescine.

KEYWORDS: Gas chromatography; histamine; seafood; AOAC method; biogenic amine

INTRODUCTION

Several authors have studied biogenic amines using gas chromatography (GC) and either did not investigate or used an alternative method for histamine quantitation (*1*−*5*). Staruszkiewicz and Bond (*4*) developed a method for GC analysis of putrescine and cadaverine using perfluoropropionyl derivatization with electron capture and nitrogen-specific detectors. They concluded histamine did not easily chromatograph and used the AOAC fluorometric method for histamine quantitation. Renon and Contoni (*6*) used trifluoroacetyl derivatization for analyzing putrescine, cadaverine, tyramine, and tryptamine, but not histamine, in tuna. Lambert and Moss (*7*) used heptafluoroylbutyric derivatives to detect putrescine and cadaverine simultaneously, but not histamine. Fardiaz and Markakis (*1*) studied amines in fermented fish paste using trifluoroacetyl derivatization and a fluorometric method for histamine analysis. Yamamoto et al. (*8*) did not include histamine in their GC study of polyamines. Kuwata et al. (*9*) and Yamanaka (*5*) studied several amines but did not include histamine. Yamanaka (*5*) used a cleanup procedure followed by ethoxycarbonyl derivatization of the amines but studied histamine independently using the AOAC method. These studies indicate there are difficulties associated with the study of histamine using GC.

Other authors report the use of GC as a tool for histamine analysis (*10*−*12*). These methods are, however, not suitable for each kind of food and often require a number of modifications (*13*). Arnold and Brown (*14*) stated that despite the possibilities for application of GC techniques, they have not achieved widespread use among researchers studying histamine intoxication. Histamine must be converted to some volatile derivative, which can then be separated by the GC (*15*). However, Mita et al. (*16*) reported that some derivatives are unsuitable for histamine quantitation. Mahy and Gelpi (*17*) reported a GC method using trimethylsilylation of histamine, and Henion et al. (*18*) reported the trimethysilyl derivative of histamine in tuna was readily resolved using a capillary column. Other derivatizing agents used are pentafluoropropionic anhydride (*19*) and heptafluorobutyryl and ethyl chloroformate (*20*), perfluoropropionyl (*21*), and others may involve a combination with 2,6-dinitro-4-trifluoromethylbenzenesulfonic acid (*10*, *12*). Each of these methods uses one of several detectors including flame ionization (FID), electron capture (ECD), nitrogen−phosphorus (NPD), and mass spectrometry (MS). However, two problems frequently cited in these methods are incomplete elution from the columns and tailing (*22*). Staruszkiewicz and Bond (*4*) and Rogers and Staruszkiewicz (*3*) used an oven temperature of 170−180 °C for peak separation and concluded histamine did not chromatograph easily by GC. While using the GC method of Rogers and Staruszkiewicz (*23*), we observed it was possible to quantify

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histamine, together with cadaverine and putrescine, using GC-FID, a temperature gradient program, and a splitless injection mode. Analyte cleanup, a short capillary column, and some other modifications are described by Antoine (*24*). Duflos et al. (*25*) and Hungerford et al. (*26*) reported on the importance of matrix effects in histamine and biogenic amines analysis.

Histamine is of interest to food scientists and government regulators because it is the single factor common to all cases of scombroid poisoning (*14*). Histamine has physiological and biological activity (*27*−*31*) and is an indicator of fish decomposition (*3*, *32*). Histamine poisoning occurs frequently and is a worldwide problem associated with the consumption of economically important fish species such as tuna, mahi-mahi, mackerel, and bluefish (*30*, *32*, *33*).

The objective of this study, therefore, was to quantify histamine levels in a complex fish matrix (mahi-mahi) using the GC-FID and to compare the measurements with the values obtained using the AOAC fluorometric method for histamine analysis.

MATERIALS AND METHODS

Reagents. Analytical grade phosphoric acid (H_3PO_4), sodium hydroxide (NaOH), hydrochloric acid, HPLC grade methanol, ethyl acetate, toluene, and hexane were purchased from Fisher Scientific (Fair Lawn, NJ). Histamine dihydrochloride, *o*-phthaldialdehyde (OPA), and pentafluoropropionic anhydride (PFPA) were bought from Sigma Chemical Co. (St. Louis, MO). Alumina-N solid phase extraction (SPE) columns (3 mL) were bought from Supelco (Bellefonte, PA). Absolute ethanol was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY). Distilled deionized water was obtained from a Photronix reagent grade water system (Photronix Corp., Medway, MA).

Instrumentation. A PE 8500 gas chromatograph (Perkin-Elmer Corp., Norwalk, CT) was fitted with a DB-1 capillary column, 15 m × 0.32 mm i.d., 3 μm film thickness (J&W Scientific, Folsom, CA). The oven temperature program was 150 °C, 0 min, 5 °C/min; 156 °C, 3 min, 5 °C/min; 161 °C, 2 min, 20 °C/min; and a final temperature of 300 °C held for 5 min. The FID and injection temperatures were 325 and 300 °C, respectively. Carrier gas flow rate was 2.5 mL/min with a column pressure of 8 psi. Injection volume was 1.0 μL with a split ratio of 18:1. All injections were done manually using a 10 μL gastight syringe (Hamilton Co., Reno, NV), and injections were held splitless for 6 s. Air and hydrogen inlet pressures were set at 22 and 12 psi, respectively.

Experimental Design. Two groups of fillets from six freshly caught, gutted, and headed mahi-mahi (*Coryphaena hippurus*), 9 to 12 kg each, were stored at 7 °C. One fillet from each fish was placed into each group. Fillets were then cut into nine portions, 350−450 g each, and the end portions were discarded. The portions of each fillet were kept together, and fillets were held in separate containers. To ensure that histamine was produced in the fish, on day 0 the portioned fillets of one group were dipped for 2 min in separate volumes (2:1 v/w) of an inoculum of *Morganella morganii* (ATCC 9237; *34*), quickly drained (3−5 min), and then placed in polyethylene bags for storage. The inoculum was prepared as described by Taylor and Woychik (*35*) and then diluted in sterile Butterfield's phosphate buffer, pH 7.2, yielding an inoculum of 10^5 colony-forming units (CFU)/mL. On each day of sampling (0, 2, 4, 6, 8, and 10), one portion of each fillet was removed and frozen at −20 °C until analyzed. Prior to analysis, the frozen samples were partially thawed, and care was taken to avoid drip loss. All glassware used was acid washed and rinsed in distilled deionized water.

Analyte Extraction Procedures. Each fish sample was chopped and homogenized in a West Bend high-performance food processor (West Bend Co., West Bend, WI). For each sample four replicates were prepared for analysis, each of 10 g of homogenized fish weighed into half-pint Mason jars. To each jar was added 40 mL of extracting solvent (75% methanol and 25% distilled deionized water) (*23*, *36*). Samples were blended for 2 min with a Hamilton Beach 14 Blend Master (Hamilton Beach/Proctor-Silex, Inc., Glen Allen, VA) set at the "liquefy" position. The extracts were transferred to 100 mL volumetric flasks, and the blades and jars were each rinsed with 3 × 15 mL of extracting solvent. The rinsings were added to the volumetric flasks, which were then heated in a water bath at 60 °C for 15 min, cooled to room temperature, and then brought up to 100 mL with extracting solvent. Extracts were centrifuged at 4 °C and 27000*g* (15000 rpm) for 40 min using an IEC refrigerated centrifuge model B20A (International Equipment Co., Needham Heights, MA), and the supernatants were filtered using 0.2 μm Gelman Acrodisc membrane filters (47 mm diameter). Filtrates were put into separate Falcon polyethylene tubes (Becton Dickinson and Co., Franklin, NJ) and stored at −20 °C until analysis.

GC Standard Preparation and Derivatization. Standard stock solution was prepared by adding the equivalent of 100 mg of histamine-free base (167 mg of histamine 2 HCl) to a 100 mL volumetric flask, dissolved in 0.1 N HCl, and brought up to volume. The stock solution was stored at −20 °C, from which fresh working solution was prepared weekly. For quantitation an external calibration curve was prepared using 10, 20, 40, 80, and 100 μg/mL histamine (free base equivalent) as described by Antoine (*24*). One milliliter of each working standard solution was added to separate 100 mL round-bottom flasks (RBF) followed by 0.5 mL of 1.0 N HCl. Each flask was swirled three times, and then the contents were evaporated to dryness on a rotary Buchi Rotavapor R114 (Brinkmann Instruments, Inc.) at 50 °C. The rotavapor was coupled to a KNF Neuberger vacuum pump, model 13-878-42 (Trenton, NJ). The residue was washed with 2 mL of distilled deionized water and again evaporated to dryness. One milliliter of ethyl acetate and 300 μL of PFPA were added to the dry residue, stoppered, mixed, and heated in a water bath (Fisher Scientific Versa-bath) at 50 °C for 30 min. The mixture was swirled at least once during heating. The PFPA-amine derivative was transferred to a 10 mL round-bottom screw-capped glass tube and the RBF rinsed three times with 1 mL of ethyl acetate. The tube was evaporated to dryness under a stream of nitrogen using an N-Evap analytical evaporator, model 111 (Organomation Associates, Inc., South Berlin, MA) and redissolved in 1 mL of ethyl acetate.

GC Sample Derivatization and Analyte Cleanup. Ten milliliters of each fish filtrate was pipetted into a 100 mL RBF, 0.5 mL of 1 N HCl was added, and the extract was prepared as per standards above. The derivatized samples, and standards, were separated on 3 mL Alumina-N SPE columns under gravitational flow. The SPE tubes were conditioned with 2 mL of hexane, which was discarded, and immediately loaded with 150 μL of the derivatized sample or standard. Eluent collection began immediately when the sample was loaded into the tube. As the sample passed through the frit, 3−4 drops of methanol were added and allowed to pass through the frit. The analyte was then eluted with 8 × 2 mL of methanol. The eluent was collected, evaporated to dryness, redissolved in 150 μL of 30% ethyl acetate in toluene (EAT), and ready for injection. All determinations were done in duplicate.

AOAC Fluorometric Determination of Histamine. The AOAC fluorometric method 977.13 (*37*) was used for analysis of histamine as a means of comparison with the GC method. The same filtered fish samples used for GC analysis were also analyzed in quadruplicate. Histamine was separated on a Dowex 1-X8 anion exchange resin column (Supelco). Chromatographic polypropylene tubes 200 × 7 mm (i.d.) (Kontes, Vineland, NJ) were each fitted with 45 cm of Teflon tubing and a flow control valve. The height of the tubes was adjusted to ensure that the gravity flow rates were the same and >3 mL/min. A Sequoia-Turner photofluorometer model 450−005 (Abbott Diagnostics, Abbott Park, IL) was fitted with a 360 nm narrow band-pass (NB) excitation wavelength and NB 440 emission wavelength filters. A 5 mL glass cuvette was used for all measurements.

Statistical Analysis. Analysis of variance and Pearson's coefficient of correlation were determined using SAS software (SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

GC Quantitation. Figure 1 shows typical GC chromatograms obtained for the standards (**Figure 1A**) and a mahi-mahi

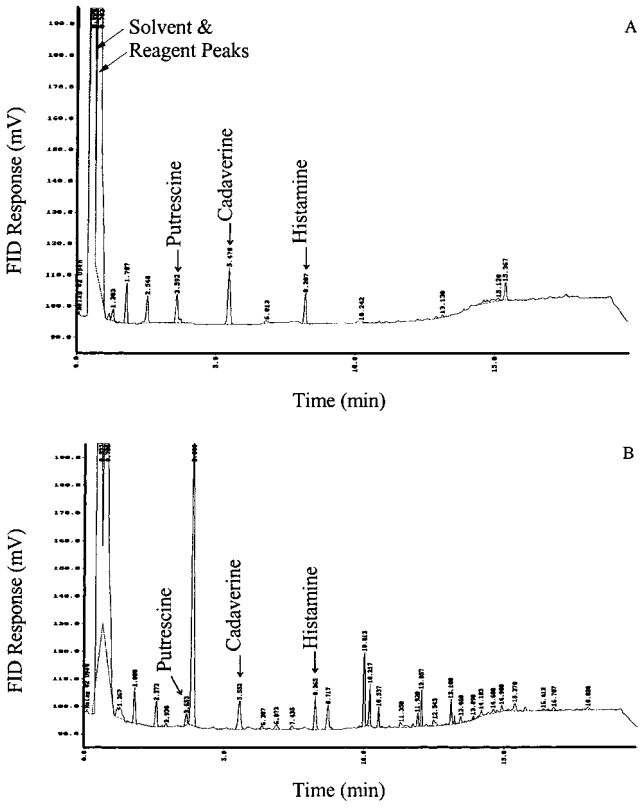

Figure 1. Gas chromatograms of (A) biogenic amines standards and (B) biogenic amines in mahi-mahi stored at 7 °C.

Table 1. Recovery Efficiency of Histamine Standards from Fortified Mahi-mahi Using GC-FID

biogenic amine	fortifn amount, mg/100 g (ppm)	recovery, %	SD, %	CV, %
low	20 (200)	66.8	7.6	11.4
medium	50 (500)	77	6.4	8.3
high	100 (1000)	58.1	8.6	14.8
mean		67.3		

sample (**Figure 1B**). Noteworthy is the absence of peak tailing, which made it possible to use peak height for quantitation. The GC intra- and interassay coefficients of variation (CV) for histamine were 2.1 and 9.3%, respectively. The minimum histamine standard detected was 7.0 ppm, and a mean recovery efficiency of 67% was found in fortified mahi-mahi (**Table 1**). The histamine calibration curve in the range of 10−100 mg/100 g (100−1000 ppm) was linear ($r^2 = 0.99$) and reproducible. The results of the GC method for histamine are comparable with reports of several authors. Mita et al. (*11*) using GC-MS reported a 70% recovery rate and concluded that this procedure would be applicable for the determination of histamine. Slemr and Beyermann (*38*) reported 92−102% recovery, and Wada et al. (*39*) reported recovery efficiencies of 104−113% for their GC methods. Davis et al. (*40*) using an HPLC method had a recovery efficiency of 54.4% for histamine, and Salazar et al. (*41*) reported 91 ± 24% for their HPLC method.

Using the AOAC (*37*) fluorometric method for histamine analysis, the intra- and interassay CVs were 0.1−2.2% and 3.2−6.7%, respectively. The mean recovery efficiency was 90% (**Table 2**). The fortification levels used for the two methods were different because of the difference in the instruments' sensitivities and the range of histamine that can be found in potentially hazardous fish. The GC-FID had lower sensitivity

Table 2. Recovery Efficiency of Histamine Standard from Fortified Mahi-mahi Using the AOAC Fluorometric Method

fortifn level	fortifn amount, mg/100 g (ppm)	concn measured, mg/100 g (ppm)	recovery, %	SD, %	CV, %
low	5 (50)	4.6 (46)	90.6	5.8	6.4
medium	10 (100)	8.9 (89)	89.1	2.9	3.3
high	50 (500)	45.4 (454)	90.6	3.3	3.6
mean			90.1		

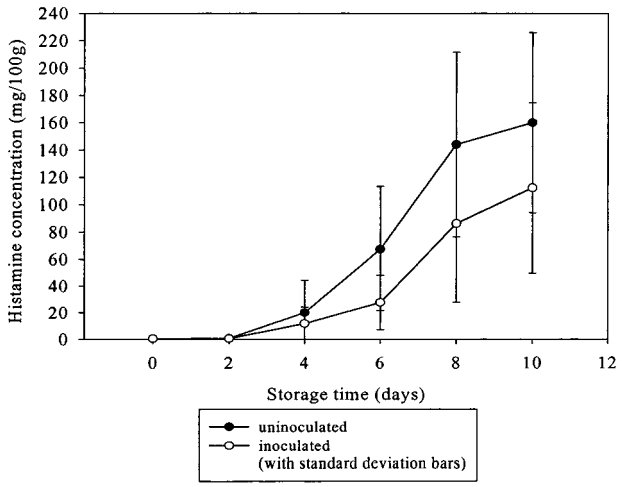

Figure 2. Changes in histamine levels during mahi-mahi storage at 7 °C.

than the AOAC method. Rogers and Staruszkiewicz (*3*) reported 84−125% recoveries for a fortification level of 5 mg/100 g (50 ppm) for fluorometric determination of histamine in tuna. **Figure 2** shows the pattern of histamine development in mahi-mahi stored at 7 °C, as determined by the AOAC method. The patterns for both the inoculated and uninoculated samples are similar. Over the storage period, the difference between the levels for the uninoculated and inoculated samples became significant ($p = 0.05$). This difference in histamine levels is probably accounted for by the difference in the enzymatic activities of the different microflora in the uninoculated and the inoculated samples (*27*, *42*). Histamine production is not a function of the number of bacteria but of the activity of the decarboxylase enzymes the bacteria produce. Like many enzymes, histamine decarboxylase from different bacterial species displays different levels of activity under the same environmental conditions of temperature, pH, substrate concentrations, etc. (*27*, *42*). High variation in histamine levels was observed between fish and agrees with reports in the literature (*3*, *43*−*46*). Takagi et al. (*47*) reported that the rate and level of histamine formation varied with fish species.

Figure 3 shows a comparison of the histamine levels as measured by the AOAC and GC-FID methods. Unlike the fluorometric method, no measurable amount of histamine was detected in mahi-mahi samples on days 0 and 2 using the GC. Both methods showed a similar pattern of histamine development during the storage period. The GC values were generally lower, although not significantly, than those of the AOAC method. This was probably reflective of the lower histamine recovery efficiency of the GC method and the lower GC-FID sensitivity. It is known, however, that the GC-ECD is more sensitive than the GC-FID, particularly toward electronegative functional groups such as halogens (*48*). Hiemke et al. (*49*) reported that the derivatized histamine is unstable in the presence of water and that up to 50% of the derivatives can be lost during

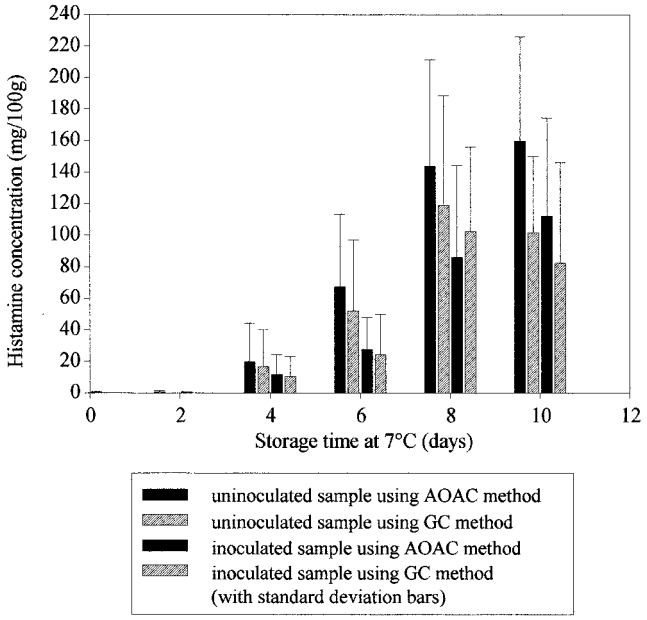

Figure 3. Comparison of histamine values in mahi-mahi using AOAC fluorometric and GC methods.

preparation. Our observations showed that the histamine derivative was stable for short periods (2−3 days at refrigeration temperature), after which time smaller and broader peaks were seen in the chromatograms. The derivatives of putrescine and cadaverine were stable for longer periods (3−5 days at refrigeration temperature). Fales and Pisano (*15*) investigated various factors that influence the successful analysis of biological amines, including histamine, using GC. They reported the injector temperature, solvent type, stationary film thickness of the column, and combinations of air, light, heat, and moisture were factors that affect recovery efficiencies. We investigated injection and detector temperatures ranging from 240 to 325 °C in order to determine which yielded the best quality signal. Fales and Pisano (*15*) demonstrated that thermal degradation during chromatography, however, was not a problem. Baker et al. (*50*) stated that peak interference arising from tissue, solvents, and reagents are problems unique to the GC compared with other techniques, but that simultaneous assay of several amines and sensitivity are advantages of using it. **Figure 1** shows the ability of the GC-FID method to separate other amines such as cadaverine and putrescine for quantitation.

Initial attempts to use hexanediamine as an internal standard proved to be frustrating because of its coelution with histamine. Different oven temperature programs were examined to enable histamine separation from hexanediamine, as well as that of cadaverine from the subsequent peak. However, external calibration was finally resorted to, using peak height for quantitation. Cleanup of the histamine−PFP derivative was necessary because of strong matrix interference, which resulted in a shorter, broader, and sometimes split histamine peak. Duflos et al. (*25*) also reported on the effects of matrix interference on amine quantitation.

The relative simplicity of this method is understood in the light of the different histamine derivatives used by various researchers. Navert (*12*) and Mahy and Gelpi (*17*) reported methods that adequately separated histamine and other imidazole derivatives using GC-FID. Wada et al. (*39*) described a method for the analysis of histamine in marine food products using ion exchange to isolate the histamine followed by derivatization with N^{α}-heptabutyryl and conversion of the N^{α}-heptafluorobutyryl derivative to N^{θ}-ethoxycarbonylhistamine using ethyl chloroformate in ethyl acetate. They reported linearity for histamine concentrations of 10−100 μg/mL. Mita et al. (*11*, *20*) used a similar derivatization procedure, although their preparatory steps were different, including cleanup after derivatization and GC-MS for quantitation. Doshi and Edward (*10*) reported good GC properties of histamine and methylhistidine using 2,6-dinitro-4-trifluoromethylbenzenesulfonic acid and GC-ECD.

Chromatographic tailing and poor peak quality were problems, especially for histamine, when samples were injected without prior SPE separation. During the developmental stages 30 m DB-5 and DB-1 columns with 0.1 μm film were used but resulted in smaller and poor peak symmetry, probably because of the longer retention times. This resulted in inconsistent quantitation values and agreed with the findings of Mita et al. (*16*), who reported that because of chromatographic tailing, histamine quantitation using the GC can be difficult. Because of the absence of peak broadening and tailing in the chromatograms using this method, when timely analysis was done, the authors chose to use peak height for quantitation as a testament to the method. Mita et al. (*20*) reported a two-step derivatization for histamine: heptafluorobutyric anhydride (HFBA) and ethyl acetate followed by treatment with ethylchloroformate to form N^{α}-heptafluorobutyryl-N^{τ}-ethoxycarbonylhistamine. The derivative was then cleaned up on a CPG-10 column prior to analysis using GC-MS.

During the preliminary stages, SPE alumina-N column cleanup of the standard and fish sample derivatives resulted in <50% recovery efficiency of the histamine−PFP derivative. Loss of the analyte during cleanup contributed toward that low recovery. Several solvents were used to elute the histamine−PFP derivative. The histamine derivative did not elute from the column using 30% ethyl acetate as the eluent, and methanol was most efficient in comparison to ethyl acetate, hexane, and acetonitrile. Attempts to chromatograph the derivative without cleanup resulted in smaller peaks, peak interference, and loss of sensitivity. Thus, matrix interference was a factor responsible for the poor chromatogram of the histamine−PFP derivative, evidence that GC analysis of acyl derivatives of histamine requires isolation of the histamine and/or sample cleanup prior to GC analysis. In addition, fish tissue is considered a complex biological material (proteins, peptides, amines, and lipids), which adds to the difficulty of GC analysis.

The chemistry of histamine derivatization and the difficulties in its GC analysis are explained by the work of several authors. Slemr and Beyermann (*38*) described a method using trifluoroacetyl derivatives for putrescine and cadaverine, but histamine was converted to N^{α}-trifluoroacetyl-N^{τ}-ethoxycarbonylhistamine (TFA-ETO). They stated that the position of the trifluorobutyrylethoxycarbonyl derivative on the imidazole ring (N^{τ} or N^{π}) is not clear. Slemr and Beyermann (*38*) reported that the N^{τ} position is more likely because of steric hindrance of the N^{π} position and that it was necessary to keep the histamine TFA-ETO derivative in excess of ethyl chloroformate in order to prevent its degradation. We used ethyl chloroformate with PFPA, but no peak quality improvement was observed and its use was abandoned after repeated trials. Cancalon and Klingman (*51*) obtained no response for their histamine−TFA and TFA−TMS derivatives.

Moodie (*52*) studied histidine using GC and provided some insight into the problems confronting the GC analysis of histamine. Histamine, like histidine, has the imidazole ring responsible for the problem of derivatization and tailing during GC analysis of histamine. Moodie (*52*) explains that histidine

(like histamine) may be derivatized to yield N^{α}- and N^{π}-bis-(trifluoroacetyl) *n*-butyl histidinate. MacKenzie and Tenaschuk (*53*) claimed the stability and suitability of the derivative in GC analysis are doubtful. Moodie also suggested the N^{α}-trifluoroacetyl-N^{π}-carbethoxybutyl histidinate derivative is possible, which is similar to the two-step derivatization procedures used by Mita et al. (*20*) and Slemr and Beyermann (*4*). Moodie (*52*) concluded that because of the chemistry of the two trifluoroacetylbutyl histidinate derivatives, successful quantitation would be doubtful. Moodie demonstrated that the stability of the diacyl derivative is poor even in light of the effectiveness of the *N*-perfluoroacyl as a potent acylating agent. MacKenzie and Tenaschuk (*53*) also reported that the procedures normally used to form *N*-perfluoroacyl alkyl esters of amino acids are not entirely satisfactory for the quantitation of histidine, because of the lability of the N^{π}-acyl bonds.

MacKenzie and Tenaschuk (*53*) provide additional evidence of the difficulty confronted when the imidazole compounds histidine and histamine are studied. In their study, MacKenzie and Tenaschuk (*53*) said acylation using ethoxyformic anhydride (EFA) was originally developed to overcome the problem of acylation in the imidazole nitrogen of histidine/histamine. This technique they concluded showed no advantage over acylation with carboxylic acid anhydrides. Our observations support this conclusion. MacKenzie and Tenaschuk (*53*) reported the imidazole nitrogen of histidine cannot be readily acylated using the perfluorocarboxylic acid anhydrides, because of the difficulty in acylation and the lability of the N^{π}-acyl bonds. They showed the effect of the proportion of various anhydrides (acetic, butyric, and propionic) to sample volume required to attain maximal response for N^{π}-acyl-N^{α}-HFB isobutyl histidine and reported, however, that the reaction of N^{α}-HFB isobutyl histidine with EFA resulted in a histidine derivative that chromatographed well.

Conclusions. This method of GC analysis of histamine is relatively easy but requires sample cleanup in order to avoid matrix interference. Methanol effectively elutes the histamine−PFP derivative, together with other derivatized amines such as cadaverine and putrescine, from the SPE alumina-N bed and yields peaks without tailing and with good symmetry. Column selection, temperature gradient program, and a splitless/split injection mode are essential to achieving good chromatograms of histamine derivatives. Further studies are needed to simplify the derivatization procedures and techniques of GC analysis of histamine.

ABBREVIATIONS USED

SD, standard deviation; CV, coefficient of variation; EAT, 30% ethyl acetate in toluene; ECD, electron capture detector; EFA, ethoxyformic anhydride; GC-FID, gas chromatograph flame ionization detector; HFBA, heptafluorobutyric anhydride; NPD, nitrogen−phosphorus detector; PFPA, pentafluoropropionic anhydride; TFA-ETO, trifluoroacetyl-ethoxycarbonyl.

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