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Gas–Liquid Chromatographic Determination of Tyramine in Fermented Food Products

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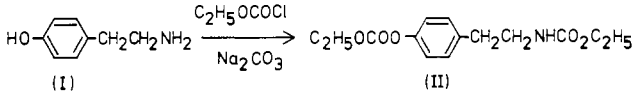
A simple and precise gas–liquid chromatographic method for the determination of tyramine in fermented food products has been described. Tyramine separated from foods, with purification by eluting through an Amberlite CG-120 column, was readily converted into the *N,O*-bis(ethyloxycarbonyl) derivative by the reaction with ethyl chloroformate, which was analyzed by gas–liquid chromatography, using 3,4-dimethoxyphenethylamine as an internal standard. Tyramine was clearly separated from other constituents in foods on a 1.5% OV-17, 0.2% SP-1000 mixed-phase column. The calibration curve for tyramine in the range of 2–100 μg is linear and sufficiently reproducible for quantitative determination. The coefficient of variation (n = 5) of the values determined is below 6.2%, and the average recovery rate throughout the procedure, including ion-exchange column chromatographic purification, derivatization, and gas–liquid chromatography, was above 94% in some kinds of foods investigated. The derivative preparation is simple and rapid, and the resulting stable derivative allowed us to develop a more precise and accurate method.

Tyramine is an indirectly acting sympathomimetic amine which releases norepinephrine from a sympathetic nerve ending, and it has been reported that tyramine-containing foods can cause unnatural and toxic effects (Stockley, 1973) when injested in large quantities. Blackwell et al. (1967) have suggested that foods containing tyramine may be particularly dangerous for patients receiving monoamine oxidase inhibitor. Tyramine and tyramine-containing foods can also cause dietary migraine patients to suffer a classical migraine attack (Hanington, 1967; Smith et al., 1971) and they produce vasoconstriction and consequently a rise of blood pressure in patients with carcinoid syndrome (Waldenstrom et al., 1956; Levine and Sjoerdsma, 1963) and with phaechromocytoma (Engelman and Sjoerdsma, 1964).

From this point of view, tyramine has been determined in foods in a variety of ways, including fluorometric (Spector et al., 1963; Price and Smith, 1971; Horwitz et al., 1964) and gas–liquid chromatographic (GLC) (Sen, 1969; Kaplan et al., 1974) methods. The presence of tyramine in foods has been reviewed (Lovenberg, 1974). In general, the fluorometric method is very sensitive but is not specific enough to determine tyramine in complex samples such as foods; the GLC technique offers a more specific method. In the GLC method described above, tyramine was analyzed as its trifluoroacetyl derivative. For sound quantitative routine GLC determination, it is desirable to convert tyramine into a more stable derivative.

We have reported that both phenolic hydroxyl and amino groups can be readily alkyloxycarbonylated with alkyl chloroformates in aqueous alkaline medium, and this reaction can be applicable to a derivatization method for microscale GLC analysis (Makita et al., 1976). On the basis of this observation, we have developed a simple and precise GLC method for the determination of tyramine in foods, and this method has been successfully applied to some Japanese fermented foods. Tyramine (I), separated from foods by using a Amberlite CG-120 column, was converted into the *N,O*-bis(ethyloxycarbonyl) (EOC) derivative (II)



(I) (II)

by reaction with ethyl chloroformate at room temperature, and the resulting derivative was determined by GLC using 3,4-dimethoxyphenethylamine as an internal standard.

EXPERIMENTAL SECTION

Materials. Each sample was purchased over the counter at local supermarkets and was treated for analysis on the same day.

Reagents. Tyramine hydrochloride and 3,4-dimethoxyphenethylamine hydrochloride, used as an internal standard, were obtained from Sigma Chemical Co. Standard solutions of tyramine (10 and 100 μg/mL, as free base) were prepared in water, and aliquots were taken for the preparation of the calibration curve and for the calculation of the recovery rate from food samples. Solutions of the internal standard in water were prepared at concentrations of 20 and 100 μg/mL, as free base, respectively. These solutions were stored in capped glass bottles at 4 °C. Ethyl chloroformate (Tokyo Kasei Kogyo, Tokyo, Japan) was used without further purification. Amberlite CG-120 resin (100–200 mesh) in the H^+ form was treated before use as follows: the resin was washed twice with 4 N HCl in a beaker and then covered with 2 N NaOH and swirled for 3 h at 70 °C after successive washing with water until approximately neutral. The resin was regenerated by washing three times with 4 N HCl and subsequently washed with water until neutral. With the use of resin as

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treated above, the recovery rate of tyramine in the standard solution from the column was checked to be above 95% every generation and washing. If the recovery rate was below 95% in this stage, the generation procedure was repeated and rechecked. After use, the resin was collected in a beaker and treated according to the procedure described above. All other chemicals and solvents were reagent grade where available commercially.

Extraction and Column Chromatography. For cheese and soybean paste samples, each known amount (2.5–5 g) was homogenized with 20 mL of 2% $HClO_4$ in a tissue grinder and then the mixture was centrifuged for 5 min. This procedure was repeated, and the supernatant fluid was removed and combined. The combined supernatants were made up to 50 mL with 2% $HClO_4$, and this solution was sampled for the column chromatography after filtration. For liquid samples except sake (Japanese alcoholic drink) and beer, each aliquot (1 mL of soya sauce and 20 mL of fermented milk) was pipetted into a 100-mL volumetric flask, directly made up to 100 mL with 5% $HClO_4$, and agitated for 3 min. For the sake and beer samples, each 50 mL was pipetted into a round-bottom flask which contained 5 drops of 6 N HCl, and this mixture was evaporated to dryness in a rotary evaporator at 60 °C in vacuo in order to remove ethyl alcohol. The oily residue was transferred into a 100-mL volumetric flask with 5% $HClO_4$ and made up to 100 mL with 2% $HClO_4$. These sample solutions prepared above were then applied to the subsequent column chromatography.

A 10-mL portion of each sample solution was loaded onto a column (9 mm i.d.) with a stopcock, containing a 3-mL bed volume of Amberlite CG-120 resin, and allowed to pass through the column at about 1 mL/min. The column was washed with 30 mL of 3.1 M sodium phosphate buffer (pH 8) containing 0.1 M NaCl and then with 30 ml of 1 N HCl. The flow rate was about 2–3 mL/min. The effluent containing the interfering substances was discarded. Forty milliliters of 6 N HCl was passed through the column at about 2–3 mL/min and the eluate containing tyramine was collected in a 100-mL, round-bottom flask with a ground-glass top. To the eluate was added 0.5 mL of the internal standard solution (10 or 50 μg) and the mixture was evaporated to dryness in a rotary evaporator at 50 °C in vacuo. The residue was transferred to a 10-mL, glass screw-top culture tube (Corning No. 9826) with a PTFE-lined cap with 2 mL of water.

Preparation of Derivative. To the solution in a glass, screw-top culture tube were added 0.5 mL of 10% Na_2CO_3 and 0.2 mL of ethyl chloroformate, and then the mixture was shaken at 300 rpm with a shaker for 10 min at room temperature. The resulting *N,O*-bis(ethyloxycarbonyl) derivative was extracted three times with 2 mL of diethyl ether, and the combined ether extracts containing the excess of ethyl chloroformate were evaporated to dryness at 50 °C under a stream of air in a draft chamber. The residue was dissolved in 0.1 mL of ethyl acetate and the solution was dried over anhydrous Na_2SO_4. A 2-μL aliquot of the solution was injected into the gas chromatograph.

Gas–Liquid Chromatography. Analyses were performed on a Shimadzu 4CM gas chromatograph, equipped with flame ionization detectors (FID) and a linear temperature programmer. Before use, a glass column (1 m × 3 mm i.d.) was silanized with 5% dimethyldichlorosilane in toluene and the mixed-phase column packing, 1.5% OV-17, 0.2% SP-1000 on 100–120 mesh Uniport HP (Gasukuro Kogyo, Tokyo, Japan), was prepared by using *n*-BuOH/$CHCl_3$ (1:1, v/v) as a coating solvent, according to the procedure of Horning et al. (1963). The packed column was conditioned at 275 °C for 15 h with a nitrogen flow rate of 30 mL/min. The operating conditions were as follows: nitrogen flow rate, 60 mL/min; injection port and detector temperatures, 280 °C; oven temperature, programmed from 170 to 245 °C at 6 °C/min; chart speed, 0.5 cm/min; FID sensitivity, 10^2 ($\times 10^6$ Ω); range, 4–32 (× 0.01 V).

Gas Chromatography–Mass Spectrometry. A Shimadzu LKB 9000 gas chromatograph–mass spectrometer was used. A glass column (1 m × 3 mm i.d.) containing 1.5% OV-17, 0.2% SP-1000 on 100–120 mesh Uniport HP was used with a helium flow rate of 20 mL/min. The operating conditions were as follows: GLC oven temperature, programmed from 150 to 250 °C at 5 °C/min; trap current, 60 μA; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion source temperature, 270 °C; separator temperature, 270 °C.

Preparation of Calibration Curves and Calculation. Calibration curves in the range of 2–10 and 10–100 μg of tyramine were prepared individually, using each 10 and 50 μg of the internal standard. In a series of five glass screw-top culture tubes, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of each standard solution were placed, respectively, and to each solution was added 0.5 mL of the internal standard solution. The total volume was then made up to 2 mL with water, and these mixtures were treated in the same manner as described above. As mentioned above, the derivatives thus obtained were dissolved in 0.1 mL of ethyl acetate, and 2 μL of each solution was injected. The peak height ratio of tyramine/internal standard was calculated by dividing the height of the tyramine peak by that of the internal standard peak and the peak height ratio was plotted against the quantity of free tyramine. In practice, the calibration curve was prepared daily, and standards were chromatographed frequently.

The calculation of the absolute amount of tyramine in foods was accomplished from the peak height ratio calculated by dividing the height of the tyramine peak by that of the internal standard peak and by interpolating on either of two calibration curves, depending on the content of tyramine in the food extract.

Derivatization Yield. In order to assess the derivatization yields for tyramine and 3,4-dimethoxyphenethylamine, pure reference derivatives were synthesized. Physical constants of derivatives were as follows: tyramine, mp 78–78 °C (uncorrected); 3,4-dimethoxyphenethylamine, bp 174–176 °C (bath temperature) (1 mmHg). One hundred micrograms of each amine was derivatized by the procedure outlined in the "Preparation of Derivative" section. The residue obtained after evaporation of diethyl ether was dissolved in 0.3 mL of methanol solution containing 300 μg/mL of phenanthrene. Each pure reference (tyramine, 20.5 mg; 3,4-dimethoxyphenethylamine, 14.0 mg) was weighed, placed in a 30-mL volumetric flask, and made up to 30 mL with methanol solution containing 300 μg/mL of phenanthrene. Each solution was then analyzed by GLC under the conditions indicated above. The derivatization yield of amine to derivative was computed by comparing the peak height ratio relative to phenanthrene obtained for the prepared derivative with that obtained for the pure reference. Phenanthrene added to each sample was employed as an external standard to correct for variations in injected sample volume, solution volume, and instrumental response.

RESULTS AND DISCUSSION

Experiments on the selection of derivative were carried out. As a result, among the *N,O*-bis(alkyloxycarbonyl) derivatives investigated, it was found that the *N,O*-bis-

(ethyloxycarbonyl) derivative is the most appropriate for the analysis of tyramine. This type of derivative of tyramine is well separated from those of amines such as tryptamine, octopamine, synephrine, and 3-methoxytyramine, which may coexist in food samples analyzed, when a mixed-phase, 1.5% OV-17, 0.2% SP-1000 column is used.

The structure of derivative II prepared by the procedure described above was established by gas chromatography–mass spectrometry (GC–MS). A molecular ion peak with the expected m/e value (281) was observed, and other significant fragment ion peaks which were useful from structure elucidation were m/e 236, 235, 192, 163, 148, 135, 120, and 107 (base peak).

The average derivatization yields (n = 4) for tyramine and the internal standard at the level of 100 μg of each with ethyl chloroformate were almost quantitative, 96.7% for the former and 95.9% for the later, respectively, with the conditions used. Both derivatives could be easily prepared in a short time without laborious work and were very stable to moisture and, therefore, no precautions are necessary in their handling and storage. Similar stability was observed for the derivatives obtained from food samples.

The calibration curve for tyramine in the range of 2–100 μg, implying sample size injected into GLC ranged from 0.04 up to 2.0 μg, was prepared by using 3,4-dimethoxyphenethylamine as an internal standard. The linearity of the calibration curve and its reproducibility were found to be satisfactory for quantitative determination. On the other hand, with use of tyramine as the internal standard, the calibration curve for 3,4-dimethoxyphenethylamine in the range of 10–100 μg was prepared in the same manner as described in the Experimental Section. Linearity was also observed and this suggested that 3,4-dimethoxyphenethylamine is suitable as an internal standard.

On the basis of these results, application of the proposed method to the determination of tyramine contents in several kinds of Japanese fermented food products was investigated. Most workers have used extraction techniques with organic solvents for separation of tyramine from foods, and we also adopted this technique in preliminary experiments. However, it gave variable results and required special care in maintaining the correct pH in order to ensure optimun extraction of tyramine. Then, for the simple and rapid separation of free tyramine from foods, the cation-exchange column chromatographic purification stage was devised. The passing of an aliquot of the solution prepared with 2% $HClO_4$ through an Amberlite CG-120 column prior to derivatization allowed the interfering substances to be excluded, and satisfactory chromatograms could be obtained, as shown in Figure 1. Separate samples to which the internal standard had not been added were also run to determine whether the internal standard was confused with another peak from foods having the same retention time, demonstrating that the internal standard region was free from interfering peaks in each food investigated. Unfortunately, in this column chromatographic stage, 3,4-dimethoxyphenethylamine as the internal standard did not show the same behavior as tyramine, that is, its recovery rate from the column was below 44% with the elution conditions used. In order to explore another suitable internal standard, an effort was directed toward testing other amines which have no possibility of coexistence in food samples. None of the amines tested was suitable with respect to low recovery from the column or confusion with interfering peaks. The internal standard 3,4-dimethoxyphenethylamine was therefore unwillingly added after the ion-exchange purification procedure although this is not the customary procedure.

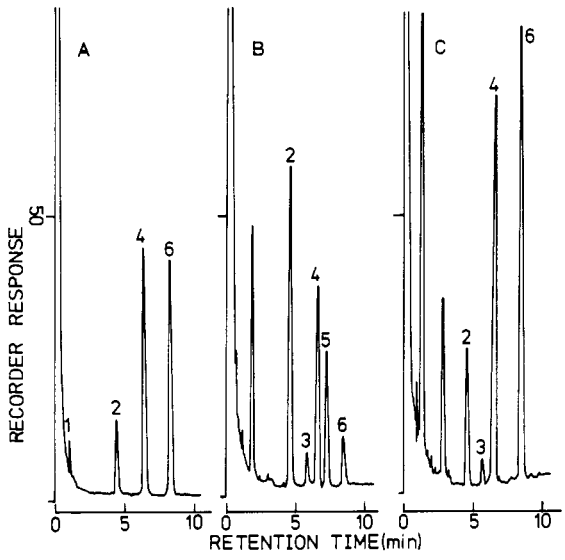

Figure 1. Typical gas chromatograms obtained from soya sauce (A), beer (B), and cheese (C). Attenuation settings are varied, depending on samples. GLC conditions are as described in the text. Peak assignments are as follows: (1) β-phenethylamine, (2) putrescine, (3) cadaverine, (4) internal standard (3,4-dimethoxyphenethylamine), (5) *N*-methyltyramine, and (6) tyramine.

Table I. Recovery Rate for Tyramine Added to Foods

sample	amount of tyramine added, μg[a]	recov rate, %		mean recov rate, %
soya sauce 1	25	93.5	95.0	
		97.8	99.4	
soya sauce 2	25	92.4	94.1	
		98.7	100.5	96.4
sake 1	5	101.9	95.6	
sake 2	5	94.7	99.7	98.0
soybean paste 1	12.5	96.8	98.4	
soybean paste 2	12.5	94.1	98.0	96.8
fermented milk 1	12.5	93.7		
2	12.5	94.4		94.1
beer 1	5	96.3		
beer 2	5	94.9		95.6
cheese 1	25	94.2		
cheese 2	25	96.3		95.3

[a] Tyramine was added to 10 mL of each sample solution prepared with 2% $HClO_4$.

Each tyramine peak obtained from samples was identified by GC–MS under the same GLC column as described above. The mass spectra obtained from the peaks due to the derivatives of tyramine from foods showed the same pattern as one from the authentic derivative, demonstrating the absence of impurity in each peak. In addition to tyramine, putrescine, cadaverine, β-phenethylamine, and *N*-methyltyramine could also be identified by GC–MS. However, quantitation of these compounds was not investigated. *N*-Methyltyramine found in all of the beers studied may have originated from barley, which contains *N*-methyltyramine, as reported by Kirkwood and Marion (1950).

The reproducibility of the method was determined by performing five separate assays on each of the foods listed in Table I. Each result gave a reasonable coefficient of variation below 6.2% (not shown). In order to evaluate the efficiency of the recovery of tyramine from foods, known amounts of tyramine were added to the food extracts prepared with 2% $HClO_4$. The recovery rate of

Table II. Tyramine Contents of Various Japanese Fermented Foods Obtained from Commercial Sources

sample no.	kind of food	brand	tyramine content,[a] μg/mL or μg/g
1	soya sauce	A	882.0
2		B	143.2
3		C	136.6
4		D	318.7
5		E	469.9
6	sake	A	0.51
7		B	0.35
8		C	0.44
9		D	0.28
10		E	0.21
11	beer	A	1.15
12		A	1.13
13		A	1.30
14		B	1.22
15		C	1.13
16		D	1.06
17	fermented milk	A	0.52
18		A	0.41
19		B	2.33
20		C	0.61
21	soybean paste	A	0.21
22		A	0.34
23		A	0.30
24		B	169.5
25		C	0.50
26		D	0.49
27	cheese	A	69.4
28		B	45.0
29		C	29.8
30		D	138.4

[a] Each value represents an average of duplicate analyses.

tyramine was reasonable, as presented in Table I. It is clear from the results that the method is precise and accurate. Tyramine contents of different foods determined by the present method are summarized in Table II. From the results it can be seen that, compared with other foods, soya sauces and cheeses generally contain relatively large amounts of tyramine. There was wide variation in tyramine contents of soya sauces and cheeses, and this suggested that the amounts of tyramine formed in the fermented food products are closely related to the conditions involved in during ripening. Tyramine contents of cheeses obtained are within the range reported by Kaplan et al. (1974) and Sen (1969), while those of beers are somewhat lower.

The most significant advantage of the method presented is that derivative preparation is simple and rapid, and the derivatives formed are very stable to moisture. Separation of tyramine and removal of interfering substances with an Amberlite CG-120 resin column are simple and reliable, and they may be applicable to various kinds of foods. This method therefore can be proposed as an alternative and routine one for the analysis of tyramine in foods.

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