Simple Pretreatment Method for Monitoring of Norephedrine in Urine and Tyramine in Food Sample Using Liquid–Liquid Extraction

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Norephedrine and tyramine are familiar as sympathetic nerve-stimulating chemicals. Analysis of norephedrine in urine sample is necessary for the evaluation of intake. Analysis of tyramine in food samples is also necessary for evaluation of its daily intake. However, both are difficult to extract by conventional liquid–liquid extraction. The present work proposed to monitor both using a small amount of a mixture of two solvents. For norephedrine, a filtrate (100 ml) of human urine sample (pH 11) was mixed with 5% sodium dodecyl sulfate solution (100 μl) and acetone (2.5 ml), then shaken with chloroform (2.5 ml). This method recovered 5–10% of norephedrine added. For tyramine, a filtrate (5 ml) of intact or concentrated sample solution (pH 11) was salted out with NaCl (1 g), then shaken with a mixture of acetone (3 ml) and ethyl ether (2 ml). This method recovered 50–60% of tyramine added.

Key words —— liquid–liquid extraction, urine sample, food sample, tyramine, norephedrine, sodium dodecyl sulfate

INTRODUCTION

In cases of drug and toxic poisoning, causal chemicals must be rapidly specified. Therefore, a simple method capable of monitoring a wide variety of chemicals is always needed. Norephedrine is a sympathomimetic ingredient of some commercially obtainable cough medicines, and has the potential to cause toxicity incidents. Under Japanese law, the legal term of erythro-2-amino-1-phenylpropane-1-ol (except materials involving 50% or less of the base type) is a regulated raw material which is also used for the illegal synthesis of stimulants. In connection with norephedrine, norpseudoephedrine is an ingredient of khat (a beverage made of Catha edulis). Tyramine is a natural ingredient contained in some fermented food products and has indirect sympathetic nerve-stimulating effects. Therefore, tyramine serves as a background chemical for the above effects in the human body, because tyramine is unconsciously ingested through diet. Detection of both prior to quantitative measurement is important. The trendy solid–liquid extraction method using a cartridge fails to treat a large volume of such mucous, turbid, or viscous samples as concentrated urine and solutions of food products, both of which clog up of the cartridge. These troublesome samples are treatable by a conventional liquid–liquid extraction method using a large quantity of solvent, however, the both are difficult to extract. The use of large quantities of solvent, particularly chloroform, is unfavorable for human health and the environment. As chloroform has excellent ability to extract a wide variety of chemicals, the previous paper proposed a monitoring method for basic chemicals using a small amount of chloroform[1]: chloroform as small as 2.5 ml treated 100 ml of human urine sample by adding 100 μl of 5% sodium dodecyl sulfate (SDS)[2] to the urine to prevent solvent-gelatinization. However, this method failed to extract norephedrine and tyramine. The present work pursued a minimum requirement of solvents available for the detection of norephedrine in urine sample and of tyramine in food sample solution. Several combinations of solvents[3]: chloroform–acetone, ethyl ether–acetone, ethyl ether–ethanol, and others were examined varying the ratio of the pair for increasing the extraction efficiency.

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MATERIALS AND METHODS

Chemicals —— Sodium dodecyl sulfate, tyramine hydrochloride, histamine, and ethanolamine are of analytical grade and were purchased from Wako Pure Chemical Industries, Ltd. A standard solution of norephedrine (phenylpropanolamine: 2-amino-1-phenyl-1-propanol) in methanol was prepared by dissolving the cough medicine Contac 600 SR: phenylpropanolamine 75 mg in two capsules, Sumitomo Pharmaceutical Co., Ltd. The other chemicals used for addition to the urine sample were also from medicines. Lexotan for bromazepam: Rosh Pharm. Co., Ltd. Cercine for diazepam: Takeda Pharm. Ind., Ltd. Hirnamin for levomepromazine: Shionogi Pharm. Co., Ltd. Akineton for biperiden: Dai-Nippon Pharm. Co., Ltd. Korgen Kowa Toroche for chlorpheniramine: Kowa Shinyaku Co., Ltd. Pabron Gold Capsule for carbinoxamine and Pabron-S Cough for dihydrocodeine and noscapine: Taisho Pharm. Co., Ltd. Avomine for promethazine: Iwaki Pharm. Co., Ltd. Inolin for trimetoquinol: Tanabe Pharm. Co., Ltd.

Materials —— A paper disk (16 mm in diameter, 1 mm thick) was made by cutting a Toyo Roshi No. 526 with a leather punch. Another filter paper was Toyo Roshi No. 101 (24 cm in diameter). Test tubes (Pyrex) were type A, 15 mm in diameter, 18 cm long; type B, 9 mm, 5 cm; and type C, 15 mm, 12 cm. High vacuum silicone grease (compound) was purchased from Torey Dow Corning Silicone, Ltd. Silicone paste (KE45T) was from Shinetsu Chemical Industries, Ltd.

Plunger-Top-Filtration (PTF) Method[4] —— For filtration of the sample solution, three paper disks were inserted into a test tube-A containing sample solution (10–15 ml) using a teflon pipe (12 mm in inner diameter, 1 mm in wall thickness, 20 cm long). The pipe was weighted down with an iron rod (*e.g.*, 0.5 kg, 15 mm in diameter, 65 cm long) until the pile of three disks came to the bottom. The filtrate (5 ml) on the pile was transferred into another test tube-A for extraction. For isolation of the solvent, a sandwich of anhydrous sodium sulfate (1 g) between two paper disks was inserted at the border of two phases, which had resulted after shaking the filtrate (5 ml) of the sample solution with a solvent mixture (5 ml) in test tube-A. The sandwich was made at the top area of the test tube-A having the two phases: one paper disk was inserted into the tube, where anhydrous sodium sulfate was mounted, and was covered with another paper disk using the rod. An apparatus (Fig. 1) was constructed as follows: the iron rod was set to be movable up and down through a cylinder (*e.g.*, 2 cm in inner diameter, 30 cm long) which was attached to a stand. Test tube-A was placed in a plastic 50 ml graduated cylinder.

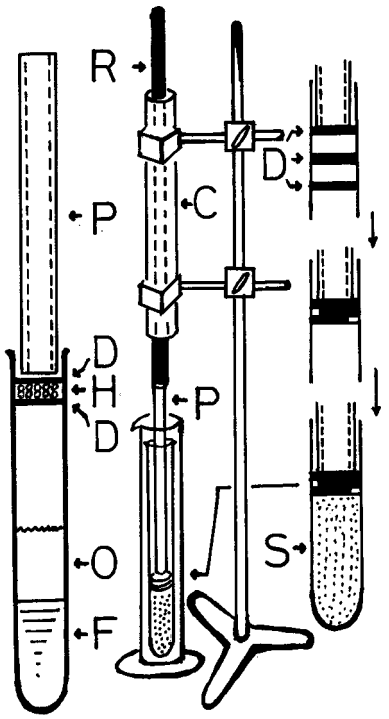

Fig. 1. Equipment for Plunger Top Filtration Method
R: rod for weight. P: teflon pipe. D: paper disk. H: anhydrous sodium sulfate, which was sandwiched with two paper disks (D). C: cylinder to move the rod (R) up and down. S: turbid sample solution. F: filtrate of sample solution (S). O: solvent layer separated after shaking with the filtrate (F).

Thin-Layer Chromatography —— Conditions were as follows: commercially available silica gel plate (No. 1.05721, Merck); isopropanol–28% ammonia solution, 95 : 5, v/v, for norephedrine; acetonitrile–28% ammonia solution, 5 : 1, v/v, for tyramine; and staining with ninhydrin reagent. The density of the stained spot of norephedrine or tyramine was measured using a Shimadzu CS-9100 chromatoscanner for conventional quantitative analysis.

Extraction Method I —— This method has been published in the previous paper,[1] and is the same as the following method II, but has no process of the addition of acetone (2.5 ml) to the urine sample (100 ml).

Extraction Method II —— To a filtrate (100 ml) of human urine sample (pH 11, NaOH) was added 5% of SDS (100 μl) and acetone (2.5 ml), and the mixture was then shaken with chloroform (2.5 ml)

for 30 s in a vessel. A clear chloroform layer was pipetted out into test tube-B, then dried in a current of air. The residue was dissolved in 50 μl of chloroform, then the resulting solution was spotted on the plate for TLC.

Extraction Method III —— An intact or concentrated sample solution (less than 10 ml) taken in test tube-A was filtered at pH 11 by the PTF method, if necessary. The filtrate (5 ml) was transferred into another test tube-A, and mixed with NaCl (1 g) and acetone (3 ml), then shaken with ethyl ether (2 ml) for 30 s. The separated solvent was isolated by the PTF method using a sandwich of anhydrous sodium sulfate (1 g). The isolated solvent was poured into another test tube-C and dried in a current of air. The residue was dissolved in methanol (100 μl), and a 10 μl portion was subjected to TLC.

Preparation of Sample Solution —— Flavoring products sample: the following samples (except sample Nos. 8, 9 and 10) were filtered through a filter paper. The filtrates of samples 1 to 7 were shaken with *n*-hexane to remove oilish ingredients prior to extraction. 1) Soybean paste[5–7] (Miso S) diluted 1 : 1 with water. 2) White soybean paste involving rice (White Miso M) diluted 1 : 1 with water. 3) Cooked cayenne leaf tsukudani (HT) (tsukudani: a food boiled down in soybean sauce). 4) Hot soybean sauce-1 (TB). 5) Hot soybean sauce-2 (BB). 6) Barbecue sauce (YT: sweet, medial, and hot taste types). 7) Vegetable sauce (TT). 8) Soybean sauce (Shoyu). 9) Grape vinegar. 10) Red wine. 11) Seaweed tsukudani cooking (EM) diluted 1 : 1 with water. 12) Water extracts (1 : 1) of dried bonito (Katsuobushi). 13) Water solution (1 : 1) of sardine powder (ID). Concentrated urine sample: human urine (500 ml, freshly collected or denatured obtained by standing at room temperature for more than one month) was evaporated to dryness *in vacuo* by floating an arched plastic tip (*e.g.*, 2 cm × 3 cm, cut from a plastic reagent container). The inside of the tip was thinly painted with a high vacuum silicone grease. The residue was transferred into test tube-A with water and adjusted to 15 ml. This was filtered by the PTF method. The filtrate (5 ml) was extracted by method III. Bovine brain sample: this sample (100 g) was homogenized with water (100 ml) and NaCl (20 g) at pH 4 (HCl), then boiled for 5–10 min. The coagulated homogenate was filtered through a filter paper. The filtrate (100 ml) was shaken with *n*-hexane (20 ml) to remove fatty materials. The separated aqueous layer was neutralized with NaOH and evaporated to dryness *in vacuo*. The residue was suspended in water to give a total volume of 15 ml. Next, 5 ml was extracted by method III. Beer sample: this sample (100 ml) was spread over two glass plates (20 cm × 20 cm) whose edges were banked with silicone paste. The beer on the plate was left standing at room temperature for 2–3 d to dry. The residue was scraped off with a single edged razor. The powder (3.3 g) was dissolved in water (10 ml) in test tube-A, and filtered by the PTF method. The filtrate (5 ml) was extracted by method III. Putrefied[8] crab sample solution: crust (1 kg) with broken meat was left standing for more than 10 d at room temperature to be putrefied. The putrefied sample was washed with water (500 ml), and the washings were filtered through a filter paper. The filtrate (5 ml) was extracted by method III.

RESULTS AND DISCUSSION

Applicability of Extraction Method I

The recovery of basic chemicals added to human urine samples was measured to determine whether a subject was extractable or not by method I. As shown in Table 1, promethazine showed a high recovery of 80–90% by single extraction. Bromazepam or dihydrocodeine was recovered at 40–50%. The other detectable chemicals were recovered at 50–70%. Recovery of 50–60% was enough for monitoring chemicals. However, norephedrine (alcoholic amine) was hardly extracted. Trimetoquinol and tyramine were not extracted by method I. Extraction of ethyl ether (20 ml) was also attempted from a human urine sample (100 ml, pH 9.5) varying the addition volume of 5% SDS. The addition of 50 μl of 5% SDS recovered only 5–6 ml of ethyl ether, and the extraction efficiency was poor for norephedrine (data not shown). Therefore, the use of small amounts of chloroform was further developed for the extraction of norephedrine in a large volume of sample solution.

Effects of Combination of Solvents on Extraction

A norephedrine-spiked urine sample was mixed with different water-soluble solvents and extracted by method I. As shown in Fig. 2, a combination of acetone and chloroform was found to exert extraction ability against norephedrine. Moreover, the volatility of both solvents was excellent for rapid dryness of the extracts. The recovery (less than 5%) of norephedrine by the chloroform–acetone system was poor. However, the fact that norephedrine could be

Table 1. Recovery of Some Chemicals Added to Human Urine Sample by Extraction with Chloroform in the Presence of SDS

Chemical (in medicine) added	Recovery (%) 5 μg	10 μg
Bromazepam (Lexotan)	32.0 ± 6.5 $n=7$	43.0 ± 5.4 $n=5$
Diazepam (Cercine)	68.2 ±10.5 $n=6$	71.0 ± 5.7 $n=6$
Levomepromazine (Hirnamin)	42.8 ± 4.2 $n=5$	51.9 ± 4.3 $n=5$
Biperiden (Akineton)	59.6 ± 8.0 $n=6$	66.2 ± 1.6 $n=6$
d-Chlorpheniramine (Korgen Kowa Toroche)	59.2 ± 6.0 $n=6$	59.5 ± 2.6 $n=6$
Carbinoxamine (Pabron Gold Capsule)	73.5 ± 5.1 $n=5$	74.7 ± 8.7 $n=6$
Dihydrocodeine (Pabron-S Cough)	52.5 ± 5.8 $n=6$	52.4 ± 5.9 $n=6$
Promethazine (Avomine)	85.3 ±11.7 $n=6$	92.0 ± 5.3 $n=6$
Noscapine (Pabron-S Cough)	—	43.7 ± 3.9 $n=6$
Norephedrine (Contac 600SR)	—	FD
Trimetoquinol (Inolin)	—	n.d.
Tyramine	—	n.d.

Data: each value is expressed as means ± S.D., for separate experiments, and n = 5–7. Conditions were mentioned in the text. FD: faintly detected. n.d.: not detected.

extracted and detected by TLC is noteworthy, because it has not been previously detected in spite of the presence of a small amount of a single solvent. Other combination systems of *n*-butanol and chloroform, or *n*-amylalcohol and chloroform were effective for the extraction of norephedrine; however, its volatility was low. A well-known combination system of chloroform (2.5 m) and isopropanol (0.5–5.0 ml)[9] recovered only 0.2–0.6% of 10 μg norephedrine added in a 100 ml urine sample (data not shown).

Modification of Extraction Method I

As shown in Fig. 3, the effects of acetone on extraction efficiency were examined. The recovery of norephedrine increased with increasing volumes of acetone added. However, the solvent layer began to be gelatinized at more than 2.5 ml of acetone. The use of larger amounts of acetone retarded progress on obtaining clear solvent, because additional operations (isolation of the gelatinized solvent layer

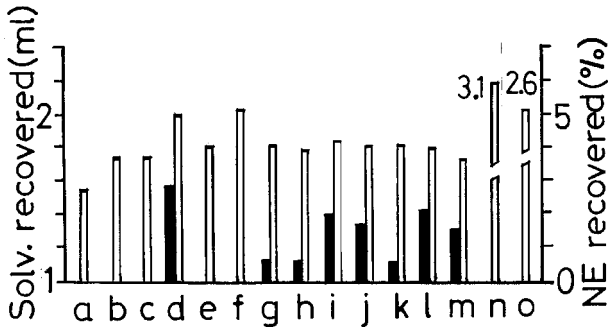

Fig. 2. Effects of Water-Soluble Solvent on Extraction Efficiency of Chloroform

Ordinate: the left for open bar, and the right for closed bar. Extraction components: 100 ml filtrate of human urine sample + 10 μg norephedrine + 100 μl 5% SDS + 2.5 ml chloroform + 2.5 ml water-soluble solvent, at pH 9.5. Water-soluble solvent: a, no use; b, methanol; c, ethanol; d, acetone; e, acetonitrile; f, 1,4-dioxane; g, *n*-propanol; h, isopropanol; i, *n*-butanol (2.0 ml); j, isobutanol; k, *tert*-butanol; l, *n*-amylalcohol (0.5 ml); m, isoamylalcohol (0.5 ml); n, ethylacetate; and o, ethyl ether.

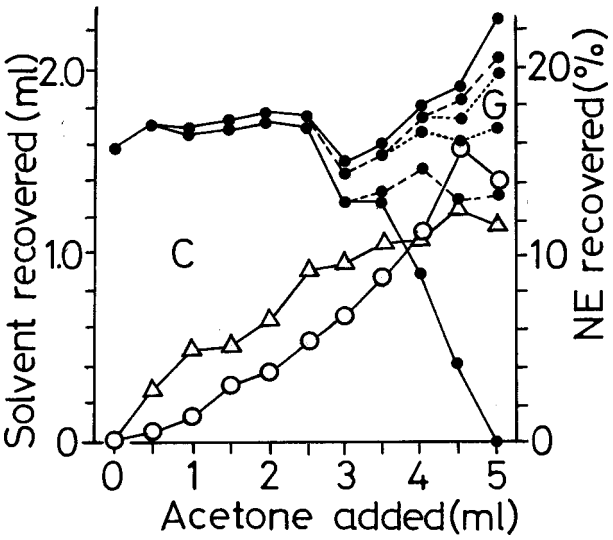

Fig. 3. Effects of Acetone on Extraction Efficiency and Recovery of Chloroform

Ordinate: the left for symbol ●, and the right for symbols ○ and △. Extraction components: 100 ml filtrate of human urine sample + 5 μg norephedrine (○) or 100 μg NE (△) + 100 μl 5% SDS + 2.5 ml chloroform + different amounts of acetone, at pH 9.5. Gel layer-1 (G) between upper and lower solid lines (●) appeared 30 min after shaking the mixture. Gel layer-2 between the two broken lines (●) appeared 3 min after shaking the isolated gel layer-1. Gel layer-3 between the two dotted lines (●) appeared 3 min after shaking the isolated gel layer-1 with anhydrous sodium sulfate (100 mg). C: clear solvent layer.

and the shaking of it with anhydrous sodium sulfate) were needed. Similarly, a counter experiment was done varying the volume of chloroform under a constant volume of acetone (2.5 ml). As shown in Fig. 4, the recovery of norephedrine or solvent be-

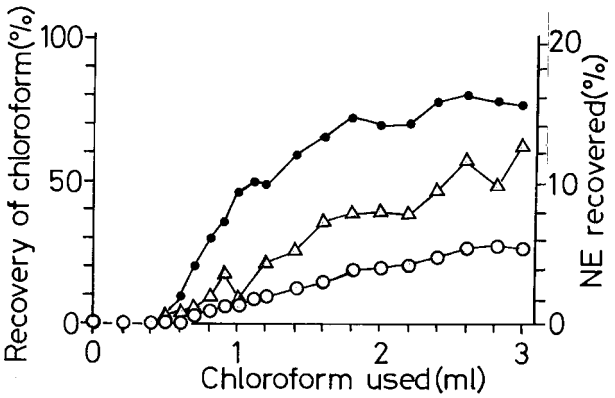

Fig. 4. Amount of Chloroform Separated and Extraction Efficiency in the Presence of Acetone

Ordinate: the left for symbol ●, and the right for symbols ○ and △. Extraction component: 100 ml filtrate of human urine sample + 10 μg (○) or 100 μg (△) norephedrine + 100 μl 5% SDS + 2.5 ml acetone + different amount of chloroform, at pH 9.5.

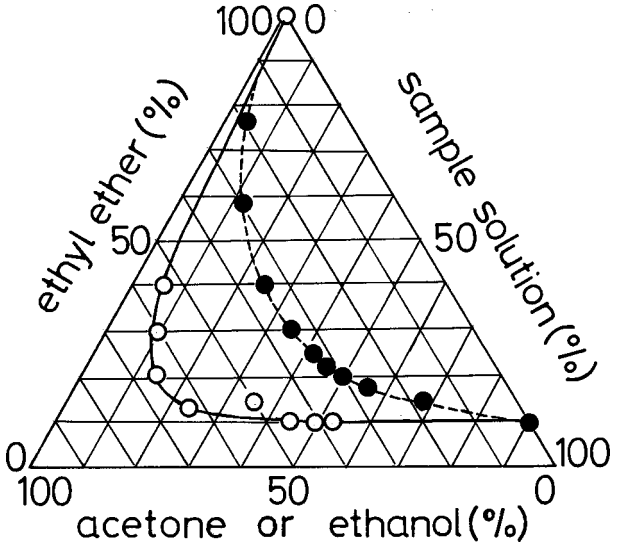

Fig. 5. Phase Separation in Three-Component System

Symbol: ○ for acetone, and ● for ethanol. Sample solution: putrefied crab sample solution (pH 11) mentioned in the text. Each curve and its inside: two-phase separation area. The outside of each curve: one phase.

gan to increase from the 0.5 ml use of chloroform, and reached a plateau (70–80% for solvent) at about 2.5 ml of chloroform. Therefore, the use of this amount was practical. Consequently, method I was modified to method II by adding 2.5 ml of acetone to 100 ml of urine sample. Method II recovered norephedrine and trimetoquinol (5–10 μg in 100 ml urine sample) in the range of 5–10%. However, tyramine failed to be extracted, even by method II.

Extraction of Tyramine

For separation of the solvent from the sample solution, a combination of ethyl ether and acetone, or ethyl ether and ethanol was examined using a putrefied crab sample solution. As shown in Fig. 5, the solid line depicted the shaking of three components of sample solution, acetone, and ethyl ether at different volume ratios which were read from each side scale of the triangle. Similarly, the broken line was obtained by using ethanol instead of acetone. The solvent layer appeared along the curve and inside the curve. The use of acetone gave a larger separation area than did the use of ethanol. This difference implied that higher solvent separation was achieved by a smaller volume ratio of ethyl ether/acetone than that of ethyl ether/ethanol under the same sample volume condition. For recovery of the solvent, sample solution (5 ml) and each volume of ethyl ether (indicated in panel) were shaken in the presence of different volumes of acetone. As shown in Fig. 6, the use of acetone gave a higher recovery of the solvent than did the use of ethanol under the same volume ratio. For example, one extraction system 5 : 3 : 2 (by volume) of sample solution, acetone, and ethyl ether (respectively) recovered 3 ml of solvent. The other system 5 : 3 : 2 of sample solution, ethanol, ethyl ether (respectively) recovered 2 ml of solvent. Extraction method III demanded as much solvent as the sample solution. Therefore, the solvent used was lessened by restricting the sample volume to 5 ml: a thin sample solution was concentrated to 5 ml.

Advantage of Method III

As shown in Table 2, a water sample (5 ml + tyramine 100 μg) was extracted in five different ways. System 2 (corresponding to the extraction method III) recovered 54% of tyramine added. The other poor recovery data demonstrated that ethyl ether could be potentiated by the addition of acetone (3 ml) and NaCl (1 g) to the sample solution. The salting out process with NaCl was essential for method III. Ethyl ether alone (system 1) or acetone alone (system 3, separable from NaCl-saturated aqueous sample) poorly recovered tyramine. However, the combination of the both (system 3) elevated the recovery. This effectiveness by both solvents is noteworthy. Method III, followed by TLC (spotted with 1/10 the solution of extracts; ninhydrin), had a detection limit of 2 μg tyramine in the 5 ml water sample.

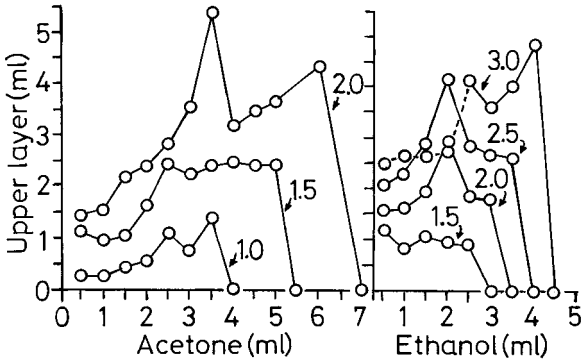

Fig. 6. Influence of Acetone or Ethanol on Recovery of Ethyl Ether

Extraction component: 5 ml putrefied crab sample solution + different amounts of acetone or ethanol + some fixed amounts of ethyl ether (ml) indicated in data curves, at pH 11.

Table 2. Extraction Rate of Tyramine

Component	Extraction system				
	1	2	3	4	5
NaCl (g)	1	1	1	0	0
Acetone (ml)	0	3	5	0	3
Ethyl ether (ml)	5	2	0	5	2
Recovery (%)	1.5	54.0	1.0	0.25	5.5

Sample: 5 ml water + 100 μg tyramine, at pH 11. System 2: corresponding to extraction method III mentioned in the text.

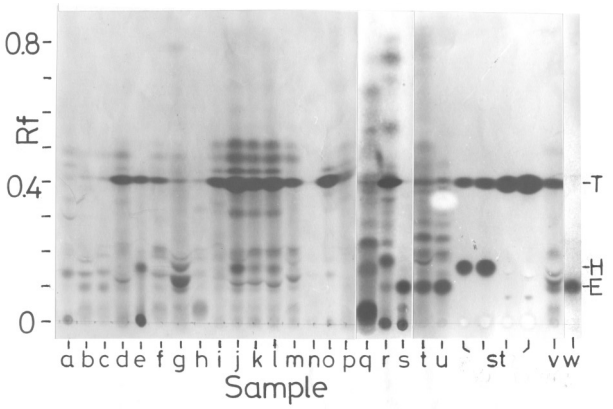

Fig. 7. Thin-Layer Chromatograms of Amine Compounds

Conditions were mentioned in the text. T, H, and E: migration sites of tyramine, histamine, and ethanolamine, respectively. Sample: a, Katsuobushi; b, white soybean paste; c, expiring white soybean paste; d, cayenne leaf tsukudani; e, hot soybean sauce-1; f, hot soybean sauce-2; g, soybean sauce; h, vegetable sauce; i, seaweed tsukudani; j, barbecue sauce (sweet taste type); k, barbecue sauce (hot taste); l, barbecue sauce (medial taste); m, expiring barbecue sauce (hot taste); n, sardine powder; o, putrefied crab sample solution; p, beer (concentration, 50 ml $\rightarrow$ 5 ml); q, grape vinegar (20 ml $\rightarrow$ 5 ml); r, red wine (20 ml $\rightarrow$ 5 ml); s, bovine brain (equivalent to 17 g); t, concentrated stored human urine; u, concentrated fresh human urine; v, soybean paste; and w, standard ethanolamine. st for four lanes: 5, 10, 50, and 100 μg of standard tyramine, respectively (lower spot: standard histamine).

Application of Method III

Profiles of tyramine in actual samples are shown in Fig. 7. Method III gave the following approximate recovery values for tyramine from 5 ml of sample solutions. Putrefied crab sample solution: 450 μg. Barbecue sauce: 400 μg. Seaweed tsukudani cooking: 300 μg. Soybean paste: 150 μg (trace for White soybean paste). Cooked cayenne leaf tsukudani: 100 μg. Hot soybean sauce-1: 50 μg. Hot soybean sauce-2: 30 μg. Soybean sauce: 20 μg. Red wine: 20 μg. Beer: 0.5 μg. Differences in content of tyramine may be attributable to species, to the amount of raw materials, and to processes of fermentation. As a biological sample, bovine brain sample failed to show tyramine. Fresh or stored human urine sample gave an ambiguous spot of tyramine. However, both urine and brain samples showed a clear spot of ethanolamine. The one detected in the brain sample seems to be almost artificially derived from phosphatidylethanolamine, which should be hydrolyzed in the boiling process (pH 4) for sample coagulation in method III.

In conclusion, chloroform (2.5 ml) recovered detectable amounts (recovery: 40–60%) of general basic chemicals added to human urine sample (100 ml), to which had been added 5% SDS (100 μl). One combination system of chloroform (2.5 ml) and acetone (2.5 ml) recovered a detectable amount (5–10%) of norephedrine added in human urine sample (100 ml) involving 100 μl of 5% SDS. The other combination system of ethyl ether (2 ml) and acetone (3 ml) recovered a detectable amount (50–60%) of tyramine in intact or concentrated sample solutions (5 ml) of fermented food products.

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