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Gas–Liquid Chromatographic Method for Analysis of Di- and Polyamines in Foods

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A gas–liquid chromatographic method for the quantitative determination of putrescine, cadaverine, spermidine, and spermine in foods has been developed. The amines were separated from foods by eluting through a cation-exchange resin column and then converted to their (ethyloxy)carbonyl derivatives by the reaction with ethyl chloroformate in aqueous medium before application to the gas chromatograph with a flame ionization detector. 1,8-Diaminooctane was used as an internal standard. Separation and determination of the resulting derivatives were performed on a 1.5% SE-30/0.3% SP-1000 on Uniport HP column (0.5 m) under the temperature-programmed condition. The calibration curves for the amines in the range of 12.5–125 nmol were linear and sufficiently reproducible for quantitative determination. The overall recovery rates were satisfactory. Putrescine and spermidine were present in all of the foods investigated. Relatively large amounts of spermidine occurred in the mushrooms and beans investigated.

The polyamines spermidine and spermine as well as the diamines putrescine and cadaverine are widely distributed in nature and have recently attracted considerable attention in the field of food chemistry from a public health standpoint since some of these amines may be nitrosated or act as potential precursors for other amines capable of forming nitrosamines. Lijinsky and Epstein (1970) postulated that putrescine and cadaverine might be converted by heating or cooking to pyrrolidine and piperidine, respectively. These amines formed are the precursors of the corresponding nitrosamines which are highly carcinogenic. Bills et al. (1973) demonstrated that *N*-nitrosopyrrolidine is produced from putrescine and spermidine when heated in the presence of nitrite. It is well-known that nitrite is widely present in nature and is also produced in human saliva. The possibility that nitrosamines can be formed in vivo, particularly in the gastrointestinal tract, has been reported (Spiegelhalder et al., 1976; Tannenbaum et al., 1974). Both spermidine and spermine contain secondary amino groups which may react with nitrite, and nitrosation might occur upon ingestion of foods containing these components. In fact, Hildrum et al. (1975, 1977) isolated 3-butenyl-2-propenyl-*N*-nitrosamine as the principal volatile product formed in the nitrosation of both spermidine and spermine and later identified three other volatile nitrosamines from spermidine. Recently, Hotchkiss et al. (1977) identified four nonvolatile nitrosamines, bis(hydroxyalkyl)-*N*-nitrosamines, formed upon nitrosation of spermidine. More recently, mutagenicity of these nonvolatile nitrosamines together with 3-butenyl-2-propenyl-*N*-nitrosamine was confirmed by using various strains of *Salmonella typhimurium* in the presence and absence of S9 mix (Hotchkiss et al., 1979).

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On the other hand, although these di- and polyamines might be present in significant amounts in food systems, investigations on quantitative methods as well as their contents in foods and food materials are relatively few. It has been reported that these amines are present in germs such as barley, rice, and wheat (Moruzzi and Caldarera, 1964) and in soybean seeds (Wang, 1972). In these papers, determinations were carried out by ion-exchange chromatographic separation, followed by spectrophotometry of the derivatives formed by the reaction with 2,4-dinitrofluorobenzene. Smith (1975) has reviewed the distribution, biosynthesis, and metabolism of di- and polyamines in higher plants, but food materials are not included. Spinelli et al. (1974) and Lakritz et al. (1975) determined the concentrations of di- and polyamines in porks with other amines, in which the amines extracted with organic solvents were dansylated and the resulting derivatives were quantified spectrofluorometrically after separation by thin-layer chromatography. However, these methods are not satisfactory because of time requirements and lack of resolving power and selectivity.

During a study of the determination of tyramine in fermented food products by gas–liquid chromatography (GLC) (Yamamoto et al., 1980), we found that putrescine and cadaverine in foods could be also derivatized with ethyl chloroformate to the corresponding (ethyloxy)carbonyl (EOC) derivatives, which showed good and reasonable peaks on the gas chromatogram. The present study, based on this observation, was undertaken in order to explore the possibility of carrying out the GLC determination of not only diamines but also polyamines such as spermidine and spermine in foods as their EOC derivatives.

EXPERIMENTAL SECTION

Chemicals and Solvents. All di- and polyamines for standards were obtained, as hydrochloride salts, from Nakarai Chemicals (Kyoto, Japan) and, prior to use, dried

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in a vacuum desiccator over phosphorus pentoxide. 1,8-Diaminooctane used as an internal standard was purchased from Nakarai Chemicals, and the dihydrochloride salt was prepared by the reaction with hydrochloric acid and recrystallized from ethyl alcohol. A standard solution of amines (each 125 nmol/mL) and an internal standard solution (125 nmol/mL) were prepared in water. These solutions were stored in capped glass bottles at 4 °C when not in use. Ethyl chloroformate (bp 95 °C) stabilized with calcium carbonate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and stored at 4 °C after distillation in an all-glass apparatus. Reagent-grade solvents as obtained commercially were used without purification except diethyl ether which was distilled in an all-glass apparatus. The regeneration procedure for Amberlite CG-120 resin (100–200 mesh) in the H^+ form was as previously reported (Yamamoto et al., 1980). All other chemicals were of reagent-grade purity and used as received from commercial sources.

Extraction and Isolation of Amines. Food samples were purchased at local retail markets and were treated for analyses on the same day without drying. For solid samples, a known amount (5–50 g) was homogenized at full speed for 15 min with 150 mL of 2% $HClO_4$ in a homogenizer (Ace Homogenizer AM-8; Nihon Seiki Kaisha Ltd., Tokyo, Japan) and then left to stand at 4 °C overnight in a centrifuge tube in order to improve the efficiency of extraction. The suspension was centrifuged for 10 min at 4000 rpm. After the precipitate was washed twice with 20 mL of 2% $HClO_4$ and centrifuged again, the supernatant and washings were combined and the total volume was made up to 200 mL with 2% $HClO_4$. This solution was filtered before application to the cation-exchange resin column when it was not clear. An aliquot of the clear solution (0.5–5 mL) was pipetted on the top of column (9-mm i.d.; 3-mL bed volume) of Amberlite CG-120 resin in the H^+ form and diluted to 10 mL with 2% $HClO_4$. For liquid samples, a 2-mL aliquot was pipetted and mixed with 2% $HClO_4$ to make up to 20 mL, and 10 mL of this solution was applied to the ion-exchange column. The elution system described in the previous paper (Yamamoto et al., 1980) was adapted for isolation and concentration of the amines from food extracts. After the column was washed successively with 0.1 M sodium phosphate buffer containing 0.1 M NaCl and with 1 N HCl, the portion eluted with 6 N HCl was collected and to this elute was added 1 mL of the internal standard solution. This mixture was evaporated to dryness in a rotary evaporator at 50 °C in vacuo, and the residue was transferred to a 10-mL, glass screw-top flat-bottom vial (50 mm × 21 mm o.d.; Mighty Vial, No. 3; Maruenu Co., Osaka, Japan) with a Teflon-lined cap with 2 mL of water.

Preparation of Derivatives. Immediately after 0.5 mL of 10% NaOH and 0.2 mL of ethyl chloroformate were added to the solution in the vial, the mixture was shaken at 300 rpm (up and down) with a flat-bed shaker (Iwaki KM Shaker VS Type; KK Iwaki, Tokyo, Japan) for 10 min at room temperature. The resulting EOC derivatives were extracted 3 times with 3 mL of diethyl ether, and the ethereal layers were then separated with a Pasteur pipet. The combined extracts were evaporated to dryness at 50 °C with a gentle current of nitrogen. The excess of reagent must be removed at this stage. After the residue was dissolved in 0.1 mL of ethyl acetate and a few grains of anhydrous Na_2SO_4 added, a 4-μL aliquot of this solution was injected for GLC analysis.

Gas–Liquid Chromatography. A Shimadzu 4CM gas chromatograph equipped with a flame ionization detector was used. Prior to use, the glass column (0.5 m × 3 mm i.d.) and quartz wool plugs placed in each end of the column were silanized with 5% dimethyldichlorosilane (DMCS) in toluene. The support, 100–120-mesh Uniport HP (Gasukuro Kogyo Co., Tokyo, Japan), was resilanized with 5% DMCS in toluene (Horning et al., 1963) after removal of grey and black particles by suspension in 36% HCl and subsequent washing with water followed by methanol. The mixed-phase column packing, 1.5% SE-30/0.3% SP-1000 on resilanized Uniport HP was prepared in our laboratory by using 1-BuOH–$CHCl_3$ (1:1 v/v) as a coating solvent according to the filtration method of Horning et al. (1963). The packed column was preconditioned at 285 °C for 20 h with a nitrogen flow rate of 30 mL/min. The operating conditions were as follows: nitrogen flow rate, 80 mL/min; H_2 flow rate, 50 mL/min; air flow rate, 0.8 L/min; injection and detector temperatures, 285 °C; oven temperature, programmed to rise linearly at 6 °C/min from 140 °C up to 285 °C; chart speed, 0.5 cm/min; sensitivity, 10^2 ($\times 10^6$ Ω); range, 4–16 (×0.01 V).

Preparation of Calibration Curves and Calculation. Calibration curves for di- and polyamines in the range of 12.5–125 nmol were constructed by using 125 nmol of the internal standard. To a series of six reaction vials, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of the standard solution were pipetted, respectively, and to each vial was added 1.0 mL of the internal standard solution. The total reaction volume was then made up to 2 mL with water, and the mixtures were treated and analyzed in the same manner as described above. The peak height ratios were plotted against the quantities of the amines. For quantitative determination of the amines in foods, the peak height ratios of identified amines were compared to those of standard prepared daily to overcome minor fluctuations in detector response.

Identification of Di- and Polyamines. Di- and polyamines in all samples were identified by coinjecting with the standard derivatives and in some samples by gas chromatography–mass spectrometry (GC–MS). The mass spectrometer was a Shimadzu-LKB 9000 operated under the following conditions: trap current, 60 μA; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion source temperature, 290 °C; separator temperature, 285 °C. GLC analyses were carried out under the same conditions described above except with a helium flow rate of 25 mL/min. Spectra of the standard derivatives and unknowns were compared for the purpose of identification.

RESULTS AND DISCUSSION

Reaction conditions for the preparation of EOC derivatives of di- and polyamines were established by using different alkaline media for varying lengths of shaking time on the basis of the previous investigation (Yamamoto et al., 1980). As a result, shaking at room temperature for 10 min in aqueous NaOH medium was found to be most satisfactory for all of the amines investigated. It was observed that the same conditions as for tyramine in the previous paper was not efficient only for spermine. This may be due to the difference of reactivity. In parallel with the investigation of reaction conditions, several liquid phases were assessed for their capacities of separating and eluting each amine derivative on GLC columns. The derivatives showed a tendency to give peaks of poor shape when less polar phases such as SE-30 and OV-17 were used under low coating in order to elute the spermine derivative which had the lowest volatility among them. With polar liquid phases such as SP-1000 and Poly-A-101A, it was difficult to elute the spermine derivative under the tem-

Table I. Recovery Rates for Di- and Polyamines Added to Food Samples

sample	added, nmol[a]	sample vol taken, mL (wt,[b] mg)	recovery rate, % put[c]	cad	spd	spn
mushrooms						
Collybia veltipes (Enokitake)[d]	50	1 (250)	100.0	102.5	102.0	102.1
	100	1 (250)	95.9	100.0	109.2	104.0
Agaricus bisporus (Tsukuritake)	100	0.5 (125)	101.4	102.4	103.1	95.3
Lentinus edodes (Shiitake)	100	1 (250)	97.3	96.4	99.0	103.7
Pleurotus ostreatus (Hiratake)	100	0.5 (125)	102.7	100.0	105.0	103.2
soybean	100	5 (125)	92.9	96.2	101.0	96.2
soybean sprouts	50	2 (500)	102.9	97.6	102.8	96.3
bamboo shoot	50	2.5 (312.5)	102.9	100.0	106.3	97.5
	50	2.5 (312.5)	102.8	95.0	103.9	93.3
pork	50	4 (100)	98.4	97.3	99.4	101.9
average			99.7	98.7	103.2	99.4

[a] Di- and polyamines were added to the aliquot of each food extract prepared with 2% $HClO_4$ and the whole was ion-exchange chromatographed. The sample volumes taken for the determination of recovery rates are indicated in the next column. [b] Equivalent to the weight of the original food sample. [c] Abbreviations: put, putrescine; cad, cadaverine; spd, spermidine; spn, spermine. [d] Japanese name.

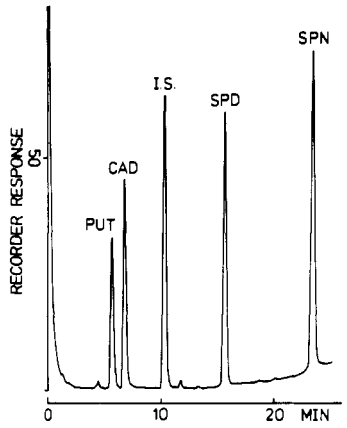

Figure 1. Standard mixture of the EOC derivatives of di- and polyamines. 100 nmol of each amine was derivatized; final volume, 100 μL; 4 μL injected. Attenuation: 8×10^2. Abbreviations: PUT, putrescine; CAD, cadaverine; SPD, spermidine; SPN, spermine; I.S., internal standard (1,8-diaminooctane; 125 nmol).

perature-programmed condition used. However, all of the peaks eluted from these polar columns were sharp and symmetrical. Finally, the best result was obtained by using a mixed phase composed with 1.5% SE-30 and 0.3% SP-1000 under the temperature-programmed condition, as shown in Figure 1. Uniport HP, which was washed with 36% HCl and resilanized with 5% DMCS in toluene before the liquid phases were applied, was used as a support. This treatment of the support was found to be more suitable for quantitative elution of the spermine derivative. One of the first observations that a chromatographic column is deteriorating was indicated by a decrease of the peak height ratio of spermine. A properly prepared column gave excellent elution for at least 2 months; however, it is recommended that the standard mixture be analyzed periodically to evaluate any change in the elution characteristics of the column.

The amine derivatives were first gas chromatographed individually to examine whether or not there was any accompanying peak and then added successively to the mixture of standard amine derivatives, thus allowing peak identification. The reaction with ethyl chloroformate produced a chromatographically single pure compound for each amine investigated, each structure of which was elucidated by GC–MS. Except for the spermine derivative, all of other derivatives had fairly prominent molecular ion peaks (M^+), and m/e agreed with the molecular weight postulated. Although the spermine derivative (molecular weight postulated = 490) did not give a molecular ion peak, a fairly prominent fragment ion peak ($M^+ - 46$) was observed at m/e 444, which was the highest mass number among the fragment ion peaks obtained from the spermine derivative. This characteristic ion peak ($M^+ - 46$) was also observed in each of the putrescine and spermidine derivatives. The GC–MS study indicated that all of the primary and secondary amino groups are substituted with EOC groups.

Having thus established suitable derivatization and gas chromatographic steps, the quantitative aspects were studied. 1,8-Diaminooctane was chosen as an internal standard because it was well separated from the amines investigated and it possesses $-NH_2$ functionality. Calibration linearity for the internal standard was observed in the range of 10–125 nmol when, with use of spermidine as an internal standard, derivatized together with the other amines, suggesting that 1,8-diaminooctane is suitable as an internal standard. The calibration curves for the amines prepared from peak height ratios relative to those of the internal standard were found to be linear in the range of 12.5–125 nmol, corresponding to 0.5–5.0 nmol of each amine in the portion injected, and the reproducibility of the calibration curves was found to be satisfactory for the quantitative determination.

The derivatives were stable, showing no signs of decomposition when kept in solution in ethyl acetate over anhydrous Na_2SO_4 in closed vials at room temperature over 3 weeks. In addition, it should be noted that this derivatization procedure does not require the absence of moisture during and after derivatization.

On the basis of these results, application of the proposed method to the determination of di- and polyamine contents in various foods was investigated. Ion-exchange chromatography was used to isolate the amines from food extracts prepared with 2% $HClO_4$. Prior to derivatization, by loading food extracts on to the Amberlite CG-120 resin column, followed by washing and eluting according to the previous paper, the interfering substances could be excluded and satisfactory gas chromatograms obtained. Typical gas chromatograms obtained from food samples are demonstrated in Figure 2. The internal standard region was almost free from interfering peaks in each food investigated. In this study, the internal standard was added to the amine fraction eluted with 6 N HCl because it did not show the same ion-exchange chromatographic behavior as the amines investigated, its recovery rates from the ion-exchange column being low under the elution

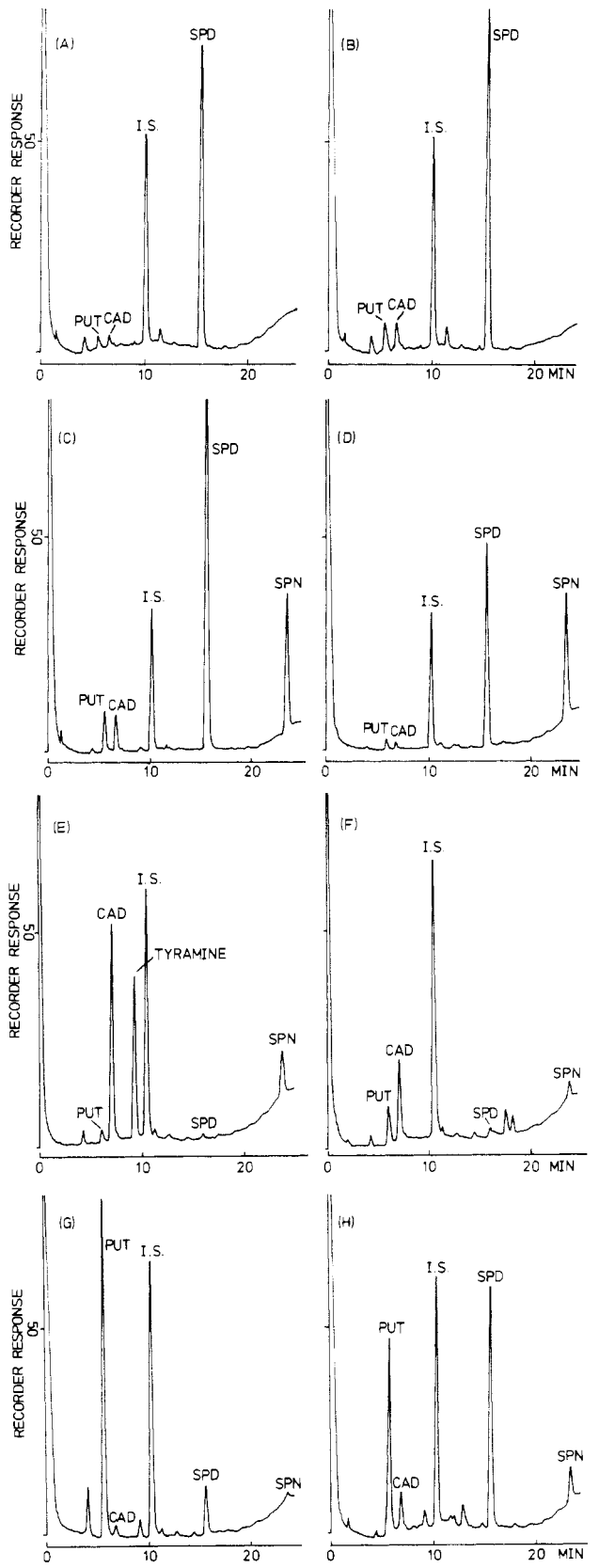

Figure 2. Typical gas chromatograms obtained from (A) *Lentinus edodes*, dried (40 mg), (B) *Collybia veltipes* (250 mg), (C) soybean (250 mg), (D) red bean (250 mg), (E) chicken ham (100 mg), (F) fish sausage (100 mg), (G) cucumber (500 mg), and (H) soybean sprouts (1 g). Abbreviations are the same as in Figure 1.

conditions used. However, it would be useful to monitor the performance of both evaporation of the effluent and derivatization and to correct for variations in the injected sample volume, solution volume, and instrumental response, thereby increasing the reliability of quantitative analysis of the amines.

Table II. Di- and Polyamine Contents of Various Foods Obtained from Commercial Sources

sample	di- and polyamines, mg/(100 g or 100 mL)[a] put[b]	cad	spd	spn
mushrooms				
Agaricus bisporus: A[c]	0.09	n.d.[d]	16.29	0.21
B	0.22	n.d.	19.22	0.30
Auricularia polytricha (Aragekikurage; dried):[e] A	1.41	n.d.	15.77	0.82
B	1.41	n.d.	15.63	0.81
Collybia veltipes: A	0.63	0.10	9.74	n.d.
B	0.59	0.52	9.33	n.d.
Pholiota nameko	2.44	0.81	8.56	0.33
Pleurotus ostreatus: A	0.21	0.06	21.34	0.10
B	1.28	0.25	17.63	0.07
Lentinus edodes: A	0.19	0.20	8.38	n.d.
B	0.14	0.15	8.00	n.d.
dried	0.38	0.09	52.24	n.d.
black soybean	2.86	0.71	17.06	5.06
mottled kidney bean	0.71	0.41	5.80	8.37
red bean: A	0.48	n.d.	8.96	7.61
B	0.65	0.26	9.49	8.79
soybean: A	2.01	1.57	15.83	7.54
B	8.43	2.35	29.51	8.08
C, germ	1.74	90.80	52.74	14.04
C, endosperm	7.81	0.63	24.02	6.16
bean: Kintokimame[e]	0.61	0.41	4.32	7.40
Daifukumame	0.61	0.51	3.98	6.23
Toramame	0.92	0.31	4.78	5.06
chicken ham	0.62	11.09	0.44	3.57
pork ham: smoke-dried	0.37	0.38	0.29	3.08
roasted	0.88	0.51	0.56	1.25
bacon: belly	0.38	0.26	0.44	2.24
picnic shoulder	0.63	0.64	0.28	1.67
fish sausage	1.61	3.57	0.44	0.95
meat sausage	1.38	3.06	0.56	2.29
pork	0.25	0.26	0.44	4.28
pork, putrefied[f]	0.91	2.32	1.14	0.80
cattle liver	0.67	n.d.	0.73	15.05
cod roe	3.00	n.d.	0.95	3.10
soybean sprouts	1.03	0.10	1.72	0.38
bamboo shoot: A	1.33	0.08	3.34	0.89
B	1.13	0.12	3.00	1.01
carrot	0.73	0.14	1.75	0.24
cucumber	4.05	0.10	0.67	0.06
Japanese radish (Daikon)	0.20	0.07	1.56	0.30
soybean sauce: A	12.97	0.49	1.69	0.40
B	6.37	0.36	1.83	0.61
alcoholic drink (Sake): A	0.16	0.05	0.07	n.d.
B	0.12	n.d.	0.07	n.d.

[a] Each value represents the average of duplicate analyses. [b] Abbreviations are the same as in Table I. [c] A, B, and C are the different samples. [d] Not detected [<0.05 mg/(100 g or 100 mL)]. [e] Japanese name. [f] Allowed to stand at 25 °C for 72 h.

For evaluation of quantitation throughout the procedure, food extracts were fortified with 50 and 100 nmol of each amine, and these were treated as described under Experimental Section. The results of these experiments showed high recovery rates for each amine, as summarized in Table I. The reproducibility throughout the procedure was checked by using five separate samples taken from 2% $HClO_4$ extracts of mushrooms, soybeans, and bacons selected as the representative foods. The results were found to be satisfactory, that is, where the relative standard deviations of the amine contents ranged from 1.1% for spermidine to 6.5% for spermine. The di- and polyamine contents in various foods determined by this method are summarized in Table II. It can readily be seen from our data that di- and polyamines are present in both animal and vegetable foods and that putrescine and spermidine

are distributed in all of the foods investigated. It is interesting to point out that mushrooms and beans contained appreciable amounts of spermidine because mutagenicity of the nitrosamines formed from spermidine has been proved as described previously. To our knowledge, this finding of the presence of large amounts of spermidine in mushrooms was not reported in the literature.

From this study, it was concluded that the accurate measurements of di- and polyamines in foods can be achieved by using GLC after derivatization. Due to simplicity and reproducibility of the derivative formation reaction, and stability of the derivatives formed, the method reported herein appears to be adequate for both qualitative and quantitative analyses of di- and polyamines in foods, although requiring ion-exchange chromatographic purification.

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Isolation and Identification of Amino Acid Derivatives from Yeast

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N-Acetylalanine and *N*-acetyltyrosine were isolated from baker's yeast. Initially the α-amino acids were quantitiatively removed from a preextract of yeast by the copper–Sephadex method. Further separation was achieved with gas chromatography, ion-exchange chromatography, electrophoresis, and paper chromatography. The isolated compounds were identified by NMR spectroscopy and mass spectrometry, respectively.

A nonprotein fraction from *Saccharomyces cerevisiae* liberating amino acids upon hydrolysis has been described (Hoehn, 1974; Ruediger, 1963). A relationship between the unpleasant taste of yeast samples and the presence of this fraction was established (Hoehn, 1974). Low molecular weight compounds, which release amino acids upon hydrolysis (e.g., peptides and amino acid derivatives), have been shown to be important for taste in foods (Erikson and Fagerson, 1976; Kirimura et al., 1969; Schiffman et al., 1975; Solms, 1969). Especially peptides contribute to bitter, sweet, and sour sensations. They can also contribute to taste-enhancing properties.

Detailed analyses of this nonprotein fraction isolated from *S. cerevisiae* have not been published. This fraction has only been isolated from *Candida utilis* (Miettinen, 1951; Ruediger, 1963; Turba and Esser, 1955) and found to contain mostly peptides, mainly sour ones. There have also been a few reports of the presence of single amino acid derivatives in microorganisms (Hall et al., 1958; Okuhara and Harada, 1971; Nakanishi, 1978; Vandecasteele et al., 1973).

The purpose of this study was to analyze the low molecular weight components from yeast (*S. cerevisiae*) extracts which liberate amino acids upon hydrolysis. The analyses were conducted on the hydrophobic fraction of the extracts because of its particular importance in the taste (Ney, 1972; Wieser and Belitz, 1975).

MATERIALS AND METHODS

Sample. The yeast sample was a *S. cerevisiae* yeast (baker's yeast) obtained from Hefefabrik Hindelbank, Switzerland.

Extraction. A total of 35 L of a 30% baker's yeast suspension was disintegrated by passing the yeast slurry 3 times through a Manton-Gaulin homogenizer (Manton Gaulin Manufacturing Co.,) Inc., Everett, MA) and freeze-dried (Hoehn, 1974).

Five-gram samples of the dry material were extracted 3 times with 90 mL of $CHCl_3$–CH_3OH (2:1 v/v) and filtered. The filtrates were pooled and extracted with 18 mL of water (Harwalkar and Elliott, 1971). After separation, the water layer was filtered through an Amicon ultrafilter (M_r cutoff 1000; Amicon Corp., Oosterhout, The Netherlands). The freeze-dried sample (93 mg) was used for further separation.

Chromatography on Copper–Sephadex. A 5-mg extract, dissolved in 1 mL of borate buffer, was chromatographed on a copper–Sephadex G-25 column as described previously (Rothenbuehler et al., 1979), giving fractions I and II. Samples were percolated over small Dowex A-1 chelating ion-exchange columns in Na^+ form at pH 11 to remove copper.

GC–MS of Derivatives. An aliquot of fraction I was directly derivatized by using the procedure of Niederwieser

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