

Determination of Ethyl Carbamate in Soy Sauce and Its Possible Precursor

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Some samples of soy sauce were examined for the presence of ethyl carbamate at a trace level. Solid-phase extraction with Celite and selected ion monitoring using a gas chromatograph-mass spectrometer were applied to analysis for ethyl carbamate in soy sauce. The method was simple and proved to be reliable for the analysis of soy sauce. Two soy sauce samples of 26 collected from Japanese and American markets had over 20 ppb of ethyl carbamate. Time course study of selected raw soy sauce samples that had been heated indicated that ethyl carbamate could be formed in the pasteurization process through ethanolysis of some precursors, as reported in wine, Japanese rice wine, or stone fruit brandy. In the case of soy sauce, citrulline would be the precursor according to examinations of a variety of experimentally fermented raw soy sauce samples. An accumulation of citrulline in raw soy sauce was concluded to be responsible for the occurrence of ethyl carbamate in soy sauce.

INTRODUCTION

Ethyl carbamate (EC) has a variety of biological activities including its ability to induce some kinds of tumors in animals (Nettleship and Henslow, 1943; Mirvish, 1968; Bateman, 1976; Field and Lang, 1988; Schlatter and Lutz, 1990). EC is known to occur naturally in various alcoholic beverages and fermented foods including soy sauce (Ough, 1976). Its Canadian limiting guidelines are 30 ppb in table wines, 100 ppb in fortified wines, 150 ppb in distilled spirits, and 400 ppb in fruit brandies and liqueurs (Conacher et al., 1987); the "no significant risk" level of California's Proposition 65 is 0.7 $\mu\text{g}/\text{day}$ (*Food Chem. News*, 1989; Kilgore, 1990). Recently, the occurrence of EC in some samples of soy sauce was reported (Canas et al., 1989; Hasegawa et al., 1990).

A reliable analysis for EC at trace level is needed because of its biological activities. For determination of EC, a number of practical gas chromatographic methods using a flame ionization detector (Kato et al., 1989), a Hall electrolytic conductivity detector (Walker et al., 1974; Dennis et al., 1986), a thermal energy analyzer with nitrogen converter (Canas et al., 1988), a mass spectrometer (Aylott et al., 1987; Gaetano and Matta, 1987; Clegg and Frank, 1988), a high-resolution mass spectrometer (Lau et al., 1987), or a tandem mass spectrometer (Cairns et al., 1987; Brumley et al., 1988) have been published. However, no simple and suitable procedures for analysis of many samples of soy sauce have been reported.

Soy sauce is a liquid seasoning made from soybeans (or defatted soybeans), wheat, and salt by a complicated fermentation (Yokotsuka, 1986). Figure 1 illustrates a typical manufacturing process. Koji mold (*Aspergillus sojae* or *A. oryzae*) is cultivated on a mixture of cooked soybeans and roasted wheat at the koji stage. Koji mold produces enzymes (proteases, amylases, etc.) that hydrolyze protein and starch in materials at the following moromi mash stage. Koji mixed with brine is called moromi mash. Yeasts (*Zygosaccharomyces rouxii*, *Candida etchellsii*, *Candida versatilis*, and so on) that produce ethanol and other flavor compounds and lactic acid bacterium (*Pediococcus halophilus*) that produces lactic acid by means of lowering of the pH are active at this same stage. Fermented and aged moromi mash is pressed within a filter cloth, and raw soy sauce is collected. It is heated at

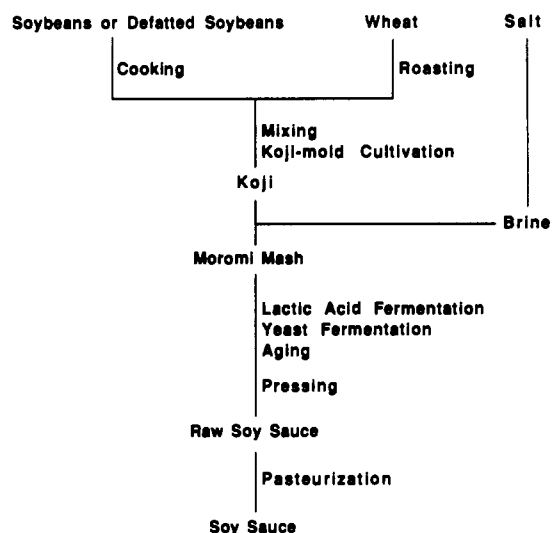


Figure 1. Manufacturing process of soy sauce.

either 70–80 or 115 °C for a short time to pasteurize the product, which completes the soy sauce process. Some stages in this manufacturing process may yield EC.

In some alcoholic beverages, EC is known to be formed by heating EC precursors with ethanol (Yoshizawa and Takahashi, 1988; Ough et al., 1988a). EC precursor is reported to be urea that is accumulated by yeast fermentation in wine (Ough et al., 1988b; Monteiro et al., 1989) and Japanese rice wine (Hara et al., 1988) but hydrocyanic acid with vicinal dicarbonyl compounds in stone fruit brandy (Baumann and Zimmerli, 1986, 1987). This paper describes research for the determination of EC and speculation on possible EC precursors in soy sauce.

MATERIALS AND METHODS

Samples. Soy sauce was collected from markets in Japan and the United States. Raw soy sauce was obtained experimentally by fermentation trials.

Reagents. Ethyl carbamate was obtained from Tokyo Kasei Kogyo Co. (Tokyo). Ethyl *N*-methylcarbamate, urea, citrulline, arginine, ethanol, sodium sulfate, Celite 545, methylene chloride, and ethyl acetate were obtained from Wako Pure Chemical Industries (Osaka). Alumina (active, neutral, for column chromatography) was obtained from E. Merck (Darmstadt). Lactic

acid was obtained from Hayashi Pure Chemical Industries (Osaka). All reagents were of analytical grade.

Extraction. The basic procedure of extraction was similar to that for alcoholic beverages described by Canas et al. (1988). Fifteen grams of sample was mixed uniformly with 15 g of Celite 545 (heated for 16 h at 700 °C and cooled) in a 200-mL beaker. The mixture was packed in a chromatographic glass column (39 mm i.d. × 400 mm) containing 10 g of deactivated alumina (adjusted to 10.0% water content by weight) capped with a 40-g layer of sodium sulfate (anhydrous); 100 mL of methylene chloride was added to the column, and the eluate was collected into a 200-mL pear-shape flask. One milliliter of ethyl acetate was added to the flask, and the eluate was concentrated to about 0.5 mL at 35 °C. The concentrate and the rinse of ethyl acetate from the flask were diluted to 1 mL with ethyl acetate in a graduated 1.5-mL vial bottle. Ten microliters of ethyl *N*-methylcarbamate solution (100 µL/mL) in ethyl acetate was added as internal standard to the vial and mixed well.

Capillary Gas Chromatography–Mass Spectrometry Analysis. Selected ion monitoring was carried out on a Hitachi M-80B double-focusing mass spectrometer equipped with a Hewlett-Packard 5890A gas chromatograph. A Supelcowax 10 bonded fused silica capillary column (0.25 mm i.d. × 60 m, film thickness 0.33 µm) interfaced directly to the mass spectrometer was used with splitless injection mode. Operating conditions were as follows: helium flow rate (as carrier gas), 1.12 mL/min; programmed oven temperature, initial hold at 40 °C for 1 min, 40–120 °C at 5 °C/min, then to 200 °C at 3 °C/min, and final hold for 50 min; injector temperature, 180 °C; purge time, 0.75 min; interface oven temperature, 220 °C; electron impact ionization mode; ionization voltage, 70 eV; ion source temperature, 140 °C; ion acceleration voltage, 3 kV; ion monitoring at *m/z* 62, 74, 75, 89. Quantitation was carried out on *m/z* 62 for EC and *m/z* 75 for internal standard (ethyl *N*-methylcarbamate). Two microliters of the sample extract was injected by a Hewlett-Packard 7673 autoinjector controlled by a Hewlett-Packard 3396A integrator.

Heating of Raw Soy Sauce. Seventeen grams of raw soy sauce in a 20-mL sealed bottle was heated at 80 °C in a dry oven. It was cooled to room temperature and analyzed.

Determination of Urea. One milliliter of raw soy sauce diluted to 5 mL with distilled water was applied to 5.5 mL of Bio-Rad AG50W-X4 cation-exchange resin (100–200 mesh, proton form) in a Bio-Rad Poly-Prep column. The column was washed with 5 mL of distilled water. The eluate from the column with the following 20 mL of distilled water was evaporated to 1 mL at 45 °C. One hundred microliters of the concentrate was analyzed using a Toyobo Dia-color UN urea analytical kit by the colorimetric determination of the resulting indophenol on 680 nm.

Determination of Ethanol. Quantitative determination of ethanol was accomplished by the gas chromatographic method of Hamano et al. (1971). The same procedure as reported by Sasaki et al. (1991) was carried out.

Determination of Citrulline, Ornithine, and Arginine. These three amino acids were determined by a Hitachi L-8500 amino acid analyzer on vital amino acids analyzing mode. Raw soy sauce was diluted 500-fold with distilled water and applied to the amino acid analyzer.

Determination of Lactic Acid. Determination of lactic acid was accomplished by an HPLC method similar to that of Yoshida et al. (1981) using postlabeled detection with bromocresol purple. A Tosoh CCPM liquid chromatographic pump, a Tosoh AS-8000 autosampler, a Tosoh UV-8010 UV-visible detector, and a Tosoh SC-8010 data processor were used. Operating conditions were as follows: column, Shodex Ionpak C-811 4.6 mm i.d. × 500 mm; mobile phase, 0.004 M HClO₄; flow rate, 1.0 mL/min; column temperature, 50 °C; postlabeled solution, 0.0002 M bromocresol purple containing 0.004 M HClO₄ and 0.025 M Na₂HPO₄; postlabeled solution flow rate, 0.5 mL/min; detection, 430 nm. Five microliters of raw soy sauce was injected to the HPLC system after filtration through a microporous filter (0.45 µm).

RESULTS AND DISCUSSION

Determination of Ethyl Carbamate. Solid-phase extraction with Celite and selected ion monitoring with a

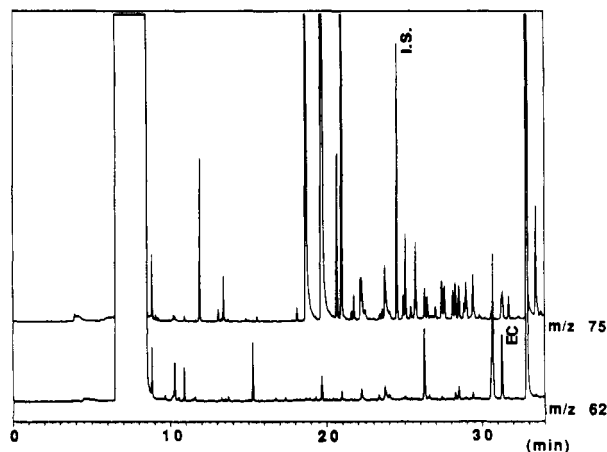


Figure 2. Mass fragmentgram for soy sauce.

Table I. Ethyl Carbamate in a Number of Soy Sauce Samples from Markets in Japan and the United States

soy sauce sample ^a	ethyl carbamate, ppb (w/w)	soy sauce sample ^a	ethyl carbamate, ppb (w/w)
A	7.3	N	1.6
B	35.2	O	0.5
C	5.2	P	1.0
D	19.3	Q	ND
E	29.9	R	3.7
F	1.4	S	2.7
G	13.7	T	3.8
H	10.2	U	6.6
I	3.0	V	ND
J	0.5	W	ND
K	ND ^b	X	5.5
L	3.1	Y	9.6
M	0.9	Z	14.8

^a Soy sauces A–T from markets in Japan; soy sauces U–Z from markets in the United States. ^b None detected.

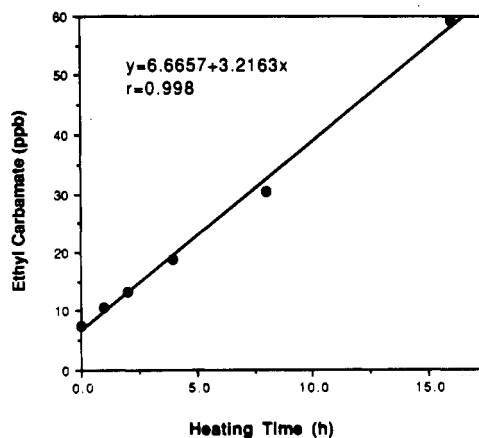


Figure 3. Ethyl carbamate formation in soy sauce during heat treatment.

mass spectrometer were employed to determine EC in soy sauce because of their simplicity and reliability. According to prior papers, selected ion monitoring was carried out on *m/z* 62, which corresponds to the intensive fragment ion generated from ethyl carbamate by electron impact ionization. Ethyl *N*-methylcarbamate selected as internal standard from nine derivatives gave an intensive fragment ion on *m/z* 75. The peak of internal standard was not overlapped on that of any components in soy sauce. Figure 2 shows a mass fragmentgram for soy sauce.

Judging from mass fragmentgrams as *S/N* > 2, the limit of detection was 7.5 ng for the injected EC amount, which corresponds to 0.5 ppb for this method. Percent recovery

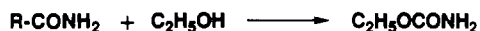


Figure 4. Formation process of ethyl carbamate.

Table II. Ethyl Carbamate Formation in Soy Sauce by a Heat Treatment with Various Additives

additive	concentration	ethyl carbamate, ^a ppb (w/w)
no addition (pH 4.79)		5.0
lactic acid	0.064% (pH 4.70)	4.7
	0.164% (pH 4.60)	4.6
	0.322% (pH 4.50)	4.7
	0.510% (pH 4.40)	4.8
ethanol	1.0%	4.6
	2.0%	6.2
	4.0%	11.4
arginine	0.1%	5.7
	0.2%	4.4
	0.4%	4.1
citrulline	50 ppm	5.5
	100 ppm	5.5
	200 ppm	7.1
	400 ppm	10.6
	800 ppm	18.0
	0.1%	24.5
	0.2%	47.4
	0.3%	69.4
0.4%	92.0	
urea	12.5 ppm	5.9
	25 ppm	7.7
	50 ppm	9.9
	100 ppm	15.5
	200 ppm	23.6

^a Determined after heating at 80 °C for 16 h.

was 93.5% on spiked tests ($n = 5$) of addition of 30 ppb of EC to soy sauce including 7.6 ppb of EC. The mean concentration was 17.7 ppb with a coefficient of variation of 2.0% on repeat analysis of soy sauce ($n = 10$).

Twenty-six soy sauce samples from Japanese and American markets were analyzed according to this method. Table I shows the EC levels. EC levels of two soy sauce samples from the Japanese market were higher than 20 ppb, as Canas et al. (1989) and Hasegawa et al. (1990) had reported. On the other hand, no soy sauce samples from the American market showed over 20 ppb of EC.

Formation of Ethyl Carbamate. After raw soy sauce in bottles had been heated at 80 °C for 0–16 h, the soy sauces were analyzed for EC. Figure 3 shows the relationship between heating time and EC levels. EC concentration was recognized to be directly proportional ($r = 0.998$) to heating time in Figure 3. This result indicated that EC is formed chemically in soy sauce from some EC precursors by heating (pasteurization). The raw soy sauce was known to contain 2.00% ethanol. Thus, the EC-forming reaction was presumed to be a sort of ethanolysis of a certain EC precursor in raw soy sauce the same as in wine, Japanese rice wine, and stone fruit brandy (shown in Figure 4).

The EC precursor in raw soy sauce was expected to be urea or other related metabolites derived from the yeast and lactic acid fermentation. Five substances possibly related to formation of EC were added to raw soy sauce. The mixtures were then heated at 80 °C for 16 h, much harsher than the usual pasteurization, and EC was determined (see Table II). Lactic acid and arginine had no effect on the formation of EC. However, ethanol, citrulline, or urea in soy sauce increased EC in proportion to their added amounts. This is explained in Figure 4. The higher concentration of the material induced more EC, but the reaction rate of formation of EC was very low, about 10^{-4} – 10^{-6} yields.

To search for an EC precursor, various raw soy sauce samples were collected from experimental fermentation trials and analyzed for their ethanol, urea, citrulline, ornithine, and arginine contents as well as for EC formed after heating at 80 °C for 16 h. Amounts of ethanol, salt concentrations, or total nitrogen values of collected raw soy sauce samples were somewhat different from each other. Table III shows the results of analysis of 20 samples of raw soy sauce. Urea in any sample of raw soy sauce having the ability to generate a higher level of EC was not beyond 4 ppm. Addition of 12.5 ppm of urea was effected only slight formation of EC as shown in Table II; it was concluded that urea did not cause a significant amount of EC. Furthermore, urea in soy sauce from markets was recognized to range from 3.5 to 7.8 ppm. However, a higher level of citrulline was observed in the raw soy sauce, which gave a high detectable level of EC after heat treatment. As the amount of EC formed also depends on the amount of ethanol, the relationship between EC after heating

Table III. Ethyl Carbamate Forming Ability and Related Components of Raw Soy Sauce Obtained by Various Fermentation Trials

raw soy sauce	components in raw soy sauce					ethyl carbamate after heating, ^a ppb (w/w)
	urea, ppm (w/v)	ethanol, % (w/v)	citrulline, mM	ornithine, mM	arginine, mM	
A	2.21	2.52	14.1	5.9	14.1	42.3
B	1.93	2.80	19.7	9.5	5.7	65.8
C	2.65	2.69	18.4	6.9	9.5	52.7
D	2.26	2.81	16.7	4.9	12.7	44.8
E	2.54	3.15	21.4	8.4	3.8	62.0
F	3.39	3.22	19.3	8.4	6.3	77.1
G	— ^b	1.37	21.5	9.5	14.6	23.3
H	—	2.15	20.3	10.8	4.3	33.3
I	3.58	2.09	13.3	6.9	9.0	21.5
J	2.58	2.07	13.2	6.9	9.0	34.1
K	—	2.73	0.0	3.7	33.6	14.9
L	—	2.49	7.1	3.8	22.5	38.9
M	—	2.35	0.0	0.7	35.4	2.3
N	—	2.40	0.6	4.7	31.4	6.4
O	—	2.49	0.0	0.6	35.9	2.9
P	—	2.82	0.4	0.6	35.9	4.7
Q	—	2.52	0.0	2.9	34.7	3.8
R	—	2.40	0.2	5.6	26.7	9.8
S	—	2.35	0.0	4.1	30.2	3.4
T	—	2.48	0.0	2.2	34.5	4.9

^a Determined after heating at 80 °C for 16 h. ^b Not analyzed.

- Cunin, R.; Glansdorff, N.; Pierard, A.; Stalon, V. Biosynthesis and metabolism of arginine in bacteria. *Microbiol. Rev.* 1986, 50, 314-352.
- Dennis, M. J.; Howarth, N.; Massey, R. C.; Parker, I.; Scotter, M.; Startin, J. R. Method for the analysis of ethyl carbamate in alcoholic beverages by capillary gas chromatography. *J. Chromatogr.* 1986, 369, 193-198.
- Field, K. J.; Lang, C. M. Hazards of urethane (ethyl carbamate): a review of the literature. *Lab. Anim.* 1988, 22, 255-262.
- Food Chem. News.* California proposes regulatory levels for 9 Prop. 65 chemicals. 1989, Dec 11, 65-66.
- Gaetano, G.; Matta, M. The dose of ethyl carbamate in wine and spirits by gas-chromatography/mass spectrometry. *Bull. O.I.V.* 1987, 671-672, 36-42.
- Hamano, M.; Okuhara, A.; Aoyama, Y.; Saito, N. Quantitative determination of acetic acid and ethylalcohol in soy sauce with gas liquid chromatography. *Chomi Kagaku* 1971, 18, 72-78.
- Hara, S.; Yosizawa, K.; Nakamura, K. Formation of ethylcarbamate in the model alcoholic beverages containing urea or its related compounds. *J. Brew. Soc. Jpn.* 1988, 83, 57-63.
- Hasegawa, Y.; Nakamura, Y.; Tonogai, Y.; Terasawa, S.; Ito, Y.; Uchiyama, M. Determination of ethyl carbamate in various fermented foods by selected ion monitoring. *J. Food Prot.* 1990, 53, 1058-1061.
- Kato, I.; Shimoi, H.; Tadenuma, M.; Hara, S.; Yoshizawa, K.; Tamura, G. Simple method for the determination of ethyl carbamate in alcoholic beverages. *J. Brew. Soc. Jpn.* 1989, 84, 349-353.
- Kilgore, W. W. California's proposition 65: extrapolating animal toxicity to human. *Am. J. Ind. Med.* 1990, 18, 491-494.
- Lau, B. P.-Y.; Weber, D.; Page, B. D. Gas chromatographic-mass spectrometric determination of ethyl carbamate in alcoholic beverages. *J. Chromatogr.* 1987, 402, 233-241.
- Mirvish, S. S. The carcinogenic action and metabolism of urethan and *N*-hydroxyurethan. In *Advances in Cancer Research*; Haddow, A., Weeinhouse, S., Eds.; Academic Press: New York, 1968; Vol. 11, pp 1-42.
- Monteiro, F. F.; Trousdale, E. K.; Bisson, L. F. Ethyl carbamate formation in wine: use of radioactivity labeled precursors to demonstrate the involvement of urea. *Am. J. Enol. Vitic.* 1989, 40, 1-8.
- Nettleship, A.; Henslow, P. S. Induction of pulmonary tumors in mice with ethyl carbamate (urethane). *J. Natl. Cancer Inst.* 1943, 4, 309-319.
- Ough, C. S. Ethyl carbamate in fermented beverages and foods. *J. Agric. Food Chem.* 1976, 24, 323-328.
- Ough, C. S.; Crowell, E. A.; Gutlove, B. R. Carbamyl compound reaction with ethanol. *Am. J. Enol. Vitic.* 1988a, 39, 239-242.
- Ough, C. S.; Crowell, E. A.; Mooney, L. A. Formation of ethyl carbamate precursors during grape juice (chardonnay) fermentation. I. Addition of amino acids, urea and ammonia: effects of fortification on intracellular and extracellular precursors. *Am. J. Enol. Vitic.* 1988b, 39, 243-249.
- Sasaki, M.; Nunomura, N.; Matsudo, T. Biosynthesis of 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone by yeasts. *J. Agric. Food Chem.* 1991, 39, 934-938.
- Schlatter, J.; Lutz, W. K. The carcinogenic potential of ethyl carbamate (urethane): risk assessment at human dietary exposure levels. *Food Chem. Toxicol.* 1990, 28, 205-211.
- Walker, G.; Winterlin, W.; Fouda, H.; Seiber, J. Gas chromatographic analysis of urethan (ethyl carbamate) in wine. *J. Agric. Food Chem.* 1974, 22, 944-947.
- Yokotsuka, T. Soy sauce biochemistry. In *Advances in Food Research*; Chichesster, C. O., Mark, E. M., Schweigert, B. S., Eds.; Academic Press: New York, 1968; Vol. 30, pp 195-329.
- Yoshida, S.; Ogura, M.; Yoshino, H. Determination of organic acids in soy sauce by high pressure liquid chromatography. (part 3) New detection system using pH indicator for organic acids in soy sauce. *J. Jpn. Soy Sauce Res. Inst.* 1981, 7, 225-229.
- Yoshizawa, K.; Takahashi, K. Effect of temperature and sake components on the production of ethylcarbamate. *J. Brew. Soc. Jpn.* 1988, 83, 69-73.

Registry No. Supplied by Author: Ethyl carbamate (urethane), 51-79-6; ethyl *N*-methylcarbamate, 105-40-8; citrulline, 372-75-8; urea, 57-13-6; arginine, 74-79-3; ornithine, 70-26-8; ethanol (ethyl alcohol), 64-17-5; lactic acid (D,L-lactic acid), 598-82-3.

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