Survey of Ethyl Carbamate in Fermented Foods Sold in the United Kingdom in 2004

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Results are presented of a survey of fermented foods and beverages sold in the United Kingdom for levels of ethyl carbamate (urethane) carried out to expand the range of food types sold in the United Kingdom for which data regarding ethyl carbamate are available. Samples were analyzed by in-house validated methods, which included measurement uncertainty estimates. The samples comprised 75 fermented liquids (beers, wines, fortified wines, spirits, liqueurs, soy sauces, and vinegars) and 25 fermented solid foods (cheeses, yogurts, soybean products, sauerkraut, yeast extract, olives, and Christmas pudding). Ethyl carbamate was not detected in the beers or the cider. Wines contained between 11 and 24 μg/kg and sake between 81 and 164 μg/kg. Fortified wines contained ethyl carbamate at levels between 14 and 60 μg/kg. Only two of five liqueurs contained ethyl carbamate. Most soy sauces and vinegars did not contain ethyl carbamate. No ethyl carbamate was detected in cheeses, yogurts, olives, or soybean-based products. Single samples of sauerkraut, yeast extract, and Christmas pudding contained low levels (29, 41, and 20 μg/kg ethyl carbamate, respectively).

KEYWORDS: Ethyl carbamate; urethane; fermented foods

INTRODUCTION

Ethyl carbamate is a known carcinogen that has been found mainly as a byproduct of fermentation, through which it is formed from various precursors, notably urea and citrulline (*1, 2*), in a reaction promoted by heat. Other sources have been identified, such as cyanide in the case of stone fruit spirits (*3*). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recently reviewed ethyl carbamate and concluded that the intake of ethyl carbamate from foods excluding alcoholic beverages would be of low concern but that "the margin of exposure for all intakes, food and alcoholic beverages combined, was of concern and therefore mitigation measures to reduce concentrations of ethyl carbamate in some alcoholic beverages should be continued" (*4*). Canada introduced guidelines and tolerance levels in alcoholic beverages in 1985, which set acceptable limits of 30 μg/L in table wines, 100 μg/L in fortified wines, 150 μg/L in distilled spirits, 200 μg/L in sake, and 400 μg/L in fruit brandies and liqueurs (*5*).

Contamination of beverages and other foods by ethyl carbamate has been reviewed (*6–9*). Most wines and stone fruit spirit drinks analyzed contained ethyl carbamate. Results for a major survey of domestic and imported beverages in Canada (*6*) showed 74 wines to contain up to 84 μg/kg and 155 stone fruit brandies up to 4330 μg/kg, the highest levels in spirits

derived from cherry, plum, and apricot. Two surveys in Korea showed that beers there contained from 0.3 to 0.8 μg/kg ethyl carbamate (*8, 9*). Wines (three samples) contained 1.7–11.7 μg/kg, whiskies (five samples) 13.9–30 μg/kg, and fruit brandies up to 690 μg/kg with an average of 197 μg/kg (*9*). Korean wines (takju) contained low (about 0.6 μg/kg) levels of ethyl carbamate, and spirits made from diluted ethanol (soju) generally contained lower levels (0.8–15.4 μg/kg) than distilled spirits (*9*).

Ethyl carbamate can be determined in distilled spirit drinks by gas chromatography with direct injection onto a polar wax column and detection by selected ion monitoring mass spectrometry (SIM). For quantification most procedures include an internal standard of propyl carbamate or butyl carbamate, although for mass spectrometric detection ethyl carbamate labeled with deuterium and/or ^{15}N is clearly preferable.

This survey was conducted to expand the range of food types sold in the United Kingdom for which data regarding ethyl carbamate are available. Established methods were used after validation using in-house reference materials, which were also employed as quality assurance samples.

For the liquid samples in this survey we used the official first action method of the Association of Official Analytical Chemists (*10*), after further in-house characterization to establish measurement uncertainty. For the solid samples we carried out a limited in-house validation of a previously published method

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for fermented foods (*11*), including characterization of the method to provide measurement uncertainty estimates.

EXPERIMENTAL METHODS

Materials. Dichloromethane and anhydrous sodium sulfate (Analar) were obtained from Fisher Chemicals, Loughborough, U.K. Ethyl carbamate 99% was obtained from Aldrich Chemicals, Gillingham, U.K., and d_5-ethyl carbamate (99.8 atom %) from C/D/N isotopes via Qm$_x$ Laboratories Limited, Thaxted, U.K. Hydromatrix was obtained from Varian U.K. Inc., Oxford, U.K. Florisil Sep-Paks were obtained from Waters (Elstree, U.K.).

GC-MS was carried out using a ThermoFinnigan Voyager instrument. The column was of 30 m × 0.25 mm i.d. fused silica, with a 0.2 μm J&W DBWax film. The carrier gas was helium with a flow rate of 1.0 mL/min at 50 °C. The split/splitless injector port and mass spectrometer interface line were heated to 200 and 250 °C, respectively. The oven temperature was programmed from 50 °C, held for 1 min, and then to 200 °C at 7.5 °C/min. Splitless injections were made with a splitless time of 1 min.

The mass spectrometer was operated in electron impact ionization mode with a source temperature of 200 °C and an emission current of 150 μA. Ethyl carbamate was detected by SIM of the major ions at m/z 62 and 74. The internal standard was detected by monitoring the equivalent ion at m/z 64.

Ethyl carbamate was quantified using calibration curves constructed from the ethyl carbamate/internal standard peak area ratios (m/z 62 vs 64) for standards containing 0.1, 0.2, 0.4, 0.8, and 1.6 μg/mL ethyl carbamate. The identity of the ethyl carbamate peak was confirmed by comparing the area ratios (m/z 62 vs 74) with those of the 10 μg/kg standards. No additional ions were of sufficient intensity to be used for confirmation purposes.

Samples. Samples were purchased according to a scheme devised by the Food Standards Agency to reflect the market share and cover a wide range of fermented foods and brands typically sold in the United Kingdom. One hundred samples were purchased at retail outlets from four major cities in the United Kingdom. The beverage samples (75) comprised beer and ale (including lager and cider), dark spirits and liqueurs (brandy and rum), fortified wines (Madeira, port, and sherry), soy sauces (focused on fermented sauces), vinegars, and wine. The solid samples (25) comprised cheeses; yogurts; soybean pastes, sauces, and curd; sauerkraut; yeast extract; olives; and Christmas pudding. The cheeses comprised single samples of Cheddar, Emmental, feta, Blue Stilton, mozzarella, goat's, soft, and semisoft. The yogurt samples were natural and fruit types (two of each). Soybean-based samples comprised black bean sauces (two), natto (one), soybean pastes (two), and tofu (two). Miscellaneous foods were olives (three samples), and single samples of Christmas pudding, sauerkraut in white wine, and yeast extract.

Samples were homogenized by blending, divided into subsamples, and kept at 4 °C prior to analysis, which was carried out within 24 h of opening the packet and within the use-by date. The subsamples were stored at −20 °C.

In-House Reference Materials. To carry out further characterization of the methods and provide measurement uncertainty estimates and also as in-house reference materials, composite samples for various matrices were prepared from samples purchased at local retail outlets. For liquid samples the composites comprised three bottles each of gin, sherry, soy sauce, wine, cider vinegar, neutralized cider vinegar, and wine. The samples were analyzed for ethyl carbamate content and portions spiked to provide method performance data.

To test the solids method, composite reference material mixtures were (1) a yogurt material prepared by mixing full-fat yogurt, various low-fat added-fruit yogurts, and added-fruit fromage frais; (2) a cheese material prepared by mixing Lancashire, Stilton, and Cheshire cheeses; (3) a pickled vegetable material prepared by mixing olives (black and green) and sauerkraut; (4) a cake material prepared by mixing three different types of fruit cake; and (5) a sauce material prepared by mixing three different types of black bean sauce, one yellow bean sauce, and one yeast extract.

Analysis of the method testing samples showed that, prior to spiking, ethyl carbamate was detected in some samples at just below the reporting limit, that is, the pickled vegetables at about 3 μg/kg, the soy sauce at about 7 μg/kg, and the sauce sample at about 9 μg/kg.

Sufficient quantities of each composite matrix sample were prepared for use as reference materials during analysis of the survey samples.

Procedure. For liquids analysis the AOAC first action method was revised in two ways. First, deuterium-labeled ethyl carbamate was used as the internal standard in preference to *n*-propyl carbamate, because it would provide more precise recovery correction. Second, the gas chromatographic conditions in the published method were revised to reduce the run time, except for the sherry samples for which an interfering compound necessitated the use of the slower published program.

For analysis of solid foods 25 g of the sample was homogenized for 3 min in a blender with 25 mL of distilled water and 40 μL of a 10 μg/mL solution of d_5-ethyl carbamate internal standard. The homogenate was mixed with 25 g of Hydromatrix solid-phase extraction material and packed into a chromatography column (40 × 2 cm) fitted with a glass frit and tap and containing 40 g of sodium sulfate. For cheeses the samples were homogenized with distilled water and internal standard as above, and the mixture was centrifuged at 3500 rpm for 15 min. Thirty milliliters of the supernatant was taken and mixed with 25 g of Hydromatrix.

Nonpolar compounds were eluted with 50 mL of hexane with an unrestricted flow. Ethyl carbamate was eluted with 150 mL of dichloromethane into a 250 mL round-bottom flask and the solvent evaporated to 5 mL, using a rotary evaporator at 30 °C. Lipid material was removed by a Florisil Sep-Pak, using the following regimen. The Sep-Pak was washed with 5 mL of 7% methanol in dichloromethane and then rinsed with 5 mL of dichloromethane. The concentrated sample extract was passed through the Sep-Pak and the round-bottom flask rinsed with 5 mL of dichloromethane. The dichloromethane was added to the Sep-Pak, and the washings were discarded. Five milliliters of 7% methanol in dichloromethane was passed through the Sep-Pak and the eluate collected in a tapered centrifuge tube. The eluate was concentrated to about 0.5 mL under a gentle stream of nitrogen and analyzed by GC-MS.

Method Validation. The methods were tested in accordance with the IUPAC Harmonized Guidelines for single-laboratory validation of methods of analysis (*12*) by the analysis of five batches for each matrix on separate days. Each batch contained one sample of each matrix spiked close to the anticipated limit of detection and one spiked at a midrange. Vinegar, which was not included in the scope of the AOAC method, was analyzed both with and without prior neutralization with dilute sodium hydroxide. Each batch also contained a sample of the AOAC method "practice sample", which is a 40% ethanol solution containing 0.1 μg/kg ethyl carbamate.

Although this study was carried out in a single laboratory, the results indicate that the limit of detection of the method for liquids has been reduced since the earlier trial. The AOAC method does not indicate limits of detection other than as ranges, that is, distilled spirits, 50−250 μg/kg; fortified wines, 40−160 μg/kg; table wines, 10−50 μg/kg; and soy sauce, 15−70 μg/kg.

For both of the methods described here a response for ethyl carbamate could be detected at m/z 62 at levels of about 1 μg/kg for all matrices studied. The peak identity could be confirmed from comparison of the m/z 62:74 ratios with those of standards at a level of 10 μg/kg for all matrices. On this basis a reporting limit of 10 μg/kg was applied.

Survey. All samples were analyzed by their best-before date. The samples were analyzed in batches, which included a reagent blank and an in-house reference material for each matrix type in that batch. Quantification was based on a calibration series of ethyl carbamate standard solutions, equivalent to a range of concentrations up to 1.6 mg/kg ethyl carbamate, measured with each batch. Quality assurance criteria were that the correlation coefficients of all calibration standard solutions should be ≥0.99, all reagent blank samples should have a response equivalent to <0.004 mg/kg, all determinations of ethyl carbamate in the reference materials should fall within two standard deviations of the characterized value, the ethyl carbamate peak area

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ratios of all samples should fall within the range of the calibration, and the retention times of the ethyl carbamate peaks of the samples should fall within ±0.2 min of those of the standards.

RESULTS

Method Testing. The in-house validation showed average recoveries for ethyl carbamate to be typically in excess of 80% with the exception of vinegar, which was lower (72−82%) regardless of neutralization. This may be due to instability of ethyl carbamate in acidic conditions, that is, decomposition during the period between spiking and analysis, the spiking having preceded the neutralization by several hours. Neutralization made little difference to the quantitative results, but it resulted in improved chromatographic peak shape. Recoveries were higher for the low-level (10−20 μg/kg) spikes, which ranged from 80 to 117% (average = 94%), than for the midrange-level (50−100 μg/kg) spikes, which ranged from 66 to 91% (average = 79%), probably because ethyl carbamate was present in the unspiked matrix at levels that were just lower than the limits of detection.

Ethyl carbamate was found to be present in the sauce reference sample at about 9 μg/kg prior to spiking and in the pickled vegetables at 3 ug/kg. The other reference samples did not contain detectable ethyl carbamate. An interfering compound with a retention time coincident with ethyl carbamate was detected in the chromatograms of the sauce reference material. The sample was analyzed using full-scan mass spectrometry, and the spectrum of the interfering compound was found to match library spectra for thiophenes. Thiophenes are reported to be present in garlic (*13*), which was an ingredient in the sauces used to make the test material. An additional strong cation exchange column step was introduced to the method, which successfully removed the compound.

In all sample matrices ethyl carbamate could be detected at 1 μg/kg, and its identity could be confirmed at a level of 10 μg/kg using the criterion that the ratio of the responses at *m/z* 62 and 74 were within 10% of those of calibration standards. Measurement uncertainty was calculated for both liquid and solid methods in accordance with Eurachem guidelines (*14*) for each matrix from the validation data. The performance of the methods and the estimated measurement uncertainty are shown in **Table 1**. The measurement uncertainty figures were updated following the survey by incorporation of additional data obtained from the analysis of the in-house reference materials, the spikes, and the duplicate analyses included in the analytical batches. The updated figures are included in the results tables.

Survey. The quality assurance criteria were met for all analytical batches. Results for the liquid survey samples are summarized in **Table 2**. Ethyl carbamate was not detected in any of the nine beers, including two lager types, or in the single apple cider sample. These results are in close agreement with those published elsewhere (*15*). The wines comprised six red wines, six white wines, one rosé, and two sakes. Seven of the European wines contained ethyl carbamate, at levels between 11 and 24 μg/kg, with no apparent difference between red and white types. These results generally agree with those published elsewhere (*11*, *16*, *17*). The two samples of sake contained relatively high levels of ethyl carbamate at 81 and 164 μg/kg. These results agree with levels (127−180 μg/L) reported from brewing experiments (*18*) and with those reported for sake obtained from retail sources (*19*).

The fortified wines all contained ethyl carbamate, at levels between 14 and 60 μg/kg (mean = 32 μg/kg), in agreement with previous surveys (*11*, *16*, *20*). There was no apparent difference between ports, sherries, and other types.

Table 1. Method Performance Data for Reference Materials Analyzed in Five Batches

reference material	spike level (μg/kg)	av	SD	RSD%	av recovery	measurement uncertainty (μg/kg)
AOAC practice sample	100	89	2.75	3.1	89	
gin	100	81	1.65	2.0	81	8
	10	11	1.75	16.2	108	4
sherry	75	63	0.61	1.0	84	6
	10	11	1.58	14.2	111	3
soy sauce[a]	57	48	0.74	1.5	84	4
	12	10	0.56	5.7	82	1
cider vinegar	50	36	0.80	2.2	72	
	20	16	0.92	5.6	82	
cider vinegar (neutralized)	50	36	0.44	1.2	72	4
	20	16	0.37	2.2	82	2
wine	50	42	1.62	3.9	84	4
	10	12	0.69	5.9	117	2
yogurt	50	35	1.52	4.3	70	9
	10	10	0.45	4.7	96	2
cheese	50	33	3.44	10.5	66	15
	10	10	3.33	32.0	104	7
vegetables[b]	53	41	8.56	20.9	82	23
	13	11	2.70	25.1	81	7
cake	50	40	0.87	2.2	80	8
	10	9	0.62	6.7	93	2
sauce[c]	59	55	9.19	16.8	91	11
	19	17	0.90	5.2	80	5

[a] Soy sauce contained 7 μg/kg ethyl carbamate before spiking. [b] Pickled vegetables contained 3 μg/kg ethyl carbamate before spiking. [c] Sauce contained 9 μg/kg ethyl carbamate before spiking.

Table 2. Ethyl Carbamate (Micrograms per Kilogram) with Measurement Uncertainty (MU) in Fermented Liquid Samples

category	no.	no. positive	EC range (positive samples)	MU range
beer (nine samples)	9	0	−[a]	
cider	1	0	−	
wine, red	6	4	11–18	2–3
wine, rose	1	0	−	
wine, white	6	3	11–24	2–4
wine, sake	2	2	81–164	13–26
fortified wine, port	5	5	14–41	4–6
fortified wine, sherry	5	5	18–37	4–5
fortified wine, Madeira	2	2	34–44	5–6
fortified wine, other	3	3	25–60	4–8
spirits, brandy	2	2	16–41	4–5
spirits, cherry brandy	3	3	34–6131	4–502
spirits, apple brandy	1	1	22	4
spirits, apricot brandy	1	1	12	4
spirits, plum brandy	1	0	−	
spirits, rum	2	2	16–72	4–7
liqueurs	5	1	170	14
soy sauces	10	1	10	3
vinegars	10	1	33	10

[a] −, <10 μg/kg.

Levels in the spirits varied considerably. Stone fruit brandies did not consistently contain more ethyl carbamate than rum. A single cherry brandy sample contained >6000 μg/kg, but another stone fruit spirit, made from plums, did not contain detectable ethyl carbamate. Levels in the liqueurs were low; however, a single U.K.-produced liqueur made from damsons contained 171 μg/kg.

In the nonalcoholic liquid samples ethyl carbamate was detected, at a very low level, in only a single sample from 10 soy sauces analyzed. This suggests that levels in this product

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Table 3. Ethyl Carbamate in Solid Food Samples

sample	no.	no. positive	EC	MU
cheeses	8	0		
yogurts	4	0		
yogurt, natural	2	0		
black bean sauce	2	0		
natto	1	0		
soybean paste	2	0		
tofu	2	0		
olives	3	0		
Christmas pudding	1	1	20	3
sauerkraut in white wine	1	1	29	13
yeast extract	1	1	41	9

may have been reduced since earlier surveys (*2, 19, 21–24*), which revealed a range of contamination up to about 70 μg/kg. Only a single sample of vinegar, produced from sherry, contained ethyl carbamate, at 33 μg/kg. Levels considerably above this, up to about 250 μg/kg, have been reported in some Asian vinegars (*25*).

Results for the solid samples are summarized in **Table 3**. Ethyl carbamate was not detected in any of eight cheeses. This was in good agreement with two previous studies in which it was reported to be present at 5–6 μg/kg in 2 of 19 cheeses, with the remainder containing <5 μg/kg (*11*), and absent from 16 cheeses of various types (*15*).

Ethyl carbamate was not detected in any of the four yogurts. It has previously been reported to be not detectable at a limit of 0.1 μg/kg in 12 yogurts of various varieties and at 0.1 and 0.4 μg/kg in 2 samples of plain yogurt (*22*). Others have found up to 2 μg/kg in 2 of 12 yogurts (*15*) and at <1 μg/kg in 9 yogurts (*11*) and in 3 of 4 yogurts at levels up to 10 μg/kg (*19*).

Ethyl carbamate was not detected in any of the seven soybean-based products. Contamination of soybean-based products has been reported previously, for example, in 6 of 14 soybean pastes (miso) at levels up to 5 μg/kg and in 3 of 6 natto samples at up to 5 μg/kg (*19*). Ethyl carbamate was found at 44 μg/kg in 1 of 9 fermented black soybean products sold in Taiwan, in 3 of 6 miso samples at 14–50 μg/kg, and at higher levels (50–148 μg/kg) in 6 of 7 samples of sufu, a cheese-like fermented soybean food product, but not in 12 soybean pastes (*25*). Seven samples of soybean paste contained up to 7.9 μg/kg, and pickled vegetables (kimchi) contained up to 16 μg/kg with a mean of 3.5 μg/kg (*21*).

No ethyl carbamate was detected in any of the three samples of olives; a single previous report described about 1 μg/kg in a single sample of olives (*26*).

Of the other foods, sauerkraut in white wine contained 29 μg/kg, yeast extract contained 41 μg/kg ethyl carbamate, and Christmas pudding (containing alcoholic spirit) contained 20 μg/kg. There are few published reports of ethyl carbamate in these foods. It has been found, at 8 μg/kg, in only 1 of 23 pickled vegetable samples (*25*) and at <0.5 μg/kg in 1 sample of sauerkraut (*27*).

DISCUSSION

Revisions to the AOAC method and improvements in instrument sensitivity have improved the limit to which ethyl carbamate can be confirmed. Limits of detection were not calculated for the AOAC procedure; however, reported ranges of ethyl carbamate for the matrices studied were 50–350 μg/kg in distilled spirits, 40–160 μg/kg in fortified wines, 10–50 μg/kg in table wines, and 15–70 μg/kg in soy sauce. Although

the current work was carried out in a single laboratory, the results indicate that the limit of detection of the method has been reduced to about 1 μg/kg for all matrices, with the peak identity being confirmed from comparison of *m/z* 62:74 ratios with those of standards at a level of 10 μg/kg, for all matrices.

The results show that ethyl carbamate continues to be present at low levels in wines and erratically and sometimes at higher levels in distilled spirits. There is potential for the reduction of these levels through changes to the production methods. Most notably, ethyl carbamate can be reduced in fermented products by the genetic modification of yeasts to produce forms that do not produce ethyl carbamate precursors such as urea while still being able to ferment sugars to alcohol; for example, sake made using such yeast does not contain ethyl carbamate (*18*). Ethyl carbamate formed in the manufacture of sherry during the prolonged "baking" periods (*28, 29*) might be reduced by adjusting the baking conditions.

The results highlight some foods and beverages in which the contribution to the intake of ethyl carbamate can be reduced or might be studied in further detail.

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