Zeitschrift: Mitteilungen aus Lebensmitteluntersuchungen und Hygiene = Travaux de chimie alimentaire et d'hygiène

Herausgeber: Bundesamt für Gesundheit

Band: 92 (2001)

Heft: 3

Artikel: Validation of a simple method for the determination of ethyl carbamate in stone fruit brandies by GC-MS

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DOI: https://doi.org/10.5169/seals-981912

Validation of a Simple Method for the Determination of Ethyl Carbamate in Stone Fruit Brandies by GC-MS

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Received 20 March 2001, accepted 20 April 2001

Introduction

Ethyl carbamate (EC) has been found to be carcinogenic in animal studies, see (1) and references therein. EC occurs in distilled spirits and wine and at lower levels in a number of other fermented foods and vegetables (2, 3). The highest levels have, however, been found in certain stone fruit brandies and this prompted the Liquor Control Board in Canada to establish regulatory levels for EC in alcoholic beverages in 1985 (400 μg/l in stone fruit brandies, 150 μg/l in spirits and 30 μg/l in wines). Since then, the US Food and Drug Administration and the US Bureau of Alcohol, Tobacco and Firearms have established voluntary programmes within the US to lower the levels of EC to the following production targets: 125 μg/l for whisky produced after 1st January 1989 and to 60 and 15 μg/l respectively for dessert and table wines after the 1988 harvest (4).

In wines and other fermented foods and vegetables, the apparent pathway to formation of EC is the degradation of amino acids; for a review of possible mechanisms, see (1) and references therein. The generally higher levels to be found in stone fruit brandies are explained by the presence of cyanide (normally in the bound form of the naturally occurring compound amygdalin, mandelic acid nitrile β-gentiobioside) in stone fruit kernels. A certain amount of the cyanide released during mashing and fermentation is normally found after distillation in the brandies. It is now rather clear that EC present in the mash and brandies is formed to its greatest extent from cyanide in the presence of ethanol. This formation involves complex and still poorly characterised reactions, in which copper clearly plays an important role (5), as well

as certain reactive or convertible intermediates (1). It seems also clear that distilled stone fruit brandies exposed to the action of light or heat, may eventually later form additional variable quantities of EC, in an equally complex way.

Several GC methods for the determination of EC have been developed over the years, including detection by flame ionisation detector, FID, nitrogen/phosphorus detector, NPD as well as mass specific detection, MSD; see table 1 in (1), as well as (6, 7). Common to many of these methods is that they employ some form of extraction as well as a concentration step prior to determination. We reasoned that the levels of EC usually present in stone fruit brandies should permit direct determination by modern GC-MS, without the need for prior extraction and concentration steps. We therefore decided to attempt to adapt the internationally collaboratively tested reference method (6) as a direct injection method. In our adaptation, the determination of EC takes place by direct injection of samples which have been adjusted to 40 volume % alcohol by dilution with pure water or by addition of EC-free ethanol. *n*-propyl carbamate is used as internal standard and the MSD is employed in selective ion detection mode (SIM). *n*-propyl carbamate was chosen as the internal standard because, in contrast to the situation a number of years ago, this substance is now available in pure form from the given manufacturer at a very reasonable price. The use of isotope-labelled (^{13}C or ^{15}N) ethyl carbamate as internal standard has the potential to increase the precision of this method, but these labelled substances are expensive. In addition, *n*-propyl carbamate was employed very successfully in the internationally collaboratively tested reference method (6), so there appears to be no problem with injector discrimination, for example.

The method described here was adapted and developed in the beverage research laboratory of the Swiss Federal Research Station for Fruit-Growing, Viticulture and Horticulture, in Wädenswil, Switzerland. It was employed in a research project into distillation methods aimed at reducing the levels of EC in stone fruit brandies (8). The latter are economically important agricultural products in this country. Since it performed well in our laboratory and a need for such a method as an addition to the Swiss collection of official analytical methods for foods was perceived, a trial study of performance was carried out in a number of laboratories in 1998 and the method collaboratively tested in 1999. A description of the method, the collaborative study design and results follows. This method has subsequently been adopted as an official method for the determination of EC in stone fruit brandies in Switzerland (9).

Reagents

Ethanol, absolute grade, free from EC
Ethanol, 40 % vol, free from EC
Water, distilled or equivalent, free from EC
Ethyl carbamate (purity ≥99%)
n-propyl carbamate (purity 98 %; Aldrich product number 42,425–0, obtainable e.g. from Fluka, CH-9471 Buchs SG)

Ethyl carbamate (EC) solutions

– (1) Stock solution of EC, approximately 0.1 g/l

Weigh out approximately 20 mg EC into a 200 ml volumetric flask, noting the *exact* weight and make up to the mark at 20° C with ethanol (40 % vol). Calculate the exact concentration of EC in this solution.

– (2) Diluted stock solution, approximately 5 mg/l

Pipette 5 ml of the above stock solution (approximately 0.1 g/l) into a 100 ml volumetric flask. Fill to the mark at 20° C with ethanol (40 % vol). Calculate the exact concentration of EC in this solution.

Solutions of n-propyl carbamate (nPC)

– (1) Stock solution of nPC, approximately 0.2 g/l

Weigh out approximately 20 mg nPC into a 100 ml volumetric flask, noting the *exact* weight and make up to the mark at 20° C with ethanol (40 % vol). Calculate the exact concentration of nPC in this solution.

– (2) Diluted stock solution of nPC, approximately 20 mg/l

Pipette 10 ml of the above stock solution (approximately 0.2 g/l) into a 100 ml volumetric flask. Fill to the mark at 20° C with ethanol (40 % vol). Calculate the exact concentration of nPC in this solution.

Calibration solutions

The range of the calibration curve is from 50 µg/l to 250 µg/l EC. The required calibration solutions are obtained by further dilution of the diluted stock solution of EC as follows: Pipette 1, 2, 3, 4, and 5 ml respectively into 100 ml volumetric flasks and fill to the mark at 20° C with ethanol (40 % vol). To 10 ml of each of the resulting solutions add 100 µl of the diluted stock solution of nPC (approximately 20 mg/l; internal standard) and mix well.

Apparatus

Temperature programmable gas chromatograph (GC) with mass spectrometric detector (MSD), capable of being operated in selective ion monitoring mode (SIM), including data station and integrating software or equivalent.

Capillary GC column, e.g. FFAP 25 m×0.32 mm i.d., film thickness 0.52 µm (Agilent Technologies) or equivalent.

Method

Sample preparation

Samples with an alcohol content of between 37.5 and 42 % vol are used directly, or otherwise adjusted to 40 % vol by appropriate adjustment (calculation via mass) or dilution (10) with EC-free ethanol or distilled water. Take into account volume contraction effects (11). Add 100 µl of diluted nPC stock solution (approximately 20 mg/l) to 10 ml of the sample (either direct or otherwise prepared as here described) and mix well.

Conditions for analysis by GC/MS

Injector 230° C, splitless, helium as carrier gas (flow-rate 1 ml/min at 25° C). Oven temperature programme: initial 60° C, then 50° C/min to 90° C and hold 3 minutes. Then ramp at 5° C/min to 140° C, followed by ramp to 215° C at 10° C/min. Hold for 5 minutes. Typical retention times for EC and nPC are 11.2 and 13.3 minutes, respectively. Transfer line temperature: 220° C. The mass spectrometer is trimmed manually using perfluorotributylamine and optimised for maximum sensitivity in the lower mass range. Other conditions: SIM; filament on after approximately 3 minutes; data acquisition after 4 minutes.

Calibration curve

Inject 1 µl aliquots of the calibration solutions. Plot the peak area ratio EC/nPC (dependent variable) for the ion $m/z = 62$ vs. the independent variable, i.e. the calculated exact concentration of EC in µg/l (approximate concentrations: 50, 100, 150, 200 and 250 µg/l).

Quantification of EC

Inject 1 µl of the sample prepared as above and calculate the peak area ratio EC/nPC for the ion $m/z = 62$. Repeat this procedure at least once. The observed difference in the calculated peak area ratios for the first and subsequent injection must not be larger than 10 %, otherwise further injections with rejection of outliers must follow.

Confirmation of the identity of EC and nPC

For each sample, the presence of the characteristic mass spectra of EC and nPC at their respective retention times should be checked. The presence of ethyl carbamate is checked on the basis of the relative intensities of the ions m/z = 62 and m/z = 74 (comparison with standard substance). However, the peak at m/z = 74 in EI mode is often very small in the case of ion trap mass spectrometers. In this case, the ion at m/z = M+1 in CI mode can be used for confirmation. In the case of contamination of the spectra with other components, the determination should be repeated

under different chromatographic conditions (e.g. temperature programme, column polarity).

Calculation and expression of results

Read the concentration of EC (µg/l) from the calibration curve of peak area ratio. If the peak area ratio EC/nPC lies outside the calibrated range, dilute the sample with EC-free ethanol (40 % vol) and analyse again. Take into account dilution factors and volume contraction effects in calculating the results. Express the results in µg/l, without a decimal place.

Remarks

The linear or useful range can extend beyond 250 µg EC/l, depending on the equipment used. In such cases, the working range may be extended by the use of appropriate calibration solutions.

Check periodically for the spontaneous production of ethyl carbamate (dirty injector lining) by injecting pure ethanol (40 % vol). In certain types of equipment, the production of a constant amount of EC was observed, although the lining was not dirty. In such cases this constant amount is subtracted in calculating the results.

Results

The collaborative study was performed in 1999, eight laboratories taking part. The aim of the study was to determine the suitability of the method for inclusion in the Swiss collection of official analytical methods for foods.

Description of samples analysed

Sample 1: Kirsch with very low, natural EC concentration
Sample 2: Same spiked with EC to approx. 800 µg/l
Sample 3: Same spiked with EC to approx. 1200 µg/l
Sample 4: Kirsch with natural EC concentration of approx. 400 µg/l
Sample 5: Kirsch with natural EC concentration of approx. 1000 µg/l
Sample 6: Kirsch with natural EC concentration of approx. 3000 µg/l

Precision of the method

The original data and the precision data determined therefrom are given in tables 1 and 2. Precision parameters were determined according to ISO 5725 (12), using an Excel™ based programme (13).

The value for the relative repeatability, r_{rel} expressed in % of the mean of all laboratory values ranged from 8 %–19 %, with a mean of 13 %.

The relative reproducibility, R_{rel}, was found to be 20 % of the measured concentration for concentrations in excess of 200 µg/l.

Table 1
Original data and calculated precision parameters for samples 1–3

	Laboratory	*Single data No. k*			*Samples analysed*	*Mean*	*Mean of all laboratories*	*Repeatability r (µg/l)*	*Reproducibility R (µg/l)*
	No. i	*1*	*2 (µg/l)*	*3*	*n(i)*	$\bar{x}$	$\bar{\bar{x}}$	(r_{rel})	(R_{rel})
	1	100	87	84	3	90.3			
	2	65	60	70	3	65.0			
	3	75.7	74	72.7	3	74.1		$r = 14.06$	$R = 29.12$
Sample	4	73.9	74.1	82	3	76.7	81.51		
No. 1	5	88	95	91	3	91.3		(17 %)	(36 %)
	6	83	82.8	84.9	3	83.6			
	7	96	91	83	3	90.0			
	8	78.1	83.6		2	80.9			
	1	604	848	774	2	811.0			
	2	875	942	976	3	931.0			
	3	873.6	836.2	837.9	3	849.2		$r = 87.73$	$R = 219$
Sample	4	915	910	930	3	918.3	893.87		
No. 2	5	881	851	853	3	861.7		(10 %)	(25 %)
	6	1024	1043	1064	3	1043.7			
	7	818	823	864	3	835.0			
	8	850	915.4	855	3	873.5			
	1	1362	1032	1086	3	116.0			
	2	1176	1279	1284	3	1246.3			
	3	1194.8	1148.7	1198.4	3	1180.6		$r = 230.67$	$R = 216.69$
Sample	4	1180	1270	1290	3	1246.7	1204.18		
No. 3	5	1693	1650	1672	0			(19 %)	(18 %)
	6	1201	1214	1200	3	1205.0			
	7	1169	1172	1142	3	1161.0			
	8	1136.3	1256.6	1296	3	1229.0			

Greyed data were outliers using Grubb's test and were not used in calculating *r* and *R*.

Table 2
Original data and calculated precision parameters for samples 4–6

	Laboratory No. *i*	Single data No. *k* 1	2 (µg/l)	3	Samples analysed *n(i)*	Mean $\bar{x}$	Mean of all laboratories $\bar{\bar{x}}$	Repeatability *r* (µg/l) (r_{rel})	Reproducibility *R* (µg/l) (R_{rel})
	1	180	482	478	2	480.0			
	2	495	527	517	3	513.0			
	3	489.3	468.3	474.6	3	477.4		$r = 41.29$	$R = 80.14$
Sample	4	508	535	545	3	529.3	489.90		
No. 4	5	480	467	469	3	472.0		(8 %)	(16 %)
	6	494.5	516.6	517.2	3	509.4			
	7	467	434	458	3	453.0			
	8	460.8	494.8	489.5	3	481.7			
	1	870	1140	982	3	997.3			
	2	996	1067	1085	3	1049.3			
	3	994.1	952.4	976.8	3	974.4		$r = 156.48$	$R = 199.57$
Sample	4	1118	1060	1140	3	1106.0	1008.00		
No. 5	5	931	901	933	3	921.7		(16 %)	(20 %)
	6	988	1026	1033	3	1015.7			
	7	971	994	995	3	986.7			
	8	995.2	1002.7	1041	3	1013.0			
	1	3120	3240	3226	3	3195.3			
	2	3245	3317	3278	3	3280.0			
	3	2853	2739.3	2858.9	3	2817.1		$r = 240.20$	$R = 640.93$
Sample	4	3563	3338	3525	3	3475.3	3189.50		
No. 6	5	2917	2975	2900	3	2930.7		(8 %)	(20 %)
	6	3312	3405	3334	3	3350.3			
	7	3208	3295	3211	3	3238.0			
	8	3116.8	3154.5	3416.4	3	3229.2			

Greyed data were outliers using Grubb's test and were not used in calculating *r* and *R*.

Discussion

The method described was tested and employed successfully within our laboratories for a number of years. It has the considerable advantage that little sample preparation is required, which is extremely useful when analysing large numbers of samples, e.g. in the distillation research project mentioned above, or in screening campaigns of public health authorities. While the precision determined in the collaborative study is not high, it is of an acceptable order of magnitude when compared with values predicted from the empirical equation of *Horwitz* (14). The method can therefore save time and effort, the reference method (6), however, being the method of choice in boundary cases or where greater precision is required.

Acknowledgements

Thanks are due to the following and their members of staff who contributed to the success of the collaborative study: *D. Andrey,* Laboratorium der Urkantonen; *A. Marti,* EAV Berne; *Th. Kaufmann,* Kantonales Laboratorium Luzern; *J. Aerny,* RAC Changins; *M.-D. Luong,* Laboratoire cantonal, Neuchâtel; *J. Ruf,* Kantonales Laboratorium, Thurgau; *O. Zoller,* Bundesamt für Gesundheit and *P. Kölbener,* Amt für Lebensmittelkontrolle, St. Gallen. We are also grateful to *H. Hohl,* Bischofszell Nahrungsmittel AG for supplying us with the Excel™ application for calculation of precision data according to ISO 5725.

Summary

A method for the direct determination of ethyl carbamate in stone fruit brandies by GC-MS is described. Its main advantage is that no prior extraction and clean up are normally necessary, thus saving time and effort. This method was collaboratively tested in eight laboratories in Switzerland (the results of the collaborative study are given). The value for the relative repeatability ranged from 8 %–19 %, with a mean of 13 %. The relative reproducibility was found to be 20 % of the mean determined concentration for concentrations of ethyl carbamate greater than 200 µg/l. The method has been adopted by the Swiss Collection of Methods for Food Analysis (Chapter "Spirituosen").

Zusammenfassung

Eine Methode für die direkte Bestimmung des Gehalts an Ethylcarbamat in Steinobstbränden mittels GC-MS wird beschrieben. Der Hauptvorteil dieser Methode ist darin zu sehen, dass vorausgehende Arbeitsschritte wie Extraktion und Aufarbeitung bei der Analyse vermieden werden können. Die Methode wurde in der Schweiz von acht Laboratorien geprüft (die Resultate des Ringversuchs werden hier wiedergegeben). Die durchschnittliche, relative Wiederholbarkeit liegt bei 13 % (Streubereich 8 %–19 %), während für Konzentrationen von Ethylcarbamat grösser als 200 µg/l die relative Vergleichbarkeit 20 % des Gesamtmittelwertes beträgt. Die

Methode wurde ins Schweizerische Lebensmittelbuch (Kapitel Spirituosen) aufgenommen.

Résumé

Une méthode par GC-MS pour le dosage direct de la teneur en carbamate d'éthyle dans les eaux-de-vie de fruits à noyaux est décrite. Le principal avantage de cette méthode est que les étapes d'extraction et de concentration ne sont en générale pas nécessaires. Cette méthode a été testée en Suisse dans huit laboratoires (les résultats des essais collaboratifs sont donnés ici). La répétabilité relative, *r*, se situe entre 8 % et 19 % avec une valeur moyenne de 13 %. La reproductibilité relative peut par ailleurs être estimée à 20 % de la concentration moyenne déterminée. La présente méthode a été adoptée par le Manuel Suisse des Denrées Alimentaires (chapitre spiritueux).

Key words

Ethyl carbamate, Stone fruit brandies, Validation, GC-MS, Collaborative study

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