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A rapid and improved method for the determination of ethyl carbamate in foodstuffs of different matrices†

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This work deals with the rapid and simple determination of the probable carcinogen ethyl carbamate (EC), which is naturally present in fermented food products. An undemanding, robust, and rapid pre-column derivatization utilizing a 9-xanthydrol reagent has been developed. The resulting derivative was subsequently analysed by reversed-phase high-performance liquid chromatography coupled with fluorescence detection. As a result of the thorough optimisation of the chromatographic conditions, the run was completed in just 5 minutes, considerably speeding up the usual time of EC separation (30–60 min). Thanks to the fast separation, satisfactory yields (around 90%), negligible matrix effects, no interfering peaks, very low detection limit, and simple sample pre-treatment (for the very first time, the derivatization was performed in the presence of light and without any extraction step), the proposed method represents a significant improvement of the EC determination protocol used so far. After method validation, a total of fifty food samples were subjected to analysis without any additional sample pre-treatment despite their diverse matrix. Due to its robustness, simplicity, and low time, cost, and manual demands, this method is suitable for rapid screening of EC in both final food products and during their production.

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1. Introduction

Ethyl carbamate (EC), also known as carbamic acid ethyl ester, urethane, or ethyl urethane, used to be commonly utilized in the textile, cosmetic, pharmaceutical, and chemical industries. However, all applications were banned after its toxicity was revealed in 1943.[1,2] Besides that, EC is naturally formed in foods and beverages during their fermentation, distillation, or storage. Therefore, EC can be detected in fermented food products, such as dairy products, pastries, sauerkraut, soy sauce, vinegar, beer, or wine. However, distilled alcoholic beverages made from stone- or pome-fruits are particularly hazardous to human health, as they often contain excessive EC concentrations.[3–6] In the Czech Republic, a decree establishing the maximum permitted EC level for wine and fruit spirits at 30 μg L^{-1} and 400 μg L^{-1}, respectively, was in force until 2012.[7] Currently, only a more lenient recommendation of the European Commission from 2010, requiring compliance with the maximum limit of 1000 μg L^{-1} for stone-fruit spirits, is applied.[8]

Several specific analytical methods based on different techniques, such as gas chromatography,[9–11] liquid chromatography,[12–15] infrared spectrometry,[16,17] nuclear magnetic resonance,[18] enzymatic approaches,[19–21] and electrochemical biosensors,[22] have already been developed for EC analysis. However, most of them require a demanding EC isolation using various extraction techniques and/or a derivatization step.[23]

The aim of this work was to simplify the current time-consuming and manually demanding chromatographic determination of ethyl carbamate in foodstuffs, both in terms of sample pre-treatment and separation. Furthermore, an effort was made to screen for this hazardous substance in commonly available Czech food products and thereby determine the level of exposure of Czech consumers to this carcinogen.

2. Experimental

2.1 Chemicals and reagents

The ethyl carbamate standard (purity ≥ 98%), 9-xanthydrol (98%), 1-propanol (99%), sodium phosphate (96%), *o*-phosphoric acid (85%, analytical grade), ethanol (96%), and acetonitrile (analytical grade) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (35%), sodium hydroxide, and sodium acetate (all analytical grade purity) were purchased from LachNer (Neratovice, Czech Republic). High-purity water was prepared using a Milli-Q purification system (Merck Millipore, Germany).

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2.2 Standards and samples

A stock solution of ethyl carbamate with a concentration of 4455 mg L^{-1} (=50 mmol L^{-1}) was prepared in 40% aqueous ethanol (v/v) and stored in a refrigerator at 5–8 °C. For derivatization purposes, the stock solution was further diluted with 40% aqueous ethanol to obtain a standard solution of 4455 μg L^{-1} (=50 μmol L^{-1}). Subsequently, an appropriate amount of 9-xanthydrol (9-XA) derivatizing agent was dissolved in 1-propanol to prepare a solution of 3964 mg L^{-1} (=20 mmol L^{-1}), which was also stored in a refrigerator.

The details of each sample examined are summarized in Table 1. A total of 19 homemade fruit spirits produced between the years 2013 and 2021 in seven different small grower Czech distilleries were subjected to the analysis. These samples were mainly based on plums (nos. 1–6), cherries (nos. 7 and 8), and pears (nos. 9–11), but also included less traditional fruits, such as mirabelle plum (no. 12), currants (nos. 13), apples (no. 14), and peaches (nos. 15 and 16). Some of the spirits were bivarietal and contained plum-gages (no. 17) or pear-carrot (no. 18). An aged spirit (no. 19) from an unknown fruit mixture was also tested. As far as the amount of alcohol was known, it was around 50%. Moreover, two producers also provided the first fraction (head fraction) of the distillation (nos. 20 and 21), which is intended for disposal and should always be removed from the main distillation fraction (heart) because of the presence of many hazardous substances. Furthermore, six commercially available plum spirits (nos. 22–27) produced by five different Czech manufacturers were analysed, all with a declared ethanol content between 40–50%. In addition to fruit spirits, EC was also monitored in other kinds of alcoholic beverages (19 samples in total). These included samples of homemade (nos. 28–31) and commercially available (nos. 32 and 33) meads, brandy (no. 34), vodka (nos. 35 and 36), whisky (nos. 37 and 38), tequila (no. 39), rum (no. 40), gin (no. 41), juniper brandy (no. 42), and white wines (nos. 43–45). One sample of concentrated grain spirit (no. 46) was also assayed. Finally, potentially hazardous foods,[24,25] such as soy sauce (no. 47), vinegar (no. 48), and balsamic vinegar (nos. 49 and 50), were also examined.

2.3 Sample pre-treatment

The EC derivatization reaction was based on a previously published procedure,[13] which was optimised and modified. The reaction scheme is shown in Fig. 1.

In 1.5 mL plastic microtubes, 600 μL 9-XA solution (c = 20 mmol L^{-1}), 100 μL hydrochloric acid (c = 1.5 mol L^{-1}), and 400 μL of sample or 12–345 μL of standard EC solution (c = 50 μmol L^{-1}; together with 388–55 μL of 40% aqueous ethanol to maintain the same final volume of the derivatization mixture) were thoroughly mixed. For the least concentrated calibration solution (c = 10.1 nmol L^{-1}), 220 μL of EC standard solution with a concentration of 50 nmol L^{-1} was pipetted together with 180 μL of 40% aqueous ethanol. The derivatization mixtures were always left at room temperature for 30 minutes, then filtered through a PTFE syringe filter (0.45 μm, 4 mm; Labstore, HPST, Prague, Czech Republic) and analysed.

2.4 Instrumentation and analysis

For EC determination, reversed-phase high-performance liquid chromatography coupled to fluorescence detection (RP-HPLC-FLD) was utilized. The system was equipped with two LC-30AD pumps, a DGU-20A_5 degasser, an RF-20A XS fluorescence detector (all Shimadzu, Kyoto, Japan), a six-port injection valve with a 20 μL external loop (Valco-Vici, Schenkon, Switzerland), and an LCO 102 column thermostat (Ecom, Prague, Czech Republic).

The optimised separation of derivatives was performed on a Luna C18 analytical column (150 × 3 mm; 3 μm particle size; Phenomenex, Torrance, USA) using a binary mobile phase consisting of sodium acetate (c = 20 mmol L^{-1}) at pH 7.2 and 100% acetonitrile. The flow rate was 0.8 mL min^{-1}, the column temperature 35 °C, and the injection volume 20 μL. The final mobile phase gradient was as follows: 0 min – 62% B, 4 min – 70% B, and 5 min – 100% B. The excitation and emission wavelengths of the fluorescence detector were set at 233 nm and 600 nm, respectively.

2.5 Method validation and data processing

Quantitative analysis was performed using the external calibration curve method. The calibration data were measured at nine concentration levels in the concentration range of 0.9–1400 μg L^{-1}; each level was five times prepared and analysed (n = 5) and fitted using linear least squares regression (QC Expert 2.9, Trilobyte, Czech Republic). Influential points were identified using graphical diagnostics (Pregibon, Williams, and L-R graphs) and potential outliers were eliminated. The linearity of the calibration curves was verified using residual plots, and the significance of the intercept of the regression straight-line was tested using Student's t-test.

The instrumental limits of detection (LOD) and quantification (LOQ) were calculated as the concentration yielded a signal-to-noise ratio of S/N = 3 and S/N = 10, respectively. The accuracy and precision of the method were verified by measuring the calibration solutions at three concentration levels (150 μg L^{-1}, 700 μg L^{-1}, and 1300 μg L^{-1}), each level with ten repetitions (ten times prepared).

3. Results and discussion

3.1 Optimisation of the derivatization procedure

Since EC lacks a fluorophore, chromophore, and electrophore in its chemical structure and interference occurs at the selected MRM transition when using the direct HPLC-MS/MS technique, a derivatization reaction is mandatory. So far, 9-XA is the only exclusively used EC derivatization reagent, applied prior to HPLC, GC, and MS analysis. The EC reaction with 9-XA in a strongly acidic environment (hitherto always performed in the absence of light) leads to ethyl-*N*-xanthyl carbamate derivative (XEC),[26] as depicted in Fig. 1. The XEC preparation had to be thoroughly optimised in terms of volumes and concentrations of individual derivatization components, derivatization time necessary for the quantitative reaction, stability of the resulting XEC derivatives, and effects of alcohol content, pH, and light

Table 1 Summary of 50 samples analysed[a]

Sample no.	Homemade distilled fruit spirits; production year	Alcohol content (%)	Producer, place of origin
1	Plum		—
2	Plum; 2013	51	Grower distillery BaKaB, Hornice, CZ
3	Plum; 2020	50	Grower distillery, Malé Hradisko, CZ
4	Plum; 2016	50	Grower distillery, Malé Hradisko, CZ
5	Plum; 2020		Grower distillery, Přerov, CZ
6	Plum; 2021		Distillery and cidery, Lipová-lázně, CZ
7	Cherry; 2019	50	Grower distillery, Veltruby, CZ
8	Cherry; 2018		Distillery and cidery, Lipová-lázně, CZ
9	Pear; 2013	50	Grower distillery BaKaB, Hornice, CZ
10	Pear; 2018	50	Grower distillery, Veltruby, CZ
11	Pear; 2021		Distillery and cidery, Lipová-lázně, CZ
12	Mirabelle plum; 2013	50	Grower distillery, Veltruby, CZ
13	Currant		Distillery and cidery, Lipová-lázně, CZ
14	Apple; 2018	50	Grower distillery, Křinec, CZ
15	Peach; 2014		Distillery and cidery, Lipová-lázně, CZ
16	Peach; 2018		Distillery and cidery, Lipová-lázně, CZ
17	Plum-Gage; 2014		Distillery and cidery, Lipová-lázně, CZ
18	Pear-carrot		Distillery and cidery, Lipová-lázně, CZ
19	Aged spirit from fruit mixture; 2021		Grower distillery, Bohdaneč, CZ
20	Head fraction of plum spirit		Grower distillery, Veltruby, CZ
21	Head fraction of plum spirit No. 6		Distillery and cidery, Lipová-lázně, CZ
Sample no.	Commercially distilled fruit spirits	Alcohol content (%)	Producer/manufacturer
22	Plum	50	Žufánek distillery, CZ
23	Plum	40	Rudolf Jelínek distillery, CZ
24	Plum	40	Rudolf Jelínek distillery, CZ
25	Plum	40	St. Nicolaus, SK
26	Plum	47	Bartida, CZ
27	Spirit from maturated plums	40	Liqui B Blatná distillery and brewery, CZ
Sample no.	Other alcoholic beverages	Alcohol content (%)	Producer/manufacturer
28	Spring homemade mead	11.5	Beekeeper, Přibyslav, CZ
29	Forest homemade mead	13.5	Beekeeper, Přibyslav, CZ
30	Medow homemade mead	13.5	Beekeeper, Přibyslav, CZ
31	Forest homemade mead	12.9	Beekeeper, Potštejn, CZ
32	Commercial mead	11	Hromčík, Nivnice, CZ
33	Commercial mead	14.5	Medovinka, CZ
34	Brandy	40	Mast-Jaegermeister, CZ
35	Vodka	40	Brown-Forman Czechia
36	Vodka	37.5	Stock Plzeň-Božkov, CZ
37	Whiskey	40	Stock Plzeň-Božkov, CZ
38	Whiskey	40	Mast-Jaegermeister, DE
39	Tequila	40	Brown-Forman Czechia, CZ
40	Rum	40	Stock Plzeň-Božkov, CZ
41	Violet gin	37.5	Stock Plzeň-Božkov, CZ
42	Juniper brandy	38	St. Nicolaus, Liptovský Mikuláš, SK
43	Pálava white wine	11.5	Vinice Hnanice, CZ
44	Cuvéé white wine	11.5	Annovino Vinařství Lednice, CZ
45	Tokaji white wine	10.5	Grand Tokaj, HU
46	Grain spirit	96	Distillery Kolín, CZ
Sample no.	Type of foodstuff		Manufacturer/distributor
47	Soy sauce		Countrylife
48	Vinegar		Kaufland
49	Wine vinegar		Lidl
50	Wine vinegar I.G.P. (from Modena)		Lidl

[a] Abbreviations: CZ = Czech Republic, DE = Germany, HU = Hungary, and SK = Slovak republic. All sample experiments were five times ($n = 5$) repeated and the results were calculated and presented as confidence intervals $\bar{x} \pm s \cdot t_{1-\alpha}$, where $\bar{x}$ is the arithmetic mean, s is the standard deviation, and $t_{1-\alpha}$ the critical value of Student's t-distribution for five repetitions (2.776) at a significance level α of 0.05 (95% probability).

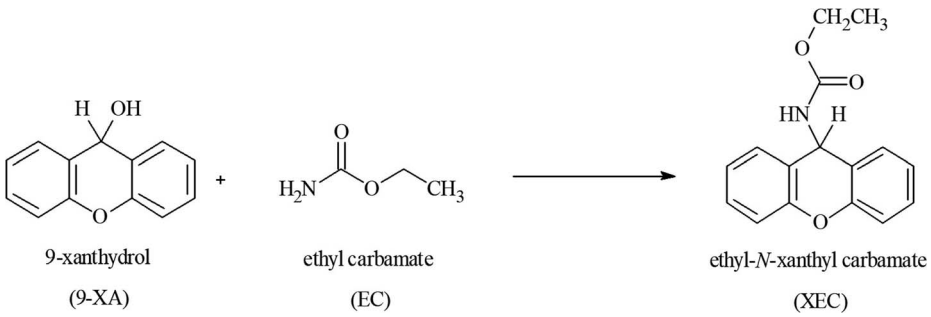

Fig. 1 Scheme of the derivatization reaction.

presence on the derivatization yields. Since the absorption spectrum of 9-XA was found to be identical to that of XEC (Fig. S1 in the ESI†), the optimisation of the derivatization could not be performed by a simple and rapid spectrophotometric technique, but chromatography had to be employed.

Information concerning the reaction time and stability of the derivatives varies between studies. However, already published studies agree[13,26,27] that with increasing acidity of the derivatization medium, the reaction kinetic accelerates, but the derivative formed is less stable. Moreover, the reaction time also depends on the sample matrix, especially on the presence of aromatics.[13] Therefore, the kinetics of the reaction and the stability of the resulting derivatives were studied in an environment of 0.15–1.5 mol L^{-1} hydrochloric acid, 1.5 mol L^{-1} acetic acid, and 0.1 mol L^{-1} phosphate buffer at pH 2.5. Using buffer, acetic acid, and less concentrated hydrochloric acid, no quantitative reaction occurred even within 24 hours (Fig. 2). Therefore, 1.5 mol L^{-1} hydrochloric acid was used for further experiments. Under these conditions, the quantitative reaction was achieved within 30 minutes at laboratory temperature and the derivative obtained was stable for at least five days (Fig. 3).

As this derivatization has so far been carried out only in the dark,[13] which requires higher demands on the operator, the effect of the presence of light on the kinetics and yield of the reaction was also investigated. It was found that light

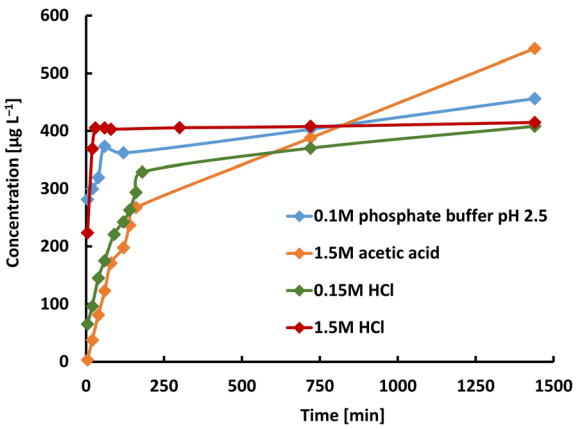

Fig. 2 Kinetics of the derivatization reaction as a function of the environment.

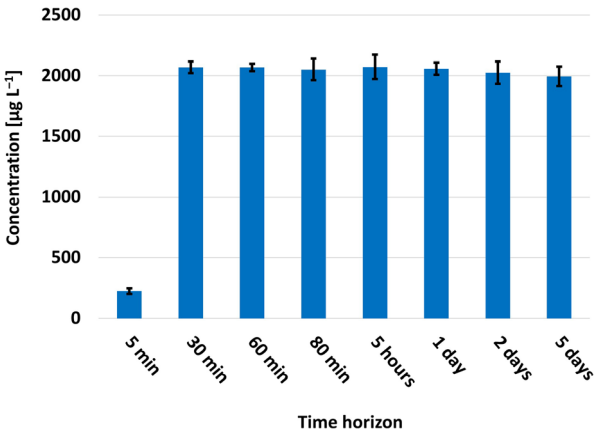

Fig. 3 Stability of the prepared XEC derivative.

elimination did not lead to a higher yield or faster reaction (Fig. 4), so subsequent experiments were conducted under light.

The literature also indicates that the derivatization yield depends on the amount of ethanol present.[13,28] For this reason, a series of spiked plum spirits/standard solutions were prepared with the same EC concentration ($c = 1300$ µg L^{-1}) but a different ethanol content, ranging between 10–60%. In the case of the EC standard, the relative yield decreased with increasing alcohol content (from 105% to 91%; Fig. S2a†). For the plum spirit, the relative yield fluctuated between 135% and 100% (Fig. S2b†). In general, spirits typically contain between 30% and 50% alcohol. In this concentration range of ethanol, there were no statistically significant changes in yields (98.1 ± 2.9%) in either case. A similar dependence has already been presented by a group of Chinese authors,[28] but according to other authors,[13] the yield of the reaction increased up to 42% ethanol and then decreased again up to 60%. For determining accurate EC concentrations in samples of different types, it would be, therefore, appropriate to always adjust the respective sample to a uniform ethanol content (corresponding to the content used to construct the calibration dependence). However, the maximum EC limits recommended by the European Commission are relatively benevolent and only applicable to distillates. Thus, it is not necessary to maintain exact ethanol concentrations for rapid EC screening of samples with different matrices in common food manufacturing companies.

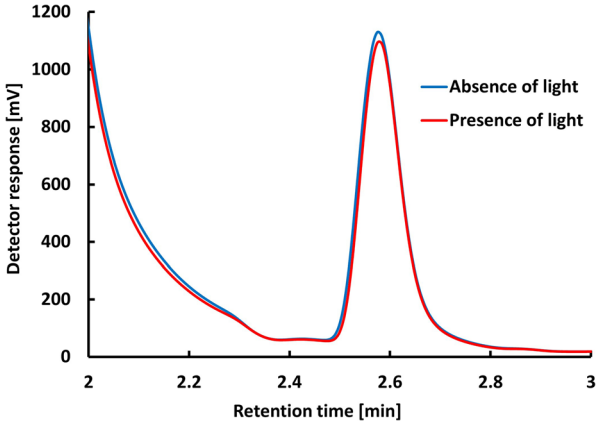

Fig. 4 Effect of the presence of light on the yield of the derivatization reaction.

3.2 Optimisation of the chromatographic separation

HPLC-FLD separation conditions were also thoroughly optimised. Since the excitation and emission wavelengths were not uniform in the literature, it was first necessary to measure the corresponding spectra of XEC derivative (Fig. S1 and S3†), based on which the excitation and emission wavelengths of 233 nm and 600 nm, respectively, were subsequently set on the detector. The choice of column type and mobile phase was inspired by previously published data.[13] In contrast, the greatest attention was paid to the optimisation of gradient elution. The aim was (i) to reduce the analysis time as much as possible so that the method is also suitable for rapid screening and routine use and (ii) to maintain sufficient peak resolution (the reaction generates a relatively large number of by-products). Thus, gradients with different initial concentrations of acetonitrile (50–62%) and different slopes were tested. The final gradient program started at 62% acetonitrile and lasted only 5 minutes (see chapter Experimental), whereas the usual analysis time has previously been between 30–50 minutes.[12,13,26–28] An example of the chromatographic separation of blank solution, EC standard solution, and samples of different types after their derivatization is shown in Fig. 5. A comparison of our EC determination parameters with previously published literature data is summarized in Table 2.

3.3 Validation of the analytical method

The developed analytical method for EC determination was validated in terms of linearity of the calibration curve (given by the coefficient of determination R^2), LOD, LOQ, accuracy (recovery), and precision (intra-day and inter-day repeatability). All the validation parameters are summarized and compared with the literature in Table 2.

Linear dependence (in the concentration range of 0.9–1400 μg L^{-1}) was characterized using the equation $A = 2.27\ (\pm 0.02)c + 204.99\ (\pm 11.44)$; where A is the peak area (mV s) and c is the concentration (μg L^{-1}) and $R^2 = 0.9991$, representing good linearity (Fig. S4†). The LOD and LOQ values reached 0.25 μg L^{-1} and 0.84 μg L^{-1}, respectively. According to the validation guideline, the recovery for our concentration range should be between

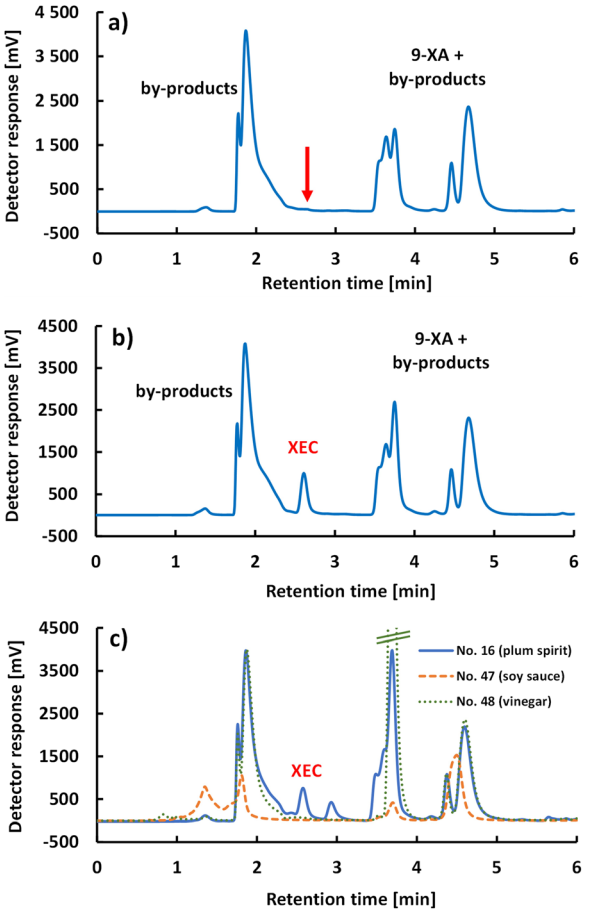

Fig. 5 Chromatographic separation of blank solution (a), the EC standard at a concentration of 400 μg L^{-1} (b), and real samples (c) of plum spirit (no. 16; blue line), soy sauce (no. 47; orange dashed line), and vinegar (no. 48; green dotted line).

80% and 110%.[29] As can be seen from Table S1,† all three matrices (plum brandy, soy sauce, and vinegar) met this range at all concentrations tested (150–1300 μg L^{-1}), and the method can be considered sufficiently accurate. Moreover, no interfering peaks causing potential co-elution with the target analyte were observed in any of the samples (Fig. 5 and S5†), despite the completely different nature of the matrices.

Intra-day repeatability and inter-day repeatability were expressed as relative standard deviation (RSD) and reached mean values of 5.7% and 6.5%, respectively (Table S2†). According to the validation guide,[29] RSD < 7.3% should be obtained for the given concentrations, which is met in both cases, and the method can thus be considered sufficiently precise. In addition, all validation data obtained are consistent with those already published or better (Table 2).

3.4 Sample analysis

EC was determined in a total of 48 food samples and 2 samples of the head fraction of the plum spirit distillation process. Out of these fifty samples analysed, EC was detected in fifteen (Table 3). The largest number of samples with detectable EC content was observed in homemade stone fruit spirits from

Table 2 Comparison of the parameters of the developed method with those of already published[a]

Number and type of samples	Sample pre-treatment	Derivatization conditions	Stability of the derivative	Separation method; analysis time	Recovery [%]	LOD [μg L^{-1}]	LOQ [μg L^{-1}]	Intra-day repeatability [% RSD]	Inter-day repeatability [%RSD]	Cost; difficulty	References
50 foodstuffs of various matrices	**Derivatization with 9-XA**	**30 min, room temperature, presence of light, smaller number of by-products**	**5 days <**	**HPLC-FLD; 5 min**	**84–104**	**0.25**	**0.84**	**5.7**	**6.5**	**Cheap; low**	**Presented study**
19 soy sauces	LLE and SPE followed by derivatization with 9-XA	30 min, higher temperature, in the dark, higher number of by-products	2 hours	HPLC-FLD; 40 min	81–95	3.9	13	<6.6	<8.9	Medium expensive; high	12
90 spirits	Derivatization with 9-XA	50 min, room temperature, in the dark, smaller number of by-products	Not given	HPLC-FLD; 30 min	96	1.8	5.3	<5	—	Cheap; low	13
34 wines	Derivatization with 9-XA	Room temperature, in the dark, different pHs	Depends on pH	HPLC-FLD; 55 min	93–104	3	—	1.8	2.1	Cheap; low	26
17 industrial and 15 experimental cider spirits	Derivatization with 9-XA	30 min, room temperature, in the dark, higher number of by-products	12 h <	HPLC-FLD; 31 min	94–98	1.64	3.56	<5	—	Cheap; low	27
26 wines and brandies	Derivatization with 9-XA	30 min, 30 °C, in the dark, higher number of by-products	60 min	HPLC-FLD; 50 min	91–105	4.8	16	<3.7	<4.8	Cheap; low	28

Number and type of samples	Sample pre-treatment	Derivatization conditions	Stability of the derivative	Separation method and analysis time	Recovery [%]	LOD [μg L^{-1}]	LOQ [μg L^{-1}]	Intra-day repeatability [%RSD]	Inter-day repeatability [%RSD]	Cost; difficulty	References
9 wines and 4 liquors	Without derivatization or purification	—	—	UHPLC-MS/MS, 5 min	107–111	1.8	4	<5		Expensive; low	14
24 fortified wines	*m*-LLE	—	—	HPLC-MS/MS; 18 min	93–114	0.17	0.52	<5.6	<8	Expensive; medium	15
35 kinds of alcoholic beverages	LLE followed by derivatization with BSTFA	30 min, 80 °C, presence of light	—	GC-MS, 24 min	71–99	0.3	5	<8.4	—	Expensive; high	9

Table 2 (*Contd.*)

Number and type of samples	Sample pre-treatment	Derivatization conditions	Stability of the derivative	Separation method and analysis time	Recovery [%]	LOD [μg L^{-1}]	LOQ [μg L^{-1}]	Intra-day repeatability [%RSD]	Inter-day repeatability [%RSD]	Cost; difficulty	References
54 stone-fruit spirits	HS-SPME at 70 °C, 30 min	—	—	GC-MS/MS; 37 min	91–109	30	110	<4.3	<8.2	Expensive; high	10
—	SPE	—	—	GC-MS; 40 min	87–93	—	—	—	—	Expensive; medium	11
100 foodstuffs of various matrices	Homogenization followed by SPE and other purification procedures (based on the AOAC official method[11])	—	—	GC-MS; separation time not given	66–117	1	—	—	—	Expensive; high	24
237 foodstuffs of various matrices	Homogenization followed by SPE and other purification procedures (based on the AOAC official method[11])	—	—	GC-MS; separation time not given	90–102	—	—	—	—	Expensive; high	25

[a] Abbreviations: 9-XA, 9-xanthydrol; BSTFA, bis(trimethylsilyl)trifluoroacetamide; FLD, fluorescence detection; GC, gas chromatography; HPLC, high-performance liquid chromatography; HS, head-space; LLE, liquid–liquid extraction; LOD, limit of detection; LOQ, limit of quantification; *m*-LLE, micro LLE; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RSD, relative standard deviation; SPE, solid phase extraction; SPME, solid phase micro-extraction; and UHPLC, ultra HPLC.

Table 3 Samples with EC content higher than LOD

Sample no.	EC quantity [μg L^{-1}]	Sample no.	EC quantity [μg L^{-1}]	Sample no.	EC quantity [μg L^{-1}]
1	81.2 ± 2.1	8	140.4 ± 1.5	16	354.0 ± 1.2
2	1503.4 ± 2.9	9	516.1 ± 2.3	19	150.4 ± 8.7
5	378.2 ± 3.3	11	<LOQ[a]	22	379.5 ± 6.8
6	<LOQ[a]	12	316.3 ± 2.2	31	467.5 ± 3.0
7	14.2 ± 1.7	15	<LOQ[a]	33	<LOQ[a]

[a] LOQ = 0.84 μg L^{-1}; values are given as confidence intervals $\bar{x} \pm s \cdot t_{1-\alpha}$, where $\bar{x}$ is the arithmetic mean, s is the standard deviation, and $t_{1-\alpha}$ the critical value of Student's t-distribution for five repetitions (2.776) at a significance level α of 0.05 (95% probability).

grower distilleries. Of these 19 samples monitored, EC was present in 12 (nos. 1, 2, 5–9, 11, 12, 15, 16, and 19). However, in three samples (nos. 6, 11, and 15), the EC concentration could not be exactly determined because its level was below the limit of quantification. The other samples contained EC at concentrations ranging from 14 μg L^{-1} to 1500 μg L^{-1}. Until 2012, a Czech decree allowed a maximum EC level of 400 μg L^{-1} in fruit spirits. This would not be met by two samples, Nos. 9 and 2, in which 516 μg L^{-1} and 1503 μg L^{-1} were found, respectively. Since 2012, a more benevolent EU recommendation has been in force in the Czech Republic, allowing EC levels up to 1000 μg L^{-1}. From this point of view, only sample no. 2 would not comply with this recommendation and is thus considered unsafe.

Since, in an earlier study,[30] a large amount of EC (up to 60 000 μg L^{-1}) was found in the first fraction of distillation, two samples of the head distillation fraction (nos. 20 and 21) were analysed in addition to alcoholic beverages and food. However, EC was not found in any of these samples. The head fractions are rich in low-boiling substances, whereas EC has a relatively high boiling point (182–184 °C) and would thus be distilled mainly in the heart or tail fractions of distillation.[8] This is also confirmed by the fact that in one distillation batch (head no. 21 and heart no. 6), EC was detected above the LOD only in the heart fraction. In the above-mentioned study,[30] high findings in the head fractions were not further commented or explained. For comparison, 6 commercially available plum spirit samples were analysed in addition to homemade fruit spirits from small grower distilleries. However, these spirits are usually not 100% fruit distillates but are fortified with ethanol and often contain flavourings, dyes, and other ingredients. According to Czech legislation, a fruit distillate is a beverage produced exclusively by alcoholic fermentation of fruit with subsequent distillation of fruit leaven. It must not be aromatized or fortified with alcohol (the exception is the addition of alcohol to the fruit distillate before the final distillation, but its concentration must not be higher than 30%).[31] Of the commercial plum spirit samples investigated, only two represent fruit distillates (nos. 22 and 26). The remaining four samples contained only a minimal quantity of fruit distillate (nos. 23–25, 27) and, therefore, were not expected to have significant EC concentrations, which was subsequently also confirmed. EC was found in only one sample of pure fruit distillate (no. 22), whose concentration (380 μg L^{-1}) did not exceed the European Commission recommended maximum level.

A diverse group of other alcoholic beverages, consisting of rum, gin, juniper brandy, vodka, tequila, whisky, brandy, wines, and meads, was also subjected to the analysis. From the total number of 19 samples, EC was quantified only in a sample of commercial mead (no. 31), with a concentration of 468 μg L^{-1}. The other commercial mead sample (no. 33) contained EC below the quantification limit. To the best of our knowledge, the determination of EC in mead has not been performed before, and therefore, our results could not be compared. EC was not detected in other alcoholic beverages, although according to the results presented by EFSA, EC concentrations for white wine samples were up to 30 μg L^{-1}, for gin, vodka, and rum up to 55 μg L^{-1}, and for whisky, tequila, and brandy up to 520 μg L^{-1}.[3] Fermented foods generally contain negligible concentrations of EC. The exceptions are soy sauces and vinegars, showing EC concentrations up to 130 μg L^{-1} [24,25] and up to 17 μg L^{-1},[32] respectively. For this reason, two balsamic vinegars, one fermented spirit vinegar, and one soy sauce were analysed. However, EC was not detected in either sample.

4. Conclusions

A derivatization of ethyl carbamate is carried out exclusively with the 9-xanthydrol reagent. By thoroughly optimizing the existing derivatization procedure, the entire process has been considerably simplified and accelerated, with the possibility of performing it in the presence of light, without an extraction step, and regardless of the matrix of the analysed sample. In addition, the optimised reaction conditions provided fewer by-products, thus exhibiting a much smaller background. After mixing 600 μL 20 mmol L^{-1} 9-XA, 100 μL 1.5 mol L^{-1} HCl, and 400 μL of the sample, this mixture was incubated in the presence of light for 30 min. The product of the derivatization reaction was a very stable fluorescent derivative (stable up to 5 days with minimal loss), which was analysed by high-performance liquid chromatography coupled with a fluorescence detector. The optimally selected separation conditions, employing a Luna C18 analytical column (150 × 3 mm; 3 μm) and a binary mobile phase consisting of 20 mmol L^{-1} sodium acetate at pH 7.2 (A) and 100% acetonitrile (B), with a flow rate of 0.8 mL min^{-1} and gradient elution of 0 min – 62% B, 4 min – 70% B, and 5 min – 100% B, significantly reduced the analysis time from the usual 30–60 min to only 5 min. The obtained validation parameters demonstrated that the developed method is sufficiently accurate, precise, robust, and sample matrix

independent, with much lower limits of detection and quantification than commonly reported. This method thus represents a very promising potential for the rapid screening of EC not only in final products of the food industry but also during their production.

The presence of ethyl carbamate in fifty samples, involving alcoholic beverages of different natures and origins, as well as fermented foods, was monitored using the developed derivatization and chromatographic method. No further sample preparation process was necessary. In eleven samples of spirits, ethyl carbamate was quantified in the concentration range of 14–1503 μg L^{-1}. In the other four samples, the presence of ethyl carbamate was observed below the limit of quantification (<0.84 μg L^{-1}).

Data availability

The data supporting this article have been included as part of the ESI.†

Author contributions

Veronika Šantrůčková: formal analysis, methodology, validation. Jan Fischer: conceptualization, writing – review & editing. Jitka Klikarová: methodology, supervision, conceptualization, data curation, writing – original draft, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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