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Rapid screening for ethyl carbamate in stone-fruit spirits using FTIR spectroscopy and chemometrics

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Abstract Ethyl carbamate (EC, urethane, $C_2H_5OCONH_2$) is a known genotoxic carcinogen of widespread occurrence in fermented food and beverages with the highest concentrations being found in stone-fruit spirits. Time-consuming procedures requiring extraction and gas chromatographic–mass spectrometric determination are regarded as reference procedures for the analysis of EC in alcoholic beverages. In this study, the rapid method of Fourier transform infrared (FTIR) spectroscopy in combination with partial least-squares (PLS) regression using selected wavelength bands is applied for the first time to the screening analysis of EC in stone fruit spirits (analysis time only 2 min). Apart from the actual content of EC in the sample, additional information was available from the FTIR spectra. This included data concerning the EC precursor hydrocyanic acid (HCN) and the maximum EC concentration which could be formed during storage. The PLS procedure was validated using an independent set of samples ($Q^2 = 0.71$ – 0.76 , SEP = 0.42 – 0.67). The method was found to lack the accuracy required for a quantitative determination; it could only be used semi-quantitatively in the context of a screening analysis. If a rejection level of 0.8 mg L^{-1} is applied as cut-off, overall correct classification rates of 85–91% for the calibration set and 77–85% for the validation set were achieved. False negative results can be avoided by lowering the cut-off to 0.6 mg L^{-1} . Through use of FTIR screening, 60–70% of all samples can be classified as negative and removed, leaving only conspicuous analysis results exceeding cut-off to be confirmed by complex and labour-intensive reference analyses.

Keywords Ethyl carbamate · Hydrocyanic acid · Stone-fruit spirits · *Prunus* ss. (L.) · PLS

Introduction

Ethyl carbamate (EC, urethane, $C_2H_5OCONH_2$) is a known genotoxic carcinogen of widespread occurrence in fermented food and beverages [1, 2, 3, 4]. Public health concern about EC in alcoholic beverages originated in 1985 when relatively high levels were detected by Canadian authorities, and included discoveries in spirit drinks imported from Germany [5]. The highest EC concentrations were found in spirits derived from stone fruit of the species *Prunus* ssp. (L.) (Rosaceae) (cherries, plums, mirabelles [yellow plums], apricots, etc.) [1, 3]. Subsequently, Canada established an upper limit of 0.4 mg L^{-1} EC for fruit spirits [5], which has since been adopted by Germany and many other countries.

The disposal of cyanogenic glycosides (such as amygdalin) in stone fruit through enzymatic action (mainly β -glucosidase) leads to the formation of cyanide, which is the most important precursor of EC in spirits. Cyanide is oxidised to cyanate, which reacts with ethanol to form EC [1, 6, 7, 8, 9]. The wide range of EC concentrations in stone-fruit spirits reflects light-induced and time-dependent formation after distillation and storage [3, 10, 11, 12, 13].

Many preventive actions have been proposed to avoid EC formation in alcoholic beverages. Self-evident measures of good manufacturing practice must be optimised. These include the use of high-quality, non-spoiled raw material, high standards of hygiene during fermentation and storage of the fruit mashes [14, 15], and mashing and distillation conditions beyond reproach. To avoid the release of cyanide, it is essential that the stones are not broken, that light irradiation is minimised, and that storage time is shortened [16]. Some researchers proposed the addition of enzymes in order to

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decompose cyanide, or complete de-stoning of the fruit prior to mashing. The mashes have to be distilled slowly, with a timely conversion (at 65% (v/v)) to the tailing-fraction [14]. Further preventive actions include the addition of patented copper salts to precipitate cyanide in the mash [16, 17, 18, 19], distillation using copper catalysts [20, 21, 22, 23] and the application of steam washers [24, 25]. It should be noted that the use of copper can create environmental problems due to hazardous waste.

According to Council Regulation (EEC) No. 315/93 (covering community procedures for contaminants in food [26]), no food items containing unacceptable contaminant amounts (according to public health standards) and in particular those at toxic levels, shall be placed on the market. Furthermore, contaminant levels shall be kept as low as can reasonably be achieved by following good practices. In our opinion, an offence against good practices can be assumed if the upper limit is exceeded more than twice. These samples would be subject to official objection due to production methods contravening European law. In consideration of lot-to-lot differences and inhomogeneities, manufacturers were advised of their duty to exercise due diligence and to use state-of-the-art measures to reduce the content of EC. In 1999, German health authorities stated that manufacturing measures undertaken at that time to reduce EC levels had led to a drop in contamination, particularly in products from large distilleries [27]. In principle, this statement is in full accordance with our previous results [28]. In 1986, more than 65% of analysed samples had to be rejected. Currently, the rejection quota varies between 25 and 40%. In particular, small distilleries that have not introduced improved technologies tend to achieve poor results. As a result, the determination of EC levels in spirit drinks is a parameter of high importance in official food control. Time-consuming procedures like gas chromatography, coupled with mass spectrometry (GC/MS) [3, 5, 10, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38] or tandem mass spectrometry (GC/MS/MS) [39, 40, 41] requiring extensive clean-up procedures (e.g. extraction over diatomaceous earth columns proposed by many authors [10, 41, 42, 43, 44, 45, 46, 47]), are regarded as reference for analysis of EC in alcoholic beverages. Increasing requirements and cost pressures have forced both government and commercial food-testing laboratories to replace traditional reference methods with faster and more economical systems. Fourier transform infrared (FTIR) spectroscopy, in combination with multivariate data analysis, has already shown great potential for expeditious and reliable screening analysis of alcoholic beverages [48, 49, 50, 51, 52, 53, 54]. The analysis of EC found in wine samples using FTIR spectroscopy was evaluated by Manley et al. [51].

In this study, FTIR in combination with partial least-squares (PLS) regression [55, 56, 57] was applied for the first time to screening analysis of EC in stone-fruit spirits.

Experimental

Sample collective

A total of 122 stone-fruit spirits submitted to the CVUA Karlsruhe were analysed for EC. This institute covers the district of Karlsruhe in North Baden (Germany) and participates in official food control in Baden-Württemberg. This area has a population of approximately 2.7 million people and includes the northern part of the Black Forest, a territory with approximately 14,000 approved distilleries (including South Baden), which produce well-known specialties like Black Forest Kirsch (cherry spirit). The sampling was conducted by local authorities, either directly from the distilleries or from retail trade. To eliminate the possibility of EC formation in samples during transport and storage, the bottles were wrapped in aluminium foil immediately after sampling.

Fourier transform infrared spectroscopy

The WineScan FT 120 instrument (Foss Deutschland, Hamburg, Germany) was used to generate the FTIR spectra. No prior preparation of the samples was required. The temperature of the samples was automatically set at 40°C in the spectrometer before analysis. The IR spectrum was scanned between 926 and 5,011 cm^{-1} (1,054 data points per spectrum). The spectral regions of water absorption between 1,447 and 1,887 cm^{-1} and 2,971–3,696 cm^{-1} were eliminated to prevent noise being included in the calculation.

The standard software FT 120 V2.2.2 was used (Foss Deutschland, Hamburg, Germany) for quantitative determination of EC and hydrocyanic acid (HCN) from the FTIR spectra (applying PLS regression). The FTIR spectra and reference results of 82 samples were used as a data set for a PLS regression (calibration set). The remaining 82 samples were used as an independent set to test the calibration (validation set). The sample grouping was done by randomisation in such a way that low, medium and high concentrations were evenly distributed between the two sets with the most extreme observations in the calibration set. Prior to calibration, the appropriate wavenumber ranges for the analytes were selected using the automatic filter selection tool of the FT 120 software, which applies multivariate data analysis. The ranges were selected based on the correlation between the reference results for the component in question and the sample variation in each wavenumber in the spectra by a non-disclosed Foss algorithm. The selected wavenumber ranges are shown in Table 1 and marked in Fig. 1. Subsequently, PLS regression of the calibration set was performed with test-set validation. The optimal number of factors, indicated by the lowest prediction error, was selected and the calibration evaluated using

Table 1 Wavenumbers selected using PLS regression with information about ethyl carbamate content in the sample (actual EC), the maximum ethyl carbamate concentration, which could be formed after UV irradiation (maximum EC), and HCN

Actual EC (cm ⁻¹)	Maximum EC (cm ⁻¹)	HCN (cm ⁻¹)
1,249–1,257	1,249–1,257	1,249–1,257
1,161–1,165	1,018–1,022	991–1,006
1,134–1,138	995–1,006	1,790–1,793
1,006–1,010	1,134–1,138	1,014–1,018
1,226–1,234	1,153	1,130–1,138
1,018	1,230–1,234	1,153
1,763–1,766	1,469	1,469
1,793–1,797	1,766–1,770	1,813
1,477	1,793	1,724
1,153–1,157	976–979	1,010
1,199	1,168–1,172	1,122

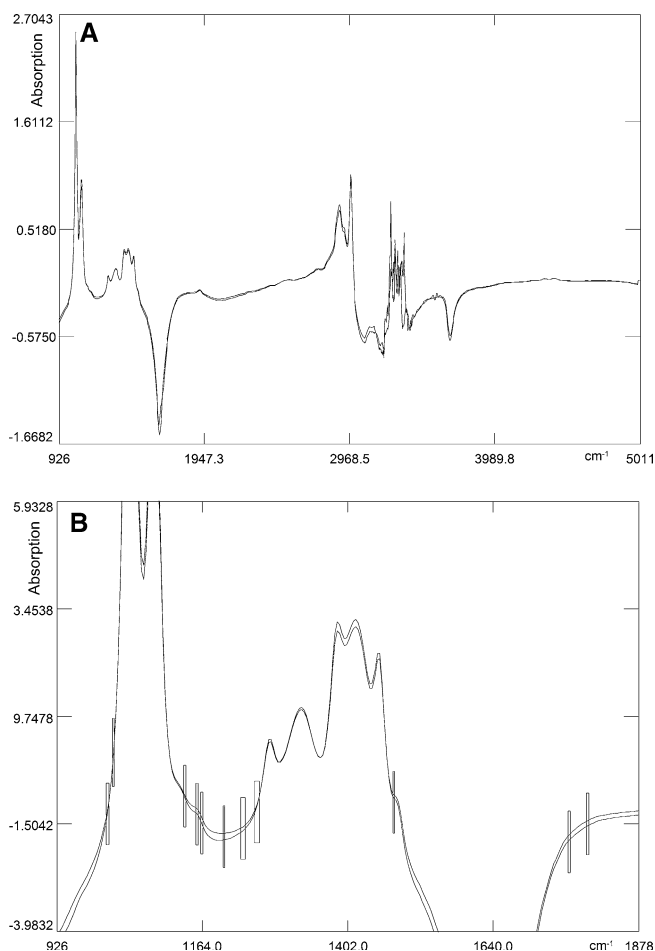


Fig. 1 FTIR spectra of two authentic stone-fruit spirits with low and high ethyl carbamate concentrations showing the total spectral range between 926 and 5,011 cm⁻¹ (a) and a strong vertical expansion of the characteristic region between 926 and 1,878 cm⁻¹ (b). Rectangles mark the spectral region used in the PLS modelling for ethyl carbamate

the independent validation set. The statistical parameters were calculated using standard formulas (e.g. ref. [57]).

Gas chromatographic and tandem mass spectrometric reference procedure

The analysis of EC was done using previously published procedures combining the extrelut extraction procedure of Baumann and Zimmerli [42] with modifications of Mildau et al. [10] and tandem mass spectrometry (GC/MS/MS) according to Lachenmeier et al. [41]. For sample preparation, 20 mL of stone-fruit spirit was spiked with 50 µL of EC-d₅ (1 mg mL⁻¹) that was synthesised according to Funch and Lisbjerg [29], and directly applied to the extraction column. The extrelut column was wrapped in aluminium foil to eliminate the possibility of EC formation during extraction. After 15 min of equilibration, the column was washed with 2 × 20 mL of *n*-pentane. Next, the analytes were extracted using 3 × 30 mL of dichloromethane. The eluates were combined in a brown flask and reduced to 2–3 mL in a rotary evaporator (30°C, 300 mbar). After that, the solution was adjusted to 10 mL with ethanol in a measuring flask and directly injected into the GC/MS/MS system. In addition to the determination of the actual EC content, the samples were exposed to UV light for 4 h using a 360-W high-pressure mercury lamp Psorilux 3060 (Heraeus, Hanau, Germany) and extracted as described above in order to evaluate the light-induced EC formation capability of the products (maximum EC). The recovery of EC was 100.4 ± 9.4%. The limit of detection was 0.01 mg L⁻¹ of EC. The precision (expressed as coefficient of variation) never exceeded 7.8% (intraday) and 10.1% (interday); the trueness (expressed as bias) never exceeded 11.3% (intraday) and 12.2% (interday) [41].

The total HCN in the stone-fruit spirits was photo-metrically determined after hydrolysis with potassium hydroxide and reaction with chloramine-T and pyridine/barbituric acid reagent using the method of Wurzinger and Bandion [58]. The limit of detection was 0.15 mg L⁻¹ of HCN.

Results and discussion

Recent developments in design and performance of FTIR spectrometers, combined with advances in chemometrics software, have provided an interesting analytical tool suitable for rapid product screening and process control [49]. In principle, EC shows characteristic IR spectra with intensive bands, especially for NH₂ and C=O absorptions [59, 60]. However, the study showed that in the spirit drink matrix, the absorptions of various functional groups of water, ethanol and volatile congeners overlapped the EC absorptions. In addition, the concentration of EC was significantly lower than other constituent levels. Stone fruit spirits display very similar bands, which cannot be assigned to EC or any other individual compound (Fig. 1). Therefore, chemometric techniques must be used to interpret the spectra. In comparing wavenumbers [selected using multivariate

data analysis (Table 1)] with EC spectra (from refs. [59, 60]) some similarities can be observed. The bands around 1,760–1,770 cm^{-1} may be explained by the C=O stretching vibrations of EC, which is typically the strongest band of EC. The bands around 1,134–1,138 cm^{-1} may have resulted from NH_2 rocking vibrations. Most of the selected wavenumbers cannot be assigned to bands from the EC spectrum. This may be explained by spectral shifts due to the ethanol–water matrix in comparison with the solid-state spectra from literature. Another possibility is that the multivariate statistics have identified wavenumbers of other compounds, which show a co-linear relationship to EC. Such compounds may be transitional or supplementary reaction products of the EC formation. The FTIR spectrum also contains information about the maximum EC content, which is normally determined after UV irradiation of the sample lasting 4 h. The FTIR calibration for this parameter appears to incorporate wavelengths of both EC (1,230–1,234, 1,766 cm^{-1}) and its precursor HCN (1,469, 1,793, and 995–1,006 cm^{-1}).

This information hidden in the FTIR spectra about the maximum content of EC and its precursor HCN elevates consumer protection. Despite the efforts of food control to prevent EC formation after sampling, this specific EC concentration (reflecting the actual status after bottling or in trade) is not entirely of concern to the consumer. Only the EC concentration at consumption would be relevant. In many cases, the content levels would have significantly increased at this point because spirit drinks are usually not stored in areas protected from light by either traders or consumers.

Calibration and validation of PLS procedure

Because FTIR is a secondary analytical technique, it was first necessary to calibrate the instrument against the

chemical reference method. Table 2 shows information concerning the reference data. Clearly, the range of reference values encompasses the characteristic appraisal of a broad range of spirit drinks. Table 3 depicts the results obtained through calibration and validation. The minimum value of standard error of prediction (SEP) determined the number of PLS factors, thus avoiding overfitting problems. The values of coefficient of multiple determination (R^2 for the calibration set) and standard error of calibration (SEC) indicate the precision achieved in calibration. In the calibration set, good quantitative information is available for both actual and maximum EC ($R^2 = 0.76$ and 0.77 , respectively). The HCN exhibited an excellent correlation ($R^2 = 0.93$). The analytes were determined with acceptable degrees of precision (SEC values between 0.29 and 0.40 mg L^{-1}).

The results of the calibration testing with the independent validation set are expressed in the statistical parameters of SEP, coefficient of multiple determination (Q^2 for the validation set) and the mean bias. The Q^2 values were significantly lower in the validation set than R^2 values in the calibration set. Values between 0.71 and 0.76 are on the boundary between the criteria proposed by Shenk and Westerhaus [61] for good quantitative information (0.7–0.9) and mere qualitative separation (0.5–0.7). However, the correlation was higher than that of the near-infrared (NIR) spectroscopic method of Manley et al. [51] ($r = 0.47$) used in wine analysis. This lesser correlation can be explained by the lower concentrations of EC in wine (in the $\mu\text{g L}^{-1}$ range).

The negative mean bias values in the validation set revealed that FTIR prediction furnishes systematically higher concentrations than the reference analyses. The validation set also showed a minor precision when compared with the calibration set as indicated by SEP values twice as high as SEC values. The fact that calibration methods will never perform better than the reference method, which has a coefficient of variation of

Table 2 Reference data for the actual ethyl carbamate concentration in the samples, the EC concentration after UV irradiation (maximum EC), and the HCN concentration

	Calibration set			Validation set		
	<i>n</i>	Range	Mean (SD)	<i>n</i>	Range	Mean (SD)
Actual EC (mg L^{-1})	82	0–5.86	0.81 ± 1.08	82	0–5.18	0.83 ± 1.12
Maximum EC (mg L^{-1})	82	0–7.30	1.11 ± 1.52	82	0–6.65	1.15 ± 1.58
HCN (mg L^{-1})	65	0–10.97	1.02 ± 2.23	62	0–4.96	0.64 ± 1.17

Table 3 Validation results of the calibration set and the independent validation set

	Calibration set				Validation set		
	PLS factors	SEC	Repeatability	R^2	SEP	Mean Bias	Q^2
Actual EC (mg L^{-1})	7	0.37	0.03	0.76	0.52	–0.12	0.75
Maximum EC (mg L^{-1})	7	0.40	0.04	0.77	0.67	–0.26	0.71
HCN (mg L^{-1})	7	0.29	0.02	0.93	0.42	–0.11	0.76

approximately 10%, must be also considered. All in all, the calibration was not accurate enough to be used in the context of a quantitative determination; however, it can be used semi-quantitatively to separate samples into high and low groups within the context of a screening analysis. This is in accordance with the results of Manley et al. [51], who found that the correlation of the NIR method lacked adequate accuracy for quantitative prediction of EC in wine, but qualitative classification was still possible.

Results for the calibration and validation sets are further broken down in Table 4 to show the percentages of correctly identified samples above and below the rejection level of 0.8 mg L^{-1} . An overall correct classification rate of 85–90% for the calibration set and 77–83% for the validation set was achieved. Of foremost importance is the number of false negative samples, which would remain undetected if no reference analyses were made in the application of this method. In the calibration set, only four false negative results were certified for both actual and maximum EC. In the validation set, false negative sample results totalled five for actual EC and three for maximum EC. As with every screening procedure, a compromise between the number of false positive and false negative results must be established and directly related to the chosen cut-off limits. In this case, it may be possible to avoid false negative results by lowering the cut-off level to 0.6 mg L^{-1} . This method, however, has the disadvantage of requiring a higher number of samples for reference analyses.

If the screening procedure was applied to stone-fruits samples, approximately 60–70% would be classified as negative and thus not be submitted for expensive GC/MS analyses.

Applicability in routine analysis

As previously mentioned, multiplying stipulations and expenses in both government and commercial fields have compelled the replacement of traditional reference methods with accelerated and less expensive processes. To this end, screening methods, which ensure a high sample throughput, seem to be most advantageous. Rapid information retrieval concerning EC and HCN within the stone-fruit spirit sample and the absence of sample preparation requirements indicate that FTIR is unique in its ability to comprehensively survey a large number of samples. A comparison between the FTIR screening procedure and the GC/MS/MS reference analyses is given in Table 5. The FTIR method is substantially faster (only 2 min per sample) and easier to use. Time-consuming sample preparation (as in extraction) is not required. Sample throughput is more than 60 times higher than results obtained by GC/MS/MS. FTIR also offers an environmentally friendly method that eliminates the use of solvents.

With information gained by FTIR screening, decisions can be made as to whether additional analyses (with more time-consuming and expensive, but more accurate, standard procedures) are required. It should be noted that the relatively high SEP values and the semi-quantitative character of the FTIR calibration demand an obligatory confirmation by GC/MS/MS before products are officially rejected.

Conclusion

This FTIR approach offers considerable advantages over conventional methods of analysis. Complex and

Table 4 Percentage correct classification of ethyl carbamate concentration ranges in stone-fruit spirits using FTIR and PLS prediction

EC concentration range (mg L^{-1})	Classification quote		Classification quote	
	Calibration set		Validation set	
	Actual EC	Maximum EC	Actual EC	Maximum EC
< 0.8	91% (51 of 56)	88% (45 of 51)	85% (51 of 60)	78% (46 of 59)
> 0.8	85% (22 of 26)	87% (27 of 31)	77% (17 of 22)	87% (20 of 23)
Overall	89% (73 of 82)	88% (72 of 82)	83% (68 of 82)	80% (66 of 82)

Table 5 Comparison between GC/MS/MS reference procedure and FTIR screening

	GC/MS/MS reference procedure	FTIR screening procedure
Sample preparation	Extrelut extraction (80–100 min)	–
Analysis	GC/MS/MS (40 min)	FTIR/PLS (2 min)
Total time	Approx. 2 h	2 min
Applicability	Accurate quantitative determination	Fast semi-quantitative determination to select conspicuous samples for confirmatory GC/MS/MS analysis

labour-intensive reference analyses are only required when conspicuous analysis results, which exceed the cut-off limit (and may lead to official rejection of the product), require confirmation. FTIR will therefore acquire increasing importance as a routine method in beverage analysis.

In the future, further quality-relevant parameters for stone-fruit spirits, such as alcoholic strength and the content of volatile congeners, may be calibrated and simultaneously determined with EC and HCN.

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