Research Article

Rapid Quantification of Ethyl Carbamate in Spirits Using NMR Spectroscopy and Chemometrics

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Ethyl carbamate (EC, urethane, C₂H₅OCONH₂) is a genotoxic carcinogen and is regularly found in fermented food products including alcoholic beverages. In this study, the rapid method of nuclear magnetic resonance (NMR) spectroscopy in combination with partial least squares (PLS) regression is applied for the first time to the analysis of ethyl carbamate in stone fruit spirits (n = 119) and unrecorded alcohols (n = 27) (analysis time only 15 min per sample). The PLS procedure was validated using an independent set of samples (n = 43, R² = 0.89, RMSE = 0.14 mg/L) in comparison to reference GC/MS/MS results. The NMR method was found to outperform other screening techniques based on NIR or FTIR regarding sensitivity and selectivity. The major advantage over GC/MS/MS, besides the reduced time of instrumental analysis, is that no sample preparation besides addition of buffer with internal standard is required, while for GC/MS/MS labour-intensive sample extraction is necessary prior to measurement.

1. Introduction

Ethyl carbamate (EC) may occur as contaminant in fermented foods and beverages with highest concentrations generally found in stone fruit spirits. Concerns over this compound were raised as it is possibly carcinogenic to humans [1] and its margin of exposure may reach public health relevant ranges [2–4]. According to a recent European commission recommendation, a level of 1 mg/L for stone fruit spirits was set as a target value [5]. In addition, the commission stated that the levels of EC in stone fruit spirits and stone fruit marc spirits should be monitored and the results should be reported.

Therefore, an increasing demand to develop a reliable, sensitive, and cheap method for ethyl carbamate quantification exists. Up to now, laborious procedures such as gas chromatography, coupled with nitrogen-phosphorus detector (GC/NPD) [6], mass spectrometry (GC/MS) [7–11], high-resolution mass spectrometry (GC/HRMS) [12], time-of-flight mass spectrometry (GC/ToFMS) [13], or tandem mass spectrometry (GC/MS/MS) [14, 15] as well as high-performance liquid chromatography with fluorescence detector (HPLC/FLD) [16] or with atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS) [17], are regarded as reference for analysis of ethyl carbamate in alcoholic beverages.

Due to the strong matrix effect of spirits, particular emphasis has been put on sample preparation and the detection after chromatographic separation. Approaches for sample preparation included Extrelut extraction [10, 14, 18], headspace solid-phase microextraction (HS-SPME) [6, 19, 20], and solid-phase extraction (SPE) [12, 17]. An alternative way to decrease matrix effects is the use of derivatization of EC with xanthydrol for HPLC/MS analysis [21].

Besides these quantitative reference procedures, spectroscopic techniques have been evaluated for screening analysis of ethyl carbamate, including FT near-infrared (NIR) and FT infrared (IR) spectroscopy [22, 23]. However, these methods were found to lack the accuracy required for a quantitative determination and could only be used semi-quantitatively in the context of a screening analysis. To the best of our knowledge, nuclear magnetic resonance (NMR)
spectroscopy has not been evaluated for EC quantification in food in general and in alcoholic beverages in particular. Therefore, in this study, NMR spectroscopy in combination with partial least squares (PLS) regression was applied for the first time for quantitative determination of ethyl carbamate in stone-fruit spirits and unrecorded alcohols.

2. Experimental Section

2.1. Sample Collective. A total of 146 samples were analyzed for ethyl carbamate. This includes stone fruit spirits \((n = 119)\) submitted to the CVUA Karlsruhe for official food control purposes in Baden-Württemberg, Germany, in the context of the current EU monitoring program \([5]\). Furthermore, unrecorded alcohol samples \((n = 27)\) were included to check the applicability for a broader range of alcoholic beverages. The samplings of this group of products were conducted in the context of international projects designed to characterize the quality and potential risks of illegally produced alcoholic beverages. To eliminate the possibility of light-induced ethyl carbamate formation in samples during transport and storage, the bottles were wrapped in aluminium foil immediately after sampling.

2.2. NMR Measurements. All NMR measurements were performed on a Bruker Avance 400 Ultrashield spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5 mm SEI probe with Z-gradient coils, using a Bruker Automatic Sample Changer (B-ACS 120). All spectra were acquired at 300.0 K. The data were acquired automatically under the control of ICON-NMR (Bruker Biospin, Rheinstetten, Germany), requiring about 15 min per sample.

The NMR protocol applied was previously described in detail \([24]\). In short, a shaped pulse sequence during the relaxation delay is applied to suppress the eight \(^1\)H NMR frequencies of water and ethanol (the OH singlet of both water and ethanol, as well as the CH\(_2\) quartet and CH\(_3\) triplet of ethanol). The sequence of reference measurement for frequency determination followed by the suppression experiment is controlled by a macro in the acquisition software so that a measurement under full automation is possible. Compared with the standard water presaturation pulse program, the eightfold suppression allowed a significantly higher setting of receiver gain without receiver overflow, which significantly increased the signal-to-noise ratio by an average factor of 10 \([24]\). In addition to this 1D experiment, an additional experiment to acquire 2D spectra was used. This two-dimensional J-resolved spectrum with multiple suppression was recorded applying the same shape during relaxation delay as in the 1D experiment described in \([24]\). After 16 dummy scans, for each of the 40 \(t_d\)-increments 4 numbers of scan were collected into a time domain of 8k complex data points during an acquisition time of 0.6144500 and hence covering a spectral width of 16.6612 ppm.

2.3. Chemometrics and Validation Studies. The resulting spectra were analyzed using the software Amix version 3.9.4 (Bruker Biospin, Rheinstetten, Germany). We tested several spectral regions of 1D spectra for calculation: aliphatic (0.25–3.0 ppm), mid-field (3.0–6.0 ppm), aromatic (6.0–10 ppm) with 0.01 ppm bucket width, and the region selective to ethyl carbamate (4.00–4.20 ppm) with a bucket width of 0.001 ppm. The bucket width of 0.04 was used to evaluate 2D J-resolved spectra in the same spectral regions. Residues of ethanol peaks at 1.32–1.08 and 3.52–3.79 ppm were excluded from the data sets if necessary.

First, PLS models for evaluation of all analyzed samples (both recorded and unrecorded alcohols, calibration set 1, \(n = 143\)) and a separate model for fruit spirits \((n = 119, \) calibration set 2) were validated by means of leave-one-out full cross-validation. To perform test set validation the NMR spectra and reference results of randomly chosen samples \((n = 76)\) from calibration set 2 were used as a data set for a separate PLS regression. The remaining 43 fruit spirits 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. The optimal number of factors, indicated by the lowest prediction error, was selected for all models.

Ethyl carbamate was purchased in proanalysis quality from Fluka (Buchs, Switzerland). Stock standard solutions were prepared at a final concentration of about 500 mg/L in distilled water. Calibration solutions were prepared by diluting the standard solution in an ethanol/water mixture (40\% v/v). The calibration curve was evaluated between 0.5 and 10 mg/L using 3-(trimethylsilyl)-propionate acid-d\(_4\) (TSP) as an intensity reference. The limit of quantification (LOQ) was calculated from the residual standard deviation of the regression line \([25]\). For all calculations statistical significance was assumed at below the 0.05 probability level.

2.4. Sample Preparation. In contrast to our previous experimental protocol \([24]\) where a dilution factor of 2 was chosen to ensure a wide variability of beverage types that can be measured, we optimised sample preparation specifically for fruit spirits in order to reach better sensitivity for ethyl carbamate. The optimized sample preparation protocol finally was as follows: 540 \(\mu\)L of beverage is mixed with 60 \(\mu\)L of pH 7.4 NMR buffer containing 0.1\% TSP as internal standard. With this measurement protocol no precipitation or signal distortions occurred for all analyzed samples.

2.5. Gas Chromatographic and Tandem Mass Spectrometric Reference Procedure. The analysis of ethyl carbamate was done using previously published procedures combining the Extrelut extraction procedure of Baumann and Zimmerli \([18]\) with modifications of Mildau et al. \([10]\) and tandem mass spectrometry (GC/MS/MS) according to Lachenmeier et al. \([14]\).

3. Results and Discussion

3.1. Direct EC Quantification. In \(^1\)H NMR of ethanol-water mixtures, only the resonances of the methylene group
of ethyl carbamate can be seen in the mid-field region (4.15–4.00 ppm) (Figure 1(a)). Signals corresponding to the presence of the amino group (7.70–7.60 ppm) cannot be seen under our experimental conditions due to fast proton exchange. The resonance of the methyl group (1.30–1.20 ppm) falls in the range of the ethanol triplet (CH₃ group, 1.32–1.08 ppm), which is hidden due to the suppression of the ethanol signals.

Attempts for direct quantification by integration (which would be the most simplistic and therefore desirable quantification method) failed due to several reasons. First and foremost, the direct quantification does not provide the required sensitivity. When determined according to DIN 32645 [25], the limit of quantification (LOQ) of ethyl carbamate was 2.7 mg/L for 1D NOESY experiment and 3.5 mg/L for 2D J-resolved spectra. Both values were definitely insufficient to control the EU target value of 1.0 mg/L [5]. Furthermore, we have observed extensive signal overlap in the region of the targeted quartet when authentic alcoholic beverages are measured (Figure 1(b)). In addition, the concentration of ethyl carbamate is significantly lower than other constituent levels (Figures 1(a) and 1(b)). This makes direct quantification impossible even in highly contaminated samples. Efforts to develop a standard addition protocol were also unsuccessful due to the same reasons. Only samples with ethyl carbamate content above 3.0 mg/L (which makes up only a small percentage of the submitted samples) can be quantified with 2D J-resolved NMR spectroscopy. But even then it was found that the same difficulties still occur and only rough quantification is possible (in some cases relative errors of quantification in comparison to GC/MS/MS reached 40%).

3.2. Calibration and Validation of the PLS Procedure. The second option was to use chemometric techniques to develop a more reliable method for ethyl carbamate quantification. The most commonly used choice in case of strong spectral overlap is PLS regression. For example, successful applications of NMR spectroscopy and PLS concerned the quantification of original gravity, ethanol, and organic acids in beer [26, 27].

In our preliminary experiments we evaluated all NMR spectral regions detailed in the Experimental section (both 1D NOESY and 2D J-resolved spectra). Results of the PLS models (Root mean square error (RMSE), correlation coefficient ($R^2$)) in different NMR regions are listed in Table 1. It came rather surprising for us that the best calibration model was constructed in the aromatic region (10–6.0 ppm). For comparison, the RMSE value for 2D J-resolved spectra in the 4.20–4.00 ppm range (actual region of EC) was 1.14 mg/L while for the aromatic range a lower error and better correlation was observed (RMSE = 0.13 mg/L, $R^2 = 0.98$) (Table 1).

| Type of spectra | NMR region | RMSE, mg/L | $R^2$ |
|----------------|------------|------------|-------|
| 2D J-resolved  | 10–6.0     | 0.13       | 0.98  |
| 2D J-resolved  | 4.20–4.00  | 1.14       | 0.65  |
| 1D NOESY      | 10–6.0     | 0.70       | 0.81  |
| 1D NOESY      | 4.20–4.00  | 0.79       | 0.82  |

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Detailed results of the PLS models in the aromatic region for 2D J-resolved spectra for calibration and independent data sets are listed in Table 2. The model where only stone fruit spirits were considered (calibration set 2) performs better than a unified model with unrecorded alcohols (calibration set 1). An independent set of samples was used
for validation (Table 2), which proved the applicability of the method as the correlation and error are judged to be acceptable for the purpose. Table 2 also provides information concerning the reference data. Clearly, the range of reference values encompasses the characteristic appraisal of a broad range of spirit drinks (both fruit spirits and unrecorded alcohol). The reference GC/MS/MS analysis has a relative error of about 8% [14], which equals 0.08 mg/L at the EU target value of 1 mg/L. As the PLS-NMR method is an indirect method based on calibration with the reference data, the RMSE of 0.14 mg/L in the validation set is higher, but still acceptable for the purpose.

Clearly, none of the chemical shifts can be assigned to resonances from the ethyl carbamate spectrum in the aromatic region where the best-fitting PLS model was constructed. Therefore, the PLS model relies not on structural characteristics of ethyl carbamate itself but derives from other reasons. Probably multivariate statistics have identified chemical shifts of other compounds, which show a colinear relationship to ethyl carbamate. Such compounds may be transitional or supplementary reaction products of the ethyl carbamate formation mechanism. The same observation was made in FTIR spectroscopy, where PLS quantification of ethyl carbamate was also based on colinear compounds [23]. In NMR, we have additionally observed that not a single substance appears to be responsible for the correlation in PLS. Resonances at 7.38, 9.98, 7.98, 6.78, and 8.50 ppm (in order of decreasing importance for the PLS model) are the key signals for differentiation. These resonances could only be identified with the aim of sophisticated techniques such as coupled high-performance liquid chromatography—nuclear magnetic resonance spectroscopy (HPLC-NMR) method. This will be a subject of further research.

### 3.3. Comparison with Other Screening Techniques and Applicability in Routine Analysis

Spectroscopic methods are nowadays widely used for the screening analysis of alcoholic beverages. In our case, we observed that NMR outperforms other spectroscopic techniques previously used for EC screening. For example, for FTIR $R^2$ and RMSE values for independent test set validation were found to be 0.71–0.76 and 0.42–0.67 mg/L correspondingly [23]. A cut-off level of 0.6 mg/L was established for this procedure which leads to a high number of samples that should be reanalyzed by reference procedures. The correlation for the Near-Infrared (NIR) spectroscopic method for wines was even worse [22]. Therefore, in spite of relatively higher cost of NMR instrumentation and longer measurement time (approximately 15 min in comparison with 2 min for FTIR), we believe that NMR can provide better quantitative results and still a high sample throughput. Considering the RMSE value, we propose a cut-off level of 0.85 mg/L for NMR. Samples below this level are clearly below the EU target value of 1 mg/L and no reference analysis is necessary in this case. Therefore, we can sort out approximately 80% of our samples, which need not to be subjected to reference analysis leading to considerable reduction of costs required for the EU monitoring program.

Furthermore it is possible to quantify other compounds such as methanol or acetaldehyde [24] from the same spectra as used for ethyl carbamate analysis, which further reduces the cost of analysis. The final advantage of application of NMR in routine analysis of foods and spirits in particular is that NMR allows a nontargeted screening for new or unknown contaminants or adulterations [28, 29]. With information gained by NMR screening, decisions can be made as to whether additional analyses (with more time-consuming and expensive reference procedures) are required.

### 4. Conclusions

In this study, an NMR method for determination of ethyl carbamate in alcoholic beverages was developed. Due to the comprehensive structural information provided by NMR spectroscopy, indirect quantification based on PLS calibration in the aromatic region is possible. The accuracy of the PLS model evaluated by test set validation was found to be sufficient to provide reliable ethyl carbamate quantification below the target level of 1 mg/L recommended by the European Commission. We are certain that NMR will acquire increasing importance as a routine method in beverage analysis.

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