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Original research

Simultaneous determination of ethanol and methanol in non-alcoholic beverages by precolumn derivatization and liquid chromatography with fluorescence detection

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ABSTRACT

The determination of ethanol content in non-alcoholic beverages is driven by health concerns and is often imposed by regulating agencies. On the other hand, methanol poisoning leads to high health risks. In addition, the application of the Halal concept in Islamic countries requires the availability of efficient analytical methods. This work reports the development of a simple and sensitive HPLC method for the simultaneous determination of ethanol and methanol in juices and beverages. The method is based on the formation of a stable derivative via the reaction of ethanol and methanol with 9-fluorenylmethyl chloroformate (Fmoc-Cl). The resulting derivatives are detected by a fluorescence detector. The method's LODs and LOQs were 0.004 g/L and 0.01 g/L, for methanol, and 0.015 g/L and 0.05 g/L, for ethanol, respectively. The mean recovery ranged from 98–109% for both methanol (RSD = 3.7%), and ethanol (RSD = 4.1%). This approach of determination is sensitive and simple in that it requires only a 100 µL sample volume and the reaction product (derivative) can be directly injected without further extraction or pre-concentration. Analysis of a number of non-alcoholic drinks from the domestic market showed that the ethanol content ranged between 0.11 - 0.71 g/L.

Keywords: 9-Fluorenylmethyl chloroformate; Food analysis; Ethanol content; Alcohol-free beverages; Energy drinks

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1. Introduction

It has been evidenced that alcohol interacts with food components leading to changes in the biological functions of food (Verplaetse & McKee, 2017; Watson et al., 2013; Zhang et al., 2022). For children and young adults, fruit juices, bananas, bread, and bakery products are a major source of food-derived alcohol (Gorgus et al., 2016). Several studies have raised concern about the toxicity of ethanol in food for children (Gaw & Osterhoudt, 2019; Vojvodić et al., 2023). In addition, information regarding ethanol contents in foodstuffs is very limited and focuses specifically on the criminal implications of these sources of exposure. Even with low ethanol content, some juices and soft drinks can pose health risks to children and young adults due to overconsumption.

On the other hand, hundreds of deaths from alcohol adulterated with methanol have been reported all over the world. For example, Iran recorded 959 cases (France-Presse, 2018), Cambodia reported 213 cases (David, 2018), India reported 95 cases (Schultz & Kumar, 2019), and Russia reported 34 cases (Russian news agency, 2021), among other places. Methanol undergoes conversion to formic acid and formaldehyde that are highly toxic substances (Garg et al., 2021). Selective detection of methanol in the presence of a much higher ethanol background is challenging (van den Broek et al., 2019). A range of analytical methods has been reported in the literature for the determination of ethanol and methanol in foodstuffs; examples include gas chromatography (GC-FID or MS) (Feng et al., 2017; Park et al., 2016), spectrophotometry (Febriani & Ihsan, 2020), enzymatic derivatization (Lacorn & Hektor, 2018; Susparini et al., 2019), and H^1NMR (Burkhardtsmaier et al., 2021; da Silva Nunes et al., 2016). However, these methods suffer from complexity, long analysis time, and low throughput. For example, spectrophotometric

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methods based on dichromate oxidation require a large sample volume with tedious sample preparation. Refractive index-based methods are simple, but are only applicable to neat matrices and cannot be used with complex food samples. Although enzymatic methods are promising, they still suffer from low stability of the enzyme-substrate, low accuracy, and poor reproducibility. Methods based on techniques such as modular Raman spectrometry, near-infrared spectroscopy, and NMR are time-consuming and require expensive hardware that not every laboratory can afford.

Direct determination of ethanol and methanol using liquid chromatography with UV or fluorescence detection is difficult. HPLC methods coupled to a refractive index or a combination of UV-flame ionization detectors were reported (Avila et al., 2018; Yarita et al., 2002). However, these methods are time-consuming and have only been applied to matrices of relatively low complexity such as alcoholic beverages and gasoline.

In the present work, we report the development of an HPLC method for simultaneous determination of ethanol and methanol after precolumn derivatization using 9-Fluorenylmethyl chloroformate (Fmoc-Cl). The resulting derivatives are then detected with a fluorescence (FL) detector. The method is easy to apply, sensitive and selective. Under optimal experimental conditions, the typical chromatographic run time is just over 4 min.

2. Material and Methods

2.1. Reagents and materials

Ethanol ≥ 99.9%, methanol ≥ 99.9%, 1-propanol ≥ 99.9%, and acetonitrile HPLC grade were obtained from Merck, KGaA, Darmstadt, Germany. 9-Fluorenylmethyl chloroformate, potassium dihydrogen phosphate, and potassium hydrogen phosphate were procured from Carl Roth GmbH, Karlsruhe, Germany. Samples of juices and soft drinks were purchased from local supermarkets. Ultrapure water was produced using a Sartorius Arium® water purification system (Sartorius AG, Goettingen, Germany). C18 solid-phase extraction cartridges (Oasis HLB 3 cc Vac Cartridge, 60 mg) and syringe filters were obtained from Waters GmbH, Helfmann-Park, Eschborn, Germany.

2.2. Instrumentation

Chromatographic analysis was performed using a Hitachi-high performance liquid chromatography (HPLC) system coupled with a photodiode array detector (PDA) and a fluorescence detector and driven by Agilent EZChrom Elite 3.2.0 software. This system was composed of a quaternary pump, an autosampler, a mobile phase degasser, and a thermostated column compartment. A reversed-phase analytical column (C18, 150 × 2.1 mm, 1.7 µm; Waters, Ireland) was used. The mobile phase used was water: acetonitrile: methanol (24:26:50, v/v). The optimal UV detection wavelength was 210 nm and the excitation and emission wavelengths were 265 and 345 nm, respectively. The injection volume was 20 µL. Elution was done under isocratic mode at a 1.0 mL/min flow rate. The analytical column compartment was maintained at 40°C.

2.3. Derivatization procedure

Aliquots of blank, calibration standards, and beverages samples (100 µL) were pipetted into 100 mm × 13 mm Pyrex® test tubes with standard ground stoppers. The samples were mixed with 100 µL of Fmoc-Cl (2 mg/mL) and 100 µL of phosphate buffer (pH 8.2). The sample mixture was gently shaken and incubated at 40°C for 40 min in a digitally controlled water bath. A volume of 20 µL of the reaction mixture was injected into the HPLC system using an autosampler.

2.4. Preparation of calibration curve

The linearity of the detector's response to the analytes' concentration was assessed using a 7-point calibration curve prepared using the procedure described above. The ethanol and methanol standard curves were made according to concentration and peak area. Precautions related to the preparation of standard solutions and spiked food samples were considered (Lacorn & Hektor, 2018). The standard solutions were prepared fresh on a daily basis.

2.5. Validation procedure

The developed method was validated according to ICH guidelines (ICH Harmonised Tripartite Guideline, 2005), in terms of the following analytical parameters: linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy.

2.5.1. Linearity

The analytical curves for methanol and ethanol samples were constructed in the range of 0.01–5.0 g/L. The linearity parameter was established by injecting triplicates of standard solutions at each point, and the least squares linear regression was used to fit the curves.

2.5.2. Limit of detection and limit of quantification

The LOD and LOQ were calculated according to equations 1 and 2.

$$LOD = 3.3\frac{\sigma_{LC}}{S} \quad (1)$$

$$LOD = 10\frac{\sigma_{LC}}{S} \quad (2)$$

where σ_{LC} is the standard deviation of the lowest concentration (LC) tested and S is the slope of the analytical curve.

2.5.3. Precision

In order to assess the method's reliability, parameters such as repeatability and intermediate precision were calculated. Six repetitions at a 100% level were used to confirm repeatability. Analytical curves created on two separate days in the same laboratory were used to assess intermediate precision, and the findings were compared using a one-way ANOVA (analysis of variance). Equation 3 showed that precision was defined as relative standard deviation (RSD, in percent).

$$RSD(\%) = \frac{\sigma}{\overline{C}} \times 100 \quad (3)$$

where σ stands for the standard deviation of the lowest concentration standard and $\bar{C}$ is the average concentration.

2.5.4. Accuracy

Accuracy was expressed as recovery (R, in %) and calculated at different concentration levels (lower, middle and higher) of methanol and ethanol (triplicate) for each analytical curve. To the first calibration curve the concentrations were 0.05, 0.1, 0.5, 1.0, 5.0 g/L. Accuracy was calculated by equation 4.

$$R(\%) = \frac{C_m}{C_{ex}} \times 100 \quad (4)$$

where Cm and Cex stand for the measured and expected concentrations, respectively.

2.5.5. Matrix effect

The matrix effect (ME) in food analysis is both common and challenging. Calculation of matrix effects is used to determine any damping or enhancement of the analyte's peak(s) in the real matrices. This effect is calculated by the quotient of the post-spike to a neat sample as follows:

$$ME = \left[1 - \left(\frac{Peak\ Area\ of\ Post - Spike}{Average\ Peak\ Area\ of\ \boldsymbol{n}\ Neat\ samples}\right)\right] \times 100 \quad (5)$$

where n ≥ 3.

3. Results and Discussion

3.1. Optimization of derivatization conditions

Fmoc-Cl is a chloroformate ester used in organic synthesis to offer the fluorenylmethyloxycarbonyl protecting group as the FMOC carbamate (Konnert et al., 2014). Solvolysis of aromatic chloroformate esters (ArOCOCl) is a substitution organic reaction (Bunton et al., 1986). In the present method, Fmoc-Cl is derived into its ethylated form by reacting with ethanol under slightly alkaline aqueous conditions. The proposed reaction is illustrated in Fig. 1.

The derivatization conditions were optimized using a sample volume of only 100 µL. The optimized factors include the amount and concentration of Fmoc-Cl, derivatization time, pH of the reaction medium, and reaction temperature. The amount of Fmoc-Cl was tested in the range of 25 – 200 µL. The reaction temperature and incubation time were tested in the ranges of 0 – 60℃ and 0 – 60 mins, respectively, using a digitally controlled water bath. The effect of medium acidity was tested in the range of pH 6.2 – 8.2 using phosphate buffer.

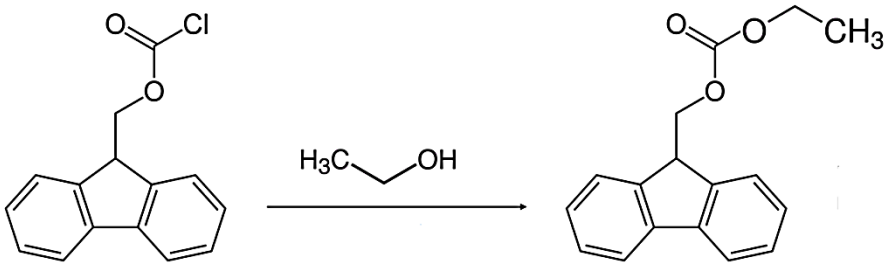

Fig. 1. The derivatization reaction.

Table 1. Validation parameters for HPLC-FL method.

Parameter	Ethanol	Methanol
Linearity range (n = 5) / g/L	0.01– 5.0	0.01– 5.0
Regression equation	y = 2E+06x - 18282	y = 2E+06x - 4654.8
Determination coefficient (R^2)	0.9992	0.9989
LOD / g/L	0.015	0.004
LOQ / g/L	0.05	0.01
Repeatability (RSD) / %	4.1	3.7
Recovery / %	98-109	98-109

3.2. *Effect of pH on Fmco-Cl derivatization*

The efficiency of the Fmco-Cl derivatization reaction was found to be pH-dependent. As can be seen from Fig. 2, the efficiency of the derivatization reaction was maximized when the pH of the medium was 8.2. This result is expected because the derivatization reaction produces hydrochloric acid as a by-product, thus an alkaline medium is necessary to allow the reaction to proceed towards completion.

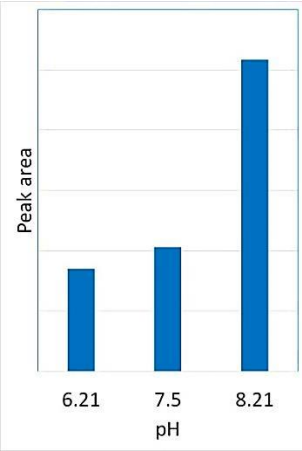

Fig. 2. Effect of acidity on the derivatization reaction.

3.3. *Effect of amount and concentration of Fmco-Cl*

For a 100 µL sample, the amount and concentration of Fmco-Cl needed to react with the full amount of ethanol and methanol in the sample was optimized. As can be seen from Fig. 3, the response increases as the amount of Fmco-Cl increases from 25 µL to 100 µL beyond which the effect becomes negligible. The concentration of Fmoc-Cl was optimized to 2 mg/mL.

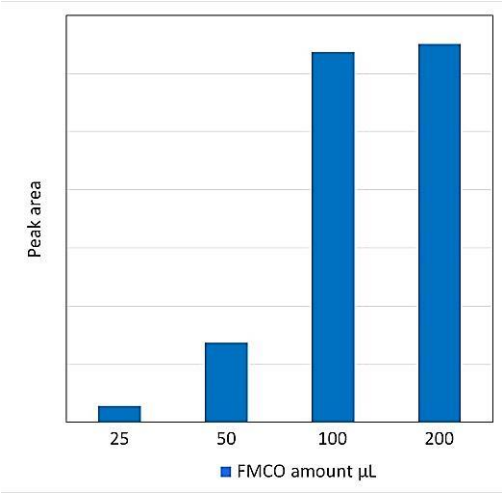

Fig. 3. Effect of amount of Fmco-Cl on the derivatization reaction.

3.4. *Effect of incubation time and temperature*

The incubation time was tested in the range of 0–60 min. Fig. 4 shows that ethanol recovery (represented by peak area) increases with increasing incubation time from 0 to 40 min, after which the increase becomes insignificant. The incubation temperature was tested in the range of 20-60°C. The ethanol recovery was maximized at 40°C.

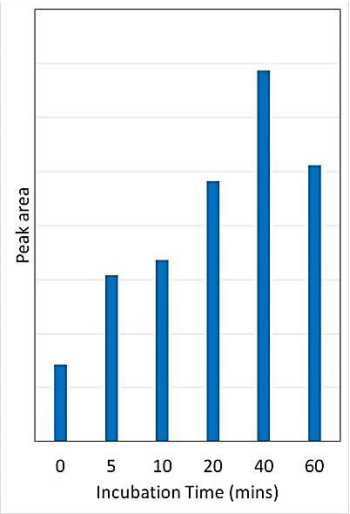

Fig. 4. Effect of incubation time on the derivatization reaction.

3.5. *Optimization of chromatographic conditions*

Factors that influence HPLC method performance such as mobile phase, column temperature, UV detection wavelength, fluorescence excitation, emission wavelengths, and flow rate were optimized for achieving optimal detection conditions. Methanol, acetonitrile, and water were used for optimizing the mobile phase. The maximum selectivity was achieved using an elution mixture of methanol, acetonitrile, and water, 50:26:24%, respectively, at a flow rate of 1.0 mL/min. The same selectivity was also achieved using an elution mixture of methanol and water in proportions of 76:24%, respectively, but with a greater retention time. A column temperature of 40 °C was found optimal, probably because it is the same as the sample incubation temperature. To achieve an acceptable detection selectivity, UV detection was tested at the wavelengths 264 nm and 210 nm based on the absorption spectrum. The best detector responses were obtained at the wavelength of 210 nm for the UV detector. Detection at the wavelength 264 nm resulted in a chromatogram with lesser background peaks but lower detector sensitivity. For optimizing FL detection, excitation and emission wavelengths were investigated in the ranges of 250 – 270 nm, and 310 – 360 nm, respectively. The best sensitivity and selectivity of the fluorescence detector were obtained with excitation and emission wavelengths of 265 nm and 345 nm, respectively. An interference test was performed by analyzing ethanol and methanol in the presence of 1-propanol. As can be seen in Fig. 5, peaks of methanol and ethanol are well separated. No peak was detected for 1-propanol.

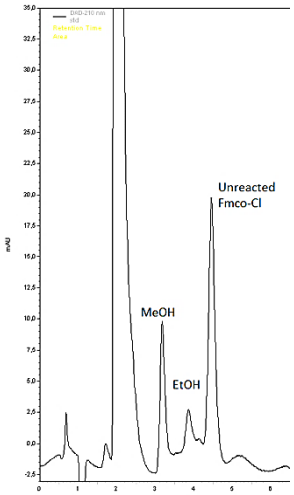

Fig. 5. A typical chromatogram of a standard sample spiked with ethanol, methanol and propanol.

3.6. *Method performance*

Sufficient peak resolution was achieved between the ethanol peak and other matrix peaks. The ethanol peak was selectively eluted with no co-eluted peaks, splits, shoulders, or other indications of co-eluting compounds. Under the optimal chromatographic and extraction conditions, the limit of detection (LOD) and limit of quantification (LOQ) were 0.004 g/L and 0.01 g/L for Methanol, and 0.015 g/L and 0.05 g/L, for ethanol, respectively. LOD and LOQ were calculated as 3 times and10 times the signal noise of the baseline, respectively (Joint Research Centre (European Commission) et al., 2016).

The linear dynamic range for this method was confirmed using a seven-point calibration curve over the range of 0.003 – 5.0 g/L and 0.01 g/L – 5.0 g/L with a square correlation coefficient (R^2) of 0.9989, and 0.9992 for methanol and ethanol, respectively. The mean recovery ranged from 98–109% with a relative standard deviation (RSD%) of 4.01% and 3.7% for methanol and ethanol, respectively. The results are summarized in Table 1.

3.7. *Analysis of real samples*

The analytical method developed in this study was applied to six samples of juices and soft drinks purchased from the local market, Rüsselsheim, Germany. The samples were stored at 4 °C until use. An amount of 5 mL of each sample was centrifuged at 2000 rpm for 5 min to remove solid particles. The clear sample was filtered through a 0.45 µm membrane. The samples were subject to derivatization reaction as detailed above, and then a volume of 20 µL of the reaction mixture was injected into the HPLC system using an autosampler. Satisfactory separation of ethanol peak from matrix peaks was achieved. To confirm the results, the real samples were spiked with ethanol and methanol at two concentration levels i.e., 0.5 g/L and 10 g/L. The results showed that the ethanol content ranged between 0.11 - 0.71 g/L. However, methanol was not detected in any of the tested beverages. The energy drink sample contained ethanol at a concentration of 0.11 g/L. A previous study showed that many energy drinks contained ethanol in the range of 0.05 to 2.3 g/L (Lutmer et al., 2009). A higher ethanol concentration of 0.71 g/L was found in the apple juice sample (100% juice), which is slightly higher than previously reported values of 0.06 – 0.66 g/L (Gorgus et al., 2016), and 0.12 g/l – 0.38 g/L (Hämmerle et al., 2011). The bio lemonade drink sample showed a concentration of 0.63 g/L, which is close to a previously reported value of 0.56 g/L found in a 10% lemon-based juice (Goldberger et al., 1996). The drink containing mainly 45% apple juice and 10% rhubarb spritzer showed an ethanol concentration of 0.31 g/L. The sample of 29% pear juice contained ethanol at a concentration of 0.45 g/L. The drink containing 2% lemon and 2% orange showed an ethanol content of 0.16 g/L, Table 2.

Table 2. Endogenous ethanol contents in selected samples of beverages.

Sample code	Main sample compositions	Ethanol content (g/L)
R-B	Energy drink	0.11
A-100	100% apple juice	0.71
A-45	45% apple juice	0.31
P-29	29% pear juice	0.45
D-2	2% lemon,	0.16
BL	bio lemonade drink	0.63

3.8. *Matrix effect*

The matrix effect was assessed by spiking real samples with ethanol at two concentration levels, i.e., 1.0 and 10.0 g/L. The results were compared with standard samples prepared at the same concentration levels. It was observed that tested matrices had different suppression matrix effects ranging from an acceptable effect (0.0 – 20%) to a strong effect of up to -41%. However, no response enhancement was observed. It is worth noting that the matrix effects were stronger with the fluorescence detector than with the UV detector.

Based on these results, it was suggested that low recoveries might be caused by matrix components that react with Fmoc-Cl leaving insufficient amount of it to react with the entire quantity of ethanol. So, to test this hypothesis, the concentration of Fmoc-Cl was increased from 1mg/mL to 2mg/mL. The results proved the hypothesis correct and satisfactory recoveries were obtained.

4. Conclusion

With the increasing consumption of non-alcoholic beverages, it is necessary to adopt a robust and versatile method for determining the ethanol and methanol content of these products to ensure public safety. The method presented here was optimized and validated, and has demonstrated reliable performance for the determination of ethanol and methanol content in non-alcoholic beverages using HPLC combined with a fluorescence detector. This method is simple and selective and can be adopted as an alternative to GC methods to monitor/control the ethanol and methanol content in beverages and ensure that regulatory requirements are met.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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