A novel, rapid and sensitive HPLC method for the determination of ethanol in non- alcoholic beverages with pre-column derivatization and simultaneous UV and fluorescence detection

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Abstract

Determination of the ethanol content in non-alcoholic beverages is driven by health concerns and is often imposed by regulating agencies. In this paper, we present the development of a simple and sensitive HPLC method for the determination of ethanol in Juices and beverages. Ethanol undergoes precolumn derivatization using 9-Fluorenylmethyl chloroformate (Fmoc-Cl). The resulting derivative has the property that it can be detected using both UV and fluorescence detectors, which makes ethanol quantification in complex matrices more accessible. LOD and LOQ were 0.004 g/L and 0.01 g/L %, for fluorescence detector, and 0.15 g/L and 0.5 g/L, for UV detector, respectively. The mean recovery ranged from 98–109% with a relative standard deviation (RSD%) of 3.7%. This approach to ethanol determination is novel and the method is so sensitive that only a 100 µL sample volume is required and the reaction product (derivative) can be directly injected without extraction or pre-concentration. The resulting derivative has the property that it can be detected using both UV and fluorescence. Ethanol is usually determined by GC, thus, the present method serves as an alternative to current GC methods.

Introduction

World Health Organization (WHO) estimated that more than 3 million deaths every year are linked to alcohol use especially among young people, with emerging evidence linking the health burden of HIV/AIDS and tuberculosis to the harmful use of alcohol (World Health Organization, 2004). Additionally, WHO estimated that approximately 13.5% of the total deaths in the age group 20–39 years are alcohol-attributable (World Health Organization, 2018). Additionally, it has been evidenced that alcohol interacts with food components leading to changes in the biological functions of food (Mayne et al., 2001; O'Brien et al., 2008, 2013; Verplaetse and McKee, 2017). For children and young adults, fruit juices, bananas, bread, and bakery products are a major source of food-derived alcohol (Gorgus et al., 2016). Although only limited data are available regarding the toxicity of ethanol to children, it has been assumed that symptoms of acute toxicity in children occur at a starting dose of 0.3 g/kg b.w (Vogel et al., 1995). In addition, information regarding ethanol contents in foodstuffs is very limited and focuses specifically on the criminal implications of these sources of exposure. Albeit containing low concentrations of ethanol, juices and soft drinks are among those food products that can pose health risks to the public, especially children and young adults, who consume a lot of non-alcoholic beverages without paying attention to their ethanol content.

Determining the amount of ethanol in commercial food products is important to quality control and public health. To effectively monitor the ethanol content in food products, a simple, fast and reliable analytical method is necessary. A range of analytical methods has been reported in the literature for the determination of ethanol in foodstuffs, some of which have employed gas chromatography (GC) with FID or MS detectors (Dorubet et al., 2009; Feng et al., 2017; Liu et al., 2014; McLachlan et al., 1999; Oliveira et al., 2005; Park et al., 2016), spectrophotometry (Febriani and Ihsan, 2020, 2020; Gros, 2011), enzymatic derivatization (Kuswandi et al., 2014; Lacorn and Hektor, 2018; Nurhidayat, 2019; Prodromidis and Karayannis, 2002), 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 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.

Due to its properties, ethanol cannot be directly determined by high-performance liquid chromatography using UV or fluorescence detection, therefore, only a few HPLC methods have been reported that use a refractive index (RI) detector (Avila et al., 2018; Martin et al., 1986), a combination of UV-flame ionization detectors (FID) (Yarita et al., 2002). Direct analysis of ethanol was reported by adding a low concentration of a UV-absorbing compound, such as acetone, that coelutes with the ethanol peak which is then detected as a negative peak (Betz and Nikelly, 1987). However, these methods have only been applied to matrices of relatively low complexity such as alcoholic beverages and gasoline. In addition, these methods require time-consuming sample preparation procedures which may increase method uncertainty. An HPLC method with fluorescence detection has employed indirect quantification of ethanol in blood samples by converting ethanol to acetaldehyde using alcohol dehydrogenase and nicotinamide adenine dinucleotide (ADH-NAD) (Chen and Peterson, 1994).

In the present work, we report the development of an HPLC method for the determination of ethanol after precolumn derivatization using 9-Fluorenylmethyl chloroformate (Fmoc-Cl). The resulting derivative has the property that it can be detected using both UV and fluorescence (FL) detectors. This feature makes the method novel, versatile, and more selective. Under optimal experimental conditions, the typical run time is just over 4 min.

Materials And Methods

Regents and materials

Ethanol ≥99.9%, methanol HPLC grade, 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.

Preparation of stock and standard solutions

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.

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.

Preparation of calibration curve

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

Results And Discussion

Optimization of derivatization conditions

Fmoc-Cl is a chloroformate ester used in organic synthesis to offer the fluorenylmethyloxycarbonyl protecting group as the FMOC carbamate (Paquet, 1982). Solvolysis of aromatic chloroformate esters (ArOCOCl) is a substitution organic reaction (Robertson, 1967). 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 C 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.

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.

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 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 2mg/mL.

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.

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.

Method performance

Sufficient peak resolution was achieved between the ethanol peak and other matrix peaks. The ethanol peak was selectively eluted with no coeluted 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 fluorescence detector, and 0.15 g/L and 0.5 g/L, for UV detector, 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 0.01 g/L – 5.0 g/L for the fluorescence detector, and 0.5 – 50 g/L for the UV detector, with a square correlation coefficient $R^2 > 0.9989$. Recoveries were calculated according to the following equation:

where C_r is the measured analyte concentration, and C_s is the corresponding concentration calculated from the standard calibration curve of spiked samples, which considers any matrix effect. The mean recovery ranged from 98–109% with a relative standard deviation (RSD%) of 3.7%.

An interference test was performed by analyzing ethanol in the presence of methanol and 1-propanol. As can be seen in Fig. 5, although both ethanol and methanol react with Fmoc-Cl, their respective peaks are well separated. No peak was detected for 1-propanol.

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

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 at two concentration levels i.e., 10 g/L and 0.5 g/L, and the results were compared with unspiked samples.

Table 1 summarizes the actual indigenous ethanol contents in the real samples. 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 1: Endogenous ethanol levels in the analyzed samples of beverages.

Sample code	The main components of the sample	Ethanol content g/L
R-B	Energy drink	0.11
A-100	100% apple juice	0.71
A-45	45% apple juice 10% rhubarb spritzer	0.31
P-29	29% pear juice	0.45
D-2	2% lemon, 2% orange	0.16
BL	bio lemonade drink	0.63

Matrix effect (ME)

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:

$$ME = \left[1 - \left(\frac{Peak\ Area\ of\ Post-Spike}{Average\ Peak\ Area\ of\ n\ Neat\ samples}\right)\right] \times 100, \text{ where } \mathbf{n} \geq 3$$

In this study, 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. However, for some samples, it might be necessary that the sample is first passed through a C18 cartridge before the derivatization reaction to remove as many organic compounds as possible

Conclusion

With the increasing consumption of non-alcoholic beverages, it is necessary to adopt a robust and versatile method for determining the ethanol content of these products. The method presented here was optimized and validated, and has demonstrated reliable performance for the determination of ethanol content in non-alcoholic beverages using HPLC combined with UV and fluorescence detectors. This method is simple and rapid and can be adopted as an alternative to GC methods to monitor/control the ethanol content in beverages and ensure that regulatory requirements are met. Although the results of this study show that ethanol content in non-alcoholic beverages is relatively low (< 1 g/L), high consumption of these products especially by kids and pregnant/ lactating women may pose a high risk to their health and the health of babies and fetuses (2004).

Declarations

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Author contribution

Saeed S. Albaseer Conceptualization, Methodology, Software, Data curation, Visualization, Investigation, Writing – original draft preparation, and final editing.

László Dören Supervision, Writing – original draft revision, Project administration, and resources.

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Data Availability All data generated or analysed during this study are included in this published article or can be obtained from the corresponding author on reasonable request.

Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Approval This article does not contain any studies with human or animal subjects performed by any of the authors.

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Figures

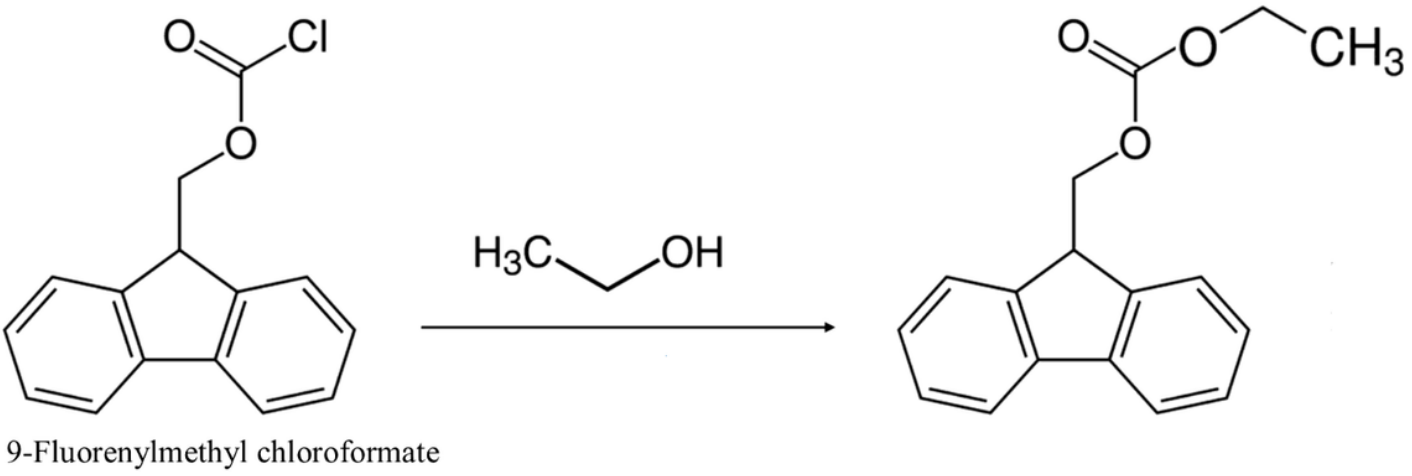

Figure 1

The derivatization reaction

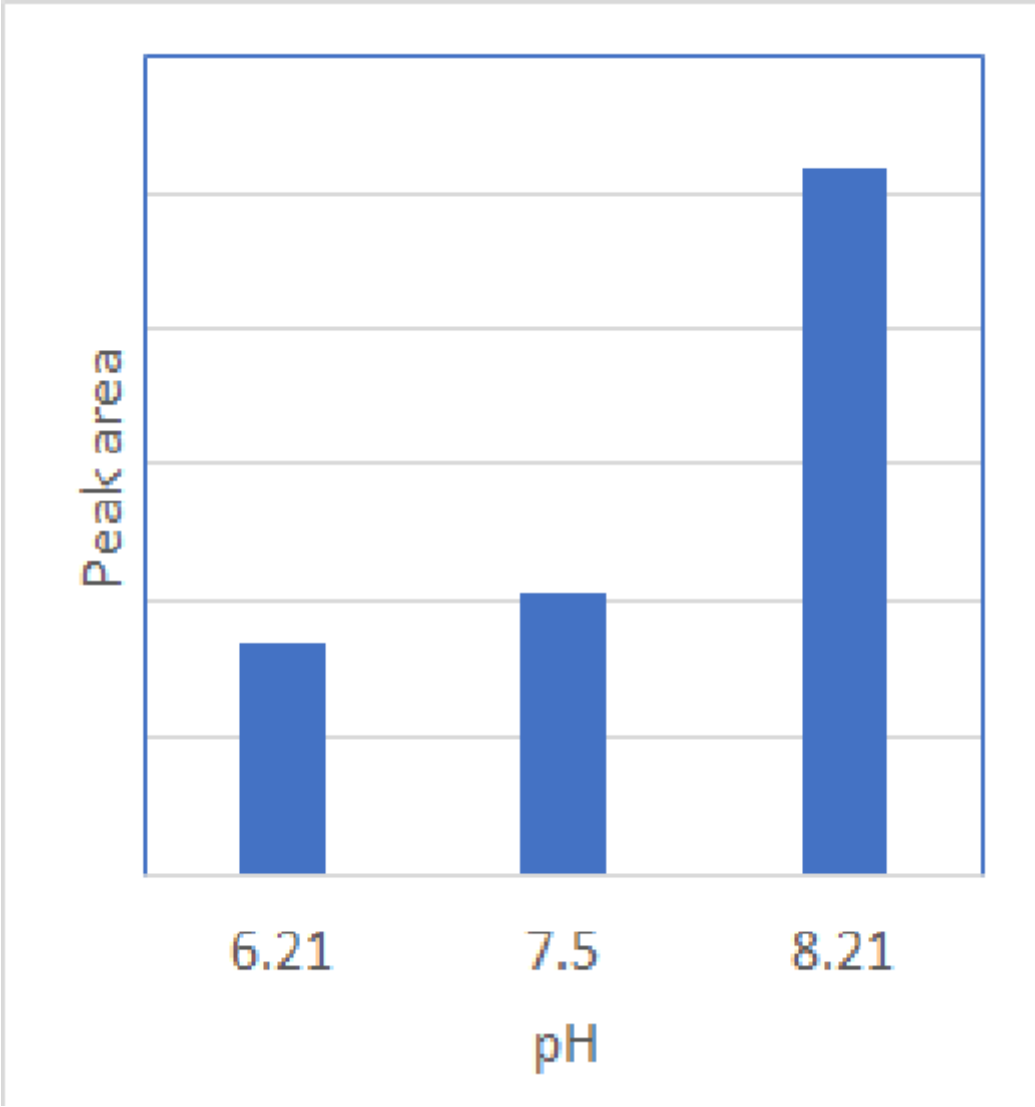

Figure 2

Effect of acidity on the derivatization reaction

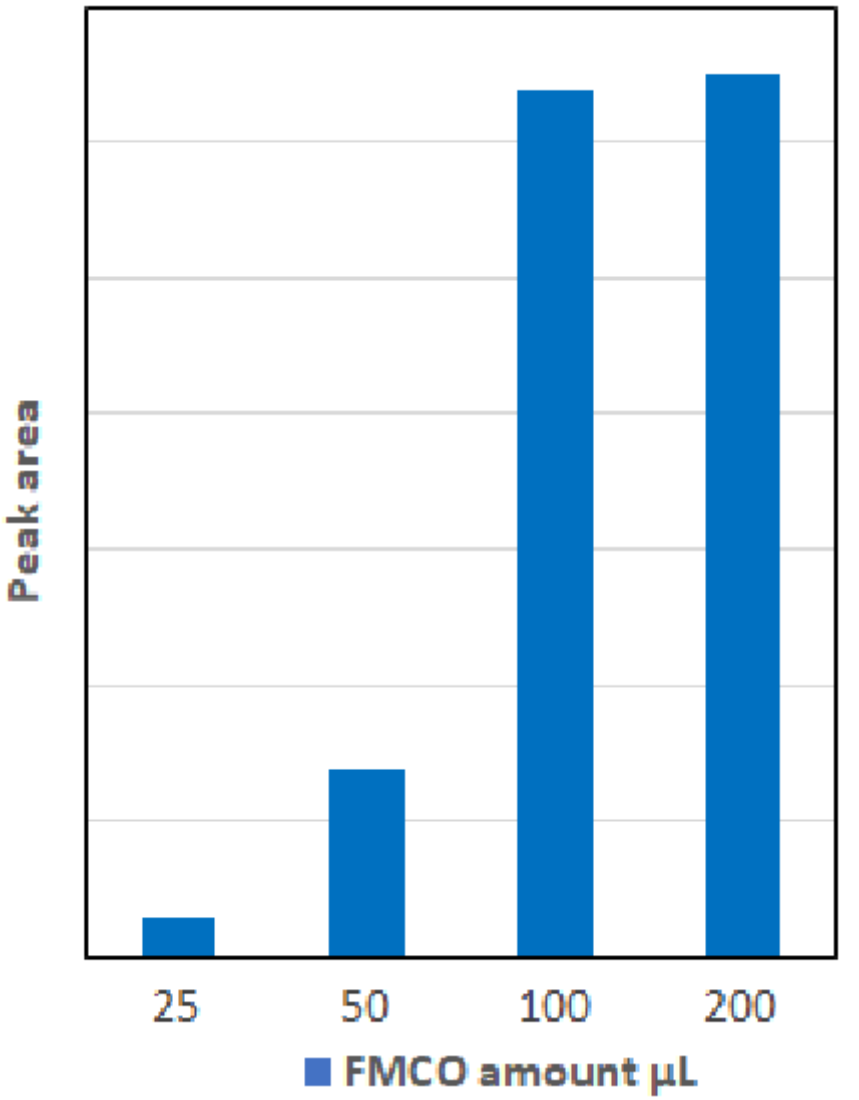

Figure 3

Effect of amount of Fmco-Cl on the derivatization reaction

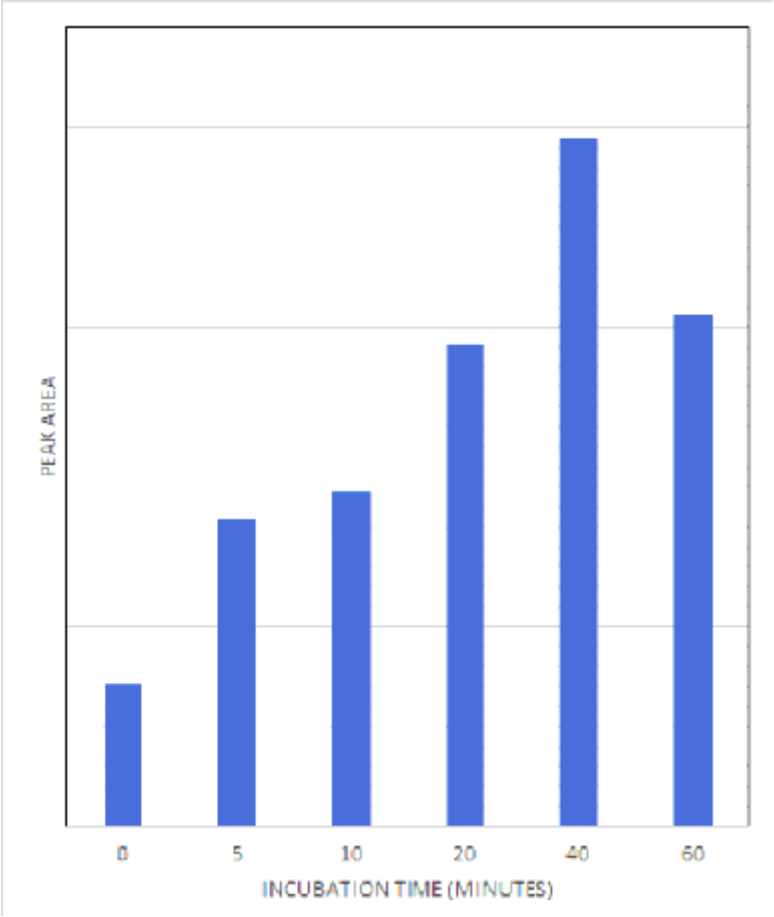

Figure 4

Effect of incubation time on the derivatization reaction

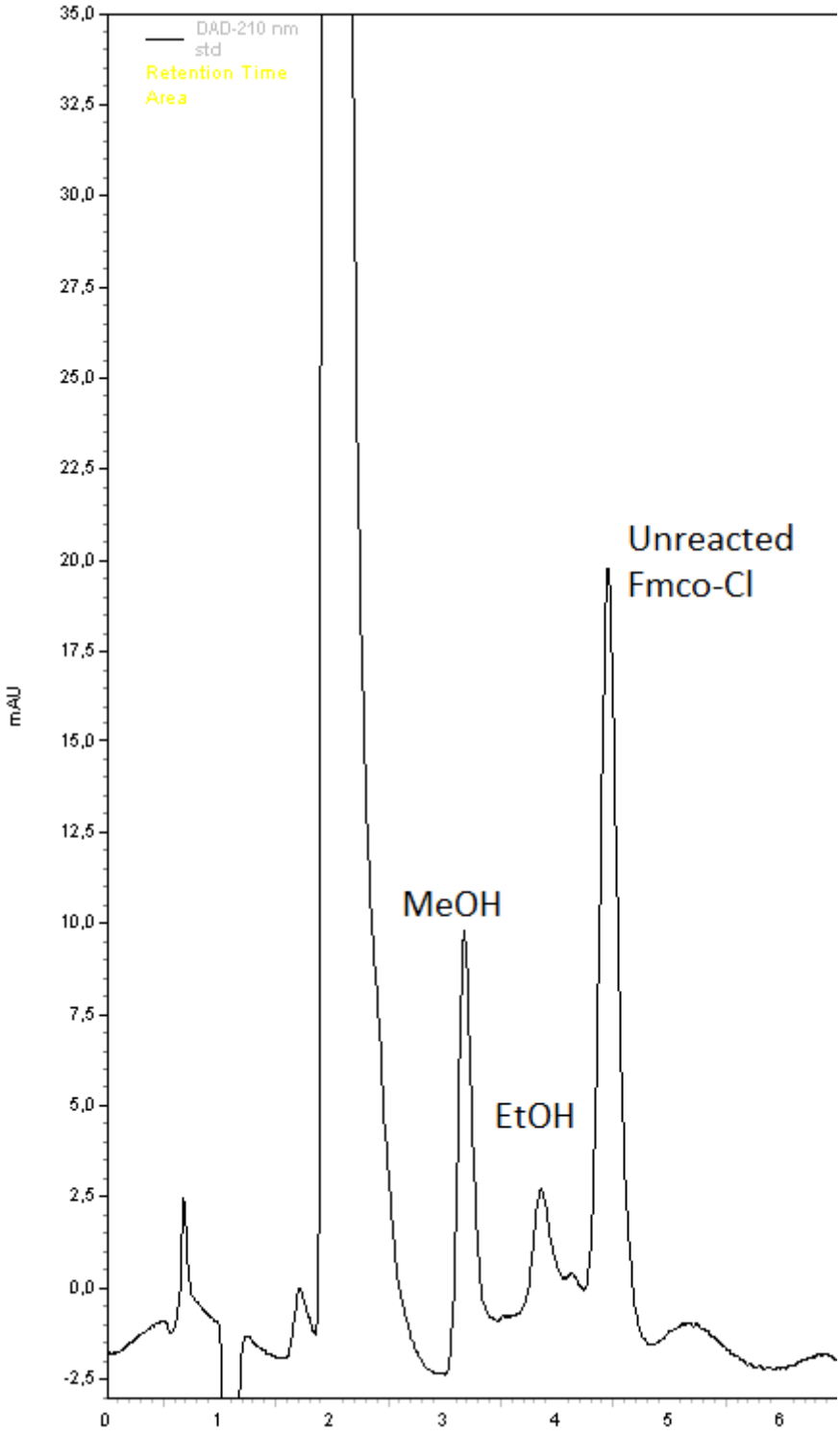

Figure 5

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