ALCOHOL IN BIOLOGICAL FLUIDS: QUANTITATIVE DETERMINATION USING RAMAN SPECTROSCOPY

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ABSTRACT

Alcohol detection in biological fluids, such as saliva, urine and blood serum, constitutes an important task, that concerns alcoholic beverages consumption, toxicology and forensic science. So far, GC or GC-MS are the most commonly used techniques, which may be accurate, but are also accompanied by high cost and require expertise personnel. The other used techniques lack in accuracy, precision or reproducibility.

In the present study we found that micro- Raman spectroscopy, a non destructive technique that can be used in vitro and in situ on biological samples, could be used to determine ethanol and methanol in synthetic and human biological fluids. Data analysis showed that the detection limit of Raman spectroscopy with our method could reach 6.82 mg/dL of ethanol in blood serum samples, a value much lower than 50mg/dL, the lowest acceptable limit of ethanol concentration in blood for drivers in Greece [1].

INTRODUCTION

Many studies have been conducted in order to define the harms caused by alcohol consumption. The most prevalent alcohol use disorders, are diseases of the internal organs (mostly connected to liver) and the behavioral disorders that deteriorate with the increase of alcohol intake [2]. On the other hand, much effort has been made to detect and precisely determine the alcohol concentration in biological fluids and tissues. These results may concern toxicological and forensic chemistry tests, and thus they need to be both precise and rapid.

The level of alcohol in blood was thoroughly studied in the past. As mentioned by Pohorecky et al., in non alcoholics, the typical effects of low alcohol levels (50mg/dL) include talkativeness, relaxation and tension reduction. Alcohol blood levels above 100mg/dL, lead to significant impairment of mental and cognitive ability that affect judgment and sensor- motor functioning. As alcohol blood levels exceed 200mg/dL, sensory and cognitive functioning is noticeably impaired and at 300mg/dL most individuals would be stuporous [3]. At concentrations higher than 400mg/dL alcohol is lethal as a result of severe depression of respiration or complications like aspiration of vomit. Methanol consumption is more dangerous than ethanol, since the side effects of methanol poisoning are more severe and chronic to those of ethanol. It may cause hemodialysis at 50mg/dL methanol blood concentration [4] and Parkinson syndrome can also be an afterwards complication [5]. The blood alcohol concentration limit for drivers varies between the countries of EU. In Greece the lowest limit is 0.5g/L (=50mg/dL)

The currently used techniques for alcohol detection are gas chromatography (GC) [6], gas chromatography-mass spectrometry (GC-MS) [7], enzymatic methods [7] [8], electrochemical oxidation [9], IR [10] and UV absorbance [9] . These techniques determine alcohol concentration by either measuring directly ethanol in biological fluids such as blood and saliva[6], or by determining ethyl glycuronide or ethanol's main metabolite acetaldehyde in urine or blood samples [7][9]. GC an GC-MS are the most precise and thus the most commonly used techniques to detect and determine ethanol, but they are time consuming, expensive and destructive. Their greatest disadvantage though, is the fact that they can only be performed by expertise personnel. The other techniques are also expensive and lack in accuracy, repeatability or speed.

In the current study we examined the usefulness and the efficiency of Raman spectroscopy, and more precisely, of the micro-Raman system, to detect and determine pure ethanol and methanol in human biological fluids (blood serum, saliva and urine). In this non destructive technique, the sample can be reused for further examination, the cost isn't as high as in GC-MS and can be performed easily by personnel that is not expertise. We propose a methodology for the estimation of limit of detection and quantitation of ethanol and methanol.

MATERIALS AND METHODS

Before examining the efficiency of our method for human biological fluids, we prepared an artificial sample of urine [11], and then added known quantities of ethanol or methanol. Ethanol and methanol samples were prepared separately. The final concentration of ethanol or methanol was 23.68mg/dL, 39.46mg/dL, 78.92mg/dL, 157.84mg/dL, 276.22mg/dL and 394.6mg/dL. Two of these concentrations are lower to the aforementioned lowest blood alcohol limit allowed for drivers in Greece, while the rest correspond to the concentrations that cause various disorders.

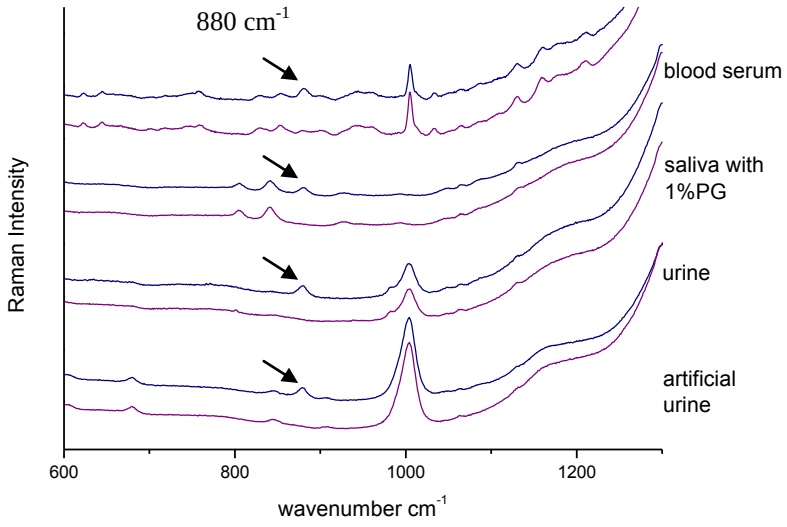
Analysis was performed by employing a Raman microscope (InVia Raman Microscope, Renishaw, UK). Spectra were acquired focusing a laser line at 785nm through an objective lens (20x) onto the sample. Each spectrum's conditions were 10 seconds exposure time with 30 accumulations. A small amount of each sample (about 30μl) was positioned into the cavity of a gold coated slide with high reflectivity, and then covered with a transparent membrane whose spectrum didn't exhibit any peaks at the region of interest. In order to obtain the best spectrum possible, we didn't focus on the surface of the membrane but deeper, at the main volume of the liquid underneath the surface. All samples' spectra were repeated 5 times, in order to ensure reproducibility.

Human biological fluids (blood serum, urine and saliva) were obtained from healthy volunteers of our lab, who abstained from alcohol for at least 3 days. The urine sample was not processed at all, while the saliva was enriched with 1% Propylen Glycerol (PG). The blood serum was isolated from whole blood that was collected by a physician. After the collection, the blood was placed in a tube without any anticoagulant factor (like EDTA) for 30 minutes and then it was centrifuged for 20 minutes at 3500RPM at 4°C. By the completion of the centrifuge we collected the blood serum from the supernatant.

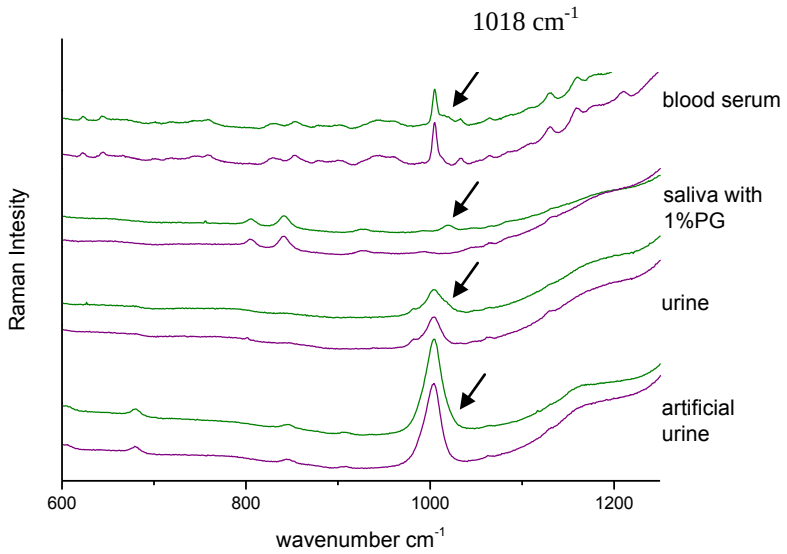
Repetition of Raman spectra acquisition for the samples of 39.46mg/dL, 157.84mg/dL and 394.6mg/dL was performed in order to examine the sample's stability and the intraday precision of our experiment [12].

RESULTS AND DISCUSSION

After the acquisition of the Raman spectra of samples they were processed with Origin Lab Pro®. As shown at graph 1 the peak of ethanol appears at 880cm^{-1}. The area where ethanol's peak appears is not overlapped by those of the sample and can be separately processed. Unfortunately except for saliva, this doesn't apply for methanol since its peak is at 1018cm^{-1} and overlaps with the main peak of our samples, appearing as a shoulder on the right side of the main peak.

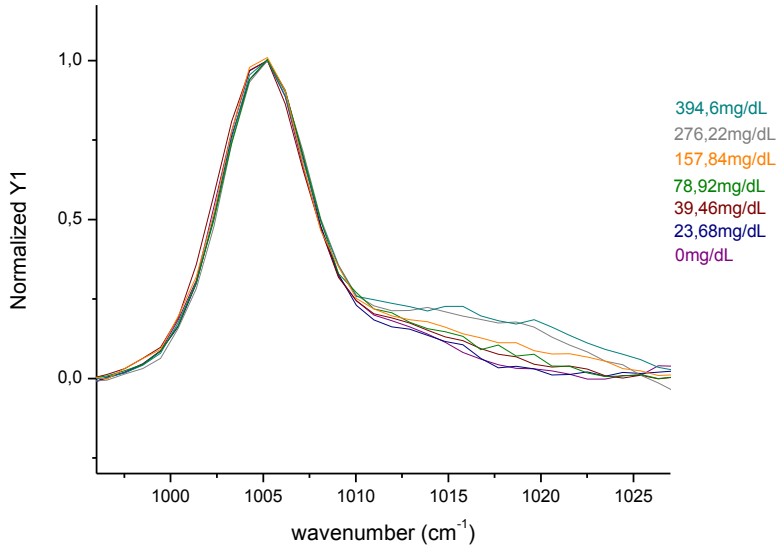


Graph 1 Raman spectra of blood serum, saliva with 1%PG, urine and artificial urine before the addition of ethanol (purple spectra) and the 394.6mg/dL solution of ethanol (blue spectra)



Graph 2 Raman spectra of blood serum, saliva with 1%PG, urine and artificial urine before the addition of methanol (purple spectra) and the 394.6mg/dL solution of methanol (green spectra).

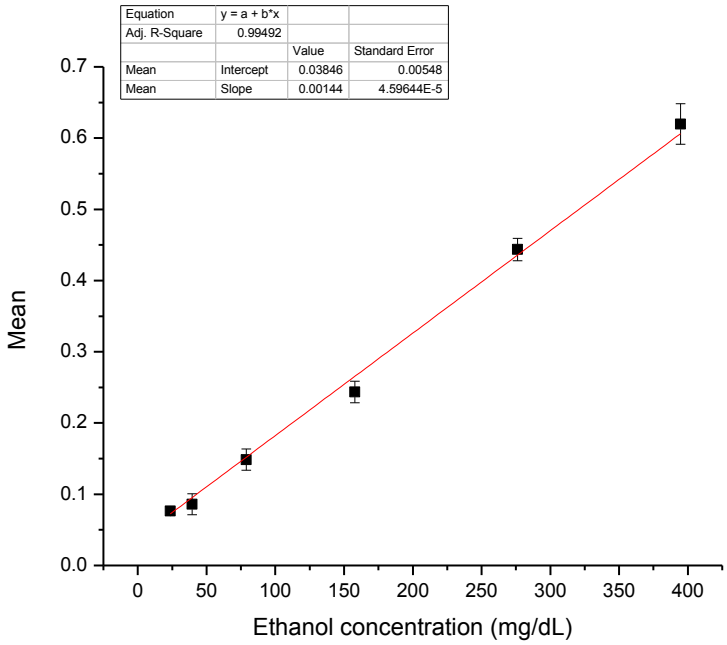
Baseline correction for the peaks of ethanol, methanol and each biological fluid was necessary for the calculation of their height, and was performed with the aid of Origin Lab Pro®. In following spectra we present methanol's peak at blood serum solutions.



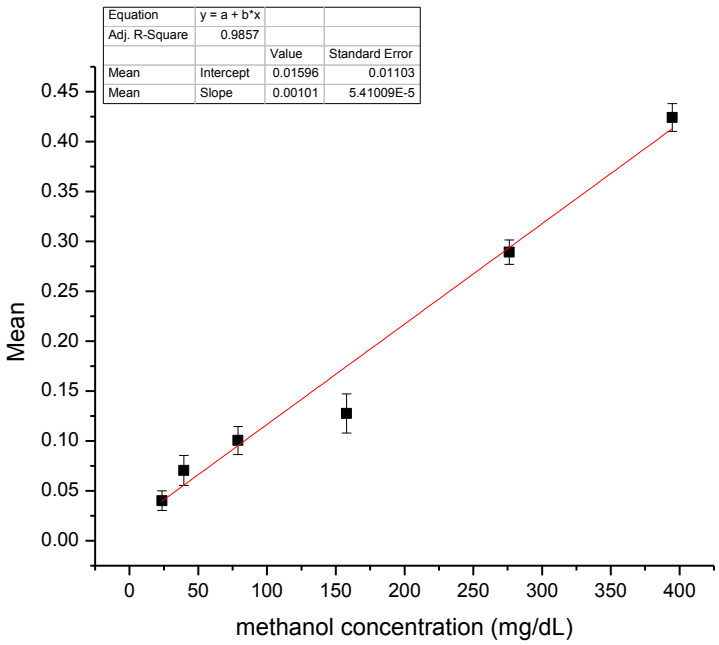
Graph 3 Normalized Raman spectra of methanol additions to blood serum. It is obvious that there is a rise of the shoulder at 1018cm^{-1} as methanol concentration increases.

Raman spectroscopy shows a linear response and thus it can be used not only for qualitative but for quantitative analysis as well. In this study we measured the intensity of ethanol and methanol in each spectrum and the intensity of the characteristic peaks of each biological fluid. These peaks are: 880cm^{-1} for ethanol, 1018cm^{-1} for methanol, 1005cm^{-1} for urine and blood serum. Saliva didn't have any characteristic peaks in its original spectrum, therefore we added 1% PG, which has sharp peaks that don't overlap with ethanol or methanol. The most characteristic one is found at 841cm^{-1}. In order to minimize inter- and intra-day errors we calculated the ratio of the ethanol's or methanol's characteristic peak height to the corresponding peak of each fluid.

As shown on graphs 4 and 5 there is a linear correlation between ethanol's or methanol's concentration and the aforementioned ratio.



Graph 4 Ratio of I^{880}/I^{841} in correlation to ethanol's concentration in saliva with 1%PG solutions.



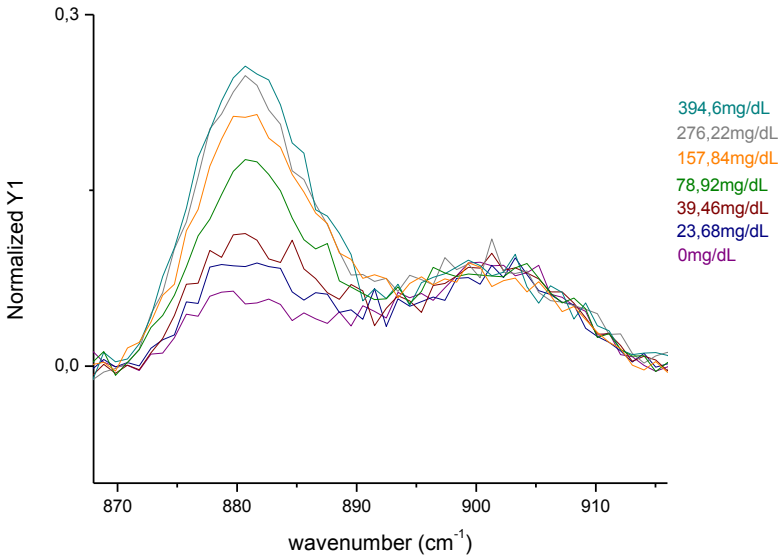
Graph 5 Ratio I^{1018}/I^{841} in correlation to methanol concentration in saliva 1% PG solutions.

The same procedure was followed for all our samples (except for the blood serum samples). The limits of detection were calculated according to literature [13].

Table 1 Limits of Detection and Limits of Quantitation, for Ethanol and Methanol solutions in saliva, urine and artificial urine.

		saliva 1%PG	urine	artificial urine
EtOH	equation	y=0.03846+0.00144x	y=0.01662+0.000741x	y=0.006560+0.000262x
	LOD (mg/dL)	23.92581409	10.18677122	20.49894783
	LOQ (mg/dL)	88.2978681	118.4548584	100.7117276
MeOH	equation	y=0.01596+0.01101x	y=-0.00808+0.0000399x	y=0.000243+0.000253x
	LOD (mg/dL)	56.27286828	76.326180	45.079841
	LOQ (mg/dL)	164.1610132	254.420601	150.266136

As far as the solutions in blood serum samples are concerned, we noticed that at concentrations higher than 157.84mg/dL, the linear correlation didn't apply. This is obvious from the normalized and baseline corrected Raman spectra of ethanol, as shown in graph 6.



Graph 6 Normalized Raman spectra of ethanol solutions to blood serum. It is obvious that ethanol's peak increases steadily until the 276.22mg/dL. Concentrations higher than 394.6mg/dL don't apply to the linear correlation noticed on lower concentrations.

In literature, interactions between ethanol and blood serum proteins have been noted [14], [15] and [16]. In order to examine whether this interaction was irreversible or not, we diluted the problematic samples, to the concentration of 78.92mg/dL. The results showed that it was inaccurate to measure high concentrations of ethanol without first diluting them, but the accurate measurement of low concentrations was possible.

Table 2 Limit of Detection and Quantitation, for Ethanol and Methanol solutions in blood serum.

		blood serum
EtOH	equation	y=0.041370+0.000158x
	LOD (mg/dL)	6.825739491
	LOQ (mg/dL)	22.75246497
MeOH	equation	y=0.024310+0.000064x
	LOD (mg/dL)	17.96288865
	LOQ (mg/dL)	59.87629551

The reproducibility of the method was verified by the extra intraday experiments that were conducted. The %RSD was lower than 2%, which, as mentioned in literature [12], is the highest acceptable value.

CONCLUSION

By evaluating these results we come to the conclusion that our method can be used to determine the ethanol and methanol content in biological fluids with accuracy and precision. The main advantage of this method is its reproducibility, its non destructive attributes, and the fact that it can be performed by personnel, that has a good perception of the technique, without the restriction of being an expert.

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