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(54) Title: ANALYSIS OF CONJUGATED METABOLITES OF ALCOHOL CONSUMPTION

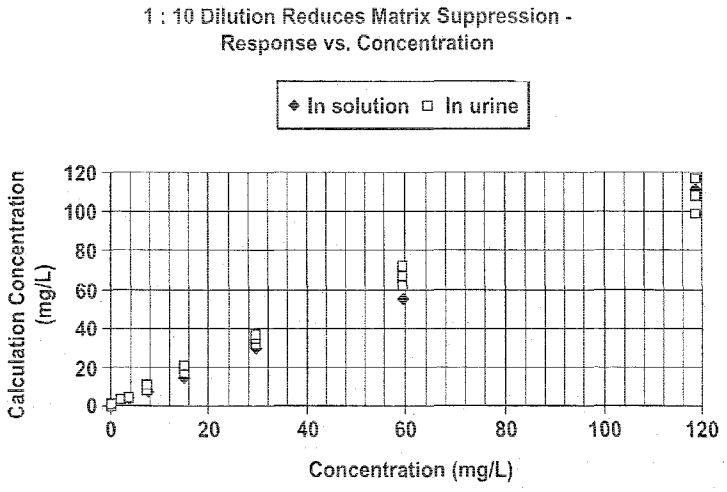

FIG. 1

(57) Abstract: A method, system, kit and uses for quantifying and normalizing at least one product of ethanol metabolism are provided. A method is provided for quantifying and normalizing at least one product of ethanol metabolism in a sample comprising creatinine. The method comprises adding a predetermined amount of at least one internal standard to the sample; adding deuterated creatinine to the sample; detecting and measuring at least one product of ethanol metabolism, the predetermined amount of at least one internal standard in the sample, the deuterated creatinine, and the creatinine. The method also comprises quantifying the amount of at least one product of ethanol metabolism in the sample using the measurement of the at least one internal standard; quantifying the amount of creatinine in the sample using the measurement of the deuterated creatinine; and normalizing the quantity of the at least one product of metabolism using the measurement of the creatinine.

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ANALYSIS OF CONJUGATED METABOLITES OF ALCOHOL CONSUMPTION

RELATED APPLICATION

The present application claims the benefit of United States Provisional Patent Application Number 60/976,539, filed October 1, 2007, the contents of which are incorporated herein by reference.

FIELD

The applicant's teachings relate to a method of quantifying and normalizing products of ethanol metabolism in a sample.

INTRODUCTION

Detection and quantification of metabolites in a sample obtained from a source can provide information about substances present in the source.

SUMMARY

In accordance with an aspect of the applicant's teachings, there is provided a method of quantifying and normalizing at least one product of ethanol metabolism in a sample comprising creatinine. The method comprises adding a predetermined amount of at least one internal standard, adding deuterated creatinine to the sample, detecting and measuring at least one product of ethanol metabolism, the predetermined amount of at least one internal standard in the sample, deuterated creatinine, and creatinine. The method also comprises quantifying the amount of at least one product of ethanol metabolism in the sample using the measurement of at least one internal standard, quantifying the amount of creatinine in the sample using the measurement of the deuterated creatinine, and normalizing the quantity of at least one product of ethanol metabolism using the measurement of creatinine.

In another aspect, there is provided a system for monitoring ethanol metabolism in a source using a mass spectrometer to analyze a sample from the source. The sample comprises creatinine which can be indicative of the physical state of the source. The system comprises a controller adapted to automatically dilute the sample by a predetermined amount at least once; add a predetermined amount of an internal standard to the at least one diluted sample; add deuterated creatinine to the sample; detect and measure at least one product of ethanol metabolism, at least one internal standard in the sample, deuterated creatinine, and creatinine; quantify the amount of at least

one product of metabolism in the sample using the measurement of at least one internal standard; quantify the amount of creatinine in the sample using the measurement of the deuterated creatinine; and normalize the quantity of at least one product of ethanol metabolism using the measurement of creatinine.

In accordance with another aspect of the applicant's teachings, there is provided a kit for quantifying and normalizing at least one product of ethanol metabolism in a sample comprising creatinine. The kit comprises at least one of the following: a sample, a deuterated internal standard, a calibration standard, a quality control check, instructions, and combinations thereof.

BRIEF DESCRIPTION OF THE FIGURES

The skilled person in the art will understand that the drawings, described below, are for illustration purposes only. The drawings are not intended to limit the scope of the applicant's teachings in any way. Like references are intended to refer to like or corresponding parts, and in which:

Figure 1 compares diluted urine matrix calculated concentrations with calculated concentration of samples in a standard matrix.

Figure 2 shows the structures of six analytes.

Figures 3 and 4 describe the automated calibration solution preparation pre-treatment method.

Figures 5 and 6 schematically illustrate the dual column plumbing configuration.

Figure 7 schematically illustrates the 10-port valve configuration.

Figures 8 and 9 show the standard drink amounts in various countries.

Figure 10 shows the production of metabolites over time after consumption of beer and red wine.

Figure 11 shows the production of metabolites over time after consumption of Brazilian rum.

Figure 12 shows the production of metabolites over time after consumption of Polish lager beer.

Figure 13 shows the production of metabolites over time after consumption of Italian red wine.

Figures 14, 15, and 16 show examples of the variation of creatinine with different volumes of urine and measured metabolite concentrations.

DESCRIPTION OF VARIOUS EMBODIMENTS

According to various embodiments of the applicant's teachings, a method for quantifying and normalizing at least one product of ethanol metabolism in a sample comprising creatinine is provided. The method can comprise adding a predetermined amount of at least one internal standard to the sample, and adding deuterated creatinine to the sample. The method can comprise detecting and measuring the at least one product of ethanol metabolism, the at least one internal standard in the sample, the deuterated creatinine, and the creatinine. The method can comprise quantifying the amount of the at least one product of ethanol metabolism in the sample using the measurement of the at least one internal standard, and quantifying the amount of creatinine in the sample using the measurement of the deuterated creatinine. The method can comprise normalizing the quantity of the at least one product of ethanol metabolism using the measurement of the creatinine.

According to various embodiments of the applicant's teachings, the sample can be obtained from a source, such as a mammal. For example, the mammal can be a human, a primate, or other lab animals and the sample can be urine, saliva, milk, blood, or other biological fluids and tissues. Samples such as milk, blood, or other biological fluids and tissues can be pre-treated to remove lipids and proteins before use in the applicant's method.

According to various embodiments of the applicant's teachings, the product of metabolism can be a metabolite of ethanol, for example, which can be indicative of ethanol present in the source. The product of metabolism can be a conjugated version of the substance present in a source. For example, if a source, such as a mammal, consumed ethanol, the product of metabolism can be ethyl sulphate and/or ethyl glucuronide.

According to various embodiments of the applicant's teachings, the detection and measurement conducted in various embodiments of applicant's teachings can be conducted using, for example, a mass spectrometer, such as, for example, a mass spectrometer comprising a triple quadrupole. Other types of mass spectrometer including various types of Ion Traps, Linear Ion Traps, Time of Flight analyzers, magnetic sector instruments all of which could also be used.

According to various embodiments of the applicant's teachings, the components of the sample can occur at varying concentrations as a result of the "thickness" or concentration of the sample. For example, the thickness of urine can reflect, for example, the source's physical state; for example, the thickness can reflect the amount of physical activity, the fluid consumption, the salt intake, muscle mass, or kidney function of the source. Certain components in the sample, such as creatinine or hydrocortisone, can be indicative of the source's physical state. The sample may comprise urine, blood or plasma. These components can be used to normalize the detected amounts of metabolites. Normalization of the detected amounts of metabolites can produce a more accurate quantification of the metabolite.

According to various embodiments of the applicant's teachings, at least one internal standard can be added to the sample before analysis of the sample. An internal standard can comprise a known quantity of a chemical having a chemical structure that mimics the chemical structure of a component of interest. The chemical of the internal standard can comprise an additional component which can be detectable by whichever mode of detection is used. For example, at least one hydrogen atom of the structure could be replaced with a deuterium atom, which allows for detection by mass spectrometry separately from the chemical that it mimics. Preferably, multiple deuterium atoms can be used. Quantification of the known quantity of the chemical of the internal standard can be used to identify and/or quantify a component of interest.

According to various embodiments of the applicant's teachings, the internal standards can be added manually or automatically by, for example, as an HPLC pre-treatment method . The internal standards can be diluted, for example, they can be serially diluted, either manually or automatically, by, for example, an HPLC method. The internal standard can comprise a chemical having a chemical structure that mimics that of a component in the sample. For example, the chemical can have a structure which mimics creatinine, hydroxycortisone, ethyl sulphate, or ethyl glucuronide. The chemical of the internal standard can be modified to be identified, detected, and/or quantified. For example, if a mass spectrometer is being used with the method, the chemical can be deuterated. Thus, the internal standards can comprise deuterated creatinine, deuterated hydroxycortisone, deuterated ethyl glucuronide, and/or deuterated ethyl sulphate.

The methods according to various embodiments of applicant's teachings can comprise at least one dilution, or serial dilutions, of the sample, either before and/or after the addition of an internal standard. The dilutions can be done manually and/or automatically. According to various embodiments of the applicant's teachings, the methods can be automated. For example, automated dilution of urine samples and automated preparation of a calibration curve sample set.

The methods according to various embodiments of applicant's teachings can be used to predict the time and level of alcohol in a source, such as a mammal, consumed as an alcoholic beverage, for example. According to various embodiments of the applicant's teachings, the methods can be used to monitor alcohol in a source, such as a mammal.

According to various embodiments of applicant's teachings, a system for monitoring ethanol metabolism in a source is provided. The system can include the use of a mass spectrometer to analyze a sample from the source. The sample can comprise creatinine indicative of the physical state of the source. The system can comprise a controller adapted to automatically dilute the sample by a predetermined amount at least once. The controller can be adapted to add a predetermined amount of an internal standard to the at least one diluted sample, and adapted to add deuterated creatinine to the sample. The controller can be adapted to detect and measure at least one product of ethanol metabolism, the at least one internal standard in the sample, the deuterated creatinine, and the creatinine. The controller can be adapted to quantify the amount of the at least one product of ethanol metabolism in the sample using the measurement of the at least one internal standard. The controller can be adapted to quantify the amount of creatinine in the sample using the measurement of the deuterated creatinine and adapted to normalize the quantity of the at least one product of ethanol metabolism using the measurement of the creatinine.

According to various embodiments of applicant's teachings, a kit of parts may be provided for quantifying and normalizing at least one product of ethanol metabolism in a sample that comprises creatinine. The kit comprises at least one of the following: a sample, a deuterated internal standard, a calibration standard, a quality control check, and combinations thereof. Typically, quality control checks can be made with predetermined low, medium, and high concentration solutions to produce certain ion counts.

Aspects of the applicant's teachings may be further understood in light of the following examples, which should not be construed as limiting the scope of the applicant's teachings in any way.

Example 1

The method used for this example detected six chemical species in less than four minutes: (1) ethyl glucuronide and (2) ethyl sulphate, conjugated metabolites of ethyl alcohol consumption in urine and their d5-deuterated internal standards, creatinine, an indicator for the

"thickness of urine", and d3-deuterated creatinine as an internal standard. These metabolite concentrations were normalized to 1 g creatinine/L urine

For example, calibration solutions were automatically prepared by serially diluting a stock solution of mixed standards in urine or in a solvent at 1:1 using a custom-configured Shimadzu pre-treatment program. Because urine can suppress ethyl glucuronide signals spiked standard solutions in undiluted urine give approximately 1/10 to 1/15 signals when compared to those in solvent only. However, 1:10 dilution restores the original signal. For this reason it was necessary to dilute the samples to reduce the matrix effect. Urine samples were treated as follows:

Each urine sample (100 μL) was mixed with 200 μL of a solution (80% water + 20% acetonitrile) containing internal standards and 700 μL of acetonitrile using a pre-treatment program as described in Figures 3 and 4 thus minimizing human error and possible contamination. Figure 1 shows a 1:10 dilution reduces matrix suppression- response vs. concentration. If there was matrix suppression, the diluted urine matrix calculated concentrations (pink) would fall below the calculated concentration of samples in a standard matrix (blue) – that was not the case in this experiment, and hence the amount of dilution is used is reasonable in analysis.

The amounts of ethyl glucuronide and ethyl sulphate were adjusted to that of creatinine (100 mg/dL or 1,000mg/L) as per "Forensic Confirmatory Analysis of Ethyl Sulphate - A New Marker for Alcohol Consumption- by Liquid Chromatography/Electrospray Ionization/Tandem Mass Spectrometer" S. Dresen, W. Weinmann, and F.M. Wurst, *J. Am. Soc. Mass. Spectrom.*, 2004, 15, 1644-1648. In this paper, the metabolites were normalized to creatinine, but the creatinine was measured using an alternative technique, whereas in the applicant's teachings, the creatinine was measured at the same time as the metabolites using the same LC-MS/MS run. Figure 2 shows the structures of six analytes.

Instruments used for this study include a Shimadzu Prominence, SIL-HT Dual Gradient System consisting of 1 x CBM-20A controller, 4 x LC-20AD pumps, 1 x SIL-20AC auto sampler, 1 x CTO-20AC column oven with 2 x FCV-20AH2 valves, and 1 x DGU-20A3 on-line degasser. An additional pump, LC-10ADvp, and a degasser, DGU14A were used to deliver a solvent to the MS source, while salts were being dumped from the line. The mass spectrometer employed for this study was an API-3200^{TM} triple quadrupole system, operated under multiple reaction monitoring mode (MRM), where a series of precursor and unique fragment ion pairs were monitored one after another in a rotating order. A minimum of 2 ion pairs were monitored per chemical species as per a European GLP Guideline, "Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC

concerning the performance of analytical methods and the interpretation of results", Official Journal of the European Communities, L221/12 17.8.2002, 2002/657/EC, for forensic MS/MS applications.

Reagents

Creatinine was available from Sigma-Aldrich, St. Louis, MO, USA" P/N C-4255 (http://www.sigmaaldrich.com). D3-creatinine was available from C/D/N Isotopes, Pointe-Claire, Quebec, Canada: P/N D-3689 (http://www.cdnisotopes.com). Ethyl glucuronide (d0 and d5) were available from Cerilliant Corporation, 811 Paloma Drive, Suite A, Round Rock, Texas 78664, USA. Ethyl sulphuric acid sodium salt was available from Tokyo Kasei Kogyo company Ltd., 6-15-9 Toshima, Kita-ku, Tokyo, Japan (E0277). D5-ethyl sulphate was synthesized by adding d5-ethanol (C/D/N Isotopes Inc., P/N:D-108 116 μL, 1.96 mM) to sulphuric acid (Sigma-Aldrich, #380075, 106 μL,, 1.93 mM) in a reacti-vial and heated at 80°C for 60 minutes. It was diluted to 1 mg/mL in water, and used to prepare a standard solution. Ammonium formate was available from Sigma-Aldrich (product #F-200 Formic acid was available from EMD (AnalaR(R), 98-100%, product # B10115). Acetonitrile (BAKER ANALYZED(R) 9017-03) was obtained from JT Baker. Millipore Q 18MΩ deionized water was used.

HPLC Method

A dual-column liquid chromatography system was used to realize high throughput analysis. The diverter valve attached to the mass spectrometer was also used to divert the early and late LC eluents to waste, while a fifth pump sent a clean solvent to the MS.

Mobile phases A, B, C, D, and Rinse 3 solution comprised 70% acetonitrile + 30% water + 10mM ammonium formate, pH adjusted to 5.0 with a small amount of formic acid at a flow rate of 0.35 mL/min (isocratic). Pump 5 used the same composition. Rinse 1 comprised 80% water + 20% acetonitrile + 500 ng/mL d5-ethyl glucuronide + 100 ng/mL d5-ethyl sulphate + 1,500 ng/mL d3-creatinine. Rinse 2 comprised acetonitrile (100%). The column was a Waters Atlantis (R) HILIC (Waters, Milford, USA) silica 3 micron, 3.0 x 100 mm with a matching guard column, heated at 50°C.

Figures 3 and 4 show the automated calibration solution preparation (1:1 dilution) pre-treatment method.

Figures 5-7 show the plumbing configuration such that the sample can be automatically injected onto column 1 or 2 (figures 5 and 6 respectively) and the valve configuration can allow the

sample to be diverted and the flow replaced by acetonitrile at times when the compound is not eluting but the urine matrix is.

Using a Shimadzu Prominence system and a standard 70-vial tray, the auto sample dilution pre-treatment shown in Table 1 are automatically done. This program can be changed to use a 105-vial tray or 175-vial tray.

Alcohol Consumption Experiments – Background readings

The determination of metabolites of alcohol can be used as an indicator of alcohol consumption, typically through consumption of alcoholic beverages. Certain other food, medicines and appliances contain alcohol that if also used could potentially become metabolites and increase the reading over and above that derived from alcoholic beverages. In order to determine how alcohol-containing medications and desserts will affect the readings for ethyl glucuronide (Et-G) and ethyl sulphate (Et-S), volunteers were asked to use (1) alcoholic gel to disinfect hands at a hospital, (2) Robitussin® cough syrup, (3) mouthwash, (4) Tiramisu cake, (5) face cleansing cloth, (6) sherry trifle (7) Irish coffee (1 measure liquor in a creamy coffee), (8) a red wine used to cook meat and (9) ham with beer glaze, all at normal usages.

Urine samples were collected before and after the use or consumption. Except for Robitussin, no measurable amounts of Et-G or Et-S were found in the urine samples of the volunteers. Urine samples collected 2 and 7 hours after taking Robitussin showed an increase in Et-S, but not Et-G.

Therefore, it is unlikely that this method will produce false-positive readings, as long as the amount of consumption is reasonable.

Alcohol Consumption Experiments

Standard drink amounts in various countries are shown in Figures 8 and 9.

In order to simulate various consumption scenarios by airline pilots, machine operators, patients undergoing an alcohol withdrawal program, volunteers were asked to consume the following drinks with meals. The selection of meals was left to the discretion of each volunteer.

(1) Red French wine (250mL, 12% alcohol content) + Portuguese red wine (100mL, 17.5%) consumed by a female volunteer.

(2) One bottle of Canadian lager beer (355 mL, 5%) + Ontario red wine (1.2L, 13.5%) consumed by a female volunteer.

(3) 2 Bottles of Ontario lager beer (710 mL, 5%) + Ontario white wine (1.2 L, 13.5%) consumed by a male volunteer.

(4) Polish lager beer (Zywiec, 5.5 %, 1L) consumed by a male volunteer.

(5) One can of Asahi lager beer (500 mL, 5%) + 400 mL Gekkeikan Japanese sake (400 mL, 16%) consumed by a male volunteer.

(6) Appleton white Jamaican Rum (20%, 180 mL over 2 hours) consumed by a male volunteer.

(7) French red wine (ca. 500 mL, 12%) consumed by a male volunteer.

(8) Pedra 90, Brazilian Rum (100 mL, 39%) consumed by a male volunteer.

(9) English Gin (50 mL, 40%) consumed by a male volunteer.

(10) Port wine (100 mL, 18%) consumed by a female volunteer.

(11) Chinese glutinous rice wine (400 mL, 14%) consumed by a male volunteer.

(12) English-made Guinness beer (600 mL, 5%) consumed by a male volunteer.

(13) Bailey's Irish Cream on ice (ca. 300 mL, 17%) consumed by a male volunteer.

(14) Single Malt Scotch Whiskey (60 mL, 40%) consumed by a male volunteer.

(15) Tequila (125 mL, 40%) consumed by a female volunteer.

Urine samples were collected before and after consumption of alcohol beverage. Volumes were recorded and a portion of urine was kept in a 15-mL centrifuge tube at 4°C for LC/MS/MS analysis. Samples were analyzed as above and plotted as concentration of metabolites of ethanol (sulphate and glucuronide) in urine over time. This shows the production of the metabolites over time after consumption. Figures 10-13 show such curves for selected cases. It is shown that the concentration of metabolites in urine increases measurably immediately after consumption, and returns to normal at least 20 hours after consumption. The elevated level of the metabolite is indicative of

consumption. The method, which normalizes the concentration to creatinine, shows good agreement between the decay curves of ethyl sulfate and glucuronide. Figure 14 shows the variation of creatinine with different volumes of urine and measured metabolite concentrations.

While the measurement of urinary concentration of metabolites of ethanol reveals elevated levels post-consumption, in order to relate this concentration to consumption volume it is necessary to perform a mass balance of the metabolite normalized to urinary output and also to the quantity of metabolite formed from the total ethanol ingested.

To evaluate the proportion of ethanol metabolized the mass balance was studied. In one case, 101 hours after the consumption of a beer and red wine (141.8 g ethanol), more than 23.84 mg of ethyl sulphate and 72.86 mg of ethyl glucuronide were formed and discharged. Stoichiometry is as follows:

$C_2H_5OH + H_2SO_4 \rightarrow C_2H_5OSO_3H$

46 98 126

46:126 = 141.8 (g):X X=(126/46) x 141.8(g)=388.4 (g)

(0.02384 g)/(388.4g) x 100 = 0.00614 (%)

Similarly, for ethyl glucuronide

46:222 = 141.8(g):Y Y=(222)/(46) x 141.8(g)=684.3 (g)

(0.07286g)/(684.3g) x 100 = 0.0106 (%)

Calculations show that 0.0061% of ethanol was converted into ethyl sulphate, and 0.0106 % of ethanol was converted into ethyl glucuronide and discharged into the urinary system. It is said the majority of alcohol is converted into carbon dioxide and water.

In another case, a female volunteer consumed French red wine (250 mL, 12%) and Portuguese port wine (100 mL, 17.5%) in 30 minutes or so.

The total amount of alcohol consumed was 37.478 grams. Urine samples were collected over 46.45 hours, volume of each discharge was measured and recorded.

Alcohol introduced: 250 mL x 12(%)/100 x 0.789 (g/mL) + 100 mL x 17.5 (%)/100 x 0.789 (g/mL) = 37.48 grams ethanol

11.091 mg ethyl sulphate detected... 0.010%

The importance of normalization was illustrated when a male volunteer consumed 1 can of chilled Asahi Super Dry beer (500 mL, 5%) followed by warm Gekkeikan Sake (400 mL, 16%) in approximately 2 hours.

The total ethanol introduced to his system was 500 x 0.05 x 0.789 + 400 x 0.16 x 0.789 = 70.211g.

As shown in Figures 15 and 16, his creatinine concentration and volume of urination (which affects concentration in the sample greatly) varied during the course of this study, thus indicating the importance of normalization.

This example showed that following consumption of alcoholic beverages it is possible to measure the quantity of the metabolites of ethanol, ethyl glucuronide and ethyl sulfate in the urine as an indicator of alcohol consumption in at least 20 hours after consumption. Various beverages and volunteers were tested. The effect of inadvertent alcohol consumption (e.g. from cough syrup or food) was evaluated and found to be quite insignificant. The effect of normalization to urinary volume and thickness of urine was demonstrated and shown to produce good results.

What is claimed is:

1. A method for quantifying and normalizing at least one product of ethanol metabolism in a sample, said sample comprising creatinine, said method comprising:

(i) adding a predetermined amount of at least one internal standard to the sample;

(ii) adding deuterated creatinine to the sample;

(iii) detecting and measuring the at least one product of ethanol metabolism, the at least one predetermined amount of internal standard in the sample, the deuterated creatinine, and the creatinine;

(iv) quantifying the amount of the at least one product of ethanol metabolism in the sample using the measurement of the at least one internal standard;

(v) quantifying the amount of the creatinine in the sample using the measurement of the deuterated creatinine; and

(vi) normalizing the quantity of the at least one product of ethanol metabolism using the measurement of the creatinine.

2. The method according to claim 1 wherein the sample is urine.

3. The method according to claim 1 wherein the sample is saliva.

4. The method according to claim 1 wherein the sample is blood or plasma.

5. The method according to claim 1 wherein the sample is obtained from a mammal.

6. The method according to claim 4 wherein the mammal is a human.

7. The method according to claim 1 wherein the detecting and measuring is performed by a mass spectrometer.

8. The method according to claim 6 wherein the mass spectrometer comprises a triple quadrupole.

9. The method according to claim 1 wherein the at least one product of metabolism is ethyl glucuronide.

10. The method according to claim 8 wherein the at least one internal standard is deuterated ethyl glucuronide.

11. The method according to claim 1 wherein the at least one product of metabolism is ethyl sulphate.

12. The method according to claim 10 wherein the at least one internal standard is deuterated ethyl sulphate.

13. The method according to claim 1 wherein the sample can be diluted before addition of the at least one internal standard.

14. The method according to claim 1 wherein the method is automated.

15. Use of the method according to claim 1 to predict the time and level of alcohol in a source.

16. The use according to claim 14 wherein the source is a mammal .

17. Use of the method according to claim 1 to monitor alcohol in a source.

18. The use according to claim 16 wherein the source is a mammal.

19. A system for monitoring ethanol metabolism in a source using a mass spectrometer to analyze a sample from the source, said sample comprising creatinine, indicative of the physical state of the source, said system comprising a controller adapted to:

(i) automatically dilute the sample by a predetermined amount at least once;

(ii) add a predetermined amount of an internal standard to the at least one diluted sample;

(iii) add deuterated creatinine to the sample;

(iv) detect and measure at least one product of ethanol metabolism, the at least one internal standard in the sample, the deuterated creatinine, and the creatinine;

(v) quantify the amount of the at least one product of ethanol metabolism in the sample using the measurement of the at least one internal standard;

(vi) quantify the amount of creatinine in the sample using the measurement of the deuterated creatinine; and

(vii) normalize the quantity of the at least one product of ethanol metabolism using the measurement of the creatinine.

20. The system according to claim 18 wherein the source is a mammal.

21. The system according to claim 19 wherein the source is a human.

22. The system according to claim 18 wherein the mass spectrometer comprises a triple quadrupole.

23. The system according to claim 18 wherein the sample is urine.

24. The system according to claim 18 wherein the sample is saliva.

25. The system according to claim 18 wherein the sample is blood/plasma

26. The system according to claim 18 wherein the at least one product of ethanol metabolism is ethyl glucuronide.

27. The system according to claim 24 wherein the at least one internal standard is deuterated ethyl glucuronide.

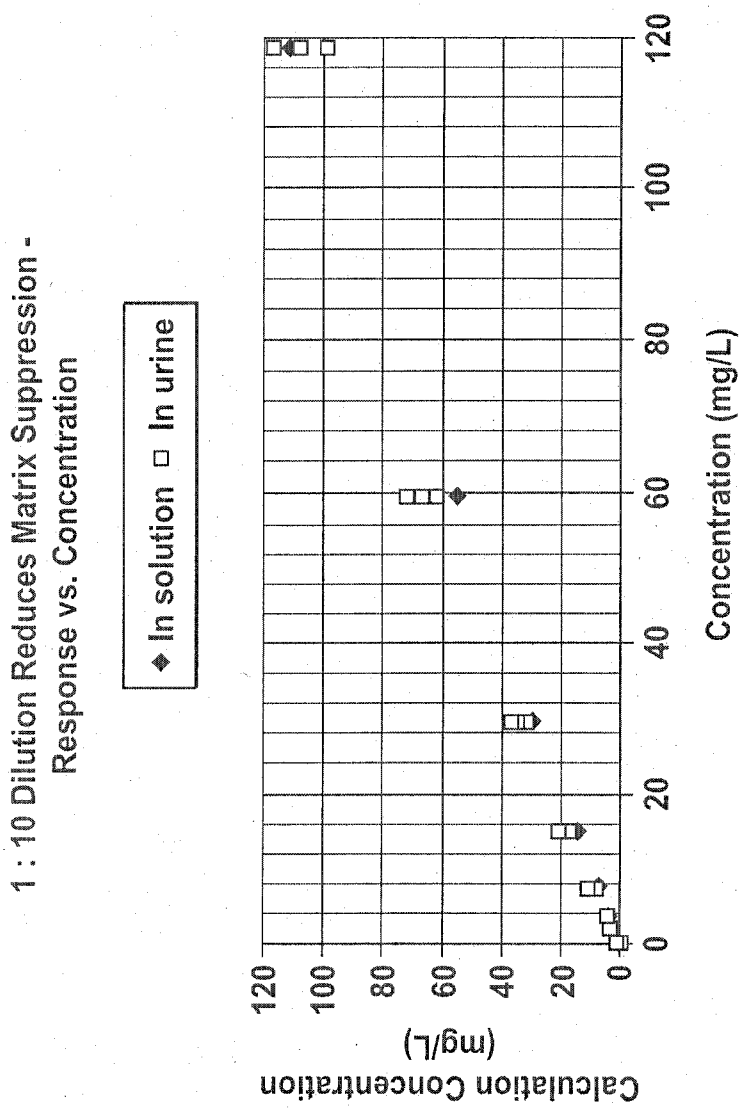
28. The system according to claim 18 wherein the at least one product of ethanol metabolism is ethyl sulphate.

29. The system according to claim 26 wherein the at least one internal standard is deuterated ethyl sulphate.

30. The system according to claim 18 wherein the sample is diluted before addition of the at least one internal standard.

31. A kit for quantifying and normalizing at least one product of ethanol metabolism in a sample, said sample comprising creatinine, said kit comprising at least one of the following: a sample, a deuterated internal standard, a calibration standard, a quality control check, instructions, and combinations thereof.

32. Any and all features of novelty described, referred to, exemplified, or shown herein and in the incorporated appendices.


1 : 10 Dilution Reduces Matrix Suppression - Response vs. Concentration
In solution
In urine
Calculation Concentration (mg/L)
Concentration (mg/L)
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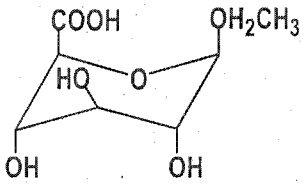
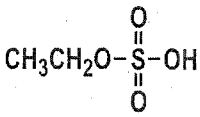
20
40
60
80
100
120

FIG. 1

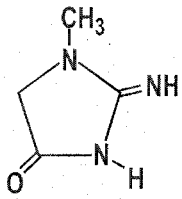
Structures of 6 Analytes



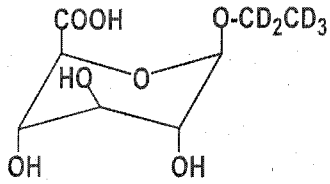
$C_3H_{14}O_7$
222.073953
Ethyl glucuronide



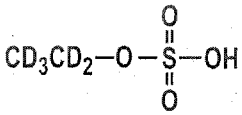
$C_2H_4O_4S$
125.998681
Ethyl sulfate



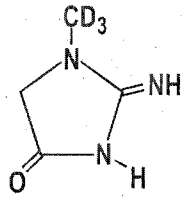
$C_4H_4N_3O$
113.058912
Creatine



$C_3H_4D_5O_7$
227.105337
d5-Ethyl glucuronide



$C_2HD_5O_4S$
131.030064
d5-Ethyl sulfate



$C_4H_4D_3N_3O$
116.077742
d3-Creatine

FIG. 2

"Automated Calibration Solution Preparation(1:1 dilution)" pre-treatment (1)

Command	Column1
• VIAL SN	Goes to Vial position sent from Analyst method
• N.STRK NS	
• ASPIR 150,150	Aspirates 150µL
• A6= SN+1	Sets the variable to determine where dilution is made
• VIAL A6	Goes to Vial position sent from method + 1
• N.STRK NS	
• DISP 150,150	Aspirated sample is delivered to dilution vial
• A0= 3	Sets valve to deliver diluent from Rinse Liquid #1
• IRINSE 500,150	Moves to rinse port and flushed MP out of loop in preparation for dilution
• VIAL A6	Moves to target dilution vial
• N.STRK NS	
• A0= 3	
• DILUTE 150,150	delivers 150µL of diluent (Rinse Liquid #1)

FIG. 3

"Automated Calibration Solution Preparation(1:1 dilution)" pre-treatment (2)

• MIX 3,50,300,150,150	Mixes liquid in dilution vial
• ASPIR IV,SS	Aspirates Injection Volume specified in the method
• RINSE 200,150	Dips needle in static rinse port
• INJ.P	Moves to Injection Port
• S.INJ	Makes injection and sends start signal to the rest of the system
• WAIT 0.1	
• V.LOAD	Rotates valve to take Sample Loop off line
• A0= 14	Sets valve to deliver diluent from Rinse Liquid #2
• IRINSE 500, RS	Flushes 500µL of Rinse Liquid #2 through sample loop, needle, injection port and valve.
• A0= 15	Sets valve to deliver diluent from Rinse Liquid #3 (initial Mobile Phase)
• IRINSE 500,RS	Flushes 500µL of Rinse Liquid #3 through sample loop, needle, injection port and valve.
• END	

FIG. 4

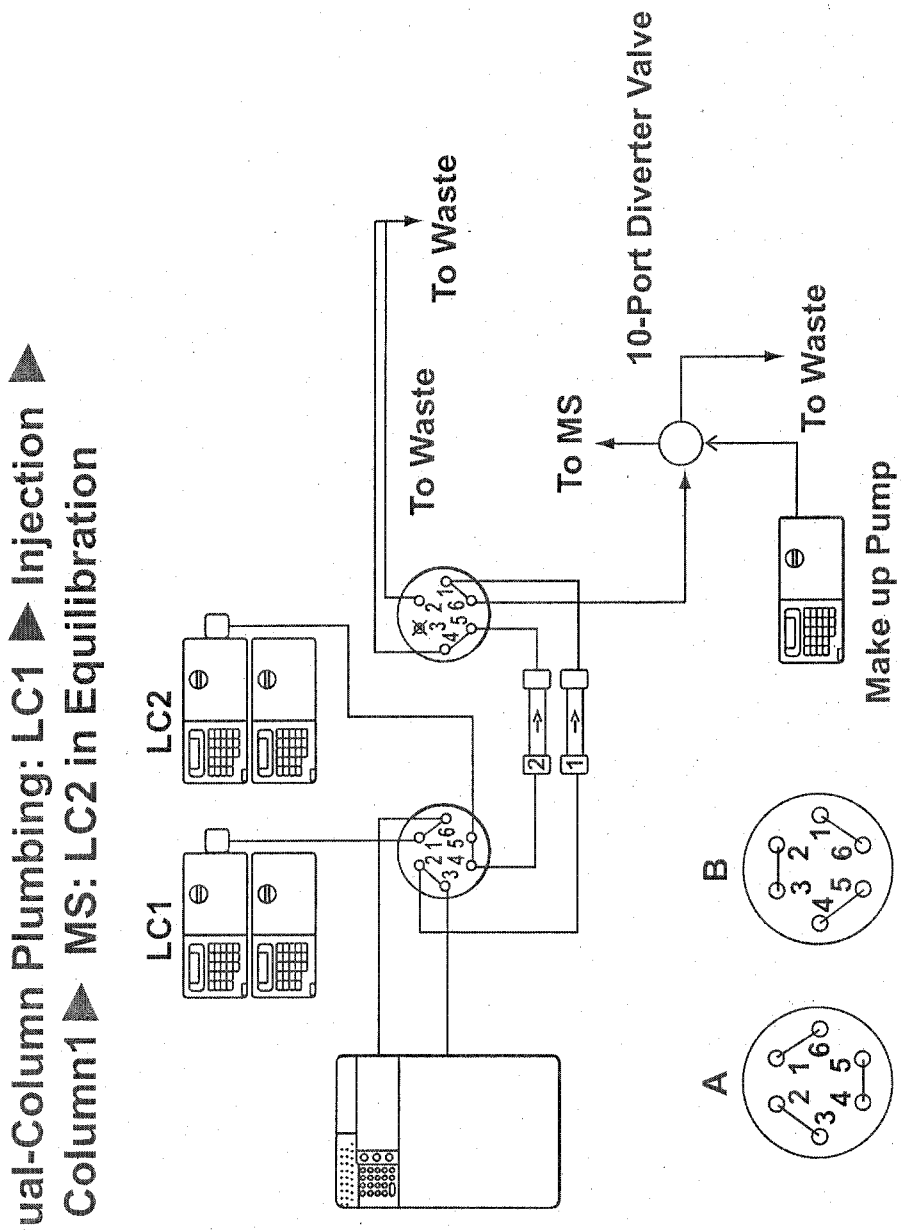

Dual-Column Plumbing: LC1 ▶ Injection ▶
Column1 ▶ MS: LC2 in Equilibration
LC2
LC1
To Waste
To Waste
To MS
10-Port Diverter Valve
To Waste
Make up Pump
A
B

FIG. 5

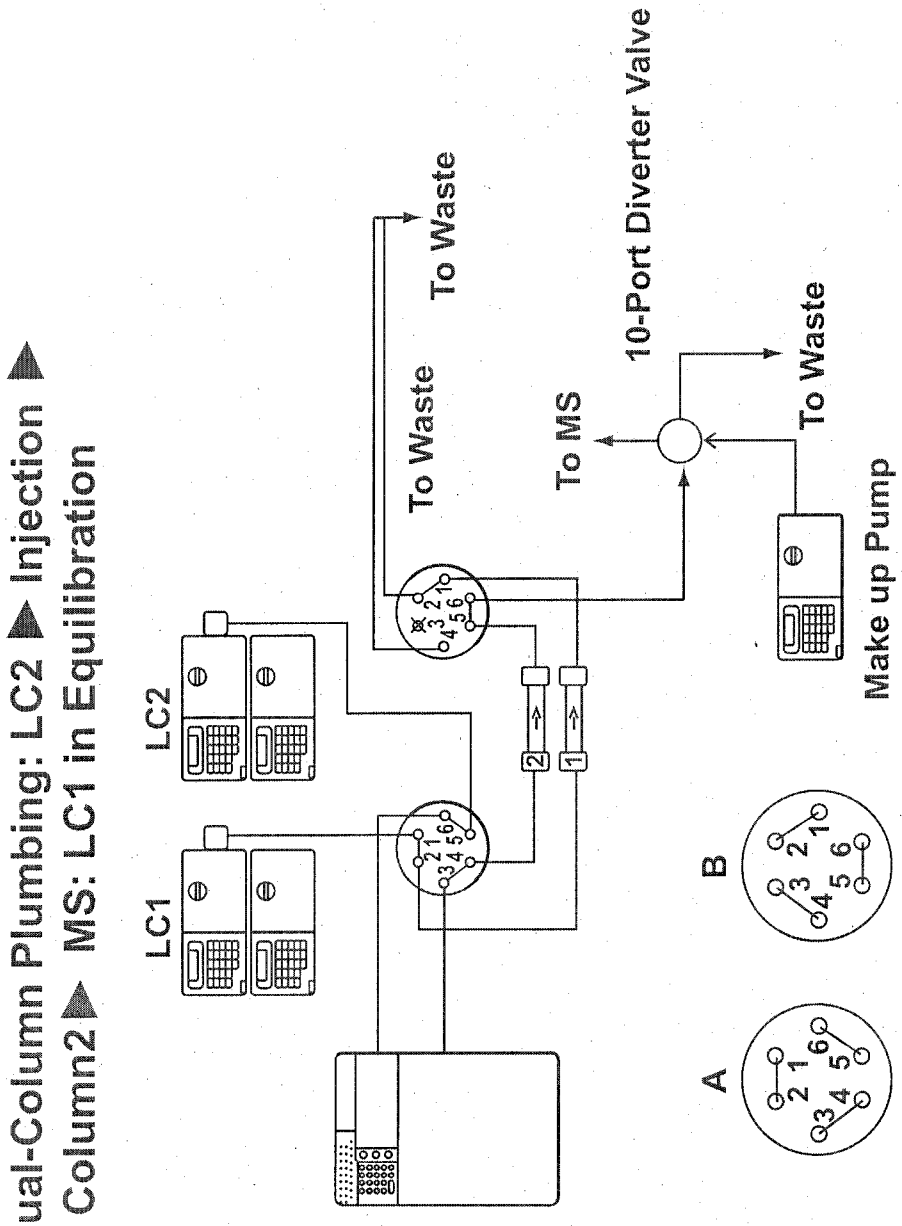

Dual-Column Plumbing: LC2 ▶ Injection ▶
Column2 ▶ MS: LC1 in Equilibration
LC2
LC1
To Waste
To Waste
To MS
10-Port Diverter Valve
To Waste
Make up Pump
A
B

FIG. 6

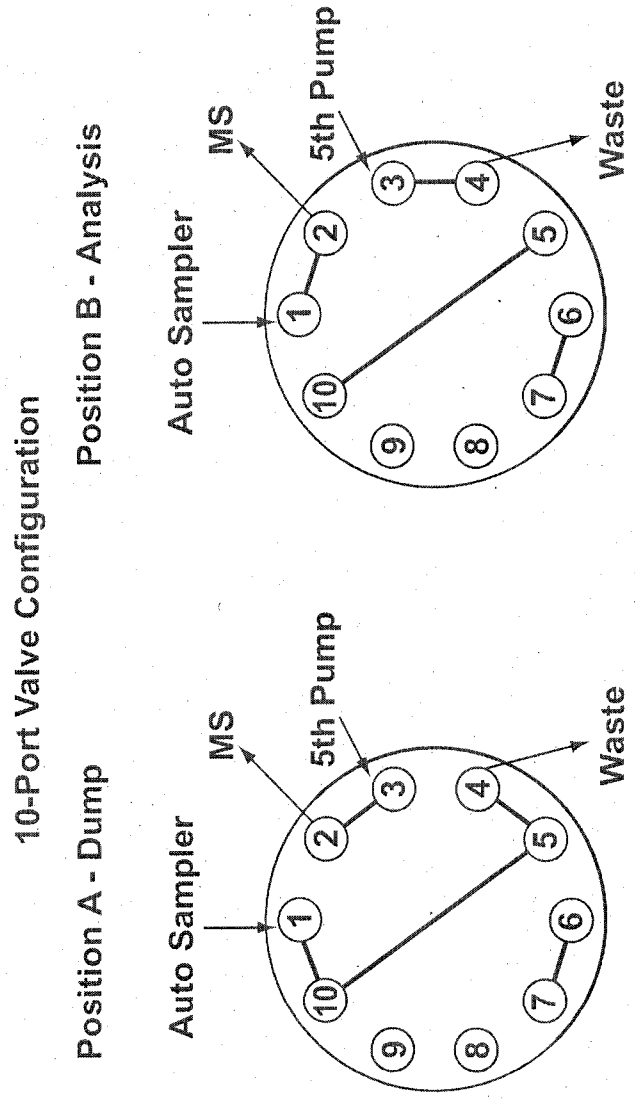

10-Port Valve Configuration
Position A - Dump
Auto Sampler
MS
5th Pump
Waste
1
2
3
4
5
6
7
8
9
10
Position B - Analysis
Auto Sampler
MS
5th Pump
Waste
1
2
3
4
5
6
7
8
9
10

FIG. 7

Standard Drink as defined by various countries - values are for pure alcohol in grams and millilitres - http://en.wikipedia.org/wiki/Standard_drink

Country	Weight	Volume
Japan	19.75 g	25 mL
Netherlands	9.9 g	12.5 mL
NewZealand	10.0 g	12.7 mL
Poland	10.0 g	12.7 mL
Portugal	14.0 g	17.7 mL
Spain	10.0 g	12.7 mL
UK	7.9 g	10.0 mL
USA	14.0 g	17.7 mL*
		(defined as 0.6 fl oz)

FIG. 8

Standard Drink as defined by various countries - values are for pure alcohol in grams and millilitres - http://en.wikipedia.org/wiki/Standard_drink

Country	Weight	Volume
Australia	10.0 g	12.7 mL
Canada	13.5 g	17.1 mL
Denmark	12.0 g	15.2 mL
Finland	11.0 g	13.9 mL
France	12.0 g	15.2 mL
Hungary	17.0 g	21.5 mL
Iceland	9.5 g	12 mL
Ireland	10.0 g	12.7 mL
Italy	10.0 g	12.7 mL

FIG. 9

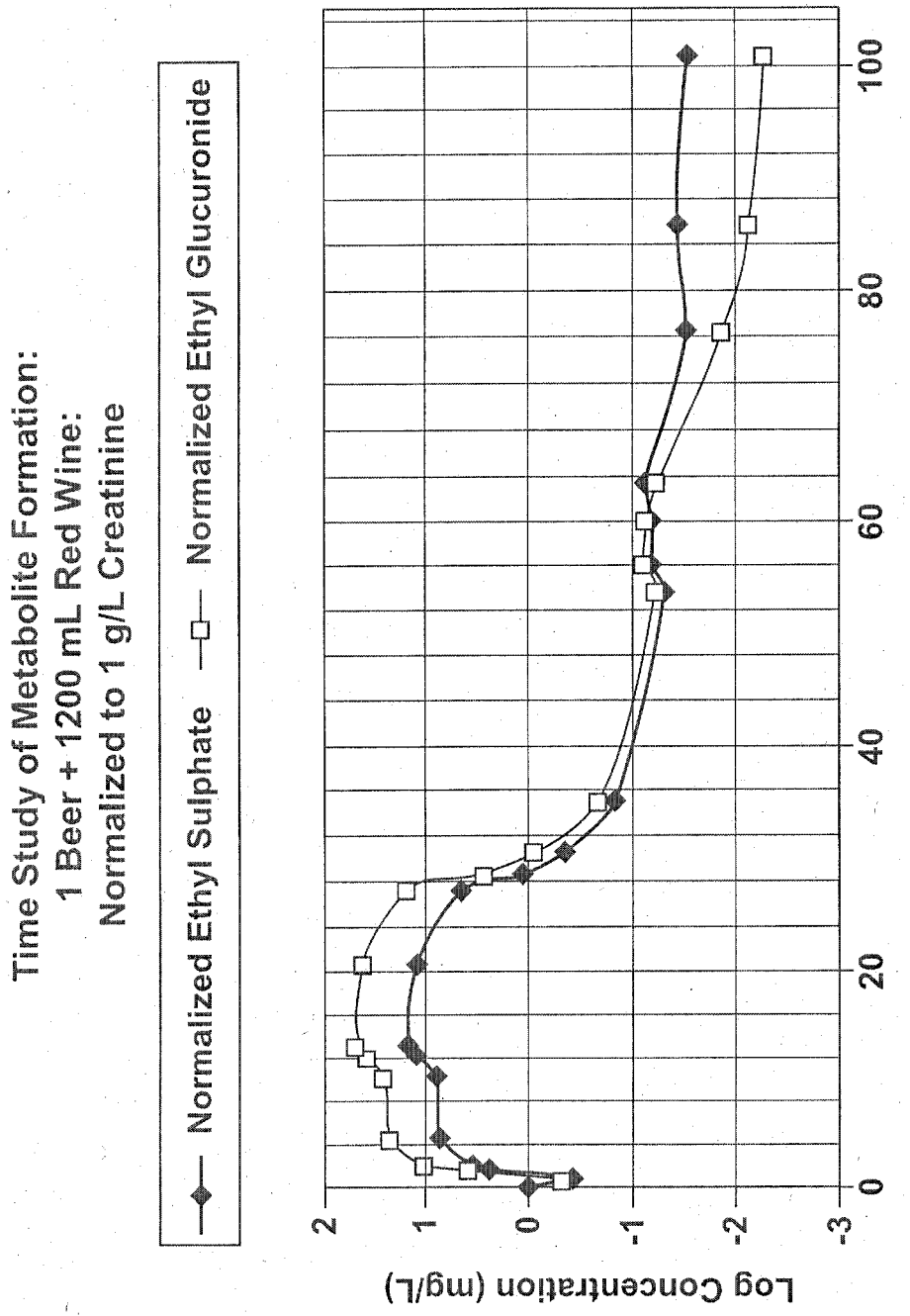

Time Study of Metabolite Formation:
1 Beer + 1200 mL Red Wine:
Normalized to 1 g/L Creatinine
Normalized Ethyl Sulphate
Normalized Ethyl Glucuronide
Log Concentration (mg/L)
2
1
0
-1
-2
-3
0
20
40
60
80
100
Time (hours)

FIG. 10

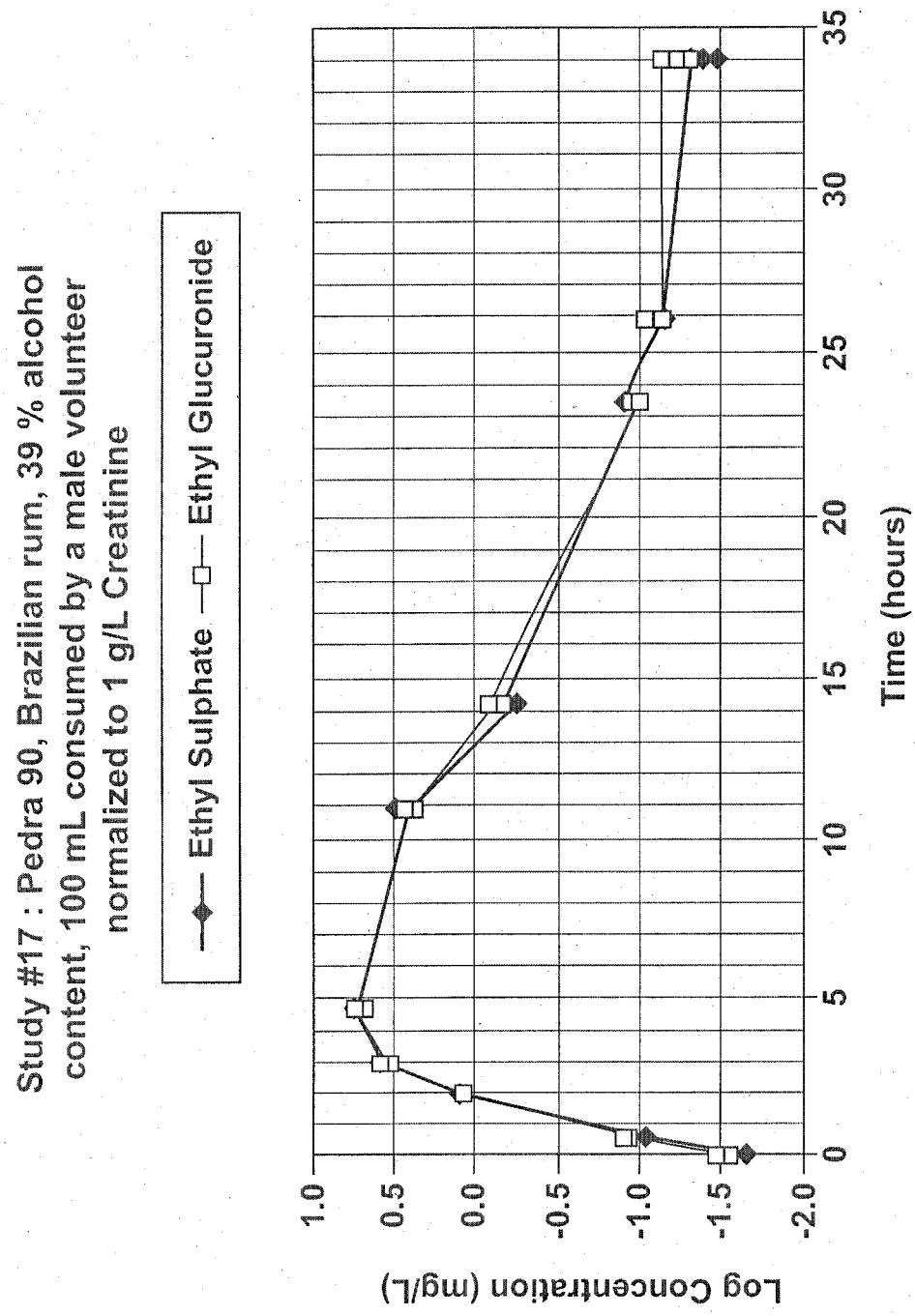

Study #17 : Pedra 90, Brazilian rum, 39 % alcohol content, 100 mL consumed by a male volunteer normalized to 1 g/L Creatinine
Ethyl Sulphate
Ethyl Glucuronide
Log Concentration (mg/L)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
0
5
10
15
20
25
30
35
Time (hours)

FIG. 11

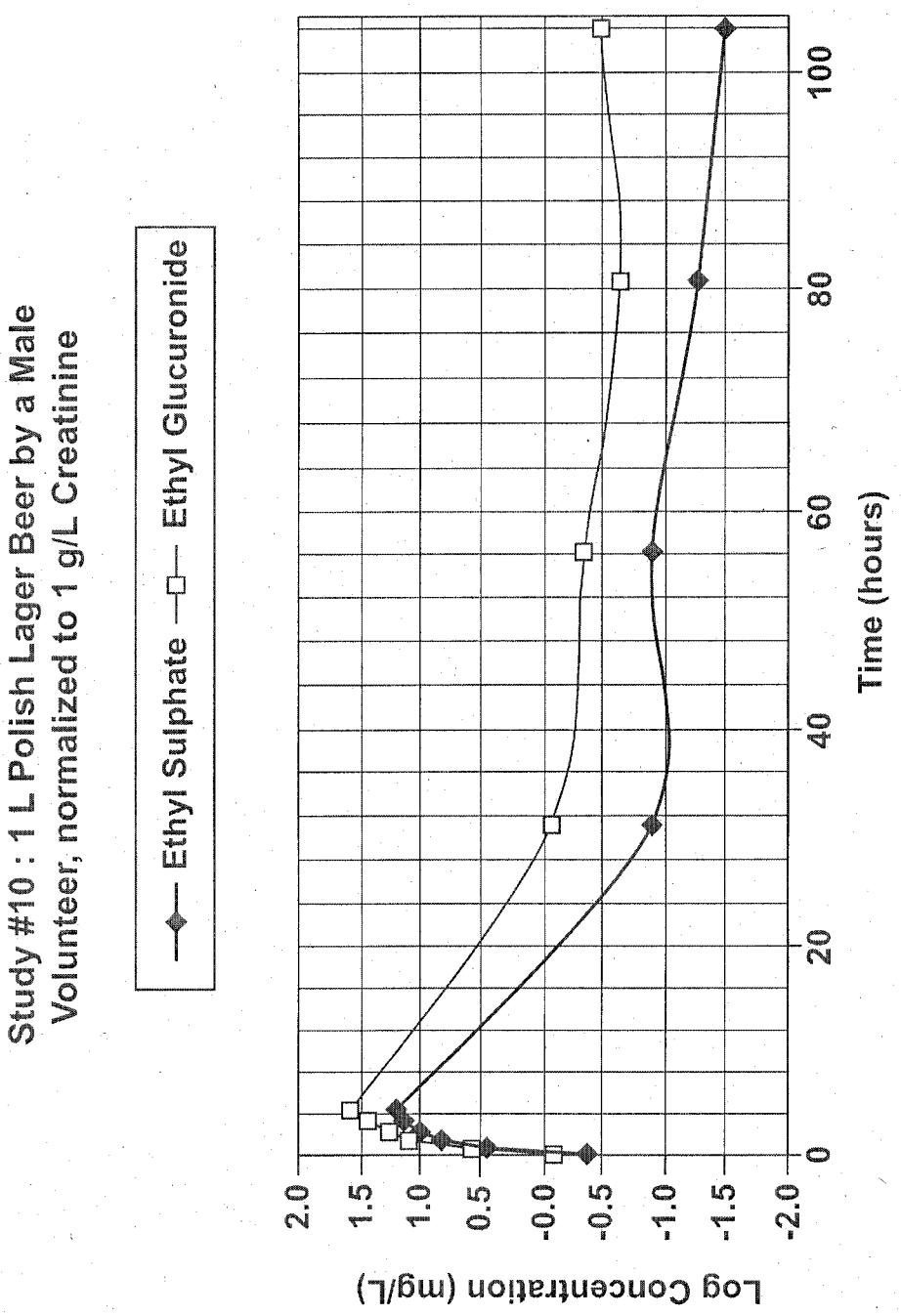

Study #10 : 1 L Polish Lager Beer by a Male
Volunteer, normalized to 1 g/L Creatinine
Ethyl Sulphate
Ethyl Glucuronide
Log Concentration (mg/L)
2.0
1.5
1.0
0.5
-0.0
-0.5
-1.0
-1.5
-2.0
0
20
40
60
80
100
Time (hours)

FIG. 12

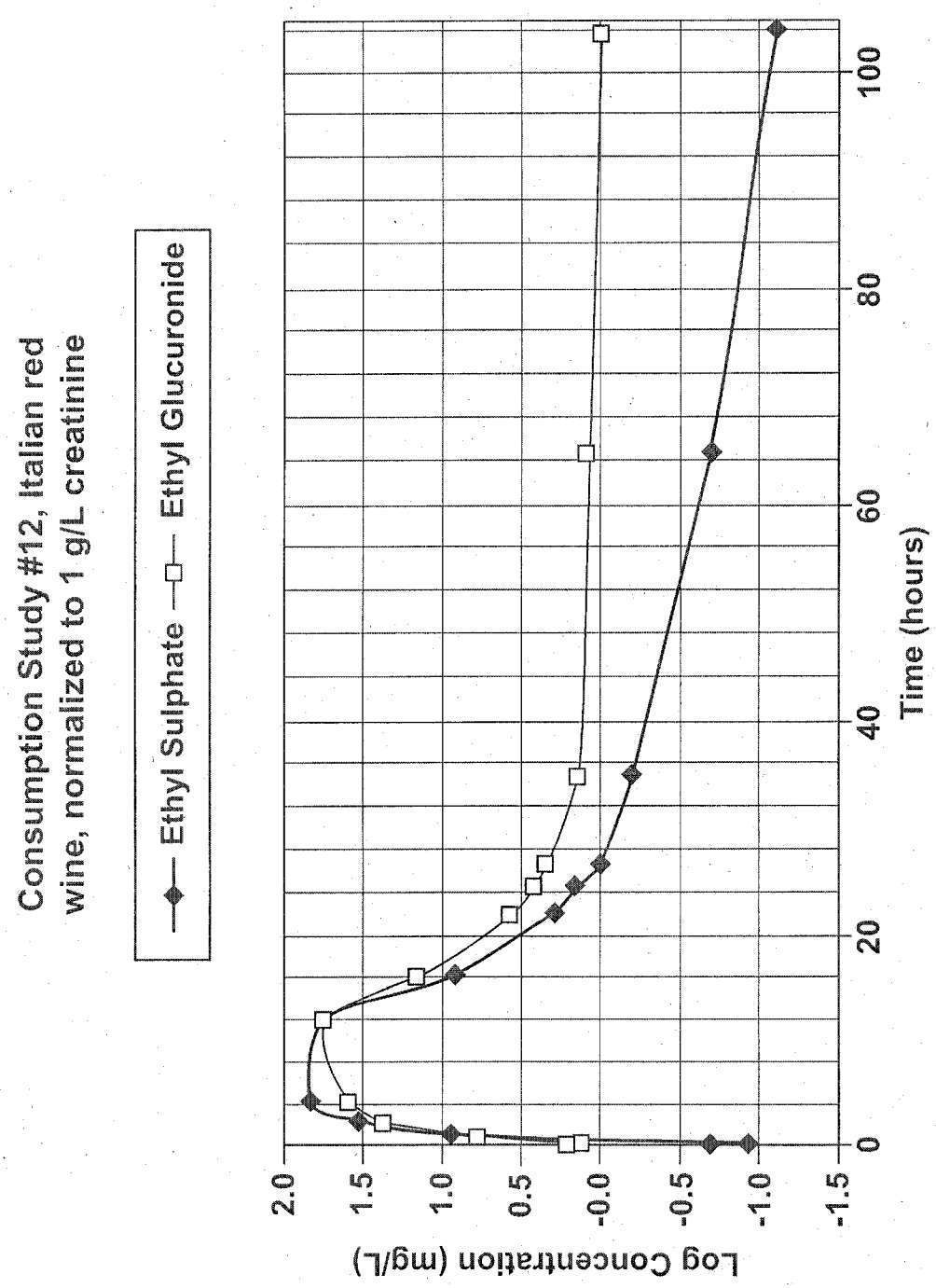

Consumption Study #12, Italian red wine, normalized to 1 g/L creatinine
Ethyl Sulphate
Ethyl Glucuronide
Log Concentration (mg/L)
2.0
1.5
1.0
0.5
-0.0
-0.5
-1.0
-1.5
0
20
40
60
80
100
Time (hours)

FIG. 13

Baileys Irish Cream on Ice
Normalized to 1 g/L Creatinine

Time	Urine Volume	Average Ethyl Sulphate	Average Ethyl Glucuronide	Average Creatinine
(hours)	(mL)	(mg/L)	(mg/L)	(mg/L)
0.00	185	0.056	0.111	1982
0.33	40	0.042	0.109	3538
1.75	125	0.990	3.711	1253
9.00	376	1.032	7.573	2525
11.00	10	0.158	2.052	4249

FIG. 14

Consumption Study #12 : Red Italian wine - tabulation

Time	Volume	Conc Et-S	Weight Et-S	Conc Et-G	Weight Et-G	Creatinine	log Average	log Average
Hours	mL	mg/L	mg	mg/L	mg	mg/L	N Et-S	N Et-G
0.00	148	0.025	0.004	2.515	0.372	1556.000	-0.942	0.209
0.25	19	0.062	0.001	2.669	0.051	2030.300	-0.687	0.119
0.92	26	2.343	0.061	11.037	0.287	1918.700	0.938	0.760
2.18	352	1.669	0.587	9.114	3.208	404.800	1.530	1.352
4.17	402	5.437	2.186	21.663	8.709	557.800	1.827	1.589
12.00	275	15.004	4.126	101.958	28.038	1876.200	1.736	1.734
16.50	242	1.496	0.362	16.931	4.097	1237.500	0.918	1.136
22.25	67	0.529	0.035	7.518	0.504	2050.400	0.283	0.563
24.75	95	0.402	0.038	4.711	0.448	1857.000	0.164	0.403
27.00	96	0.252	0.024	3.947	0.379	1841.000	0.001	0.331
35.00	238	0.200	0.048	3.210	0.764	2309.300	-0.199	0.143
65.00	175	0.063	0.011	2.587	0.453	2172.800	-0.671	0.076
104.00		0.031	0.000	2.492	0.000	2235.600	-1.069	0.047

FIG. 15

Consumption Study #15
(Beer + Sake) - Tabulation

Hours	log Time(hrs)	Volume (mL)	Ave Et-S (mg/L)	ave log Et-S	ave Et-G (mg/L)	ave log Et-G	Creatinine (mg/L)	log C
0.00		110	0.000		0.000		2434.400	3.386
0.25	-0.6021	750	0.624	0.1767	0.980	0.382	415.330	2.618
0.50	-0.3010	300	1.751	0.6782	3.548	0.984	367.293	2.565
1.00	0.0000	250	3.258	0.8935	8.745	1.322	417.177	2.620
2.00	0.3010	300	5.627	0.9828	18.495	1.500	584.917	2.767
4.00	0.6021	150	15.091	0.9275	55.803	1.496	1783.300	3.251
66.00	1.8195	150	0.000		0.000		395.053	2.597
96.00	1.9823	150	0.000		0.000		967.413	2.986
120.00	2.0792	150	0.000		0.000		1829.700	3.262

FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/001728

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***G01N 33/98*** (2006.01) , ***G01N 33/483*** (2006.01) , ***G01N 33/50*** (2006.01) , ***G01N 33/70*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***G01N 33/98*** (2006.01) , ***G01N 33/483*** (2006.01) , ***G01N 33/50*** (2006.01) , ***G01N 33/70*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
CPD, Delphion, Derwent, Pubmed
Keywords: ethanol, alcohol, creatinine, normalizing, normalisation, deuterated, ethyl sulphate, ethyl glucuronide, consumption

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Bergstrom et al., "Ethyl glucuronide concentrations in two successive urinary voids from drinking drivers: relationship to creatinine content and blood and urine ethanol concentrations", Forensic Science International, 2003, 133:86-94 Whole document	1-32
Y	Dresen et al., "Forensic confirmatory analysis of ethyl sulfate - A new marker for alcohol consumption - by liquid-chromatography/electrospray ionization/tandem mass spectrometry", J. Am. Soc. Mass Spectrom., 2004, 15:1644-1648 Whole document	1-32
Y	Dahl et al., "Comparison of urinary excretion characteristics of ethanol and ethyl glucuronide", Journal of Analytical Toxicology, 2002, 26:201-204 Whole document	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 December 2008 (01-12-2008)	16 January 2009 (16-01-2009)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Isabelle Gagne 819- 997-2743

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/001728

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Takahashi et al., "Tandem mass spectrometry measurements of creatinine in mouse plasma and urine for determining glomerular filtration rate", Kidney Int., February 2007, 71(3):266-271 Whole document	1-32
Y	Huskova et al., "Determination of creatinine in urine by tandem mass spectrometry", Clin. Chim. Acta, December 2004, 350(1-2):99-106 Whole document	1-32