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Simultaneous determination of GHB and EtG in hair using GCMS/MS

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A gas chromatographic tandem mass spectrometric (GCMS/MS) method for simultaneously determining trace concentrations of gamma-hydroxybutyrate (GHB) and ethyl glucuronide (EtG) in hair has been developed. Multiple reaction monitoring (MRM) was used to detect precursor and product ions of GHB, (233 and 147) and EtG (261 and 143) following anion exchange solid phase extraction and derivatization with *N,O-bis*[trimethylsilyl]trifluoroacetamide (BSTFA). Deuterated standards of GHB and EtG were used as internal standards. The assay produced excellent linearity ($r^2 > 0.99$) and sensitivity. The lower limit of quantitation (LLOQ) was 10 pg/mg for EtG assuming a 20 mg hair sample. The method has been used to investigate cases of suspected drug facilitated assault as well as being used to identify heavy alcohol consumption in a group of volunteers.

Keywords: GHB; ethyl glucuronide; GCMS/MS; hair

Introduction

Gamma-hydroxybutyrate (GHB) is a powerful central nervous system depressant originally developed as an anaesthetic drug in the 1960s. It is an analogue of the endogenous neurotransmitter gamma-aminobutyric acid (GABA) and is present in regions of the mammalian brain although its functions in this capacity are not completely understood.[1]

GHB has found legal uses as an anaesthetic agent and in the treatment of alcohol withdrawal and narcolepsy.[2] However, the drug has gained widespread illicit use due to its euphoric and sedative properties. GHB has been alleged to increase production of growth hormone leading to its misuse by bodybuilders as a steroid alternative.[3] In low doses GHB is a popular club drug, sometimes known as 'liquid ecstasy' or 'scoop' due to the stimulant, euphoric effects at this concentration. At higher concentrations the drug is a hypnotic with powerful sedative properties. Unfortunately such properties have led to a recent rise in the use of GHB in drug-facilitated sexual assault, also known as 'date rape'. This problem is further exacerbated by the fact that GHB can be totally excreted from the body within hours, making timely analysis a crucial factor in such cases. The half-life of GHB is approximately 30 min and is non-linear in that it increases at higher doses.[4,5] Studies have shown that GHB is undetectable in plasma after 6–8 hours.[6,7] The detection of GHB in hair significantly increases the detection window and can be a useful alternative to the traditional matrices of blood and urine. GHB detection in hair represents a significant challenge to the toxicologist. There are complicated issues of endogenous GHB in hair, which require careful interpretation. It is necessary to use each subject as their own control when analyzing GHB in hair; this enables the analyst to distinguish the basal GHB level found in hair from a possible exogenous administration of the drug. To accomplish this, multiple sections of hair are analyzed from the individual to determine their endogenous GHB range. Once this is complete, a section of hair with a significant deviation from this concentration range would strongly suggest exogenous administration. The concept of segmentation has been discussed by several authors, notably Goulle, Kalasinsky, and Kintz.[8,9,10]

In the study by Goulle *et al*.[8] sample preparation is achieved through liquid-liquid extraction; the research found an interesting observation relating to GHB levels in beard hair. It was found that GHB levels in beard hair could be 10–60 times higher than concentrations observed in head hair. This was said to be a consequence of the elimination of GHB via sweat, causing contamination of the beard hair. For this reason it is important that GHB concentrations from the proximal segment of head hair be treated with caution as an elevated level might not be a positive indicator of GHB exposure. GHB contamination due to sweat is more likely to occur in the proximal section than in the distal section. Kalasinsky *et al*.[9] produced the first study to attempt the analysis of single dose GHB administration. A single dose of GHB was believed to be responsible for the overdose and death of a 22-year-old woman. Analysis of segmental hair sections did not identify GHB above the cut-off concentration of 1 ng/mg. However the analysis of the root bulbs identified very high levels of GHB at 47.4 ng/mg and 2221 ng/mg, washed *versus* unwashed hair samples respectively. Kintz *et al*.[10] evaluated hair digestion parameters and discovered that NaOH when used at a lower concentration (0.01 M) rather than the more commonly used 1 M concentration produced a cleaner extract, with less breakdown products. This extract was used to successfully identify a single dose GHB exposure.

When analysis of GHB in hair is necessary as part of the investigation of drug-facilitated assault, the challenge is even greater, as it is becomes necessary to be able to detect a single dose of the drug. Due to difficulties of drug incorporation in hair, and the resulting low concentrations, this part of the work requires great attention to develop a fast, accurate, and sensitive method to determine just a single dose of the drug in hair. Drug-facilitated assault is commonly associated with excess alcohol

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consumption, and for this reason the development of an assay to simultaneously determine GHB in addition to the ethanol metabolite ethyl glucuronide (EtG) was undertaken. The ability to combine the two assays (GHB and EtG simultaneously) represents a significant development in this field, as it provides a simple and fast method to characterize the presence of GHB and can also show evidence of heavy drinking from the same hair sample.

Ethanol itself is not a suitable compound for hair analysis due to its high volatility, and for this reason research has focused on other alcohol markers, particularly fatty acid ethyl esters (this group comprises over 20 compounds), ethyl glucuronide, and cocaethylene (a mixed metabolite resulting from combined use of ethanol and cocaine).[11,12,13] Other less common direct alcohol markers included ethyl phosphate, ethyl sulphate, phosphatidyl ethanol, and ethyl phenidate.

After alcohol is ingested, it is metabolized by various pathways to a number of secondary products. A two-stage oxidation process converts a proportion of the ethanol to acetaldehyde by alcohol dehydrogenase and then on to acetic acid via aldehyde dehydrogenase.[14] A very small fraction of the ethanol may also be converted to ethyl glucoronide (EtG) after a phase II conjugation reaction with glucuronic acid which is catalyzed by endoplasmic reticulum UDP – glucuronosyltransferase.

The EtG can then be excreted in the urine, and a small fraction will be incorporated into the hair. EtG is an interesting and useful ethanol metabolite as it is only produced *in vivo* following ethanol consumption. This has allowed its use as a marker for chronic alcohol use.

Several authors have commented on the suitability of EtG in hair as a reliable marker for alcohol consumption. Politi *et al.* investigated the correlation between ethanol daily intake and the concentration of EtG in hair.[15] Their results suggested an EtG concentration of up to 434.7 pg/mg in the hair of alcoholics whilst no EtG was detected in the hair of teetotalers. Skopp *et al.*[16] detected EtG in hair at concentrations as high as 13.8 ng/mg from alcohol abusers, and approaching 2.2 ng/mg in the hair of social drinkers. Research has focused on detection methods utilizing GCMS (gas chromatography mass spectrometry), or LCMS (liquid chromatography mass spectrometry) technologies both of which have been combined with tandem mass spectrometry in an effort to reduce detection limits.[17–20] Headspace solid phase microextraction (HS-SPME) has also been used by researchers in an effort to improve the detection limits for EtG. Agius *et al.*[21] developed a method based on hair extraction with water, followed by clean-up with solid phase extraction, derivatization with HFBA, and HS-SPME in combination with GCMS/MS (gas chromatography tandem mass spectrometry). This produced an LLOQ of 2.8 pg/mg. In addition to technological improvements, new research has suggested the usefulness of applying EtG hair testing in the field of abstinence monitoring of suspected drinking drivers. Liniger *et al.*[22] utilized the detection of EtG in hair to monitor a group of 154 people whose fitness to drive had been questioned due to suspected drinking problems. The group had all claimed to be tee-total. The research showed that two thirds of the group actually displayed EtG levels consistent with excessive drinking (EtG concentrations >30 pg/mg).

A number of researchers have commented on the suitability of EtG as a biomarker for the detection of alcohol[23,24,25] and our understanding of the interpretation of this technique is rapidly growing stronger. In support of the growing trend towards the use of hair biomarkers for alcohol detection Pragst *et al.*[26] have suggested that the combined use of fatty acid ethyl esters in conjunction with EtG provides a great increase in accuracy for the diagnosis of chronic alcohol abuse.

Experimental

Solvents and reagents

GHB (1.0 mg/ml) was purchased from LGC Promochem (Teddington, UK). EtG (1.0 mg/ml) was purchased from Medichem (Steinenbronn, Germany). Methanol, ammonia and acetic acid were purchased from Fisher Scientific (Loughborough, UK. BSTFA with 1% trimethylchlorosilane was purchased from Sigma Aldrich (Poole, Dorset, UK).

Preparation of standards

The EtG and GHB stock solutions were diluted with methanol to prepare a working solution which was then stored at $-20\,^{\circ}$C. A working solution of GHB-D6 and EtG-D5 was prepared in the same way. On a daily basis, a sub-dilution of the working solution was prepared in de-ionized water. This solution was then used to prepare a five-point calibration range and three quality control samples. A daily sub-dilution of GHB-D6 and EtG-D5 was prepared in the same manner from its working solution. During method development it was noted that GHB has poor long-term stability in water, thus it was necessary to prepare the water sub-dilution for calibrators on a daily basis.

Preparation of hair samples

A minimum of 20 mg of hair was used for this assay. Hair was divided into multiple sections of 5 mm and then thoroughly washed with methanol. The methanol wash was then removed and transferred to a separate test tube for storage. 1 ml of de-ionized water and 200 µl of the GHB-D6/EtG-D5 daily sub-dilution were then added to the hair sample, which then underwent overnight sonication. It has been shown by Jurado *et al.*[19] that extraction of EtG from hair using water is preferable to extraction using methanol, methanol/water mixes, and aqueous trifluoroacetic acid.

Solid-phase extraction

Following sonication, samples were prepared for analysis using anion exchange solid-phase extraction. Waters Oasis MAX (sorbent mass 30 mg and reservoir volume 1 ml) were conditioned with 1 ml methanol and 1 ml de-ionized water. 500 µl of sample (calibrator, quality control, blank or hair sample) was then loaded onto the column. Interference elution was achieved through sequential washes of 1 ml 5% ammonia solution in water, 1 ml water, and finally 1 ml methanol. The column was then dried under full vacuum for 5 min. 1 ml 5% acetic acid in methanol was used to elute the analyte. Samples were then evaporated to dryness and reconstituted in 10 µl ethyl acetate and 10 µl BSTFA and thoroughly vortex mixed. To ensure complete derivatisation of GHB and EtG, the samples were heated on a hot plate set to $80\,^{\circ}$C for 20 min. Samples were then allowed to cool for 10 min before 2 µl was injected on to the GCMS/MS system.

Table 1. Summary statistics for GHB validation

Spiked amount of GHB	3.0 ng/ml		6.0 ng/ml		9.0 ng/ml	
	Intraday	Interday	Intraday	Interday	Intraday	Interday
N	3	9	3	9	3	9
Mean	3.03	3.04	6.10	6.08	8.93	9.04
S.D	0.07	0.11	0.07	0.13	0.15	0.12
% C.V	2.31	3.62	1.15	2.14	1.68	1.33

Table 2. Summary statistics for EtG validation

Spiked amount of EtG	0.6 ng/ml		1.2 ng/ml		1.8 ng/ml	
	Intraday	Interday	Intraday	Interday	Intraday	Interday
N	3	9	3	9	3	9
Mean	0.56	0.60	1.15	1.20	1.86	1.84
S.D	0.08	0.06	0.03	0.06	0.10	0.06
% C.V	14.28	10.00	2.61	5.00	5.38	3.26

Table 3. EtG levels in volunteers

Volunteer no.		EtG ng/mg	Volunteer no.		EtG ng/mg
Heavy drinker	**1**	0.07	Social drinker	**5**	0
		0.07			0
		0.09			0
		0.05			0.03
		0.12			
		0.1	Light drinker	**6**	0.05
		0.1			0.03
		0.11			0
					0
Moderate drinker	**2**	0.02			
			Teetotaler	**7**	0
Moderate drinker	**3**	0.07			0
					0
Social drinker	**4**	0.03			0
		0.03			
		0.02			
		0.03			

Instrumentation and analysis

Analysis was performed on a Varian CP3800 gas chromatograph equipped with a 1200L series triple quadrupole mass spectrometer (Yarnton, Oxford, UK). 2 µl of sample was injected in splitless mode. The column used was a Varian Factor IV (15m × 0.25 mm DF = 0.25), (Yarnton, Oxford, UK). The injector and detector temperatures were set to 280 °C. Column flow rate was 1 ml/min, carrier gas was helium. The column temperature was initially set at 50 °C for 1 min before increasing to 120 °C at a rate of 20 °C per minute. The temperature was then increased to 300 °C at 75 °C per minute before a final temperature ramp to 320 °C at 50 °C per minute, taking the total run time to 7.30 min.

Multiple reaction monitoring (MRM) was used in the Electron Impact mode (EI) for quantitative analysis. The precursor ions of EtG (261) and EtG-D5 (266) were selected in the first quadrupole before fragmentation in the collision cell at an energy of 10.0 eV, giving product ions of 143 for both EtG and EtG-D5. The precursor ions of GHB (233) and GHB-D6 (239) were selected in the first quadrupole before fragmentation in the collision cell at an energy of 9 eV, giving product ions of 147 for both GHB and GHB-D6. All data was acquired and analyzed using Varian 1200 software.

Results and Discussion

The analytical method was fully validated. The results from the validation are summarized in Tables 1 and 2. The limit of detection (LOD) for EtG was 5 pg/mg. The lower limit of quantification for EtG was 10 pg/mg assuming a 20 mg hair sample. An upper limit of quantification (ULQ) of 10.0 ng/mg was set for this assay. Spiked concentrations higher than the upper limit were found to be accurate when extrapolated up using the standard plot data. No carryover was detected following injection of the ULQ. It is not appropriate to state the LOD or LLOQ for GHB due to the endogenous presence of GHB in hair. There will always be a background level of GHB in hair samples so calculating the true limit is not possible.

The intra-day variability was determined by the analysis of three control samples run in triplicate. The EtG control samples were spiked at 0.6, 1.2 and 1.8 ng/ml and run on a single day, as were the GHB control samples which were spiked at 3.0, 6.0 and 9.0 ng/ml. The inter-day variability was determined using the same concentrations in triplicate over three separate days during a period of three weeks. Five calibrators and two blank samples were analyzed with each validation run. No EtG was found in the blank controls above the cut-off level. Standard curves were linear ($r^2 = >0.99$).

EtG levels from volunteers

This method was also used to define the relationship between the EtG concentrations found in hair and the amount of alcohol consumed by an individual on a regular basis. The hair of seven volunteers was taken in varying amounts (dependant on amount of head hair available). Volunteer 1 was a 38-year-old male; volunteers 2–5 were male and between 30 and 40 years of age; volunteer 6 was a 54-year-old female; and volunteer 7 was a 14-yaer-old male. The amount of alcohol that the volunteers drank on a regular basis was reported in approximate units (10 ml pure alcohol in UK law) of alcohol per week and from this a classification scheme was devised producing five categories:

Heavy drinker: >25 units per week
Moderate drinker: >15 units per week
Social drinker: >10 units per week
Light drinker: >5 units per week
Teetotal: 0 units per week

Head hair from the seven volunteers was prepared and analyzed according to the procedure described above. Hair was sectioned into multiple 1-cm sections where possible, according to the length and amount of hair available. Not all hair samples were cut into the same number of sections. The results are shown in Table 3.

It can be seen from these results that the relationship between alcohol consumed and the corresponding EtG level in hair is difficult to define. Any relationship between these variables will not be consistent between individuals as everyone metabolizes alcohol at a different rate. It is important to note that these results

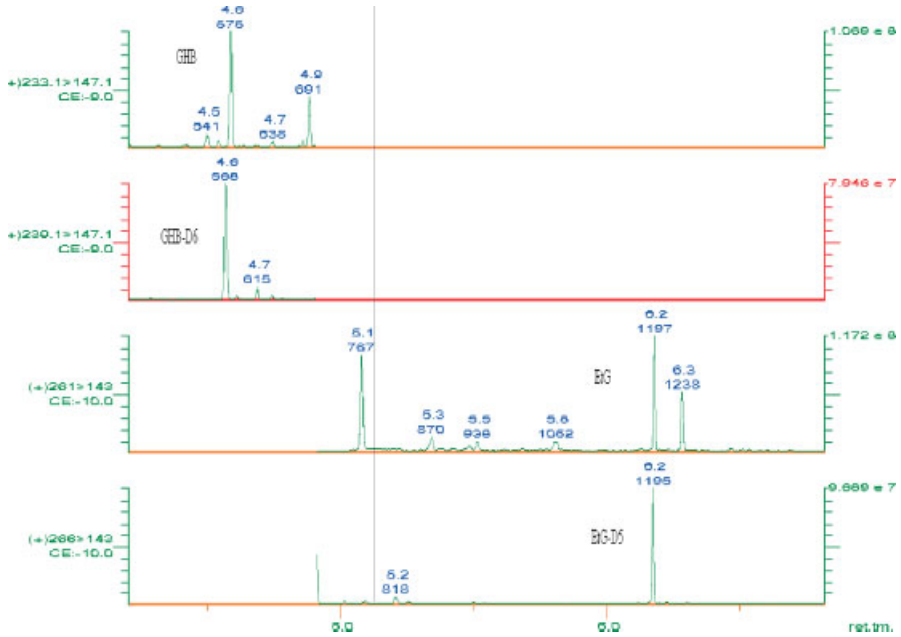

Figure 1. Chromatogram from combined EtG/GHB analysis. GHB concentration from hair sample was measured at 3.23 ng/mg.

only apply to the small volunteer group studied, and in general it is difficult to relate an EtG concentration in hair to a quantitative measure of alcohol consumption. It can be seen that the level of EtG found in the self-reported heavy drinker, volunteer 1, falls above the recently agreed cut-off value of 30 pg/mg of EtG to document chronic excessive alcohol consumption.[27]

Determination of exogenous GHB in a recreational drug user

Head hair was sampled from a 25-year-old male who admitted recreational use of GHB on one occasion. The hair was 5-cm long and therefore segmented into 10 sections of 5 mm. Analysis using the described methodology showed a level of EtG in all sections. Endogenous GHB along the hair shaft was documented at levels between 0.3 ng/mg and 0.11 ng/mg, but a significant rise in concentration was noted in sections of hair 2–3 cm along the hair length. This corresponded approximately with the history of the recreational user who admitted using GHB approximately seven weeks prior to the hair sampling. GHB in hair sections 5 and 6 of the 10 samples was detected at 4.5 ng/mg and 3.81 ng/mg respectively. Whilst a single exposure to GHB may result in a tight band of the drug being observed in only one 5-mm hair section only, the observation of exogenous GHB levels in two sections could be due to drug migration through a small section of the hair, or a possible inaccuracy in the drug-use history of the subject.

Application of method to drug-facilitated assault cases

Following the successful validation and testing of the method to simultaneously detect EtG and GHB, a partnership was established with a local police force, whereby all cases of suspected drug-facilitated assault over a period of months were referred to TrichoTech for analysis using this method.

Four cases of suspected drug-facilitated assault were referred to TrichoTech. Three were cases of alleged drug-facilitated sexual assault. Hair from these three cases was cut into multiple sections of 5 mm and analyzed using the described method. EtG was found in all samples, but GHB concentrations were deemed to be endogenous.

The fourth case involved a male who was arrested for grievous bodily harm following an assault at a party. The man arrested for the offence claimed no knowledge of this, stating that he had lost all memory from half-way through the party. The suspects hair was 4-cm long, and was therefore prepared into 8 sections of 5-mm lengths. Analysis of the hair showed evidence of alcohol use, but more importantly, on first examination it demonstrated exogenous exposure to GHB in the hair section corresponding to the approximate date of the incident. It therefore appeared possible that the suspect's drink had been spiked with GHB, which would account for the anterograde amnesia and mood-altering effect. Endogenous concentrations along the hair shaft were in the range 0.1 ng/mg to 0.19 ng/mg whereas the hair segment corresponding to the month of the assault showed a concentration of 3.23 ng/mg. To confirm this result, an additional sample of hair was taken from the suspect two months later. Theoretically, if the previously found exposure to GHB was the result of exogenous exposure, the 'spike' of GHB should have travelled down the hair shaft approximately 2 cm in accordance with an average growth rate of 1 cm per month. However, when this additional hair sample was analyzed, the GHB spike had not shifted down the hair shaft. A significant rise in GHB concentration was again observed in this sample, but had remained in the proximal section. We concluded that this rise in GHB concentration was due to increased GHB incorporation in the proximal segment due to sweat, rather than due to exogenous GHB exposure. For this reason it is advisable to wait at least 4–6 weeks after a possible GHB exposure to avoid issues of contamination due to sweat.[8,10] An example of the chromatography from this sample is shown in Figure 1.

Conclusions

The detection of GHB and EtG in hair is a complex procedure, often requiring state-of-the-art tandem mass spectrometry and careful

interpretation of analytical data. The combined detection of EtG and GHB into a single, rapid and reliable method represents an extremely useful method in the field of drug analysis. The success of this combined method is important as it allows the analyst to interpret GHB results in the context of an individual's alcohol consumption. This will prove a great advantage during analysis of hair samples to aid in the investigation of drug-facilitated crime, as alcohol is thought to be the most commonly used drug in this type of offence.

References

[1] E. Haas, H. Fung, S. Fung, J. Raybon, J. Xu, *J. Chromatogr. B.* **2004**, *807*, 287.
[2] G. P. Wedin, C. S. Hornfeldt, L. M. Ylitalo, *Current Drug Safety* **2006**, *1*, 99.
[3] Anon., *MMWR* **1997**, *46*, 281.
[4] S. D. Ferrara, S. Zotti, L. Tedeschi, G. Frison, F. Castagna, L. Gallimberti, G. L. Gessa, P. Palatini, *Brit. J. Clin. Pharm.* **1992**, *34*, 231.
[5] R. Brenneisen, M. A. Elsohly, T. P. Murphy, J. Passarelli, S. Russmann, S. J. Salamone, D. E. Watson, *J. Anal. Toxicol.* **2004**, *28*, 625.
[6] S. Elliott, GHB. *Drug Monitor* **2003**, *4*, 1.
[7] F. Couper, B. Logan, National Highway Traffic Safety Administration. Drugs and Human Performance Fact sheets, **2000**, Available at: www.nhtsa.dot.gov/people/injury/research/job185drugs/gamma-hydroxybutyrate.htm [14 August 2010].
[8] J. Goulle, M. Cheze, G. Pepin, *J. Anal. Toxicol.* **2003**, *27*, 574.
[9] M. Kalasinsky, G. Dixon, G. Schmunk, S. Kish, *J. Forensic Sci.* **2001**, *46*, 728.
[10] P. Kintz, V. Cirimele, C. Jamey, B. Ludes, *J. Forensic Sci.* **2003;**, *48*, 1.
[11] V. Auwärter, F. Sporkert, S. Hartwig, F. Pragst, H. Vater, A. Diefenbacher, *Clin. Chem.* **2001**, *47*, 2114.
[12] B. Appenzeller, R. Agirman, P. Neuberg, M. Yegles, R. Wennig, Ethyl Glucuronide determination in segmental hair analysis of alcoholics. Presented at the Communication XIIIeme congres annuel de la Societe Francasie de Toxicologie Analytique, Pau, France, 8–10 June **2005**.
[13] L. Politi, A. Zucchella, L. Morini, C. Stramesi, A. Polettini, *Forensic Sci. Int.* **2007**, *172*, 23.
[14] A. Jones, *Forensic Sci. Int.* **1990**, *45*, 217.
[15] L. Politi, L. Morini, F. Leone, A. Polettini, *Addiction* **2006**, *101*, 1408.
[16] G. Skopp, G. Schmitt, L. Potsch, P. Dronner, R. Aderjan, R. Mattern, *Alcohol* **2000**, *283*.
[17] I. Janda, W. Weinmann, T. Kuehnle, M. Lahode, A. Alt, *Forensic Sci. Int.* **2008**, *128*, 59.
[18] L. Morini, L. Politi, A. Groppi, C. Stramesi, A. Polettini, Direct determination of ethyl glucuronide in hair samples by liquid chromatography electrospray tandem mass spectrometry, presentation at the workshop of the Society of Hair Testing, Strasbourg 28–30 September **2005**.
[19] C. Jurado, T. Soriano, M. Giménez, M. Menéndez. *Forensic Sci. Int.* **2004**, *145*, 161.
[20] M. Yegles, A. Labarthe, V. Auwärter, S. Hartwig, H. Vater, R. Wennig, F. Pragst, *Forensic Sci. Int.* **2004**, *145*, 167.
[21] R. Agius, T. Nadulski, HG. Kahl, J. Schrader, B. Dufaux, M. Yegles, F. Pragst, *Forensic Sci. Int.* **2010**, *196*, 3.
[22] B. Liniger, A. Nguyen, A. Friedrich-Koch, M. Yegles, *Traffic Inj. Prev.* **2010**, *2*, 123.
[23] R. B. Palmer. A review of the use of ethyl glucuronide as a marker for ethanol consumption in forensic and clinical medicine. *Semin. Diagn. Pathol.* **2009**, *1*, 18.
[24] G. Hosieth, L. Morini, A. Polettini, A. Christophersen, J. Morland, *Alcohol Clin. Exp. Res.* **2009**, *33*, 812.
[25] M. Concheiro, A. Cruz, M. Mon, A. de Castro, O. Quintela, A. Lorenzo, M. López-Rivadulla, *J. Anal. Toxicol.* **2009**, *33*, 155.
[26] F. Pragst, M. Rothe, B. Moench, M. Hastedt, S. Herre, D. Simmert, *Forensic Sci. Int.* **2010**, *196*, 1.
[27] P. Kintz, *Forensic Sci. Int.* **2009**, *196*, 2.