EtG and EtS in autopsy blood samples with and without putrefaction using UPLC-MS-MS

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Abstract

Analytical challenges related to post-mortem specimens are well known. The degree of putrefaction of the corpse will influence the quality of the blood samples, and both the efficiency of sample preparation and the subsequent chromatographic performance can be affected. An ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS) method was developed and validated for the determination of ethyl glucuronide (EtG) and ethyl sulfate (EtS) in postmortem whole blood. Sample preparation prior to UPLC-MS-MS analysis consisted of protein precipitation and filtration through a phospholipid removal plate. Chromatography was achieved using an HSS T3 column and gradient elution with formic acid in water in combination with methanol. The injection volume was 0.5 µL. Negative electrospray ionization was performed in the multiple reaction monitoring (MRM) mode. Two transitions were monitored for the analytes and one for the internal standards. The between-assay relative standard deviations were in the range of 1.7% – 7.0% and the limits of quantification were 0.025 mg/L and 0.009 mg/L for EtG and EtS, respectively. Recovery was 51-55% and matrix effects ranged from 98 to 106% (corrected with internal standard). Blood samples from nine autopsy cases with various extents of putrefaction were analyzed. The sample preparation efficiently removed the phospholipids from the blood specimens. The samples were clean and the analytical quality of the chromatographic performance was satisfactory for both analytes irrespective of the degree of putrefaction.

Introduction

The possibility of postmortem formation of ethanol poses a significant challenge in forensic toxicology. After death, ethanol may be produced as a putrefactive product from glucose by microorganisms penetrating from the skin or intestines, particularly after severe trauma and in high temperature conditions (1). Production of ethanol may also occur *in vitro* in a sample obtained at autopsy if not handled properly. In the interpretation of a postmortem ethanol finding, criteria like specific case information and comparisons of ethanol levels in blood, urine and vitreous humor are often used, but are not always reliable (2, 3). A supplementary method to distinguish antemortem ethanol consumption from postmortem ethanol formation is the analysis of the ethanol metabolites ethyl glucuronide (EtG) and ethyl sulfate (EtS) (4-7). These metabolites are produced by enzymatic conjugation of ethanol in living individuals (8, 9). However, as EtG and EtS represent only a small fraction (i.e. <0.1%) of the total ethanol dose ingested, sensitive analytical methods are needed to be able to detect these substances (10, 11). EtG has in some cases been shown to be unstable in postmortem blood due to bacterial degradation (12, 13), but this instability seems to demand heavy decomposition and high temperatures. More importantly, no formation of EtG has been observed in samples spiked with ethanol either with or without preservatives added (14), although from a theoretical point of view, postmortem production of EtG cannot be completely excluded (15). EtS appears to be even more stable than EtG (5); however, under extreme conditions some instability of EtS has also been reported (16).

Protein precipitation with methanol or acetonitrile has previously been used as sample cleanup for EtG and EtS in blood (4, 17-20). Such precipitation does not remove phospholipids, which can build up on the analytical column and pollute the mass spectrometer source, and thus contributes to matrix effects and reduced sensitivity. In addition, samples tend to be greasy even with an extra centrifugation step. Recently, several sample preparation products for cleanup of phospholipids in blood have become commercially available. EtG and EtS are highly polar metabolites requiring a very low proportion of organic modifiers (< 5%) for elution from a reversed phase column. Hydrophilic interaction liquid chromatography (HILIC) has previously been shown to be suitable for separation of EtG and EtS (21). However, the run time was unfavorably long (20 minutes including time for equilibration), rendering the method less favorable for routine analysis. High strength silica columns have previously been shown to give reproducible retention times, good separation and short run times (< 5 min) for EtG and EtS in blood and urine (22, 23).

The aim of the present study was to develop a method for the analysis of EtG and EtS in whole blood from autopsies, using protein precipitation and phospholipid removal plates. Particular emphasis was given to develop a method suitable also for samples with heavy putrefaction.

Method

Chemicals and reagents

EtG, EtS, EtG-d$_5$ and EtS-d$_5$ were obtained from Lipomed GmbH (Weil am Rhein, Germany). LC-MS grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany)

and formic acid Aristar 98% was from VWR (Leuven, Belgium). All water used was provided from a Millipore A10 Synthesis filtering system (Billerica, MA).

Preparation of solutions
, Two separate stock solutions (1 mg/mL) were prepared for each compound using the same lot numbers from the same manufacturer, identified as calibration and quality control (QC), respectively. EtG and EtS purchased from Cerilliant (Round Rock TX) were used to control the concentrations of the stock solutions. Sample material was weighed and resolved in methanol. These solutions were further diluted with methanol and used for calibration and QC samples prepared in whole blood with concentrations of 0.08, 0.15, 0.30, 0.5, 1.0 and 2.0 mg/L for EtG and 0.025, 0.075, 0.15, 0.5, 1.0 and 2.0 mg/L for EtS. The internal standards EtG-d_5 and EtS-d_5 were diluted with water to a concentration of 2.5 mg/L. The stock solutions and standards in blood were stored at -20°C and internal standard at 4°C.

Sample treatment
Aliquots of blood specimens (100 µL) were mixed with 25 µL of internal standard in plastic tubes (1.5 mL Safe-Lock Tube, Eppendorf, Hamburg, Germany). Five hundred microliters of ice-cold methanol were added to each tube, followed by shaking on a vortex mixer. The tubes were capped and placed in a freezer at -20°C for a minimum of 10 min, then centrifuged at 8900 × g (8600 rpm at a Hettich Mikro20 centrifuge, Tuttlingen, Germany) for 10 min. The supernatant was transferred to a glass tube and subsequently to the phospholipid removal plate (Ostro Protein Precipitation & Phospholipid Removal Plate, 25 mg, Particle Size, 1/pkg, Waters, Milford , MA, USA). The latter step was performed by a Tecan Freedom Evo 100 pipetting robot (Tecan Nordic, Mölndal, Sweden). Samples were then eluted into a 96-well collection plate (96-well Square collection plate, Waters) by a Positive Pressure-96 Processor (Waters), evaporated to dryness under compressed air at 40°C (Ultravap, Portvair science, North Wales, UK) and reconstituted in 100 µL water. After sealing the collection plate (Cap-mat square plugs, silicone/PTFE treated, pre-slit, Waters), the samples were mixed (Multi vortexer).

Instruments
A Waters Acquity UPLC I-Class FTN system (Waters) was used for separation, applying an Acquity HSS T3 column (2.1 × 100 mm, 1.8 μm; Waters) maintained at 50ºC. A pre-column (HSS T3, 2.1 × 5 mm, 1.8 μm; Waters) was used in prior to the analytical column. A flow rate of 0.6 mL/min with the following binary solvent system was used: 0.1% formic acid in water (A) and 100% methanol (B). The gradient was run as follows: 0 min, A 99%, B 1%; 1.2 min, A 80%, B 20%; 1.5 min, A 10%, B 90%; 1.9 min, A 99%, B 1%. Total run time was 2.5 min. The post-inject wash was performed with methanol/ acetonitrile/ isopropanol/ water/ formic acid (25/25/25/24/1, v/v) for 6 s. The injection volume was 0.5 µL.

For the detection of EtG and EtS, a Xevo TQ-S tandem-quadrupole MS (Waters) equipped with a Z-spray electrospray interface was used. Negative electrospray ionization was used. The capillary voltage was set to 1.0 kV, the source block temperature was 120˚C, and the

desolvation gas nitrogen was heated to 650°C and delivered at a flow rate of 1000 L/h. The m/z 221.0 > 85.1 and 221.0 > 75.1 transitions (cone voltage: 40 V, collision energy: 15 eV) were monitored for EtG. The m/z 125.0 > 97.0 (cone voltage: 50 V, collision energy: 15 eV) and 125.0 > 80.0 (cone voltage: 50 V, collision energy: 22 eV) transitions were monitored for EtS. The m/z 226.0 > 85.1 transition (cone voltage: 40 V, collision energy: 15 eV) was monitored for EtG-d_5 and the m/z 130.0 > 97.8 transition (cone voltage: 50 V, collision energy: 15 eV) was monitored for EtS-d_5. System operation and data acquisition were controlled using the Mass Lynx 4.1 software (Waters). All data were processed with the Target Lynx quantification program (Waters).

Method validation

The six-point calibration curves (three replicates of each standard) were based on peak-area ratios of the analyte relative to the internal standard using a weighted (1/x) linear line, excluding the origin. Within-assay precision was estimated by analysis of six separate replicates of QC samples at three concentrations in a single assay (EtG: 0.12, 0.6 and 1.5 mg/L, EtS: 0.04, 0.6 and 1.5 mg/L). Between-assay precision and accuracy were determined by analysis of one replicate at the same concentration levels on six different days. Recovery was determined at two concentration levels (lowest and highest QC sample) with six replicates at each level. Total recovery was estimated by comparison of the peak areas obtained when the analytes were added before sample preparation with those obtained when the analytes were added after the extraction step. In both cases, the internal standards were added after the extraction step. Matrix effects (ME) were evaluated at the lowest and highest QC level, the analyte signal in spiked water was compared with the analyte signal in the matrix, and the ME was defined as ME% = (matrix area/water area) x 100. Five replicates of blood samples (from five different individuals) were analyzed. To define the limit of quantification (LOQ), the signals to noise criteria should be ≥ 10 for both transitions and the precision of the calculated concentrations should be within ± 20%. Several concentrations were tested, and 0.025 mg/L of EtG and 0.009 mg/L of EtS were chosen and included in the calibration curve. These concentrations were run in one replicate on six different days. A standard sample with concentrations identical to the LOQ sample was included in the calibration curve. The limit of detection (LOD) was determined by dilution and evaluation of signal to noise (S/N ≥ 3).

Sample specimen

Post-mortem blood samples were collected from the femoral vein at autopsy. The autopsies were performed at the Department of Forensic Pathology and Clinical Forensic Medicine, The Norwegian Institute of Public Health (NIPH), Oslo, Norway. The samples were received in 25 mL Sterilin tubes (Sterilin, Caerphilly, U.K.) containing 200 mg potassium fluoride.

Classification of putrefaction

The classification of putrefaction of samples was based on the evaluation of the forensic pathologist obtaining the sample and the detection of n-propanol. If the forensic pathologist considered the sample being putrefied, it was classified as "definite putrefaction." If the forensic pathologist did not notice any putrefaction but n-propanol was detected in the sample, it was classified as "some putrefaction". N-propanol was analyzed by using headspace gas chromatography equipped with a flame ionization detector as described previously (24).

Ethics

The project was approved by the Regional Committee for Medical and Health Research Ethics (approval No. 2015/636) and the Director General of Public Prosecution.

Results and discussion

Method development

The validated sample preparation procedure efficiently removed the phospholipids from the postmortem blood samples analyzed. Prepared samples were clean and easy to solute in water prior to analysis, giving a longer analytical column life and less mass spectrometer source maintenance.

The presented method was initially validated using an injection volume of 5 μL. This volume gave satisfactory chromatographic performance for both analytes and internal standards spiked in whole blood samples from healthy donors and post mortem specimens tested during method development and validation. However, during the validation process, we became aware that a previously described method (19), which included protein precipitation and an injection volume of 3 μL, demonstrated poor chromatography for both for EtG, EtS and deuterated analogues in some postmortem samples (Figure 1; right panel, Sample No. 6 in Table 3). Further research revealed that these samples were often heavily putrefied. As EtG and EtS are small polar compounds that elute early in the chromatogram, the observed splitting peaks might be explained by a competition between the analyte and the matrix on the analytical column in putrefied samples. A selection of these samples was sent to our laboratory and reanalyzed with our method. When using an injection volume of 5 μL, the chromatographic separation was still not acceptable with the recently developed method (Figure 2; right panel, Sample No. 6 in Table 3). In an attempt to resolve the case, lower injection volumes were tested, a procedure considered feasible since the S/N ratio for both compounds at the lowest calibration level were >200 with a volume of 5 μL. Figure 3 (right panel) shows a significantly better chromatographic peak shape, especially for EtG, with an injection volume of 0.5 μL. Consequently, the method was revalidated with the injection volume reduced from 5 μL to 0.5 μL and with samples with various degrees of putrefaction included (Table 3).

Method validation

The calibration range, LOD, LOQ, within-assay precision, between-assay precision, bias and recovery for EtG and EtS are presented in Table 1. The within-assay coefficients of variation (CVs) were 2.7%-4.6%, and the between-assay CVs were 1.7%-7.0%. The bias was in the range of -2.4% to 1.8%. The recoveries varied between 51% and 55%. The matrix effect ranged from 81% to 92% (Table 2), indicating some ion suppression for both analytes. However, when corrected with the internal standard, the observed matrix effects were reduced for both compounds.

Application

Analytical problems related to post-mortem samples are well known. The matrices are complex and the quality varies due to the progressive nature of decomposition . To date, no quantifiable measure of the extent of putrefaction of a body does exists. Postmortem changes and signs of putrefaction in autopsy cases are observed by pathologists during autopsy. The assessment of the degree of putrefaction is subjective by nature. The present method was applied to nine samples that had also been analyzed with the previously published method (19). We classified these cases as no putrefaction (n = 5), some putrefaction (n = 1) and definite putrefaction (n = 3) based on information given by the forensic pathologist and detection of n-propanol in the samples (Table 3). The extracted samples were analyzed and injection volumes of 0.5 µL and 5 µL were used. For samples no. 7 and 9 the blood concentration of EtG was above the highest calibration level. These samples were therefore diluted ten times (1:10) using negative whole blood and reanalyzed.

The quality of the chromatographic performance was acceptable for all samples when 0.5 µL was injected. With an injection volume of 5 µL, all the putrefied samples as well as two samples with no apparent purification (No. 4 and No. 5) demonstrated noticeable problems with the chromatographic performance of EtG. All samples gave acceptable results for EtS with both injection volumes (Table 3). In these analyses, the chromatographic performance of EtG/EtG-d$_5$ was more affected by the matrix compared to EtS/EtS-d$_5$, as shown in Figure 2.

Conclusion

This study indicates a relationship between putrefaction and poor chromatographic performance of EtG and EtG when using 5 µL as injection volume. Because of a limited number of samples available, we cannot exclude other factors than putrefaction that may have an impact on chromatographic performance of EtG and EtS. However, a combination of phospholipid removal plates and reduced injection volume was shown to improve the quality of the analytical results of EtG and EtG determined in blood with various degrees of putrefaction.

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Table 1. Calibration range, correlation coefficient, limit of detection (LOD), limit of quantification (LOQ), within-assay and between-assay precisions, bias and recovery for EtG and EtS in whole blood. The injection volume was 0.5 µL.

Analyte	Calibra-tion range (mg/L)	Correlation coefficient (r value) (n=3)	LOD (mg/L)	LOQ (mg/L) (n=6)	Mean concentration found (mg/L)[a]	Within-assay CV (%) (n=6)	Between-assay CV (%) (n=6)	Bias (%) (n=6)	Recovery (%) (n=6)
EtG	0.08-2.0	0.999	0.004	0.025	0.12	2.9	3.1	1.8	52
					0.6	4.6	3.8	-1.1	-
					1.5	3.8	7.0	-1.7	51
EtS	0.025-2.0	0.999	0.003	0.009	0.04	4.5	1.7	-1.6	55
					0.6	4.6	2.8	-1.3	-
					1.5	2.7	5.1	-2.4	53

[a] For all concentrations, the means found were the same as the expected theoretical concentrations .

Table 2. Evaluation of matrix effects (ME) for EtG and EtS in whole blood. The injection volume was 0.5 µL.

Analyte	Concentration (mg/L)	ME (%)	Relative ME (CV %)	ME corrected with IS[a] (%)	Relative ME corrected with IS (CV %)
EtG	0.12	89	2.3	98	3.2
	1.5	90	0.9	101	1.3
EtS	0.04	92	3.0	106	2.9
	1.5	81	0.9	98	0.9

[a] IS = internal standard

Table 3. Chromatographic performances for EtG and EtS using injection volumes of 5 and 0.5 µL in 15 whole blood samples with no, some and definite putrefaction (for definitions, see footnote 2). Sample No. 6 is the sample for which the chromatogram is shown in Figures 1-3.

Sample No.	Putrefaction	Concentration found (mg/L) [a]		Chromatographic performance, injection volume 5 µL		Chromatographic performance, injection volume 0.5 µL	
		EtG	EtS	EtG	EtS	EtG	EtS
1	No	1.28	1.16	Acceptable	Acceptable	Acceptable	Acceptable
2	No	0.82	0.64	Acceptable	Acceptable	Acceptable	Acceptable
3	No	0.15	0.25	Acceptable	Acceptable	Acceptable	Acceptable
4	No	0.29	1.17	Not acceptable[c]	Acceptable	Acceptable	Acceptable
5	No	1.63	1.10	Not acceptable[c]	Acceptable	Acceptable	Acceptable
6	Definite[b]	0.15	0.058	Not acceptable[c]	Acceptable	Acceptable	Acceptable
7	Definite[b]	2.81	1.25	Not acceptable[c]	Acceptable	Acceptable	Acceptable
8	Definite[b]	1.00	0.66	Not acceptable[c]	Acceptable	Acceptable	Acceptable
9	Some[b]	7.45	1.91	Not acceptable[c]	Acceptable	Acceptable	Acceptable

[a] Based upon an injection volume of 0.5 µL

[b] Classified on the basis of the evaluation of the forensic pathologist obtaining the sample (classified as "definite") and the detection of n-propanol (classified as "some" if the forensic pathologist did not notice any putrefaction)

[c] Poor chromatography (cf. Figure 2)

Figure legends

Figure 1. MRM-chromatograms of ethyl glucuronide (EtG) and ethyl sulfate (EtS) of quality control (QC) 1 (EtG; 0.13 mg/L, EtS; 0.04 mg/L, left panel) and an authentic sample with definite putrefaction (Sample No.6, right panel), using a previously published method (19) with an injection volume of 3 μL. From above: EtG, m/z 221.0 > 85.1; EtG, m/z 221.0 > 75.1; EtG-d$_5$, m/z 226.0 > 85.1; EtS m/z 125.0 > 97.0; EtS, m/z 125.0 > 80.0; EtS-d$_5$, m/z 130.0 > 97.8.

Figure 2. MRM-chromatograms of ethyl glucuronide (EtG) and ethyl sulfate (EtS) (5 μL injection volume) of quality control (QC) 1 (EtG; 0.12 mg/L, EtS; 0.04 mg/L, left panel) and an authentic sample with definite putrefaction (Sample No.6, right panel). From above: EtG, m/z 221.0 > 85.1; EtG, m/z 221.0 > 75.1; EtG-d$_5$, m/z 226.0 > 85.1; EtS m/z 125.0 > 97.0; EtS, m/z 125.0 > 80.0; EtS-d$_5$, m/z 130.0 > 97.8.

Figure 3. MRM-chromatograms of ethyl glucuronide (EtG) and ethyl sulfate (EtS) (0.5 μL injection volume) of quality control (QC) 1 (EtG; 0.12 mg/L, EtS; 0.04 mg/L, left panel) and an authentic sample with definite putrefaction (Sample No.6, right panel). Determined concentrations were: EtG 0.15 mg/L, EtS 0.058 mg/L. From above: EtG, m/z 221.0 > 85.1; EtG, m/z 221.0 > 75.1; EtG-d$_5$, m/z 226.0 > 85.1; EtS m/z 125.0 > 97.0; EtS, m/z 125.0 > 80.0; EtS-d$_5$, m/z 130.0 > 97.8.
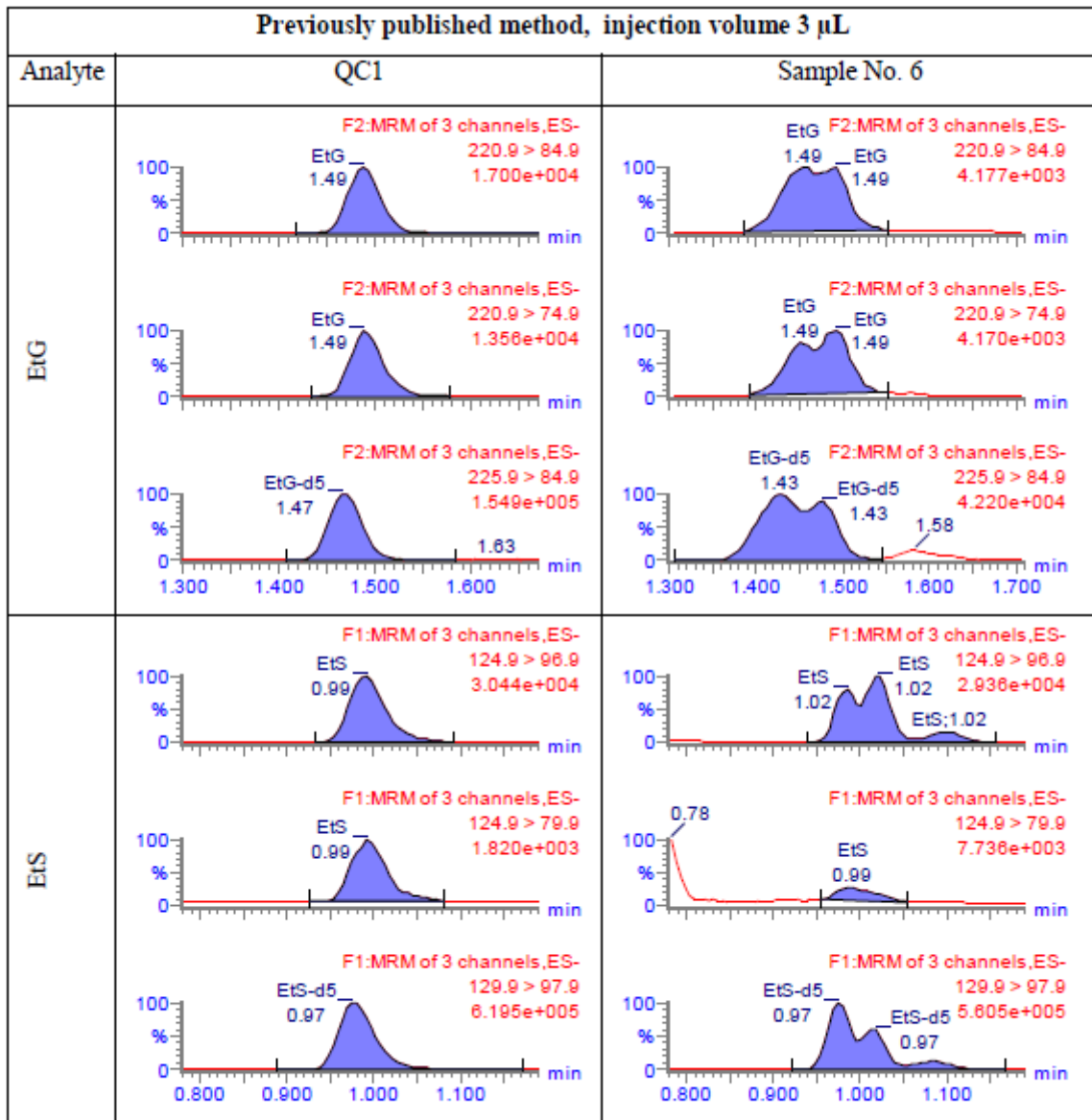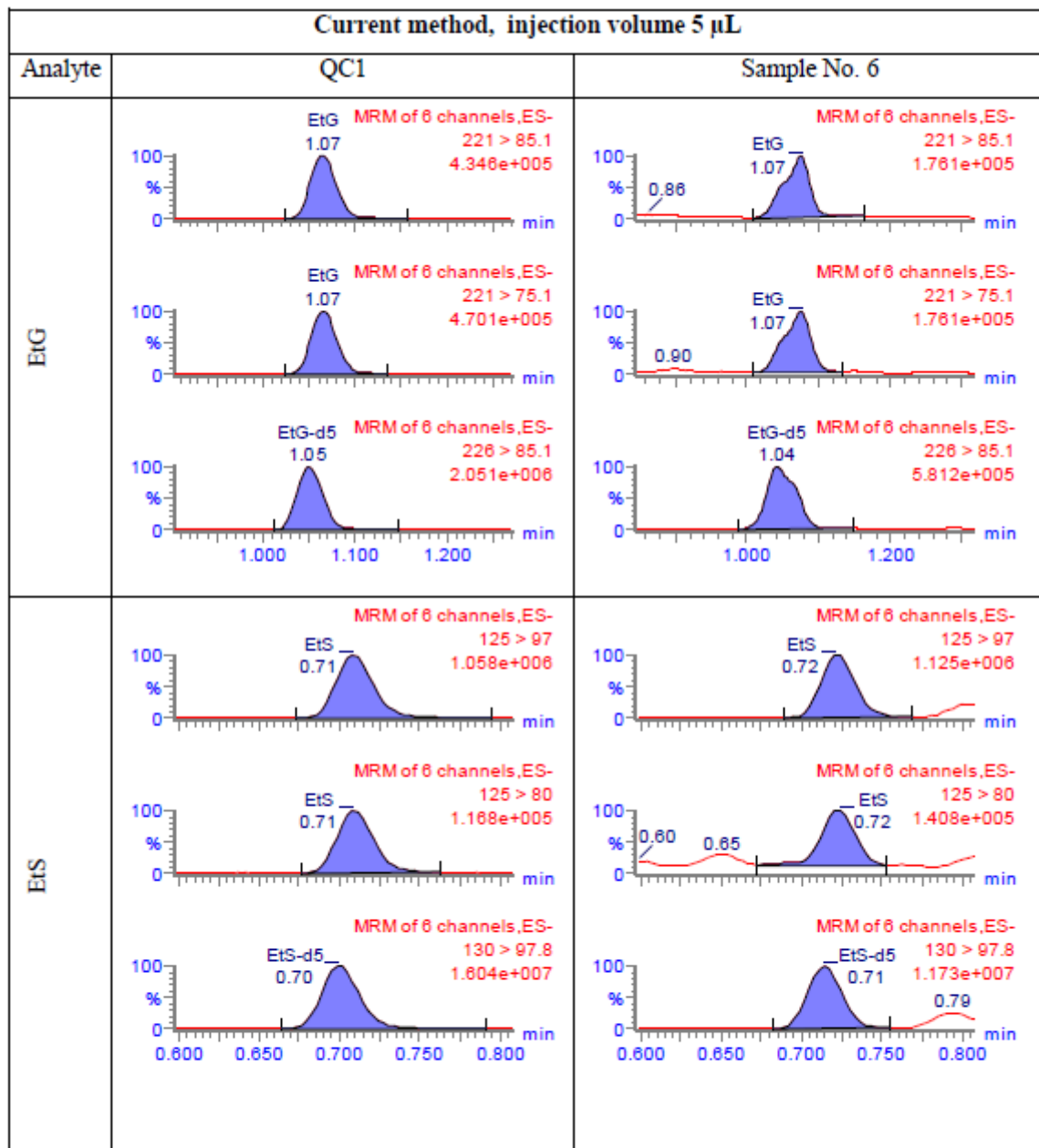
Figure 1

Figure 2