Application note

Screening and confirmation of a glycerol positive case

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Introduction

Propane-1,2,3-triol is a widely occurring substance and is better known by its trivial names glycerol or glycerin(e). The most important natural source is in lipids. It is present in some types of explosives and cosmetics. From the field of food-chemistry, glycerol can be categorized as a sugar-alcohol (poly-ol) because each carbon bears a hydroxyl-function and has a sweet taste [1]. Therefore it is used as an additive (E422) to sweeten and to preserve food. Nevertheless, overdosing glycerol can cause headaches, dizziness, thirst, and diarrhea. Some sources report it can be used in the treatment of bacterial meningitis [2], in Menière disease (diagnostic agent) [3], stroke [4] or brain edema [5]. Unfortunately, convincing evidence for a benefit of these applications is missing. A more common use in the field of medicine is the application as lubricant, additive for medication and as laxativum.

Like other products, glycerol triggered attention from athletes or their physicians for its capability to hyper-hydrate which might yield an ergogenic effect. However scientific research showed controversial results regarding its ergogenic potency [6-12].. Another reason why athletes might use glycerol is for its capability to retain water which can result in a reduced hematocrit-value [14,15]. But these effects are also limited. Nevertheless, glycerol was included in the WADA-prohibited list from January 2012 on with a threshold of 1.3 mg/mL[16]. In September 2013, the threshold was further increased to 4.3 mg/mL (Decision limit (DL) 5.3 mg/mL) [17] because research has shown that the previous threshold might yield false positive findings [18]. Because of the aforementioned questionable and moderate benefits of glycerol, it was not expected to have an Adverse Analytical Finding (AAF). However, recently a routine doping sample in our laboratory was found to contain glycerol in concentrations higher than the decision limit. This application note describes the screening by LC-HRMS and confirmation by a GC-EI-MS method.

Materials and methods

Chemicals and reagents

All the reagents were analytical or HPLC grade. Reference standard of glycerol (electrophoresis-grade) and internal standard (ISTD) glycerol-d_5 were purchased from Sigma-Aldrich (Bornem, Belgium). Beta-glucuronidase (containing aryl-sulphatase activity) from *Helix Pomatia* (HP) was from Roche (Mannheim, Germany). Buffer pH 5.2 was obtained by dissolving 136 g sodium acetate (NaOAc) (Merck, Darmstadt, Germany) into 800 mL of double-distilled water. The pH was adjusted to 5.2 by adding acetic acid (HOAc) (Merck). Then the final volume was made to 1 L. Buffer pH 9.5 was prepared by dissolving 45 g potassium carbonate (K_2CO_3)(Merck) and 37 g sodium hydrogencarbonate ($NaHCO_3$)(Merck) in 300 mL of H_2O. Formic acid (HCOOH) (99%) and ammonium formiate (NH_4OOCH) were from Fisher-Scientific (Aalst, Belgium). Water and acetonitrile (ACN) were obtained from J.T. Baker-Avantor (Deventer, The Netherlands). *tert*-butyl methyl ether (TBME) for extraction was from Macron-Avantor (Deventer). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was obtained from Karl-Bucher GMBH (Waldstetten, Germany).

Screening (LC-HRMS)

To 50 µL of urine, 450 µL of dilution-solution is added. The dilution solution is composed of 95/5 (H_2O/ACN) containing 0.01% HCOOH/1mM NH_4OOCH and glycerol-d_5 (300 µg/mL). Finally, the sample is centrifuged 5 min. at 7900 RCF. Meanwhile, to 3 mL of the same urine, 50 µL beta-glucuronidase and 1 mL of the acetate-buffer pH 5.2 is added. The sample is hydrolysed for 1h at 56°C. After adding 1 mL of the buffer (pH 9.5 and 5 mL of TBME, the sample is extracted for 20 min on a CAT RM5-roller (Staufen, Germany). After extraction, the sample is centrifuged 5 min at 600 RCF. Then, the organic layer is transferred to a separate tube and is evaporated under oxygen free nitrogen (OFN). 200 µL of the dilute-and-shoot fraction was added to the dried residue.

Analysis is done on a Thermo Exactive-system. Chromatography is performed on a Surveyor (pump/autosampler)-system equipped with a Zorbax-RX C8 column (50mm, ID= 2 mm, particle size 1,8 µm). A: H_2O (0,01% NH_4OAc, 1mM NH_4OOCH)/ B: ACN (0,01% HCOOH). The solvent program was as follows: 0.0 - 0.5 min: isocratic 100 % A; 0.5 - 7.5

min: decrease to 20 % A; 7.5 – 8.0 min: decrease to 0 % A; 8.0 - 9.5 min: isocratic 0 % A; 9.5 – 9.6 min: increase to 100 % A; 9.6 - 12 min: isocratic 100% A. Flow-rate is 0.250 µL/min, injection volume 20 µL. ESI (3 kV) ,Sheath gas: 50, aux gas: 20/ Capillary temperature: 350°C; Capillary voltage 30 V or -25 V(pos/ neg-mode respectively). 4 experiments are acquired simultaneously: Full scan MS+, Full scan MS-, all ion fragmentation (HCD), Full scan MS+, in source CID (80 eV), Full scan MS+. Glycerol and glycerol-d_5 are detected in the Full scan MS+ by monitoring respectively *m/z* 115.03650 $[glycerol+Na]^+$ and $[glycerol-d_5+Na]^+$ *m/z* 120.06790 with a mass window of 5 ppm.

Confirmation (GC-MS)

The confirmation was performed with a validated GC-MS method. A 6-point calibration curve spiked at 1.25; 2.5 4.5; 6; 8; 10 mg/mL and quality control sample was co-analysed with the suspicious sample. Sample preparation is as follows: To 200 µL of urine, 25 µL IL-ISTD-solution (10 mg/mL glycerol-d5) and 5 mL TBME is added followed by a liquid-liquid extraction for 20 minutes by rolling Then, the organic phase was evaporated under OFN. 100 µL MSTFA was added to the dried sample. Derivatization time was 10 min at 80°C +/- 5°C. Analysis was done on an Agilent GC-MSD (HP6890) in full scan MS: scan range *m/z* 50-800, Injection mode: Splitless (0.2µL)/ Column: 15 m, HP-5MS (0.25 mm; 0,25 µm), carrier gas: Helium. Oven program: 0-0.5 min: 90°C; 0.5-4.1 min: linear increase to 108°C; 4.1-4.7 min: 108°C; 4.7-7.5 min: linear increase to 320°C; 7.5-8.78 min: 320°C. Flow program: 0-4 min: 1 mL/min; 4-6 min: linear increase to 2 mL/min. The ions used for quantitation are *m/z* 205 for glycerol and *m/z* 208 for glycerol-d_5. The additional ions used for qualitative purposes are *m/z* 218, 103, 117, 133, 175 for glycerol and *m/z* 222, 298 for glycerol-d_5

Results and discussion

Screening

Both GC-MS and LC-MS are the standard screening-techniques for small molecules (< 1,000 Da) in the field of anti-doping analysis. Papers describing glycerol detection by GC-MS are ubiquitous [13,19-23]. Hence the first idea was to screen for glycerol by this latter technique by incorporating glycerol into a GC-MS method used for the detection of anabolic

steroids[24]. Unfortunately, initial tests show an interfering peak in blank urine as well as in the blank control sample (H_2O). Hence, it was concluded that the background originated from one of the reagents. Indeed, glycerol was found to be present as a stabilizing agent in the liquid beta-glucuronidase (BG) preparations from E.Coli, independent from the brand. Unfortunately, this enzyme can't be replaced because it is the only enzyme permitted to be used for the analysis of endogenous anabolic steroids (AAS) in combination which GC-MS [25]. If screening by GC-MS would need to be performed, a separate (extra) method –without hydrolysis- would be needed. Instead, it was considered to include this substance into the LC-HRMS screening method. Indeed, previous research has shown that glycerol can be detected by LC-MS by monitoring the metal adducts $[M+Cs]^+$ [26] or $[M+Na]^+$ [27]. Based upon these findings a method consisting of a simple 10-fold dilution step of the urines followed by injection into the LC-HRMS method was developed in our laboratory [28] following a so called dilute-and-shoot strategy (DS-LC-MS) [29]. Recently, a similar detection strategy was developed by Gorgens et al. [30].

Because the 10-fold diluted urine from the original method [28] is currently used to reconstitute the dried extract (see experimental section) the glycerol from the BG from E. Coli would also interfere. Therefore several other lyophilised BG-preparations from E. Coli. were evaluated. Beside the high price, the activity (measured against androsterone-glucuronide, results not shown) was poor compared to readily dissolved BG-preparations from E. Coli. Finally, liquid BG from *Helix Pomatia* -not containing glycerol- was incorporated in the sample preparation showing good activity .

Due to the polar nature of glycerol, chromatographic retention is limited (k = 0,2). Therefore, the use of an IL-ISTD was considered to be mandatory. However, spiking glycerol-d_5 in the same concentration range of the target compound (> mg/mL range), was deemed economically unacceptable. Therefore, the IL-ISTD was only added to the dilute-and-shoot-fraction of the method.

The semi-quantitative detection in the screening (1 point curve, forced through zero) was validated by analysing 11 samples (10 urines + H_2O) spiked with glycerol at 3 mg/mL. Calculating the detected concentrations against the water sample allowed to evaluate the effect of the matrix. Results of the validation-experiments are depicted in table 1. Maximum error observed was 18% for urine 10. To assess the reproducibility, 10 different urines, spiked at different levels, were extracted and analysed on 3 consecutive days. Also these results were

satisfying (Table 2). In both table 1 and 2 a negative deviation (underestimation of the concentration) was observed. Therefore the cut-off for the screening method was set to 1.5 mg/mL, corresponding to the maximum endogenous urinary concentration which was previously encountered in blank urines (99.9 percentile of male and female athletes) [18]. Using the described screening protocol, a suspicious sample with an estimated concentration of 26 mg/mL was detected in a routine batch. The screening –result is presented in figure 1.

Confirmation and interpretation

For confirmation purposes, multiple (product) ions have to be monitored [31]. These are generally obtained by fragmentation of the intact molecule by MS/MS or EI. Because of the simplicity of its structure and its poor ionisation behaviour, tandem mass spectrometry does not yield usable diagnostic ions for glycerol in LC-MS/MS. Confirmation by LC-MS/MS can only be done after derivatization of the glycerol [32,33]. Therefore an in house GC-EI-MS confirmation method was developed based on previous work [13,19-22] showing both good ionization and acceptable fragmentation. Quantitative confirmatory analysis of the suspicious sample by GC-MS showed a final concentration of 12.6 mg/mL, which is greater than the DL. Additionally, the mass spectrum fulfilled the criteria as described by WADA taking into account 3 diagnostic ions [31]. In figure 2, the extracted chromatograms for a blank urine, QC-sample (4.3 mg/mL) and the positive finding are presented.

Finally, the sample was reported as an AAF to NADO-Flanders. Several possible sources for the AAF were considered. Because the sport was kick-boxing, an attempt to manipulate blood-parameters [15] was unlikely. Glycerol is also used as a lubricant for self–catheterization in particular by impaired athletes suffering from neurogenic bladder dysfunction [34] Because the sample was obtained from a non-impaired athlete this latter possibility could be excluded. The only remaining explanation was that the athlete tried to hyper hydrate [9]. However, during the hearing for the disciplinary commission, the athlete declared to have used glycerol by drinking 1/5 diluted aqueous solutions (frequency unknown) for several weeks in an attempt to lose weight. This therapy was recommended by his pharmacist. Indeed, Glycerol can dehydrate a small portion of the body when applied local (brain, ear) [3]. However, proof for a systemic dehydrating effect, could not be found. Oppositely, taking into account the ability to retain fluid when glycerol is consumed [35], the athlete might have gained weight instead of losing. In conclusion, this work shows that the

combination of LC-HRMS and GC-MS is a good strategy for screening/confirmation of glycerol.

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References

[1] Glycerol: A Jack Of All Trades http://www.chem.yorku.ca/hall_of_fame/essays96/glycerol.htm. Accessed (Accessed 15th September 2015).

[2] D. van de Beek, M.C. Brouwer, G.E. Thwaites, A.R. Tunkel. Advances in treatment of bacterial meningitis. *Lancet* **2012**, 380, 1693.

[3] S. Padoan. Oral versus i.v. administration of the glycerol test: Side-effects and usefulness. *Acta Oto-Laryngol.* **2003**, 123, 482.

[4] Y.L. Yu, C.R. Kumana, I.J. Lauder, Y.K. Cheung, F.L. Chan, M. Kou, K.Y. Fong, R.T.F. Cheung, C.M. Chang. treatment of acute cortical infarct with intravenous glycerol - a double-blind, placebo-controlled randomized trial. *Stroke* **1993**, 24, 1119.

[5] C. Berger, O.W. Sakowitz, K.L. Kiening, S. Schwab. Neurochemical monitoring of glycerol therapy in patients with ischemic brain edema. *Stroke* **2005**, 36, E4.

[6] P. Montner, D.M. Stark, M.L. Riedesel, G. Murata, R. Robergs, M. Timms, T.W. Chick. Pre-exercise glycerol hydration improves cycling endurance time. *Int. J. Sports Med.* **1996**, 17, 27.

[7] M.J. Anderson, J.D. Cotter, A.P. Garnham, D.J. Casley, M.A. Febbraio. Effect of glycerol-induced hyperhydration on thermoregulation and metabolism during exercise in the heat. *Int. J. Sport Nutr. Exerc. Metab.* **2001**, 11, 315.

[8] T.P. Scheett, M.J. Webster, K.D. Wagoner. Effectiveness of glycerol as a rehydrating agent. *Int. J. Sport Nutr. Exerc. Metab.* **2001**, 11, 63.

[9] A. Coutts, P. Reaburn, K. Mummery, M. Holmes. The effect of glycerol hyperhydration on Olympic distance triathlon performance in high ambient temperatures. *Int. J. Sport Nutr. Exerc. Metab.* **2002**, 12, 105.

[10] F.E. Marino, D. Kay, J. Cannon. Glycerol hyperhydration fails to improve endurance performance and thermoregulation in humans in a warm humid environment. *Pflugers Arch.* **2003**, 446, 455.

[11] E.D.B. Goulet, R.A. Robergs, S. Labrecque, D. Royer, I.J. Dionne. Effect of glycerol-induced hyperhydration on thermoregulatory and cardiovascular functions and endurance performance during prolonged cycling in a 25 degrees C environment. *Appl. Physiol. Nutr. Metab.* **2006**, 31, 101.

[12] J.L. Nelson, R.A. Robergs. Exploring the potential ergogenic effects of glycerol hyperhydration. *Sports Med.* **2007**, 37, 981.

[13] M. Thevis, S. Guddat, U. Flenker, W. Schanzer. Quantitative analysis of urinary glycerol levels for doping control purposes using gas chromatography-mass spectrometry. *Eur. J. Mass Spectrom.* **2008**, 14, 117.

[14] T.P. Polyviou, C. Easton, L. Beis, D. Malkova, P. Takas, C. Hambly, J.R. Speakman, K. Koehler, Y.P. Pitsiladis. Effects of Glycerol and Creatine Hyperhydration on Doping-Relevant Blood Parameters. *Nutrients* **2012**, 4, 1171.

[15] K. Koehler, M. Thevis, W. Schaenzer. Meta-analysis: Effects of glycerol administration on plasma volume, haemoglobin, and haematocrit. *Drug Test. Anal.* **2013**, 5, 896.

[16] The 2012 Prohibited List, International Standard (2012). World Anti-Doping Agency (WADA). https://wada-main-prod.s3.amazonaws.com/resources/files/WADA_Prohibited_List_2012_EN.pdf. Accessed 11.08.2015.

[17] Technical Document – TD2014DL, Decision Limits for the confirmatory quantification of threshold substances (2014). World Anti-Doping Agency (WADA). https://wada-main-prod.s3.amazonaws.com/resources/files/WADA-TD2014DL-v1-Decision-Limits-for-the-Quantification-of-Threshold-Substances-EN.pdf. Accessed 07/08/2015.

[18] B.N. Kelly, M. Madsen, K. Sharpe, V. Nair, D. Eichner. A population study of urine glycerol concentrations in elite athletes competing in North America. *Drug Test. Anal.* **2013**, 5, 890.
[19] M. Adiels, T. Larsson, P. Sutton, M.R. Taskinen, J. Boren, B.A. Fielding. Optimization of N-methyl-N- tert-butyldimethylsilyl - trifluoroacetamide as a derivatization agent for determining isotopic enrichment of glycerol in very-low density lipoproteins. **2010**, 24, 586.
[20] Y.X. Shen, Z.M. Xu. An improved GC-MS method in determining glycerol in different types of biological samples. *J. Chromatogr. B* **2013**, 930, 36.
[21] F. Magni, L. Arnoldi, L. Monti, P. Piatti, G. Pozza, M.G. Kienle. Determination of plasma glycerol isotopic enrichment by gas-chromatography mass-spectrometry - an alternative glycerol derivative. *Anal. Biochem.* **1993**, 211, 327.
[22] M.T. Ackermans, A.F.C. Ruiter, E. Endert. Determination of glycerol concentrations and glycerol isotopic enrichments in human plasma by gas chromatography mass spectrometry. *Anal. Biochem.* **1998**, 258, 80.
[23] V.F. Sardela, F.B. Scalco, K.M. Cavalcante, R.E. Simoni, D.R. Silva, H.M.G. Pereira, M.L.L. Costa de Oliveira, F.R. Aquino Neto. Systematic analysis of glycerol: colourimetric screening and gas chromatography–mass spectrometric confirmation. *Drug Test Anal* **2015**, Published online 26th of June 2015/ DOI: 10.1002.
[24] P. Van Eenoo, W. Van Gansbeke, N. De Brabanter, K. Deventer, F.T. Delbeke. A fast, comprehensive screening method for doping agents in urine by gas chromatography-triple quadrupole mass spectrometry. *J. Chrom. A.* **2011**, 1218, 3306.
[25] Technical Document – TD2014EAAS,Technical Document for endogenous anabolic androgenic steroids. (2014). World Anti-Doping Agency (WADA). https://www.wada-ama.org/en/resources/science-medicine/td2014-eaas. Accessed 07/08/2015.
[26] E. Rogatsky, H. Jayatillake, G. Goswami, V. Tomuta, D. Stein. Sensitive LC MS quantitative analysis of carbohydrates by Cs+ attachment. *J. Am. Soc. Mass Spectrom.* **2005**, 16, 1805.
[27] T.S. McIntosh, H.M. Davis, D.E. Matthews. A liquid chromatography-mass spectrometry method to measure stable isotopic tracer enrichments of glycerol and glucose in human serum. *Anal. Biochem.* **2002**, 300, 163.
[28] A. Jimenez, K. Deventer, K. Roels, P. Van Eenoo. Development and validation of an open screening method for diuretics, stimulants and selected compounds in human urine by UHPLC-HRMS for doping control. *Anal. Chim. Acta.* **2012**, 721, 137.
[29] K. Deventer, O.J. Pozo, A.G. Verstraete, P. Van Eenoo. Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology. *Trac-Trends Anal. Chem.* **2014**, 55, 1.
[30] C. Gorgens, S. Guddat, A.K. Orlovius, G. Sigmund, A. Thomas, M. Thevis, W. Schanzer. "Dilute-and-inject" multi-target screening assay for highly polar doping agents using hydrophilic interaction liquid chromatography high resolution/high accuracy mass spectrometry for sports drug testing. *Anal. Bioanal. Chem.* **2015**, 407, 5365.
[31] Technical Document – TD2010IDCR, Identification criteria for qualitative assays incorporating column chromatography and mass spectrometry (2010). World Anti-Doping Agency (WADA). https://www.wada-ama.org/en/resources/science-medicine/td2010-idcr. Accessed 13.08.2015.
[32] Y. Dong, Y. Ma, K. Yan, L. Shen, X. Wang, Y. Xu, G. He, Y. Wu, J. Lu, Z. Yang, F. Feng. Quantitative analysis of glycerol levels in human urine by liquid chromatography–tandem mass spectrometry. *J. Chromatogr. B* **2014**, 957, 30.
[33] A. Borno, L. Foged, G. van Hall. Glucose and glycerol concentrations and their tracer enrichment measurements using liquid chromatography tandem mass spectrometry. *J Mass Spectrom.* **2014**, 49, 980.
[34] M. Okano, Y. Nishitani, S. Kageyama. Possibility of analytical finding of glycerol caused by self-catheterization in doping control. *Drug Test. Anal.* **2014**, 6, 1151.
[35] K. Koehler, H. Braun, M. de Marees, H. Geyer, M. Thevis, J. Mester, W. Schaenzer. Urinary excretion of exogenous glycerol administration at rest. *Drug Test. Anal.* **2011**, 3, 877.

Table 1: Evaluation of the matrix effect for the LC-HRMS-screening of glycerol, using the described sample preparation.

	Blank	Spike	
Sample	C (µg/mL)	C (µg/mL)	Error (%)*
H2O	0,01	3,00	0,0
urine1	0,01	2,85	-5,0
urine2	0,02	2,56	-14,5
urine3	0,02	2,79	-7,0
urine4	0,02	2,60	-13,2
urine5	0,02	2,81	-6,3
urine6	0,02	2,85	-5,0
urine7	0,01	2,93	-2,3
urine8	0,02	2,67	-10,8
urine9	0,01	2,61	-13,0
urine10	0,01	2,46	-18,0

*spiked H2O vs urine

Table 2: Evaluation of the reproducibility of the LC-HRMS method in 10 spiked urines. The average concentration was obtained from 3 extractions and analyses performed on 3 consecutive days.

Sample	C_{spike}**	$C_{average}$**	bias (%)	RSD (%)
urine1	1,5	1,4	-7	14,4
urine2	5	4,7	-5	5,0
urine3	7,5	6,6	-12	5,6
urine4	4	3,6	-9	6,6
urine5	19	16,8	-11	8,0
urine6	3	2,9	-5	5,8
urine7	9	8,5	-5	8,5
urine8	7	6,3	-10	5,5
urine9	5	4,8	-5	7,0
urine10	4	3,9	-2	3,8

** mg/mL

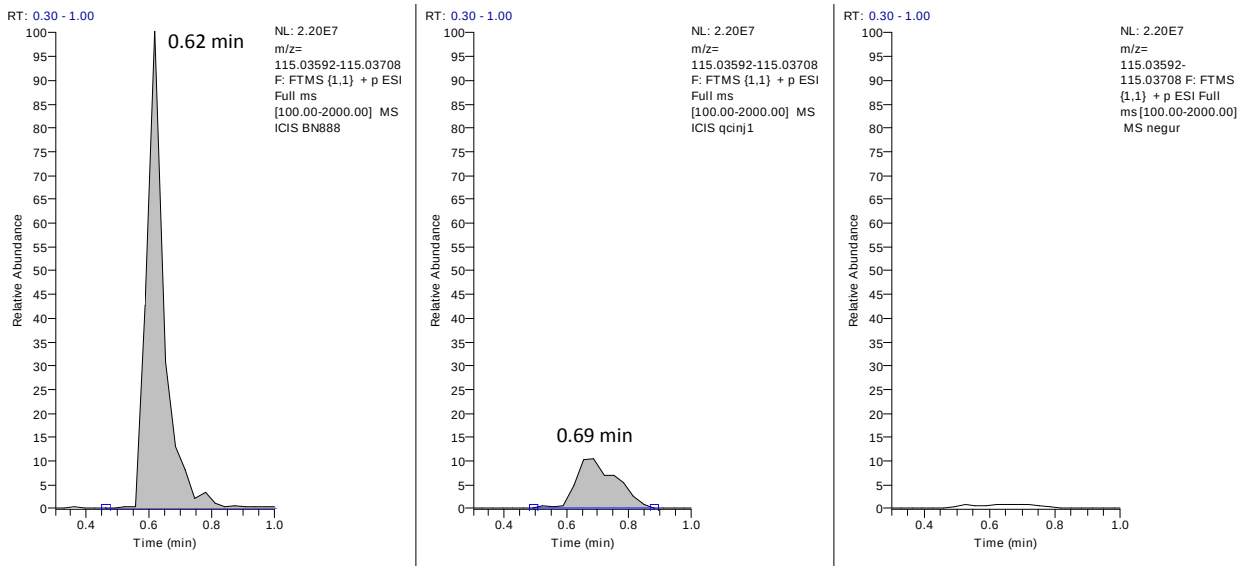

Figure 1: Extracted chromatograms for glycerol from the screening; (a) Suspicious sample (estimated concentration 26 mg/mL), (b) Quality-control sample spiked at 3 mg/mL, (c) blank urine sample.

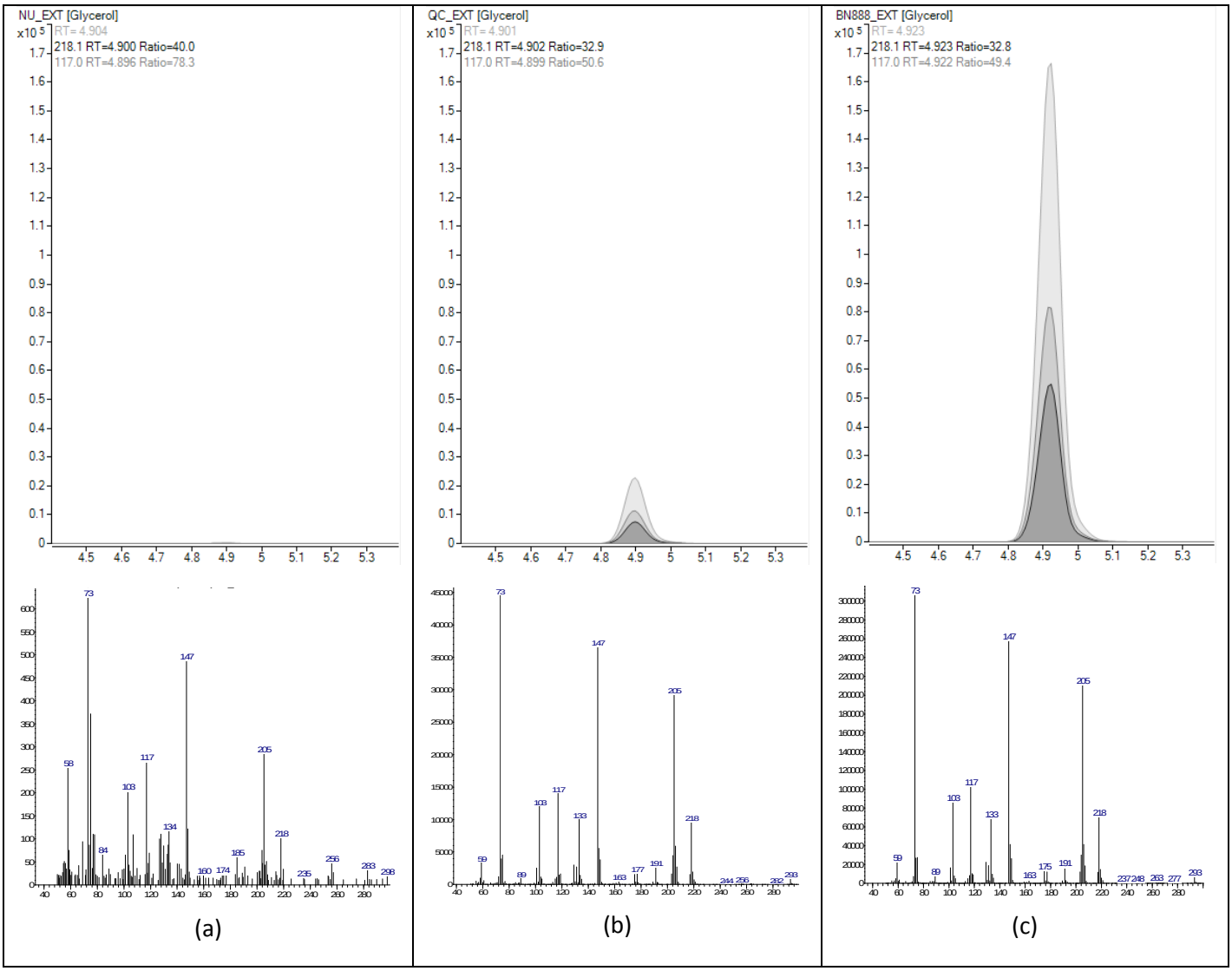

Figure 2: Extracted ion chromatograms and corresponding full scan mass spectra for glycerol after confirmatory GC-MS analysis: (a) blank sample; (b) QC-sample spiked at 4.3 mg/mL; (c) AAF-sample. All chromatograms were scaled to the peak found in the AAF (c) to illustrate the low background originating from the endogenous occurrence of glycerol in blank urine [13,18,23].