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A versatile UHPLC–MSMS method for simultaneous quantification of various alcohol intake related compounds in human urine and blood†

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Alcohol intake has been associated with preventive as well as negative effects on health. However, the intake estimates are often based on subjective reporting and therefore biased and the types of beverages consumed are often inaccurately reported. Accurate and specific quantification of alcohol related compounds in biological samples may help to understand dietary exposure and metabolic kinetics. The aim of this study was to develop a simple, rapid and versatile UHPLC–MSMS method capable of quantifying various alcohol derived compounds or potential effect markers. The method was thoroughly validated for L-tartaric acid, ethyl sulphate, ethyl-β-D-glucuronide, indoxyl sulphate, *p*-cresol sulphate, resveratrol, estrone sulphate and dihydroepiandrosterone sulphate. Isocohumulone and isoxanthohumol related to beer intake were also evaluated and the former was found to be detectable but no standards were available for final analytical validation. All selected analytes were analyzed within 6 minutes using negative ionization mode and multiple reaction monitoring.

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1. Introduction

The search for answers regarding the effects of alcoholic beverage consumption on human health is hampered by insufficient compliance control during the trials and lack of detailed information about biochemical mechanisms involved in its metabolism and actions in the body. Recent advances in metabolomics research indicate that monitoring of compounds strictly related to specific food or beverage consumption in biological samples may help to control compliance and understand dietary exposure.[1,2] In order to study the effect of consuming alcohol and related beverages along with the kinetics of consequent metabolite changes, it is essential to develop a versatile analytical method. This method should allow for simultaneous quantification of diverse metabolites affected by alcohol intake or other compounds which can be specifically present in certain alcohol beverages or affected after alcohol intake. Most of the analytical tools described in the literature have been developed for the analysis of single compounds. However, their ability to simultaneously identify, quantitate and compare metabolite ratios could increase their relevance and we have therefore set out to develop a method that incorporates a number of relevant metabolites in a single run. Due to the generally low concentrations of metabolites in clinical samples, the method of choice has been UHPLC coupled with multiple-reaction monitoring (MRM) tandem mass spectrometry[3] (MSMS). The main objective was to design a versatile, simple and rapid method, which within a single run will separate compounds across a wide polarity range and will be applicable to biological samples such as urine or blood. The method has been validated for well described alcohol biomarkers such as ethyl-β-D-glucuronide (EtG) and ethyl sulphate (EtS),[4–6] as well as putative alcohol intake related compounds such as L-(+)-tartaric acid (TaA)[7] and resveratrol[8] proposed as wine biomarkers. Their specificity for wine intake is however questionable since they are also present in grapes and grape juice.[9] For beer several hop related compounds such as isoxanthohumol and isocohumulone may be useful markers; however, labelled standards are unavailable for quantitative analyses. In this study we included the steroid hormones, dehydroepiandrosterone (DHEAS) and estrone sulphate (EsS), which may be linked to the risk of cardiovascular diseases, oxidative stress,[10] or, in the case of estrone sulphate, to cancer risk in both women[11] and men.[12] These compounds were included since alcohol intake has also been linked to cardiovascular diseases[13,14] and cancer[15] and since both have previously been observed to increase after even moderate alcohol intake.[16] We have also included some common uremic toxins, indoxyl sulphate (InS) and cresol sulphate (CrS), formed by the microbiota since a previous study indicated that moderate wine intake affects other gut microbial metabolites, including hippurate.[17] Other possible metabolites to be included in the proposed method such as isoxanthohumol or isocohumulone (putative beer intake biomarkers), 3-nitrotyrosine (marker of high NO and possibly related to alcohol-

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induced changes in blood pressure), indole-3-lactic acid (a putative marker of hepatic damage), and cortisol or cortisol sulphate (stress markers) were additionally tested in this study to potentially increase its versatility.

2. Materials and methods

2.1 Materials

The chemicals used in this research were purchased from the following vendors: TaA, EtG, ethyl-β-D-glucuronide-d_5 (EtG-d_5), ethyl sulphate sodium salt, estrone 3-sulphate sodium salt, estrone-2,4,16,16-d_4 3-sulphate sodium salt (EsS-d_4), dehydroepiandrosterone 3-sulphate sodium salt, dehydroepiandrosterone-d_5-3-sulphate sodium salt (DHEAS-d_5), indoxyl sulphate potassium salt, resveratrol, resveratrol-(4-hydroxyphenyl-$^{13}C_6$), isoxanthohumol, 3-nitro-tyrosine, indole-3-acetic acid, hydrocortisone, and formic acid (eluent additive for LC-MS) from Sigma (Schnelldorf, Germany). Ethyl-d_5 sulphate (EtS-d_5) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany), *p*-cresol sulphate ammonium salt (CrS) from Alsa Chim (Illkirch, France), indoxyl sulphate-d_4 potassium salt (InS-d_4) and cortisol sulphate from TRC (Toronto, Canada), tartaric-2,3-d_2 acid (TaA-d_2) from QMX (Thaxted, UK), *p*-cresol sulphate potassium salt-d_7 (CrS-d_7) from Cambridge Isotope Laboratories (Massachusetts, USA), and acetonitrile (ACN) and methanol (MeOH) Optima® LC/MS Grade from Fisher Chemical (Leicestershire, United Kingdom). Deionized water from a Millipore system (mQ) was used for the preparation of all aqueous solutions (conductivity 2.3 mS cm^{-1} and resistivity 18.2 mΩ cm).

Stock solutions were either obtained as methanol solutions or prepared by dissolving a powder substance in mQ water, methanol or in a mixture of MeOH : mQ (1 : 4, v/v), depending on the polarity of the compound to the concentration *ca.* 1–1.5 mg mL^{-1}.

A mixture of working solutions was prepared by diluting the stock solutions in MeOH : mQ (1 : 1, v/v) to avoid precipitation of non-polar compounds (especially isoxanthohumol).

2.2 Methods

2.2.1 LC-MSMS analysis. The chromatographic separation was performed on an Acquity™ UPLC System using a Waters Acquity™ UPLC HSS T3 1.8 μm 2.1 × 100 mm column connected with an Acquity™ UPLC HSS T3 1.8 μm VanGuard pre-column 2.1 × 5 mm purchased from Waters (Milford, USA). 0.075% (v/v) formic acid in mQ water as solvent A and MeOH as solvent B were mobile phases used for the gradient elution. 50% acetonitrile in 0.1% formic acid (v/v) was used as strong needle wash solvent; 10% acetonitrile (v/v) in mQ water as seal wash solvent and 10% acetonitrile in 0.1% formic acid (v/v) as weak needle wash solvent. The injection volume was 7.5 μL (partial loop with needle overfill). An overview of the gradient steps ran at a column temperature of 40 °C is presented in Table S-1.†

2.2.2 MSMS method. MSMS parameters were optimized for single analytes by individual direct infusions of MeOH solutions with a compound concentration of approximately 10 μg mL^{-1} and an infusion flow rate of 5–10 μL min^{-1}. The target analytes were detected in MRM negative electrospray ionization mode using argon collision gas. The capillary voltage was set to 3.50 kV, source temperature to 150 °C, desolvation temperature to 350 °C, desolvation gas flow to 900 L h^{-1} and cone gas flow to 50 L h^{-1}. The primary transition was used for the quantification and the secondary for the confirmation of the compound identity (Table S-2†). Peak integration was performed in QuanLynx Application Manager included with MassLynx Software 4.1 by Waters. Further calculations and quantifications were done in Microsoft Excel 2010.

2.2.3 Sample source and preparation. The human samples were obtained from the METABEER study (accepted by the regional ethics committee for Copenhagen and Frederiksberg with # H-1-2013-029 and registered in clinicaltrials.gov NCT02449577) where 19 healthy volunteers after two days of abstaining from alcohol consumed a beer or a control drink; blood as well as urine samples were collected before and at 45, 120 and 180 min; and in the intervals, 0–1.5, 1.5–3, 3–21, respectively. This study was approved according to existing Danish regulations by the Ethical Committee of the Copenhagen Region with approval number H-15016961 and all participants who provided samples have given their prior informed consent.

Urine samples were frozen and stored at −80 °C for 36 months. The urine samples were thawed on ice, shaken, and centrifuged at 10 000 crf for 3 min and the supernatant was 15× diluted in mobile phase A spiked with the internal standards prior to analysis.

Blood samples used for validation experiments were centrifuged directly after the sampling and serum/plasma were extracted and frozen at −20 °C for 1–14 weeks until the analyses were performed. Plasma and serum samples were pre-processed according to the method described previously by our group with minor modifications.[18] Briefly, in order to remove proteins, the samples were thawed on ice and 40 μL of sample was added into a 96-well Sirocco™ plasma protein filtering plate (Waters) containing 180 μL of MeOH : ACN (1 : 1, v/v). The plates were vortexed for 5 min, afterwards kept in a refrigerator at 4 °C for 10 min to promote more protein precipitation followed by 5 min re-equilibration at room temperature. A 96 × 1 mL plate for the UPLC autosampler (Waters) was placed underneath the protein filtering plate and vacuum was applied to the manifold. When the filtering plates were dry, 90 μL of MeOH was added to each well to further extract compounds from the precipitated protein and vacuum was connected until dryness. This step was repeated one more time. The solvent was evaporated from the UPLC plates by using a cooled vacuum centrifuge and the dry samples were re-dissolved in 200 μL in mobile phase A spiked with the internal standards prior to analysis.

2.2.4 Validation experiments. The analyte signal was corrected by means of internal standards and the response value was calculated according to the formula: analyte peak area × (internal standard concentration/internal standard peak area).

The optimal concentration of formic acid in mobile phase A was investigated in the range of 0.05–1.0% (v/v) and injection volumes from 5 to 10 μL by comparing total ion currents of all

compounds obtained by repeated injections of the same standard solution.

The selectivity of the method is an intrinsic feature of the MRM technique and was demonstrated by MS transitions and illustrative chromatograms in Table S-2 and Fig. S-1.†

2.2.4.1 Linearity. Once the linear and dynamic ranges had been roughly estimated by injection of several standards with different concentration levels, the final mixture of calibrants appropriate to cover the maximal linear range was prepared in MeOH : mQ (1 : 1, v/v) at the following concentrations (μg mL^{-1}): TaA (4.37), EsS (0.95), EtG (5.00), InS (9.86), CrS (12.47), resveratrol (3.54), EsS (2.63) and DHEAS (10.00). Thus, the prepared mixture was sequentially diluted 2-, 4-, 8-, 16-, 64-, 512-fold with the same solvent. From these calibrants, 12 μL were pipetted directly into the plate wells containing 168 μL of MPA and internal standards at final concentrations (μg mL^{-1}): TaA-d_2 (1.43), EtS-d_5 (0.47), EtG-d_5 (0.50), CrS-d_7 (0.26), resveratrol-$^{13}C_6$ (0.93), EsS-d_4 (0.17) and DHEAS-d_5 (0.056). Linearity was determined by injection of at least 6 concentration levels (7 for EtG, CrS, InS, resveratrol and DHEAS) analyzed in triplicate. Additionally, calibration curves with 6 points (injected in triplicates) were constructed and linearity was determined independently in solvent, urine and plasma also during the matrix effect experiments (see 2.2.4.6 below).

2.2.4.2 Concentrations of internal standards. A mixture of TaA-d_2 (2.8), EtS-d_5 (1.0), EtG-d_5 (1.0), CrS-d_7 (0.5), resveratrol-$^{13}C_6$ (2.0), EsS-d_4 (0.35) and DHEAS-d_5 (0.1) μg mL^{-1} was diluted 2-, 4-, 8-fold. Linear response behavior was investigated by injection of the prepared calibrants analyzed in triplicate.

2.2.4.3 Repeatability and intermediate precision. Repeatability was investigated by consecutive injection ($n = 6$) of the mixture of calibrants, namely TaA ($c = 1.0$ μg mL^{-1}); EtS ($c = 0.24$ μg mL^{-1}); EtG ($c = 1.3$ μg mL^{-1}); InS ($c = 2.5$ μg mL^{-1}); CrS ($c = 0.88$ μg mL^{-1}); resveratrol ($c = 0.89$ μg mL^{-1}); EsS ($c = 1.5$ μg mL^{-1}) and DHEAS ($c = 2.5$ μg mL^{-1}). The same concentration levels were used for spiking the urine and plasma.

Intermediate precision was calculated from the response of the standard solution and spiked matrixes analyzed in triplicate on three different days ($n = 9$; mobile phase A prepared separately each day).

2.2.4.4 Recovery. Recovery was evaluated by duplicate analysis of spiked urine and plasma samples at 4 concentration levels. The urine matrix was spiked with the final mixture of calibrants in order to achieve its dilution by 5- and 50-fold and 10- and 25-fold for plasma, representing levels of target compounds below the upper limit of quantitation (ULOQ) and above the lower limit of quantitation (LLOQ), further referred to as high- and low-levels. The measured concentrations were compared with the nominal values (measured concentration values in the non-spiked sample), and the recovery percentage was calculated for each concentration.

2.2.4.5 Limit of detection and lower limit of quantification. The theoretical limit of detection (tLOD) was estimated by the methodology described in the technical note by Wells *et al.*[19] The following formula was proposed:

$$\mathrm{tLOD} = t_\alpha \times S\bar{x}$$

where t_α comes from a table of the Student *t*-test using $n - 1$ (number of measurements minus one) and $S\bar{x}$ is used as an estimate of the true standard deviation of the distribution of sample means. The measured sample should contain the analyte of interest at a concentration close to the tLOD. The limit of detection (LOD) was also determined experimentally by injection of standard solution containing the analyte at tLOD levels. The tLOD of the method was determined using sample solutions prepared in the mobile phase since it was not possible to obtain a representative blank matrix free of all analytes.

LLOQ was defined as the lowest point of the calibration curve with $r^2 > 0.99$.

2.2.4.6 Matrix effect. The matrix effect was evaluated by comparing the slopes of calibration curves obtained in pooled urine, plasma or serum spiked with the same concentration levels of calibrants as we used for the solvent calibration. The following formula for calculating the percent difference was used: |((slope in matrix/slope in solvent) × 100%) − 100%|.

2.2.4.7 Stability of compounds in standard solutions and samples. The stability of the compounds was monitored in the calibrants prepared in mobile phase A, spiked urine and plasma samples containing low (just above the LLOQ), and high (just below the ULOQ) analyte levels. All samples underwent at least 3 full thaw/freeze cycles. The integrated peak areas without signal correction by the internal standard were compared to the one measured in the beginning after the first freeze/thaw cycle, which was considered as our baseline value.

3. Results and discussion

The method was validated according to the ICH,[20] EMA[21] and FDA[22] guidelines. Since these documents differ in terminology and purpose of use, customization and selection of validation design was necessary.

All experiments were performed with an optimized injection volume of 7.5 μL and 0.075% (v/v) formic acid was used as mobile phase A, which was found to be stable for at least 7 days at room temperature.

3.1 Selectivity

The selectivity was demonstrated by MS transitions in Table S-2† and illustrative chromatograms in Fig. S-1† for all compounds. In the case of resveratrol a double peak was observed after injection of the standard, which is most probably attributed to the separation of *cis*- and *trans*- isomers on the column. The same peak distribution was observed when a sample of red wine was injected (data not shown) indicating that the natural source of resveratrol also includes both isomers.

3.2 Linearity

The linear ranges (μg mL^{-1}) estimated from the average responses ($n = 12$) by least-squares regression calculation were as follows: 0.068–4.37 for TaA ($r^2 = 0.9991$); 0.015–0.95 for EtS

($r^2 = 0.9952$); 0.0098–5.0 for EtG ($r^2 = 0.9981$); 0.019–9.86 for InS ($r^2 = 0.9992$); 0.024–12.47 for CrS ($r^2 = 0.9960$); 0.0069–3.54 for resveratrol ($r^2 = 0.9987$); 0.041–2.63 for EsS ($r^2 = 0.9991$) and 0.020–10 for DHEAS ($r^2 = 0.9992$). CV (%) of r^2 was <1% in all cases.

3.3 Concentrations of internal standards

The costs of isotopically labeled internal standards significantly contribute to the financial burden, both for method development and for the further routine analyses. Therefore, the intention was to choose the minimum concentration, which would either provide integrated peak area values corresponding to unlabeled standards' peak areas in the upper half of the linear range, or give integrated area values >1000. Additionally, the linear relationship between the signal and the added internal standard concentration was investigated in the range from 4× lower to 2× higher concentrations compared with the finally selected one (see 2.2.4.2). This was done in order to ensure that ion-suppression as well as ion enhancement caused by the matrix would be adequately corrected. The average ($n = 3$) correlation coefficients for the concentration *vs.* signal experiments of the internal standards in the tested ranges were >0.99 for TaA-d_2, EtS-d_5, EtG-d_5, for InS-d_4, CrS-d_7, resveratrol-$^{13}C_6$, >0.98 for EsS-d_4 and >0.97 for DHEAS-d_5. Overall, the achieved linearity covering a wide range of internal standard concentrations ensures that a similar signal correction may be expected from any internal standard concentration within this range. Initial studies with higher levels of internal standards provided similar quantitative results (data not shown) indicating that we could reduce the costs without sacrificing accuracy.

3.4 Repeatability and intermediate precision

Repeatability with CV below 5% was achieved in all cases except EtS. The CVs of the intermediate precision exceeded 5% in the cases of EtS, CrS and resveratrol but 10% only in the case of EtS. It is assumed that some of the larger CVs (>10%) may be caused by the condition of the sample cone on particular days and therefore, it is necessary to inject calibrants individually on each plate and for each analysis. The results are displayed in detail in Table S-3.†

3.5 Recovery

In urine the recovery values for high-level spiked samples varied between 90% (CrS) and 114% (InS). For samples spiked at the lowest level, the recoveries were in the range of 103% (resveratrol and EsS) – 137% (EsS). In plasma, the recovery values for samples spiked at the highest level tested varied between 82% (CrS) and 114% (ESS and resveratrol). At the lowest level tested, the recoveries were found in the range of 128% (EtS)–218% (TaA) underlining that the level of added spike was selected to be very low compared to the nominal concentration of the analyte (<5%) and actually represents an increment just above the LLOQ. Taking into account CV% (10%) of the measurement repeatability validated above, the calculated value can result in a high relative error although it is nominally low. Additionally, ion-suppression depending on the nature of the real matrix in each individual sample may lead to lowering the response close to or below the LLOQ. The relative difference (%) of 2 recovery values was in the range of 1–10% for high-spiked and 1–20% for low-spiked samples.

3.6 Limit of detection and limit of quantitation

The authors of the approach described in 2.2.4.5 reasonably argue that it is unclear what region of the baseline should be selected to estimate the background noise and therefore, significantly different values of the LOD may be obtained when there is low and highly variable noise. On the other hand, modern mass spectrometers may limit background noise nearly to zero thus making it impossible to integrate the noise area in order to obtain a numerical value for calculating a real LOD based on the signal-to-noise ratio approach. Even though LODs are conventionally reported as calculated values, in our study we also tested them experimentally. The calculated and experimentally found LODs are compared in Table 2.

In mass spectrometry, ion-suppression may be expected when analyzing a matrix so the LOD values obtained from the experiments using standard solutions prepared in solvent can be overestimated. Therefore, we chose the lowest point of the calibration curve as the LLOQ instead of calculation based on signal-to-noise or on the standard deviation of the response and the slope.[20] As demonstrated in "matrix effect" experiments, the

Table 1 Comparison of average slopes ($n = 3$) ± S.D. of calibration curves made in mobile phase A (*i.e.* 0.075% (v/v) formic acid), 15× diluted pooled urine ($n = 80$), pooled plasma ($n = 3$) and a serum sample. Differences were calculated in relation to the reference slope values obtained in mobile phase A considered as reference values

Analyte	0.075% HCOOH	Urine	Difference (%)	Plasma	Difference (%)	Serum	Difference (%)
L-Tartaric acid	1.43 ± 0.05	1.43 ± 0.03	0	1.53 ± 0.05	7.0	1.46 ± 0.05	2.1
Ethyl sulphate	2.37 ± 0.03	1.97 ± 0.04	16.9	2.16 ± 0.01	8.9	2.01 ± 0.01	15.2
Ethyl-β-D-glucuronide	0.82 ± 0.01	0.91 ± 0.03	11.0	0.97 ± 0.05	18.3	0.86 ± 0.02	4.9
Indoxyl sulphate	0.90 ± 0.03	0.99 ± 0.04	10.0	1.04 ± 0.02	15.6	0.99 ± 0.01	10.0
Cresol sulphate	0.052 ± 0.004	0.04 ± 0.02	23.1	0.066 ± 0.002	26.9	0.057 ± 0.002	9.6
Resveratrol	0.88 ± 0.02	0.90 ± 0.02	2.3	0.99 ± 0.0007	12.5	0.93 ± 0.01	5.7
Estrone sulphate	1.25 ± 0.01	1.27 ± 0.01	1.6	1.38 ± 0.05	10.4	1.29 ± 0.02	3.2
DHEAS	1.18 ± 0.01	1.21 ± 0.02	2.5	1.34 ± 0.01	13.5	1.21 ± 0.02	2.5

Table 2 Comparison of calculated and experimentally found detection limits (ng mL^{-1})

Analyte	Calculated	Experimentally found
L-Tartaric acid	45	41
Ethyl sulphate	0.36	0.40
Ethyl-β-D-glucuronide	1.1	5.0
Indoxyl sulphate	0.22	0.20
Cresol sulphate	5.0	3.0
Resveratrol	9.7	50
Estrone sulphate	1.3	5.0
DHEAS	22	10

linearity for all concentration levels was confirmed in all matrixes, *i.e.* urine, plasma and serum. The LLOQ values (ng mL^{-1}) were as follows: 68 for TaA; 2.0 for EtS; 10 for EtG; 19 for InS; 24 for CrS; 55 for resveratrol; 41 for EsS and 20 for DHEAS.

3.7 Matrix effect and sample dilution

Bioanalysis of human samples using LC-MS based methods is hampered by the composition of the matrix, which affects the repeatability and the extent of ionization of the analytes compared to water or solvent based solutions. Many discussions and studies about matrix effects in LC-MS bioanalysis exist in the literature, but there is no consensus on how these matrix effects should be evaluated.[23–28] In our opinion the most relevant approach seems to be the one which compares the slopes of calibration curves measured in the matrix with those prepared in solvent since the signal correction by using isotopically labelled internal standards does not guarantee the elimination of matrix effects.[29,30] As illustrated in Table 1, the slopes of calibration curves obtained by analyses of spiked matrices, *i.e.* pooled urine, plasma and serum differed in some cases more than 10% from the calibration slope obtained in solvent A. This finding indicates that signal corrections by means of isotopically labelled internal standards do not always completely eliminate the matrix effect, as already discussed.

In the study by Stahnke *et al.*,[31] it was found that the extent of matrix effects in QuEChERS extracts (European standardized procedure for the determination of pesticide residues using GC-MS and/or LC-MS (/MS) following acetonitrile extraction/partitioning) depends on the logarithm of matrix concentration. It was also found that suppressions between 25% and 50% were eliminated by a 10-fold dilution. In order to double the effect of a 10-fold dilution, a 100-fold was required. However, this can represent a serious obstacle due to sensitivity limitations of the instruments currently used. Additionally, this finding cannot be applied to different biological matrixes as a universal rule. Therefore, we investigated the effect of 15-, 30- and 50-fold dilution on the signal for all eight analytes in 8 urine samples ($n = 8$). Of these 64 possible analytical results, only 38 gave a result above the LOD. It was observed that the signal for internal standards, except for DHEAS-d_5, was slightly increasing by dilution, *i.e.* for TaA-d_2, EtS-d_5, EtG-d_5, CrS-d_7, and InS-d_4. The average r^2 value of the linear relationship of the dilution factor *vs.* calculated response for all detected compounds in all samples was >0.99 with CV 0.57% ($n = 38$).

3.8 Stability of compounds in standard solutions and samples

No decrease of the integrated peak area value was observed in any sample after 4 weeks storage at −20 °C (undergoing at least 3 thaw/freeze cycles), as well as when samples were stored at 5 °C for 15–20 hours in the LC sample manager. The stock standards stored under conditions recommended by the supplier were stable for at least 9 months.

Table 3 Comparison of LC-MSMS analytical parameters of our method with those found in the literature

Analyte	Retention time (min)	LOD [ng mL^{-1}]	LLOQ [ng mL^{-1}]	Linearity [μg mL^{-1}]	Tested samples	Reference
(L)-Tartaric acid	0.55	41	68	0.068–4.370	Urine/plasma/serum	Our work
	1.09	25.4[b]	7[a]	0.007–15.000	Human urine	7
Ethyl sulphate	0.80	0.4	15	0.015–0.950	Urine/plasma/serum	Our work
	4.1	50	110	0.10–5.00	Human urine	5
Ethyl-β-D-glucuronide	1.39	5	9.8	0.0098–5.0000	Urine/plasma/serum	Our work
	5.08	52	152	0.05–2.00	Human urine	32
Indoxyl sulphate	2.54	0.2	19	0.019–9.860	Urine/plasma/serum	our work
	ca. 6	23	75	0.1–40.0	Human serum	33
Cresol sulphate	2.68	3	24	0.024–12.470	Urine/plasma/serum	Our work
	1.54	Not provided	0.001[a]	0.001–300.000	Human plasma	34
Resveratrol	3.03 + 3.26	50	55	0.055–3.540	Urine/plasma/serum	Our work
	2.21	1.8	5.9	0.01–2.50	Rat tissues	35
Estrone sulphate	3.78	5	41	0.041–2.630	Urine/plasma/serum	Our work
	2.42	Not provided	0.07	0.00007–0.01	Human plasma	12
DHEAS	4.16	10	20	0.020–10.000	Urine/plasma/serum	Our work
	ca. 1	Not provided	18.8	0.013–6.690	Human serum	36

[a] LLOQ was not explicitly mentioned and was assumed by us from the linearity range. [b] Reported LOD was higher than the lowest point of the linear range.

3.9 Comparison with the literature

As illustrated in Table 3, this versatile method provided in most of the cases analytical performance comparable to methods published recently by others. Instrument sensitivity may be an issue explaining the somewhat higher LLOQs for some compounds, most notably EsS.

3.10 Real sample analysis

In order to test the method on a large set of real samples, 80 urine samples from 4 different subjects together with 6 calibrants were injected in random order in one batch. The retention times of all compounds were stable during the batch analysis (±0.02 min) and no carry-over was observed in calibrants or blanks (mobile phase A) injected during and at the end of the plate analysis. Neither was any carry-over detected in the blank injected after the most concentrated calibrant mixture. EsS and resveratrol were not detected in any urine sample; the latter result was expected since the study only provided beer and volunteers were asked to abstain from all other alcoholic beverages. TaA was detected in 71 samples (67 above the LLOQ), EtS in 41 (39 above the LLOQ), EtG in 42 (28 above the LLOQ), and DHEAS in all samples (71 above the LLOQ). InS and CrS were in all samples above the LLOQ. Pools ($n = 5$) of all samples ($n = 80$) were injected after approx. every 20 injections as a control for uniformity during the batch analysis and the CV% for the response for all the detectable analytes in the pools was below 3.4%.

3.11 Additional compounds

The method can also be used for the separation and detection of other compounds such as isoxanthohumol or isocohumulone, 3-nitrotyrosine, indole-3-lactic acid, and cortisol or cortisol sulphate (Fig. S-2a–d†). This may be useful for further alcohol intake cohort studies or industrial applications. However, further validation and selection of the optimal target matrix is necessary.

4. Conclusion

A versatile UHPLC–MSMS method for simultaneous quantification of L-tartaric acid, ethyl sulphate, ethyl-β-D-glucuronide, indoxyl sulphate, *p*-cresol sulphate, resveratrol, estrone sulphate and dihydroepiandrosterone sulphate has been developed and validated. The liquid chromatography gradient allows separation of various compounds across a wide polarity range within 6 min. However, for certain analytes which have been suggested in this paper, more sensitive mass spectrometers may be required in order to detect and quantify them in biological matrices. Overall, this method may be an efficient tool for targeted metabolomics, especially for alcohol intake related research.

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