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Determination of alkylresorcinol metabolites in human urine by gas chromatography–mass spectrometry

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ABSTRACT

Alkylresorcinols (ARs) are phenolic lipids present at high concentrations in the outer parts of rye and wheat kernels and have been proposed as biomarkers for intake of whole grain and bran products of these cereals. AR are absorbed in the small intestine and after hepatic metabolism two major metabolites, 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA), are excreted in urine either as such or as conjugates. Urine samples from nine individuals were incubated with different enzymes to assess type and extent of conjugates. In comparison with DHBA, which was mostly found in the free form, the less polar DHPPA was conjugated to a greater extent and the major conjugates were glucuronides. In this method, urine samples were hydrolyzed using β-glucuronidase from *Helix pomatia* and syringic acid was used as internal standard. Samples, silylated with BSTFA, were analyzed by GC–MS utilizing a BP-5 fused silica capillary column and single ion monitoring of molecular ions (*m/z* 370 [DHBA], *m/z* 398 [DHPPA]). Recoveries of DHBA and DHPPA were estimated to be 94% and 93%, respectively. The average intra-assay/inter-assay coefficients of variation were 4.9/5.7% for DHBA and 7.6/9.3% for DHPPA.

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1. Introduction

A diet including whole grain products is considered to be part of a healthy life style, and epidemiological studies have shown relationships between whole grain consumption and decreased risks of several chronic diseases, e.g. obesity [1], type 2 diabetes [2], coronary heart disease [3], and some cancers [4]. However, the diet–disease association might be over- or underestimated due to the effect of relatively large measurement errors inherent in traditional dietary assessment methods used in epidemiology [5]. Establishment of complementary or alternative measurements of whole grain intake, through the use of a biomarker, is expected to overcome some of these obstacles [6].

A group of phenolic lipids, alkylresorcinols (ARs), have been suggested as useful biomarkers for whole grain rye and wheat intake [7]. Alkylresorcinols (1,3-dihydroxy-5-alkylbenzene derivatives) are found in high amounts in the outer parts of rye and wheat kernels [8], and are therefore present in products containing bran or whole grains of rye or wheat, but are generally absent or found in very small amounts in other commonly consumed foods [9,10]. Ingested AR, absorbed in the small intestine and distributed to the systemic circulation by the lymphatic pathway, have been found in several biological compartments including plasma [11], erythrocytes [12], and adipose tissue [13]. Hepatic metabolism of AR is suggested to consist of phase I metabolism, including ω- and β-oxidation, resulting in the formation of two major metabolites: DHBA (3,5-dihydroxybenzoic acid) and DHPPA (3-(3,5-dihydroxyphenyl)-1-propanoic acid) [14]. These metabolites are excreted in urine either as such or as more polar conjugates (e.g. glucuronide or sulfate conjugates) after phase II metabolism.

AR metabolites have been previously quantified in plasma [15] and urine [16] by HPLC coupled to coulometric electrode array detector. In this study, a highly specific gas chromatographic–mass spectrometric method was developed for quantification of the two main AR metabolites in human urine. Furthermore, the distribution of free and conjugated urinary DHBA and DHPPA was investigated in nine human subjects after consumption of rye bran flakes.

2. Materials and methods

2.1. Chemicals and tubes

Type H-1 β-glucuronidase/sulfatase from *H. pomatia*, type B-1 β-glucuronidase from bovine liver, N-methyl-N-

Abbreviations: AR, alkylresorcinol; BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; CV, coefficient of variation; DHBA, 3,5-dihydroxybenzoic acid; DHPAA, 3,5-dihydroxyphenylacetic acid; DHPPA, 3-(3,5-dihydroxyphenyl)-1-propanoic acid; HMDS, hexamethyldisilazane; LOD, limits of detection; LOQ, limit of quantification; MSTFA, N-methyl-N-(trimethylsilyl)trifluoroacetamide; QSM, quick silylation mixture; SIR, selected ion recording; SPE, solid phase extraction; TIC, total ion count; TMCS, trimethylchlorosilane.

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(trimethylsilyl)trifluoroacetamide (MSTFA), and syringic acid were obtained from Sigma Chemicals (St. Louis, MO, USA). Standards of the two metabolites DHBA and DHPPA were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) and Isosep AB (Tullinge, Sweden), respectively. DHPAA (3,5-dihydroxyphenylacetic acid) was purchased from Apin Chemicals Ltd. (Abingdon, UK). Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were purchased from Supelco (Bellafonte, PA, USA). Oasis® Max 60 mg solid phase extraction cartridges were obtained from Waters (Milford, MA, USA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% TMCS was purchased from Thermo Scientific (Rockford, IL, USA). Ethyl acetate, hexane, methanol, and pyridine were obtained from Merck (Darmstadt, Germany). All tubes used were obtained from VWR International (Stockholm, Sweden): hydrolysis and extraction were performed in 15 ml glass tubes, purified AR metabolites were eluted into 6 ml glass tubes and derivatized samples were transferred to 0.1 ml inserts in 1.5 ml GC vials.

2.2. Samples

Urine samples were obtained from nine healthy individuals (five women and four men) who took part in an alkylresorcinol dose–response study in Uppsala, Sweden [17]. The subjects avoided products containing whole grain or bran of rye and wheat during a 1-week run-in period before the treatment period. The treatment period consisted of one week where the subjects had a daily intake of 45 g rye bran flakes as part of their diet. The daily total AR intake during the treatment period was 66 mg (170 μmol). Two continuous 24 h urine collections were taken separately at the end of the treatment period.

The samples used for screening for endogenous concentrations of 3,5-dihydroxyphenylacetic acid (DHPAA) and syringic acid were randomly chosen 24 h urine collections from free-living female ($n = 17$) and male ($n = 3$) subjects, consuming their habitual diet. The urine was collected on two occasions, approximately three months apart. To all urine collections, 9 ml 20% HCl was added to inhibit microbial growth and samples were stored at −80 °C until analysis.

2.3. Internal standard

DHPAA and syringic acid were evaluated as internal standards by screening urine samples for the endogenous content of these substances. Screening for endogenous DHPAA was performed as single measurements on 40 individual samples using syringic acid as internal standard (2.25 nmol in 15 μl methanol added to 50 μl urine). Hydrolysis, extraction, derivatization steps, and GC–MS analysis were performed according to the final protocol (Fig. 1). All samples were analyzed by single ion monitoring (SIM).

Endogenous concentrations of syringic acid in urine were estimated in 20 samples (one from each subject) using external calibration. To 100 μl urine, 1.5 ml hydrolysis solution, containing 0.1 M sodium acetate buffer (pH 5.0) and β-glucuronidase/sulfatase from *H. pomatia* (50 mU β-glucuronidase and >320 mU aryl sulfatase), were added and the samples were incubated overnight at 37 °C. Hydrolysis was terminated by addition of 30 μl concentrated HCl. Extraction and derivatization were performed according to the final protocol. After transferring the samples to GC vials, analysis in SIM mode on GC–MS was performed according to the final protocol.

Both standard solutions and spiked urine were used to evaluate the upper limit of detector linearity for DHPAA and syringic acid. Pooled urine extracts were divided into 2 ml aliquots, to which eight levels (2.25–45.00 nmol, equivalent to 45–900 μM urinary concentration) of DHPAA or syringic acid were added. After evaporating the spiked urine extracts, derivatization and analysis were performed according to the final protocol. Spiked samples were analyzed in duplicate. Within the batch of samples, a seven-point standard curve (2.25–75.00 nmol, equivalent to 45–1500 μM urinary concentration, of each DHPAA and syringic acid) was prepared. The measured peak areas were plotted against the urinary concentrations of added substance and linear regression of the four lowest standard levels (2.25–15.00 nmol, 45–300 μM urinary concentration) was forced through origin. The measured peak areas of DHPAA and syringic acid at each level (both in the standards and spiked urine) were compared with the theoretical peak areas (extrapolation of the linear regression). The upper limit of linearity was determined as the highest level of added standard resulting in a measured peak area ≥95% of the theoretical peak area.

In the final protocol, syringic acid (15 nmol in 10 μl methanol) was added as internal standard to 50 μl urine.

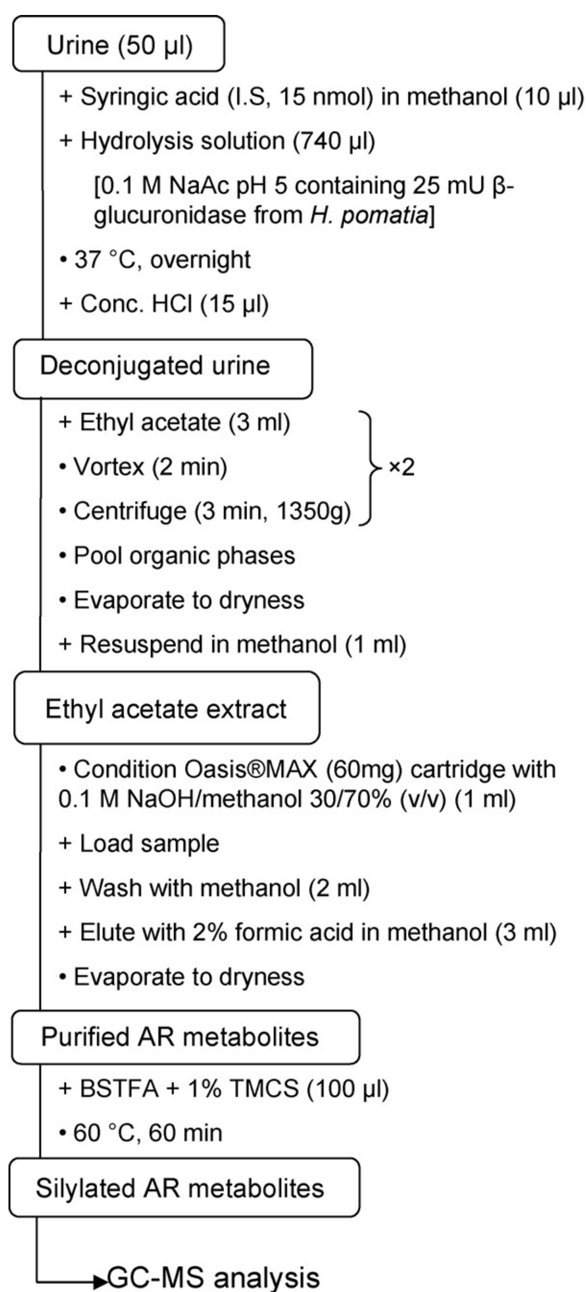

Fig. 1. Scheme of the final protocol for analysis of alkylresorcinol metabolites in urine.

2.4. Hydrolysis

To evaluate the most suitable hydrolysis enzyme, a sample with high endogenous concentration (>150 μM total metabolites [17]) was hydrolyzed by incubation with different enzymes (pure β-glucuronidase and β-glucuronidase with sulfatase activity) overnight at 37 °C. As internal standard, 2.25 nmol syringic acid (dissolved in 15 μl methanol) was added to thawed urine samples (50 μl). The urine was hydrolyzed at 37 °C overnight by incubation with 735 μl 0.1 M sodium acetate buffer (pH 5.0), containing different amounts of β-glucuronidase from bovine liver (0–400 mU) or β-glucuronidase/sulfatase from *H. pomatia* (0–100/0 to >140 mU). Hydrolysis was terminated by addition of 15 μl concentrated HCl. Extraction, derivatization, and analysis of unconjugated AR metabolites were performed according to the final protocol.

In the final protocol, the urine with syringic acid was diluted by addition of 735 μl hydrolysis solution, containing 0.1 M sodium acetate buffer (pH 5.0) and β-glucuronidase/sulfatase from *H. pomatia* (25 mU β-glucuronidase and >160 mU aryl sulfatase). Enzymatic hydrolysis was performed overnight at 37 °C and was followed by addition of concentrated HCl (15 μl).

2.5. Liquid–liquid extraction

As the next step in the final protocol, deconjugated samples were extracted with ethyl acetate (3 ml) by vortexing (2 min at maximum speed) with a Vortex-Genie 2 from Scientific Industries (Bohemia, NY, USA). Phases were separated by centrifugation (1350 × *g*, 3 min) on a Heraeus Multifuge 3 S from Thermo Scientific (Waltham, MA, USA), followed by freezing the water phase in a dry ice-ethanol bath. The organic phase was collected. This extraction procedure was repeated once and the organic phases were pooled. The samples were evaporated to dryness under a stream of nitrogen before purification on Oasis® Max extraction cartridges.

2.6. Solid phase extraction (SPE)

Prior to loading the samples in 1 ml methanol, Oasis® Max (60 mg) extraction cartridges were conditioned with 1 ml of 0.1 M sodium hydroxide/methanol (30/70, v/v). After washing with 2 ml methanol, the compounds were eluted in 3 ml of 2% (v/v) formic acid in methanol. In the final protocol, SPE was performed as described above with a GX-274 ASPEC automated SPE instrument from Gilson (Middleton, WI, USA). Sample application and elution were executed at a flow rate of 1 ml/min, while both cartridge conditioning and washing were performed at flow rate of 3 ml/min. Standards of DHBA and DHPPA were used to investigate possible losses of analyte during solid phase extraction by comparing area ratios (analyte/internal standard) of standards subjected to SPE before derivatization to area ratios of standards directly derivatized and injected (without SPE). To 0.45 μg syringic acid (in 15 ml methanol), eight different amounts (0.002–1.000 μg) of DHBA or DHPPA dissolved in methanol were added. This was done in duplicates and one of each duplicate was evaporated to dryness in a SpeedVac Concentrator SVC100 H coupled to a refrigerated condensation trap, both from Savant/GMI (Ramsey, MN, USA) and redissolved in 1 ml methanol. Solid phase extraction was performed according to the final protocol. Both elutes from cartridges and standard solutions not subjected to solid phase extraction were then evaporated to complete dryness and incubated with 100 μl BSTFA + 1% TMCS for 60 min at 60 °C. The samples were transferred to GC vials before analysis and resulting area ratios were compared.

2.7. Derivatization

To determine a suitable derivatization procedure, three different derivatization reagents were tested. To 0.2 μg of DHBA or DHPPA (dissolved in 100 μl methanol), 2.25 nmol of syringic acid (0.45 μg dissolved in 30 μl methanol) was added. The samples (8 replicates/derivatization reagent) were evaporated to dryness under a stream of nitrogen and 100 μl BSTFA, MSTFA or quick silylation mixture (pyridine:HMDS:TMCS 9:3:1 (v/v/v)) (QSM) were added. Samples with BSTFA and MSTFA were incubated for 60 min at 60 °C, while samples with QSM were incubated for 60 min at room temperature. Half the samples from each treatment were directly transferred to GC vials for analysis, while the other half were evaporated to dryness under a stream of nitrogen and then redissolved in 100 μl hexane before transfer to GC vials. Treatments were compared based on the resulting peak areas of the molecular ions of DHBA, DHPPA, and syringic acid. Temperature effects on silylation with QSM were then investigated by comparing the resulting peak areas of the tested substances (DHBA, DHPPA, and syringic acid) after incubation at two different temperatures (room temperature and 60 °C).

Derivatization in the final protocol was performed as follows: solid phase extracts were evaporated to complete dryness under a stream of nitrogen and 100 μl BSTFA was added. The samples were incubated for 60 min at 60 °C and finally transferred to GC vials for analysis.

2.8. GC–MS analysis

The GC–MS system consisted of a Finnigan™ TRACE GC Ultra Gas Chromatograph coupled to a Finnigan TRACE DSQ II mass detector (Thermo Fischer Scientific, Waltham, MA, USA). Separation of DHBA, DHPPA, syringic acid (internal standard), and DHPAA (internal standard candidate) was performed on a BP-5 fused silica capillary column (15 m × 250 μm I.D., 0.25 μm film thickness). The oven temperature was initially held at 100 °C for 1 min, raised by 8.6 °C/min to 250 °C, then by 40 °C/min to 300 °C, and held for 2 min. The temperatures for the inlet, transfer liner, and ion source were set at 300 °C, 310 °C and 250 °C, respectively. Sample injection volume was 1 μl and split-less injection was used. Helium was used as carrier gas with a flow rate of 1 ml/min. Samples were analyzed in both full scan mode (m/z 50–650) and selected ion recording (SIR). Molecular ions and selected fragments were detected in SIR as follows: DHBA: m/z 370 (molecular ion) and m/z 355; DHPPA: m/z 398 (molecular ion), m/z 293, and m/z 281; DHPAA: m/z 384 (molecular ion), m/z 369 and m/z 252; and syringic acid: m/z 342 (molecular ion), m/z 327, and m/z 312.

The analytes were identified by comparing the gas chromatographic retention times with retention times of standards of the two metabolites and by studying the mass spectra of DHBA and DHPPA. Molecular ions were used for quantification of DHBA, DHPPA, DHPAA, and syringic acid.

2.9. Calibration

AR metabolites were quantified by preparing eight-point calibration curves (2–600 ng of each AR metabolite), randomly analyzed within each batch. Known amounts of analyte were linearly regressed against the resulting peak area ratio (analyte/internal standard). Analyte amounts in urine samples were quantified by comparing peak area ratio with the equation from the calibration curve.

Endogenous DHPAA was quantified by comparing peak area ratio of DHPAA and syringic acid in the urine samples with a linear regression (extrapolated) of a four-point calibration curve (0.15–1.50 nmol DHPAA).

Endogenous syringic acid was quantified by preparing a multipoint calibration curve (0.015–0.750 nmol, $n = 8$) randomly analyzed within the batch. Known amounts of analyte were linearly regressed against the resulting peak area and amounts of syringic

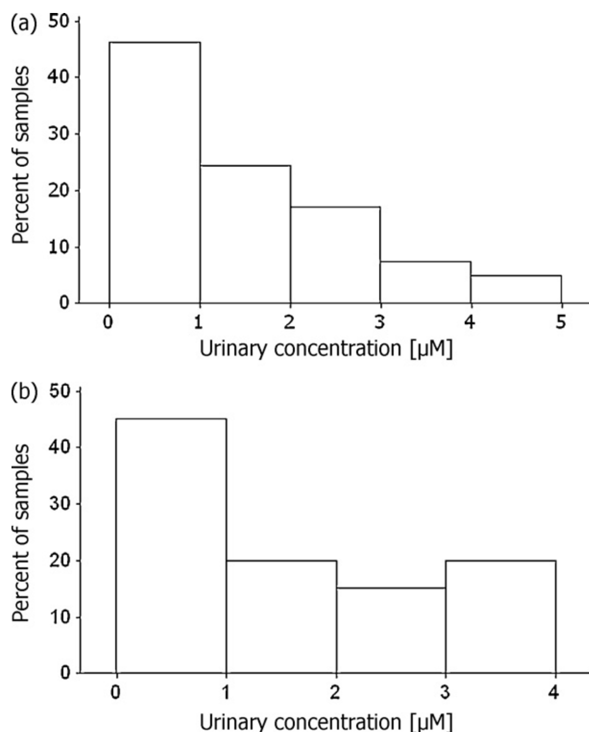

Fig. 2. Endogenous concentrations of (a) DHPAA ($n = 40$) and (b) syringic acid ($n = 20$) in urine samples from free-living men and women.

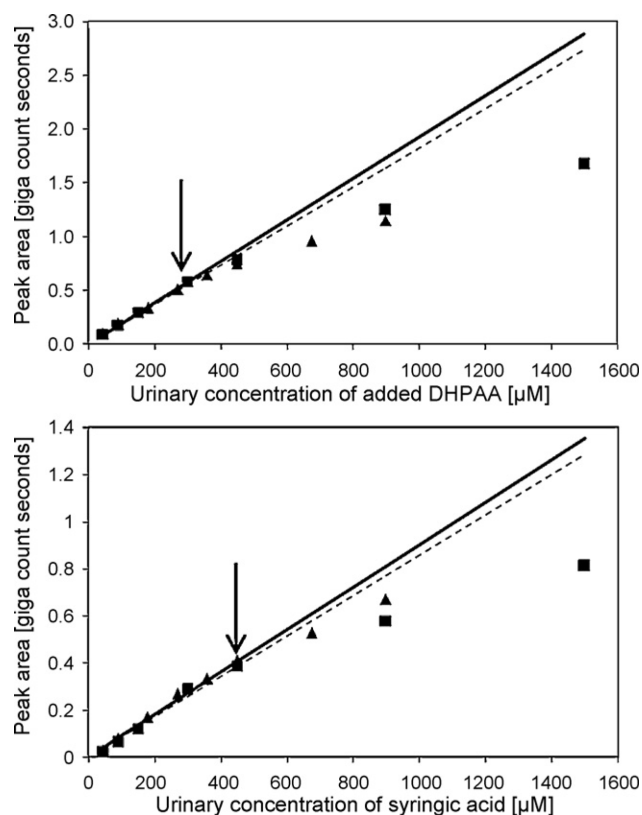

Fig. 3. Upper limit of linear range (↓) for the internal standards DHPAA and syringic acid. Urinary concentrations of added substance analyzed without purification (squares) and added to purified urine (triangles) was plotted against resulting peak areas. Extrapolation of the linear regression of the four lowest standard levels (unbroken line) was used to calculate the theoretical peak areas. The upper limit of linearity was determined as the highest level of added standard resulting in a measured peak area ≥95% of the theoretical peak area (broken line).

acid in urine samples were quantified by comparing peak area with the equation of the regression.

Solid phase extracted standards of DHBA and DHPPA were quantified by comparing the area ratio to an eight-point calibration curve (standards not exposed to solid phase extraction).

2.10. Recovery and precision

The recoveries of DHBA and DHPPA were measured in triplicate by adding four levels of both compounds (equivalent to 12–94 μM DHBA and 10–80 μM of DHPPA urinary concentrations) to urine samples (50 μl) from three subjects with varying endogenous concentrations of the AR metabolites. The measured concentrations were plotted against the theoretical concentrations (endogenous concentration + added amount) and mean recovery were determined as the slope of linear regressions made from the plots.

The precision of the method was evaluated by measuring 3–5 replicates of three different samples in a single analysis (intra-assay) and on three separate occasions (inter-assay). Intra-assay standard deviation represents a mean value of the standard deviations from three occasions. Inter-assay standard deviations were calculated based on the intra-assay mean values from the three occasions. To determine the precision of the GC–MS analysis, two pre-treated samples were silylated and analyzed repeatedly. Hydrolysis, liquid extraction, and solid phase extraction of the two urine samples were performed on 10 replicates. The replicates were pooled and the two samples were derivatized and analyzed according to the final protocol on five separate occasions.

2.11. AR metabolite conjugates

In order to investigate the extent of conjugation of urinary DHBA and DHPPA, samples from nine subjects were incubated with different hydrolysis buffers. To 50 μl urine, 2.25 nmol syringic acid (446 ng in 15 μl methanol) was added. Each urine sample was incubated with 735 μl 0.1 M sodium acetate buffer (pH 5) containing no enzyme (control buffer), 25 mU β-glucuronidase from bovine liver (β-glucuronidase buffer) or β-glucuronidase with sulfatase activity [25 mU β-glucuronidase and ≥34 mU sulfatase] from *H. pomatia* (β-glucuronidase/sulfatase buffer). Extraction, derivatization, and analysis of unconjugated AR metabolites were performed according to the final protocol and samples were analyzed in triplicate.

Analyte concentrations quantified in samples incubated with control buffer were defined as free aglycones, while analytes quantified in samples hydrolyzed with β-glucuronidase/sulfatase from *H. pomatia* were defined as total metabolites. Concentrations of glucuronides were calculated by subtracting the free aglycone concentrations from the analyte concentration quantified in samples hydrolyzed with β-glucuronidase from bovine liver. Concentrations of other, non-classified, conjugates were determined by subtracting the concentrations of glucuronides and free metabolites from total metabolites.

2.12. Statistical analysis

All statistical analyses were performed with SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Differences in silylation yield between different derivatization reagents were tested using a mixed linear model with derivatization reagents, analyte and injection procedure as fixed factors and replicate as random factor. Least-squares means were calculated for silylation agents and p values were Bonferroni-corrected. A mixed model was also used to address the temperature dependence of incubation with QSM. In this model, temperature and analyte were set as fixed factors and replicate as

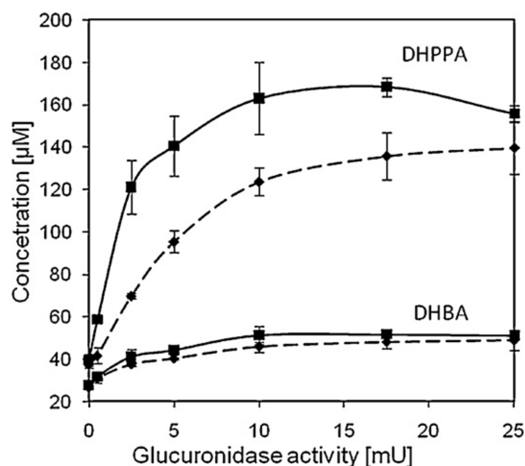

Fig. 4. Concentration of urinary DHBA and DHPPA in a urine sample after hydrolysis with enzymes at different concentrations (mean ± SD, $n = 3$). β-glucuronidase/sulfatase (—) and pure β-glucuronidase (– –).

random factor. Least-squares means were calculated for temperatures.

A general linear model was used to compare conjugation distribution between metabolites and sexes. Metabolite and sex were set as fixed factors, while subject was a random factor.

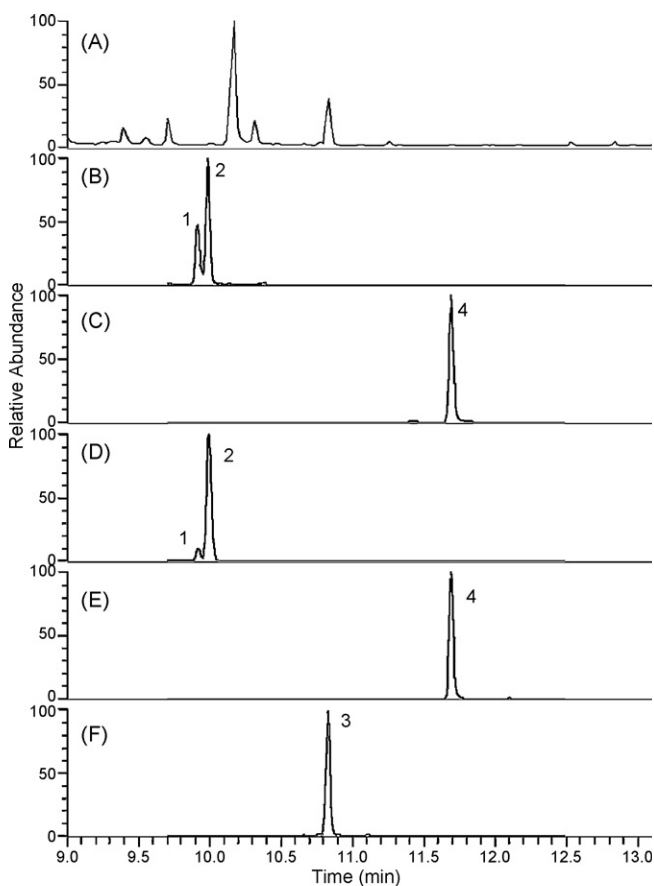

Fig. 5. Chromatograms of urine samples treated accordingly to the experimental protocol. (A) Total ion count (m/z 50–600) chromatogram of a urine sample with low AR metabolite concentration. Same urine sample analyzed with selected ion recording (SIR); (B) m/z 370 and (C) m/z 398. Chromatograms (SIR) of a urine sample with a normal concentration: (D) m/z 370; (E) m/z 398 and (F) m/z 342. Peaks: (1) 3,4-dihydroxybenzoic acid, (2) DHBA, (3) syringic acid and (4) DHPPA.

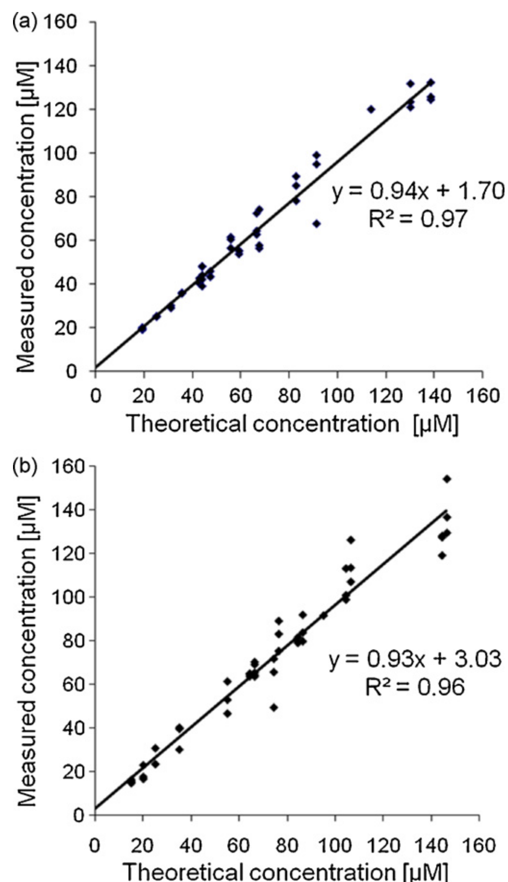

Fig. 6. Recovery of (a) DHBA and (b) DHPPA in spiked urine samples. Theoretical values are plotted against measured values and linear regressions estimate recovery. Theoretical concentration of the samples is defined as the endogenous concentration plus the added amount of analyte.

3. Results and discussion

3.1. Method development

The final protocol for the analysis of urinary AR metabolites (Fig. 1) consisted of four sample treatment steps (deconjugation, liquid–liquid extraction, solid phase extraction, and derivatization) prior to analysis with GC–MS.

Previously, syringic acid has been used as internal standard for the quantification of AR metabolites by HPLC coupled to a coulometric electrode array detector [16]. However, syringic acid is present in a number of cereal products [18] and in a recent study, subjects excreted small amounts of syringic acid after consuming berry-rich meals [19]. Another possible internal standard for quantification of AR metabolites, 3,5-dihydroxyphenylacetic acid (DHPAA), has been previously found in rat urine after oral administration of myrecetin and related compounds [20]. In order to evaluate the utility of syringic acid and/or DHPAA as internal standards, the endogenous concentrations and the upper limit of detector linearity of the two substances were investigated. In this study, endogenous concentrations of DHPAA and syringic acid in the screened samples were ≤4.4 μM ($n = 40$) and ≤3.5 μM ($n = 20$), respectively (Fig. 2). The upper limits of the linear range of DHPAA and syringic acid were estimated to be 0.150 nmol/injection (equivalent to a urinary concentration of 300 μM) and 0.225 nmol/injection (equivalent to a urinary concentration of 450 μM), respectively (Fig. 3). Due to variations in endogenous concentration between samples, the added amount

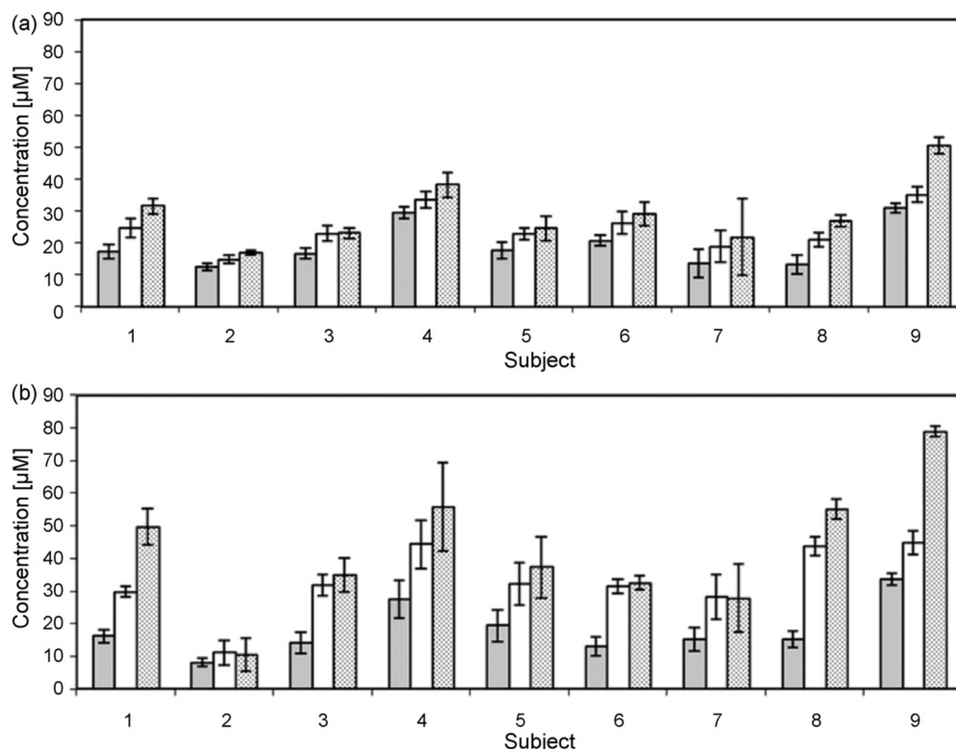

Fig. 7. Concentration of (a) DHBA and (b) DHPPA in urine samples after treatment without (grey) and with deconjugating enzymes: pure β-glucuronidase (white) and β-glucuronidase with sulfatase activity (dotted). Values are mean ± SD, $n = 3$.

should be as high as possible within the linear range. The higher upper limit of detector linearity together with the narrower range of endogenous concentrations suggest that syringic acid is a more suitable internal standard than DHPAA. The amount of syringic acid (15 nmol) added as internal standard in the final protocol is equivalent to a urinary concentration of 300 μM.

By hydrolyzing a urine sample that contains high amounts of AR metabolites with different concentrations and types of enzymes, the crude mixture from *H. pomatia*, containing both β-glucuronidase and sulfatase activity, was found to deconjugate DHBA and DHPPA to a higher extent than the pure β-glucuronidase from bovine liver (Fig. 4). Different enzyme concentrations were tested and it was found that 25 mU β-glucuronidase was sufficient for 50 μl of the urine sample (highly concentrated with AR metabolites) to reach optimal deconjugation during 16 h (overnight) at 37 °C. This was observed for both the enzyme mixture and the pure β-glucuronidase.

Oasis® MAX cartridges used in this protocol are mixed mode polymetric cartridges with anion-exchange and reversed phase functionalities and have been previously used to remove neutral and basic hydrophobic compounds from human urine [21,22]. No analyte loss was observed during solid phase extraction (SPE) when comparing standards subjected to SPE and standards directly injected on GC–MS (data not shown). The use of an automated SPE instrument decreased the time and labor involved in sample treatment.

Derivatization of the analytes and internal standard with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) yielded significantly larger peak areas ($p < 0.02$) compared with N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and quick silylation method (QMS). Concerning silylation with QSM at different temperatures, a significant increase in peak area due to increased incubation temperature could only be observed for DHPPA ($p < 0.02$). However, this difference due to temperature was smaller than the difference between reagents (BSTFA vs. QSM), so BSTFA was kept as silylation reagent in the final protocol. Furthermore, peak areas of silylated standards redissolved in hexane and injected into GC–MS corresponded to 1–13% of the peak areas from silylated standards injected in the silylation solution. Despite the possibility of increased contamination of the GC–MS, in the final protocol samples were injected in BSTFA, as it allowed higher sensitivity and was less time-consuming than redissolving them in hexane. Fig. 5 shows total ion count (TIC) and molecular ion chromatograms of AR metabolites and syringic acid resulting from analysis of urine samples treated according to the final protocol.

3.2. Linearity, limit of detection and limit of quantification

Reference standards were used to calculate the limits of detection (LOD), limits of quantification (LOQ) and linearities. LOD was defined as the amount of injected analyte resulting in a signal to noise ratio of 3:1 and LOQ was defined as the amount of injected analyte giving rise to a signal to noise ratio of 10:1. A DHPPA concentration of 0.112 μM resulted in a signal to noise ratio of 13:1, which gives an estimate of 0.1 μM as LOQ. DHBA with the measured concentration 0.019 μM showed a signal to noise ratio of 194:1. This shows that DHBA can be detected at much lower concentrations than DHPPA and that both metabolites can be quantified at concentrations much lower than can be expected in normal samples. Linearity was determined in the range where a linear detector response could be obtained (pg/injection). Linearity ranged all over the tested interval (LLOQ-3077 ng/injection) for both analytes.

3.3. Recovery and precision

The mean recovery of all concentrations was 94% for DHBA and 93% for DHPPA (Fig. 6). Intra- and inter-assay coefficient of variation (CV) values for both AR metabolites were less than 9% at all concentrations tested except the inter-assay CV for DHPPA at the lowest concentration tested, which was 14%. For DHBA, the intra-assay and

inter-assay CV values were 4.9 ± 1.4% and 5.7 ± 2.5%, respectively. The corresponding values for DHPPA were 7.6 ± 1.2% and 9.3 ± 4.1%, respectively. A small part of the inter-assay variation in the method can be explained by between-day variations in the GC–MS analysis, since the mean CV value of the two samples silylated and analyzed on five separate occasions was 2.2% for DHBA and 2.7% for DHPPA.

3.4. Conjugate distribution in urine from human volunteers

The total amounts of DHBA and DHPPA in the nine human urine samples were in the range 17–50 μM and 11–79 μM, respectively, and the DHPPA/DHBA ratio was 1.5 ± 0.4 (Fig. 7). Quantification of unconjugated and deconjugated metabolites showed that urinary AR metabolite DHPPA was conjugated to a significantly greater extent than the more hydrophilic DHBA ($p < 0.01$). Free aglycones of DHBA corresponded to 67 ± 10% (mean ± SD) of the total amount of DHBA, whereas glucuronides constituted 18 ± 7% of the total pool of DHBA. In contrast, 39 ± 13% of the total amount of DHPPA consisted of glucuronides, while 46 ± 14% consisted of free aglycones. Other conjugates, e.g. sulfates, comprised only a small fraction (15 ± 11%) of the total metabolites. These results indicate that the difference in conjugate distribution between the two metabolites was mostly due to glucuronide conjugates, since the proportion of glucuronides was significantly higher in DHPPA than in DHBA ($p < 0.01$), while no significant difference was observed between the two metabolites when comparing the amounts of other conjugates. No significant differences in conjugate distribution due to sex could be observed in the limited number of samples analyzed.

Concerning DHBA, these results agree with conclusions by Koskela et al. that AR metabolites are mainly present as free aglycones in urine [16]. On the other hand, the results in the present study indicate that urinary excretion of DHPPA, which is the more hydrophobic of the two AR metabolites, is dependent on conjugation to a greater extent than urinary excretion of DHBA. The samples used for the investigation of conjugation came from subjects with relative high intake levels (66 mg/d) which should be compared to the average population (median intake 10–20 mg/d) [23]. Recovery of ingested AR, as DHBA and DHPPA in 24-h urine collection, decreased as the daily intake of AR increased, which could be due to alternation in the elimination of AR [17]. Furthermore, dose-dependent shifts in conjugation, from sulfation at low doses to glucuronidation at high doses, have been observed for several phenolic compounds [24].

4. Conclusions

An alternative method for sensitive and accurate determination of the AR metabolites DHBA and DHPPA in urine was developed, using GC–MS for quantification. Generally, analysis with GC–MS is associated with a number of advantages (e.g. compound identification, relative short run-time and high number of theoretical plates). Furthermore, GC–MS equipment is more common in laboratories than HPLC–CEAD, the equipment used in the previously published method for quantification of AR metabolites in urine. The method presented here was successfully applied to investigate the extent of metabolite conjugates in human subjects after consumption of rye bran flakes. The major conjugates were glucuronides and the more hydrophobic AR metabolite, DHPPA, was conjugated to a greater extent than DHBA, which was mostly present in the free form.

In order to investigate the relationships between ingested dose levels and shifts in metabolism, conjugation and/or excretion, further studies are needed. New experiments could also further address the distribution of sulfate and sulfoglucuronide conjugates in urine. Furthermore, the method described here should be compared to the previously published HPLC–CEAD method [16].

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