Determination of alkylresorcinols and their metabolites in biological samples by gas chromatography–mass spectrometry

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1. Introduction

Epidemiological studies have consistently shown inverse relationships between consumption of whole grain cereals and the risk of developing several chronic diseases such as cardiovascular disease, type 2 diabetes and colorectal cancer[1–3]. Most studies rely on self-reported intakes from food frequency questionnaires (FFQ), which are known to suffer from relatively large systematic and random measurement errors[4]. Accurate whole grain intake data acquisition via FFQ may be hampered by having few questions on cereal foods, difficulties among consumers in recognizing whole grain products, large variations in whole grain content in cereal foods and lack of food composition data[5]. Therefore, dietary biomarkers may provide an independent tool to overcome some of these obstacles and a better way for objective ranking of whole grain intake in epidemiological studies[6,7].

Alkylresorcinols (AR), a group of amphiphilic 1,3-dihydroxy-5-alkyl phenolic lipids, have been suggested and evaluated as biomarkers of whole grain wheat and rye intake in different populations[8–10] due to their almost exclusive presence in the outer layer of wheat and rye grains among commonly consumed foods[11,12]. Homologues with odd alkyl chains of 17–25 carbons atoms are the most common AR compounds in cereal grains. The source of these AR biomarkers may provide an independent tool to overcome some of these obstacles and a better way for objective ranking of whole grain intake in epidemiological studies[6,7].

Abbreviations: AR, alkylresorcinol; AT, adipose tissue; BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; CV, coefficient of variation; DHBA, glycine, 2-(3,5-dihydroxybenzamido) acetic acid; DHBA, glycine, 2-(3,5-dihydroxybenzamido) acetic acid; DHCA, 3,5-dihydroxycinnamic acid amide; DHPPA, 3-(3,5-dihydroxyphenyl)-propanoic acid; DHPPA, 3-(3,5-dihydroxyphenyl)-propanoic acid; EI, Electron impact ionization; FFQ, food frequency questionnaires; GC–MS, gas chromatography–mass spectrometry; GLM, general linear model; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; NCL, negative chemical ionization; SIM, selected ion monitoring; SPE, solid phase extraction; MS, mass spectrometry; TFAA, trifluoroacetic anhydride; TMCS, trimethylchlorosilane.

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liver into two main metabolites, which are present in free or conjugated forms [16]. These metabolites are 3-(3, 5-dihydroxyphenyl)-1-propanoic acid (DHPPA) and 3,5-dihydroxycinnamic acid (DHBA).

In a recent study, 3-(3, 5-dihydroxyphenyl) pentan-2-one tetrafluorooxycarbonyl (DFPTA) and 2-(3, 5-dihydroxybenzamido) acetic acid (DHBA-glycine) were also described as urinary metabolites derived from AR [17]. Moreover, 3,5-dihydroxycinnamic acid (DHCA) and 3,5-dihydroxycinnamic acid amide (DHCA-amine) have recently been identified in urine and suggested as AR metabolites [18].

To allow determination of AR in plasma, adipose tissues and their metabolites in urine samples for epidemiological studies, rapid and sensitive methods are needed. In this study, we sought to develop optimized gas chromatography–mass spectrometry (GC–MS) methods for rapid, sensitive and robust determination of AR in plasma and small adipose tissue biopsies and for the first time for simultaneous determination of all currently reported AR metabolites in urine (DHBA, DHPPA, DHPPTA, DHBA-glycine, DHCA and DHCA-amine).

2. Materials and Methods

2.1. Chemicals and standards

All reagents used were of HPLC or gradient grade. Diethyl ether, ethanol, ethyl acetate, formic acid, hexane and methanol were purchased from Merck (Darmstadt, Germany). Alkylresorcinol reference compounds (C17:0, C19:0, C21:0, C23:0 and C25:0) and AR internal standards (C20:0, C22:0, C24:0 and C26:0) were of >95% purity and were purchased from ReseaChem Life Sciences (Burgdorf, Switzerland). An AR internal standard (IS) mixture was prepared by mixing AR C20:0, C22:0, C24:0 and C26:0 in methanol (300 µg L⁻¹ for each homologue). The AR metabolites (DHBA, DHPPA, DHPPA, DHPPTA and DHBA-glycine) were of >95% purity and were purchased from ReseaChem (Burgdorf, Switzerland), while DHCA (98% purity) and DHCA-amine (78% DHCA-amine, 17% DHCA and 5% other unidentified compounds) were synthesized at Vontor (Ing. T. Vontor, Pardubic 628, Hradec Kralove IV, Czech Republic) as described by Wierzbicka et al. [18]. Impurities of DHCA-amine were corrected for in all analyses. Syringic acid, used as an internal standard for the determination of AR metabolites in urine samples (297 µg mL⁻¹), and Type H-1 β-glucuronidase/β-sulphatase from Helix pomatia, used for hydrolysis of AR metabolites in urine, were purchased from Sigma Chemicals (St. Louis, MO, USA). Oasis® Max 60 mg solid phase extraction (SPE) cartridges were obtained from Waters (Milford, MA, USA) and N, O bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Thermo Scientific (Rockford, IL, USA). Stainless steel beads (diameter 2.8 mm) were purchased from VMR, Sweden. Trifluoroacetic anhydride (TFAA) (99%, ReagentPlus®) and undecane (99%) were purchased from Sigma–Aldrich, Sweden.

2.2. Sample analysis

2.2.1. Determination of AR in plasma samples

Plasma sample extraction and cleanup was conducted as described previously [19], but a new method for derivatization and quantification using gas chromatography–mass spectrometry (GC–MS) was developed in order to obtain more stable samples, improve method precision and shorten GC–MS run time. In brief, plasma (200 µL) was pipetted into a disposable test tube and 15 µL of AR internal standard mixture were added. The sample was vortexed for 1 min, followed by extraction with diethyl ether (3 mL) for 2 min on a multi-sample vortexer. The water phase was frozen in an ethanol-dry ice bath, while the organic phase was poured off into a new tube. The liquid–liquid extraction was repeated in total three times and organic extracts were combined and evaporated to dryness at 35 °C under a gentle stream of nitrogen. Methanol (1 mL) was added, and the test tube was vortexed before sample cleanup with SPE. Sample extracts from all matrices reported in this paper were purified on an SPE-robot as follows: Cartridges were conditioned with 1 mL 0.1 M NaOH in MeOH (3:7, v/v) at a flow rate of 1 mL min⁻¹, sample extract was applied (1 mL min⁻¹), neutral lipophilic compounds were washed away with 3 mL MeOH (1 mL min⁻¹) and AR were eluted with 2% formic acid in MeOH (2 mL). Eluates were evaporated to complete dryness under a nitrogen stream at 60 °C.

Evaporated plasma extracts were derivatized with TFAA (200 µL) for 30 min in sealed test tubes. Following derivatization, the solution in the test tube was transferred to a GC-vial with insert and TFAA was removed to avoid column damage by evaporation at 60 °C until dryness (usually around 10 mins). The sample was reconstituted in 20 µL undecane and sealed with a cap.

Derivatised samples and AR reference compounds were analysed on GC–MS (Finnigan TM Trace GC Ultra Gas chromatograph coupled to a Finnigan Trace DSQ II mass spectrometer, Thermo Fisher Scientific, Waltham, MA, USA). A ZB-5MS column (15 m × 0.25 mm × 0.25 µm, Zebron) was used. Helium (1.0 mL min⁻¹) was used as the carrier gas. The temperature of the transfer line was 310 °C and that of the ion source 250 °C.

A 2 µL sample was injected into a programmed temperature vaporizing (PTV) injector with a steel liner set at 320 °C. The oven temperature program was as follows: 200 °C was maintained for 2 min and then raised to 310 °C within 2.2 min. The temperature was kept at 310 °C for 5 min.

Electron impact ionization (EI) and negative chemical ionisation (NICI) with methane as reagent gas were tested. The EI at 70 eV was chosen for the applications described in this work due to considerably higher abundance and less frequent cleaning of the ion source required to ensure high sensitivity. Derivatized AR reference compounds were analyzed in scan-mode (m/z 50–660) to characterize fragmentation pattern and select suitable ions for quantification. Samples and standards were analyzed in selected ion monitoring (SIM) mode with 2–3 ions per acquisition window to ensure highest sensitivity. In samples, AR homologues were identified by their characteristic base ion at m/z 316, their molecular ions and by comparing their retention times with those of a mixture of synthetic AR standards (C17:0–C25:0). The base ion m/z 316, rather than the molecular ions m/z 540 (C17:0), 568 (C19:0), 596 (C21:0), 624 (C23:0) and 652 (C25:0), was used for quantification of AR due to higher selectivity and sensitivity for that ion compared with the molecular ions. Peak width was recorded and dwell time was carefully evaluated to ensure adequate sensitivity and to ensure that more than 10 data points were collected for each peak. The dwell time of molecular ions and base ion was set to 10 ms and 100 ms, respectively. A multipoint standard curve (5–500 pg µL⁻¹, n = 9, corresponding to sample concentration range 1–300 nmol L⁻¹ for each AR homologue) was used for quantification of AR homologues C17:0–C25:0 in each sample batch. Known AR concentrations were linearly regressed against the ratio of AR/internal standard, C20:0 was used as internal standard for all homologues in plasma samples.

2.2.2. Determination of AR in adipose tissue samples

A pig adipose tissue sample (about 50 g) was homogenized by Lourdes Multi-mix homogenizer and used for method evaluation. Different amounts of homogenized pig adipose tissue (10.2, 16.9, 26.4, 30.6, 35.0 and 54.0 mg) were transferred to six disposable plastic vials (screw-top micro-tube, 2 mL, PP, Sarstedt AG, Nürnberg, Germany) randomly loaded with different numbers of 2.8 mm diameter stainless steel beads (1–6 per vial), in order to
evaluate the effect of sample amount and number of beads needed for quantitative extraction of AR. The AR internal standard mixture (15 μL) and diethyl ether (1 mL) were added and the vials were sealed and extracted three times in a FastPrep®-24 Instrument (MP Biomedicals, Solon, OH, USA) at a speed of 4.5 for 20 s with 1 min rest interval between runs. The samples were transferred to Eppendorf tubes and centrifuged for 10 min at 1792 × g in an Eppendorf 5417C centrifuge (Hamburg, Germany) to separate connective tissue and organic solvent. The supernatants were transferred to new test tubes and evaporated to dryness at 35 °C under a gentle stream of nitrogen. Methanol (1 mL) was added and the test tubes were vortexed for 30 s and stored at 4 °C for 30 min to crystallise fat. Sample extracts were filtered through a 0.45 μm syringe filter (GHP Acrodisc® PallGelman Laboratory, USA) to remove fat crystals and other solid particles, followed by sample cleanup with SPE as described for plasma samples (see Section 2.2.1). The evaporated adipose tissue sample extracts eluted from SPE were derivatised with TFAA (300 μL) for 1 h at 40 °C in test tubes sealed with caps. After derivatisation, the solutions were transferred to GC vials with inserts and TFAA was removed to avoid column damage by evaporation at 60 °C until dryness (~15 min). Samples were then reconstituted in 30 μL undecane and sealed with a cap. GC–MS analysis of AR in adipose tissue samples was conducted as described above for plasma samples with a few exceptions: 2 μL was injected into a split–splitless injector with a glass liner (unpacked, straight design) set at 300 °C. The oven temperature program was as follows: 170 °C was maintained for 2 min, and then raised to 310 °C within 2.8 min. This temperature was held for 5 min. Individual AR internal standards were used for quantification of C17:0 (C20:0), C19:0 (C20:0), C21:0 (C22:0), C23:0 (C24:0) and C25:0 (C24:0) in adipose tissue samples. Human subcutaneous adipose tissue biopsy samples (10–50 mg) were extracted similarly as for pig adipose tissue, with six steel beads per sample. After suspension in undecane, the sealed GC vials were heated for 10 min at 60 °C and vortexed for 20 s to ensure that derivatised AR were recovered in the undecane.

2.2.3. Determination of AR metabolites in urine samples

Three different urine samples from a previous study [20] were processed in quadruplicate using a modification of a previously reported method for determination of DHBA and DHPPA [21]. The procedure comprises enzymatic hydrolysis of conjugated AR metabolites, extraction of the deconjugated compounds by liquid–liquid extraction, purification on SPE columns and derivatisation of AR metabolites before quantification by GC–MS.

To evaluate the most suitable hydrolysis procedure for all reported AR metabolites, different enzyme concentrations (12.2, 36.6, and 54.8 mU) were used in a modification of the previous methodology. To determine a suitable derivatisation procedure, a standard sample (100 μL) was mixed with four different ratios of BSTFA and TMCS (70:30; 80:20; 90:10; 99:1) and incubated for 60 min, 90 min and 120 min. The experiment was conducted in duplicate. In the final protocol, derivatisation was performed by adding BSTFA + TMCS (99:1, v/v, 100 μL) and incubating for 70 min at 60 °C. The entire volume was transferred to GC vials for analysis.

Human urine samples were analysed by GC–MS as described for plasma samples with a few modifications: The sample volume injected was 1.5 μL and the injector temperature was 250 °C. The oven temperature programme was as follows: 100 °C was maintained for 2 min and increased to 300 °C within 5 min. This temperature was held for 5 min. Samples were quantified in EI-SIM mode using molecular ions and at least one confirmatory ion.

2.3. Evaluation of methods

2.3.1. Determination of AR in plasma

Specificity was evaluated as non-interference at the retention times of the respective analyte from endogenous matrix components and internal standard using the proposed sample preparation procedure and GC–MS conditions. The linearity was evaluated using calibration standards (5–500 pg μL–1 for each AR homologue, n = 9, corresponding to plasma AR concentration in the range 3–300 nmol L–1 for each AR homologue). The theoretical limits of detection (LOD) and quantification (LOQ) were determined as the concentration where signal to noise ratio (S/N) was 3 and 10, respectively, based on determined S/N of AR homologues in a plasma sample with low endogenous analyte concentration (total AR concentration was 35 nmol L–1).

The recovery was assessed by spiking a plasma quality control sample with three different amounts of AR homologues at final concentrations corresponding to expected low, medium and high analyte concentrations, corresponding to 150, 300 and 450 nmol L–1, respectively, of total AR in authentic samples. Four replicate samples were prepared at each spiking level.

Recovery was calculated according to equation:

\[
\% \text{Recovery} = \left( \frac{\text{Analysed content} - \text{Endogenous content}}{\text{Spiked content}} \right) \times 100
\]

Precision was estimated by replicate determinations (n = 4) of AR homologues and their sum in a plasma quality control sample performed over a one-week period. Intra- and inter-batch coefficients of variation (CVs) were calculated. The stability of TFAA derivates of AR in undecane was tested by re-injecting a batch of samples (n = 200) after storage for two weeks at room temperature. The new modified method presented here was compared with a previous method used in our laboratory [19] by analysing 76 plasma samples with both methods. Method agreement for AR homologues was evaluated by Bland–Altman plots.

2.3.2. Determination of AR in adipose tissue samples

Specificity, the theoretical limits of detection (LOD) and quantification (LOQ) and precision were evaluated as for plasma samples. The recovery was assessed by spiking a human adipose tissue sample with three different amounts of AR homologues at final concentrations corresponding to expected low, medium and high authentic samples analyte concentrations (8–600 ng g–1 for each AR homologue in sample mass range 10–50 mg). Four replicate samples were prepared for each spiking level.

2.3.3. Determination of AR metabolites in urine

The linearity, theoretical limit of detection (LOD), limit of quantification (LOQ), recovery, and precision were assessed as described above for plasma and adipose tissue samples. An eight-point standard calibration curve (0.8–103, 0.7–88, 0.6–76, 0.7–89, 0.7–89 and 0.6–76 μmol L–1 for DHBA, DHPPA, DHCPA, DHCA, DHCA-amide and DHBA-glycine) was used to evaluate linearity. Stability of the metabolites in human urine samples was assessed by analyzing four
different standard samples corresponding to low, medium and high concentrations at four different occasions during one week.

2.4. Statistical analysis

General linear model (GLM) was used to evaluate silylation yield between different BSTFA/TMCS ratios and incubation times entered as fixed factors. Linear regression was applied to examine the stability of the AR metabolites by entering time (days) as a continuous variable and sample concentration (n = 4 levels) as a fixed factor. Minitab 16 (State College, PA, USA) was used for statistical analysis and P < 0.05 was considered statistically significant.

3. Results and discussion

Slightly different GC–MS methods were developed or modified to allow rapid and repeatable quantification of the five most common AR homologues in plasma and adipose tissue and six AR metabolites in human urine samples. The methods developed were evaluated according to the European Union decision 2002/657/EC [22]. To our knowledge, this is the first published report of an evaluated GC–MS method for determination of AR in small sample adipose tissue biopsies and for simultaneous determination of all currently reported AR metabolites in human urine.

3.1. Determination of AR in plasma

The extraction and clean-up of AR in plasma samples has been thoroughly evaluated before [19], thus these steps were applied without any changes in the current procedure. However, a new derivatization technique using TFAA was evaluated in the current method to increase sample stability, yield and thereby increase overall precision and sensitivity. A faster GC-program was also used (10 min instead 30 min). The molecular ions m/z 540 (C17:0), 568 (C19:0), 596 (C21:0), 624 (C23:0) and 652 (C25:0) and a common base ion at m/z 316 were found on analyzing TFAA-AR derivatives in a blank and in a plasma sample after whole grain wheat and rye consumption are shown in Fig. 2a and b. The molecular ions m/z 540 (C17:0), 568 (C19:0), 596 (C21:0), 624 (C23:0) and 652 (C25:0) and a common base ion at m/z 316 were selected for identification and quantification of AR in plasma based on their abundance in the reference standard mixture (Fig. 2). The AR homologues eluted within 10 min and were well separated, with no obvious interferences for either base ion or molecular ions (Figs. 1 and 2a and b). The abundance of molecular ions ranged from 1.6 to 8.3% of base ion for the different AR homologues under EI conditions (data not shown).

The method developed showed good linearity (r² > 0.99) for all AR homologues in the concentration range tested (5–500 pg μL⁻¹ for each AR homologue, corresponding plasma total AR concentrations in the range 15–1500 nmol L⁻¹). This covers most real sample conditions (geometric mean total AR is 35–41 nmol L⁻¹ in the Central European and Scandinavian countries and <23 nmol L⁻¹ in the Mediterranean countries [23]). The LOD for AR homologues was 0.04 (C17:0), 0.05 (C19:0), 0.02 (C21:0), 0.02 (C23:0) and 0.09 (C25:0) nmol L⁻¹ and LOQ was 0.13 (C17:0), 0.17 (C19:0), 0.06 (C21:0), 0.06 (C23:0) and 0.30 (C25:0) nmol L⁻¹. The intra- and inter-batch CV for determination of AR homologues in a plasma quality control sample was <10% and <15%, respectively (Table 1). Recovery was between 76 and 118% for all homologues (Table 1). Maintenance of the GC system, including cutting the column 30–40 cm at the inlet side, changing liner and septum and cleaning the ion source, was typically conducted after injection of about 200 samples, in order to ensure adequate LOD and LOQ.

Results for AR concentrations in 200 samples determined immediately and after 2 weeks at room temperature showed excellent agreement (r > 0.996, P < 0.001 for C17:0–C25:0, data not shown). This shows that TFAA-derivatised AR homologues are stable in undecane for at least two weeks. The average difference and limits of agreement (bias ± 1.96 × SD) between the previous method for determination of AR in plasma [19] and the slightly modified method presented here was 1.6 ± 4.4, −3.2 ± 9.1, 1.1 ± 14.5, −0.5 ± 6.2 and −2.2 ± 10.5 nmol L⁻¹ for C17:0, C19:0, C21:0, C23:0 and C25:0, respectively (Fig. 3). There was no significant proportional error (P > 0.05 for slope in the Bland–Altman plot). The small bias and absence of proportional error show that the two methods can be used interchangeably.

![Fig. 1. Formation of trifluoroacetic anhydride (TFAA) derivatised 5-n-alkylresorcinols and their detected fragments in electron impact ionisation (EI), R = C16H33–C24H49.](image-url)
Fig. 2. Electron impact-selected ion monitoring (EI-SIM) of the base ion (\( m/z \) 316 for C17:0, C19:0, C20:0 (internal standard), C21:0, C22:0 (internal standard), C23:0, C24:0 (internal standard), C25:0 and C26:0 (internal standard)), and the molecular ions of AR homologues (\( m/z \) 540, 568, 582, 596, 624 and 652 for C17:0, C19:0, C20:0 (internal standard), C21:0, C23:0, C25:0, respectively). (a) blank, (b) human plasma sample with total AR concentration 134.8 nmol L\(^{-1}\) and (c) human adipose tissue with total AR concentration 0.840 nmol g\(^{-1}\).

Table 1

Within- and between-batch precision for determination of alkylresorcinols (AR) in a plasma sample used as an in-house quality control sample (\( n = 4 \) per batch, 3 batches in total) and recovery (\( n = 4 \) for each spiking level) after spiking with 10, 20 and 30 ng of total AR as the sum of equal amounts of spiked C17:0, C19:0, C21:0, C23:0 and C25:0.

| AR     | Average concentration (nmol L\(^{-1}\)) | Within-batch CV (%) | Between-batch CV (%) | Recovery after spiking (%) |
|--------|----------------------------------------|---------------------|----------------------|--------------------------|
|        |                                       |                     |                      | 10 ng\(^a\)   | 20 ng\(^b\)   | 30 ng\(^c\)   |
| C17:0  | 5.7                                    | 3.3                 | 5.9                  | 110          | 109          | 110          |
| C19:0  | 17.2                                   | 1.8                 | 8.5                  | 97           | 91           | 118          |
| C21:0  | 18.8                                   | 2.6                 | 12.4                 | 90           | 94           | 110          |
| C23:0  | 6.6                                    | 3.2                 | 2.6                  | 83           | 76           | 98           |
| C25:0  | 7                                      | 7.5                 | 8.8                  | 90           | 106          | 101          |

\(^a\) Sum of spiked amount of AR C17:0–C25:0 (2 ng per homologue), corresponding to plasma total AR concentration 150 nmol L\(^{-1}\).

\(^b\) Sum of spiked amount of AR C17:0–C25:0 (4 ng per homologue), corresponding to plasma total AR concentration 300 nmol L\(^{-1}\).

\(^c\) Sum of spiked amount of AR C17:0–C25:0 (6 ng per homologue), corresponding to plasma total AR concentration 450 nmol L\(^{-1}\).

3.2. Determination of AR in adipose tissue biopsies

A typical GC–MS (EI-SIM) chromatogram of the base ion and the molecular ions of TFAA-AR derivatives in an adipose tissue sample after whole grain wheat and rye consumption is shown in Fig. 2c. Adipose tissue needle aspiration biopsy samples are typically in the range 10–50 mg and the sample amount varies between collections. Different amounts of adipose tissue sample in combination with different numbers of steel beads were therefore evaluated in order to ensure quantitative extraction, irrespective of variations in sample amount used. Different organic solvents and their mixture were previously evaluated for extraction of AR and pure diethyl ether showed the best results [19]. Determined AR content in pig adipose tissue samples (\( n = 6 \), 10.2–54.0 mg) homogenized with a random number of stainless beads (\( n = 1–6 \)) in pure diethyl ether (1 mL) was excellently correlated with the corresponding sample mass (Fig. 4), suggesting that any combination of sample amount and number of beads in the tested range could be used. In the final protocol for human adipose tissue, six steel beads were used to ensure disintegration of connective tissue, which can be abundant in some needle aspiration biopsy samples. In comparison with a previous method where a Heidolph Diax 600 homogeniser (Heidelhol, Kelheim, Germany) [15] was used for extraction of each sample, the current steel bead extraction method allowed simultaneous analysis of 30 samples. This makes the current method considerably faster and less prone to contamination errors. Heating derivatised AR in undecane resulted in improved precision for determination of all homologues (Table 2). The heating probably improved the solubility of derivatised AR homologues in undecane and this step was incorporated in the final protocol.

Internal standards with different chain lengths (C20:0, C22:0, C24:0, C26:0) were evaluated to provide a method with high precision, since the losses of AR homologues during sample preparation differed due to their diverse lipophilicity and/or polarity. The AR homologues C22:0, C24:0 and C26:0 were evaluated as internal standards. C22:0 worked well for C21:0 and C23:0; while C24:0 and
Table 2

Alkylresorcinols (AR) determined in a human adipose tissue sample without and with heat treatment after derivatisation.

| AR     | Without heating \((n=4)\) | With heating \((n=4)\) |
|--------|---------------------------|------------------------|
|        | Mean (nmol g\(^{-1}\)) | SD                     | CV, (%)       | Mean (nmol g\(^{-1}\)) | SD                     | CV, (%)       |
| C17:0  | 0.04                      | 0.009                  | 21.2          | 0.04                      | 0.003                  | 9.7           |
| C19:0  | 0.58                      | 0.075                  | 12.8          | 0.66                      | 0.009                  | 1.3           |
| C21:0  | 1.2                       | 0.103                  | 8.5           | 1.24                      | 0.047                  | 3.8           |
| C23:0  | 0.12                      | 0.025                  | 20.3          | 0.15                      | 0.005                  | 3.2           |
| C25:0  | 0.08                      | 0.022                  | 28.9          | 0.09                      | 0.003                  | 3.2           |
| Total  | 2.03                      | 0.205                  | 10.1          | 2.18                      | 0.045                  | 2.1           |

AR, alkylresorcinol; CV, coefficient of variation; SD, standard deviation.

Table 3

Within- and between-batch precision for determination of alkylresorcinols (AR) in adipose tissue sample used as an in-house quality control sample \((n=4\) per batch, 3 batches in total) and recovery \((n=4\) for each spiking level) after spiking with 2, 10 and 30 ng of total AR as the sum of equal amounts of C17:0, C19:0, C21:0, C23:0 and C25:0.

| AR     | Average Concentration (pmol g\(^{-1}\)) | Within-batch CV (%) | Between-batch CV (%) | Recovery, (%) |
|--------|-----------------------------------------|----------------------|-----------------------|---------------|
|        | 2ng\(^a\) | 10 ng\(^b\) | 30 ng\(^c\)           |               |
| C17:0  | 4.7      | 13.7      | 16.4                  | 109           |
| C19:0  | 30.7     | 4.4       | 8.3                   | 88            |
| C21:0  | 86.8     | 3.5       | 11.3                  | 61            |
| C23:0  | 53.2     | 4.4       | 14.2                  | 81            |
| C25:0  | 88.8     | 4.1       | 12.0                  | 105           |

\(^a\) Sum of spiked amount of AR C17:0–C25:0 (0.4 ng per homologue), corresponding to adipose tissue total AR concentration 40 ng g\(^{-1}\) in 10 mg of adipose tissue or 8 ng g\(^{-1}\) in 50 mg of adipose tissue.

\(^b\) Sum of spiked amount of AR C17:0–C25:0 (2 ng per homologue), corresponding to adipose tissue total AR concentration of 200 ng g\(^{-1}\) in 10 mg of adipose tissue or 40 ng g\(^{-1}\) in 50 mg of adipose tissue.

\(^c\) Sum of spiked amount of AR C17:0–C25:0 (6 ng per homologue), corresponding to adipose tissue total AR concentration of 600 ng g\(^{-1}\) in 10 mg of adipose tissue or 120 ng g\(^{-1}\) in 50 mg of adipose tissue.

C26:0 worked well for C25:0. However, C24:0 was chosen, since it showed higher S/N ratio than C26:0.

The LOD for AR homologues in adipose tissue was 0.04 (C17:0), 0.05 (C19:0), 0.02 (C21:0), 0.02 (C23:0) and 0.09 (C25:0) fmol injection\(^{-1}\) and LOQ was 0.12 (C17:0), 0.16 (C19:0), 0.08 (C21:0), 0.07 (C23:0) and 0.31 (C25:0) fmol injection\(^{-1}\). Recovery was between 81 and 109% for all homologues, except for C21:0 at low spiking concentration (Table 3). Intra- and inter-day AR homologue CV was below 5% and 15%, respectively, except for homologue C17:0, where the corresponding CV was 13.7 and 16.4%, respectively (Table 3). The high inter-batch CV for C17:0 is probably due to the low concentration of C17:0 in the human tissue sample used for method evaluation.

3.3. Determination of AR metabolites in urine

A slightly modified protocol based on a previous method [21] was developed to allow accurate quantification of four novel AR metabolites recently identified (DHPPTA, DHBA-glycine, DHCA and DHCA-amide) along with DHBA and DHPPA in urine samples [17,18]. Enzymatic hydrolysis, extraction and SPE of DHBA and DHPPA have been optimized previously [21], and similar conditions were applied in the present method. However to evaluate the most suitable hydrolysis procedure in order to ensure complete hydrolysis for the newly identified AR metabolites, five levels of enzymes were tested. The importance of the de-conjugation step...
Table 4
Concentrations of AR metabolites (μmol L⁻¹) in a urine sample (n = 3), without and with deconjugation. CV represents the within-batch coefficient of variation (n = 3).

| Metabolite   | Concentration after deconjugation, μmol L⁻¹ (CV, %) | Concentration without deconjugation, μmol L⁻¹ (CV, %) |
|--------------|-----------------------------------------------------|-----------------------------------------------------|
| DHBA         | 80.8 (8.0)                                          | 44.3 (8.2)                                          |
| DHPPA        | 220.8 (8.7)                                         | 68.4 (7.2)                                          |
| DHPPTA       | 17.8 (12.2)                                         | 2.2 (13.4)                                          |
| DHCA         | 95.6 (7.2)                                          | 43.8 (8.3)                                          |
| DHCA-amide   | 254.0 (14.3)                                        | 22.8 (12.5)                                        |
| DHBA-glycine | 271.0 (13.3)                                        | 300.1 (14.2)                                       |

was confirmed for DHPPTA, DHCA and DHCA-amide by comparing urine concentrations before and after deconjugation (Table 4). The amount of enzyme optimized for deconjugation of DHBA and DHPPA [21] was also found to be suitable for the new metabolites by testing four different levels (data not shown). Deconjugation did not affect DHBA-glycine concentration, which shows that this molecule is not sulphated or glucuronidated. The obtained results demonstrated that DHPPTA, DHCA and DHCA-amide are excreted in urine in free form and as conjugates, as shown previously for DHBA and DHPPA [21,24,25]. Similar extraction and SPE conditions as for the previous method [21] were used.

The efficiency of derivatisation of OH- groups and the COOH-group of AR metabolites may differ between metabolites [26]. Therefore we optimized the derivatisation procedure to ensure appropriate derivatization of the novel AR metabolites. The results showed, that when comparing derivatization conditions, i.e., the proportion of BSTFA/TMCS in the silylation mixture and the duration of the reaction, significantly larger peak areas were obtained for DHCA (P < 0.009) and DHPPTA (P < 0.01) when using 70:30 BSTFA/TMCS, whereas no significant difference was found for DHCA-amide (P < 0.383) and DHBA-glycine (P < 0.347). However, the differences were ≤ 20% and since a BSTFA/TMCS ratio of 70:30 would cause more extensive contamination of the GC–MS instrument and cause problems with the injection syringe, the ratio 99:1 was used as before for DHBA and DHPPA [16,21]. Moreover, there was no significant effect of silylation time, suggesting that 70 min, which is 10 min longer compare to previous method, was sufficient for complete silylation of all metabolites. When analysing several batches, the derivatisation step should preferably be conducted for all batches at the same time, in order to obtain satisfactory precision (Table 6) whereas silylation at different occasions give higher CVs (data not shown).

Fig. 5. Total ion chromatogram (1) and selected ion monitoring (SIM) chromatograms for molecular ions from DHBA (2), SA (3), DHPPA (4), DHPPTA (5), DHCA (6), DHCA-amide (7) and DHBA-glycine (8) in (A) standard solution and (B) a blank urine sample treated according to the experimental protocol.
A GC–MS (EI-SIM) chromatogram of the profile of AR metabolites in a standard solution and in a typical urine sample after whole grain wheat and rye intake is shown in Fig. 5. The current method has been modified in order to obtain faster run times, good separation and adequate precision for simultaneous quantification of already reported and novel AR metabolites. After optimization of the temperature programme, all metabolites were well separated within 7 min. This is 9 min faster than in the previ-
ous method for determination of DHBA and DHPPA [21]. Silylated derivatives of DHBA, DHPPA, DHPPTA, DHCA, DHCA-amide and DHBA-glycine in human urine samples were identified by comparing their retention times and EI spectra with those of standard derivatives of DHBA, DHPPA, DHPPTA, DHCA, DHCA-amide andous method for determination of AR metabolites in three different human urine samples (n = 3).a

| Metabolite  | Sample | Batch 1 Mean (μmol L⁻¹) | CV (%) | Batch 2 Mean (μmol L⁻¹) | CV (%) | Batch 3 Mean (μmol L⁻¹) | CV (%) |
|-------------|--------|-------------------------|--------|-------------------------|--------|-------------------------|--------|
| DHBA        | 1      | 35.5                    | 6.1    | 42.3                    | 6.6    | 54.3                    | 3.7    |
|             | 2      | 7.9                     | 7.8    | 10.1                    | 2.6    | 7.5                     | 18.5   |
|             | 3      | 30.1                    | 12.7   | 28.3                    | 26.8   | 29.8                    | 25.7   |
| DHPPA       | 1      | 76.1                    | 2.7    | 74.1                    | 3.1    | 77.4                    | 5.3    |
|             | 2      | 21.3                    | 2.5    | 22.7                    | 2.6    | 14.3                    | 21.2   |
|             | 3      | 112.2                   | 3.5    | 117.9                   | 4.9    | 116.6                   | 3.4    |
| DHCA        | 1      | 20.5                    | 5.1    | 19.2                    | 2.7    | 21.5                    | 8.3    |
|             | 2      | 5.1                     | 6.7    | 5.2                     | 1.4    | 3.7                     | 8.8    |
|             | 3      | 19.9                    | 2.7    | 23.6                    | 9.2    | 23.1                    | 7.2    |
| DHPPTA      | 1      | 4.3                     | 6.1    | 3.9                     | 4.26   | 4.3                     | 7.0    |
|             | 2      | 0.6                     | 14.4   | 0.6                     | 18.6   | 0.2*                    | 4.5    |
|             | 3      | 7.2                     | 5.8    | 7.6                     | 6.9    | 7.1                     | 3.5    |
| DHCA-amide  | 1      | 41.8                    | 7.2    | 44.3                    | 3.1    | 44.4                    | 8.1    |
|             | 2      | 54.5                    | 12.7   | 55.5                    | 13.9   | 45.7                    | 5.4    |
|             | 3      | 57.0                    | 7.1    | 64.7                    | 3.4    | 63.8                    | 13.8   |
| DHBA-glycine| 1      | 33.9                    | 8.7    | 37.7                    | 3.2    | 33.6                    | 12.3   |
|             | 2      | 7.1                     | 9.4    | 7.3                     | 6.6    | 4.4                     | 22.9   |
|             | 3      | 25.3                    | 4.1    | 26.6                    | 16.9   | 25.8                    | 16.4   |

*Error during the sample preparation, value not included in estimation of inter-batch CV.

† Mean concentration (μmol L⁻¹) between days.

CV, coefficient of variation between days.

4. Conclusions

High-throughput GC–MS methods for quantification of AR homologues C17:0–C25:0 in human plasma and adipose tissue and all currently reported AR metabolites (DHBA, DHPPA, DHPPTA, DHBA-glycine, DHCA and DHCA-amide) in human urine were developed and evaluated. All methods showed good sensitivity, accuracy and precision. Methods for determination of AR in plasma and adipose tissue samples also had considerably shorter GC–MS run-time, considerably higher sample extraction through put (adipose tissue), improved precision and lower LOD/LOQ than previous methods. In summary, these methods could be used for assessment of whole grain wheat and rye intake biomarkers in large-scale epidemiological studies.

Author contributions

HW conducted laboratory work, data analysis and drafted the sections about AR in plasma and adipose tissue. RW conducted laboratory work, data analysis and drafted the sections about AR metabolites in urine. HW and RW contributed equally to the work presented in this manuscript and therefore share first authorship. MF and AK-E supervised the manuscript preparation. RL conceived the study and supervised the sample and data analysis and manuscript preparation. All authors contributed to interpretation of the data and approved the final content.

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