Simplified Method for the Measurement of Plasma Alkylresorcinols: Biomarker of Whole Grain Intake

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Short title: Simplified method to measure plasma alkylresorcinols

Abbreviations: AR: Alkylresorcinol; BMI: Body mass index; CVD: Cardiovascular disease; LDL: Low density lipoprotein; Q-TOF/MS: Quadrupole time-of-flight mass spectrometer; UHPLC: Ultra-high performance liquid chromatography.
Abstract

Rationale: Consumption of whole-grains is negatively associated with cardiovascular disease (CVD) risk but quantification of whole-grain intake is challenging. Alkylresorcinols (ARs) are biomarkers of whole-grain intake. Current methods for AR quantification involve a time consuming multi-step separation process that hampers applicability in large-scale studies.

Methods: We report a streamlined method to quantify ARs in human plasma based on protein precipitation and direct injection into an ultra-high performance liquid chromatography coupled to atmospheric pressure chemical ionization operating in negative ionization mode and using a quadrupole time-of-flight mass spectrometer.

Results: Separation of 5 major ARs was achieved, with linearity in the 5 to 550 nmol/L range and a lower limit of detection of 0.5 nmol/L (LOD) and quantification (LOQ) of 5 nmol/L. Within-run and between-run precision and accuracy were below 15%, and recoveries above 90%. Once validated, the method was applied by measuring plasma ARs concentrations of subjects who participated in a randomized, cross-over trial evaluating the effect of carbohydrate-type on CVD risk factors. The unrefined-carbohydrate diet with the highest fiber content resulted in the highest plasma AR concentration (93±78 nmol/L), and was significantly different (p<0.01) from the other lower fiber diets (18±26 nmol/L and 19±26 nmol/L, simple and unrefined-carbohydrate, respectively).

Conclusions: This method offers a simplified approach to measure plasma ARs concentrations as an objective biomarker of whole-grain intake that can be applied to large-scale cohort studies.

Keywords:
Alkylresorcinols; Biomarkers; Liquid chromatography; Randomized clinical trial; Whole-grains.
1. Introduction

The consumption of whole grains is associated with improvements in cardiometabolic risk factors and is inversely associated with cardiovascular disease and cancer risk. According to a recent World Health Organization report on Healthy Diet, the majority of people do not eat the recommended amounts of fruit, vegetables and fiber-containing foods such as whole grains. The estimation of whole grain intake is one of the major challenges encountered in both epidemiological studies that assess whole grain-related health benefits and the health claims based on these studies. The accurate measurement of whole grain intake is challenging due to differences in the definition and interpretation of the terms “whole grain” and “whole grain foods”, the diversity among whole grains and whole grain products, the limitations of self-reported intake questionnaires (including under- and overestimate food intakes), and limited accuracy of current food composition tables.

One way to overcome these challenges is by measuring an objective biomarker of compliance (in the case of intervention studies) and/or intake (in the case of free-living population studies). Alkylresorcinols (AR) are a group of phenolic lipid compounds that contain a resorcinol (benzene ring with two hydroxyl groups in positions 1 and 3) and an odd-numbered alkyl chain (varying from 17 to 25 carbons long) at position 5. They are abundant in the outer layer and bran fractions of wheat, rye and barley grains and generally not present in refined grain products, although small amounts of AR can be present in refined grains due to contamination of bran parts. Additionally, minor AR with monoenoic, dienoic, and/or oxygenated side-chains have been described in cereal grains. Because ARs are absorbed and can be measured in plasma they are considered objective biomarkers of whole grain wheat, barley and rye intake. A moderate correlation between self-reported whole grain wheat and rye intake and plasma AR concentrations has been previously documented. Recently, even-numbered alkylresorcinols have been found in quinoa and have been suggested as potential biomarkers of quinoa intake. Several analytical methods are available for the analysis of AR in plasma. These methods are based on either gas chromatography coupled to mass spectrometry (GC-MS) or liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS). Current methods for ARs quantification involve a multi-step separation, extraction and purification processes that are time-consuming, limiting applicability in large-cohort studies. Sample preparation prior to GC-MS analysis involves a triplicate liquid-liquid extraction with
diethyl ether, solid-phase extraction, and chemical derivatization. In an attempt to simplify the sample preparation process, some LC/MS/MS methods have been developed that eliminate the solid-phase extraction or use a hybrid solid-phase extraction. With the exception of a method that does not separate ARs prior to analysis, chromatographic methods for the analysis of ARs in plasma typically have long run times (between 10 and 20 minutes per injection) and require a sample volume of 50 to 500 µL of plasma.

Our aim was to develop a rapid and simple method for the measurement of ARs in small volumes of plasma that requires a relatively short preparation and run time, hence, would be cost effective when applied to large cohort studies. The method developed is based on UHPLC/Q-TOF-MS and was validated using plasma samples from a controlled human intervention trial that evaluated the effect of diets that differed in carbohydrate type; simple, refined, or unrefined carbohydrate.

2. Materials and methods

2.1. Chemicals and reagents

5-Heptadecylresorcinol (AR C17:0; >95%), 5-nonadecylresorcinol (AR C19:0), 5-nonadecylresorcinol-D₄ (AR C19:0-D₄; >98%), 5-heneicosylresorcinol (AR C21:0; >98%), 5-heneicosylresorcinol-D₄ (AR C21:0-D₄; >98%), 5-tricosylresorcinol (AR C23:0), 5-tricosylresorcinol-D₄ (AR C23:0-D₄; >98%), 5-pentacosylresorcinol (AR C25:0), and 5-pentacosylresorcinol-D₄ (AR C25:0-D₄; >98%) were purchased from ReseaChem GmbH (Burgdorf, Switzerland). HPLC grade methanol and water were purchased from J.T.Baker (Center Valley, PA, USA). HPLC grade dichloromethane was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Standards and solutions

Stock solutions of all ARs (1 mg/mL) were prepared by weighing 10 mg of powdered standard and dissolving them in 10 mL of methanol. Gentle sonication was needed to achieve complete dissolution of AR C23:0 and AR C25:0 due to their high lipophilicity. Working solutions were prepared by serial dilutions with methanol in a concentration range from 5 to 550 nmol/L. The ISTD working solution (containing a mixture of AR C19:0-D₄, AR C21:0-D₄, AR C23:0-D₄ and
AR C25:0-D₄) was prepared at a concentration of 65 nmol/L by carrying out serial dilutions with methanol.

2.3. Sample preparation and extraction

A dilute and shoot strategy was developed to analyze the 5 predominant ARs in human plasma samples. An aliquot of 20 μL of plasma was spiked with 20 μL of internal standard solution (containing a mixture of AR C19:0-D₄, AR C21:0-D₄, AR C23:0-D₄ and AR C25:0-D₄ at 65 nmol/L) and diluted with 160 μL of methanol. After vortexing and centrifugation (5 min; 3500 g; RT) the supernatant was transferred into an Agilent autosampler vial and directly injected into the UHPLC/Q-TOF-MS system. A detailed description of the chromatographic and mass spectrometric conditions is detailed below.

2.4. Liquid chromatography and mass spectrometry conditions

An Agilent 1290 Infinity LC system with a cooled autosampler, quaternary pump, and heated column compartment was interfaced with an Agilent 6550 QTOF with an ion funnel and dual APCI and APPI sources (Agilent Technologies, Santa Clara, CA, USA). Liquid chromatographic separation of AR was performed using a ZORBAX RRHD Eclipse Plus C18 column: 2.1 x 50mm, 1.8 μm, 1200 bar (Agilent Technologies, Santa Clara, CA, USA), at a flow rate of 0.5 mL/min.

The mobile phases used were A: methanol, B: water, and C: 10% of dichloromethane in methanol (the latter used as a wash between runs to elute retained compounds in the column). The initial proportion was 95% A and 5% B, and it was increased up to 100% A over 2 minutes and kept at 100% A for an additional 2 minutes. Then the column was flushed with 50% A and 50% C for 6 minutes, and with 100% A for 3 minutes, then returned to initial conditions (95%A/5%B). The total run time was 15 minutes per sample. The injection volume was 20 μL. Atmospheric pressure chemical ionization (APCI) was selected, and the following settings were applied: gas temperature, 290 °C; vaporizer temperature 500 °C, nebulizer pressure, 60 psig; capillary voltage, 3800 V; fragmentor voltage, 365 V; skimmer voltage, 65 V; and corona voltage, 20V. Nitrogen was used as the nebulizing gas. The gas flow rate was set at 14 L/min. Targeted mass spectra were acquired in negative ion mode over an m/z range of 50-3200 without applying any collision energy and with an MS scan rate of 1 spectra/sec. The column
temperature was set to 30°C and the samples to 4°C. The peak areas under the chromatograms and isotope ratios were calculated automatically using the Agilent MassHunter software system. In order to obtain robust performance, the Agilent APCI needle, the APCI sprayer, the entrance to the QTOF, and the ion source drain area were cleaned daily with methanol and an optical grade fiber optic cleaning wipe (Kimwipes, Fisher Scientific). This step is highly recommended especially when analyzing large number of samples.

2.5. Method validation

The following characteristics of the method were evaluated: linearity of the calibration curves, lower limit of quantification, within- and between-run accuracy, and within- and between-run precision.

In order to evaluate the linearity of the method, calibration standards with a mixture of the 5 AR (AR C17:0, AR C19:0, AR C21:0, AR C23:0; AR C25:0) the analytes were prepared in methanol at 7 concentrations (5, 12, 25, 62, 125, 250 and 550 nmol/L). Calibration curves with their corresponding slope (s), intercept, correlation coefficient (r), and coefficient of determination ($r^2$) were calculated by weighting (1/x) least squares linear regression of the peak area ratio (analyte/internal standard) versus the concentration of the standards using SPSS for Windows, (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY, USA). The method was considered linear in the selected range if a correlation of determination ($r^2$) greater than 0.98 was obtained and if the concentration residuals were ≤20%. ARs were identified on the basis of their molecular formula, retention time and mass using Agilent MassHunter Qualitative Analysis software and quantified with the use of the respective standard curves. Concentration residuals (%) were calculated as follows: (interpolated concentration–nominal concentration)*100/interpolated concentration.

The lower limit of detection (LOD) was calculated using an empirical approach consisting of measuring progressively more dilute concentrations of the AR (concentration range 0.1 to 540 nmol/L). Additionally, it was confirmed that the LOD was at least 3 times the signal-to-noise ratio. The lower limit of quantification (LOQ) was the lowest calibration standard used in the calibration curve that presented acceptable accuracy and precision (see below).
Quality control (QC) samples were prepared in order to evaluate the precision and accuracy of the proposed method. Known amounts of the five ARs (AR C17:0, AR C19:0, AR C21:0, AR C23:0, and AR C25:0) were spiked into pooled human plasma samples to achieve three different AR concentrations: low QC (5 nmol/L), medium QC (50 nmol/L) and high QC (525 nmol/L). Three replicates of each QC (low, medium, and high) were analyzed against the calibration curve, and the same experiments were performed on three different days to assess between-run accuracy and precision.

The accuracy of the method was calculated using the replicates of the QC samples by dividing the calculated concentration (estimated from the interpolation of the calibration curve) by the known concentration of the corresponding QC, and expressed as a percentage. Nine replicates of spiked plasma samples were analyzed on three different days. Within-run accuracy was calculated as the average of the individual accuracy values that were analyzed within one day (first validation assay). Within-run precision was calculated as the coefficient of variation (standard deviation/mean) of the spiked plasma samples that were analyzed on the first validation day. Between-run accuracy of the method was calculated as the average of the nine individual accuracy values of the nine plasma samples analyzed over the three validation days. Between-run precision was calculated using the mean and standard deviation of the means for each of the 3 days, i.e. the standard deviation of the three mean values, and it was expressed as coefficient of variation (standard deviation/mean*100). The method was considered accurate and precise if accuracies were in the range 85–115% and the coefficients of variation were below 15%.

The recovery efficiencies for each AR homologue were calculated by comparing analytical results from pooled QC plasma samples spiked with the AR standard mixture (deuterated and non-deuterated) before and after extraction using 3 different concentrations (5 nmol/L, 50 nmol/L and 525 nmol/L) over the calibration range. This experiment was performed in triplicate.

2.6. Method application: Subjects and Study Design

Archived plasma samples from a controlled human intervention trial were used for method validation. Briefly, participants [postmenopausal women and men (N = 11, 65 ± 8 years, BMI 29.8 ± 3.2 kg/m², LDL-C ≥2.6 mmol/L)] consumed each of 3 isocaloric diets (60% energy from carbohydrate, 15% energy from protein, 25% energy from fat) enriched in either simple, refined,
or unrefined carbohydrate-containing foods for 4.5 weeks in a randomized crossover design, with 2-week washout periods. The simple carbohydrate–enriched diet included a high proportion of foods made with sucrose or high-fructose corn syrup. The refined carbohydrate–enriched diet included a high proportion of foods made with white rice (milled rice that has had its husk, bran, and germ removed), white bread and white pasta (both made using white flour containing only the endosperm portion of wheat after removal of bran and germ). The unrefined carbohydrate–enriched diet included foods similar to the refined carbohydrate diet but made with whole grains and whole wheat flour (includes the bran, endosperm, and germ of the wheat grain). All foods and beverages were provided to study participants. Participants visited the Metabolic Research Unit three times per week and were provided with one meal for consumption on-site and additional meals for consumption off-site. The study was conducted in accordance with the Declaration of Helsinki guidelines. All procedures were approved by the Institutional Review Board of Tufts University/Tufts Medical Center and written informed consent was obtained from the study participants. The trial was registered at clinicaltrials.gov as NCT01610661 on 7 November 2011. The study was conducted between 2012 and 2015.

2.7. Data treatment and statistical analysis

Data analysis was performed using the Agilent MassHunter Qualitative Analysis software package (Agilent Technologies, Santa Clara, CA, USA). Targeted identification and integration of the compounds of interest was completed by using the algorithm “Find Compounds by Formula” included in the software package. MassHunter PCDL (Personal Compound Database and Library manager; Agilent Technologies, Santa Clara, CA, USA) was used to create a library of compounds of interest for the identification of 5 ARs as well as the internal standards. Data were exported into Excel and further processed using SPSS for Windows (version 25.0) and GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA). Data were tested for normality (Shapiro-Wilk test) before statistical analysis. A repeated measures ANOVA model was used to test the differences in AR concentrations between diet phases. The Tukey-Kramer method was used for post hoc analyses.

3. Results

3.1. Chromatographic separation
Five ARs were clearly resolved using a ZORBAX RRHD Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm; Figure 2). Of note, we initially used an isocratic method (100% methanol), which allowed the separation of the 5 ARs within 6 minutes but, the elution of the more polar analyte (AR C17:0) was close to the solvent front. Starting with a gradient using 5% water and 95% methanol allowed a better separation of the 5 ARs in a 15 minute window (Figure 2).

3.2. Ionization method and mass spectrometric conditions

APCI was used as the ionization method because of the low polarity of the AR and less sensitivity to matrix effects than electrospray. Amongst the different ionization parameters, the vaporizer temperature was found to be of special relevance. Since a gradient with 5% water was employed at the beginning of the run, a vaporizer temperature of 500°C was needed to facilitate the evaporation in the ion source, thereby bringing both the mobile phase and analytes into the gas phase. The use of lower temperatures hindered the evaporation resulting in lower signal intensity of the first two eluting compounds (AR C17:0 and AR C19:0).

Quadruple-time-of-flight mass spectrometry (Q-TOF-MS) was chosen because it provides high mass accuracy, good isotope ratio fidelity, and full-scan sensitivity over a wide mass of range. Table 1 shows the resulting AR library.

Identification of compounds was achieved with pure standards, which were used to determine the retention time and ionization behavior and to verify the exact mass and the isotopic abundance and pattern (i.e. isotope peak ratios). All the ARs studied had a similar behavior and generated 3 characteristic ions corresponding to deprotonated monoisotopic peak and their corresponding most abundant $^{13}$C isotopes. A representative chromatogram illustrates the mass analysis (counts versus mass-to-charge ratio) of AR C25:0 which contains an ion cluster containing the monoisotopic peak $[\text{M-H}]^-$ with only $^{12}$C (459.4), and the stable isotopes with a single $^{13}$C (460.4) and with two $^{13}$C (461.4) (Figure 3).

3.3. Method validation

The method was found to be linear for the five AR within the range of 5 and 550 nmol/L and satisfactory determination coefficients ($r^2 > 0.98$) were obtained for the 5 AR (Table 2).
The lower LODs were 0.6 nmol/L for AR C17:0; 0.5 nmol for AR C19:0, AR C21:0, and AR C23:0; and 0.4 nmol/L for AR C25:0. The lower LOQs were 5.7 nmol/L for AR C17:0; 5.3 nmol/L for AR C19:0; 4.9 nmol/L for AR C21:0; 4.6 nmol/L for AR C23:0; and 4.3 nmol/L for AR C25:0.

Both within- and between-run accuracies were commonly in the range 99–115% (Table 2) for all analytes. The method was also found to be precise, as within-run and between-run assays had coefficients of variation (CV) below 12% for all AR in plasma. These data indicate complete extraction, minimal losses, good alignment between spiking and calibration solution and also the analytical system. The dilute-and-shoot strategy does not require an extraction step, thus eliminating the need to evaluate the recovery of the analytes.

It is worth mentioning that, in order to obtain acceptable values of within- and between-run accuracy for AR C23:0 and AR C25:0, the use of their corresponding deuterated internal standards (AR C23:0-D₄ and AR C25:0-D₄) was required. In our first validation approach, we employed AR C19:0-D₄ as internal standard for all five AR. While AR C17:0, AR C19:0, and ARC21:0 have acceptable values of accuracy and precision, AR C23:0 and ARC25:0 had unacceptable accuracy values (>120%) (data not shown). When the corresponding deuterated compounds were used as internal standards, the accuracy and precision improved and met the acceptance criteria (Table 2).

Extraction recovery using this dilute and shoot method was above 90% for all AR homologues (and their corresponding internal standards). This high recovery was observed at low (5 nmol/L), medium (50 nmol/L), and high concentrations (525 nmol/L) (Table 3).

3.4. Method application

To validate the analytical application of the method, we measured AR in plasma from 11 subjects who participated in a randomized, cross-over trial evaluating the effect of carbohydrate quality on cardiovascular disease risk factors. Fasting plasma total AR concentrations were significantly higher after consumption of the unrefined diet (93±78 nmol/L), compared to the simple (18±26 nmol/L) and refined diets (19±26 nmol/L) (Figure 4). AR C17:0 and AR C23:0 were present at lower concentrations whereas AR C21:0 and AR C19:0 were responsive to the dietary intervention. The observed increase in AR plasma concentrations following consumption
of the unrefined carbohydrate diet is consistent with the higher intake of predominantly whole-wheat containing foods provided to the study participants.

4. Discussion

Over the last two decades, ARs have received increasing attention as potential biomarkers of whole grain wheat, rye and barley intake \(^{25}\). Current methods for the measurement of ARs in human plasma are based on GC/MS or LC/MS and require time-consuming sample preparation, a fact that hampers its applicability in large-scale studies \(^{20-23}\). In this work, we developed a simple, direct, and rapid method for the measurement of five major ARs in human plasma using UHPLC/QTOF/MS. Application of this method to a randomized crossover nutritional intervention study indicated that plasma ARs were higher after consumption of a diet enriched in whole grains, compared to a diet enriched in simple sugar or refined grains.

The development of analytical detectors with high specificity and sensitivity, together with the use of four isotopically labeled internal standards, has enabled the possibility of reducing or avoiding some steps of the sample preparation. Our method bypassed the extraction step (liquid-liquid extraction and/or solid-liquid extraction) prior to sample analysis using a strategy known as dilute-and-shoot \(^{27,28}\). This method represents an important reduction of consumables, reagents, and labor and overcomes low extraction recoveries. The use of APCI provided good ionization for the non-polar ARs, and the coupling of UHPLC with a QTOF/MS detector allowed for accurate identification of ARs. When this method was applied to a human clinical trial of carbohydrate type, total ARs plasma concentrations were higher after subjects consumed the unrefined diet, rich in whole grains, compared to the simple and refined carbohydrate diet, both low in fiber. Of note, AR C19:0 and AR C21:0 were the major plasma ARs detected and were also those that increased the most after the unrefined-carbohydrate diet. The increase could also be observed, although to a lower extent, in AR C25:0, AR C23:0 and AR C17:0. These concentrations were also in a similar range to previous reports that measured ARs by GC-MS \(^{29-31}\). It has been documented that the dominant ARs in wheat are AR C19:0 and AR C21:0, in barley is AR C25:0, and rye are AR C17:0, ARC19:0 and AR C21:0 \(^{32}\). Our plasma AR results reflect the main food sources of whole grains, wheat (whole wheat muffins and rolls), with smaller amounts from barley and brown rice. These foods were chosen since they reflect food
sources of whole grains typically consumed in the U.S, unlike European populations with higher rye consumption.

Despite the significant advantages of the proposed method in terms of sample volume, sample preparation and run time, some limitations must also be acknowledged. Other methods can reach lower limits of detection using a triple quadrupole, but we consider that the current method is sufficiently sensitive for the purpose described: primarily measuring plasma alkylresorcinols as a biomarker of whole-grain intake. Additionally, we acknowledge decreased sensitivity in the quantification of minor ARs in the diet: specifically AR C17:0 and AR C23:0. While other methods use a single internal standard (typically AR C19:0-D<sub>4</sub>), our method requires the additional use of AR C23:0-D<sub>4</sub> and AR C25:0-D<sub>4</sub> in order to provide an accurate quantification of these compounds. Finally while plasma ARs reflected wheat containing sources of whole grains in our study, and rye-based foods in others<sup>15,33</sup>, plasma ARs will be of limited value as a biomarker of whole grain intake in populations with high intake of other grain species that do not contain ARs, such as brown rice or oats<sup>34</sup>.

5. Conclusion
This simplified method, which is the first four isotopically labeled internal standards, offers a direct and rapid strategy to measure ARs as a biomarker of whole grain intake, using small amounts of plasma and can be scaled up for large studies.

Acknowledgments
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Clinical Trial Information: ClinicalTrials.gov no. NCT01610661 (registered 7 November
Disclosure Summary: The authors have no disclosures.
References


Tables

**Table 1.** Library created to search for alkylresorcinols (AR) in human plasma samples.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>Exact Molecular mass</th>
<th>Retention time (min)</th>
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<tr>
<td>AR C17:0</td>
<td>C_{23}H_{40}O_{2}</td>
<td>348.3028</td>
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<td>376.3341</td>
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<td>AR C19:0-D_{4}</td>
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<td>380.3592</td>
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<td>C_{27}H_{48}O_{2}</td>
<td>404.3654</td>
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<td>AR C21:0-D_{4}</td>
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<td>408.3905</td>
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<td>C_{31}H_{52}D_{4}O_{2}</td>
<td>464.4531</td>
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Table 2. Linearity range, within- and between-run accuracy and precision for 5 major alkylresorcinols (AR) in human plasma. CV: Coefficient of variation; QC: Quality Control; Low QC: 5 nmol/L; Medium QC: 50 nmol/L; High QC: 525 nmol/L.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Internal standard</th>
<th>Range (nmol/L)</th>
<th>$r^2$</th>
<th>Within-run accuracy (%)</th>
<th>Between-run accuracy (%)</th>
<th>Within-run error (%CV)</th>
<th>Between-run error (%CV)</th>
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<tr>
<td>AR C17:0</td>
<td>AR C19:0-D$_4$</td>
<td>5 to 550</td>
<td>0.995</td>
<td>100% 100% 99%</td>
<td>106% 103% 105%</td>
<td>2% 2% 2%</td>
<td>6% 3% 5%</td>
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<td>AR C19:0</td>
<td></td>
<td></td>
<td>0.997</td>
<td>102% 105% 100%</td>
<td>104% 100% 108%</td>
<td>1% 12% 1%</td>
<td>6% 5% 8%</td>
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<tr>
<td>AR C21:0</td>
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<td>0.998</td>
<td>109% 103% 103%</td>
<td>109% 101% 109%</td>
<td>7% 7% 8%</td>
<td>1% 2% 5%</td>
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<tr>
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<td>113% 108% 107%</td>
<td>3% 1% 3%</td>
<td>1% 6% 7%</td>
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<tr>
<td>AR C25:0</td>
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<td>0.987</td>
<td>110% 103% 102%</td>
<td>105% 106% 106%</td>
<td>5% 6% 5%</td>
<td>5% 7% 7%</td>
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</table>
**Table 3.** Percent recovery (%) of non-deuterated and deuterated alkylresorcinols (AR) in pooled plasma samples. This experiment was performed in triplicate.

<table>
<thead>
<tr>
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<th>Non-deuterated standards</th>
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<tr>
<td></td>
<td>AR C17:0</td>
<td>AR C19:0</td>
<td>AR C21:0</td>
<td>AR C23:0</td>
<td>AR C25:0</td>
</tr>
<tr>
<td>5 nmol/L</td>
<td>91%</td>
<td>93%</td>
<td>91%</td>
<td>101%</td>
<td>95%</td>
</tr>
<tr>
<td>50 nmol/L</td>
<td>105%</td>
<td>105%</td>
<td>107%</td>
<td>118%</td>
<td>109%</td>
</tr>
<tr>
<td>525 nnol/L</td>
<td>114%</td>
<td>104%</td>
<td>114%</td>
<td>106%</td>
<td>105%</td>
</tr>
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<table>
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<th>Deuterated internal standards</th>
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<tr>
<td></td>
<td>AR C17:0</td>
<td>AR C19:0-D₄</td>
<td>AR C21:0-D₄</td>
<td>AR C23:0-D₄</td>
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<tr>
<td>5 nmol/L</td>
<td>-</td>
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<td>93%</td>
<td>93%</td>
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<tr>
<td>50 nmol/L</td>
<td>-</td>
<td>116%</td>
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</tr>
<tr>
<td>525 nnol/L</td>
<td>-</td>
<td>106%</td>
<td>98%</td>
<td>108%</td>
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