Analytical Biochemistry 499 (2016) 1–7

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A high-throughput method for liquid chromatography–tandem mass spectrometry determination of plasma alkylresorcinols, biomarkers of whole grain wheat and rye intake

Alastair B. Ross [a, *], Cecilia Svelander [a], Otto I. Savolainen [a], Mads Vendelbo Lind [a, b], John P. Kirwan [c], Isabelle Breton [d], Jean-Philippe Godin [d], Ann-Sofie Sandberg [a]

[a] Division of Food and Nutrition Science, Department of Biology and Biological Engineering, Chalmers University of Technology, 412 96 Gothenburg, Sweden
[b] Department of Nutrition, Exercise, and Sports, Faculty of Science, University of Copenhagen, 1870 Frederiksberg, Denmark
[c] Department of Pathobiology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, USA
[d] Nestlé Research Centre, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

ARTICLE INFO

Article history:
Received 20 July 2015
Received in revised form
24 November 2015
Accepted 30 December 2015
Available online 28 January 2016

Keywords:
Alkylresorcinols
Biomarkers
Blood plasma
Whole grain
Liquid chromatography–tandem mass spectrometry

ABSTRACT

Plasma alkylresorcinols are increasingly analyzed in cohort studies to improve estimates of whole grain intake and their relationship with disease incidence. Current methods require large volumes of solvent (>10 ml/sample) and have relatively low daily sample throughput. We tested five different supported extraction methods for extracting alkylresorcinols from plasma and improved a normal-phase liquid chromatography coupled to a tandem mass spectrometer method to reduce sample analysis time. The method was validated and compared with gas chromatography–mass spectrometry analysis. Sample preparation with HybridSPE supported extraction was most effective for alkylresorcinol extraction, with recoveries of 77–82% from 100 μl of plasma. The use of 96-well plates allowed extraction of 160 samples per day. Using a 5-cm NH_2 column and heptane reduced run times to 3 min. The new method had a limit of detection and limit of quantification equivalent to 1.1–1.8 nmol/L and 3.5–6.1 nmol/L plasma, respectively, for the different alkylresorcinol homologues. Accuracy was 93–105%, and intra- and inter-batch precision values were 4–18% across different plasma concentrations. This method makes it possible to quantify plasma alkylresorcinols in 100 μl of plasma at a rate of at least 160 samples per day without the need for large volumes of organic solvents.

High intake of whole grains has been frequently associated with reduced risk of some types of cancers, in particular cancers of the gastrointestinal tract [1]. However, estimating intake of whole grains in population studies is difficult due to their diversity in both type and products in which they can be found [2]. Using biomarkers to improve estimates of whole grain intake is recommended [2], and recently the combination of plasma alkylresorcinols and estimated whole grain wheat and rye intake was associated with a decreased risk of colon cancer, whereas estimates of whole grain wheat and rye intake alone were not [3]. An increasing body of data suggests that plasma alkylresorcinols are well associated with whole grain intake [4], can distinguish between whole grain wheat and rye intake [5], and can be used to complement and possibly substitute [6] for questionnaire- or diary-based estimates of whole grain intake in clinical and observational studies.

Alkylresorcinols are long-chain phenolic lipids found almost exclusively in wheat, rye, and barley among the edible parts of plants [4]. Because they are located in the outer layers of the kernel, their intake is effectively only from the whole grain or bran of wheat, rye, and barley, with minimal amounts present in the white flour of these cereals [7,8]. Ingested alkylresorcinols are absorbed from the small intestine and can be measured in plasma, which has

Abbreviations: GC, gas chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography; APCI, atmospheric pressure chemical ionization; LLE, liquid–liquid extraction; SLE, supported liquid extraction; EDTA, ethylenediaminetetraacetic acid; MRM, multiple reaction monitoring; qTOF–MS, quadrupole time-of-flight mass spectrometer.

* Corresponding author.
E-mail address: alastair.ross@chalmers.se (A.B. Ross).

http://dx.doi.org/10.1016/j.ab.2015.12.023
0003-2697/

led to investigations on their use as biomarkers of the intake of these cereals [9–11]. Alkylresorcinols have not been demonstrated to have any bioactive effect in humans, although in vitro and rodent studies suggest a role in inhibition of some cancer cells [12] and alteration to lipid [13–15] and glucose metabolism [16]. To date, there is a growing body of literature supporting their application as food intake biomarkers because they are correlated with measures of whole grain intake and repeated plasma alkylresorcinol measurements are reproducible under controlled conditions [4,17] and fairly reproducible under free-living conditions when wheat and rye intake is stable and frequent [18].

Current methods for alkylresorcinol analysis are based on gas chromatography–mass spectrometry (GC–MS) [10,19], gas chromatography–tandem mass spectrometry (GC–MS/MS) [20], and normal-phase liquid chromatography–tandem mass spectrometry (LC–MS/MS) with atmospheric pressure chemical ionization (APCI) [21]. All methods are based on the same sample preparation principle developed by Linko and coworkers [10] and improved by Landberg and coworkers [19], involving triplicate liquid–liquid extraction (LLE) with diethyl ether and then removal of neutral steroids (mainly cholesterol) by solid-phase anion exchange chromatography. The amount of plasma used is generally 200 μl, although as little as 80 μl can be used if concentrations of alkylresorcinols are expected to be high [19]. Using normal-phase LC–MS/MS removes the need for the anion exchange chromatography step because the interfering lipids are separated chromatographically, although this also uses hexane as a mobile phase [21]. The use of large volumes of either diethyl ether or hexane in a method is not ideal from a safety and environmental standpoint.

Sample throughput with both the GC–MS and LC–MS/MS methods is on the order of 60–80 samples per day, which is a limiting factor if plasma alkylresorcinols are to be widely applied in observational studies with thousands of samples. To improve the sample throughput of plasma alkylresorcinol analysis, and reduce the amount of organic solvents used during the analytical process, we have tested plasma phospholipid removal plates and supported liquid extraction (SLE) plates for plasma extraction and have improved an LC–MS/MS method to reduce environmental impact, reduce required sample volume, and greatly increase sample throughput.

Materials and methods

Chemicals and sample preparation plates

All solvents were HPLC (high-performance liquid chromatography) or LC–MS grade from Sigma–Aldrich (Stockholm, Sweden). Alkylresorcinol standards—heptadecylresorcinol (C17:0), nonadecylresorcinol (C19:0), $^{2}H_{4}$-nonadecylresorcinol ($^{2}H_{4}$–C19:0), heneicosylresorcinol (C21:0), tricosylresorcinol (C23:0), and pentacosylresorcinol (C25:0)—were purchased from ReseaChem (Burgsdorf, Switzerland). The phospholipid removal plates tested were Oasis (Waters, Manchester, UK), Phree (Phenomenex, Værløse, Denmark), and HybridSPE (Supelco, Sigma–Aldrich). SLE plates were Isolute (Biotage, Uppsala, Sweden) and ChemElute (Agilent, Kista, Sweden).

Clinical samples

A pooled plasma sample collected with citrate anticoagulant used for method development was obtained from the Blood Transfusion Unit of the Sahlgrenska University Hospital (Gothenburg, Sweden). A pooled ethylenediaminetetraacetic acid (EDTA) plasma sample from U.S. donors (Innovative Research, Novi, MI, USA), and EDTA plasma from a Swedish donor with celiac disease (no intake of wheat, rye, or barley) were used for method validation.

After development of the method, the protocol was tested using samples from two clinical intervention studies where subjects were fed whole grains. The two studies were registered at ClinicalTrials.gov (NCT01411540 and NCT02462798). These samples were used to provide extended data on intra- and inter-batch variation with several different people using the method. These data represent 92 different subjects and 192 samples from a crossover intervention study ($n = 32$) and a parallel design intervention study ($n = 60$). Data from one study (NCT01411540, 72 samples) were also analyzed using GC–MS (see below) and were used as a comparison with the current method.

Sample preparation method development

The results generated with this new method were compared with previous results for the sample preparation method of Landberg and coworkers [19], which is based on triplicate LLE with 3 ml of diethyl ether followed by removal of neutral steroids by anion exchange solid-phase extraction. Three phospholipid removal plates and two SLE plates were tested using a standardized protocol comparing recovery of alkylresorcinols based on the volume of plasma (100 or 200 μl), the type of elution solvent (ethyl acetate, acetonitrile, methanol, and acetone for phospholipid removal plates; ethyl acetate, heptane, methyl-*t*-butyl-ether, and dichloromethane for SLE plates), and the volume of elution solvent (1000 or 1600 μl). Each experimental permutation was carried out in triplicate on the same day. The best conditions were selected based on the area of the stable isotope-labeled internal standard (i.e., nonadecylresorcinol-d_4, C19:0 d_4), the total area of five alkylresorcinol homologues, and the variation of both total area and homologue to internal standard ratio over triplicate extractions. Plates were also qualitatively evaluated for the ease of flow and precipitation of particles in the extraction eluate. Because not all solvent–plate combinations were possible due to solvent incompatibility, the final comparison was based on an overall ranking for 11 plate–solvent combinations.

The best performing conditions were further optimized for elution solvent volume (0.8, 1.6, or 2.4 ml) and solvent additive (no additive, 1% formic acid, or 2% acetic acid). The final protocol was as follows. First, 100 μl of plasma was pipetted into the wells of a HybridSPE 96-well plate (Sigma–Aldrich). Then, 20 μl of internal standard solution (prepared with labeled alkylresorcinols) in acetone (100 ng/ml) was added to each well, and the plate was mixed gently using a vortex mixer on a low rotation setting. Plasma was extracted using 2 × 800 μl of acetone without further mixing, and the extracts were pulled through the SPE manifold over 5–10 min under 5 mm Hg pressure. The two extraction eluates were collected into a 96-deep-well plate and evaporated until dryness under reduced pressure using a vacuum centrifuge evaporator (MiVac Duo, Genevac, Ipswich, UK) at 50 °C. Samples were resuspended in 100 μl of 95% heptane/5% ethanol (v/v), covered with an ethylene vinyl acetate sealing mat (Porvair Sciences, Norfolk, UK), and vortexed at high speed on a multivortex mixer prior to analysis by LC–MS/MS.

Alkylresorcinols were quantified based on an 8-point matrix matched calibration curve, using a plasma sample with a low basal amount of alkylresorcinols, spiked with 0.05–10 ng (i.e., 0.14–28 pmol, equivalent to 1.4–287 nmol/L) of each alkylresorcinol homologue (see Fig. 1). Stock standard solutions contained all unlabeled alkylresorcinol homologues and were spiked into plasma in 800 μl of acetone. Within this range, which covers the usual concentration of alkylresorcinols in fasting plasma, the calibration curve was linear, with the standard error of the