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Journal of Chromatography B, 809 (2004) 125–130
JOURNAL OF CHROMATOGRAPHY B
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Identification of cereal alkylresorcinol metabolites in human urine—potential biomarkers of wholegrain wheat and rye intake

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Received 29 January 2004; received in revised form 3 June 2004; accepted 10 June 2004

Available online 13 July 2004

Abstract

Alkylresorcinols, phenolic lipids present in high amounts in wholegrain wheat and rye, are of interest as potential biomarkers of the intake of these cereals. Alkylresorcinols are known to be absorbed by humans and animals, but little is known about their metabolism or resulting metabolites. A preliminary human study was carried out to identify alkylresorcinol metabolites in human urine. Urine samples, collected before and after a wheat-bran based meal, were deconjugated with β-glucuronidase/sulphatase and then extracted with ethyl acetate. Extracts were separated by thin-layer chromatography, and fractions containing alkylresorcinols and possible metabolites were identified by retention on the plate compared to standard compounds, and staining with fast blue B. These fractions were further analysed by gas chromatography-mass spectrometry. Deconjugated human urine after the wheat-bran based meal contained two alkylresorcinol metabolites, 3,5-dihydroxybenzoic acid and 3-(3,5-dihydroxyphenyl)-1-propanoic acid, as well as smaller amounts of unchanged alkylresorcinols, confirming the hypothesis that alkylresorcinols are metabolised in humans via β-oxidation of their alkyl chain.

Keywords: Biomarkers; Alkylresorcinol

1. Introduction

Epidemiological studies suggest that wholegrain cereal foods have many beneficial health effects, including reducing the risk of obesity, diabetes, heart disease and some cancers [1–5]. However, there are concerns about the methodology used to determine dietary intake in epidemiological studies [6], and many consumers have difficulty in identifying what cereal products are wholegrain [7]. Hence, there is some doubt about the validity of estimates of wholegrain cereal intake in epidemiological studies and therefore the health claims based on these studies. The strength of these epidemiological studies could be increased by the use of a biomarker of wholegrain cereals as an objective measure of their intake. A group of compounds present in high levels in wholegrain wheat and rye, the alkylresorcinols, may be suitable candidates as biomarkers of their intake [8,9].

Alkylresorcinols (1,3-dihydroxyl-5-*n*-alkylbenzene derivatives) are phenolic lipids present in plants as odd-numbered hydrocarbon side chain homologues (in wheat and rye the side chain is C15:0–C25:0). Of the plants commonly used for human food, alkylresorcinols are only present in significant amounts in wholegrain wheat and rye [10]. These two cereals can be differentiated on the basis of their alkylresorcinol homologue content [10]. Alkylresorcinols are absorbed to a significant extent by rats, pigs and humans [11–13], and can be measured in human plasma [14]. However, no studies have been carried out on the human metabolism of cereal alkylresorcinols. Two studies on alkylresorcinol absorption in rats have suggested that urinary alkylresorcinol metabolites are considerably more polar than intact alkylresorcinols [11,12], though the metabolites were not identified.

Previously we proposed a scheme for mammalian metabolism of alkylresorcinols [9] based on their similar structure to other amphiphilic compounds; i.e. tocopherols [15,16] and 4-*n*-nonylphenol [17]. These compounds are metabolised by conjugation of the hydroxyl groups on the phenolic ring, and degradation of the alkyl tail by

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doi:10.1016/j.jchromb.2004.06.015

ω-oxidation, conversion of the ω-hydroxyl group to a carboxylic acid, followed by successive β-oxidation, thus making them water soluble and excretable via the urine. The metabolism of olivetol (C5:0 alkylresorcinol), by the fungus *Syncephalastrum racemosum* was suggested to follow a similar pathway, resulting in the formation of 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) [18].

This paper describes a preliminary study to identify potential metabolites of cereal alkylresorcinols in human urine, with a view to their use as biomarkers of wholegrain wheat and rye intake.

2. Experimental

2.1. Diet, chemicals and reagents

The wheat bran meal used as a source of alkylresorcinols was a commercial wheat bran-based breakfast cereal (WB) obtained from a local supermarket (Uppsala, Sweden). The alkylresorcinol content as determined by GC [10] was 1 mg/g dry matter. β-Glucuronidase/sulphatase (EC 3.2.1.31; Sigma, St. Louis, MO, USA) was used for deconjugation of urine samples. The synthetic standards 1,3-dihydroxybenzoic acid and 3-(3,5-dihydroxyphenyl)-1-propanoic acid were purchased from Aldrich (Steinheim, Germany), and ISOSEP AB (Tollinge, Sweden) respectively. Purified rye alkylresorcinols, a mixture of alkylresorcinol homologues C15:0–C25:0, used as a standard for TLC and GC–MS analysis were purified from rye bran as per reference [19]. This standard mixture was >99% pure alkylresorcinols (all homologues together) as determined by NMR [20]. Fast blue B (Zn salt) (Fluka Chemie GmbH, Buchs, Switzerland) was used to stain the TLC plates. Samples were silylated using BSTFA + 1% TMCS (Pierce Chemical Company, Rockford, IL, USA) prior to GC–MS analysis. All chemicals and solvents not described here were of analytical grade (E Merck, Darmstadt, Germany) and used without further purification.

2.2. Study design

One 26-year-old male followed a five day alkylresorcinol free diet (avoiding all foods containing wholegrain wheat, rye and barley, and wheat, rye and barley bran). At the end of this period, an overnight urine sample was collected, which was taken as the blank sample. Then the subject ate WB (150 g) over a 1-h period (19.00–20.00). An overnight urine sample was taken the following morning. Urine samples were stored at −20 °C until analysed.

2.3. Extraction of the urine

To 100 mL of each urine sample, 30 000/1000 U β-glucuronidase/sulphatase in 100 mL of 0.1 M acetate buffer (pH 5) was added to deconjugate any conjugated metabolites, and the urine sample incubated overnight in a shaking waterbath at 37 °C. Urine samples were then acidified to pH < 1 with 6 M hydrochloric acid, and extracted with 3 × 100 mL ethyl acetate. The ethyl acetate extracts were pooled and evaporated to a thick brown oil (∼1 mL). The oil was dissolved in 2 mL ethyl acetate to enable spotting on the TLC plates.

2.4. Separation of potential urinary alkylresorcinol metabolites

The ethyl acetate extracts from deconjugated and un-deconjugated blank and WB urine (∼5 μL) were spotted on silica gel 60 TLC plates (5 cm × 20 cm) (Merck), together with purified rye bran alkylresorcinols, synthetic 3,5-dihydroxybenzene and synthetic DHPPA. The TLC plates were developed using chloroform:methanol (4:1, v/v) as the mobile phase. Seperated spots were visualised by spraying with 0.05% aqueous fast blue B, a dye that specifically stains compounds with a resorcinolic ring pink-crimson [21]. Seven spots were apparent in the deconjugated WB urine (Table 1) that were not present in the deconjugated blank urine or un-deconjugated samples. About 60 μL of the deconjugated blank and both deconjugated and un-deconjugated WB urine extracts were then streaked as 15 cm bands on three separate 20 cm × 20 cm TLC plates. The extracts were spotted on the left and right edges of the plates for later visualisation. The plates were developed using the same conditions as before. Six fractions of interest (Table 1), identified by spraying the sides of the plate with fast blue B, were scraped off and extracted with 3 × 10 mL chloroform:methanol (2:3, v/v). The extracts were pooled and dried using a vacuum rotoevaporator, silylated and analysed using GC–MS.

2.5. Identification of potential urinary alkylresorcinol metabolites by GC–MS

Fractions from TLC separation of deconjugated WB urine, un-deconjugated WB urine, and deconjugated blank urine were run on an HP 5890 series II gas chromatograph coupled to a TRIO-1000 mass spectrometer with Mass Lab version 1.3 software (Fisons PLC, Manchester, UK). An HP-5 MS column (length 25 m, inner diameter 330 μm, film thickness 0.25 μm; Hewlett-Packard, Avondale, PA, USA) was used for separation with the following temperature programme: 120 °C (0 min), 200 °C (6 min), 260 °C (14 min), 260 °C (65 min). Injector temperature was 250 °C, and interface temperature was 280 °C. The mobile gas flow rate was 1 mL/min. The mass spectra were recorded at an electron energy of 70 eV. Samples were scanned in total ion count (TIC) mode between *m*/*z* 100 and 650. For samples that possibley contained intact alkylresorcinols, samples were also scanned using selective ion recording (SIR) mode at *m*/*z* 268 and the molecular ions for the five main alkylresorcinols, as this mode is more sensitive than

Table 1
Identification of alkylresorcinol metabolites in human urine after consumption of a wheat bran meal was carried out by staining TLC plates with fast blue B

Possible AR metabolites as indicated by TLC (Rf value)[a]	Rf range of corresponding fractions scraped off for GC–MS analysis	Alkylresorcinols/metabolites present as analysed by GC–MS
0.04	0.01–0.05 (fraction 1)	DHBA[b], DHPPA[c,d]
0.10	0.06–0.14 (fraction 2)	DHBA[d]
0.20[e]} 0.26[f]	0.18–0.33 (fraction 3)	DHBA and DHPPA
0.61	0.56–0.71 (fraction 4)	No metabolites identified
0.78	0.71–0.89 (fraction 5)	No metabolites identified
1.00	0.89–1.0 (fraction 6)	Native alkylresorcinols

[a] Rf values for standard compounds: DHBA[b], 0.20; DHPPA[c], 0.26; purified rye alkylresorcinols, 1.0.
[b] DHBA = 1,3-dihydroxybenzoic acid.
[c] DHPPA = 3-(3,5-dihydroxyphenyl)-1-propanoic acid.
[d] Peaks only visible when scanning for ions related to the metabolites, see text for details.
[e] Same Rf as synthetic DHBA.
[f] Same Rf as synthetic DHPPA.

TIC. Silylated alkylresorcinols produce a strong molecular ion, along with the base ion at *m/z* 268, with little other fragmentation [14,22]. Alkylresorcinols in urine extracts were identified by comparing mass spectra and retention time to those alkylresorcinols present in the WB diet and purified rye bran alkylresorcinols. The identity of the two metabolites was confirmed by comparison of retention time and mass spectra to synthetic 3,5-dihydroxybenzoic acid (DHBA) and DHPPA.

3. Results and discussion

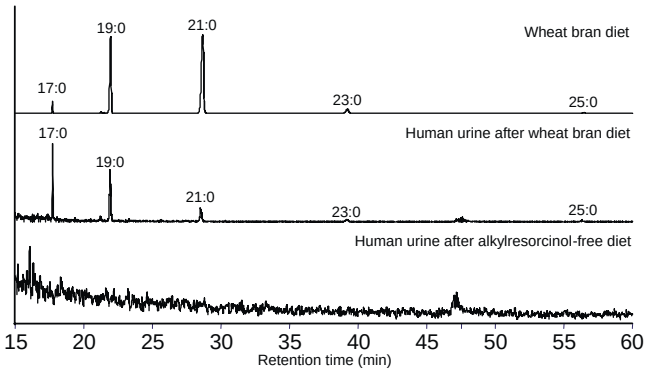
3.1. Deconjugation and extraction of potential metabolites

The extraction procedure used in this study is similar to those used in many studies to isolate phenolic compounds from urine, e.g. [17,23]. It is likely that alkylresorcinols and their metabolites are conjugated with glucuronide and/or sulphate groups to increase their solubility in urine. Treatment with β-glucuronidase/sulphatase allowed extraction of radioactivity from radiolabelled alkylresorcinol in rat urine [12], and resorcinol was recovered in rat urine as glucuronide, sulphate, or glucuronide and sulphate metabolites [24]. All urine samples were extracted with ethyl acetate as McClanahan and Robertson [18] found that olivetol metabolites were extractable in ethyl acetate, but not in chloroform.

3.2. Identification of potential metabolites

The results from the TLC are described in Table 1, showing the Rf values for the initial spots identified as possible alkylresorcinol metabolites, the Rf values of the three standards used (purified rye bran alkylresorcinols, DHPPA and 1,3-dihydroxybenzoic acid), the fractions scraped off from the preparative TLC plates, and what metabolites were identified in each fraction by GC–MS.

Fraction six (Rf 0.89–1.0) (Table 1) from the TLC plate of the deconjugated WBM urine contained a small crimson spot, which was not present in the other urine extracts. This fraction (Fig. 1) contained the most lipophilic substances extracted, and included intact alkylresorcinols (log octanol/water partition coefficient (log *P*): 9.4–13.4, [9]). The intact alkylresorcinols were present in minute amounts, but could be identified by their Rf value on TLC plates, GC retention time, and similar mass spectra compared to wheat bran alkylresorcinols. Fig. 1 shows GC–MS TIC



	Wheat bran diet			Human urine after the wheat bran diet		
Alkylresorcinol homologues	RT[1] (min)	M^{+b}	%BP[c]	RT (min)	M^{+}	%BP
17:0	17.71	492	15.0	17.72	492	14.5
19:0	21.93	520	17.1	21.90	520	16.6
21:0	28.65	548	18.0	28.54	548	15.0
23:0	39.23	576	18.3	39.30	576	17.0
25:0	56.34	604	20.3	56.15	604	15.5

[a] RT = GC-MS retention time.
[b] M^{+} = Molecular ion of alkylresorcinol
[c] %BP = Percentage of molecular ion with respect to the base peak at *m/z* 268 (100 %) after subtraction of background peaks

Fig. 1. Total ion count (TIC) GC–MS chromatograms at *m/z* 268, indicating the presence of native alkylresorcinols in the wheat bran diet and deconjugated human urine after consumption of the wheat bran diet, and lack of alkylresorcinols in deconjugated human urine after a four day alkylresorcinol free diet. The table with the retention times and diagnostic ions refers to the chromatograms above. Note the difference in alkylresorcinol homologue composition between the urine and the wheat bran diet.

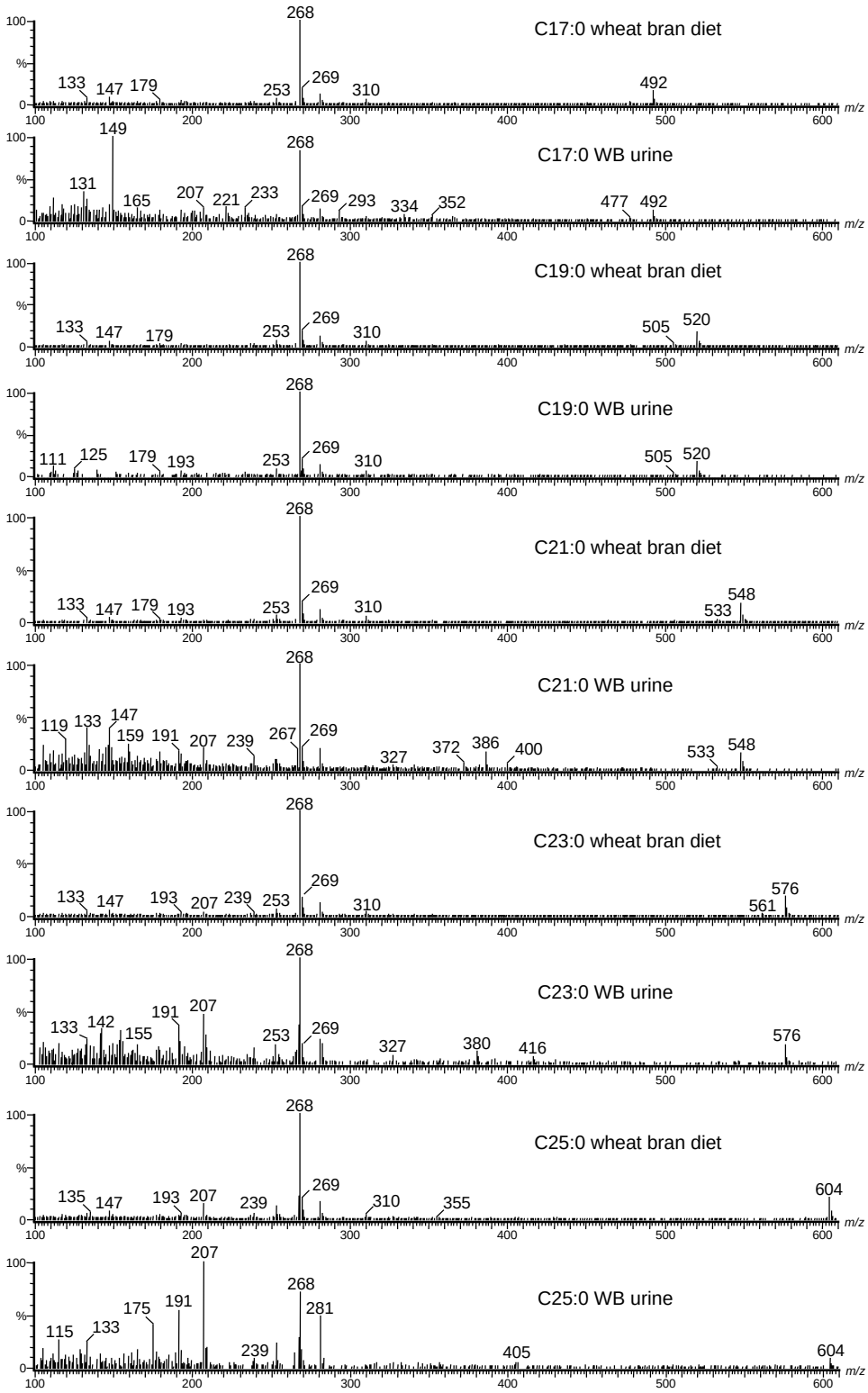


Fig. 2. Mass spectra of the intact alkylresorcinols from the wheat bran diet, and those extracted from deconjugated human urine after consumption of the wheat bran diet (WB urine).

chromatograms with the main diagnostic ion for alkylresorcinols, *m*/*z* 268, extracted, along with the relative abundance of the molecular ion to the base peak (*m*/*z* 268). Fig. 2 shows the mass spectra of each of the main alkylresorcinol homologues (C17:0–C25:0) from WB and those found in WB urine. The mass spectra for WB is comparable to those previously published [14,22,25]. The reason for the high amount of background ions, especially for the longer chain alkylresorcinols, is probably due to the very low amounts of intact alkylresorcinols present in the extract. The relative homologue composition of urinary alkylresorcinols differed from that of WB (Fig. 1); C17:0 was the most predominant homologue in the WB urine, but C21:0 was the predominant homologue in WB, and in wheat in general [10]. This indicates that long chain alkylresorcinols might be preferentially metabolised, or incorporated into adipose tissue or lipid membranes [20,26].

The intact alkylresorcinols observed in the deconjugated WB urine were not detected in the un-deconjugated WB urine, confirming that they are conjugated prior to excretion via the urine. This is in contrast to plasma, where the recovery of intact alkylresorcinols was not increased by deconjugation [14], though to date no studies on the presence alkylresorcinol metabolites in plasma have been carried out.

Fraction three (Rf 0.18–0.33) from the preparative TLC plate streaked with deconjugated WB urine extract contained two pink-red spots with Rf values that corresponded with those of synthetic DHBA (Rf = 0.20, calculated log *P* 0.91) and DHPPA (Rf = 0.26, calculated log *P* 1.33) (Table 1). GC–MS analysis of this fraction showed two peaks corresponding to two possible short-chain alkylresorcinol metabolites with the same retention times and mass spectra as synthetic DHBA and DHPPA (Fig. 3). As these peaks are not completely separated from surrounding peaks,

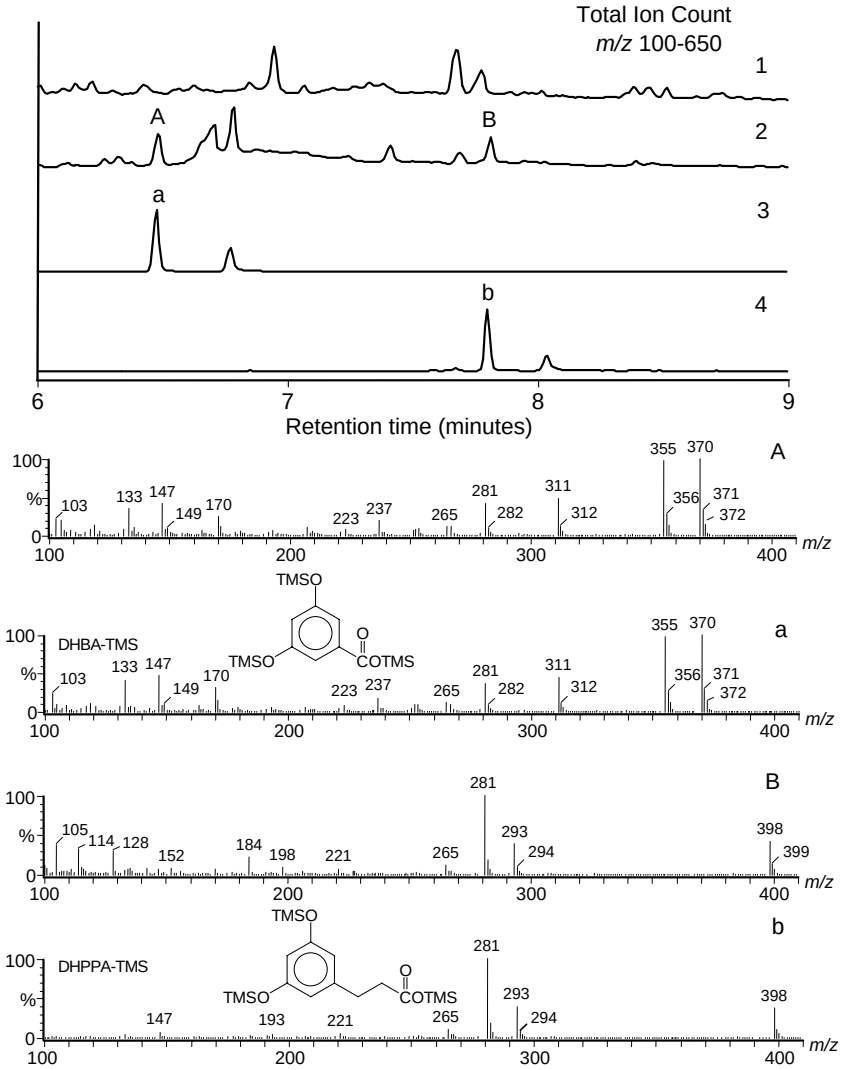


Fig. 3. Gas chromatography-mass spectrometry chromatograms and spectra of (1) human urine extract after an alkylresorcinol-free diet, compared to human urine extract after a meal rich in wheat bran (2), containing the alkylresorcinol metabolites DHBA (A) and DHPPA (B). Chromatograms and mass spectra of synthetic standards of 3,5-dihydroxybenzoic acid (3, a) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (4, b) are shown for comparison.

it is likely that some of the minor contaminating ions present in the spectra are due to co-eluting compounds (Fig. 3). The Rf value and mass spectrum for DHPPA was similar to that reported for the same compound characterised as a fungal metabolite of olivetol [18]. The alkyl chain of the phenolic lipid 4-*n*-nonylphenol is also metabolised to one and three carbon carboxylic acids [17]. Analysis of fractions 1 and 2, indicated the presence of both DHBA and DHPPA, on the basis of both retention time, and a similar, though not pure, mass spectra to the standard compounds. The presence of these metabolites, not visible as peaks on the TIC chromatogram, in these two fractions is probably due to overloading of the TLC plate.

Finding DHPPA and DHBA in deconjugated urine after the wheat bran diet, but not from the blank urine suggests that these compounds are metabolites of alkylresorcinols. Alkylresorcinols are not present in significant amounts in foods other than those that contain wholegrain or bran of wheat, rye or barley [10], and the presence of a single 3,5-dihydroxybenzene ring is not common in food plants, so the likelihood of these metabolites coming from other phenolic compounds is low. Other similar compounds such as 2,3- and 2,4-dihydroxybenzoic acids, present in plasma, have different mass spectra [27] compared to 3,5-dihydroxybenzene. Further evidence that these metabolites come from alkylresorcinols is that γ-tocopherol metabolism by Hep G2 cells in vitro is competitively inhibited by alkylresorcinols, suggesting that both compounds are metabolised in a similar manner [20].

3.3. Conclusions

From the metabolites detected in this study, it appears that alkylresorcinols are metabolised via conjugation with glucuronide and/or sulphate groups and shortening of the alkyl tail via β-oxidation as previously hypothesised [9]. As these metabolites appear in urine, it is possible that they are also present in plasma. Further studies are needed to confirm that these metabolites come from alkylresorcinols, and to develop quantitative methods to test for their suitability as biomarkers of wholegrain wheat and rye intake. As these metabolites are water-soluble, HPLC coupled with mass or electrochemical detectors may be more suitable for their quantitative analysis than GC–MS. Knowledge of these metabolites will aid in the study of alkylresorcinol bioavailability and their development as possible biomarkers of wholegrain wheat and rye intake.

Acknowledgements

ABR gratefully acknowledges the financial support of the Swedish Nutrition Foundation.

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