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Review

Chromatographic analysis of alkylresorcinols and their metabolites

Alastair B. Ross*, Per Åman, Roger Andersson, Afaf Kamal-Eldin

Department of Food Science, Swedish University of Agricultural Sciences (SLU), Box 7051, S-750 07 Uppsala, Sweden

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Abstract

Alkylresorcinols (AR) are phenolic lipids present in high amounts in the bran fraction of wheat and rye. AR are of scientific interest as bioactive compounds, as markers for the presence of wholegrain or bran fractions of wheat and rye in food products, and as possible biomarkers of intake for wholegrain wheat and rye. This review discusses their extraction from cereal grains, food and biological fluids, and the various chromatographic methods used in their analysis.
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Keywords: Reviews; Food analysis; Wheat; Rye; Biomarkers; Alkylresorcinols

Contents

1. Introduction 157
2. Standards 159
3. Extraction of alkylresorcinols 159
 3.1. Extraction of alkylresorcinols from cereal grains 159
 3.2. Extraction of alkylresorcinols from cereal products 159
4. Chromatographic analysis of alkylresorcinols 159
 4.1. Thin layer chromatography 159
 4.2. Column chromatography 160
 4.3. Gas chromatography 160
  4.3.1. GC–MS analysis of alkylresorcinols 160
 4.4. High performance liquid chromatography 161
5. Analysis of alkylresorcinols in human samples 162
 5.1. Analysis of intact alkylresorcinols in blood 162
 5.2. Analysis of alkylresorcinols and their metabolites in urine 162
 5.3. Analysis of alkylresorcinols in illeal digesta 163
6. Needs for future method development 163
Acknowledgements 163
References 163

1. Introduction

Epidemiological studies strongly indicate that consumption of wholegrain cereals is linked to a decreased risk of diseases, such as obesity [1], diabetes [2,3], coronary heart disease [4,5], stroke [6], and some cancers [7–9]. However, the mechanisms behind the health benefits of wholegrain cereals are not well-known due to the multifactorial nature of these diseases, relatively low incidences of certain diseases that make it hard to get statistically significant results, the difficulty that consumers have in identifying wholegrain products [10], and possible weaknesses of food frequency questionnaires in the estimation of food intake in epidemiological studies [11]. One way of improving the proposed link between consumption of wholegrain cereals and a decreased incidence of disease is to use a biomarker,

* Corresponding author. Present address: Nestlé Research Center, CH-1000 Lausanne 26, Switzerland. Tel.: +46-18-67-1000; fax: +46-18-67-2995.
E-mail address: Alastair.Ross@lmv.slu.se (A.B. Ross).

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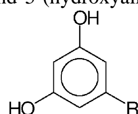
i.e. specific compounds that can be measured in a biological fluid (e.g. plasma or urine), and can be related to the intake of the food(s) in question which is linked to a biological activity and/or decreased risk of disease.

5-*n*-Alkylresorcinols (AR) are a group of phenolic lipids suggested to have potential as markers for the presence of wholegrain wheat and rye and/or the bran fractions of these cereals, and as biomarkers of wholegrain wheat and rye intake [12]. AR, found in high amounts in the bran of wheat and rye (0.03–0.15% of whole kernel weight), consist of a phenolic ring with two hydroxyl groups in the *meta* position, and an odd numbered alkyl chain at position 5. The alkyl chain in cereal AR varies from 15 to 25 carbons long [13,14], and is most commonly saturated (Table 1). Minor AR derivatives have also been identified in wheat and rye, including AR with unsaturated alkyl chains [15], keto groups [16], a combination of keto groups and unsaturation [17,18], or a combination of hydroxyl groups and unsaturation [18]. Rye contains 15–30% of these minor AR derivatives [19–21]. Alkylresorcinols and their derivatives are claimed to have a wide range of biological activities, especially related to their amphiphilic properties. The detailed chemistry, nutritional effects and bioactivities of AR are described in more detail in reviews by Kozubek and Tyman [14] and Ross et al. [12].

According to recent studies, AR may have potential as biomarkers of wholegrain wheat and rye intake [12]. Currently, it is known that AR are absorbed by humans [22] and

Table 1
Structures of 5-*n*-alkyl-, 5-alkenyl-, 5-(oxoalkyl)-, 5-(oxoalkenyl)-, and 5-(hydroxyalkenyl)-resorcinols isolated from wheat and rye grains



Alkylresorcinol derivative (R)	Structure	Molecular weight (Da)	Isomers (position of double bond)	Reference
5-*n*-Alkylresorcinols				
C17:0	5-*n*-Heptadecylresorcinol	348	–	[13,14]
C19:0	5-*n*-Nonadecanylresorcinol	376	–	[13,14]
C21:0	5-*n*-Heneicosylresorcinol	404	–	[13,14]
C23:0	5-*n*-Tricosylresorcinol	432	–	[13,14]
C25:0	5-*n*-Pentacosylresorcinol	460	–	[13,14]
5-Alkenylresorcinols				
C17:1	5-(Heptadecenyl)-resorcinol	346	8*Z*, 10*Z*, 12*Z*	[15]
C19:1	5-(Nonadecenyl)-resorcinol	374	10*Z*, 12*Z*, 14*Z*	[15]
C21:1	5-(Heneicosenyl)-resorcinol	402	12*Z*, 14*Z*, 16*Z*	[15]
C23:1	5-(Tricosenyl)-resorcinol	430	14*Z*, 16*Z*, 18*Z*	[15]
C25:1	5-(Pentacosenyl)-resorcinol	458	16*Z*, 18*Z*, 20*Z*	[15]
C19:2	5-(Nona-10*Z*,13*Z*-decadienyl)-resorcinol	372	–	[15]
C21:2	5-(Henei-12*Z*,15*Z*-cosadienyl)-resorcinol	400	–	[15]
C23:2	5-(Tri-14*Z*,17*Z*-cosadienyl)-resorcinol	428	–	[15]
C25:2	5-(Penta-16*Z*,19*Z*-cosadienyl)-resorcinol	456	–	[15]
5-Oxoalkylresorcinols				
C19:Oxo	5-(2-Oxononadecanyl)-resorcinol	390	–	[16]
C21:Oxo	5-(2-Oxoheneicosanyl)-resorcinol	418	–	[16]
C23:Oxo	5-(2-Oxotricosanyl)-resorcinol	446	–	[16]
C25:Oxo	5-(2-Oxopentacosanyl)-resorcinol	474	–	[16]
5-Oxoalkenylresorcinols				
C19:1,Oxo	5-(2-Oxononadecenyl)-resorcinol	388	10*Z*, 12*Z*, 14*Z*	[18]
C21:1,Oxo	5-(2-Oxoheneicosenyl)-resorcinol	416	12*Z*, 14*Z*, 16*Z*	[18]
C23:1,Oxo	5-(2-Oxotricosenyl)-resorcinol	444	14*Z*, 16*Z*, 18*Z*	[18]
C25:1,Oxo	5-(2-Oxopentacosenyl)-resorcinol	472	16*Z*, 18*Z*, 20*Z*	[18]
C19:2,Oxo	5-(2-Oxo,10*Z*,13*Z*-nonadecadienyl)-resorcinol	386	–	[18]
C21:2,Oxo	5-(2-Oxo,12*Z*,15*Z*-heneicosenyl)-resorcinol	414	–	[18]
C23:2,Oxo	5-(2-Oxo,14*Z*,17*Z*-tricosadienyl)-resorcinol	442	–	[18]
C25:2,Oxo	5-(2-Oxo,16*Z*,19*Z*-pentacosadienyl)-resorcinol	470	–	[18]
5-Hydroxyalkenylresorcinols				
C19:1,Hydroxy	5-(2-Hydroxynonadecenyl)-resorcinol	390	10*Z*, 12*Z*, 14*Z*	[18]
C21:1,Hydroxy	5-(2-Hydroxyheneicosenyl)-resorcinol	418	12*Z*, 14*Z*, 16*Z*	[18]
C21:1,Hydroxy	5-(4-Hydroxyheneicosenyl)-resorcinol	418	12*Z*, 14*Z*, 16*Z*	[18]
C23:1,Hydroxy	5-(4-Hydroxytricosenyl)-resorcinol	446	14*Z*, 16*Z*, 18*Z*	[18]
C25:1,Hydroxy	5-(4-Hydroxypentacosenyl)-resorcinol	474	16*Z*, 18*Z*, 20*Z*	[18]
C21:2,Hydroxy	5-(4-Hydroxy,12*Z*,15*Z*-heneicosenyl)-resorcinol	416	–	[18]
C23:2,Hydroxy	5-(4-Hydroxy,14*Z*,17*Z*-tricosadienyl)-resorcinol	444	–	[18]

that they are present in human plasma [23] and as metabolites in urine [24]. In order to establish AR as biomarkers of wholegrain wheat and rye intake, more needs to be understood about the kinetics of AR absorption and metabolism, and storage in the body. This will require rapid and sensitive analytical methods, capable of being used for a number of different matrices, including foodstuffs and human samples. This review will focus on the analysis of AR in cereal grains and food products, and the analysis of AR and their metabolites in human blood and urine.

2. Standards

One of the main limiting factors for the analysis of AR is the lack of commercial standards, which is a problem for laboratories wishing to start work on AR. Currently only olivetol (C5:0), is available (95% purity, Sigma–Aldrich, St Louis, MO, USA). Previously, C15:0 was also available from Aldrich at 85% purity, but in order to use this material as a quantitative standard or for biological studies, it needs to be purified [25]. Other options for obtaining standard AR material is to purify them from raw material (normally rye or wheat bran, or cashew nut shell liquid) using column chromatography (see later) or to synthesise them. There are many published methods for AR synthesis [26–31] but the procedure requires expensive reagents and chromatographic purification afterwards. Synthesis of radiolabelled AR has also been reported, though it is inefficient, with only 2–3% yield [31].

3. Extraction of alkylresorcinols

3.1. Extraction of alkylresorcinols from cereal grains

AR from cereal grains have a $\log P$ (log octanol:water partition coefficient) of 8.5–13.4 [12], meaning that they are essentially insoluble in water, but are soluble in more hydrophobic solvents such as: acetone, ethyl acetate, methanol, ethanol, diethyl ether, chloroform, cyclohexane, and *n*-hexane [14,32]. Most AR homologues are soluble in methanol, although the longer chain homologues (C23:0 and C25:0) are not extractable to the same extent as in acetone and ethyl acetate [20], so the use of methanol as an extraction solvent might lead to some discrimination against these homologues.

Methods for extraction of AR from grains tend to be fairly simple, using 0.1–5 g raw material, and extracting with acetone, methanol or ethyl acetate at room temperature for 16–24 h [20,33–36] or for 2 h in a Soxhlet apparatus with acetone or cyclohexane [32,37]. The amount of solvent used varied, but the ratios of sample to solvent were mostly between 0.02 and 0.03 (w/v). Methods using lower amounts of solvent (<5 ml/g) tend to also use multiple extractions, while those using larger amounts of solvent (40–50 ml/g) require only one extraction step. As AR are only located in the outer layers of cereal grains, and not in the starchy endosperm or germ [38], they can be extracted from whole grains without milling [20]. Milling of grains decreases extraction time and reduces the amount of sample required, but also increases the amount of co-extracted materials, making later chromatographic analysis more difficult [20]. More hydrophilic solvents such as methanol and 80% ethanol also co-extract more compounds that may interfere with later chromatographic analysis [20,36].

3.2. Extraction of alkylresorcinols from cereal products

A number of papers described the analysis of AR in food products using methods developed for analysis of AR in cereal grains, utilising acetone as extraction solvent [39,40]. These papers described the destruction of AR by the baking process on the basis that bread contained 0–70% less AR than the flour it was baked from. However, around 90% of the AR present in the flour could be recovered when bread was extracted with methanol [21]. Complete recovery of AR from bread was obtained using hot propanol:water (3:1, v/v) extraction [21], a method previously used for total lipid extraction from starch [41]. This difference in recovery is most probably due to AR being bound in difficult to extract complexes with starch, in a similar manner to other polar lipids [21].

4. Chromatographic analysis of alkylresorcinols

Extracted AR can be analysed by chromatographic methods such as paper chromatography [42], thin layer chromatography (TLC), gas chromatography (GC) or high performance liquid chromatography (HPLC) [14]. Although direct analysis of AR in extracts by fluorescence [33,34,39] and colourimetry [43] have also been reported, these methods are unspecific and unable to differentiate between different AR homologues. Moreover, there is particular doubt about the reliability of the fluorescence method for AR concentrations <200 μg/g [36], an important aspect to consider when reviewing old analytical data. Chromatographic analysis of AR is complicated by the fact that they are amphiphilic with a broad $\log P$ range, so a portion of the AR analysed tend to strongly adsorb to many common column stationary phases unless conditions are optimised. Because of this, many different chromatographic methods have been developed for the analysis of AR.

4.1. Thin layer chromatography

TLC is useful for rapid qualitative detection and isolation of AR, and can be used to identify different homologues [14] but is limited quantitatively. TLC is normally carried out using plates covered with silica gel 60. Mobile

phases vary depending on the matrix in which the AR are present, but some examples are methanol:water (90:10, v/v), chloroform:ethyl acetate (9:1, v/v), benzene:ethyl acetate (85:15, v/v) [14,44], chloroform:methanol (85:15, v/v) [45] and chloroform:methanol (4:1, v/v) [46]. With normal-phase chromatography, all AR form one spot together. Addition of formic acid to the mobile phase sharpens the AR band (Tyman, personal communication). Impregnation of the silica TLC plate with 20% silver nitrate in 50% methanol, and developing with benzene:ethyl acetate (85:15, v/v) separates AR on the basis of unsaturation, while impregnation of the silica TLC plate with 5% paraffin oil in *n*-hexane and development with acetone:methanol:water (60:15:25, v/v/v) allows separation on the basis of chain-length [19,44,47].

AR develop colours with several spray reagents. They form a red colour with vanillin–HCl reagent, a purple-grey to blue colour in ammonia vapour with Gibb's reagent (2,6-dichlorobenzoquinone-4-chloromide, 0.3% in methanol) [48], and different colours with a number of diazonium compounds, e.g. an orange colour with diazotized-*p*-nitroaniline [49]. AR can be visualised by iodine vapour and they turn a bright-red colour with anisaldehyde–sulphuric acid reagent [45]. Fast Blue B (methoxy-4-nitroaniline), a dye that stains phenolic compounds with a resorcinolic ring pink to deep crimson depending on chain-length, is commonly used for the visualisation of AR on TLC plates [14]. Two Fast Blue B salts are available; BF_4 and Zn; the former gives approximately twice the colour intensity of the later [50].

4.2. Column chromatography

Traditionally, large-scale purification of AR has been carried out using silica column chromatography [25,51]. Column chromatography can be used for large-scale (several grams) non-quantitative isolation of AR from wheat or rye bran. Kozubek [51] describes the isolation of AR from rye bran using silica gel suspended in chloroform, starting with one column volume of chloroform, followed by 10 column volumes of chloroform:ethyl acetate (9:1, v/v) to elute the AR. The resulting purity of the combined AR was found to be 99% by NMR [52] although the homologue composition was slightly different from that normally found in rye. AR tend to bind strongly to silica making quantitative recovery, e.g. by solid-phase extraction (SPE) as a method for sample pre-treatment, not achievable even when using strong solvents such as chloroform and methanol (Tyman, personal communication; Ross and Chen, unpublished results).

Bruce et al. [53] described the use of preparative HPLC to isolate individual AR homologues from cashew nut shell liquid but no methods have been yet published for large-scale isolation of individual AR homologues. A method for large-scale separation of AR by centrifugal partition chromatography is being developed, which could prove useful for the isolation of large amounts (>100 mg) of individual AR homologues [54].

4.3. Gas chromatography

As AR are mostly lipophilic, most quantitative studies using chromatography have used gas chromatography. GC analysis of AR is fairly rapid, and allows good separation of AR chain-length homologues. As packed GC columns are rarely used now, methods involving their use have not been included in this review. Capillary GC methods, developed for AR analysis, generally use non-polar stationary phases, e.g. 100% dimethylpolysiloxane or 5% phenyl-methylpolysiloxane [15,20,23,36,37,55]. Injection methods vary, but on-column injection allows analysis of dilute samples and avoids the risk of solvent explosion or part of the sample being trapped in the injector. However, this can require more sample purification prior to injection. Quantitative analysis with split injection is also possible, provided the injector temperature is high (325 °C) [20]. Using lower injector temperatures with a split injector may lead to part of the AR in the sample remaining in the injector/liner after injection yielding poorer analytical results [20].

AR can be analysed without derivatisation [20] or after converting them to their trimethylsilyl (TMS) ethers [20,23], though ethylation has also been used [37,45]. If AR have been derivitised prior to analysis, lower GC temperatures can be used [23,37]. Derivatisation also reduces the retention time of AR, and combined with a short column (DB1, 12 m), all AR from cereals (C15:0–C25:0) can be analysed in less than 20 min [23]. Using the method for the analysis of AR in cereals by GC–flame ionisation detector (FID), the detection limit was as low as 5 μg/g sample [21]. AR were found to have equal response factors using GC–FID [21].

4.3.1. GC–MS analysis of alkylresorcinols

AR give characteristic electron impact mass spectra, with distinct molecular ions and a base fragment at *m/z* 124, due to McLafferty rearrangement of the phenolic ring, and other minor fragments at *m/z* 123 due to the dihydroxytropylium ion formed by direct β-cleavage, *m/z* 137 due to γ-cleavage, and *m/z* 166 of unknown origin [15,56]. The *m/z* abundance ratio of 123/124 (Fig. 1) is *ca* 1:5 in accordance with *meta*-dihydroxy substitution in the benzene ring [57,58]. The mass spectra of the TMS derivatives of alkylresorcinols show the McLafferty base fragment at *m/z* 268 and another

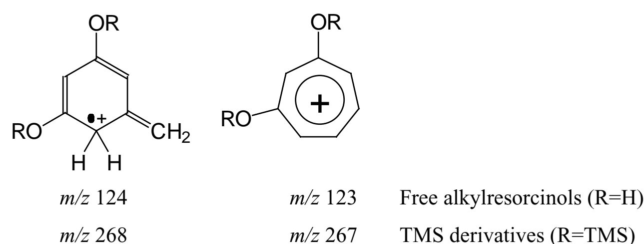
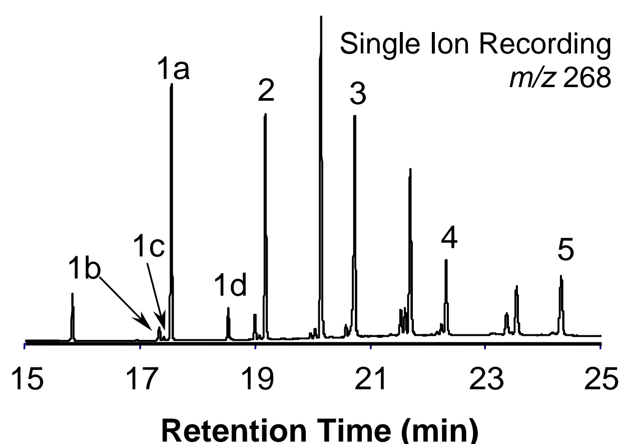


Fig. 1. Characteristic MS fragments for alkylresorcinols and their trimethylsilyl (TMS) derivatives from GC-electron impact MS detection.



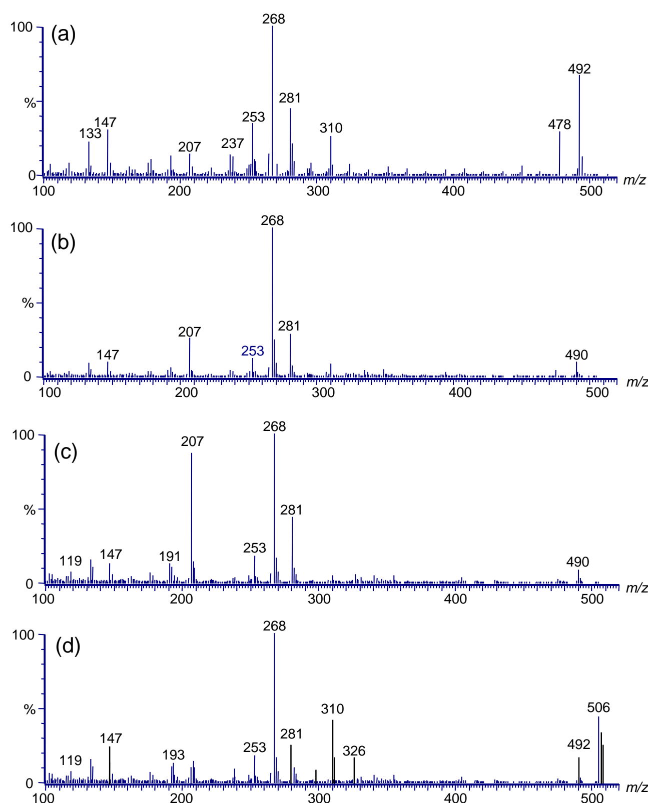


Fig. 2. GC–MS chromatogram of the TMS derivatives of alk(en)yl-resorcinols extracted from rye bran enriched in keto-alkylresorcinols. The mass spectra suggest different derivatives of heptadecylresorcinol (C17:0): (1a) heptadecylresorcinol; (1b) heptadecenylresorcinol a; (1c) heptadecenylresorcinol b; and (1d) keto-heptadecylresorcinol. The other labelled alkylresorcinols in the GC–MS chromatogram are: (2) nonyldecylresorcinol; (3) henicosylresorcinol; (4) tricosylresorcinol; (5) pentacosylresorcinol. Note that normally, keto derivatives are only present as a very small fraction of total alkylresorcinols in wheat and rye.

small fragment at *m/z* 281, though this derivatisation sacrifices some fragmentation and structural information [16].

Fig. 2 shows GC–MS chromatogram of rye bran AR enriched in keto derivatives. Peak 1a is C17:0 AR (with a saturated alkyl chain), peaks 1b and 1c are isomeric alkenylresorcinols with similar mass spectra but slightly different retention times, and peak 1d is a keto AR derivative. As this sample has been silylated, there is limited structural information from the mass spectra [15], and it is not possible to deduce the location of the double bond of 1b and 1c. Moreover, silylation seems to compromise the relative intensity of the fragment due to β-cleavage (*m/z* 123 for underivatised AR, *m/z* 268 for TMS derivatives) because of facile fragmentation between the α-methylene group and the β-keto group [16]. AR were detected at ng/g levels in the analysis of plasma by GC-electron impact MS using selective ion monitoring [23]. Fast atom bombardment mass spectrometry (FAB-MS) has also been used to structurally characterise AR [17,55].

4.4. High performance liquid chromatography

HPLC has also been applied to the analysis of AR [16,31,36,45,53,59–62]. As AR are mostly lipophilic, they do not lend themselves to easy analysis by HPLC. Prior to HPLC analysis, extracts may be filtered or purified by solid-phase extraction on silica [60], C8 or C18 [63]. Many of the methods using these clean up steps have not been validated for quantitative analysis. Unfortunately, injection volumes for HPLC have rarely been reported, but in the cases where this has been done, 50 μl of sample is injected [36,64]. While this large injection volume will compromise peak shape and separation, it might be necessary when solublising AR in polar solvents such as methanol.

Seitz [16] used a column temperature of 50 °C. The mobile phases previously used for AR analysis have mostly been various gradients starting with 100% water or a combination of water and methanol, increasing to 100% methanol at the end of the gradient program. Reusch and Sadoff [45] analysed AR from *Azotobacter vinelandii* using normal-phase HPLC with chloroform:methanol (90:10, v/v) as mobile-phase, or reversed-phase HPLC with methanol:water (99:1, v/v) as mobile phase. Bruce et al. [53] used tetrahydrofuran in a 0–100% gradient with acetonitrile/water/acetic acid (66:33:2, v/v/v) solution for preparative HPLC, while Paramashivappa et al. [62] used acetonitrile/water/acetic acid (80:20:1, v/v/v) with no gradient for their analysis of cardol (C15:0) from cashew nut shell liquid. Fig. 3 presents a HPLC chromatogram of saturated and unsaturated AR in rye obtained using a C18 column and a step-wise gradient of 85–100% methanol [60]. No AR with a C20:2 alkyl chain (Fig. 3), or even any AR with an even-numbered alkyl chain have been reported elsewhere in literature (Table 1).

The UV absorption for C15:0 in ethyl acetate has two absorption maxima, λ_{max} (nm) (ε_{max}) 275 (1569), and 282 (1571) [25]. Detection of AR with HPLC has been performed with either UV or diode array detectors set at 275–280 nm. In the study by Hengtrakul et al. [60], both orcinol (C1:0) and pentadecylresorcinol (C15:0) had the same response when detected at 280 nm indicating that the absorption is apparently due to the resorcinolic ring. No studies have employed other types of detectors although

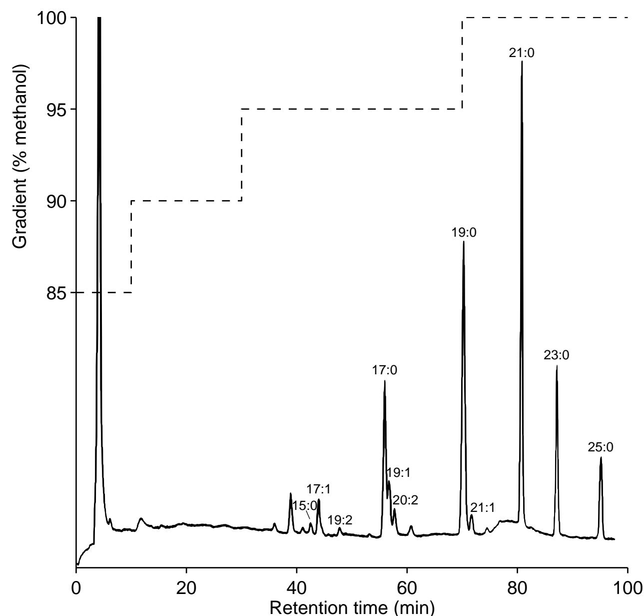


Fig. 3. HPLC chromatogram of alk(en)ylresorcinols extracted from rye bran. The figure is re-drawn with permission from the authors and Elsevier.

the fact that fluorescence methods have been previously used to analyse AR suggests that a fluorescence detector may also be useful for AR detection. To our knowledge, no published studies on AR have used LC–MS. Initial analysis of AR using HPLC coupled to a coularray detector in our laboratory used a C18 column and a solvent gradient from 90 to 100% methanol over 30 min. Peaks were detected between 550 and 750 mV.

Based on comparison of published chromatograms, current GC methods (see above) are superior to HPLC in their ability to separate different AR homologues, though HPLC offers more possibilities as it can be used for purification and may be more appropriate for analysis of the more water-soluble AR metabolites in biological fluids. Moreover, HPLC detectors coupled with post-column derivatisation may offer better selectivity than GC flame ionisation detectors with respect to keto derivatives of alkylresorcinols. Future method development for AR analysis by HPLC should test new types of column materials.

5. Analysis of alkylresorcinols in human samples

5.1. Analysis of intact alkylresorcinols in blood

Linko et al. [23] described a method for the analysis of AR in human plasma. In this method, the plasma is incubated with water to release AR from complexes of an unknown nature, possibly with proteins. AR are then extracted with diethyl ether and separated from neutral sterols in plasma by ion exchange chromatography with DEAE Sephadex in chloride form. AR are then silylated and analysed by GC–MS. When Rauls and Penney [64] analysed olivetol (pentylresorcinol) in rabbit serum using a simple HPLC method, diethyl ether extracts of serum were dried and dissolved in 50 μl acetonitrile, and injected onto a C18 HPLC column using acetonitrile–water (40:60, v/v) as the mobile phase and UV detection at 280 nm. Olivetol is less lipophilic than cereal AR (log P C5:0 = 3.5, cf. C17:0 = 9.4), but such a method may be suitable for AR metabolites. A problem with this analysis was that serum components, including olivetol are not very soluble in acetonitrile, and lipid build-up necessitated the frequent cleaning of the HPLC system.

5.2. Analysis of alkylresorcinols and their metabolites in urine

AR were recently found in intact and metabolised forms in ethyl acetate extracts of deconjugated human urine [24]. After deconjugation using β-glucourinidase/sulphatase, urinary extracts were separated on silica 60 TLC plates using chloroform:methanol (4:1, v/v) as a mobile phase, and then fractions were analysed using GC–MS. Intact AR were detected at the top of the TLC plate (R_f 1.0), while two AR metabolites, 3-(3,5-dihydroxyphenyl)-1-propanoic acid and 1,3-dihydroxybenzoic acid, were detected at R_f 0.24 indicating that AR are metabolised via β-oxidation of their alkyl chain. Further purification, in a larger scale than TLC, can be obtained from a semi-purified extract by anion exchange chromatography (Fig. 4). A urine sample (100 ml) was treated with β-glucuronidase/sulphatase to

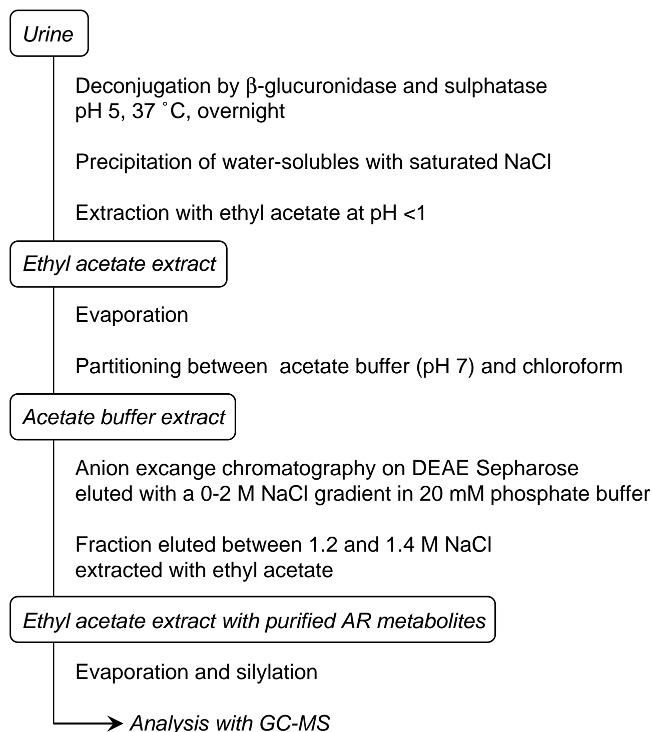


Fig. 4. Method for purification of alkylresorcinol metabolites in human urine for analysis by GC–MS.

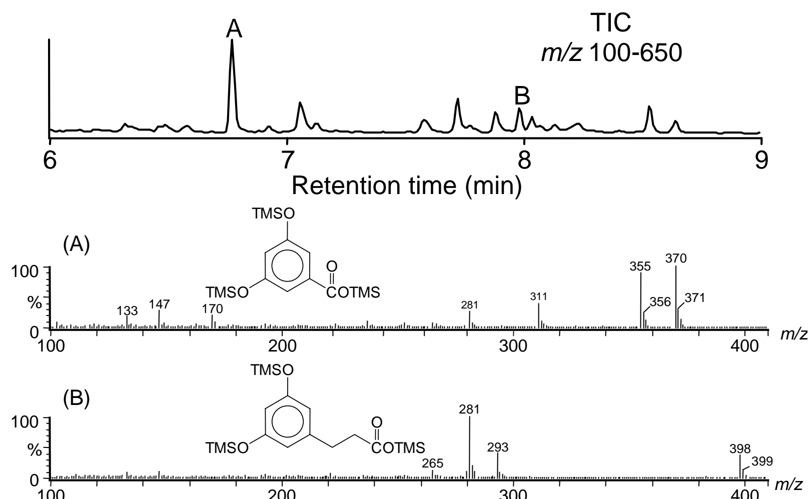


Fig. 5. GC–MS chromatogram and mass spectra of alkylresorcinol metabolites 1,3-dihydroxybenzoic acid (A) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (B), purified from human urine extract using ion exchange chromatography (Fig. 4).

allow deconjugation followed by addition of NaCl to saturation to salt out part of the water-soluble material present. The mixture was acidified and the AR metabolites extracted with ethyl acetate. This extract was then concentrated into a brown oil that was partitioned between acetate buffer (pH 7) and chloroform. The buffer phase was applied to the anion exchange column and eluted as described in Fig. 4. Fractions with AR metabolites were identified by Fast Blue B staining on a TLC plate, pooled and extracted with ethyl acetate. Results from GC–MS showed that the urinary metabolites 3-(3,5-dihydroxyphenyl)-1-propanoic acid and 1,3-dihydroxybenzoic acid were isolated with reasonable purity (Fig. 5) compared to fractionation with TLC [24].

5.3. Analysis of alkylresorcinols in illeal digesta

Illeostomy studies, where the content of a compound(s) of interest is measured in the illeal digesta from illeostomy operated subjects, are commonly used to estimate absorption in humans and pigs. AR can be extracted from the illeal digesta of humans and pigs fed rye, using the same extraction procedure as for whole cereal grains [22,31]. Although some other lipid soluble compounds are co-extracted with AR, particularly cholesterol and plant sterols, the peaks do not interfere with each other, allowing analysis with GC–FID as for cereal grains, though some optimisation (e.g. split ratio, concentration of samples) is required to prevent overloading of the column. For analysis by TLC, AR were also extracted from lyophilised rat faeces using 1 g faeces and 10 ml methanol:chloroform (1:1, v/v) and extracting for 24 h [65].

6. Needs for future method development

While current methods for analysis of AR in grains and foodstuffs using GC appear to be adequate, there is room for improvement and adaptation of new technologies. Analysis using HPLC, in particular, provides poorer separation and requires long run times. This may be improved by the use of newly developed stationary phases. The use of LC–MS is also yet to be explored, as it may prove useful for the analysis of AR and their metabolites in biological samples. One problem with the analysis of AR in these samples is to separate them from other lipids with similar properties. While methods such as saponification may separate sterols from other lipids, they are not suitable for AR as they appear to be difficult to extract from the soap fraction. When analysing AR from adipose tissue and erythrocyte samples, Ross et al. [52] used normal silica solid-phase extraction to separate AR from fats. However, while this method was useful qualitatively, the recovery of AR was variable due to incomplete elution of AR from the silica. As AR appear to be taken up via the lymph and stored in fatty tissues, it is important that a quantitative method for their analysis in fatty samples is developed.

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