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CONTENT OF ALKYLRESORCINOLS IN CEREALS GROWN IN LATVIA DETERMINED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV

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Cereal alkylresorcinols (ARs) are a group of phenolic lipids mainly found in the outer parts of grains of rye and wheat. They have been suggested for use as selective biomarkers for intake of whole grain and bran products of these cereals. Consumption of whole grains and whole grain products has been associated with reduced risk of developing chronic diseases, such as cardiovascular disease, diabetes type 2, obesity and some types of cancer. In this article a sensitive and rapid method of High Performance Liquid Chromatography with UV detection for quantitative determination of ARs in the cereals grown in Latvia is described. Instrument detection limits (IDL) were determined for C17:0, C19:0 and C21:0 homologues (coefficient of variation < 3%). According to the results of these studies, ARs were found in rye (87.1–112.0 mg/100 g), wheat (24.0–40.2 mg/100 g), triticale (32.1–74.4 mg/100 g), and in small amounts in barley (2.2–3.7 mg/100 g), but not in oats.

Key words: *alkylresorcinols, HPLC-UV, whole grain cereal.*

INTRODUCTION

Epidemiological studies have associated a diet rich in whole grain and cereal products with decreased risk of several chronic diseases, e.g. coronary heart disease (Liu *et al*., 1999), diabetes type 2 (Munter *et al*., 2007), obesity (Fung *et al*., 2001) and some cancers (Jacobs *et al*., 1998), although the mechanisms are poorly understood (Slavin, 2003). Also, there are concerns about the methodology used to determine dietary intake in epidemiological studies (Bingham *et al*., 2003). Studies are further complicated by the fact that consumers might have difficulty in identifying whole grain products (Lang and Jebb, 2003). Whole grain intake assessment also shares the common weaknesses, such as poor precision and bias, with assessment methods for other dietary constituents (Kaaks, 1997; Bingham *et al*., 2003). The use of a biomarker can establish complementary or alternative measurements of whole grain intake and is expected to overcome some of these obstacles (Ocke and Kaaks, 1997; Ross *et al*., 2004a). Alkylresorcinols (ARs) have been proposed to be biomarkers of whole grain wheat and rye intake (Ross *et al*., 2004b; Linko and Adlercreutz, 2005).

Alkylresorcinols are amphiphilic 1,3-dihydroxybenzene derivatives with an odd-numbered alkyl chain at position 5 (Fig. 1). The alkyl chain in cereal ARs varies from 15 to 27 carbon atoms. They are located in the intermediate layers between pericarp and testa in the grain (Landberg *et al*., 2008), and are found in high concentrations only in whole grain and/or bran products of wheat (31.7–143.9 mg/100 g dry matter, DM) and rye (36.0–320.0 mg/100g DM), and in very small amounts in refined flour (white bread, most breakfast cereals, pasta) or in any other commonly consumed foods (Ross *et al*., 2003; Ross *et al*., 2004a; Ross and Kochhar, 2009; Annica *et al*., 2010). They are also present in triticale (43.9–64.7 mg/100 g DM), and in small amounts in barley (4.2–5.1 mg/100 g DM), but not in oats (Ross *et al*., 2003).

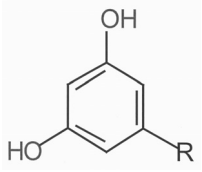

Fig. 1. Structures of alkylresorcinols commonly found in cereal grains.

R	ARs names	Short names	Molecular weight
$C_{15}H_{31}$	5-n-pentadecylresorcinol	C15:0	320
$C_{17}H_{35}$	5-n-heptadecylresorcinol	C17:0	348
$C_{19}H_{39}$	5-n-nonadecylresorcinol	C19:0	376
$C_{21}H_{43}$	5-n-heneicosylresorcinol	C21:0	404
$C_{23}H_{47}$	5-n-tricosylresorcinol	C23:0	432
$C_{25}H_{51}$	5-n-pentacosylresorcinol	C25:0	460

In vitro, ARs have been reported to have anticancer, enzyme-inhibiting, and DNA-cleaving properties (Kozubek and Tyman, 1999). ARs are reported to be antioxidants (Winata and Lorenz, 1996), although they are weak antioxidants *in vitro* compared with α-tocopherol (Kamal-Aldin *et al*., 2001; Ross *et al*., 2004b; Korycińska *et al*., 2009). Another *in vitro* study (Ross *et al*., 2004c) showed that ARs significantly inhibited the conversion of γ-tocopherol to its water-soluble hydroxychroman metabolite, indicating that ARs may increase γ-tocopherol's levels via inhibition of tocopherol metabolism *in vivo*. A recent *in vitro* study (Stasiuk *et al*., 2008) showed that ARs isolated from rye grain decreased the enzymatic activity of acetylcholinesterase of erythrocytes. These findings are of interest due to the growing evidence that acetylcholinesterase could participate in the pathological processes of Alzheimer's disease. The biological activities of dietary and purified ARs have been previously reviewed (Kamal-Aldin *et al*., 2001; Ross *et al*., 2004b).

Different chromatographic methods for analysis of ARs in whole grain and cereal products have been developed over years. Gas chromatography — mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) are commonly used for the routine quantitative determination of ARs (Ross *et al*., 2001; Mattila *et al*., 2005; Landberg *et al*., 2006). For the rapid determination of total ARs content in cereal grain products, colorimetric method after formation of intense red complex with fast blue salt (the diazonium salt Fast Blue B) has been used (Mattila *et al*., 2005; Kulawinek and Kozubek, 2008). Fast Blue B has been used for quantitative determination of ARs in cereal grain extracts, but without reported validations of the methods used (Andersson *et al*., 2008). Recently, the HPLC method for the analysis of intact ARs in cereals using coularray detection (HPLC-CA) has been suggested. Use of the CA detector allowed detection of low concentrations of ARs in white wheat flour, which had not been reliably detected using previous methods (Ross and Kochhar, 2009).

In this paper we present a rapid and sensitive HPLC method for the analysis of ARs in whole grain cereals with ultra violet (UV) detector. This new method was developed based on method by Ross *et al.* (2001). HPLC-UV method is rather simple and does not require derivatization or any other sample pretreatment. However, validation parameters should be performed in next research.

MATERIALS AND METHODS

Chemicals and standards. The Alkylresorcinol standard C17:0 used had ≥ 95% purity as determined by the company from where it was purchased (Sigma-Aldrich, St.Luis, USA). Homologues C19:0 and C21:0 were of > 98% purity as determined by the company from where they were purchased (Reseachem Life Science, Burgdorf, Switzerland). All standards were prepared as stock solutions at 2.5 mg/ml in methanol/ethanol mixture and were stored in a freezer at –18 °C. All solvents were of HPLC-grade (Sigma-Aldrich, St.Luis, USA) and were used without further purification.

Cereal samples. Rye samples (n = 2) and triticale samples (n = 2) were provided by the State Priekuļi Plant Breeding Institute (Priekuļi, Latvia), and grains were harvested in 2010. Common spring wheat samples (n = 2) and barley samples (n = 2) were provided by the State Stende Cereals Breeding Institute (Stende, Latvia), and grain were harvested in 2010. Common winter wheat samples (n = 2), triticale samples (n = 1), rye samples (n = 4) and oat samples (n = 2) were also provided by the State Stende Cereals Breeding Institute (Stende, Latvia), and grains were harvested in 2011.

Extraction of samples. According to a slightly modified method of Ross *et al.* (2001), alkylresorcinols were extracted from 1.00 g cereal grains (coarsely ground in a coffee grinder, then milled with a mortar and pestle) with 40 ml of ethyl acetate for 24 h with continuous shaking at room temperature. The extracts were centrifuged at 4400 rpm for 10 min and the supernatants (4 ml) were then evaporated to dryness in a rotary evaporator (Heidolph Laborata 4001 Efficient System, USA). Methanol (1 ml) was added and samples were filtered through 0.45 μm filters before injection into the HPLC. All samples were extracted in duplicates, and the results are reported on a basis of fresh weight (FW). Alkylresorcinol homologues C17:0–C21:0 were quantified using an external standard method. Calibration curves were prepared with the following concentrations: 0, 5, 20, 50, 70 and 100 μg/ml. Alkylresorcinol homologues C15:0, C23:0 and C25:0 were identified according to their spectra (Kulawinek and Kozubek, 2008) and determined semiquantitatively by method of internal standard, with C21:0 homologue as an internal standard (10 μg/ml). Relative response factors for C15:0, C23:0 and C25:0 homologues were calculated by taking into account the amount of homologue (based on molecular weight) injected into chromatographic column versus homologue C21:0. All quantifications were based on peak area.

HPLC-UV analysis. Method development and analysis were carried out on a Shimadzu Prominence HPLC with a SPD-20A Diode Array Detector scanning between 260 and 295 nm. Separation of alkylresorcinol homologues was done with a Symmetry C-18 (4.6 × 150 mm, 5 μm) column from Waters. The gradient programme was used at a flow rate 1.00 ml/min starting with a mobile phase of methanol/water (80/20) for 5 min, followed by methanol/water (99.2/0.8) for 25 min. The temperature of the column oven was set on 30 ^{o}C and 40 μl of each sample were injected into the chromatographic column. Instrument detection limits (IDL) were determined for C17:0 homologue (0.48 μg/ml), for C19:0 homologue (0.44 μg/ml) and for C19:0 homologue (0.47 μg/ml), with a coefficient of variation < 3%. IDL was determined to be signal (peak area) that is greater than the system noise. Within a specified probability, IDL was calculated according to the formula (Wells *et al*., 2011):

$X_{IDL} = t_{\alpha,n} \cdot$ relative standard deviation $\cdot$ amount standard / 100%, where

t ($\alpha = 0.05$; n = 8) = 2.3646 (Student T-table).

RESULTS

In the present study, a developed HPLC-UV method for quantitative determination of ARs in the cereals is described. A good homologue separation (an appropriate gradient), IDL lower then 0.5 mg/100 g and a relatively short time of total analysis, showed that this method is sensitive and rapid (Fig. 2).

The UV spectrum of the homologues in methanol is shown on Fig. 3. All the homologues showed characteristic spectra with maximum absorbance at $\lambda = 275$ nm and $\lambda = 280$ nm.

Quantification of C15:0, C23:0 and C25:0 alkylresorcinol homologues was problematic due to the lack of these standards. We attempted to overcome this by using C21:0 as an internal standard. All data were converted to fresh weight (FW), considering that the moisture content of cereals is about 14% (Anonymous, 1992). Alkylresorcinols were found in rye, wheat, triticale, and barley, but not in oats (Table 1).

DISCUSSION

Results of the analysis of rye grains showed that ARs levels varied from 87.1 to 112 mg/100 g FW. These values are comparable to literature: 61.9–65.5 mg/100 g FW by Ross *et al.* (2003), 92.7 mg/100 g FW by Mattila *et al.* (2005), 134.2 mg/100 g FW by Landberg *et al.* (2009).

Average ARs concentration in the wheat samples analysed in this study was 34.8 mg/100 g FW. This value is comparable to the previously reported data: 28.8–42 5 mg/100 g FW by Chen *et al.* (2004), 42.0–42.1 mg/100 g FW by Landberg *et al.* (2009). However, our results for wheat samples are

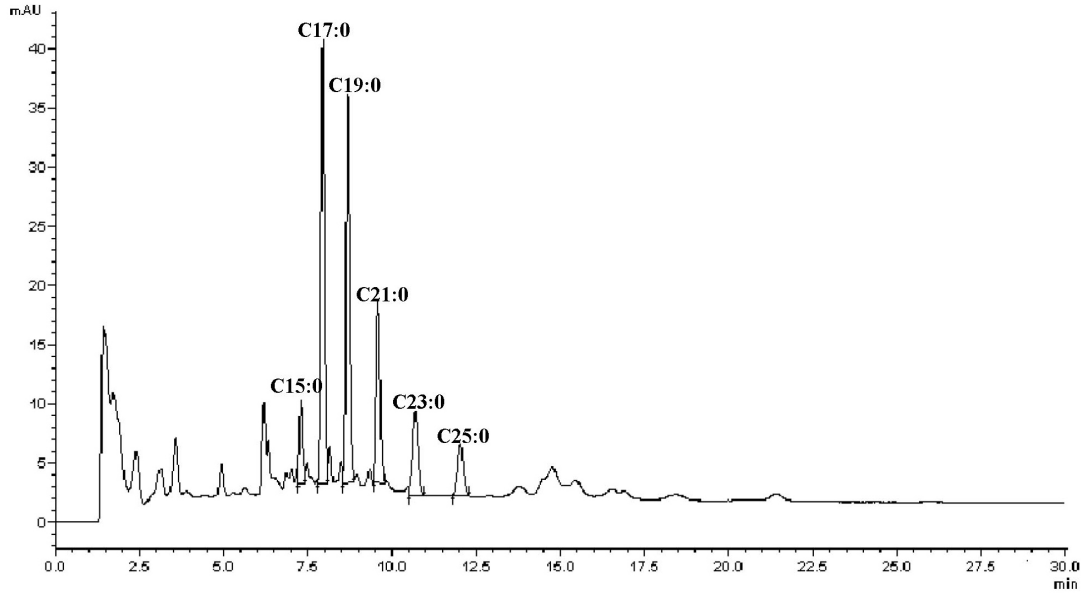

Fig. 2. HPLC-UV chromatogram of alkylresorcinol homologues in rye (Amilo) after extraction with ethyl acetate for 24 h at room temperature, centrifuged, evaporated and redissolved in methanol (1 ml). See text for HPLC conditions.

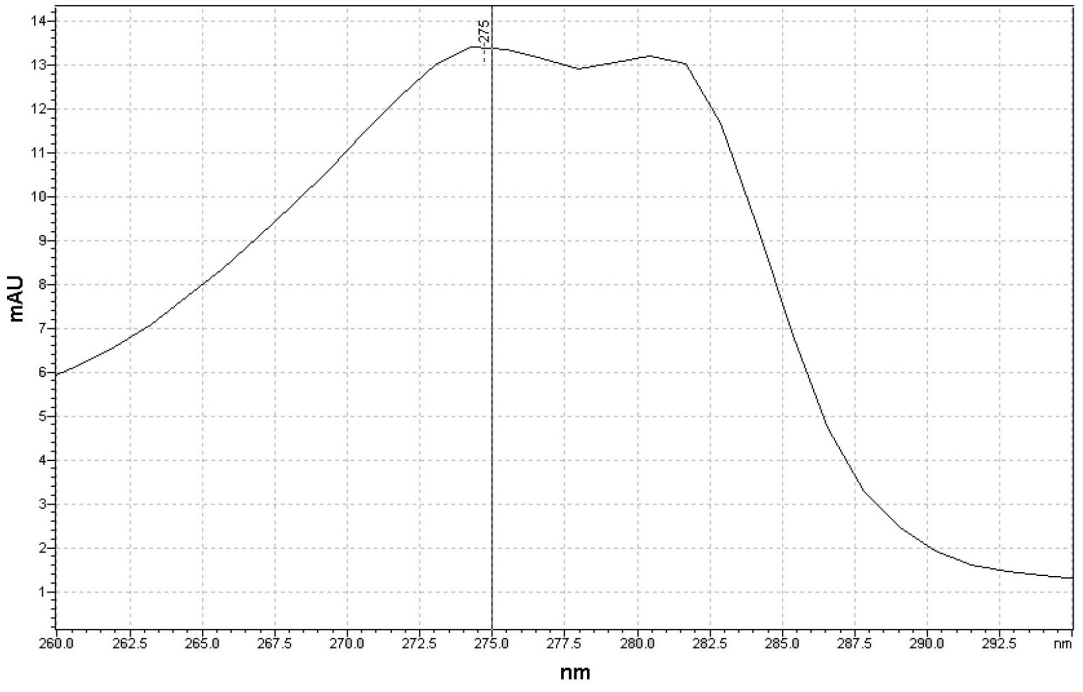

Fig. 3. The characteristic spectrum of alkylresorcinol homologues in methanol.

Table 1

CONTENT OF ALKYLRESORCINOLS IN CEREAL GRAINS (mg/100 g of fresh weight)[1]

Cereal	Cultivar	C15:0	C17:0	C19:0	C21:0	C23:0	C25:0	Total ARs
Rye	04014-4	0.8 ± 0.4[2]	24 ± 4	28±2	21.8 ± 0.3	21 ± 2 [2]	12 ± 1 [2]	107.6
	Amilo	2.18 ± 0.05	30.5 ± 0.7	26.8 ± 0.5	14.3 ± 0.5	9.6 ± 0.7	4.6 ± 0.5	88.0
	Kaupo	1.14 ± 0.07	31.8 ± 0.1	29.5 ± 0.1	19.7 ± 0.2	14.6 ± 0.7	8.9 ± 0.1	105.6
	Dankovskie Diament	2.8 ± 0.3	34 ± 2	30 ± 2	19 ± 1	14.5 ± 0.3	8.8 ± 0.6	109.1
	9918	1.75 ± 0.02	25 ± 2	25 ± 1	16.3 ± 0.8	10.9 ± 0.9	8.1 ± 0.4	87.1
	Picaso	2.0 ± 0.4	34 ± 4	31 ± 2	22 ± 2	14 ± 2	9 ± 1	112.0
Common spring wheat	L920 Uffo	nd[3]	1.96 ± 0.05	13.7 ± 0.2	20.2 ± 0.6	4 ± 1	0.38 ± 0.03	40.2
	L934 Eminent	nd	nd	5 ± 1	11 ± 2	7.7 ± 0.2	0.343 ± 0.004	24.0
Common winter wheat	Spelta (ID)	nd	3.9 ± 0.1	8.3 ± 0.6	18.3 ± 0.4	5.8 ± 0.2	0.97 ± 0.09	37.3
	96-58	nd	5 ± 1	13.6 ± 0.2	16.7 ± 0.1	2.1 ± 0.1	0.34 ± 0.02	37.7
Triticale	Falmoro	nd	nd	3.2 ± 0.3	9.5 ± 0.7	16 ± 3	3.4 ± 0.2	32.1
	Nazaret	nd	4 ± 2	12 ± 4	15 ± 2	11.1 ± 0.4	2.0 ± 0.2	44.1
	9402-3	nd	12.1 ± 0.7	23 ± 1	21.0 ± 0.7	15 ± 2	3.28 ± 0.01	74.4
Barley	Jet	nd	nd	nd	1.5 ± 2	0.17 ± 0.02	2.0 ± 0.8	3.7
	1196	nd	nd	nd	1.2 ± 0.8	nd	1.0 ± 0.7	2.2
Oats	S-156	nd	nd	nd	nd	nd	nd	nd
	Arta	nd	nd	nd	nd	nd	nd	nd

[1] Values are average ± standard deviation

[2] C15:0, C23:0 and C25:0 – semiquantitative results.

[3] nd = not detected (IDL of C17: – C21:0 < 0.5 mg/100 g).

lower than those obtained by Ross *et al.* (2003) — ARs levels in common wheat grains varied from 42 to 122.9 mg/100 g FW, by Mattila *et al.* (2005) — whole wheat flour contained 75.9 mg/100 g FW of ARs and, also by Kulawinek and Kozubek (2008) ground wheat grains contained 57.8 mg/100 g FW of ARs (for C15:0–C 19:0 homologues). This variation is probably due in part to differences in analytical methods and also the ARs content of cereals appears to be highly variable, depending on cultivar and environmental conditions.

Alkylresorcinol concentration in triticale varied from 32.1 to 74.4 mg/100 g FW, and is comparable to those reported previously (37.8–55.6 mg/ 100g FW) by Ross *et al.* (2003). Unfortunately, this cereal is not commonly consumed by humans.

The total ARs concentration in barley varied from 2.2. to 3.7 mg/100 g FW. These values are in line with those reported by Ross *et al.* (2003 — from 3.6 to 4.4 mg/100 g FW, by Mattila *et al*. (2005) — 3.2 mg/100 g FW, although lower than reported by Landberg *et al.* (2009) — 7.7 mg/100 g FW. The difference is probably because of use of different extraction solvents and, of course, different environmental conditions. Also, a cereal specific correction factor was used by Landberg *et al.* (2009). The factor was calculated by taking into account differences in molecular weight and the average relative homologue composition (determined by GC) of the particular cereal. None was found in oats.

According to our results, the main ARs homologues in rye are C17: 0 and C19:0; in wheat are C19:0 and C21:0; in triticale are C21:0 and C23:0; in barley are C21:0 and C25:0. In addition, the mean total content of ARs in analyzed grain samples was in the following sequence: rye > triticale > wheat > barley > oats. Nonetheless, more data need to be collected about ARs concentration in different cereals breed in Latvia.

This HPLC-UV method is appropriate for ARs determination in cereals, due to its simplicity, rapidity and sensitiveness.

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ALKILREZORCĪNU SATURA NOTEIKŠANA LATVIJĀ AUDZĒTOS GRAUDOS AR AUGSTI EFEKTĪVU ŠĶIDRUMU HROMATOGRĀFIJU AR ULTRAVIOLETU DETEKTORU

Graudaugu alkilrezorcīni (AR) ir savienojumu grupa, kur viena molekulas daļa ir fenola atlikums, bet otra ir lipīdu atlikums. AR lielā daudzumā atrodas grauda ārējos slāņos (graudapvalkos, aleirona slānī) galvenokārt rudzu un kviešu graudos, kā arī nelielos daudzumos miežos. Rafinētos produktos AR ir niecīgos daudzumos vai vispār nav. Tieši tāpēc AR tiek piedāvāts lietot par selektīvu pārtikas biomarķieri, lai patērētājiem būtu vieglāk saprast, kurš produkts ir pilngraudu vai satur pilngraudus. Pilngraudu produktu patērēšana ir saistīta ar samazinātu risku saslimt ar dažādām hroniskām slimībām: aptaukošanās, 2. tipa diabēts, sirds slimības un vēzis. Šajā publikācijā tiek prezentēta ātra un jutīga augsti efektīvu šķidrumu hromatogrāfija ar ultravioletu detektoru – metode alkilrezorcīnu noteikšanai Latvijā audzētos graudaugos. Instrumenta detektēšanas robeža tika noteikta C17:0, C19:0 un C21:0 homologiem (variācijas koeficients < 3%). AR saturs rudzos bija robežās no 87,1 līdz 112,0 mg/100g, kviešos no 24,0 līdz 40,2 mg/100g, tritikālē no 32,1 līdz 74,4 mg/100g, nedaudz miežos no 2,2 līdz 3,7 mg/100g, bet auzās netika atrasts.