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High-performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid glucosides in flaxseed by alkaline extraction

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Abstract

A HPLC method was developed for the analysis of secoisolariciresinol diglucoside (SDG) and hydroxycinnamic acid glucosides in milled defatted flaxseed flour. Direct extraction by 1 *M* NaOH for 1 h at 20 °C resulted in a higher yield than that obtained by hydrolysis of alcoholic extracts. An internal standard, *o*-coumaric acid, was used and the method was found to be easy, fast, and with good repeatability. On dry matter basis, different samples of flaxseeds varied considerably in their content of (+)-SDG (11.9–25.9 mg/g), (−)-SDG (2.2–5.0 mg/g), *p*-coumaric acid glucoside (1.2–8.5 mg/g), and ferulic acid glucoside (1.6–5.0 mg/g).

Keywords: *Linum usitatissium*; Plant materials; Secoisolariciresinol diglucoside; Glucosides; Hydroxycinnamic acid glucosides; Lignans; Coumaric acid glucoside; Ferulic acid glucoside

1. Introduction

Flaxseed (*Linum usitatissium*) is a rich source of the plant lignan secoisolariciresinol (SECO) [1–3] and other phenolic compounds including *p*-coumaric and ferulic acids [4]. In flaxseed, SECO is present as a glycoside, secoisolariciresinol diglucoside (SDG) [2], which is further linked with 3-hydroxy-3-methylglutaric acid (HMGA) to form oligomers [5,6] (Fig. 1). *p*-Coumaric and ferulic acids are also present in glucosidic forms as part of larger compounds of yet an unknown structure [2].

In humans and animals, SECO is transformed by the anaerobic intestinal microflora into the mammalian lignans, enterolactone (EL) and enterodiol (ED) [7]. Mammalian lignans are capable of producing weak estrogenic or antiestrogenic effects by binding at low levels to estrogen receptors [8,9]. The antioxidant activities of EL, ED and their precursors, SECO and SDG, have been established in several assays in both lipid and aqueous in vitro systems [10–13]. SDG was claimed to be effective in reducing hypercholesterolemic atherosclerosis by reducing oxidative stress and lowering serum levels of cholesterol and low-density lipoprotein (LDL)-cholesterol and raising serum levels of high-density lipoprotein (HDL)-cholesterol [14]. Additionally, low serum EL concentration in man has been associated with

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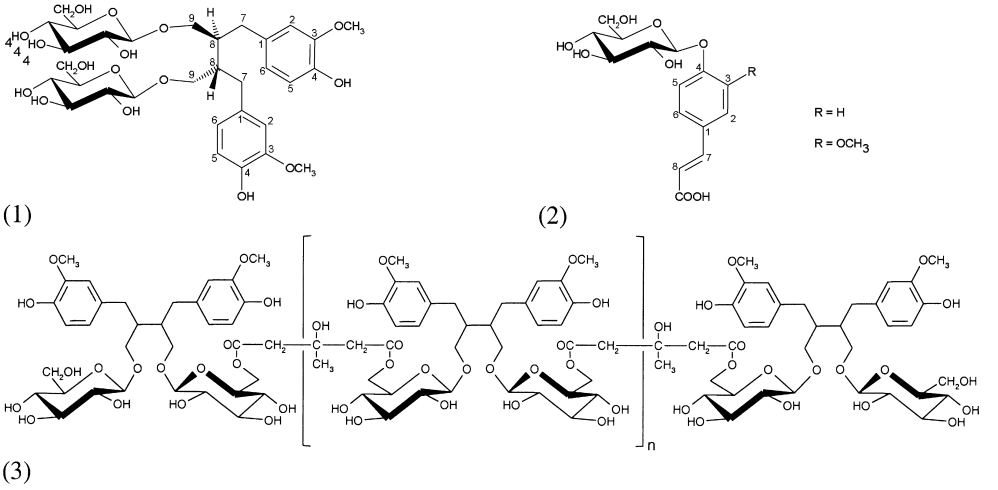

Fig. 1. The chemical structures of (+)-secoisolariciresinol diglucoside {SDG; 2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediglucoside} (1) and 4-*O*-β-D-glucopyranosyl-*p*-coumaric acid; R=H, and 4-*O*-β-D-glucopyranosyl ferulic acid; R=OCH_3 (2). The chemical structure of the oligomers composed of (+)-SDG and HMGA residues in flaxseed (3).

enhanced lipid peroxidation in vivo and might, therefore, be a risk factor for cardiovascular disease [15,16]. Dietary supplementation with SDG was shown to be effective in inhibiting the development of type-1 [17,18] and type-2 diabetes [19,20]. With respect to cancer, supplementary SDG was reported to inhibit mammary tumorigenesis in rats [21], and pulmonary metastasis of melanoma cells in mice [22]. EL and ED were claimed to significantly inhibit the growth of human colon tumor cells [23].

Analysis of SDG in milled defatted flaxseed flour has been performed by high-performance liquid chromatography (HPLC) after hydrolysis of extracted oligomers with base, acid, or enzyme. Extraction of the oligomers was performed with dioxane–ethanol (1:1, v/v) [3], aqueous ethanol [24], or aqueous methanol [25–28]. Alkaline hydrolysis of the extract releases SDG by cleavage of the ester linkages in the SDG–HMGA oligomer [3,24–26]. Transmethylation of carboxylic groups of the hydroxycinnamic acid glucosides to less polar derivatives occurs during hydrolysis with alkali in methanol [3]. The phenolic compounds in base hydrolysate are kept stable by adjusting the pH to 3. When aqueous acid hydrolysis is used, the aglycone (SECO) is released from the oligomers by deglycosylation, and is transformed into its anhydrous form anhydrosecoisolariciresinol [25]. Obermeyer and co-workers [29,30] used enzymatic hydrolysis with a mixture of glucuronidase and sulfatase to mildly release SECO from the glucose units.

Quantitative determination of SDG, and hydroxycinnamic acid glucosides in flaxseed is restricted by incomplete extraction from the matrix, inefficient sample clean-up, inability to measure naturally occurring aglycones or extensive losses during analysis due to formation of artefacts and aglycones. Due to the growing interest in flaxseed phenols, there is a need for development of simple, fast and reliable methods for the quantitative determination of SDG and hydroxycinnamic acid glucosides in flaxseeds and flaxseed products. No data have hitherto been provided for the content of hydroxycinnamic acid glucosides in flaxseed. This paper presents a new HPLC method for the analysis of SDG and other phenolic compounds in flaxseed eliminating the alcoholic extraction step by using direct alkaline hydrolysis.

2. Experimental

2.1. Materials

A pressed flaxseed cake (Alternativ Förädling,

Glanshammar, Sweden) was used for method development after milling (Retsch type ZM 1, Haan, Germany, 0.5 mm screen) and defatting with *n*-hexane (1:4, w/v, 2×24 h). Seventeen flaxseed samples were obtained from Hushållningssällskapet (harvest 2001, Örebro, Sweden) and 10 flaxseed samples were obtained from Svalöf Weibull (harvest 2000, Svalöv, Sweden). For analysis, samples (5 g) were milled and defatted with *n*-hexane (30 ml) in steel tubes together with steel balls during intense shaking for 1 h [31]. The milled and defatted flaxseed flour (DFF) was filtered and washed with *n*-hexane (30 ml). Dry matter was determined by drying 0.2 g sample at 105 °C for 17 h in triplicates.

SDG was isolated as described by Johnsson et al. [3]. Syringaresinol, pinoresinol, and matairesinol (gifts from Lennart Lundgren, SLU, Uppsala, Sweden) were used to identify chromatographic peaks. Ferulic acid, *p*-coumaric acid, *o*-coumaric acid, protocatechuic acid, *p*-hydroxybenzoic acid, chlorogenic acid, (+)-catechin, vanillic acid, caffeic acid, and syringic acid (Sigma–Aldrich, Taufkirchen, Germany) and sinapic acid (Fluka, Buchs, Switzerland) were used to identify chromatographic peaks and select an internal standard. Solvents and reagents for chromatography were of analytical grade and were used without further purification (E. Merck, Darmstadt, Germany).

2.2. Extraction with dioxane–ethanol followed by alkaline hydrolysis

SDG and hydroxycinnamic acid glucosides were analyzed by a method slightly modified from that previously reported [3] by introduction of an internal standard and omission of the solid-phase extraction step. DFF (500 mg) was mixed in a 35-ml centrifuge tube with 10 ml 1,4-dioxane–95% aq. ethanol (1:1, v/v) and 0.2 ml internal standard (*o*-coumaric acid, 3.8 mg/ml methanol) and shaken for 16 h at 60 °C in a water bath. After two centrifugations (900 *g*, 20 min) with intermittent washing with 10 ml 1,4-dioxane–95% aq. ethanol (1:1, v/v), liquid phases were combined and evaporated at 40 °C using a rotavapor. The dried extracts were hydrolysed using 0.3 *M* aqueous sodium hydroxide (10 ml) at room temperature under constant rotation. After hydrolysis, samples were acidified to pH 3 using 2 *M* sulfuric acid, and the volume was made to 25 ml. Samples were filtered through 0.45 μm Puradisc 25 PP (Whatman, Kent, UK) before injection into the HPLC system. Effect of extraction and hydrolysis on yield was studied with a full factorial experimental design, involving four extraction times (1, 4, 16, 48 h) and four hydrolysis times (1,4, 16, 48 h). General linear model analysis was performed using Minitab 13.31 for Windows (Minitab, State College, PA, USA).

2.3. Extraction by direct alkaline hydrolysis

DFF (100 mg), vortexed together with 1.0 ml internal standard (*o*-coumaric acid, 0.8 mg/ml methanol), was continuously mixed with 4 ml distilled water and 5 ml 2 *M* aqueous sodium hydroxide for 1 h at 20 °C by constant rotation. The hydrolysate was acidified to pH 3 using 2 *M* sulfuric acid and centrifuged (1700 *g*, 10 min). The supernatant was recentrifuged in microcentrifuge tubes (11 000 *g*, 5 min) to a clear liquid phase. The liquid phase (0.6 ml) was mixed with 95% aq. ethanol (0.9 ml) in 1.5-ml microcentrifuge tubes, left at room temperature for at least 10 min and centrifuged (11 000 *g*, 5 min) to precipitate and remove water-soluble polysaccharides and proteins. Before HPLC analysis, the sample was filtrated through 0.45 μm Whatman Puradisc 25 PP (Fig. 2b).

Experimental designs were created to find conditions giving the highest yields of phenolic compounds. The effect of temperature and concentration of alkali on the yield of phenolic compounds was studied using a full factorial experimental design involving three temperatures (20, 30, 40 °C) and three concentrations of alkali (0.3, 1 and 1.7 *M*) with two replicates. The effect of the hydrolysis time on yield was studied during five time periods (0.5, 4, 16, 32, and 48 h) at 20 °C with two replicates. Matrix effects were studied using a full factorial experiment design involving three levels of DFF (100, 200, 300 mg) with three levels of spiked I.S. (0.1, 0.3, 0.5 mg). General linear model analysis was performed using Minitab 13.31. The repeatability of the method was evaluated by two analysts, each carrying out six replicate analyses per day for 3 days.

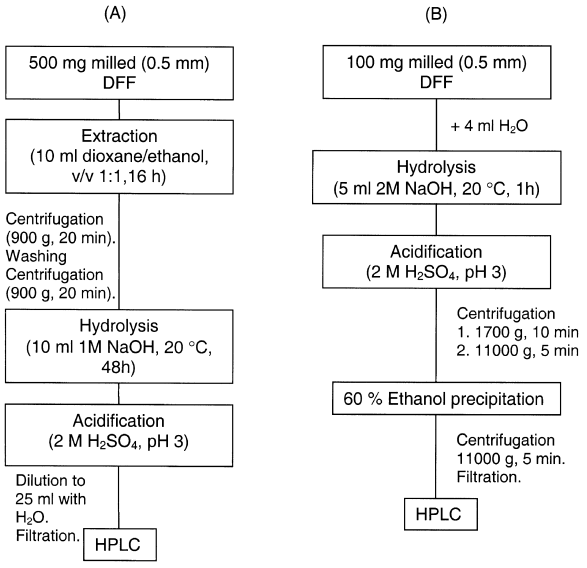

Fig. 2. Scheme of the two methods used for analysis of phenolic compounds in flaxseed. (A) Dioxane–ethanol extraction followed by alkaline hydrolysis. (B) Direct alkaline hydrolysis.

2.4. High-performance liquid chromatography

Dionex PDA-100 (Dionex, Sunnyvale, CA, USA) HPLC equipment with a UV–Vis diode array detector and Chromelion software was used for the analysis. The separation was performed at 25 °C on an Econosil HPLC column RP C_{18} (5 μm, 250×4.6 mm, Alltech, Deerfield, IL, USA). The injection volume was 10 μl for the dioxane–ethanol method and 30 μl for the method using direct alkaline hydrolysis. The mobile phase consisted of two solvents (A) 5% acetonitrile in 0.01 *M* phosphate buffer, pH 2.8, and (B) acetonitrile, and the separation was performed using the following gradient of A–B: 0 min (100:0, v/v), 30 min (70:30, v/v), and 32 min (30:70, v/v) at a flow-rate of 1 ml/min. Standard solutions of *p*-coumaric acid, ferulic acid, SDG, and *o*-coumaric acid were prepared in concentration levels (10, 20, 30, 40, 50, 70, 100, 150 μg/ml) and used to construct calibration curves of the standards. The phenolic compounds were expressed as mg phenolic compound per g sample on dry matter basis. The yields of *p*-coumaric acid glucoside and ferulic acid glucoside were calculated using the slopes of the calibration curves of the aglycones corrected for the molecular mass of the glucosides and (−)-SDG was quantified using the same slope as (+)-SDG. The relative retention time (RRT) was determined by calculating the retention time of each phenolic compound against the retention time of the internal standard (*o*-coumaric acid).

3. Results and discussion

3.1. Identification and quantification of phenolic compounds and choice of internal standard

Fig. 3 presents a typical HPLC chromatogram, obtained after direct alkaline hydrolysis, showing peaks corresponding to *p*-coumaric acid glucoside (13.3 min, RRT=0.49), ferulic acid glucoside (15.2 min, RRT=0.56), (+)-SDG (21.0 min, RRT=0.77), (−)-SDG (22.5 min, RRT=0.83), and other minor unknown peaks. The chromatograms obtained by direct alkaline hydrolysis were similar to those obtained by the method developed by Johnsson et al. [3]. *p*-Coumaric acid glucoside, ferulic acid glucoside, and (+)-SDG were previously isolated and their structures were confirmed by nuclear magnetic resonance (NMR) spectroscopy [3,4]. The peak eluting at 21.0 min was identified as (+)-SDG by comparison with the authentic standard [3] and by liquid chromatography–mass spectrometry (LC–MS) where it showed an ion at *m*/*z* 709, corresponding to the molecular mass of SDG plus sodium [4]. The peak eluting at 22.5 min showed a similar MS spectra to that of SDG (results not published) and was, therefore, identified as (−)-SDG, a diastereoisomer of SDG at C-8, previously described in literature [32,33].

The retention times of several standard phenolic compounds were compared with those of peaks resulting from flaxseed samples. *o*-Coumaric acid (27.2 min, RRT=1.00), a compound not naturally found in flaxseed, was the most suitable internal standard among the tested compounds [protocatechuic acid (RRT=0.40), *p*-hydroxybenzoic acid (RRT=0.55), chlorogenic acid (RRT=0.58), (+)-catechin (RRT=0.56), vanillic acid (RRT=0.63), caffeic acid (RRT=0.65), and syringic acid (RRT=0.67)]. The slopes (Table 1) were calculated from

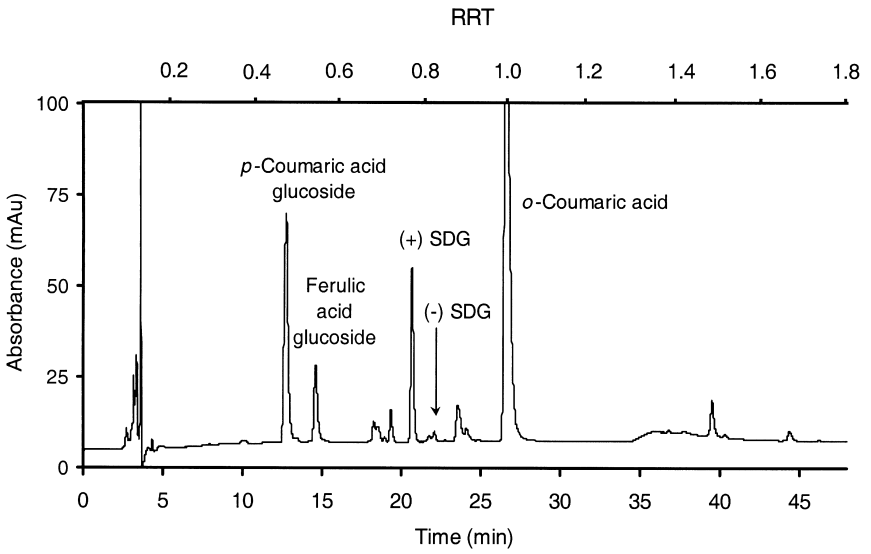

Fig. 3. A typical HPLC chromatogram of a flaxseed sample after direct alkaline hydrolysis (280 nm).

the calibration curves of ferulic acid, *p*-coumaric acid, (+)-SDG and *o*-coumaric acid. The correlation coefficients (R) for calibration curves were >0.99 (Table 1), indicating linearity for all analyzed phenolic compounds in the used concentration range.

Other compounds previously reported in flaxseed include other phenolic acids, e.g., sinapic acid, ferulic acid, and *p*-coumaric acid [34], and other lignans, e.g., pinoresinol [33,35], isolariciresinol [35] and matairesinol [34,35]. Standard solutions of several compounds were injected into the HPLC system, but their relative retention times [sinapic acid (RRT=0.91), ferulic acid (RRT=0.89), *p*-coumaric acid (RRT=0.82), pinoresinol (RRT=1.29), and matairesinol (RRT=1.35)] and UV spectra did not agree with any of the chromatographic peaks obtained using this method.

Table 1
Standard calibration curves for phenolic compounds in the range 10–150 μg/ml

Phenolic compound	Standard curve	R^2
p-Coumaric acid	$y=0.7329x-2.8477$	0.9902
Ferulic acid	$y=0.4107x-0.793$	0.9905
(+)-SDG[b]	$y=0.0469x+0.3401$	0.9976
o-Coumaric acid	$y=0.8364x-1.1002$	0.9957

[a] Correlation coefficients of equation $y=kx+m$, where x is yield (μg/ml methanol), y is the peak area (mAu min), k is the slope and m is the intercept.

[b] (+)-Secoisolariciresinol diglucoside.

3.2. Analysis by extraction and hydrolysis versus direct alkaline hydrolysis

The effect of dioxane–ethanol extraction and alkaline hydrolysis on the yield of the analyzed phenolic compounds was determined by an experimental design. The models describing these effects are shown as response surfaces in Fig. 4. The longer the extraction time, the higher the yield of phenolic compounds up to 48 h [log (time) equals 1.7]. The hydrolysis time is less important since the yield of phenolic compounds reached a high level already after 1 h [log (time) equals 0] of hydrolysis. The hydrolysis time affected the yield of hydroxycinnamic acid glucosides more than the yield of (+)-SDG (Fig. 4) which could indicate that the hydrocinnamic acid glucosides and (+)-SDG are released from their combined forms differently.

The effect of temperature and concentration of alkali used in direct alkaline hydrolysis on the yield of all phenolic compounds was small. Therefore, the practical temperature of 20 °C and 1 M of NaOH were used for direct alkaline hydrolysis. Under these

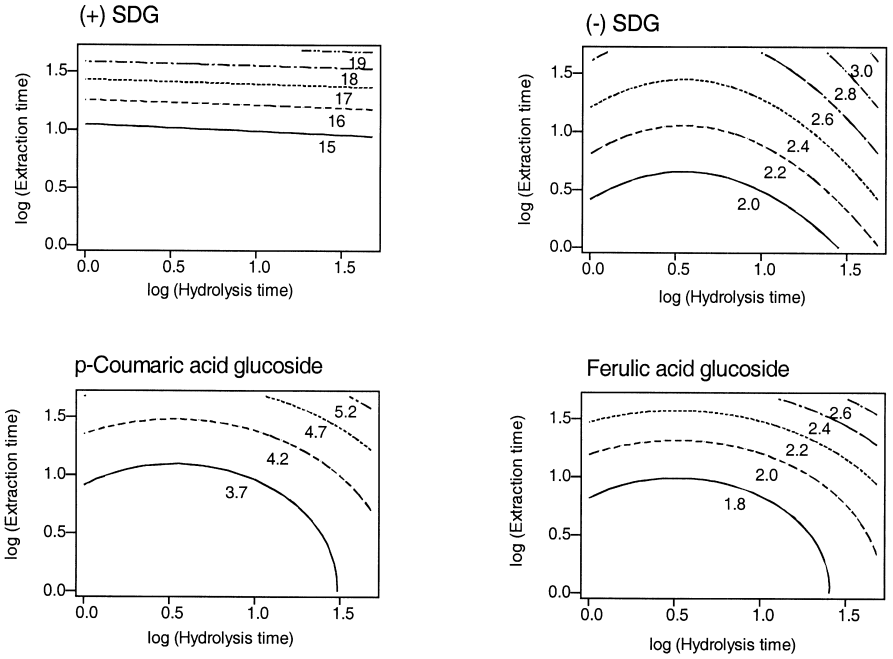

Fig. 4. Response surfaces of models showing the effect of dioxane–ethanol extraction and alkaline hydrolysis times on yield (mg/g DFF) of (+)-SDG, (−)-SDG, *p*-coumaric acid glucoside, and ferulic acid glucoside.

conditions, high yield of the phenolic compounds was obtained after half an hour of hydrolysis and the yield did not change by a prolonged hydrolysis time. The recoveries of phenolic compounds were affected by the alcohol concentration during precipitation of polysaccharides and protein. The yield obtained by using 60% aq. ethanol was almost equal to the yield obtained using 90% aq. methanol. Ethanol concentrations higher than 60% lowered the yield and produced extra peaks in the chromatogram.

The direct alkaline hydrolysis resulted in a higher yield of (+)-SDG (22.3±1.4, six replicates) than obtained with alkaline hydrolysis after dioxane–ethanol extraction (19.1±0.9, three replicates), but the removal of the dioxane–ethanol extraction step did not affect the yield of (−)-SDG and the hydrocinnamic acid glucosides.

Matrix binding effects of *o*-coumaric acid were investigated by determining the yield of phenolic compounds using different levels of *o*-coumaric acid. Negligible variations suggest that the yield was not affected by the amount of *o*-coumaric acid used in the analysis. The repeatability of the method was checked by two analysts. The yield of the phenolic compounds was similar for both analysts and the relative standard deviations were <7% except for (−)-SDG (Table 2). The minor component (−)-SDG had a high RSD because of an interfering peak.

3.3. Variation in phenolic compounds in flaxseed samples

The direct alkaline hydrolysis method was used to study the variation in the content of phenolic compounds in flaxseed samples from two different locations, Svalöv in the south of Sweden and Örebro in the middle of Sweden (Table 3). Considerable variation was obtained for (+)-SDG (11.9–25.9 mg/g), (−)-SDG isomer (2.2–5.0 mg/g), *p*-coumaric acid glucoside (1.2–8.5 mg/g), and ferulic acid glucoside (1.6–5.0 mg/g). Weak correlations were found between (+)- and (−)-SDG (R=0.59), and between ferulic acid glucoside and *p*-coumaric acid glucoside (R=0.53). The highest content of SDG was in the samples Jupiter (25.9 mg/g), Barbara

Table 2
Repeatability of two analysts analyzing samples of DFF with direct alkaline hydrolysis[a]

	(+)-SDG	(−)-SDG	*p*-Coumaric acid glucoside	Ferulic acid glucoside
Analysts 1				
Yield±SD	22.3±1.4	3.3±0.2	5.3±0.2	2.7±0.1
RSD (%)	6.1	7.0	3.8	3.7
Analysts 2				
Yield±SD	22.9±0.8	3.2±0.3	5.5±0.2	2.9±0.1
RSD (%)	3.4	10.5	3.6	3.4

[a] Mean value, standard deviation and relative standard deviation of six replicates per day analyzed on 3 different days.

Table 3
Levels of phenolic compounds analyzed by direct alkaline hydrolysis in Swedish flaxseed samples

Flaxseed[a]	Oil in seed (% d.m.)	Content of phenolic compounds in seeds (mg/g d.m.)			
		(+)-SDG	(−)-SDG	*p*-Coumaric acid glucoside	Ferulic acid glucoside
Svalöv					
Antares 1	40.7	14.8	3.2	1.7	1.6
Antares 2	42.7	15.5	3.3	1.9	1.7
Barbara	39.7	25.0	3.7	4.2	2.0
Flanders	44.9	14.8	2.5	3.9	2.6
Jupiter	40.4	25.9	3.4	6.7	3.1
Niagara	43.8	13.3	2.5	1.2	2.2
Oscar	42.1	18.5	2.9	2.6	1.8
SWA9101	41.9	22.8	5.0	4.9	2.5
SWB9104	44.3	24.3	3.8	5.2	1.8
Zoltan	46.0	15.7	3.2	3.0	5.0
Örebro					
Agraf 17	45.2	16.5	3.1	2.8	2.1
Antares	43.5	16.0	3.8	3.3	2.3
Bethune	44.7	15.6	3.2	6.0	3.5
Biltstar	45.9	14.2	4.1	3.1	4.1
Capricorn	41.4	19.8	3.0	7.6	4.3
Early Bird	47.2	15.7	3.0	6.7	3.9
Flanders	43.0	13.1	2.6	4.6	3.2
Lager	39.2	11.9	2.6	4.4	3.0
Linus	45.7	12.6	2.4	2.9	2.6
Lagoon PI	46.3	13.9	2.6	3.4	2.8
Lagon	46.3	12.6	2.5	4.5	3.1
Niagara	43.7	13.3	2.2	1.4	2.6
Oscar	43.8	18.3	3.4	4.4	2.5
Sky	44.2	14.1	2.6	3.0	2.5
Talon	46.7	18.7	2.3	2.5	1.8
Taurus	43.8	17.3	3.9	5.0	3.0
Windermere	44.8	19.5	3.4	8.5	3.8
RSD (%)[b]	–	2.9	17.0	4.7	3.8

Note: d.m. = dry matter.
[a] Svalöv is in the south and Örebro is in the middle of Sweden.
[b] Samples were analyzed in triplicates. RSD indicates the average relative standard deviation obtained for each analyte.

(25.0 mg/g) and the lowest was in Lager (11.9 mg/g).

Levels of (+)-SDG obtained in this study (11.9–25.9 mg/g dry matter) were higher compared to levels reported by other authors [1,3,28,30]. Johnsson et al. [3] obtained a range of 6.1–13.3 mg/g dry matter in whole flaxseeds grown in Sweden and Denmark, and Westcott and Muir [24] reported a range of 13.6–20.0 mg/g DFF in cultivars grown in Canada. Charlet et al. [25] found 16.6 μmol/g dry mass in cultivar Barbara, the same content of SDG as found by Johnsson et al. [3]. Very low yields were obtained by Thompson et al. [30] using microbial fermentation (0.7–2 mg/g in whole seeds), and Obermeyer et al. [29] using an enzyme mixture (2.3 mg/g flaxseed meal). Differences in results between studies could be due to inefficient extraction of oligomers from flaxseed matrix with different organic solvents, degradation of oligomers during extraction at high temperature, losses during sample clean-up, or degradation of SECO during acid hydrolysis, but might also be due to differences in cultivars and growing conditions.

4. Conclusions

A fast HPLC method based on direct alkaline hydrolysis and use of internal standard is developed for the quantitative determination of (+)-SDG, (−)-SDG, and hydroxycinnamic acid glucosides in flaxseed. The hydroxycinnamic acid glucosides and (−)-SDG were quantified for the first time. Large variations in the content of (+)-SDG and these phenolic glucosides were found in Swedish flaxseed samples.

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