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Separation and determination of secoisolariciresinol diglucoside oligomers and their hydrolysates in the flaxseed extract by high-performance liquid chromatography

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Abstract

Flaxseed contains the largest amount of lignan secoisolariciresinol diglucoside (SDG) oligomers and is the richest dietary source of SDG. SDG oligomers in the flaxseed extract are often hydrolyzed to break the ester linkages for the release of SDG and the glycosidic bonds for the release of secoisolariciresinol (SECO). The hydrolysates of SDG oligomers are complicated because of the production of esters in an alcohol-containing medium. In this study, a new gradient reversed-phase high-performance liquid chromatography (HPLC) method has been developed to be suitable for the separation and determination of: (1) SDG oligomers extracted from the defatted flaxseed powder by a 70% aqueous methanol solution; (2) SDG oligomers and their alkaline hydrolysates, including SDG, *p*-coumaric acid glucoside and its methyl ester, ferulic acid glucoside and its methyl ester in an alkaline hydrolytic solution; and (3) the succedent acid hydrolysates, including secoisolariciresinol monoglucoside (SMG), SECO, anhydrosecoisolariciresinol (anhydro-SECO), *p*-coumaric acid and its methyl ester, ferulic acid and its methyl ester, 5-hydroxymethyl-2-furfural (HMF) and its degradation product in an acid hydrolytic solution. The content of SDG oligomers in a defatted flaxseed powder was found to be 38.5 mg/g on a dry matter basis, corresponding to a SDG content of 15.4 mg/g, which was determined after alkaline hydrolysis. Furthermore, this study presented a major reaction pathway for the hydrolysis of SDG oligomers.
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Keywords: Flaxseed; SDG oligomers; Secoisolariciresinol diglucoside; Secoisolariciresinol monoglucoside; Secoisolariciresinol; Anhydrosecoisolariciresinol; *p*-Coumaric acid; Ferulic acid

1. Introduction

Lignans are a large group of phenolic compounds widely distributed in edible plants [1], and belong to the group of phytoestrogens [2]. Flaxseed (*Linum usitatissimum*, Linn., Linaceae) contains the largest amount of lignan, secoisolariciresinol diglucoside (SDG) among all the grains, legumes, fruits, and vegetables [3,4], and is the richest dietary source of the plant-based SDG [3–18], which can be metabolized to the mammalian lignans, enterodiol and enterolactone by human intestinal microflora [2,18–21]. Lignans are becoming increasingly important for their possible application in the fields of pharmacy and nutrition, and have been found to possess a variety of biological properties [22]. Secoisolariciresinol (SECO) and SDG are known to have a number of potential health benefits, including reduction of serum cholesterol levels, delay in the onset of type II diabetes, and decreased formation of breast, prostate and colon cancers [15,16,21,23–26], which may be partially attributed to their antioxidant properties [16,27,28].

SECO and SDG cannot be directly determined in the flaxseed extract because SECO is present as a glycoside, SDG, which is further ester-linked with 3-hydroxy-3-methyl-glutaric acid

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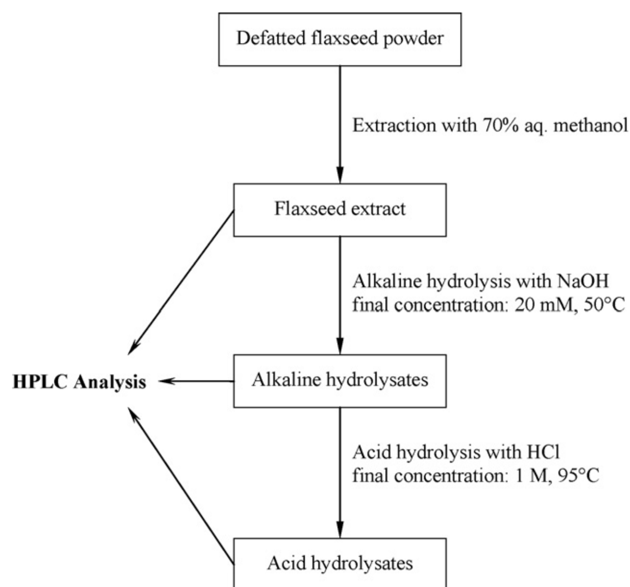

Fig. 1. Summary of the application of the developed HPLC method on the analysis of SDG oligomers and their hydrolysates.

(HMGA) to form SDG oligomers with a molecular weight of around 4000 [4]. Other phenolic compounds such as *p*-coumaric acid and ferulic acid are also present in glucosidic forms as a part of oligomers [4,6–8,11,15–17,22]. Hydrolysis, by which both ester linkages and glycosidic bonds are broken [29], is often done to simplify the subsequent chromatographic analysis of the extracts containing SDG oligomers [22]. Although strong acid hydrolysis breaks both ester linkages and glycosidic bonds whereas alkaline hydrolysis breaks ester linkages, strong acid treatment is destructive to some lignans or causes transformation reactions from one lignan to another lignan [30]. Therefore, it is necessary at first to transform the complex SDG oligomers by alkaline hydrolysis into free SDG, *p*-coumaric acid glucoside, and ferulic acid glucoside, and then an additional acid hydrolysis should be carried out to release free aglycone SECO from SDG [9,10,22]. The instability of SECO during the acid hydrolysis step results in the conversion of SECO to anhydrosecoisolariciresinol (anhydro-SECO) as an artifact by elimination of a water molecule from the diol structure of SECO [9,10,12,22,31]. In addition, secoisolariciresinol monoglucoside (SMG) has been found to be an intermediate product during the deglycosylation of SDG to form SECO by human intestinal bacteria [20]. Furthermore, Luyengi et al. [32] found a phenylpropanoid glucoside, which was a cinnamic acid methyl ester, from the defatted flaxseed. This indicates that the esters of *p*-coumaric acid and ferulic acid glucosides are formed when hydrolysis of flaxseed oligomers is performed in alcohols.

A number of analytical methods have been reported for the determination of the hydrolysates of SDG oligomers in the flaxseed extracts mainly by gas chromatography (GC) [22] and high-performance liquid chromatography (HPLC) [5–11,14,17,20,33]. The SDG oligomers in the flaxseed extracts [6–8] and their alkaline hydrolysates including SDG, *p*-coumaric acid glucoside, and ferulic acid glucoside [5–8,11,17] have been separated by the gradient reversed-phase HPLC. The acid hydrolysates of SDG oligomers including SECO, *p*-coumaric acid, and ferulic acid have been separated by the isocratic reversed-phase HPLC [10]. Charlet et al. [9] developed an isocratic reversed-phase HPLC procedure for the quantification of anhydro-SECO. Sicilia et al. [12] separated SECO, anhydro-SECO, and other lignans in the flaxseed extract after acid hydrolysis by a gradient reversed-phase HPLC.

To our knowledge, there is no previous information concerning the simultaneous determination of SDG oligomers, their alkaline hydrolysates, and the succedent acid hydrolysates. In this study, a new gradient reversed-phase HPLC method was developed to be suitable for the simultaneous separation and determination of SDG oligomers and their alkaline and acid hydrolysates, including SDG, SMG, SECO, anhydro-SECO, *p*-coumaric acid glucoside and its ester, ferulic acid glucoside and its ester, *p*-coumaric acid and its ester, ferulic acid and its ester, 5-hydroxymethyl-2-furfural (HMF) and its degradation product.

2. Experimental

2.1. Materials and reagents

The flaxseed sample, grown in Wulanchabu, Inner Mongolia, was purchased from Inner Mongolia Flaxseed Oil Health Promotion Center (Hohehot, Inner Mongolia, China). The commercial flaxseed lignan (40% of lignan) was obtained from Hunan Deray Biological Industry Group Co. Ltd. (Huaihua, Hunan, China). HPLC-grade methanol was purchased from Merck KGaA (Darmstadt, Germany). SECO (95% purity), *p*-coumaric acid (98% purity), ferulic acid (98% purity), and HMF (99% purity) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Water was purified using a Millipore Simplicity system (Billerica, MA, USA). Acetic acid, sodium hydroxide, and hydrochloric acid were of analytical grade. Preparative thin-layer chromatography (TLC) plates were prepared by depositing 10 g of silica gel (GF254, Qingdao Haiyang Chemical Co. Ltd.; Qingdao, China) onto a glass plate (20 cm × 20 cm, 2 mm). The thickness of the adsorbent (silica gel) layer is about 0.5 mm.

2.2. High-performance liquid chromatography

HPLC was conducted on a Waters liquid chromatograph equipped with a 1525 binary pump and a 2996 photodiode array detector from Waters Corporation (Milford, MA, USA). SDG oligomers and their alkaline and acid hydrolysates (Fig. 1) were separated and analyzed by using a Waters XTerra® RP18 column (4.6 mm × 250 mm, 5 μm) and a guard column (4.6 mm × 12.5 mm, 5 μm) at 30 °C. The mobile phase consisted of solvent A (0.5% of acetic acid in water) and solvent B (methanol). The following linear gradient procedure was used: 0–20 min, 15% of B; 20–30 min, 15–28% of B; 30–40 min, 28% of B; 40–60 min, 28–55% of B; 60–70 min, 55–85% of B; 70–80 min, 85% of B; and back to 15% of B within 1 min. The system was equilibrated with 15% of B for 10 min at the end of each run. The flow rate was 1.0 ml/min. The detecting wavelength was set between 210 and 400 nm, and the chromatographic peaks were measured at a wavelength of 280 nm to

facilitate the detection of SDG and its hydrolysates. Aliquots of 20 μl were directly injected into the HPLC for the determination. All injections were repeated three times.

The purity of the purified compounds which would be used as standards such as SDG oligomers, SDG, SMG, and anhydro-SECO was determined on the basis of the area percentage of the main peak absorbed at 280 nm [8,34] by this HPLC method.

2.3. Extraction of SDG oligomers

The flaxseed sample was ground into powder with the help of a mortar and a pestle, and then defatted with petroleum ether. A 50 g amount of the milled and defatted flaxseed powder was accurately weighed and extracted with 300 ml of 70% aqueous methanol solution with continuous stirring for 4 h followed by sonication for 10 min and centrifugation at 10,000 × *g* for 5 min. The extraction procedure was repeated three times and the total extract was sampled for HPLC analysis to detect SDG oligomers in the flaxseed extract.

2.4. Alkaline hydrolysis of SDG oligomers

The flaxseed extract was subjected to an alkaline hydrolysis (final NaOH concentration of 20 mM) at 50 °C for hydrolyzing SDG oligomers. The reaction mixture was directly sampled to HPLC for monitoring the alkaline hydrolysis of SDG oligomers and the release of SDG and other glycosides during the process of alkaline hydrolysis.

2.5. Acid hydrolysis of SDG and other glycosides

After 15 min of alkaline hydrolysis, SDG oligomers were completely hydrolyzed. SDG in the alkaline hydrolytic solution was subjected to an acid hydrolysis (final HCl concentration of 1 M) at 95 °C. The acid hydrolytic reaction mixture was directly sampled to HPLC for monitoring the acid hydrolysates of SDG and other glycosides during the process of acid hydrolysis.

2.6. Purification and identification of SDG oligomers, SDG, SMG, and anhydro-SECO

2.6.1. Purification of SDG oligomers

The flaxseed extract (250 ml) was dried and dissolved in 30 ml of water, and then aliquots of 3 ml were loaded on C18 column (Supelclean ENVI-18 SPE, 3 ml, 0.5 g, Supelco, Bellefonte, PA, USA) preconditioned with 5 ml of methanol and 5 ml of water, washed with 5 ml of 50% aqueous methanol solution, and then eluted with 5 ml of 60% aqueous methanol solution to obtain the purified SDG oligomers [4].

2.6.2. Purification of SDG

A 10 g amount of the commercial flaxseed lignan (40%) was dissolved in 100 ml of ethanol at room temperature. The lignan solution was filtered and concentrated using a rotary evaporator (Laborota 4001-efficient, Heidolph Instruments, Schwabach, Germany). A 5.6 g amount of dried extract was obtained, and then dissolved in 80 ml of ethanol and mixed with a 21 g amount of silica gel (GF 254). After dried, this mixture (∼4 g each) was subjected to a flash column chromatography (a glass tube, 20 mm × 300 mm) and washed with 100 ml of ethyl acetate/ethanol (8.5:1.5, v/v), and then, SDG adsorbed by silica gel was eluted with 180 ml of ethyl acetate/ethanol (8:2, v/v). Six fractions (30 ml each) were collected and detected by HPLC. The SDG-containing fractions were combined and then concentrated. SDG was further purified by the preparative TLC, which was prewashed by ethyl acetate, with ethyl acetate/ethanol (8:2, v/v) as the developing solvent followed by HPLC analyses to confirm its purity. The fraction with the R_f value of 0.28 corresponding to SDG was cut and extracted with methanol. After filtered and dried, an 1 g amount of SDG was obtained.

2.6.3. Purification of SMG

A 500 mg amount of SDG was dissolved in 25 ml of water and hydrolyzed with hydrochloric acid (final HCl concentration of 1 M) at 95 °C for 100 min to convert SDG into SMG. The acid hydrolysates were extracted with ethyl acetate. The water phase, which contains SMG, was loaded on C18 column (Supelclean ENVI-18 SPE) preconditioned with 5 ml of methanol and 5 ml of water. After washing with 10 ml of water, SMG was eluted with 5 ml of methanol. SMG was purified by the preparative TLC with ethyl acetate/ethanol (8.5:1.5, v/v) as the developing solvent followed by HPLC analyses to confirm its purity. The fraction with the R_f value of 0.50 corresponding to SMG was cut and extracted with methanol. After filtered and dried, a 20 mg amount of SMG was obtained.

2.6.4. Purification of anhydro-SECO

A 300 mg amount of SDG was dissolved in 25 ml of water and hydrolyzed with hydrochloric acid (final HCl concentration of 3 M) at 95 °C for 3 h to convert SDG into anhydro-SECO. The acid hydrolysates were extracted with ethyl acetate. The anhydro-SECO-containing organic phase was concentrated and purified by the preparative TLC with ethyl acetate/petroleum ether (4:6, v/v) as the developing solvent followed by HPLC analyses to confirm its purity. The fraction with the R_f value of 0.61 corresponding to anhydro-SECO was cut and extracted with ethyl acetate. After filtered and dried, a 25 mg amount of anhydro-SECO was obtained.

2.6.5. Identification of SDG oligomers, SDG, SMG, and anhydro-SECO

The structures of SDG oligomers, SDG, SMG, and anhydro-SECO were identified by nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS). The NMR spectra were recorded at 500 MHz on a Varian INOVA-500NB NMR spectrometer from Varian Inc. (Palo Alto, CA, USA). FAB mass spectra for SDG oligomers, SDG, SMG, and anhydro-SECO were obtained using a double-focusing mass spectrometer (VG-ZAB-HS, Micromass, Manchester, UK) coupled with a MASPEC II data system. The samples dissolved in 3-nitrobenzyl alcohol were introduced into the mass spectrometer.

Table 1
^{1}H NMR spectroscopic data for SDG, SECO, and anhydro-SECO

Position	SDG[a] δ_H (multiplicity, J Hz)	SECO[a] δ_H (multiplicity, J Hz)	Anhydro-SECO[b] δ_H (multiplicity, J Hz)
1/1′	–	–	–
2/2′	6.59 (d, 1.8)	6.59 (d, 1.9)	6.84 (d, 1.7)
3/3′	–	–	–
4/4′	–	–	–
5/5′	6.64 (d, 8.0)	6.66 (d, 7.9)	7.13 (d, 8.0)
6/6′	6.56 (dd, 1.8, 8.0)	6.54 (dd, 1.9, 7.9)	6.92 (dd, 1.7, 8.0)
7/7′a	2.61 (dd, 7.9, 13.8)	2.56 (dd, 7.5, 13.7)	2.85 (dd, 7.8, 13.8)
7/7′b	2.68 (dd, 6.9, 13.8)	2.66 (dd, 6.8, 13.8)	2.92 (dd, 6.6, 13.7)
8/8′	2.12 (m)	1.90 (br t, 6.1)	2.50 (m)
9/9′a	4.06 (dd, 5.6, 9.9)	3.58 (m)	4.25 (dd, 6.5, 8.6)
9/9′b	3.47 (dd, 6.4, 9.9)	3.31 (m)	3.86 (dd, 5.6, 8.7)
OCH_3	3.73 (s)	3.74 (s)	4.16 (s)
1a/1a′	4.23 (d, 7.8)	–	–
2a/2a′	3.21 (t, 7.8, 9.0)	–	–
3a/3a′	3.28–3.37	–	–
4a/4a′	3.28–3.37	–	–
5a/5a′	3.25 (m)	–	–
6a/6a′a	3.84 (br d, 2.3, 11.8)	–	–
6a/6a′b	3.68 (dd, 5.5, 11.8)	–	–

[a] Measured in CD_3OD, δ in ppm.
[b] Measured in $CDCl_3$, δ in ppm.

2.7. Method validation

The stock standards of SDG oligomers, SDG, SMG, SECO, anhydro-SECO, *p*-coumaric acid, ferulic acid, and HMF were prepared at 300 mg/l, and the additional calibration levels were prepared by a serial dilution with ethanol. The standard calibration curves were constructed using these standard solutions. The linear regression analysis was carried out by plotting the peak areas (A) against the concentrations (C) of these standard solutions. The linearity was demonstrated by a correlation coefficient (r^2) greater than 0.999. The limit of detection (LOD) and the limit of quantification (LOQ) were determined based on signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively.

The precision and accuracy were determined by analyzing standard solutions to evaluate the repeatability for this HPLC method. Precision was calculated as a relative standard deviation (RSD) for the repeated measurements. For recovery studies on added SDG oligomers and SDG, known volumes of standard solutions were added to 100 mg of defatted flaxseed powder at three levels (3, 5.3, and 11.6 mg/g for SDG oligomers, and 3.5, 6.9, and 9.0 mg/g for SDG). The spiked samples were extracted following the described procedure. SDG oligomers in the flaxseed extract and the total SDG in an alkaline hydrolytic solution were determined. Background levels were subtracted in all recovery determinations.

3. Results and discussion

3.1. Purification and identification of SDG oligomers, SDG, SMG, and anhydro-SECO

SDG oligomers were purified from the defatted flaxseed powder, SDG was purified from the commercial flaxseed lignan (40%), and SMG and anhydro-SECO were purified from the acid hydrolysates of the purified SDG. The purities of the purified SDG, SMG, and anhydro-SECO were determined by HPLC. On the basis of the area percentage of the main peak absorbed at 280 nm [8,34], SDG, SMG, and anhydro-SECO were 96%, 97%, and 95% pure, respectively, and were available for NMR and MS analysis. Although SDG oligomers are the heterogenic macromolecular compound [6,8], the “purity” of the isolated SDG oligomers was also determined by HPLC and found to be 98% on the basis of the area percentage of the main peak responded at 280 nm.

SDG oligomers, SDG, SMG, and anhydro-SECO were identified by NMR and MS. SDG and anhydro-SECO were subjected to ^{1}H NMR spectroscopic analyse, and their ^{1}H chemical shifts are shown in Table 1 (see Fig. 6 for the annotation). The ^{1}H NMR spectrum of SECO was also determined for the purpose of comparison (Table 1). The results showed that the ^{1}H NMR spectrum of SDG was essentially identical to that reported by Ford et al. [7]. SMG was subjected to ^{1}H and ^{13}C NMR spectroscopic analyses, and both ^{1}H and ^{13}C chemical shifts of SMG are shown in Table 2. SDG oligomers were also subjected to ^{1}H NMR spectroscopic analyse, and their ^{1}H NMR spectrum showed, in the aromatic region, peaks from SDG (δ 6.3–6.5 ppm) and peaks from other phenolic compounds (δ 6.3–7.7) such as *p*-coumaric acid and ferulic acid, which were essentially identical to that reported by Kamal-Eldin et al. [4].

Spectroscopic evidence for the identity of SDG, SMG, and anhydro-SECO was obtained from positive FAB-MS which exhibited ions at *m/z* 686, 524, and 344, corresponding to $[M]^+$ ions for SDG ($C_{32}H_{46}O_{16}$), SMG ($C_{26}H_{36}O_{11}$), and anhydro-SECO ($C_{20}H_{24}O_5$), respectively. For SDG oligomers, FAB-MS exhibited ions at *m/z* 369, 341, 307, 289, 279, 219, 205, 154, 149, 137, 136, 123, and 107, which were identical to that of

Table 2
^{1}H NMR and ^{13}C NMR spectroscopic data for SMG

Position	SMG[a]	
	δ_C	δ_H (multiplicity, J Hz)
1	134.0	–
1′	133.8	–
2/2′	113.5	6.59 (dd, 1.8, 8.0)
3/3′	148.7	–
4/4′	145.4	–
5/5′	115.7	6.65 (d, 7.9)
6	122.8	6.56 (dd, 1.9, 5.3)
6′	122.8	6.54 (dd, 2.0, 5.3)
7b	35.9	2.67 (dd, 7.3, 13.6)
7a	35.9	2.57–2.61
7′a,7′b	35.5	2.57–2.61
8	41.4	2.10 (m)
8′	43.9	1.94 (m)
9a	71.1	4.05 (dd, 6.1, 9.8)
9b	71.1	3.47 (dd, 6.2, 9.9)
9′a	62.6	3.64 (dd, 6.4, 11.2)
9′b	62.6	3.55 (dd, 6.0, 11.0)
1a	104.7	4.21 (d, 7.8)
2a	75.2	3.20 (t, 7.9, 9.0)
3a	78.2	3.27–3.36
4a	71.7	3.27–3.36
5a	77.9	3.23 (m)
6a-a	62.8	3.84 (dd, 2.4, 11.9)
6a-b	62.8	3.68 (dd, 5.6, 11.9)
OCH_3	56.2	3.74 (s)

[a] Measured in CD_3OD, δ in ppm.

SDG. This indicates the presence of SDG in the oligomers. In addition, FAB-MS of SDG oligomers also exhibited ions at m/z 327 and 357, corresponding to $[M-H]^+$ ions for p-coumaric acid glucoside and ferulic acid glucoside, respectively, indicating the presence of p-coumaric acid glucoside and ferulic acid glucoside in the oligomers.

3.2. HPLC method validation

The XTerra® RP18 column and the gradient elution procedure were used to separate SDG oligomers and their alkaline and acid hydrolysates. Lignans all absorb UV light, and UV detection may offer sufficient selectivity and sensitivity for the determination of lignans [22]. SDG, SMG, SECO, and anhydro-SECO present maximum absorptions at ∼280 nm, which is attributed to the aromatic chromophore and the substituents of –OH and $-OCH_3$ on aromatic ring [3]. The inset of Fig. 2 shows the UV absorption spectrum of SDG oligomers. Using a photodiode array detector, peaks were identified by taking the spectra of each peak during elution. The identifications of SDG, SMG, SECO, anhydro-SECO, p-coumaric acid, ferulic acid, and HMF were achieved by comparing their retention time and spectra against the known standards.

The calibration curves of the peak-area (A) against the concentration (C) for these standards at 280 nm gave the good linear responses over a wide range of concentrations (Table 3). The precision and accuracy were determined, and the results were shown in Table 4. The recoveries of SDG oligomers and SDG were

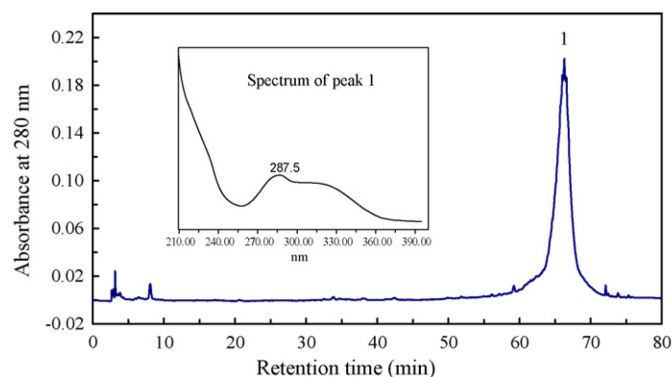

Fig. 2. HPLC chromatogram of SDG oligomers (peak 1) in the intact flaxseed extract. The insert shows the UV absorption spectrum of SDG oligomers.

found to be between 94.8% and 107.9%, and between 93.6% and 102.0%, respectively.

3.3. Separation of SDG oligomers and their alkaline hydrolysates

The intact flaxseed extract was analyzed by this developed HPLC method, and the HPLC chromatogram was shown in Fig. 2. The result indicated that no free SECO and SDG were detected, and SDG oligomers were the major fraction in the flaxseed extract. As can be seen from Fig. 2, SDG oligomers, designated as peak 1, eluted between 60 and 70 min and showed a relatively broad peak, suggesting the structural heterogeneity or differences in their molecular weights [4,6,8]. Johnsson et al. [6] fractionated the whole oligomers by solid phase extraction into three oligomeric fractions, which contained substantial amounts of SDG. Kamal-Eldin et al. [4] and Johnsson et al. [6] used nuclear magnetic resonance spectroscopy to assign an average straight-chain oligomeric structure composed of five SDG residues interconnected by four HMGA residues to the main phenolic polymer in flaxseed.

SDG oligomers were hydrolyzed by adding freshly prepared methanolic NaOH (final NaOH concentration of 20 mM) at 50 °C. The reaction mixture was sampled to HPLC during the process of alkaline hydrolysis to monitor the change in alkaline hydrolysates including SDG and other glycosides. Fig. 3 shows the HPLC chromatograms for the analysis of the alkaline hydrolysis solution. It had been established that SDG and the glucosides of p-coumaric acid and ferulic acid could be released from SDG oligomers following alkaline hydrolysis [6–8].

During the initial stage (0–15 min), alkaline hydrolysis resulted in hydrolytic cleavage of SDG oligomers with a concomitant increase in peak intensities of peaks 2, 3, and 4, and decrease in peak 1 (Fig. 3a and b). During the process of the alkaline hydrolysis, the content of SDG (peak 4) released from SDG oligomers kept increasing up to ∼15 min and then leveled off, indicating that SDG oligomers had been almost completely hydrolyzed after 15 min of alkaline hydrolysis (20 mM, 50 °C) and no SDG oligomers were found (Fig. 3b).

The succedent alkaline hydrolysis resulted in decrease in the peak intensities of peaks 2 and 3, and increase in the peak inten-

Table 3
Linear regression data, LOD and LOQ of SDG oligomers, SDG, SMG, SECO, anhydro-SECO, *p*-coumaric acid, ferulic acid, and HMF at 280 nm ($n = 3$)

Analyte	Linear range (μg/ml)	Linear regression equation	r^2	LOD (μg/ml)	LOQ (μg/ml)
SDG oligomers	20–1000	$y = 10828x - 67110$	0.9992	0.065	0.217
SDG	5–300	$y = 8113x - 4937$	0.9998	0.087	0.288
SMG	5–300	$y = 9524x - 1432$	0.9998	0.073	0.245
SECO	5–300	$y = 17948x - 9839$	0.9999	0.039	0.130
Anhydro-SECO	5–300	$y = 18547x - 10547$	0.9999	0.038	0.126
p-Coumaric acid	5–300	$y = 98646x - 101673$	0.9999	0.007	0.023
Ferulic acid	5–300	$y = 54227x - 29760$	0.9997	0.013	0.043
HMF	5–150	$y = 136762x + 47634$	0.9998	0.005	0.017

Table 4
Precision and accuracy for the determination of SDG oligomers, SDG, SMG, SECO, anhydro-SECO, *p*-coumaric acid, ferulic acid, and HMF

Analyte	Concentration (μg/ml)	Found (μg/ml)	RSD (%)	Accuracy (%)
SDG oligomers	100.0	95.7	3.9	95.7
	500.0	487.3	0.7	97.5
SDG	30.0	29.5	1.3	98.3
	90.0	89.9	0.5	99.9
SMG	40.0	39.6	0.5	99.0
	80.0	80.6	1.2	100.8
SECO	30.0	29.0	1.1	96.7
	90.0	87.3	1.7	97.0
Anhydro-SECO	36.0	36.0	0.7	100.0
	108.0	105.4	0.9	97.6
p-Coumaric acid	30.0	27.9	1.8	93.0
	90.0	88.1	1.1	97.9
Ferulic acid	30.0	28.5	0.6	95.0
	90.0	88.2	0.9	98.0
HMF	30.0	30.8	0.6	102.7
	90.0	90.8	0.3	100.9

Table 5
The contents (mg/g) of SDG oligomers and their hydrolysates in the defatted flaxseed powder on a dry matter basis ($n = 3$)[a]

Analyte	Flaxseed extract	Alkaline hydrolytic solution (20 mM, 50 °C)		Acid hydrolytic solution (1 M, 95 °C, 210 min)
		15 min	7 h	
SDG oligomers	38.5	–	–	–
SDG	–	15.4	15.2	0.2
SMG	–	–	–	2.5
SECO	–	–	–	2.1
Anhydro-SECO	–	–	–	2.7
p-Coumaric acid glucoside ester	–	1.8[b]	–	–
Ferulic acid glucoside ester	–	1.1[b]	–	–
p-Coumaric acid glucoside	–	0.3[b]	1.9[b]	–
Ferulic acid glucoside	–	0.2[b]	1.2[b]	–
p-Coumaric acid	–	–	–	0.2
Ferulic acid	–	–	–	0.2
p-Coumaric acid ester	–	–	0.1	1.0[b]
Ferulic acid ester	–	–	0.4	0.8[b]
HMF	–	–	–	0.8

–, not detected.
[a] The mean values (RSDs <5%) of three determinations are presented.
[b] The contents of the esters and glucosides of *p*-coumaric acid and ferulic acid were quantitated as free *p*-coumaric acid and ferulic acid, respectively.

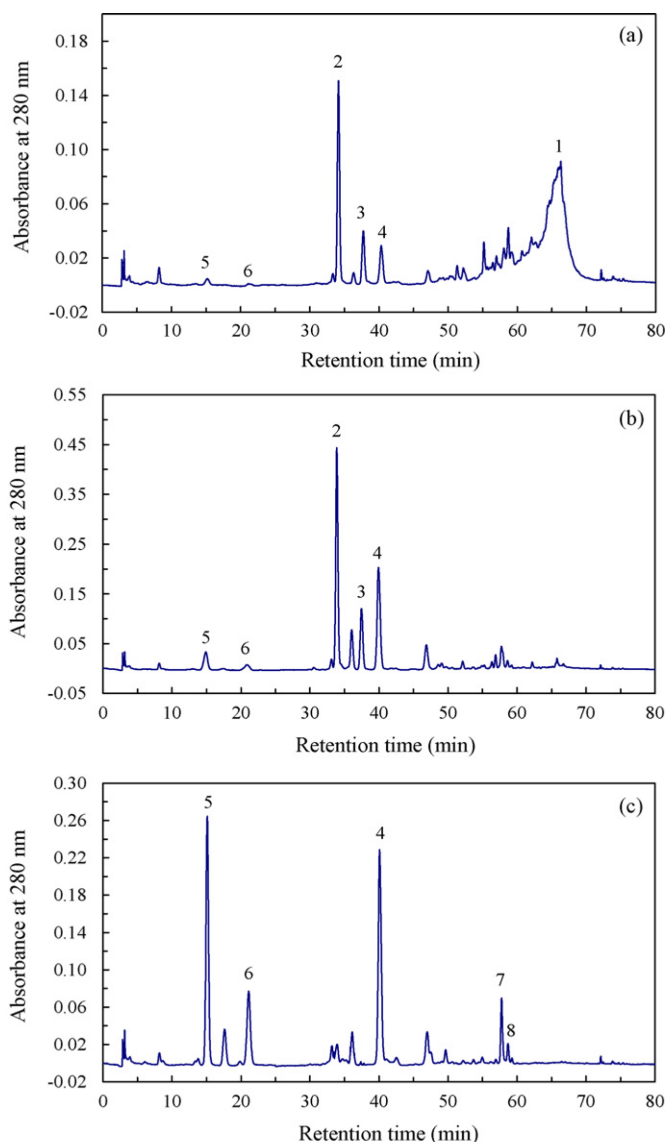

Fig. 3. HPLC chromatograms of the alkaline hydrolysates in the alkaline hydrolytic solution after 1 min (a), 15 min (b), and 7 h (c) of alkaline hydrolysis (final NaOH concentration of 20 mM) at 50 °C. Peak: 1, SDG oligomers; 2, the methyl ester of *p*-coumaric acid glucoside; 3, the methyl ester of ferulic acid glucoside; 4, SDG; 5, *p*-coumaric acid glucoside; 6, ferulic acid glucoside; 7, the methyl ester of ferulic acid; and 8, the methyl ester of *p*-coumaric acid.

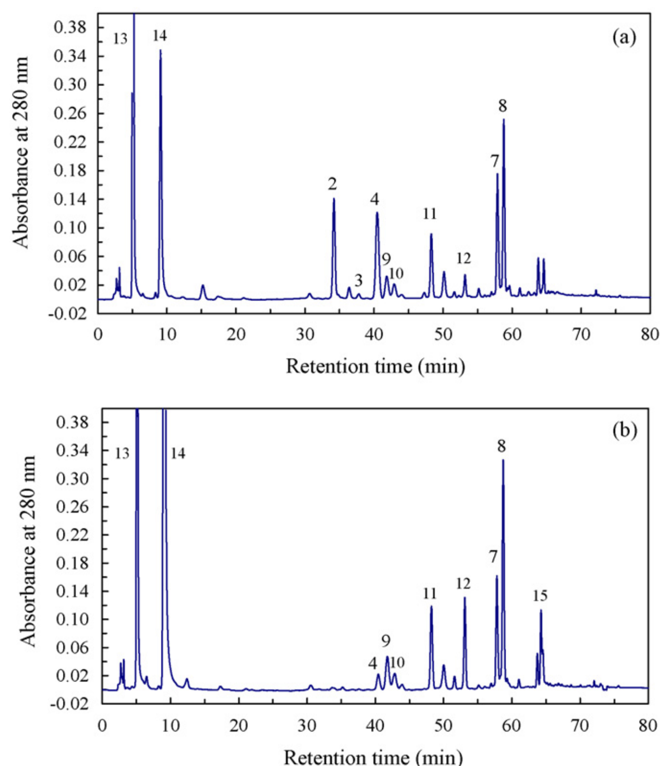

Fig. 4. HPLC chromatograms of the acid hydrolysates in the acid hydrolytic solution after 40 min (a) and 140 min (b) of acid hydrolysis (final HCl concentration of 1 M) at 95 °C. Peak: 2, the methyl ester of *p*-coumaric acid glucoside; 3, the methyl ester of ferulic acid glucoside; 4, SDG; 7, the methyl ester of ferulic acid; 8, the methyl ester of *p*-coumaric acid; 9, *p*-coumaric acid; 10, ferulic acid; 11, SMG; 12, SECO; 13, HMF; 14, the degradation product of HMF; and 15, anhydro-SECO.

sities of peaks 5 and 6. The results showed that, after 7 h of alkaline hydrolysis (20 mM, 50 °C), the peak intensities of *p*-coumaric acid glucoside (peak 5) and ferulic acid glucoside (peak 6) reached a maximum and almost no peaks 2 and 3 were found (Fig. 3c).

The present study showed that the alkaline hydrolysis process performed in methanol might be divided into two stages: (1) the hydrolysis of SDG oligomers (Fig. 3a), and the release of SDG (peak 4) and the methyl esters (peaks 2 and 3) of *p*-coumaric acid glucoside and ferulic acid glucoside (Fig. 3b); and (2) the release of *p*-coumaric acid glucoside (peak 5) and ferulic acid glucoside (peak 6) from their respective precursors (Fig. 3c).

It has been reported that the SDG oligomers also contain flavonoids such as herbacetin diglucoside (HDG) isolated from flaxseed hulls (0.2%), which might be a richer source of HDG compared to flaxseed (0.01%) [8,35]. According to the result of Struijs et al. [8] that HDG eluted before SDG, it is possible that one of the peaks eluting between 32 and 37 min is HDG, which was not investigated in the present study (Fig. 3c).

3.4. Separation of the acid hydrolysates of SDG

After 15 min of alkaline hydrolysis, SDG in the alkaline hydrolytic solution was further hydrolyzed with hydrochloric acid. The acid hydrolytic reaction mixture was sampled to HPLC during the process of acid hydrolysis to monitor the change in SDG and its acid hydrolysates. The typical HPLC chromatograms of acid hydrolysates during the process of acid hydrolysis were shown in Fig. 4.

In order to identify the acid hydrolysates of SDG, the standard solution of SDG was also hydrolyzed with a final HCl concentration of 1 M at 95 °C. The result showed that only SDG, SMG, SECO, and anhydro-SECO were detected in the acid hydrolysates of SDG by HPLC. As can be seen from Fig. 4, SMG (peak 11), SECO (peak 12), and anhydro-SECO (peak 15) were the main acid hydrolysates of SDG (peak 4).

During the process of acid hydrolysis, SDG was firstly deglycosylated into SMG, and then SECO, which could be transformed in anhydrous form, depending on the acid concentration

used [9] and hydrolysis time. Under mild acid hydrolytic conditions, SMG was found to be the major acid hydrolysate of SDG. The further acid hydrolysis resulted in increase in the peak intensities of peaks 12 and 15. Although Clavel et al. [20] found that the deglycosylation of SDG by *Clostridium* sp. led to the formation of SMG, which was an intermediate product to produce SECO and corresponded to SDG with one glucose molecule removed, to our knowledge, there was no previous information concerning the detection of SMG under acid hydrolytic conditions. Under drastic acid hydrolytic conditions, SECO was unstable and would be transformed mainly to anhydro-SECO [10], which was relatively stable in the hot acid solution [9,18] and chemically identical to another naturally occurring lignan called shonanin (3,4-divanillyltetrahydrofuran) [18]. It could also be transformed into a mammalian lignan similar to enterodiol and enterolactone, potentially beneficial in prostate cancer prevention [9]. Charlet et al. [9] reported a method based upon a total acid hydrolysis, which not only liberated SECO from SDG but also converted all of the SECO into its anhydrous form anhydro-SECO. After 2.5 h of 2 M HCl hydrolysis, the highest amount of anhydro-SECO was obtained without any trace of SECO [9].

3.5. *Production of esters of p-coumaric acid, ferulic acid, and their glucosides during the process of hydrolysis*

It has been well established that the glucosides of *p*-coumaric acid and ferulic acid may be involved in formation of SDG ester-linked oligomers [6,7]. Johnsson et al. [6] suggested *p*-coumaric acid glucoside, ferulic acid glucoside, and other phenolic compounds were present most probably in acylated forms. It was reported that a phenylpropanoid glucoside, which was a cinnamic acid methyl ester, had been isolated from the defatted meal of flaxseed [32]. Thus it was presumed that, in SDG oligomers, the glycosidic unit of SDG was esterified by *p*-coumaric acid glucoside or ferulic acid glucoside, which was easily released by alkaline hydrolysis to form the methyl ester of *p*-coumaric acid glucoside or ferulic acid glucoside in a reaction medium containing a large amount of methanol. Therefore, peaks 2 and 3 in Fig. 3 were tentatively identified as the methyl esters of *p*-coumaric acid glucoside and ferulic acid glucoside, respectively. Johnsson et al. [5] also found that when the SDG polymer was base-hydrolyzed in methanolic rather than aqueous medium, the first two peaks (*p*-coumaric acid and ferulic acid glucosides) were diminished and some extra peaks eluted after the SDG peak. In the present study, the “extra peaks” (peaks 2 and 3) eluted before the SDG peak (Fig. 3b).

The results also suggested that peaks 7 and 8 in Fig. 3c were the deglycosylation products of the methyl esters of ferulic acid glucoside and *p*-coumaric acid glucoside, respectively, by alkaline hydrolysis. As shown in Fig. 3c, only a smaller amount of *p*-coumaric acid methyl ester (peak 8) was found, indicating that the methyl ester of *p*-coumaric acid glucoside was more difficult to be deglycosylated by alkaline hydrolysis in comparison with the methyl ester of ferulic acid glucoside.

While SDG oligomers were dissolved in an aqueous ethanol solution and hydrolyzed by adding ethanolic NaOH, as described

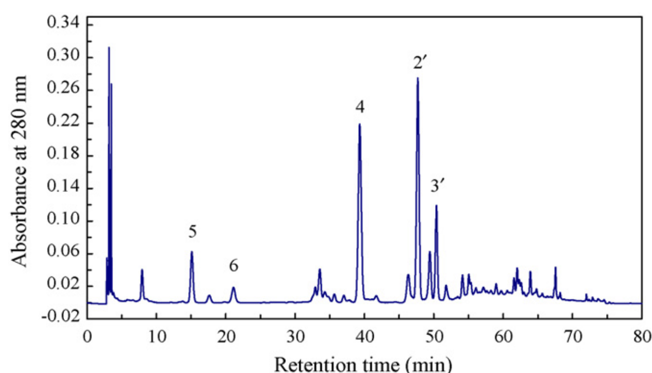

Fig. 5. HPLC chromatogram of the alkaline hydrolysates of SDG oligomers dissolved in the aqueous ethanol solution after 15 min of alkaline hydrolysis (final NaOH concentration of 20 mM) at 50 °C. Peak: 2′, the ethyl ester of *p*-coumaric acid glucoside; 3′, the ethyl ester of ferulic acid glucoside; 4, SDG; 5, *p*-coumaric acid glucoside; and 6, ferulic acid glucoside.

by Struijs et al. [8], two new precursors (peaks 2′ and 3′) of *p*-coumaric acid glucoside and ferulic acid glucoside, whose retention times markedly increased in comparison with peaks 2 and 3 in Fig. 3 (increasing from 33.9 and 37.5 min to 47.7 and 50.4 min, respectively), were found (Fig. 5). This indicates that, in a reaction medium containing a large amount of ethanol, *p*-coumaric acid glucoside and ferulic acid glucoside released from SDG oligomers were firstly esterified by ethanol to produce the ethyl esters of *p*-coumaric acid glucoside and ferulic acid glucoside, which subsequently were further hydrolyzed by alkali to produce *p*-coumaric acid glucoside and ferulic acid glucoside. Fig. 5 shows a similarity with the previous HPLC profiles obtained by Struijs et al. [8], who had also found two unidentified peaks eluting after SDG peak in the alkaline hydrolysates of SDG oligomers in an aqueous ethanol solution.

The experiments have shown that, under acid conditions, *p*-coumaric acid or ferulic acid standard dissolved in an aqueous methanol solution could be esterified by methanol to produce *p*-coumaric acid methyl ester or ferulic acid methyl ester. As shown in Fig. 4, after acid hydrolysis, comparable high amounts of *p*-coumaric acid methyl ester (peak 8) and ferulic acid methyl ester (peak 7) could be found, and only a small amount of free *p*-coumaric acid (peak 9) and ferulic acid (peak 10) were detected (Fig. 4). This indicates that, in a reaction medium containing large amounts of methanol and hydrochloric acid, *p*-coumaric acid and ferulic acid have been almost completely esterified.

While SDG oligomers were dissolved in water and hydrolyzed by adding an aqueous NaOH solution, as described by Johnsson et al. [5] and Eliasson et al. [11], the alkaline hydrolysis resulted in the immediate production of *p*-coumaric acid glucoside and ferulic acid glucoside (data not shown). While the alkaline hydrolysates were further hydrolyzed by acid, *p*-coumaric acid glucoside and ferulic acid glucoside were completely hydrolyzed to produce *p*-coumaric acid (peak 9) and ferulic acid (peak 10). The results also showed that two unspecified peaks with the retention times of 63.8 and 64.6 min (Fig. 4) were the derivatives of the methyl esters of *p*-coumaric acid and ferulic acid, respectively, which were not found in methanol-free medium.

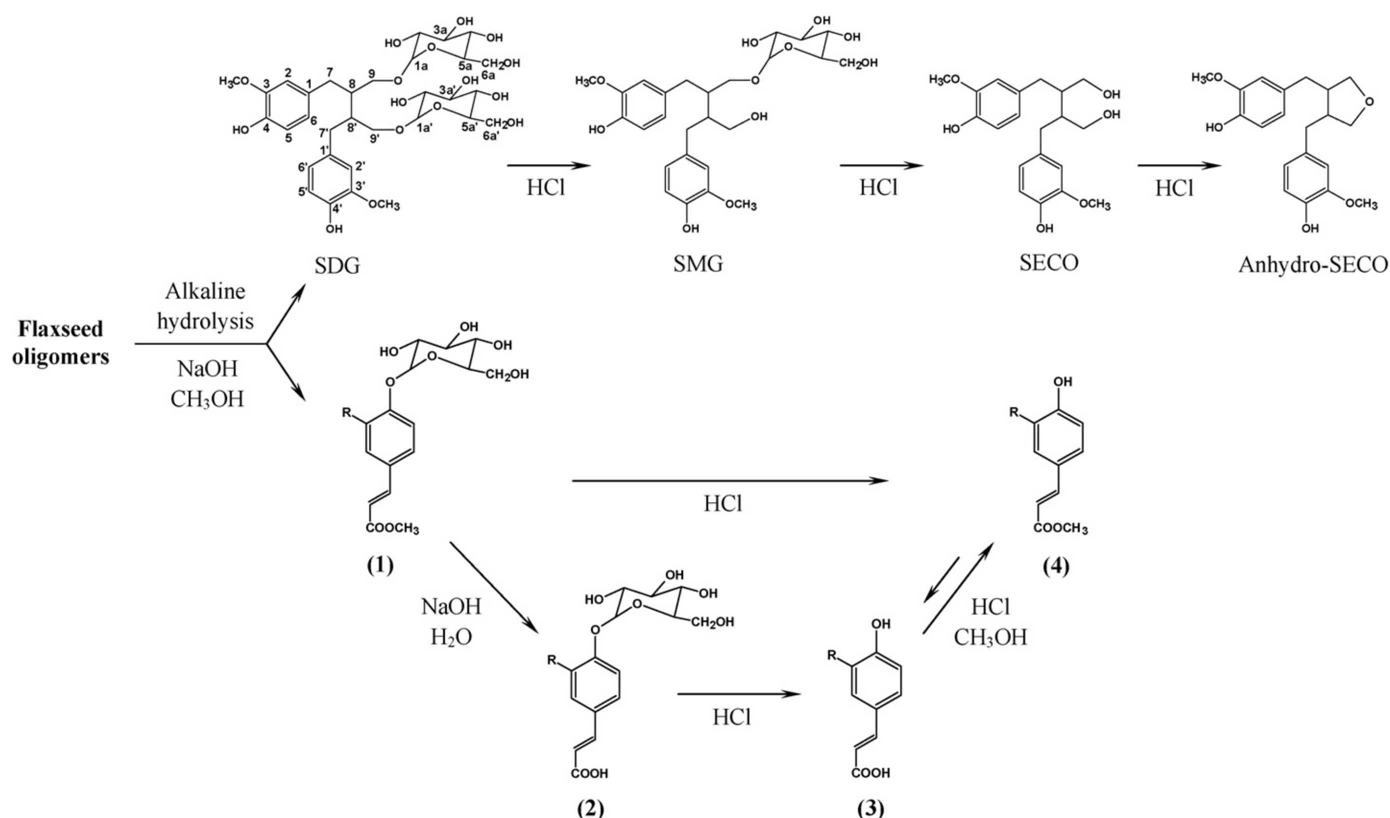

Fig. 6. A proposed major hydrolytic reaction pathway of SDG oligomers. (**1**) R═H: the methyl ester of *p*-coumaric acid glucoside, R═OCH_3: the methyl ester of ferulic acid glucoside; (**2**) R═H: *p*-coumaric acid glucoside, R═OCH_3: ferulic acid glucoside; (**3**) R═H: *p*-coumaric acid, R═OCH_3: ferulic acid; and (**4**) R═H: the methyl ester of *p*-coumaric acid, R═OCH_3: the methyl ester of ferulic acid.

3.6. Identification of HMF in the acid hydrolysates of SDG oligomers

As can be seen from Fig. 4, two largest peaks (13 and 14) were detected after the alkaline hydrolysis solution was further hydrolyzed with hydrochloric acid at 95 °C. Peak 13 was identified as HMF by comparing its retention time and spectrum against HMF standard. It has been established that HMF is the degradation product of the hydrolysis of hexoses [36]. The previous study had shown that the only sugar residue present in the SDG oligomers was glucose [6], and sucrose had been separated from the flaxseed extract [32]. It is possible that HMF is the degradation product of glucose from glucoside and other hexose carbohydrates, such as sucrose in the flaxseed extract.

As shown in Fig. 4, the further acid hydrolysis resulted in the increase in peak intensity of peak 14, and decrease in peak 13, indicating that peak 14 was the degradation product of HMF in hot acid solution, which has been validated by heating HMF standard dissolved in HCl solution. It has been reported that, under acidic conditions, HMF in water decomposes to levulinic acid [37].

3.7. Determination of contents of SDG oligomers and their hydrolysates

The contents of SDG oligomers and their hydrolysates in the flaxseed extract and the hydrolytic solutions were determined and the results on a dry matter basis are shown in Table 5.

The content of SDG oligomers in the defatted flaxseed powder was found to be 38.5 ± 0.5 mg/g on a dry matter basis. The SDG content was found to be 15.4 ± 0.3 mg/g in the defatted flaxseed powder on a dry matter basis (Table 5). The result is in agreement with the previous findings that the SDG content varied between 6 and 29 mg/g in the defatted flaxseed powder [5,6,9,11,17]. Struijs et al. [8] obtained 29.9 g of lignan macromolecule from 400 g of flaxseed hulls, indicating that flaxseed hulls were enriched in SDG compared to the cotyledons.

The contents of SDG oligomers and SDG in the commercial flaxseed lignan (40%) were also determined by dissolving 10 mg of this sample in 10 ml of 70% aqueous methanol solution. The results showed that the commercial flaxseed lignan (40%) contained both free SDG and SDG oligomers, and the contents of SDG and SDG oligomers in the lignan sample were 380.9 ± 9.8 and 363.3 ± 9.2 mg/g, respectively, on a dry matter basis. After the alkaline hydrolysis, the SDG level increased to 479.2 ± 9.6 mg/g on a dry matter basis (i.e. 47.9% of SDG).

4. Conclusion

In the present study, a new gradient reversed-phase HPLC method was developed to be suitable for the simultaneous separation and determination of: (1) SDG oligomers; (2) SDG oligomers and their alkaline hydrolysates including SDG, *p*-coumaric acid glucoside and its methyl ester (or ethyl ester), ferulic acid glucoside and its methyl ester (or ethyl ester); and (3)

the succedent acid hydrolysates including SDG, SMG, SECO, anhydro-SECO, *p*-coumaric acid and its methyl ester, ferulic acid and its methyl ester, HMF and its degradation product. This HPLC method allows direct injection of the flaxseed extract, its alkaline or acid hydrolysis reaction mixtures without further sample pretreatment to prevent from unwanted sample losses.

According to these results given above, a major hydrolytic reaction pathway of SDG oligomers in a reaction medium containing a large amount of methanol was proposed and the reaction pathway is shown in Fig. 6. Under the acidic conditions, SDG released from the SDG oligomers by the alkaline hydrolysis was firstly deglycosylated into SMG and SECO, and then could be further transformed to anhydro-SECO. In addition, the methyl esters of *p*-coumaric acid and ferulic acid were the main acid hydrolysates of the *p*-coumaric acid glucoside and ferulic acid glucoside released from the SDG oligomers and only a small amount of free *p*-coumaric acid and ferulic acid were present in the reaction medium containing hydrochloric acid and a large amount of methanol.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2008.01.066.

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