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On-line liquid-chromatography–nuclear magnetic resonance spectroscopy–mass spectrometry coupling for the separation and characterization of secoisolariciresinol diglucoside isomers in flaxseed

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Abstract

Two secoisolariciresinol diglucoside (SDG) diastereomers were extracted from flaxseed and liberated through alkaline hydrolysis. Anion-exchange and reversed-phase chromatography were successfully employed to purify the hydrolyzed flaxseed extract. On-line LC–NMR–MS analyses revealed the structure of the isolated and purified SDG diastereomers, [2*R*,2′*R*]-2,3-bis[(4-hydroxy-3-methoxyphenyl)-methyl]-1,4-butanediyl-bis-β-glucopyranoside the predominant flaxseed lignan and [2*R*,2′*S*]-2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediyl-bis-β-glucopyranoside, a previously incompletely characterized minor flaxseed lignan. Circular dichroism (CD) analyses confirmed the presence of two distinguished optically active compounds present in the flaxseed extract.

Keywords: Flaxseed; Functional food; Liquid chromatography–NMR spectroscopy mass spectrometry; Nuclear magnetic resonance spectroscopy; Secoisolariciresinol diglucoside

1. Introduction

The application of new coupled analytical techniques is becoming increasingly important for the investigation of natural products from plant origin. Several methods for coupling LC and GC with MS have been developed, allowing the detection and identification of a broad range of natural compounds in plant materials and foods [1–4]. However, a complete characterization of the chemical composition of complex natural products is usually not possible by LC–MS and GC–MS analysis due to the occurrence of complex mixtures of conjugates (glycosides) and aglycones present in natural extracts. The latter cannot be sufficiently distinguished in all cases by sole use of mass spectrometry. Moreover, many natural products can undergo severe degradation when subjected to the elevated temperatures required for some MS interfaces, and sometimes the molecular ions have a low abundance or are even not detectable at all [5]. High-resolution NMR is the most important method for the structure elucidation of complex compounds. Especially for the structure elucidation of unstable compounds such as caro-

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tenoid stereoisomers on-line LC–NMR excluding light and oxygen is the method of choice. For these samples LC–MS cannot distinguish between the different geometrical and optical stereoisomers [6,7]. The direct coupling of LC–NMR–MS is one of the most promising tools that have recently been developed for the fast and unambiguous analysis of natural products [8–10]. These combined techniques give in many cases complete structural information of complex plant compounds without the risk of oxidation and isomerization during time-consuming off-line analyses.

Stereospecific identification of bioactive compounds from natural sources is considered as a prerequisite for the physiological and toxicological evaluation of compounds that maybe of interest for the development of nutraceuticals and functional foods. A recent example for different physiological properties of individual isomeric forms has been shown for isomers of conjugated linoleic acid [11,12].

The occurrence of secoisolariciresinol diglycoside (SDG) (Fig. 1) was first described by Bakke and Klosterman 1956 [13]. Flaxseed (*Linum usitatissimum*) has been identified as the predominant source of this lignan which is a precursor for certain mammalian lignans, such as enterolactone and enterodiol [14,15]. Various physiologically effects have been observed in animal studies after SDG consumption. For example, an SDG-rich, high cholesterol diet reduced the serum cholesterol, LDL-cholesterol, and hypercholesterolemic atherosclerosis in rabbits by 33, 35, and 73%, respectively. Moreover, HDL cholesterol increased by more than 140% whilst the total cholesterol–LDL-cholesterol ratio decreased by 64% [16]. In addition, SDG has been shown to have an antitumor effect when provided at the early promotion stage of tumorigenesis [17].

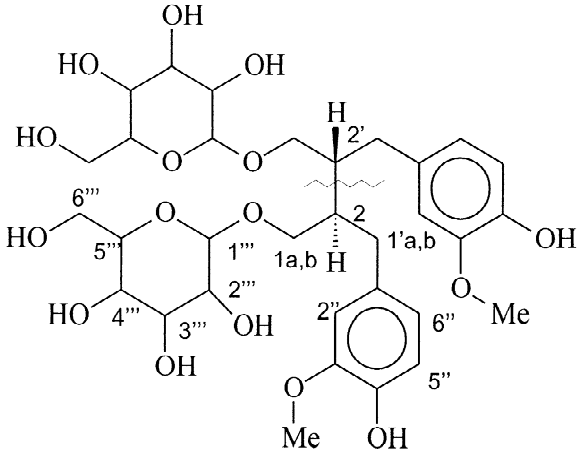

Fig. 1. Chemical structure and nomenclature of secoisolariciresinol diglucoside (SDG, $M_w = 686$). Nomenclature according to Chimini et al. [24].

Previous analytical work with flaxseed suggested the existence of two diastereoisomers of SDG, due to the two equivalent stereogenic centers present in the molecule [18]. Different extraction and separation techniques have been described [19–21]. Up to now, mainly GC–MS and LC–MS have been employed to study SDG [22,23]. Off-line NMR experiments to investigate flaxseed constituents were performed by Chimini et al. [24] and Qui et al. [25]. However, no complete structure elucidation of minor SDG isomers has been achieved until now.

In this article the application of on-line LC–NMR–MS is described for the structure elucidation of SDG diastereoisomers occurring in flaxseed. Moreover, CD measurements were performed to confirm the presence of optically active compounds.

2. Experimental

2.1. Materials

Defatted and crushed flaxseed meal was obtained from Thywissen (Neuss, Germany). Hexane, ethyl acetate, acetonitrile (ACN), methanol, deuterated water (D_2O), acetic acid glacial, and sodium hydroxide were purchased from Fisher Scientific (s'Hertogenbosch, The Netherlands) (all solvents were HPLC grade). Q Sepharose Fast Flow was obtained from Pharmacia (Woerden, The Netherlands) for anion exchange chromatography.

2.2. Purification equipment

XK26 LC columns for anion exchange chromatography were purchased from Pharmacia, semi-preparative LC columns (Merck LiChroprep RP-18, particle size 40–63 μm) for purification purpose

were obtained from Merck (Darmstadt, Germany). Two peristaltic pumps (Pharmacia, and Ismatec MS-Reglo, De Bilt, The Netherlands) were used as LC equipment.

2.3. Extraction

In the present work, the method applied for the extraction and further purification of the flaxseed extract is based on a procedure described by Johnsson et al. [21] and Westcott and Muir [26] with slight modifications.

About 100 g flaxseed meal was transferred into a beaker filled with methanol–water (75:25, v/v) and stirred continuously for 24 h at 65 °C. Afterwards the extract was filtered through a paper filter to remove insoluble material. The crude extract was concentrated by means of vacuum evaporation.

2.4. Hydrolysis

The flaxseed concentrate was hydrolyzed by addition of 0.3 *M* NaOH in a ratio of 1:1 (w/w). After hydrolysis the extract was neutralized with glacial acetic acid and adjusted to pH 6. The extract was filtered through a couple of filters in series (cotton candlefilter and Nylon Cuno 0.2 μm) and then concentrated by means of vacuum evaporation.

2.5. Anion-exchange chromatography

The concentrate was further applied on an anion-exchange column. The anion-exchanger was previously conditioned with acetate buffer adjusted to pH 6. Flaxseed lignans (and other organic material) were eluted with water, while anionic material was retarded on the column. The anion-exchanger was recovered by washing with acetic acid–ethanol (50:15, v/v) and water.

2.6. Reversed-phase chromatography

Further purification of SDG was accomplished by (semi)preparative reversed-phase chromatography (Merck LiChroprep RP-18; 40–63 μm). The column (length 310 mm, I.D. 25 mm) was filled with reversed-phase material (~70 g) and first conditioned with methanol to remove possible organic contamination and finally flushed with water. The purified extract was applied to the column and washed with water to elute the organic and inorganic salts (including buffer salts from the anion-exchange procedure). SDG was eluted with methanol–water (50:50, v/v). The purity of the obtained extracts was monitored by LC–MS analysis.

2.7. Characterization of flaxseed lignans

Analytical separations were carried out on a 250× 4.6-mm stainless steel LiChrospher C_{18} reversed-phase column (Merck, Darmstadt, Germany) with a particle size of 5 μm and an average pore diameter of 100 Å. The HPLC system used was an HP 1100 equipped with a quaternary pump G1311A and a UV/DAD detector G1315A (Agilent, Waldbronn, Germany). The column thermostat was set to 25 °C. The injection volume was set to 30 μl. Chromatograms were recorded at 280 nm. The flaxseed extract was separated using a binary mixture of (A) D_2O and (B) ACN. A linear gradient from 95% (A) (0 min) to 40% (A) (44 min) at a flow-rate of 1 ml/min was applied. Although highly efficient solvent suppression methods have been developed for NMR and deuterated solvents are no longer mandatory for on-line LC–NMR analyses, it is still convenient to use a deuterated mobile phase without any buffer systems.

2.8. LC–NMR–MS coupling

The on-line LC–NMR–MS experiments were conducted using a 600-MHz AVANCE NMR spectrometer (Bruker, Rheinstetten, Germany) and a mass spectrometer G1946A with an ESI interface (Agilent Technologies, Waldbronn, Germany). The NMR spectrometer was equipped with an LC inverse gradient probe with a detection volume of 120 μl. The chromatographic equipment and the Bruker BPSU-36 interface including a post-column Bruker BNMI flow splitter with a split ratio MS–NMR (1:20, v/v) were controlled by Hystar software (Bruker, Rheinstetten, Germany). Thus, in this experimental layout MS and NMR data were acquired in parallel, rather than in series [27,28]. For the MS analyses the non-deuterated solvent was used by

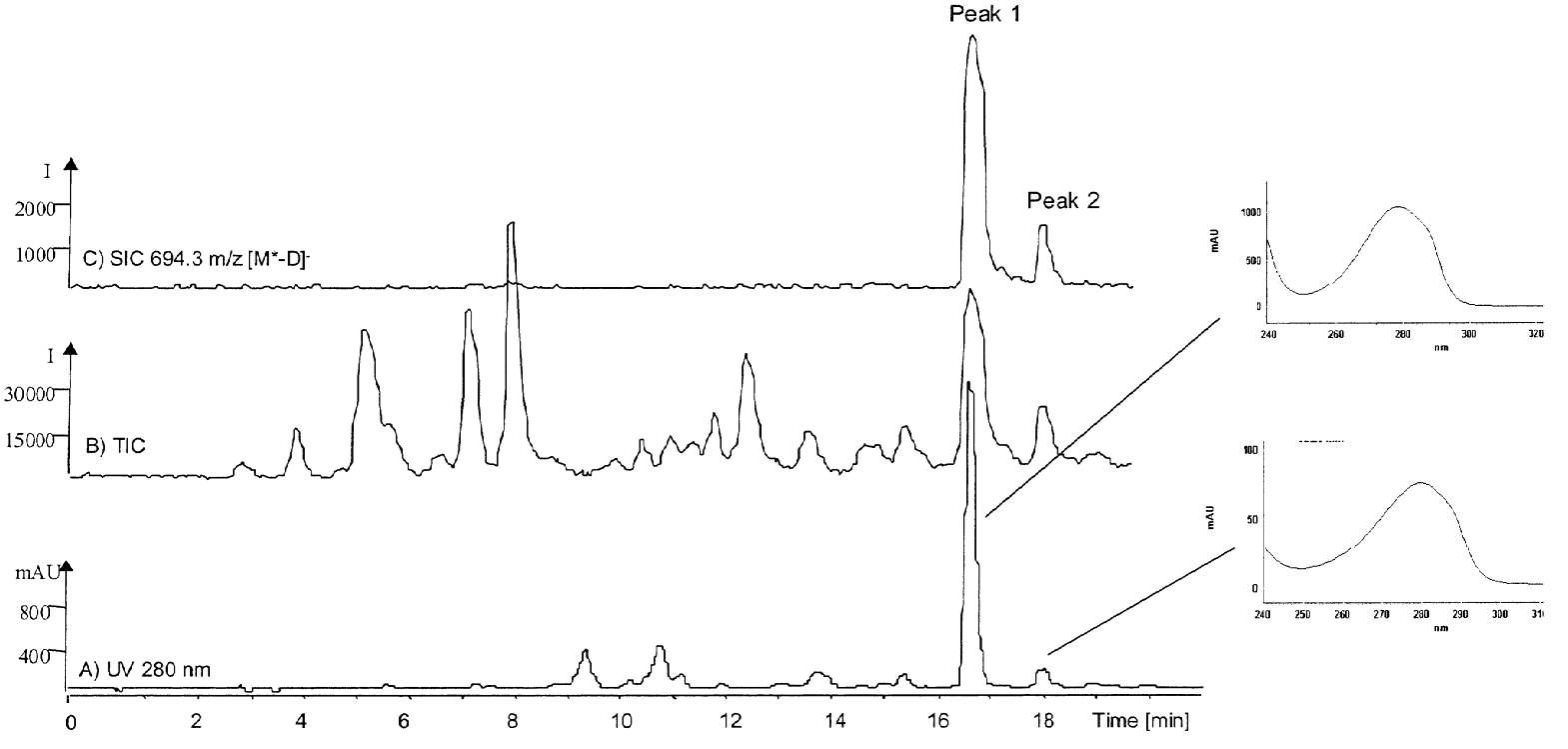

Fig. 2. On-line LC–NMR–MS analysis of a flaxseed extract: (A) UV spectrum and chromatogram recorded at 280 nm; UV spectra (240–340 nm) of peaks 1 and 2 are superimposed. (B) Total ion chromatogram (TIC). (C) Selected ion chromatogram (SIC) of m/z 694.3. Peaks 1 and 2 indicate the two diastereoisomers of secoisolariciresinol diglucoside.

adding 150 μl/min of a mixture ACN–H_2O (20:80, v/v) after splitting the solvent.

ESI mass spectra were acquired in the positive and negative ion mode in the mass range of m/z 100–1000 at fragmentor voltages of 100 and 300 V, respectively. Nitrogen was used as drying gas at a flow-rate of 10.0 l/min and 300 °C. Nebulizer pressure was set to 4 bar. The capillary voltage was optimized to 3500 V. For all spectra manual baseline subtraction was performed.

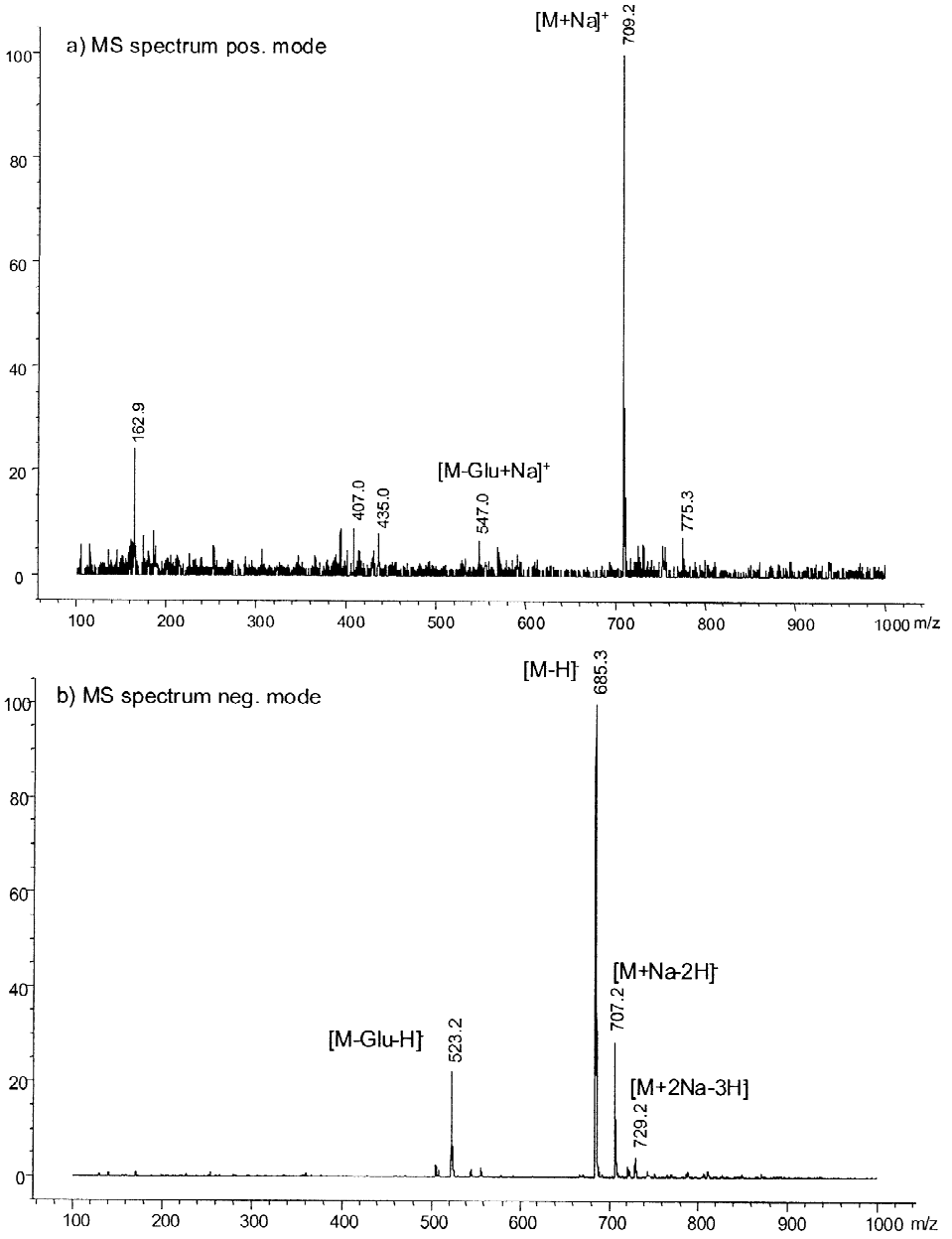

Fig. 3. ESI–MS spectra of secoisolariciresinol diglucoside extracted from flaxseed obtained at a fragmental voltage of 100 V: (a) positive mode, (b) negative mode.

For ^{1}H NMR stopped-flow measurements, 2 K (peak 1) and 22 K (peak 2) transients were accumulated at a constant temperature of 27 °C, using a time domain of 16 K and a sweep width of 20 ppm. Solvent suppression was performed using the WET sequence. Processing was performed with 1D XWINNMR software (Bruker). For all spectra, zero filling up to 32K data points and an exponential multiplication of the FID with a line broadening of 0.5 Hz was performed before Fourier transformation.

2.9. CD analyses

Circular dichroism (CD) spectra were recorded to confirm the presence of optically active species in the flaxseed extract. To this end, peaks 1 and 2 were fractionated after the NMR analysis, evaporated and re-dissolved in MeOH. The CD spectra were recorded on a Jasco J700 CD spectrometer (Oklahoma City, USA). The calibration and validation of the instrument was performed with standard solutions of 1.25 g/l isoandrosteron in dioxane and 1 g/l camphorsulphonic acid in milli-Q water. Each spectrum was collected 16 times, co-added, calculated in Theta units (Molar ellipticity in Deg M^{-1} cm^{-1}) and corrected for the blank. Analysis was done in cylindrical cuvettes with pathlengths of 0.02 cm. During acquisition the temperature of the cuvette-holder was kept constant at 20 °C. The optical activity was determined within the spectral region between 190 and 260 nm at 100 nm/min and a fixed bandwidth of 1 nm.

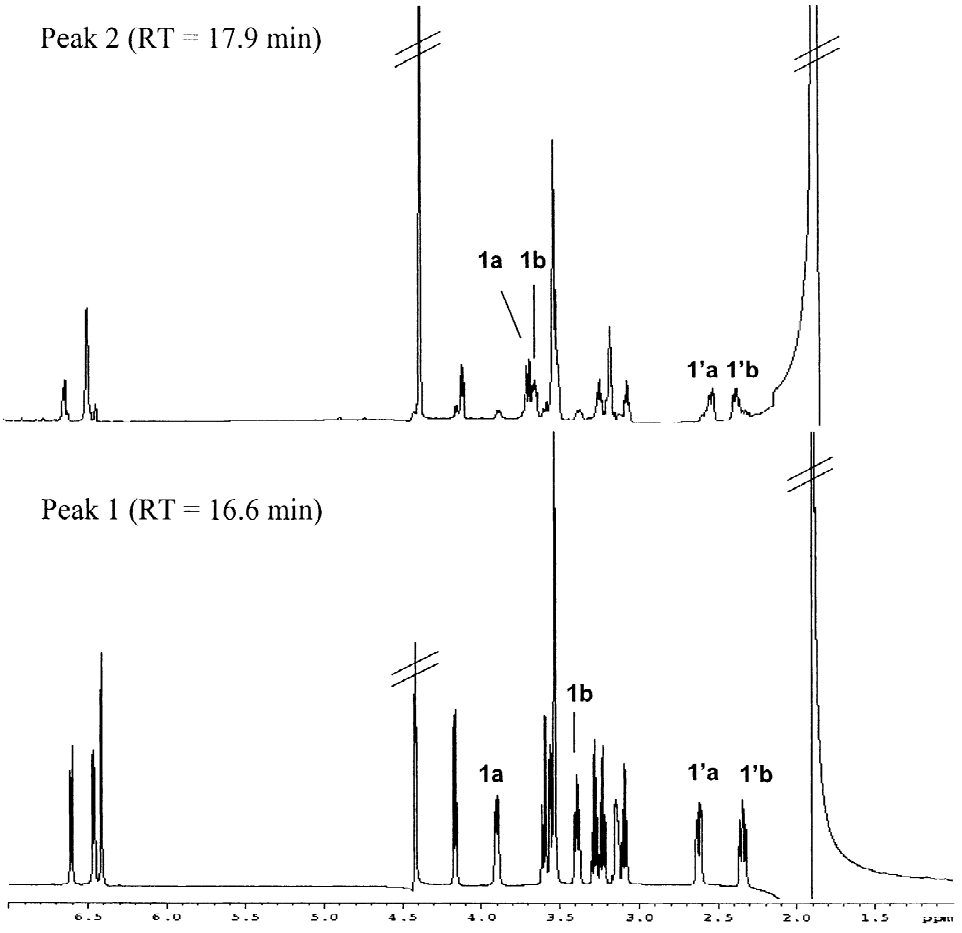

Fig. 4. On-line ^{1}H NMR (600 MHz) spectra of the two diastereoisomers of SDG.

3. Results and discussion

For the structure elucidation of flaxseed SDG diastereomers (Fig. 1) on-line LC–NMR–MS analysis was performed. Fig. 2a shows the UV spectra of compound 1 and compound 2 as well as the UV chromatogram recorded at 280 nm, the observed absorption maximum of both compounds. The total ion chromatogram (TIC) is depicted in Fig. 2b, as well as the selected ion chromatogram (SIC) of m/z 694.3 in Fig. 2c, both detected in the negative ESI mode. Two MS signals at retention times 16.6 and 17.9 min are visible corresponding to the deuterated molecular mass $[M^*-D]^-$ of SDG. For the detection of the non-deuterated molecular mass of the molecule solvent exchange was performed. The resulting MS spectra are shown in Fig. 3. Both compounds show identical mass spectra of the non-deuterated forms with the same fragmentation patterns, indicating two isomeric forms of the compound (spectra not shown).

The mass spectra of SDG shows a $[M+Na]^+$-peak at m/z 709.2 in the ESI+ mode corresponding to the molecular formula $C_{32}H_{46}O_{16}$. In addition, the mass fragment m/z 547.0 and m/z 162.9 obtained at a fragmentor voltage of 100 V indicate the loss of a sugar unit (Fig. 3a). At higher fragmentor voltages also the loss of the second sugar unit as well as a fragment ion m/z 136.9 corresponding to a (4-hydroxy-3-methoxyphenyl)methyl moiety can be observed (spectra not shown). However, it cannot be excluded from our data that the fragment ion m/z 136.9 may be attributed to a 4-methoxy-3-hydroxy unit. Additional two-dimensional NMR experiments are necessary to overcome this uncertainty [24,25].

Moreover, in the ESI-mode an $[M-H]^-$-peak at 685.3 m/z is visible as shown in Fig. 3b. Comparison to the detected $[M-D]^-$-peak at 694.3 m/z measured in deuterated solvents indicates the existence of 10 D/H-exchangeable protons in the molecule. The MS signal 523.2 m/z can be assigned to $[M-Glu\text{-}H]^-$. Additionally, the Na-adducts $[M+Na\text{-}2H]^-$ at 707.2 m/z and $[M+2Na\text{-}3H]^-$ 729.2 m/z can be detected. At higher fragmentor voltages a set of additional peaks appear in the MS spectrum: m/z 361 $[M-2Glu\text{-}H]^-$, m/z 545 $[M-Glu+Na\text{-}2H]^-$, and m/z 567 $[M-Glu+2Na\text{-}3H]^-$ (spectra not shown). Minor fragment ions correspond to fragments of sugar and (4-hydroxy-3-methoxyphenyl)methyl units.

Fig. 4 depicts the ^{1}H NMR spectra of the two peaks at 16.6 and 17.9 min detected in UV and MS. Both peaks can be unequivocally assigned as SDG isomers. Off-line NMR data of Peak 1 were already reported by Chimini et al. [24]. The obtained on-line NMR data are listed in Table 1. The chemical shift values as well as coupling constants are in good agreement with the reported data. Small shift differ-

Table 1
On-line ^{1}H NMR (600 MHz) data of the two diastereoisomers of SDG

Proton	SDG peak 1 (δ in ppm)	SDG peak 1 (J in Hz)	SDG peak 2 (δ in ppm)	SDG peak 2 (J in Hz)
H-2″	6.41	–	6.48	
H-5″	6.60	8.1 (H-6″)	6.62	8.1 (H-6″)
H-6″	6.46	8.1 (H-5″)	6.48	8.1 (H-5″)
3″-OMe	3.53	–	3.56	–
H-1‴	4.16	7.3 (H-2‴)	4.13	7.3 (H-2‴)
H-2‴-H-6‴	3.09–3.60	n.d.	3.10–3.60	n.d.
H-1′a	2.62	13.2 (H-1′b)	2.57	13.2 (H-1′b)
		5.1 (H-2)		5.1 (H-2)
H-1′b	2.34	13.2 (H-1′a)	2.42	13.2 (H-1′a)
		10.3 (H-2)		10.3 (H-2)
H-2, H-2′	n.d.	n.d.	n.d.	n.d
H-1a	3.90	10.3 (H-1b)	3.72	10.3 (H-1b)
		4.4 (H-2)		4.4 (H-2)
H-1b	3.39	10.3 (H-1a)	3.67	10.3 (H-1a)
		8.1 (H-2)		8.1 (H-2)

n.d., not detectable.

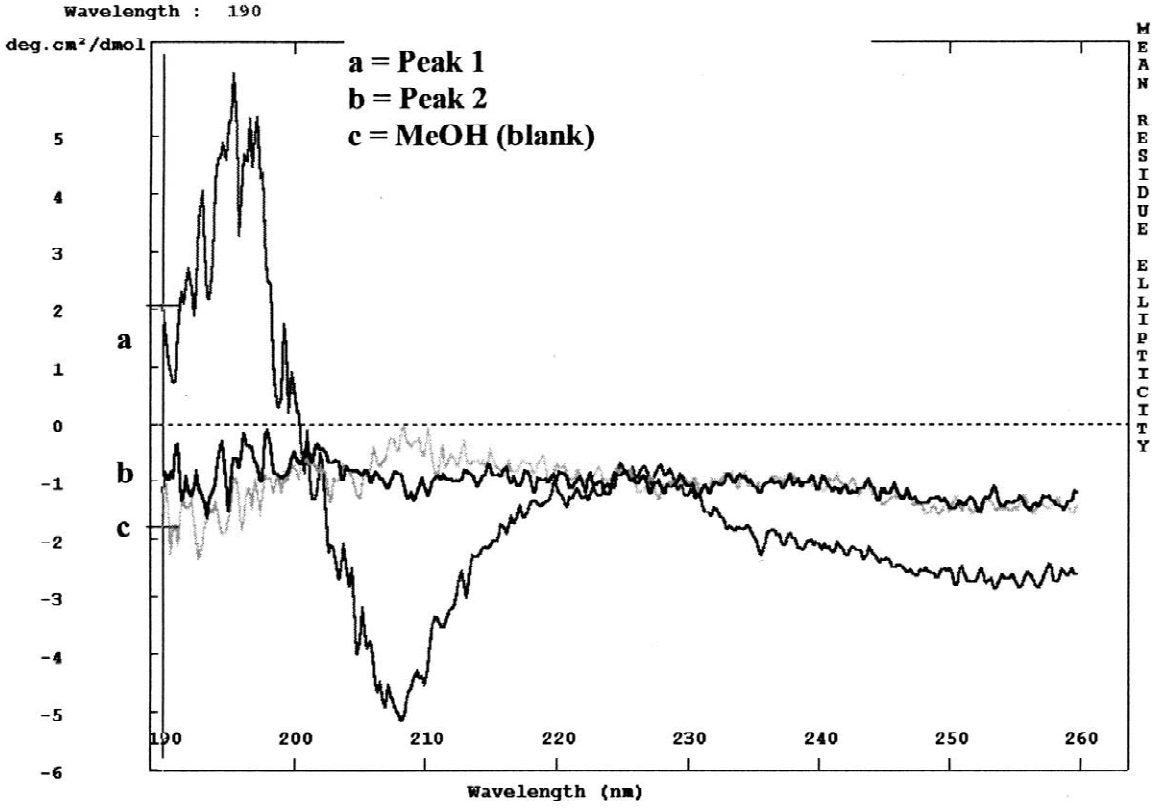

Fig. 5. CD spectra of the two SDG diastereomers compared with a blank solution (MeOH). Peak 1 (a) [2*R*,2′*R*]-2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediyl-bis-β-glucopyranoside, Peak 2 (b) [2*R*,2′*S*]-2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediyl-bis-β-glucopyranoside.

ences were observed due to the use of a different solvent system [27,28]. The on-line ^{1}H NMR spectrum displays an anomeric proton system at 4.16 ppm with a coupling constant of 7.3 Hz, which is characteristic of an axial orientation of the anomeric proton (β-configuration). Thus, compound 1 (peak 1) at retention time of 16.6 min was identified as [2*R*, 2′*R*] - 2, 3 - bis[(4 - hydroxy - 3 - methoxyphenyl) - methyl] - 1, 4 - butanediyl - bis - β - glucopyranoside. Significant different chemical shifts of prochiral protons H-1a (3.90 ppm) and H-1b (3.39 ppm) were observed. The other prochiral protons H-1′a and H-1′b also show a difference in the chemical shift of $\Delta\delta = 0.28$.

The on-line spectrum of compound 2 (peak 2) at a retention time of 17.9 min can be assigned to [2*R*, 2′*S*] - 2, 3 - bis[(4 - hydroxy - 3 - methoxyphenyl) - methyl] - 1, 4 - butanediyl - bis - β - glucopyranoside. As shown in Fig. 4 the prochiral protons have significantly different chemical shifts in comparison to the stereoisomer of peak 1.

In addition, CD measurements confirm the presence of optically active species (Fig. 5). A 0.02-cm cylindrical cell was used for the CD measurements instead of a 1-cm pathlength to reduce the absorbance of methanol. The use of these small pathlengths allows measurements even in the far-end of the UV region. As can be seen from Fig. 5 compound 2 (peak 2) showed a less intense optical activity than compound 1 (peak 1) which supports the [2*R*,2′*S*] configuration of the minor flaxseed lignan isomer.

4. Conclusion

On-line LC–NMR–MS was applied for the struc-

ture elucidation of two diastereomeric 2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediyl-bis-β-glucopyranosides extracted from flaxseed. The hyphenated technique allowed the separation and characterization of two flaxseed lignan diastereoisomers within one single chromatographic run. Using LC–MS sole it was not possible to distinguish between the two SDG isomers. CD analysis supported the occurrence of two distinguished optically active compounds.

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