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SE-HPLC-DAD Analysis of Flaxseed Lignan Macromolecule and its Hydrolysates

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A lignan macromolecule (LM) was extracted from defatted flaxseeds using an ethanol-dioxan system (1:1, v/v) and purified using Amberlite column chromatography with water and methanol as mobile phases. The LM was subjected to chemical hydrolysis (base, acid, base & acid), as well as to enzymatic processing using pepsin, pancreatin, cellulase, and β-glucuronidase.

The study revealed that lignan macromolecule in flaxseed was not homogenous. The chemical hydrolysis as well as enzymatic treatment using β-glucuronidase and cellulase released low molecular phenolic compounds from the lignan macromolecule. The liberation of secoisolariciresinol (SECO) and free phenolic acids (*p*-coumaric and ferulic acids) from flaxseed lignan macromolecule as a result of the base and acid hydrolyses was noted. The application of pepsin and pancreatin did not change the composition of the lignan macromolecule.

INTRODUCTION

Lignans are a class of diphenolic compounds generally containing a dibenzylbutane skeleton structure. In human nutrition the richest source of lignans is flaxseed (*Linum usitatissimum* L.) [Eldin-Kamal *et al.,* 2001]. The main lignan of flax is secoisolariciresinol diglucoside (SDG) [Meagher *et al.*, 1999]. On consumption of flaxseeds, SDG is converted by bacteria to mammalian lignans: enterodiol (ED) and enterolactone (EL) [Borriello *et al*., 1985].

The antioxidant activity of flaxseed extracts and SDG was assessed by several authors using different experimental models [Amarowicz *et al.,* 1993, 1997; Niemeyer & Metzler, 2003; Hosseinian *et al.*, 2006; Hu *et al.*, 2007]. The potential role of lignans in reducing the risk of mammary and prostatic tumours was confirmed in several studies [Jenab & Thompson, 1996; Adlercreutz, 2002; Lin *et al.,* 2002; Boccardo *et al*., 2004, Lainé *et al.*, 2009]. The similarity in the chemical structure of ED and EL and oestradiol led to the suggestion that they can act as weak oestrogenic/antioestrogenic compounds [Nesbitt & Thompson, 1999].

The analysis of lignans and other phenolic compounds present in flaxseed was performed using different chromatographic methods: silica gel, Sephadex LH-20, RP-8 column chromatography, TLC, RP-HPLC [Amarowicz *et al*., 1993, 1994, 1997; Johnsson *et al*., 2000; Struijs *et al*., 2008; Lorenc-Kukuła *et al*., 2005, 2009]. The present study reports on the application of size exclusion high performance liquid chromatography with a diode array detection (SE-HPLC-DAD) in analysis of lignan macromolecule (LM) and phenolic compounds liberated from LM after chemical and enzymatic hydrolysis.

MATERIAL AND METHODS

Material

Ground, partially defatted flaxseeds were purchased from the "Ekoprodukt" company (Częstochowa, Poland).

Preparation of crude extract

The material was defatted with hexane and then phenolic compounds were extracted using a dioxane:ethanol (1:1; v:v) mixture [Johnsson *et al*., 2000]. The extraction was carried out for 16 h, at 60°C with continuous shaking in a water bath. Then, solvent was evaporated using Büchi Rotavapor R-200 at 40°C.

Amberlite column chromatography

The flaxseed phenolic compounds extract was purified using column chromatography on Amberlite XAD-16 (34 mm i.d.; 170 mm length) [Srivastava *et al*., 2010]. A 5-g portion of the extract was suspended in 5 mL of distilled water and loaded on the column. Firstly, water soluble compounds, mainly sugars, were eluted using distilled water and discarded, then solvent was changed over to methanol which eluted phenolic compounds. The solvent was removed using Büchi Rotavapor R-200.

Base hydrolysis

The purified extract was subjected to base hydrolysis. Briefly, the purified extract was suspended in 0.3 mol/L NaOH,

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and left for 2 days at room temperature under continuous stirring. The obtained hydrolysate was acidified to pH 3 using 2 mol/L HCl [Johnson *et al.,* 2000] and subjected to column chromatography on RP-18 gel (Lichrosper 100 RP-18, Merck, Darmstadt, Germany). Water soluble compounds were eluted with distilled water and discarded, whereas compounds of interest were eluted with methanol. The solvent was removed using Büchi Rotavapor R-200.

Acid hydrolysis

The purified extract was suspended in 2 mol/L HCl and heated for 2 h at 100°C. Then the hydrolysate was cooled down, neutralized with 6 mol/L NaOH and subjected to column chromatography on RP-18 gel (Merck) according to the procedure described above.

Base and acid hydrolysis

The purified extract was suspended in 0.3 mol/L NaOH, and left for 2 days at room temperature under continuous stirring. Then pH was adjusted to pH 2 with 2 mol/L HCl and subsequently 6 mol/L HCl was added to the final concentration of 2 mol/L in the mixture. After 2 h of heating at 100°C the mixture was cooled down and purified in the same manner as in the case of base hydrolysate.

Enzymatic hydrolysis by pepsin

The suspension of 200 mg of purified extract in 8 mL of distilled water was mixed with 1 mL of a pepsin solution (5 mg/mL 2500 FIP – U/g, Merck, Darmstadt, Germany). The mixture was adjusted to pH 2.0 with 2 mol/L HCl and the samples were incubated at 37°C for 30 min with continuous stirring. After incubation the mixture was transferred onto RP-18 (Merck) column equilibrated with distilled water. Firstly, the column was rinsed with 100 mL of water and then the compounds of interest were eluted with 100 mL of methanol. The solvent was evaporated using Büchi Rotavapor R-200.

Enzymatic hydrolysis by pancreatin

The portion of 100 mg of purified extract was suspended in 10 mL of acetic buffer (0.1 mol/L, pH 5.0) and then mixed with 125 μL of a pancreatin solution (40 mg/mL, pancreatin from porcine pancreas grade VI, Sigma-Aldrich, Poznań, Poland). The mixture was incubated at 37°C for 16 h with continuous stirring. The compounds of interest were separated in the same manner as in the case of pepsin hydrolysis.

Enzymatic hydrolysis by cellulase

Purified extract was dissolved in acetic buffer (0.1 mol/L, pH 5.0) and 100 μL of cellulase from *Aspergillus niger* (4 mg/mL, 0.8 U/mg, Sigma-Aldrich) was added [Åkerberg *et al.*, 1998]. The mixture was incubated at 37°C for 16 h. Then, the hydrolysate was subjected to RP–18 column chromatography and purified.

Enzymatic hydrolysis by β-glucuronidase

β-Glucuronidase from *Helix pomatia* (4 mg/mL, type H-5, Sigma-Aldrich) was used for hydrolysis. The procedure was the same as for cellulase.

SE-HPLC-DAD analysis

The analysis of lignan macromolecule and its hydrolysates was performed using a TSK G2000SW$_{XL}$ column (7.8 × 300 mm, 5 μm; TosoHaas) and a Shimadzu system (Shimadzu Corp., Kyoto, Japan) consisting of LC-10AD pump, SCL 10A system controller, SPD-M 10A diode array detector. The mobile phase, consisting of 45% (v/v) acetonitrile and 0.1% TFA (v/v), was delivered at a rate of 0.2 mL/min. Samples (20 μL) at a concentration of 2 mg/mL were injected manually onto the column. The detection was performed at 280 nm and 320 nm.

RESULTS AND DICUSSION

The chromatogram depicted in Figure 1 shows an excellent baseline separation of the mixture of four standards (cytochrome C – 12,300, aprotinin – 6,500, epigallocatechin gallate – 458, and gallic acid – 170) on TSK G2000SW$_{XL}$ column. The results confirmed usability of SE column for HPLC analysis of flaxseed phenolic compound which are characterised by a broad range of molecular weights. In our laboratory this kind of column was used before for separation of a tannin fraction of bearberry (*Arctostaphylos uva-ursi* L. Sprengel) leaves [Pegg *et al*., 2008], its complex with myosin [Amarowicz *et al*., 2009], and phenolic compounds from rapeseed [Karamać *et al*., 2007].

SE-HPLC chromatograms of lignan macromolecule (Figure 2) showed one main broad peak with retention time of 56.61 min (Table 1). According to chromatograms depicted in Figures 1 and 2, the molecular weight of lignan macromolecule seems to be <6,500. These results are in accordance with findings of Struijs *et al*. [2009]. In the cited work the molecular weight of LM determined using MALDI-TOF MS ranged from 1,500 (for a molecule consisting of 2SDG + 1 hydroxymethyl-glutaric acid [HMGA)]) to 4,300 (for a molecule consisting of 5SDG + 6HMGA).

It is very interesting that UV spectra for eluates recorded at different retention times were not the same. At the be-

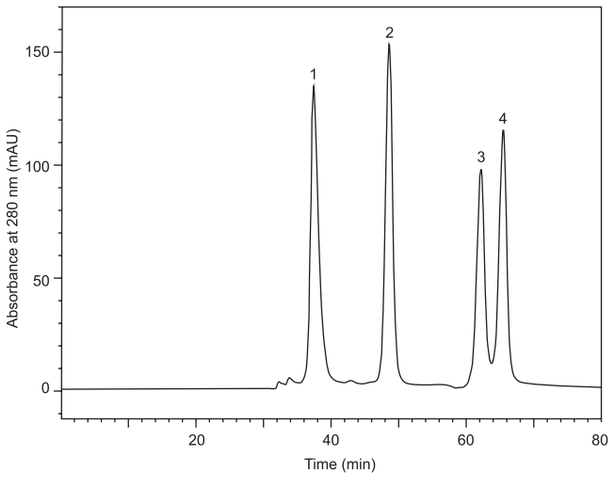

FIGURE 1. SE-HPLC chromatogram of the molecular weight standards; 1 – cytochrome c (12,300), 2 – aprotinin (6,500), 3 – epigallocatechin gallate (458), 4 – gallic acid (170).

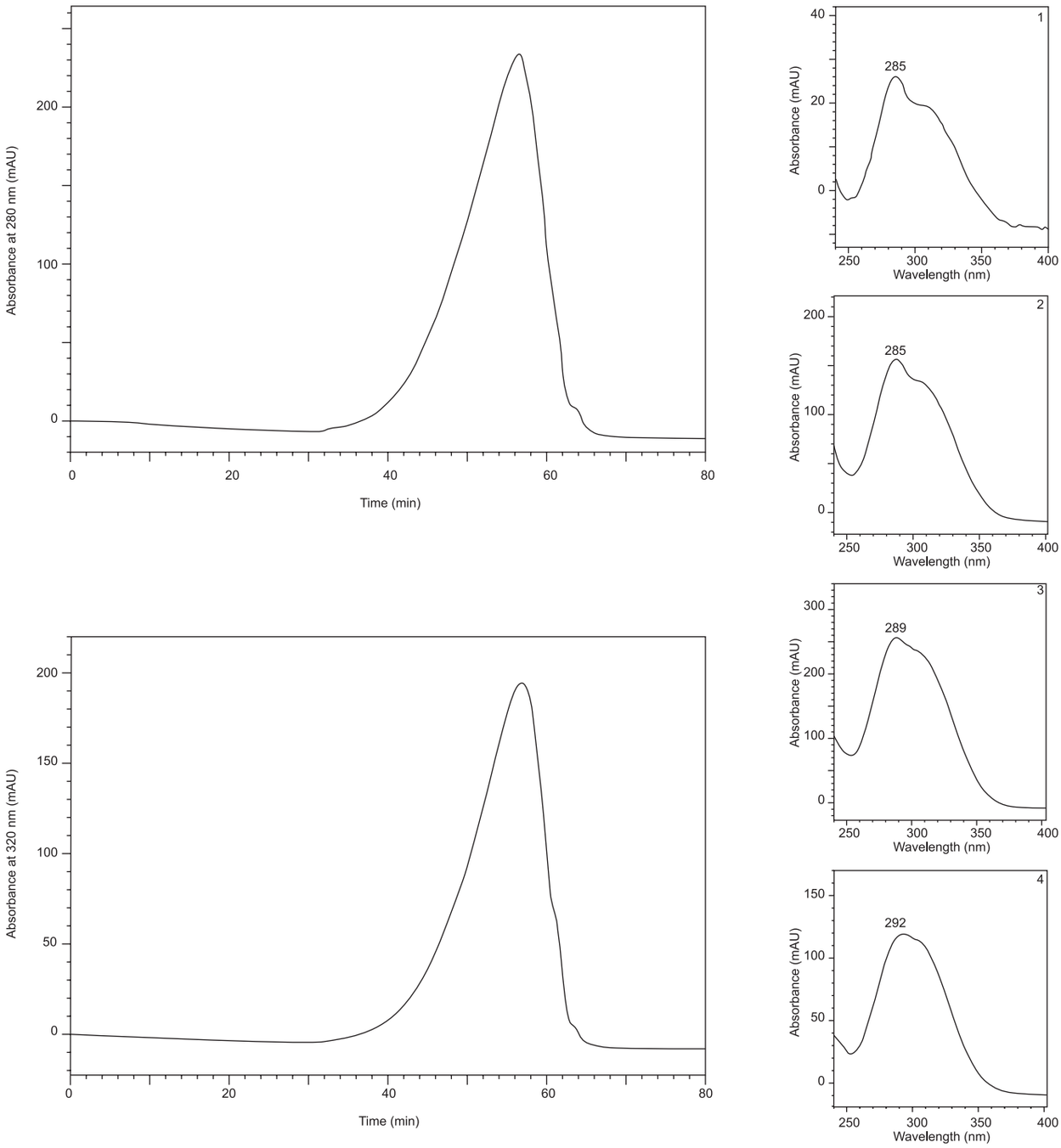

FIGURE 2. SE-HPLC chromatogram of the lignan macromolecule and UV spectra at retention time of 42.02 min (1), 50.94 min (2), 56.61 min (3), and 60.13 min (4).

ginning, the spectrum was characterised by a maximum at 285 nm and a shoulder (Figure 2, spectra 1 and 2). With a longer retention time the maximum was shifted in direction of the longer wavelengths and the shoulder disappeared (Figure 2, spectra 3 and 4). Similar UV spectra of phenolic fractions separated from the crude flaxseed extract using Sephadex LH-20, RP-8 and silica gel column chromatography were described by Amarowicz *et al*. [1993, 1994, 1997].

The UV spectra obtained in this study indicated that lignan macromolecule in flaxseed was not homogenous. This is rather a mixture of molecules with similar molecular weight but with a slightly different composition of individual phenolic compounds. According to Johnson *et al*. [2002] and Struijts *et al*. [2008], SDG ester linked to hydroxymethyl-glutaric acid (HMGA) forms the backbone of the lignan macromolecule. The hydroxycinnamic acids: *p*-coumaric acid glucoside and ferulic acid glucoside, are also part of a lignan macromolecule.

Figure 3 depicts the chromatograms of the extracts after base hydrolysis. The absence of a broad peak is an evidence for the incomplete process of hydrolysis. The macromol-

TABLE 1. Characteristics of the separation of lignan macromolecule and its hydrolysates using SE-HPLC-DAD method.

Hydrolysis	Peak number	Retention time (min)	UV max (nm)
Native lignan macromolecule	Main peak	56.61	295
Chemical hydrolysis			
Base	1	57.64	334
	2	59.61	317
	3	61.96	287
Acid	1	56.92	284
	2	59.58	282
	3	61.32	286
	4	63.40	288
Base & acid	1	58.50	282
	2	60.59	288, 310
	3	63.29	282
Enzymatic hydrolysis			
Pepsin	Main peak	56.71	295
Pancreatin	Main peak	56.71	295
Cellulase	1	58.04	289
	2	61.45	290
	3	62.23	289, 308
β-Glucuronidase	1	57.77	288
	2	61.45	288, 307
	3	62.23	289, 308

ecule was hydrolysed to small phenolic molecules such as SDG, 4-*O*-β-glucopyranosyl-*p*-coumaric acid (CouAG), 4-*O*-β-glucopyranosyl-ferulic acid (FeAG), and herbacetin diglucoside (HDG) [Johnson *et al*., 2002]. The UV spectra were typical of CouAG (max at 334 nm) and FeAG (290 and 317 nm) [Lorenc-Kukuła *et al.*, 2009]. The absorption band at 287 nm, originated probably from SDG derivative, was noted at UV-DAD spectra (Figure 3, spectrum 3).

After acid hydrolysis four peaks (1–4) were recorded on the chromatograms (Figure 4). The maxima of UV spectra were noted at 284 nm (peak 1) 282 nm (peak 2), 286 nm (peak 3), and 288 nm (peak 4) (Table 1). The occurrence of peak 1 on the chromatogram suggests the presence of small amounts of the fragments of the lignan macromolecule with molecular weight >458 in the acid hydrolysate of the flaxseed extract. The chemical bonds between *p*-coumaric and ferulic acids and glucose were hydrolysed during the acidic process. Furthermore, UV-DAD spectra corresponding to individual peaks were more typical of SDG [Lorenc-Kukuła *et al.*, 2009].

The application of the base and acid hydrolyses resulted in the presence of three peaks (1–3) on the chromatogram recorded at 280 nm (Figure 5). Only one peak was revealed on the chromatogram recorded at 320 nm. The maxima of UV spectra were noted at 282 nm (peak 1), 288 and 310 nm (peak 2), and 282 nm (peak 3) (Table 1). The UV spectra depicted in Figure 5 show the liberation of secoisolariciresinol (SECO) and

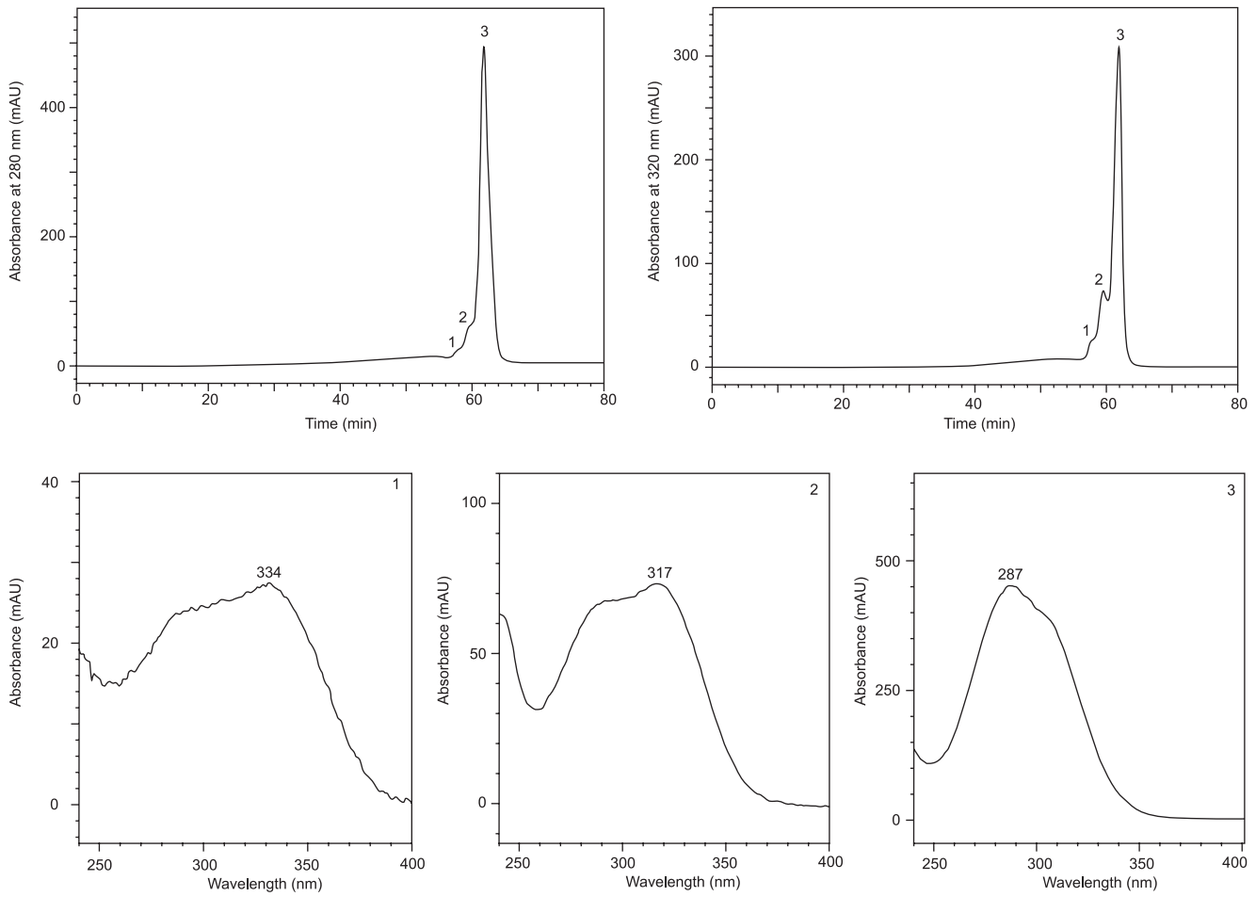

FIGURE 3. SE-HPLC chromatogram of the lignan macromolecule after base hydrolysis and UV spectra relevant to the peaks.

free phenolic acids (*p*-coumaric and ferulic acids) from flaxseed lignan macromolecule as a result of the combination of base and acid hydrolyses.

The application of pepsin and pancreatin did not change the LM chromatographic profile (Figures 6 and 7). However, in the case of pepsin the peak was more sharp than that of native LM (Figures 6 and 2). The change can be caused by a low pH value applied for pepsin hydrolysis.

The chromatograms of cellulase and β-glucuronidase hydrolysates (Figures 8 and 9) were characterised by the presence of the peak originating from the lignan macromolecule (peak 1) and additional peaks (2 and 3) originating from phenolic compounds liberated from LM during the enzymatic process. Peaks 2 and 3 in Figure 9 were higher than those depicted in Figure 8, which can indicate deeper hydrolysis of LM by β-glucuronidase than by cellulase.

The enzyme-assisted release of low molecular phenolic compounds from the extracts of flaxseed hulls was investigated by several authors [Obermeyer *et al*., 1995; Mazur *et al*., 1996; Kraushofer & Sontag, 2002; Milder *et al*., 2004; Krajčová *et al.*,

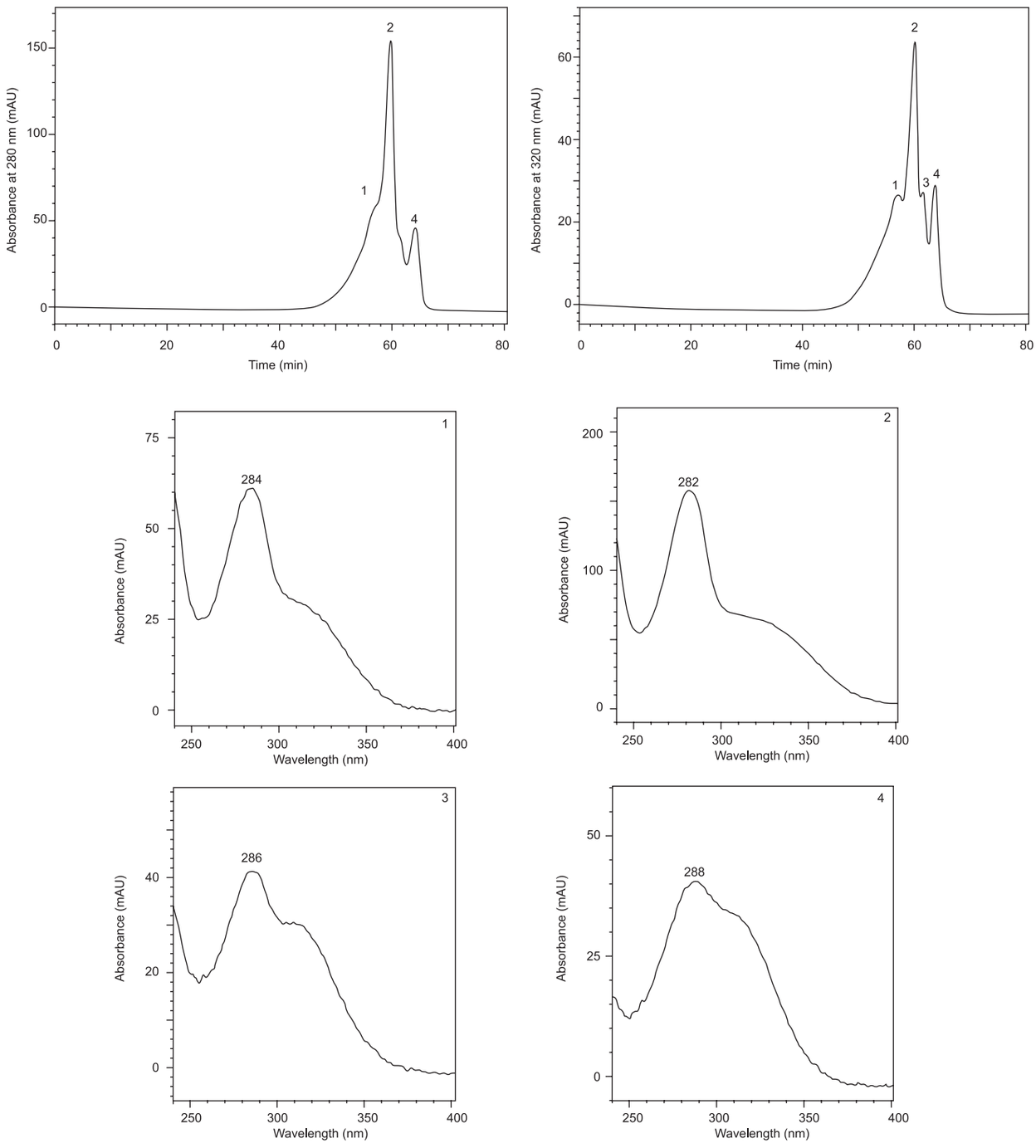

FIGURE 4. SE-HPLC chromatogram of the lignan macromolecule after acid hydrolysis and UV spectra relevant to the peaks.

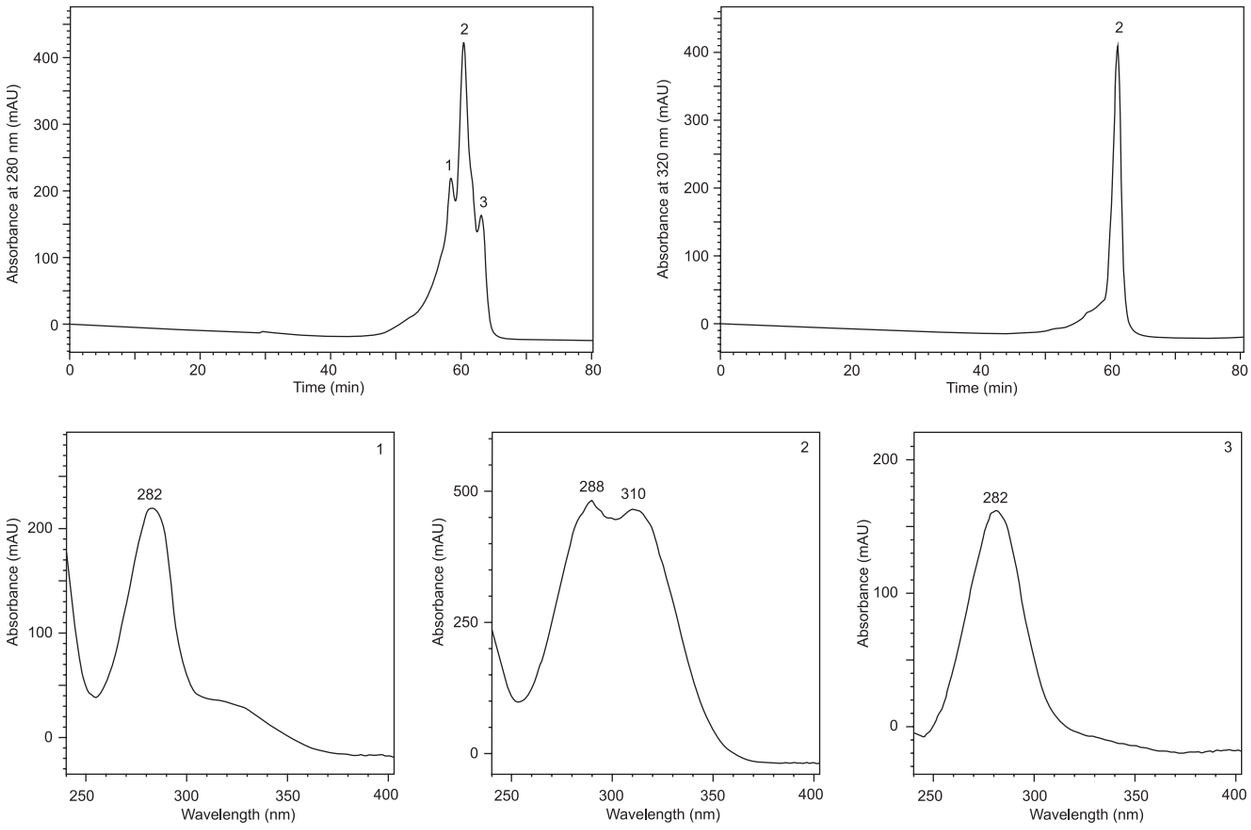

FIGURE 5. SE-HPLC chromatogram of the lignan macromolecule after base and acid hydrolyses and UV spectra relevant to the peaks.

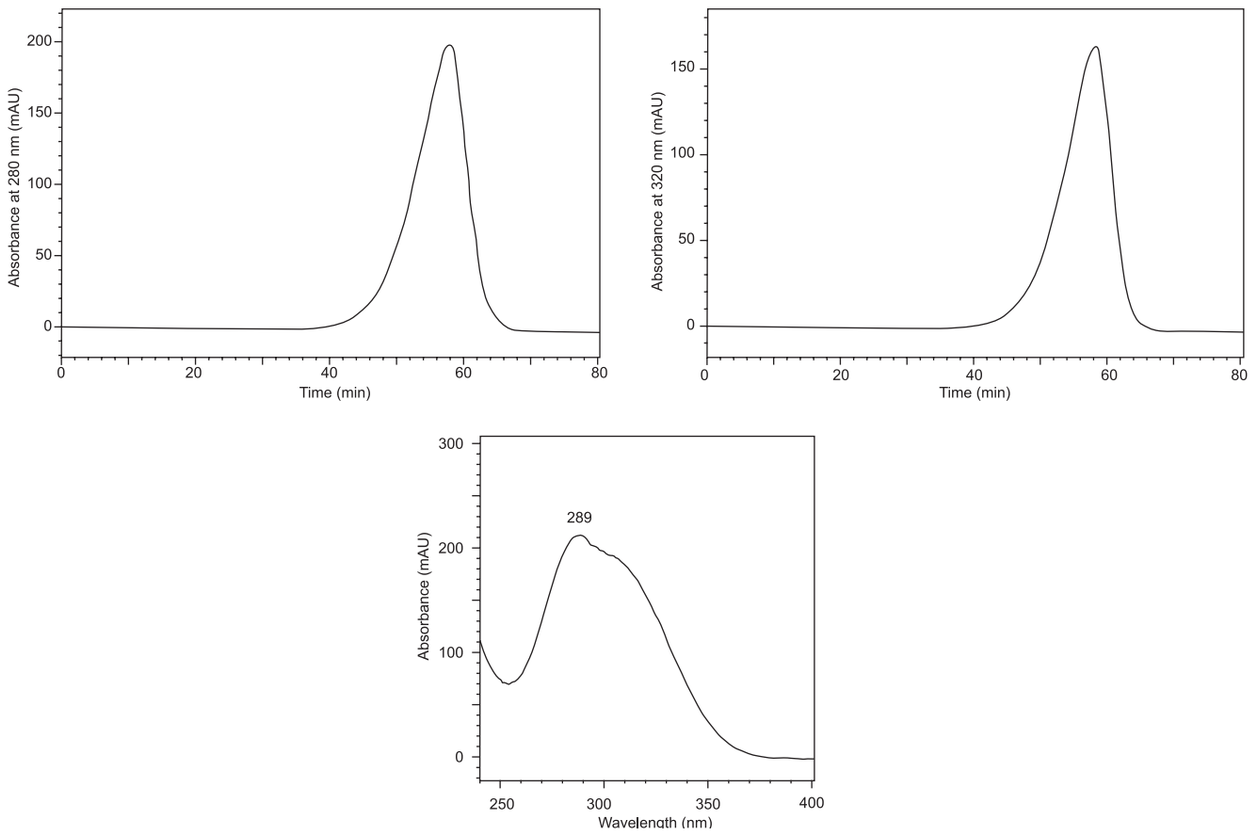

FIGURE 6. SE-HPLC chromatogram of the lignan macromolecule after treatment with pepsin and UV spectrum relevant to the peak.

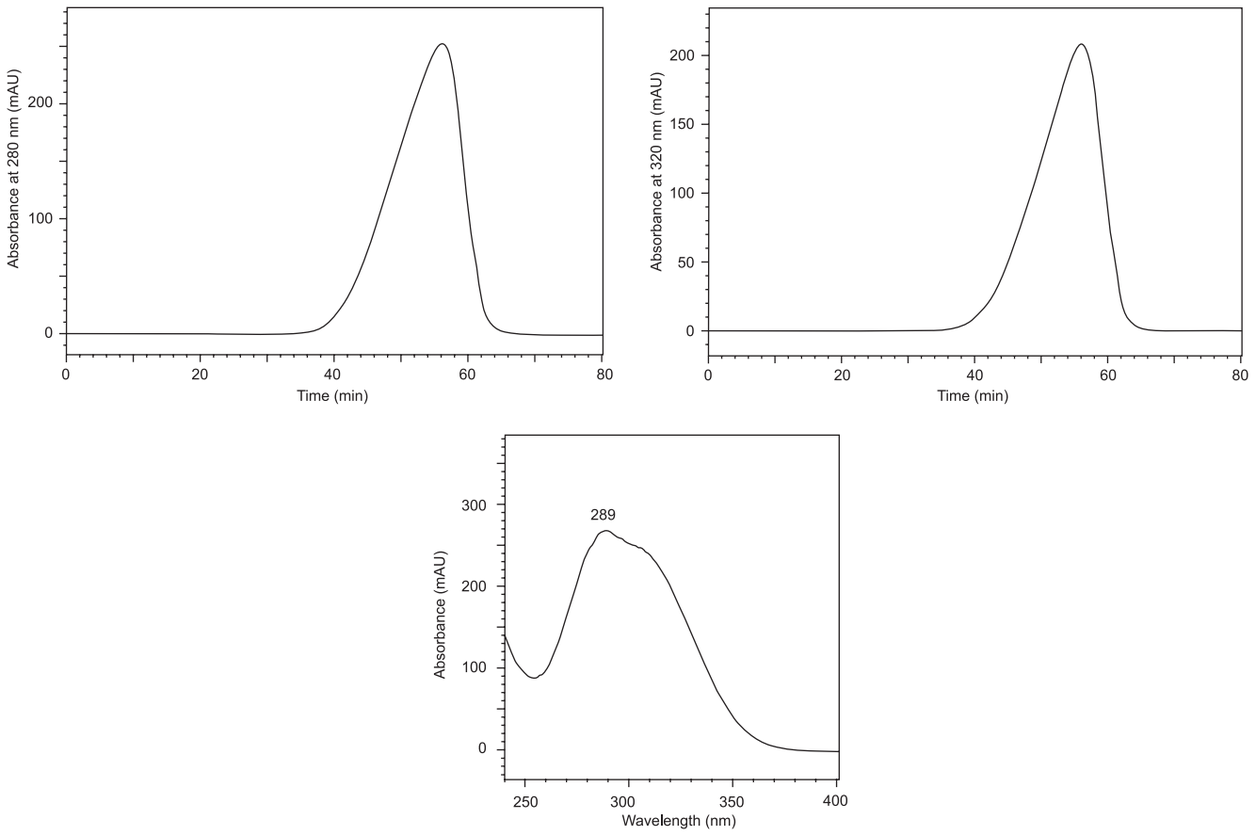

FIGURE 7. SE-HPLC chromatogram of the lignan macromolecule after treatment with pancreatin and UV spectra relevant to the peak.

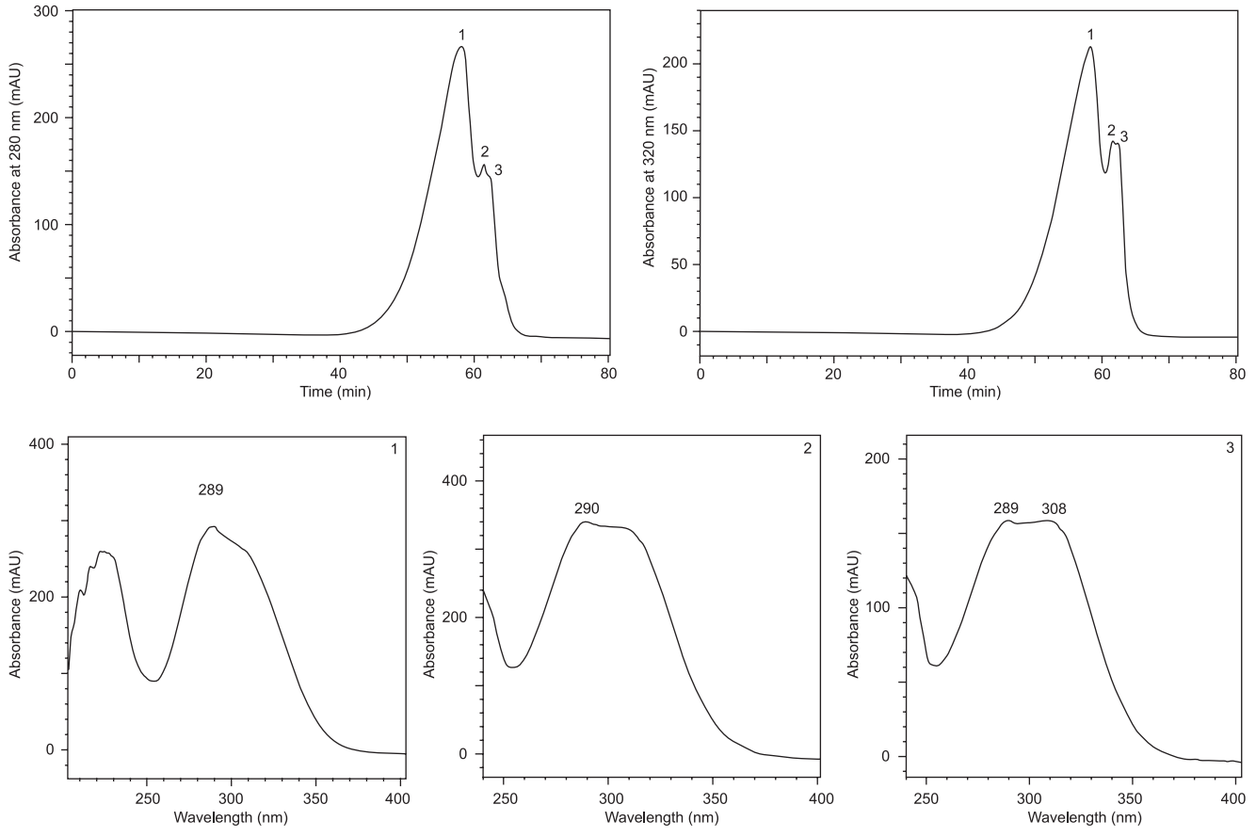

FIGURE 8. SE-HPLC chromatogram of the lignan macromolecule after treatment with cellulose and UV spectra relevant to the peaks.

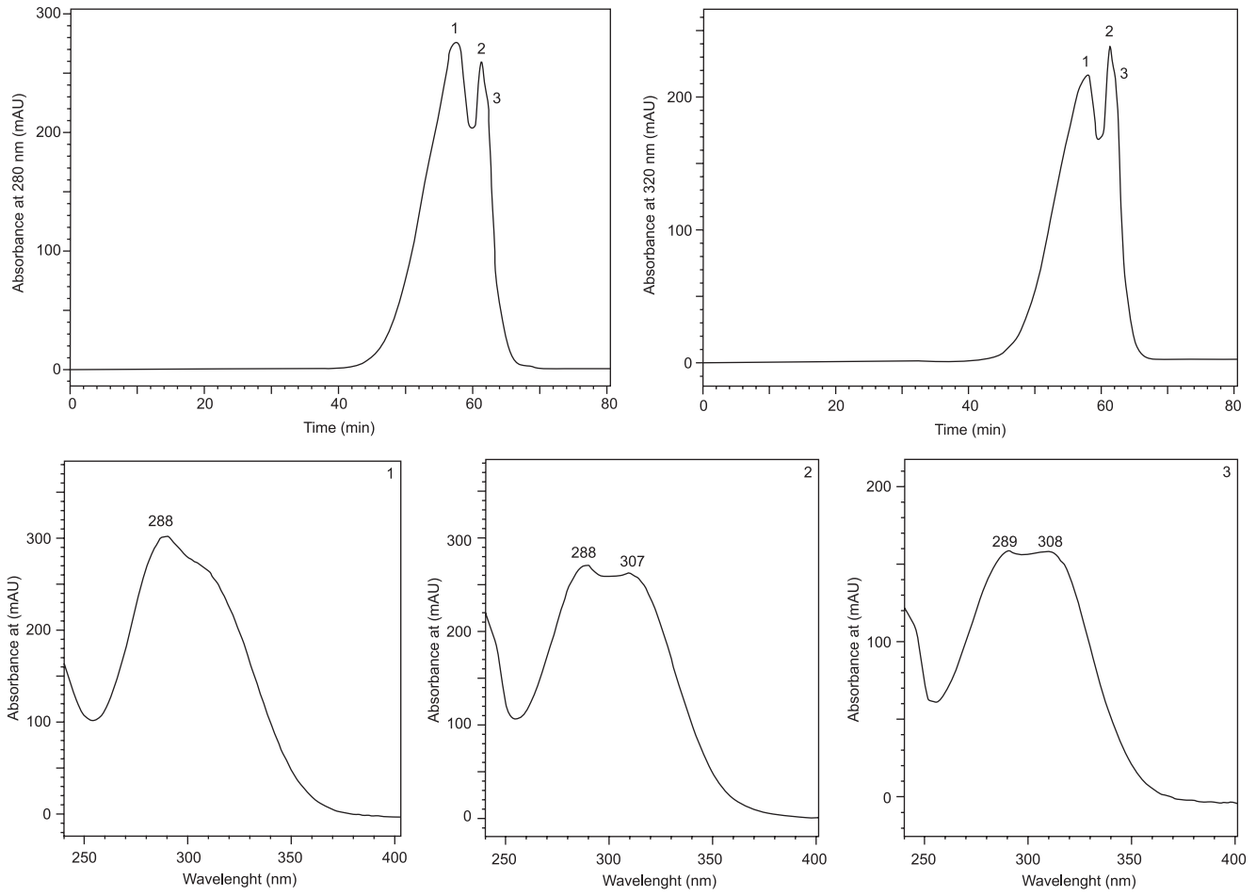

FIGURE 9. SE-HPLC chromatogram of the lignan macromolecule after treatment with β-glucuronidase and UV spectra relevant to the peaks.

2009; Renouard *et al*., 2010]. They used different solvent systems for extraction and different enzymes (β-glucuronidase, sulfatase, cellulase) for hydrolysis. In the study of Renouard *et al.* [2010], the aglycone form of flaxseed lignan (SECO) was released after hydrolysis using cellulase.

CONCLUSIONS

SE-HPLC-DAD analysis using a TSK G2000SW$_{XL}$ columns enables a good separation of lignan macromolecule (LM) and low molecular weight phenolic compounds liberated from LM after chemical and enzymatic hydrolysis. Pepsin and pancreatin under conditions applied in the presented study did not hydrolyse LM. The molecular weight of LM seems to be lower than 6,500.

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