*Bulgarian Chemical Communications, Volume 46, Number 3 (pp. 640 – 644) 2014*

# Sephadex LH-20 column chromatography of the hydrolysed lignan macromolecule of flaxseed

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Received July 26, 2013; Revised December 13, 2013

The lignan macromolecule (LM) was extracted from defatted flaxseeds using ethanol:dioxane (1:1, v/v) and then purified by Amberlite XAD-16 column chromatography with water and methanol as mobile phases. The LM was subjected to base hydrolysis (0.3 M NaOH, 2 days at room temperature under continuous stirring). Four general fractions (I-IV) containing phenolic compounds were obtained from the hydrolysate using Sephadex LH-20 column chromatography with methanol as mobile phase. Secoisolariciresinol diglucoside (SDG) was present in fractions I and II. The applied technique is useful prior to semi-preparative HPLC in the purification procedure of SDG to be used as a standard.

**Keywords:** flaxseed; lignan; macromolecule; chromatography; Sephadex LH-20

## 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.) [1]. The main lignan of flaxseed is secoisolariciresinol diglucoside (SDG) [2]; it occurs in the plant in the form of a lignan macromolecule (LM). When flaxseeds are consumed, SDG is essentially converted by bacteria to "mammalian" lignans, namely enterodiol (ED) and enterolactone (EL) [3].

The antioxidant activity of extracts from flaxseed and isolated SDG has been confirmed by several authors using different experimental models [4-7]. The potential role of lignans in risk reduction of mammary and prostatic tumors has been confirmed in several studies [8-11]. The similarities in the chemical structures of ED and EL to estradiol have led to the suggestion that both ED and EL can act as weak estrogenic/antiestrogenic compounds [12].

Several chromatographic methods have been employed for the separation and determination of flaxseed phenolic compounds. These include the following: Sephadex LH-20, RP-8, and silica gel column chromatography; TLC; RP-HPLC; and SE-HPLC [3, 13-19].

The aim of this work was the application of a Sephadex LH-20 column with methanol as mobile phase for the separation of phenolic compounds liberated from a flaxseed extract after base hydrolysis. This chromatographic method can be used prior to semi-preparative HPLC in the purification of a SDG standard.

## MATERIALS AND METHODS

### *Chemicals*

All solvents used were of analytical grade unless otherwise specified. Methanol, hexane, and acetonitrile were acquired from the P.O.Ch. Company (Gliwice, Poland). Sephadex LH-20 and Amberlite XAD-16 were obtained from Sigma-Aldrich. RP-18 gel (40-63 μm) was purchased from Merck (Darmstadt, Germany).

### *Plant material*

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

### *Extract preparation*

The material was defatted with hexane, after which the phenolic compounds were extracted using dioxane:ethanol (1:1, v/v) [17]. The extraction was carried out for 16 h at 60°C under continuous shaking in a water bath. Then, the solvent was evaporated using a Büchi Rotavapor R-200 at 40°C.

### *Extract purification*

The extract of phenolic compounds was purified using column chromatography on Amberlite XAD-16 [20]. A 1.0 g portion of the extract was suspended in distilled water and was loaded on the column. Firstly, water-soluble compounds, mainly

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sugars and low-molecular-weight organic acids, were eluted with distilled water and discarded. The solvent was then changed over to methanol which eluted the phenolic compounds. The solvent was removed from the collected fraction using the Rotavapor.

*Hydrolysis*

The purified extract was subjected to base hydrolysis. Briefly, the purified extract was suspended in 0.3 M NaOH, and left for 2 days at room temperature under continuous stirring. The obtained hydrolysate was acidified to pH 3.0 using 2 M HCl [17] and was subjected to column chromatography on RP-18 gel. Water-soluble compounds were eluted with distilled water and discarded, whereas compounds of interest were eluted with methanol. The solvent was removed from the collected fraction using the Rotavapor.

*Sephadex LH-20 column chromatography*

A 0.6 g portion of the hydrolysate obtained was dissolved in 8 ml of methanol and was applied to a chromatographic column (2 × 60 cm) packed with Sephadex LH-20 and was eluted with methanol. Fractions (5 ml) were collected using a fraction collector and their absorbance was measured at 280 and 320 nm, which are the characteristic wavelength maxima for SDG and hydroxycinnamates, the major phenolic constituents of flaxseed. Eluates were then pooled into major fractions, the solvent was evaporated and the residues were weighed.

*UV spectra*

UV spectra of individual fractions dissolved in methanol were recorded with a Beckman DU 7500 diode array spectrophotometer.

*RP-HPLC*

The separated fractions were analysed using a Luna C18 (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA) column and a Shimadzu system consisting of two LC-10AD pumps, a SCTL 10 A system controller, and a SPD-M 10 A diode array detector. Gradient elution with acetonitrile:water:acetic acid (5:93:2, v/v/v) [solvent A] and acetonitrile:water:acetic acid (40:58:2, v/v/v) [solvent B], at 0-50 min from 0 to 100% solvent B was employed [16]. The concentration of the sample dissolved in methanol was 2 mg/ml, the injection volume was 20 μl, the flow rate was 1 ml/min. The separation of the compounds was monitored at 280 and 320 nm. The standard of SDG was separated from the hydrolysed LM using a semi-preparative Luna C18 (250 × 10 mm, 5 μm; Phenomenex) column. Flow rate of 3 ml/min and the same gradient elution were used. A volume of 500 μL was injected into the column.

## RESULTS AND DISCUSSION

Four fractions (I-IV) containing phenolic compounds were obtained from the hydrolysed purified extract of flaxseed using Sephadex LH-20 column chromatography with methanol as the mobile phase (Fig. 1).

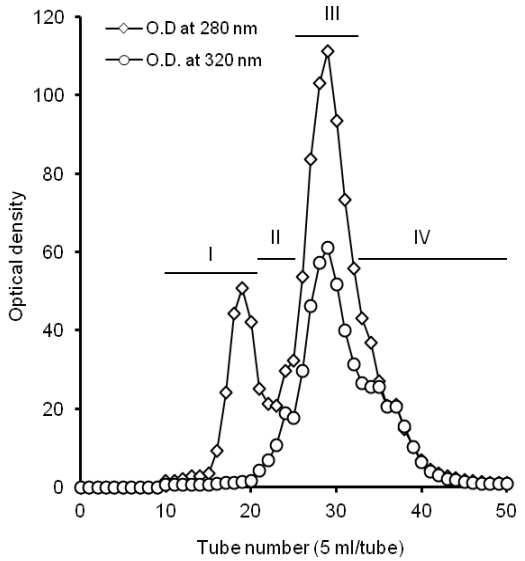


**Fig. 1.** Separation of phenolic compounds from a hydrolysed flaxseed molecule on a Sephadex LH-20 column with methanol as mobile phase.

The highest relative content of fraction I in the separated hydrolysate was at 53.5%, whereas the smallest content at 13.2% was determined for fraction IV (Table 1).

**Table 1.** Relative content of individual fractions separated using Sephadex LH-20 column chromatography and their UV spectral data

| Fraction | Relative content (%) | $\lambda_{max}$ (nm) | $\lambda_{sh}$ (nm) |
|---|---|---|---|
| I | 53.5 | 284 | - |
| II | 12.9 | 284 | 308 |
| III | 21.1 | 286 | 306 |
| IV | 12.5 | 286, 308 | - |

It is interesting to note that the highest content of total phenolic compounds was present in fraction III; the lowest content of total phenolics was found in fraction I.

The UV spectrum of fractions I and II exhibited a maximum at 284 nm. The absorption maxima for fractions III and IV were observed at the longer wavelengths of 286 and 308 nm, respectively (Fig. 2, Table 1).

These results demonstrate the absence of phenolic acids in the first two fractions.

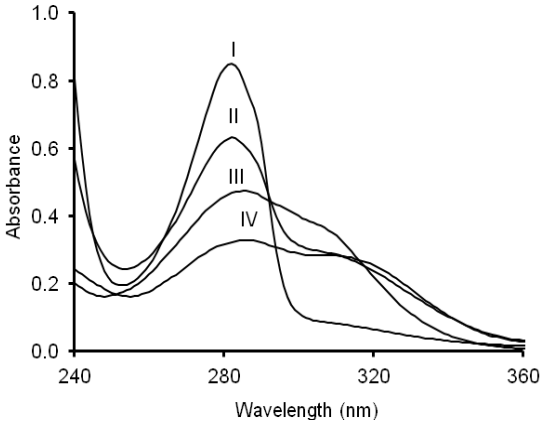


**Fig. 2.** UV spectra of the individual fractions separated on a Sephadex LH-20 column with methanol as mobile phase.

The RP-HPLC chromatogram of the hydrolyzed LM recorded at 280 nm is characterized by three main peaks (Fig. 3).

According to UV-DAD spectra and literature data [17], the first two peaks originated from glucosides of *p*-coumaric and ferulic acids (CoAG and FeAG). The peak with a retention time of 20.32 min originated from SDG. After Sephadex LH-20 column chromatography, SDG was observed in fractions I and II (Fig. 3). The content of SDG in fractions I and II was found to be534 and 464 mg/g, respectively. Approximately 82% of SDG was found in fraction I (Table 2). CoAG and FeAG were the dominant phenolic compounds present in fractions III and IV (Fig. 4).

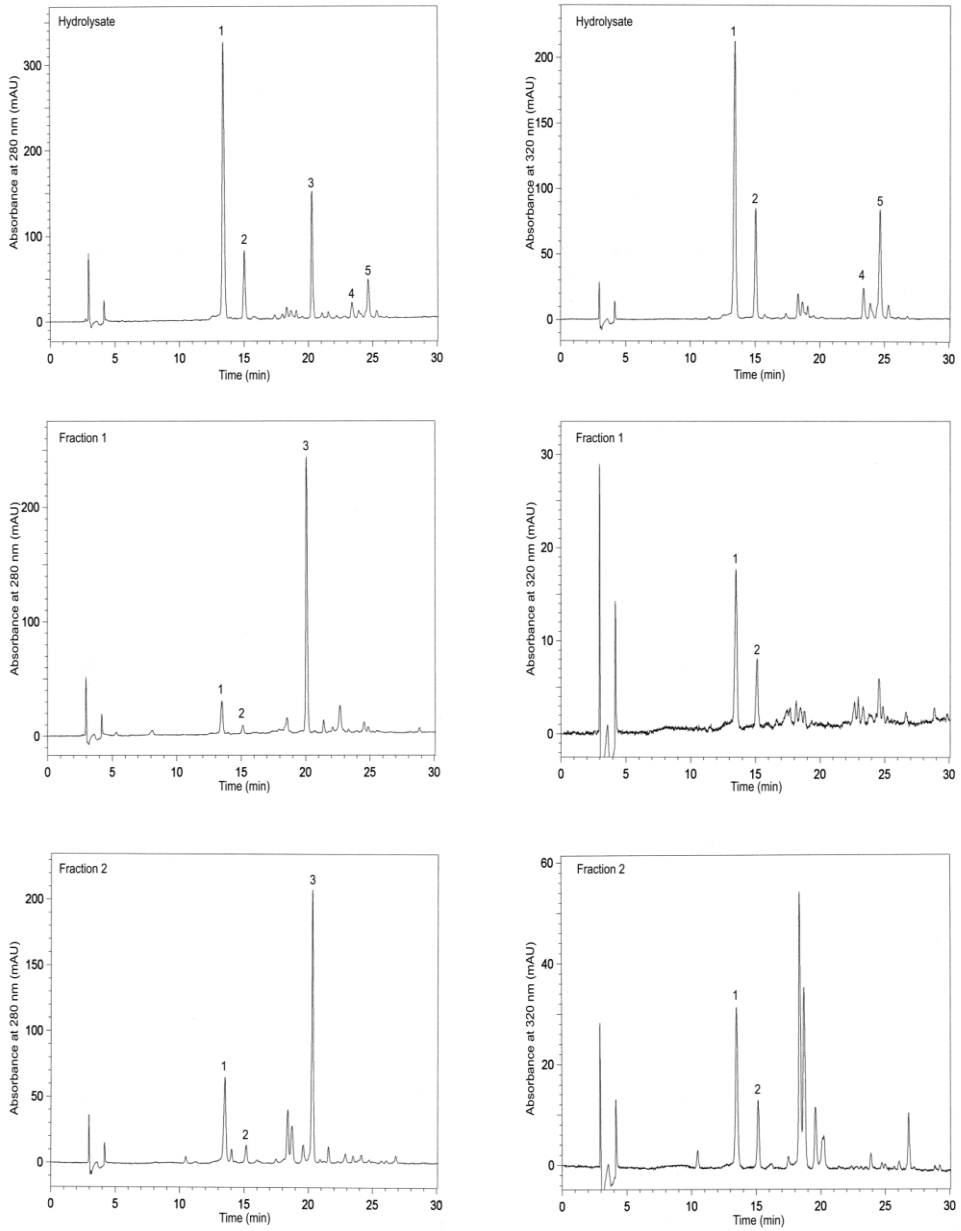


**Fig. 3.** RP-HPLC chromatograms of the extract and fractions I and II separated on a Sephadex LH-20 column with methanol as mobile phase; 1 – CoAG, 2 – FeAG, 3 – SDG, 4 and 5 – other phenolic compounds.

**Table 2.** Content of SDG in fractions separated using a sephadex LH-20 column

| Fraction | Content of SDG (mg/g) | Relative content (% of total) |
|---|---|---|
| I | 533 | 82.1 |
| II | 464 | 17.9 |
| III | - | - |
| IV | - | - |

## CONCLUSION

Sephadex LH-20 column chromatography with methanol as mobile phase provides good separation of SDG and other low-molecular-weight phenolic compounds liberated from the flaxseed macromolecule after base hydrolysis. This chromatography seems to be a useful method prior to semi-preparative HPLC in the purification of SDG to be used as a standard.

***Acknowledgments:*** *Financial support of Polish Academy of Sciences and Bulgarian Academy of Sciences for exchange visits are gratefully acknowledged.*

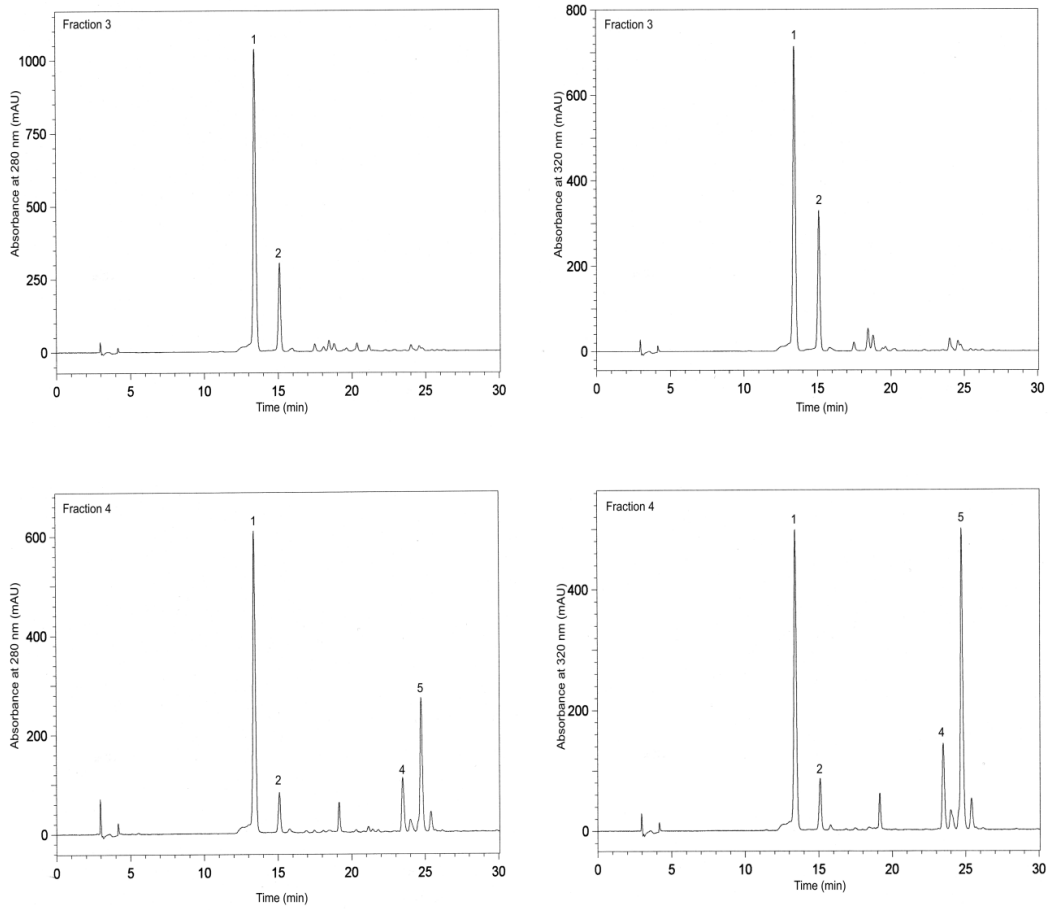


**Fig. 4.** RP-HPLC chromatograms of fractions III and IV separated on a Sephadex LH-20 column with methanol as mobile phase; 1 – CoAG, 2 – FeAG, 3 – SDG, 4 and 5 – other phenolic compounds.

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# КОЛОННА ХРОМАТОГРАФИЯ СЪС SEPHADEX LH-20 НА ХИДРОЛИЗИРАНА ЛИГНАНОВА МАКРОМОЛЕКУЛА ОТ ЛЕНЕНО СЕМЕ

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(Резюме)

Лигнановата макромолекула (ЛМ) е екстрахирана от обезмаслено ленено семе, използвайки етанол/диоксан (1:1, v/v) и след това е пречистена с Amberlite XAD-16 колонна хроматография с вода и метанол като мобилни фази. Лигнановата макромолекула, ЛМ, е подложена след това на алкална хидролиза. Получени са четири фракции (I-IV), съдържащи фенолни съединения от хидролизирания пречистен естракт на ленено семе, използвайки Sephadex LH-20 колонна хроматография с метанол като мобилна фаза. Секоизо-ларицирезинол диглюкозид (СДГ) присъства във фракции I и II. Тази хроматография е полезна техника преди полу-препаративна високоефективна течна хроматография (ВЕТХ) при пречиднването на СДГ, използван като стандарт.