

## HYDROLYSIS STUDIES OF FLAXSEED EXTRACT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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*In memory of Acad. Cristofor I. Simionescu,  
a man of outstanding qualities as a teacher,  
researcher and school founder*

Flaxseed is the richest dietary source of enterolignan precursors, primarily secoisolariciresinol diglucoside (SDG), with potential health benefits and an excellent nutrient profile. In this study, SDG oligomers in a flaxseed extract were hydrolysed to break first ester linkages for the release of SDG and then glycosidic bonds for the release of secoisolariciresinol (SECO). The hydrolysates of SDG oligomers are complex, therefore, different pathways were investigated and evaluated by high-performance liquid chromatography (HPLC) in order to improve the separation and determination of flaxseed components: acidic and enzymatic hydrolysis using ten different enzymes, such as  $\beta$ -glucuronidase, sulphatase, laccase, lignin peroxidases, Bakezyme<sup>®</sup> Concreate, Panamore<sup>®</sup> Golden, Veron<sup>®</sup>292, Veron<sup>®</sup>393, Gluzyme<sup>®</sup>10000 BG and Fungamyl<sup>®</sup>2500 SG. The obtained results revealed that the chemical hydrolysis, as well as the enzymatic treatment using laccase, determined the liberation of SDG oligomers from flaxseed lignan macromolecules in different quantities. The application of all the other enzymes did not change significantly the composition of the flaxseed extract.

**Keywords:** flaxseed extract, SDG, acidic hydrolysis, enzymatic hydrolysis, HPLC

### INTRODUCTION

Plants are a rich source of biologically active substances of special importance to both humans and animals. Records of the using plants for their therapeutic effects date back to ancient times, but without knowledge of their composition or mechanism of action.<sup>1</sup> Flaxseed is one of the oldest crops, having been cultivated since the beginnings of civilization, primarily to produce fibres and food. In the last years, flaxseed has been the focus of increased interest in the field of diet and disease research due to the potential health benefits associated with some of its biologically active components.<sup>2</sup> The features that contribute to the health benefits include naturally bioactive compounds with aromatic structure, such as polyphenols (phenolic acids, flavonoids and lignans).<sup>3</sup> All these constituents are associated with several functional properties of flaxseed, including antioxidant<sup>4</sup> and anticancer activity,<sup>5,6</sup>

cardioprotective,<sup>7</sup> anti-inflammatory and antidiabetic effects,<sup>8</sup> the ability to lower blood glucose,<sup>9</sup> serum lipids, serum total cholesterol,<sup>10,11</sup> etc. A growing body of evidence suggests that flaxseed lignans, in particular, are responsible for health benefits preventing some chronic diseases, specifically, being associated with a decreased risk of various cancers (prostate, colon, skin, ovarian and endometrial, breast),<sup>12,13</sup> as well as with positive effects on cardiovascular diseases, fertility, thyroid activity, menopausal symptoms and postprandial blood glucose reduction, antioxidant and anti-inflammatory potential,<sup>14,15</sup> etc. In recent years, various preclinical and clinical studies have demonstrated the health benefits of both flaxseeds and flaxseed lignans by *in vitro* or *in vivo* administration in different dosages.<sup>16,17</sup> For example, Prasad proved first that dietary flaxseed has an important role in

preventing hypercholesterolemic atherosclerosis in rabbits;<sup>18</sup> then, he isolated SDG from flaxseed and revealed in the following publications the protective effect of SDG against streptozotocin-induced diabetes and its mechanism.<sup>16</sup>

While the number of publications is continually growing in support of flaxseed consumption, many more studies are needed in order to improve the assessment of lignans, phenolic acids or flavonoids and to determine the role of each component.

Flaxseed is one of the richest plant sources of lignans, particularly the lignin secoisolariciresinol diglucoside (SDG), followed by secoisolariciresinol (SECO), matairesinol (MATA), pinoresinol (PINO), lariciresinol (LARI), hidrossimataresinol (HYDMA) and isolariciresinol (ISO).<sup>19</sup> SDG is the most abundant lignan in flaxseeds, which was isolated for the first time by Bakke and Klosterman in 1956.<sup>20</sup> In the presence of colonic gut microflora, SDG converts into two major mammalian lignans, enterodiol (ED) and enterolactone (LD), first identified in urine samples at the beginning of 1980.<sup>21,22</sup> SDG has a parent structure, containing two cinnamyl units linked together by an 8-8'-( $\beta$ ,  $\beta'$ ) carbon-carbon single bond in the side chains.<sup>23</sup> SDG is found in plants in the form of oligomeric polymer complexed with hydroxymethyl-glutaric acid (HMGA) and hydroxycinnamic acids, p-coumaric acid glucoside, ferulic acid glucoside and the flavonoid herbacetin diglucoside (HDG).<sup>24</sup> SDG lignan macromolecule is attached within this complex *via* ester-linkages between its glucosyl moiety and HMGA and directly linked with hydroxycinnamic acid glucosides through the glucosyl moiety of SDG.<sup>25</sup> After ingestion, the SDG-HMGA glucoside complex suffers a series of changes in order to generate other lignans that will be metabolized into mammalian lignans.

The fact that the major part of SDG is retained in the flaxseed during the pressing process has triggered the development of various methods for determining aglycones (free lignans) using complete hydrolysis of the SDG-HMGA complex. Several approaches have been reported, such as microwave-assisted extraction, alkaline hydrolysis,<sup>26</sup> enzymatic hydrolysis with  $\beta$ -glucuronidase,  $\beta$ -glucosidase or sulfatase and bacterial fermentation under mild conditions, in order to achieve the cleavage of the glycoside complex and the conversion of SDG to its aglyconic form, SECO, but with no marked yield

improvements.<sup>27</sup> Also, acidic hydrolysis is reported as a step that allows the recovery of the aglycone form secoisolariciresinol, but this method could be destructive if a too long heating period or a too high hydrochloric acid concentration is used.<sup>28</sup> Therefore, the determination of the corresponding aglycones depends on the hydrolysis method applied, indicating that the cleavage of the conjugates is the crucial point of the analysis method. Thus, the main goal of this study was to evaluate the enzymatic capacity of ten different enzymes for aglyconic SDG conversion (SECO) and compare it with acidic hydrolysis from a hydroalcoholic extract of flaxseeds using HPLC analysis.

## EXPERIMENTAL

### Materials

Flaxseed extract powder was obtained in our laboratory by cold pressing of raw flaxseeds (*Linum usitatissimum*) belonging to a Romanian variety, called "Cosmin", sown and harvested in 2011 and received from Agricultural Research and Development Institute, Livada, Satu-Mare, Romania. Ethanol was purchased from Carl Roth GmbH+Co. KG, Germany, and methanol, sodium hydroxide, hydrochloric acid, acetic acid and sodium acetate from Merck, Germany. HPLC phenolic standards (SDG, SECO, LARI, MATA, PINO, ferulic acid, galic acid and p-coumaric acid),  $\beta$ -glucuronidase from *Escherichia coli*, sulphatase from *Helix pomatia*, laccase from *Trametes versicolor* and lignin peroxidases were purchased from Sigma Aldrich. Bakezyme<sup>®</sup> Concreate enzymes (cellulase from *Trichoderma reesei*) and Panamore<sup>®</sup> Golden (lipase from *Aspergillus sp.*) were acquired from DSM, Netherlands, Veron<sup>®</sup>292 enzymes and Veron<sup>®</sup>393 (concentrated xylanase from *Aspergillus niger*) from AB Enzymes GmbH, Germany, Gluzyme<sup>®</sup>10000 BG (glucose oxidase from *Aspergillus niger*) and Fungamyl<sup>®</sup>2500 SG (amylase from *Aspergillus oryzae*) were supplied from Novozymes<sup>®</sup>, Denmark. Only cellulase, lipase Veron<sup>®</sup>292, Veron<sup>®</sup>393, Gluzyme<sup>®</sup>10000 BG and Fungamyl<sup>®</sup>2500 SG were food grade products. Analytical grade chemicals were used as received, without further purification. All solutions were prepared with double distilled water.

### Flaxseed extraction

The flaxseed samples were extracted as reported in a previous published paper.<sup>29</sup> Briefly, the flaxseeds were air-dried, ground in an electric mill to a fine powder of 0.5 mm and freeze dried. Solvent defatting was performed using Soxhlet extraction in hexane. The defatted seeds, at a ratio of 5:100 (w/v) were stirred for 4 h at 60 °C in ethanol/water 80/20 (v/v). The obtained light yellow solution was then filtered, concentrated at 40 °C under pressure (175 mbar), followed by freeze

drying. The resulted powder was then submitted to acidic and enzymatic hydrolysis.

#### Acidic hydrolysis

The flaxseed extract powder (0.5 g) was solubilized in a mixture of ethanol/water 70/30 (v/v) and hydrolyzed with HCl for 2 hours at 80 °C, up to 2 M final concentration. After the hydrolysis, the reaction medium was neutralized with NaOH (1 M). An aliquot of the hydrolysate was filtered through a 0.45 µm filter membrane before HPLC analysis.

#### Enzymatic hydrolysis

An amount of 100 mg of flaxseed extract powder was solubilised in 10 mL acetic acid buffer. The solution pH was adjusted with acetic acid or sodium acetate, depending on the enzyme hydrolytic activity. Thus, β-glucuronidase enzyme from *E. coli* has a high rate of hydrolytic activity at pH 6.8, sulphatase at pH 5, laccase at pH 4.5, lignin peroxidases at pH 3 and Bakezyme® Concreate, Panamore® Golden, VERON®292, VERON®393, Gluzyme®10000 BG and Fungamyl®2500 SG at pH 5. A volume of 100 µL of enzyme (3 mg/mL for the analytic enzymes and 15 mg/mL for food grade enzymes) was added to the solution. The mixture was incubated at 37 °C for 24 h under continuous shaking. During the hydrolysis, aliquots of the hydrolysate were taken at 4 h time intervals and filtered through a 0.45 µm filter membrane before HPLC analysis.

#### Chromatographic analysis of flaxseed lignans

The chromatographic separations and identification of lignans were carried out using a suitable slightly modified method optimized by Popova *et al.*<sup>30</sup> on a Shimadzu LC-SPD20AD system with a diode array detection (DAD). The HPLC optimal conditions were set as follows: Agilent Zorbax RX – C18 (5 µm, 4.6 x 150 mm) column, thermostated at 30 °C, with an injection volume of 20 µL; mobile phase consisted of water with 1% acetic acid (A) and methanol (B). The analysts delivered at a flow rate of 1 mL/min were eluted in the isocratic mode with 10% mobile phase B for 5 min, followed by a linear gradient to 76% B from 5 to 40 min, 10% B up to 45 min and maintained constant up to 50 min. The chromatograms were acquired at 280 nm. The peaks were identified and quantified by comparison with those of standard polyphenols and lignans.

## RESULTS AND DISCUSSION

The hydrolysis of lignan glycosides and oligomers represents the key step in sample preparation for identification and quantitation of lignan aglycones. This research was conducted in order to develop and optimize an improved methodology to quantify the content of SDG, the main lignan present in flaxseed.

The chromatographic separations and identification of lignans and phenolic compounds, namely, SDG, LARI, SECO, MATA, PINO, GAE, p-coumaric acid and t-ferrulic acid, were based on standards analysis (Fig. 1 A and B) determined from accurate mass measurements and comparison with literature data.<sup>31</sup> All experiments were performed at least in triplicate and presented as means ± standard deviation.

Figure 2 shows the chromatograms of the analysed lyophilized flaxseed extract before and after acidic hydrolysis. All the lignans and polyphenol compounds identified in the standard solution were also found in the flaxseed sample. In order to simplify the subsequent chromatographic analysis of the extracts and to liberate the bound compounds from the complex matrix, both ester linkages and glycosidic bonds were broken by acidic hydrolysis before the analysis.<sup>32</sup>

As observed in all the spectra, the chromatographic separation of SDG oligomers could not be performed on the raw lyophilized flaxseed extract, therefore it was necessary to carry out acidic hydrolysis with 2M HCl, followed by NaOH neutralization, as demonstrated also in Figure 2.

In the case of SDG, acid hydrolysis of the flaxseed extract could be destructive under drastic conditions, such as long heating periods or high hydrochloric acid concentration, therefore, it was necessary to find new ways to cleave the ester bonds under mild conditions.

Enzymatic hydrolysis evolution of the flaxseed extract was observed through HPLC analysis as shown in Figure 3 to Figure 7. All the enzymes, namely, β-glucuronidase, sulphatase, laccase, lignin peroxidases, Bakezyme® Concreate, Panamore® Golden, Veron®292, Veron®393, Gluzyme®10000 BG and Fungamyl®2500 SG were used for the hydrolysis of the flaxseed extract and the obtained chromatographic profile was compared with the chromatograms of the raw material and of the extract after the acidic hydrolysis.

Figure 3 shows the chromatographic profile of the flaxseed extract after enzymatic hydrolysis using β-glucuronidase and sulphatase enzymes, compared with that of the raw lyophilized flaxseed extract and that of the extract after acid hydrolysis. It could be noted that β-glucuronidase enzyme hydrolysed the raw flaxseed extract and released low molecular mass phenolic compounds from SDG.

An incomplete hydrolysis was observed in Figure 3B, for sulphatase enzyme, with partially hydrolysed compounds. According to literature data, the enzymatic hydrolysis with  $\beta$ -

glucuronidase, sulphatase or cellulase is only partial for the compounds of interest,<sup>33,34</sup> therefore, the obtained data are in accordance with the results from the mentioned studies.

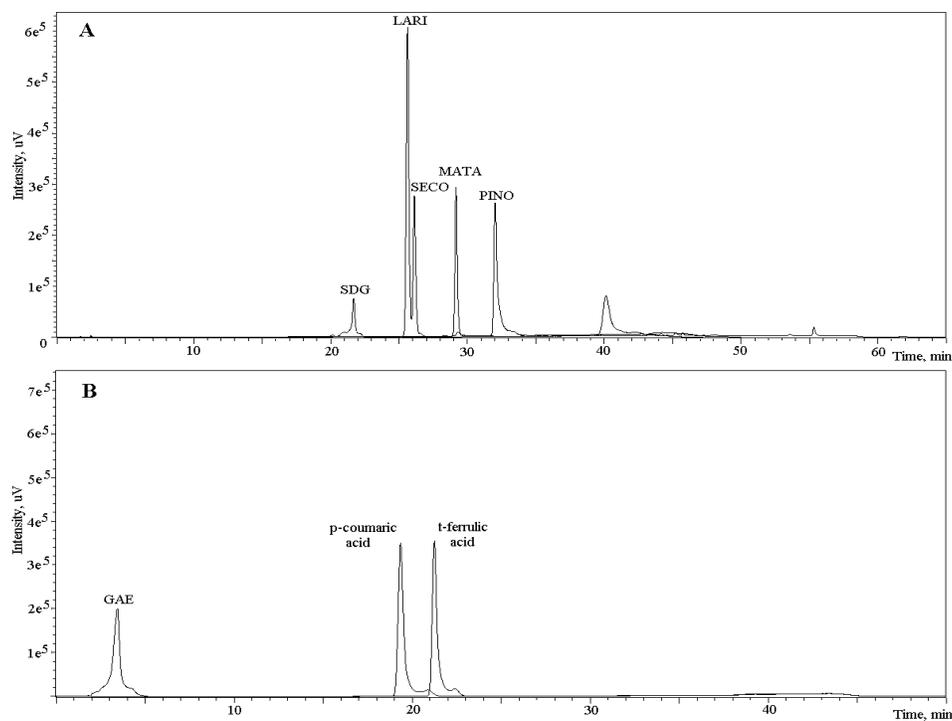


Figure 1: HPLC chromatograms for flaxseed standards: A. lignans, and B. phenolic compounds

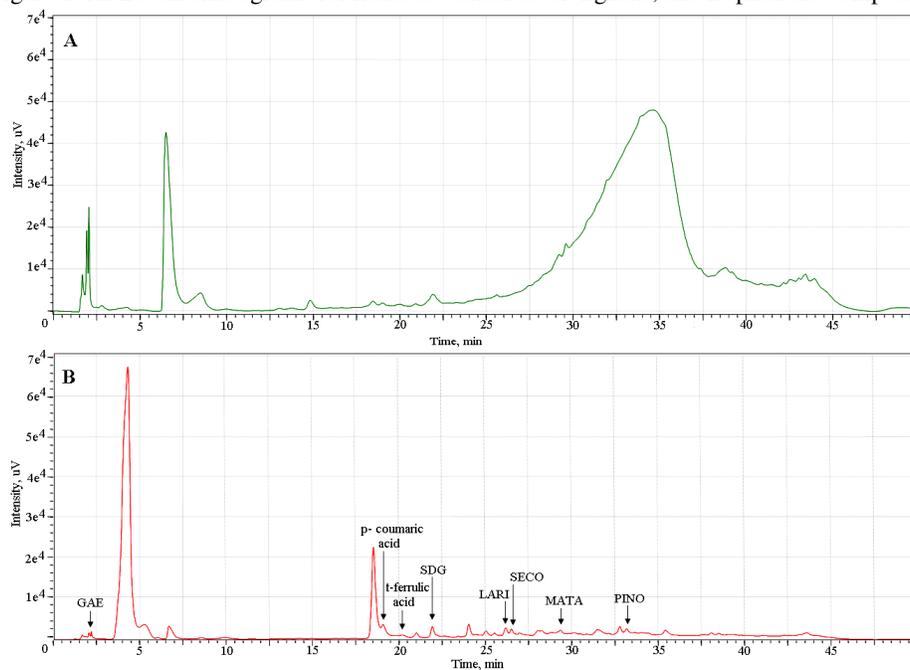


Figure 2: Chromatographic profiles of A. raw lyophilized flaxseed extract, and B. acid hydrolysed flaxseed extract

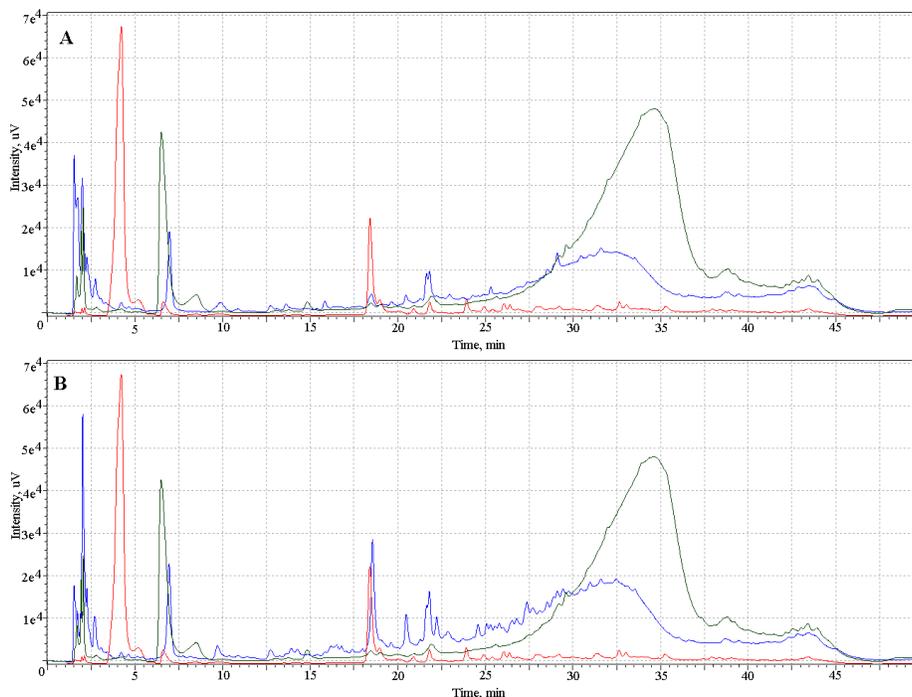


Figure 3: Chromatographic profile of the flaxseed extract subjected to enzymatic hydrolysis using: A.  $\beta$ -glucuronidase and B. sulphatase enzymes (■), compared with those of raw lyophilized flaxseed extract (■) and acid hydrolyzed flaxseed extract (■)

The contents of SDG hydrolysates in the flaxseed extract after hydrolysis with lignin peroxidases enzyme increased significantly, based on the chromatographic peak height, when compared with the raw lyophilized flaxseed extract, indicating an advanced, but incomplete process (Fig. 4A).

As can be seen from Figure 4B, all the peaks present after the acidic hydrolysis were also detected after the enzymatic hydrolysis of the flaxseed extract with laccase. The results suggested that both ester linkages and glycosidic bonds were broken, simplifying the subsequent chromatographic analysis of the extracts containing SDG oligomers, even after 4 h of treatment. According to the results presented in the literature, laccases act on phenolic substrates with an enhanced yield under optimal conditions.<sup>35</sup>

A low enzymatic activity was observed after hydrolysis with food grade enzymes, namely Veron<sup>®</sup>292 and Veron<sup>®</sup>393 (Fig. 5), Panamore<sup>®</sup> Golden and Gluzyme<sup>®</sup>10000 BG (Fig. 6) and Bakezyme<sup>®</sup> Concreate and Fungamyl<sup>®</sup>2500 SG (Fig. 7), when compared with the acidic SDG hydrolysates. The absence of a broad peak is

evidence of an incomplete process of hydrolysis. The chromatograms evidenced that the food grade enzymes could not break the ester linkages nor the glycosidic bonds from the flaxseed extract, therefore, the bioactive compounds were not determined. This could be caused by the low enzymatic activity of the used food grade enzymes on the glycoside complex under the proposed conditions. It can be deduced that these enzymes may act predominantly on  $\alpha$ -bonds, therefore, the enzymatic hydrolysis of the SDG oligomer, which contains two cinnamyl units linked together by an 8-8'-( $\beta$ ,  $\beta'$ ) carbon-carbon single bond in the side chains, is low.

Taking into consideration the results acquired after the enzymatic hydrolysis of the flaxseed extract using all the discussed enzymes, only the chromatographic profile obtained after laccase hydrolysis was further analysed in order to quantify the separated compounds. All the lignans and polyphenol compounds identified and quantified in the flaxseed extract samples subjected to acidic and enzymatic hydrolysis with laccase are summarised in Table 1.

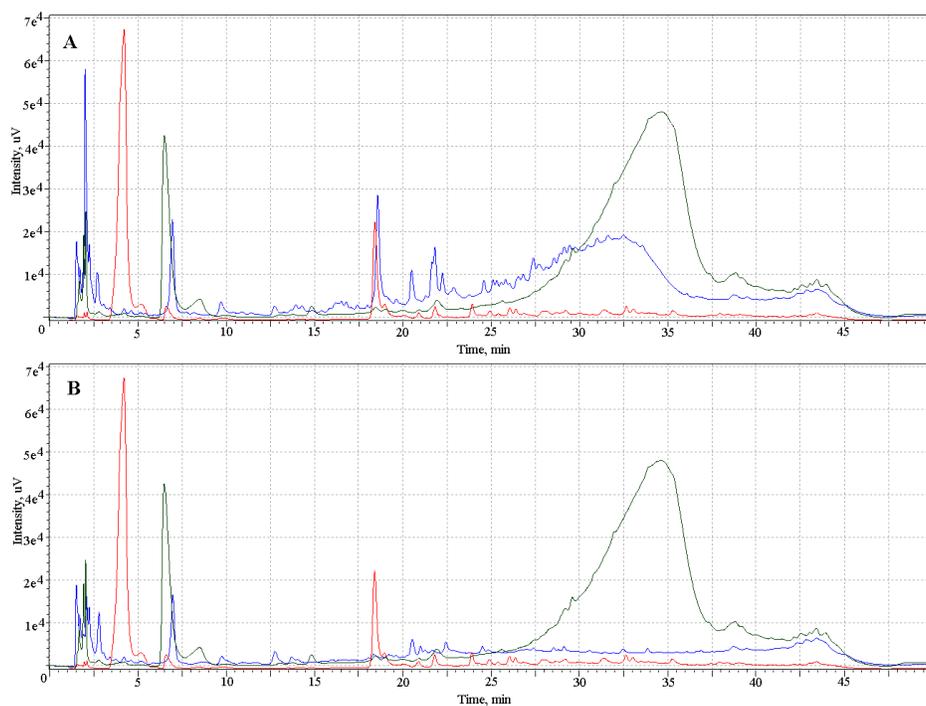


Figure 4: Chromatographic profile of the flaxseed extract subjected to enzymatic hydrolysis using: A. lignin peroxidases and B. laccase (■) enzymes, compared with those of raw lyophilized flaxseed extract (■) and acid hydrolyzed flaxseed extract (■)

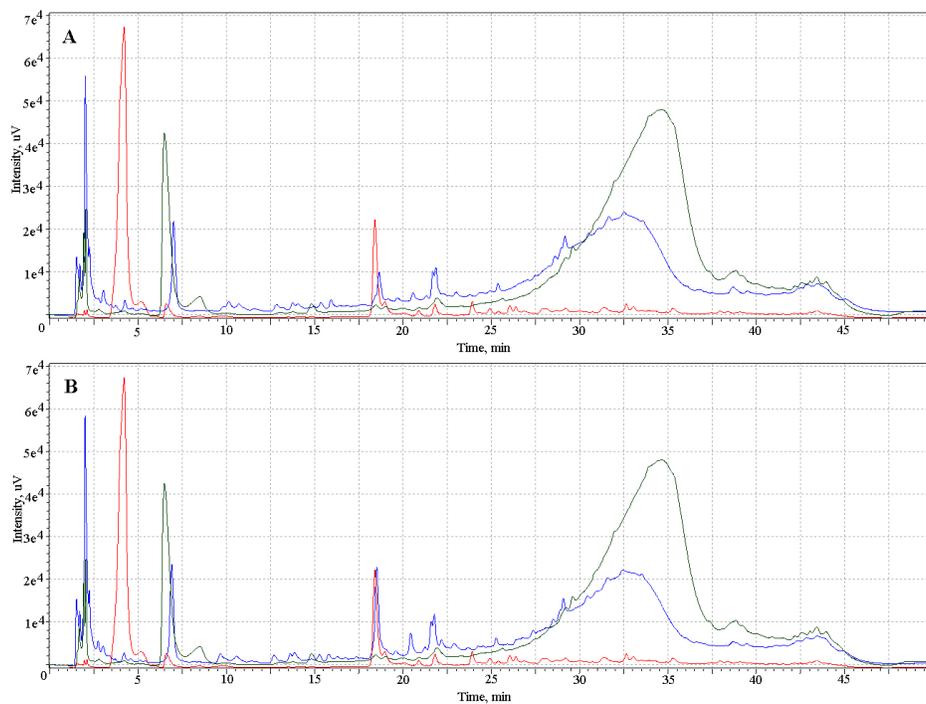


Figure 5: Chromatographic profile of the flaxseed extract subjected to enzymatic hydrolysis using: A. Veron®292 and B. Veron®393 (■) enzymes, compared with those of raw lyophilized flaxseed extract (■) and acid hydrolyzed flaxseed extract (■)

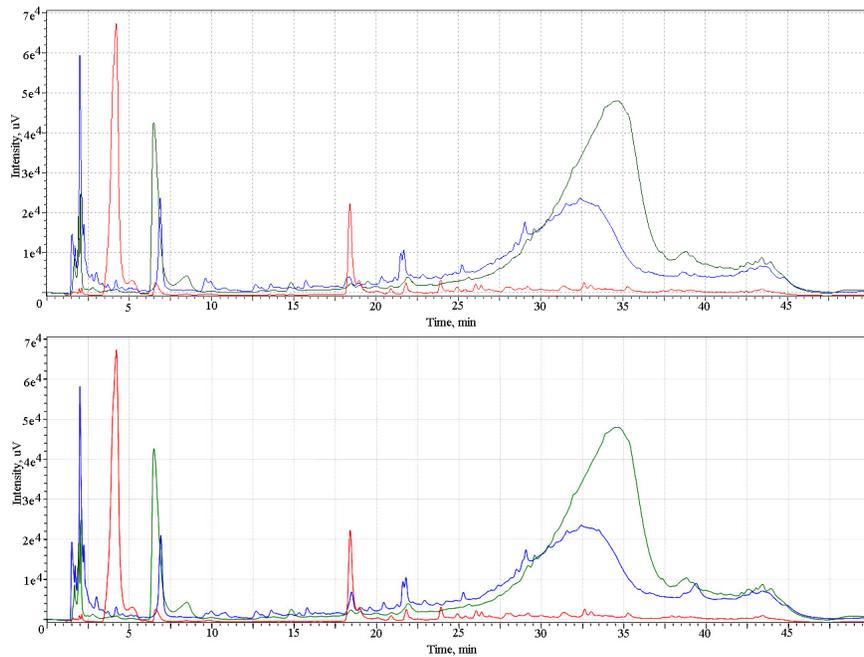


Figure 6: Chromatographic profile of the flaxseed extract subjected to enzymatic hydrolysis using: A. Panamore® Golden and B. Gluzyme®10000 BG (■) enzymes, compared with those of raw lyophilized flaxseed extract (■) and acid hydrolyzed flaxseed extract (■)

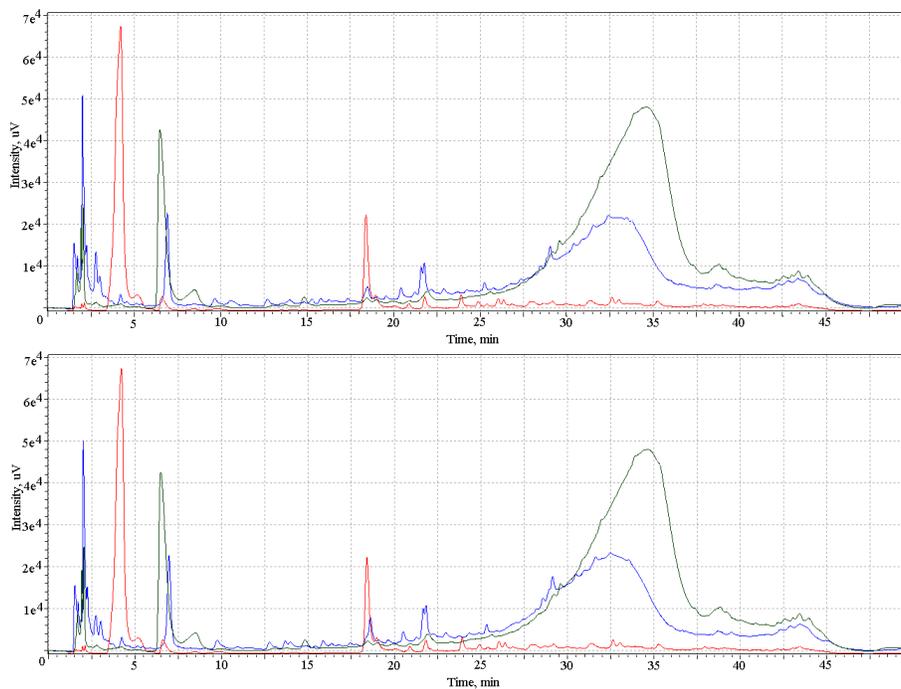


Figure 7: Chromatographic profile of the flaxseed extract subjected to enzymatic hydrolysis using: A. Bakezyme® Concrete and B. Fungamyl®2500 SG (■) enzymes, compared with those of raw lyophilized flaxseed extract (■) and acid hydrolyzed flaxseed extract (■)

The concentrations were calculated based on the standard peak area integration. No significant differences were observed between the values calculated for the flaxseed extract sample after acidic hydrolysis and those of standards, as also reported in our previously published paper.<sup>29</sup> The concentrations of lignans and polyphenol compounds identified after laccase hydrolysis are slightly different from those obtained after acidic hydrolysis. Thus, according to Table 1, the amounts of phenolic compounds separated and quantified by laccase hydrolysis were significantly higher, with a value of 200.4 mg of analyte per 100 g<sup>-1</sup> dry sample, than the ones

calculated after acidic hydrolysis (14.6 mg phenolic compounds per 100 g<sup>-1</sup> dry sample). GAE was more abundant than p-coumaric or ferulic acid. After laccase hydrolysis, lignans were found in a lower concentration, of about 98.1 mg of analyte per 100 g<sup>-1</sup> dry sample, compared with 154.8 mg of analyte per 100 g<sup>-1</sup> dry sample obtained after acidic hydrolysis.

It can be summarized from the comparative results that the concentration of lignans and phenolic compounds can be modulated by the hydrolysis type.

Table 1  
Quantitative assessment of lignans and phenolic compounds using acidic and enzymatic hydrolysis with laccase

Compound	Concentration (mg/100 g <sup>-1</sup> dry material) after acidic hydrolysis <sup>29</sup>	Concentration (mg/100 g <sup>-1</sup> dry material) after laccase hydrolysis
SDG	9.3	7.5
SECO	110.8	42.9
LARI	4.9	10.1
MATA	6.9	10.5
PINO	22.9	27.1
t-ferulic acid	4.8	63.4
GAE	4.6	107
p-coumaric acid	5.2	30

## CONCLUSION

In the present study, the enzymatic hydrolysis of a flaxseed extract was conducted in order to identify and quantify its main lignans. Various hydrolysis pathways, using 10 different enzymes, namely,  $\beta$ -glucuronidase, sulphatase, laccase, lignin peroxidases, Bakezyme<sup>®</sup> Concreate, Panamore<sup>®</sup> Golden, Veron<sup>®</sup>292, Veron<sup>®</sup>393, Gluzyne<sup>®</sup>10000 BG and Fungamyl<sup>®</sup>2500 SG, were evaluated and a quantitative assessment of the compounds of interest was performed by high performance liquid chromatography. Under the chosen conditions, only laccase enzyme proved to be effective in extracting secoisolariciresinol, leading to a similar chromatographic profile to that corresponding to acidic hydrolysis. A comparison with a conventional extraction method demonstrated that laccase hydrolysis is effective and eco-friendly. The presented process is a valuable method for efficient extraction and quantification of the main flaxseed lignan, SDG, and its metabolite, SECO. Moreover, this improved method of separation will facilitate *in vivo* and *in vitro* experiments aimed at elucidating the biological activity of these promising compounds.

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