Preparation of Flaxseed for Lignan Determination by Gas Chromatography-Mass Spectrometry Method

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Abstract

Sarajlija H., Čukelj N., Novotni D., Mršić G., Brnčić M., Ćurić D. (2012): **Preparation of flaxseed for lignan determination by gas chromatography-mass spectrometry method**. Czech J. Food Sci., **30**: 45–52.

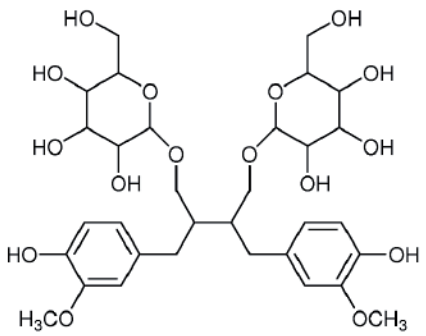
Since 1980s, several methods for the determination of lignans in food samples have been developed depending on the types of lignans and foods analysed, but mostly on flaxseed as a reference food. In this work, specific steps in flaxseed preparation for lignan secoisolariciresinol analysis by gas chromatography-mass spectrometry method were examined. Ethanol extraction of lignan from defatted and non-defatted flaxseed before acid hydrolysis yielded significantly lower concentrations (5172 ± 49 μg/g; 5159 ± 83 μg/g, respectively), when compared to the direct acid hydrolysis (8566 ± 169 μg/g; 8571 ± 192 μg/g, respectively). In the analysed samples of defatted and dried flaxseed, no significant difference in lignan content was observed when compared to non-defatted flaxseed samples.

Keywords: defatting; extraction; GC/MS; hydrolysis; lignans

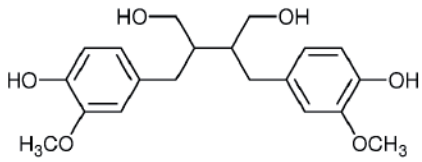
Lignans are defined as a group of phenylpropanoid dimers, in which the phenylpropane units are linked by the central carbon (C_8) of their propyl side chains. They are classified into eight subgroups based upon the way in which oxygen is incorporated into the skeleton, and the cyclisation pattern: furofuran, furan, dibenzylbutane, dibenzylbutyrolactol, dibenzylbutyrolactone, aryltetralin, arylnaphtalene, and dibenzocyclooctadiene (Umezawa 2003). In the intestines, lignans are converted to enterolactone and enterodiol which are thought to have beneficial effects on human health, due to (anti)estrogenic, antinflammatory, and antioxidative effects as well as the ability to reduce cancer and cardiovascular risks (Adlercreutz 2007). Thus in the recent years many studies on lignans have arisen, giving focus to all the aspects of lignan analysis – from their occurrence in nature to their bioactivity in the human body (Muir 2006).

Even so, considering the large number of molecules covered by lignan definition, there is still a plenty of work for the scientists interested in this type of compounds. The same goes for the development and optimisation of the extraction, separation, and identification methods. Few works have been done in this area, mostly on flaxseed as a reference, being the food most abundant in lignans. The most investigated lignan, secoisolariciresinol (SECO, Figure 1), has been proven to occur in an oligomeric structure bonded to glucose molecules into secoisolariciresinol diglu-

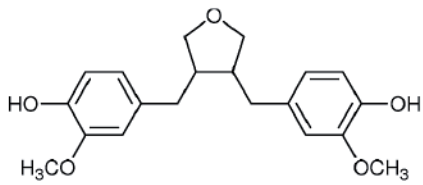
Supported by the Ministry of Science, Education and Sport of the Republic of Croatia, Project No. 058-0580696-0702.



secoisolariciresinol diglucoside (SDG)



secoisolariciresinol (SECO)



anhydrosecoisolariciresinol diglucoside (ANHSECO)

Figure 1. Scheme of secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO) and anhydrosecoisolariciresinol (ANHSECO)

coside (SDG; Figure 1). SDG is further bonded via ester bonds to 3-hydroxy-3-methyl-glutaric acid molecules (Kamal-Eldin *et al.* 2001; Li *et al.* 2008). The methods for the lignans extraction contain more or less the same basic steps in the preparation: the extraction of lignan glycosides and oligomers, hydrolysis of glycosides to aglycones, and extraction of aglycones. Many steps in the preparation are the consequence of complex lignan structure occurrence within the plant tissue. The existence of other lignan polymers is still unknown; therefore, lignans are usually translated and measured in aglycone forms. Lignan extraction procedures differ in chemicals and methods used to separate and concentrate the required molecules. The extraction of lignan oligomers is usually done with an aliphatic alcohol solvent, e.g. a mixture of methanol or ethanol with water. Up to now, acid or alkaline hydrolysis or combinations of these with enzyme hydrolysis have been applied to obtain lignan aglycones (Table 1). For the identification and quantification of lignans from food, high performance liquid chromatography (HPLC) with diode array detection (DAD) (Meagher *et al.* 1999; Johnsson *et al.* 2000; Eliasson *et al.* 2003; Zhang & Xu 2007), coulometric electrode array (CEA) detection system (Kraushofer & Sontag 2002; Schwartz & Sontag 2006), and MS/MS detectors (Milder *et al.* 2004; Smeds *et al.* 2007) or GC/MS (Mazur *et al.* 1996; Meagher *et al.* 1999; Liggins *et al.* 2000; Sicilia *et al.* 2003; Peñalvo *et al.* 2005; Thompson *et al.* 2006; Popova *et al.* 2009) have been used. In Table 1, the overview is given of the methods developed for flaxseed lignan gas chromatography-mass spectrometry (GC/MS) analysis.

As can be seen, a lot of work has been done in this area, however, no definitive and unique method exists for lignan analysis in food, which would provide optimal and iterative conditions, including minimum of working steps, minimum time, and valid results. Due to a broad range of lignans with diverse chemical structures and physicochemical properties (e.g., pH sensitivity and diversity of chemical bonds within the polymers), complexity of food matrix, and ambiguous ideas of the matrix position into which lignans are incorporated, it is nearly impossible to have any definitive procedure or protocol for lignan analysis. Despite that, there is still enough space and a need to improve the existing methods in order to develop the most suitable one. The aim of this work was to study the necessity of lignan glycoside extraction in obtaining aglycones from flaxseed for GC/MS analysis, comparing it with direct acid hydrolysis. The need for flaxseed defatting prior to lignan isolation was examined as well, since up-to-now the analyses have been performed both on defatted and fresh flaxseed.

MATERIAL AND METHODS

The standard of secoisolariciresinol (> 95% purity) was a kind gift from Oy Separation Research Ab (Turku, Finland) while anhydrosecoisolariciresinol (90–95% purity) was purchased from Plantech UK (Berkshire, England). Ethyl-acetate, methyl-*tert*-butyl ether, hydrochloric acid, sodium hydroxide, *n*-hexane, and pyridine were purchased from J.T.Baker (Griesheim, Germany) while the derivatising agent *N,O*-bis(trimethylsilyl)trifluoro-

acetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (99.4%) was purchased from Sigma Aldrich (Taufkirchen, Germany). The institute for Seeds and Seedlings (Osijek, Croatia) kindly provided flaxseed, type Recital.

Sample preparation. Prior to the sample preparation, all glassware was silanised in a 5% solution of dimethyldichlorosilane in heptane, followed by deactivation of excess reagent in methanol. Flaxseed was milled in a coffee grinder and either used for analysis or additionally defatted by Soxhlet extraction in *n*-hexane for 8 h (ISO 659:1998) and oven dried until constant mass. Defatting was also done by magnetic stir mixing of milled flaxseed in *n*-hexane (1:5 w/v) at room temperature for 1 h, and repeated once, referring to previous works that used stirring with *n*-hexane to remove the oil (Sicilia *et al.* 2003; Popova *et al.* 2009). The content of oil in flaxseed before and after stir mixing was determined by Soxhlet extraction (ISO 659:1998).

Direct acid hydrolysis. Direct acid hydrolysis was carried out according to Liggins *et al.* (2000), with minor modifications. To 0.5 g of ground, fresh or defatted and dried flaxseed, 5 ml of 1.5 mol/l hydrochloric acid was added to obtain aglycones from glycone oligomers. The samples were heated in dry block (Reacti-Therm I, Thermo Scientific, Barrington, USA) under constant stirring at 100°C for 3 hours. After hydrolysis, the samples were cooled down to room temperature, and pH was adjusted to 5–6 by adding 10 mol/l sodium hydroxide. Secoisolariciresinol (SECO) and anhydrosecoisolariciresinol (ANHSECO, Figure 1) were extracted for 60 min under constant stirring with 3 ml of methyl-*tert*-butyl ether (MTBE) and ethyl acetate (1:1, v/v), followed by centrifugation (2000 rpm, 10 min) to separate the organic layer. The organic layer was transferred to a separate flask and the remainder was washed twice with 2 ml of MTBE:ethyl acetate mixture. The supernatants were combined and the organic extract

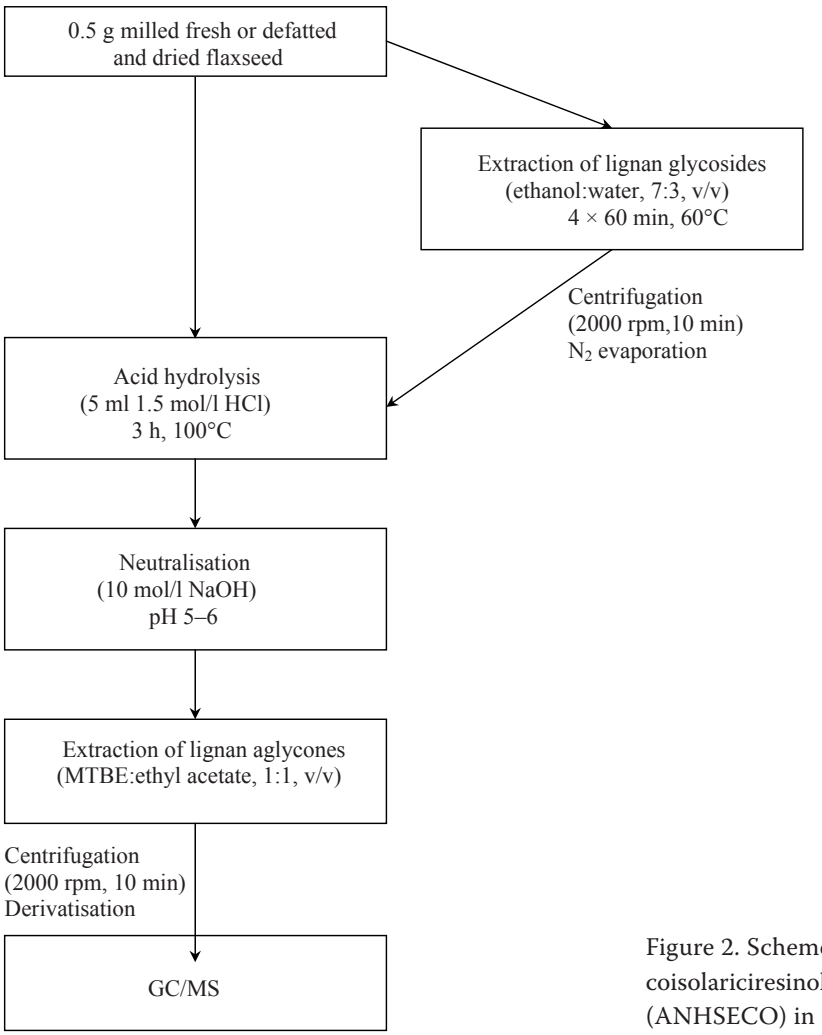


Figure 2. Scheme of the protocol used for analysis of secoisolariciresinol (SECO) and anhydrosecoisolariciresinol (ANHSECO) in flaxseed

was evaporated to dryness under nitrogen stream. The dry sample extracts were stored in a freezer at –20°C until analysis.

Acid hydrolysis of ethanol extracts. The extraction was performed prior to hydrolysis in the following way: to 0.5 g of ground, fresh or defatted and dried flaxseed 5 ml of ethanol:water (7:3, v/v) was added and the mixture was heated at 60°C for 60 minutes. Ethanol was chosen over methanol due to its less hazardous effect on the environment (Zhang *et al.* 2007). The samples were shaken every 15 min for 20 s because of the solids settling out. After 60 min, the samples were centrifuged at 2000 rpm for 10 min and the extraction was repeated three times in the same way. The supernatants were combined and evaporated in nitrogen stream under gentle heating to obtain syrupy mass and a volume of about 500 µl. Then, 5 ml of 1.5 mol/l hydrochloric acid was added to obtain aglycones from glycones. Further sample treatment was the same as that applied in direct acid hydrolysis. When evaluating the extraction efficiency, each of the four ethanol extracts were analysed separately for SECO content.

GC-MS analysis. The dried flaxseed extracts were derivatised with 1 ml (4:1 v/v) of the mixture of pyridine and BSTFA with 1% trimethychlorosilane at 70°C for 60 min to form trimethylsilyl derivatives of lignans. The standards of SECO and ANHSECO used for calibration were derivatised in the same manner. GC/MS analysis was performed on a Shimadzu 2010 gas chromatograph equipped

Table 1. Methods for preparation of flaxseed lignans for GC/MS analysis

Flaxseed preparation	Extraction of oligomers	Hydrolysis	Extraction of aglycones	Sample purification/preparation	Lignans detected*	Reference
Drying, milling, defatting	–	enzymatic: 2500 fishman units of *H. pomatia* juice (2 h, 60°C) followed by acid: 2 mol/l HCl (2.5 h, 100°C)	diethyl ether	anion exchange chromatography; HMDS derivatisation (0.5 h, 25°C)	SECO, MATA	Mazur *et al.* (1996)
Milling, defatting	methanol:water (8:2, v/v) (4 h, 55°C)	acid: 1 mol/l HCl (1 h, 100°C)	ethyl acetate: *n*-hexane (1:1 v/v)	RP C18, BSTFA derivatisation (20 min, room temperature)	ANHSECO, SECO, MATA, PINO, ISOLARI	Meagher *et al.* (1999)
Drying	–	acid: 1,5 mol/l HCl (3 h, 100°C)	ethyl acetate: methy-*tert*-butyl ether (1:1, v/v)	BSTFA derivatisation (1 h, 60°C)	ANHSECO, SECO, MATA	Liggins *et al.* (2000)
Drying, milling, defatting	methanol:water (7:3, v/v) (2 h, 60-70°C)	alkaline: 1 mol/l NaOH (3 h, room temperature) followed by enzyme: β-glucuronidase (overnight, 37°C)	–	SPE C18, Tri-Sil Reagent derivatisation (0.5 h, 60°C)	SECO, MATA, PINO, LARI	Thompson *et al.* (2006)
Milling, defatting	ethanol:water (8:2, v/v) (4 h, 55°C)	enzymatic: β-glucuronidase and β-glucosidase (14 h, 37°C) and acid: 1 mol/l HCl (1 h, 95°C)	ethyl acetate: *n*-hexane (1:1 v/v)	BSTFA derivatisation (overnight, 20°C)	ANHSECO, SECO, MATA, PINO, ISOLARI, LARI	Sicilia *et al.* (2003)
–	methanol:water (7:3, v/v) with 0.3 mol/l NaOH (1 h, 60°C)	enzymatic: β-glucuronidase (overnight, 37°C)	–	SPE C18, ion exchange chromatography, HMDS derivatisation	SECO, MATA, LARI, PINO	Peñalvo *et al.* (2005)
Milling, defatting drying	methanol:water (8:2, v/v) (8 h, 55°C)	acid: 2 mol/l HCl (2 h, 100°C)	dichloromethane: water (86:14 v/v)	BSTFA derivatisation (1 h, 60°C)	ANHSECO, SECO, ISOLARI, ENL	Popova *et al.* (2009)

*SECO – secoisolariciresinol; MATA – mataires inol; PINO – pinoiresinol; ISOLARI – isolariciresinol; ANHSECO – anhydrosecoisolariciresinol; LARI – lariciresinol; ENL – enterolactone

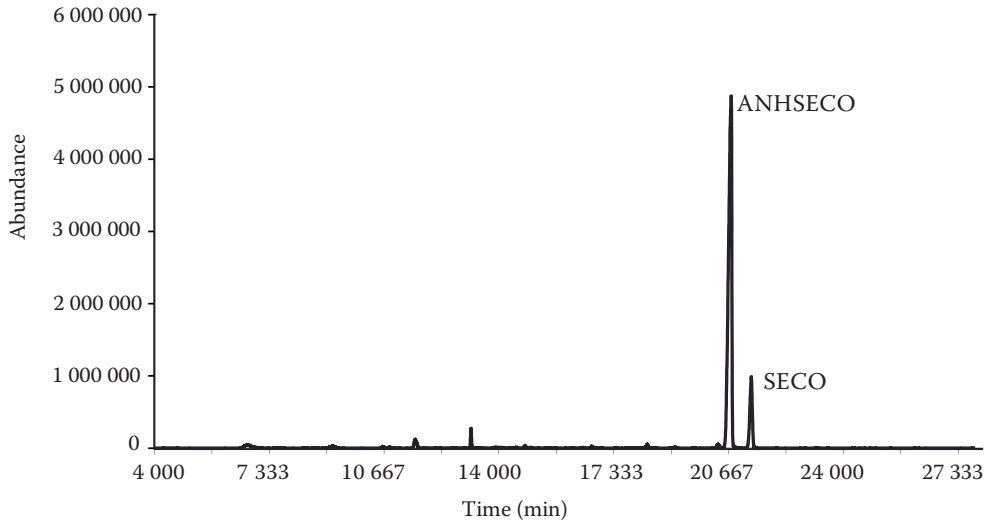


Figure 3. Total ion chromatograms of lignan extracts from non-defatted (fresh) flaxseed with applied direct acid hydrolysis

with an auto sampler (AOC-20i) and QP-2010 Plus mass selective detector. A 30 m HP-5 ms capillary column with 0.25 mm *i.d.* and 0.25 µm film thickness (J&W Scientific, Folsom, USA) was used. One µl of the sample was introduced via injection port operated in splitless mode at 285°C, with splitless time of 30 seconds. The initial column temperature was 175°C. After 6 min it was increased to 270°C at 15°C/min rate, and held for 15.68 min (total run time was 28 min). The interface temperature was 285°C. The analyses were performed in the total ion current (TIC) mode and/or selected ion monitoring (SIM) mode. In SIM mode two ions, one target ion and one reference ion for each compound, were monitored at a particular retention time windows. The quantification curve was constructed by external standard method using four concentrations of ANHSECO and SECO ranging from 40 µg/ml to 150 µg/ml and from 10 µg/ml to 100 µg/ml, respectively. The authenticity of lignans coming from the sample was identified and quantified by comparison with the known quantities of the reference standards analysed in the same run by means of the combination of their chromatographic retention times and the ratio of two mass ions.

RESULTS AND DISCUSSION

Flaxseed samples were hydrolysed, extracted and analysed as shown in Figure 2. The method of direct acid hydrolysis by Liggins *et al.* (2000) was chosen since the literature data showed this method to result in the highest amounts of flaxseed SECO (12 617 µg/g and 7400 µg/g) detected. When applying ethanol or methanol extraction prior to acid, alkaline, or enzymatic hydrolyses, the detected amounts were in the range of 1570–6994 µg/g for SECO and 600–900 µg/g for ANHSECO (Milder *et al.* 2004; Peñalvo *et al.* 2005; Thompson *et al.* 2006; Smeds *et al.* 2007; Krajčová *et al.* 2009; Popova *et al.* 2009). Altough the discrepancy between the results of different methods could be explained by the differences in the plant variety and growth conditions (Johnsson *et al.* 2000), the work comparing alkaline hydrolysis of dioxane/ethanol extracts with direct alkaline hydrolysis showed better recovery of SDG using direct hydrolysis (Eliasson *et al.* 2003). Thus, in this work acid hydrolysis was applied directly on flaxseed and on ethanol extracts to see if the differences in quantified SECO amount could be linked to the step of ethanol extraction prior to acid hydrolysis. The concentration of ethanol (70%) was chosen based on the work of Zhang *et al.* (2007) while the temperature on the previous works that used extraction temperatures ranging from 55°C to 70°C (Table 1). To eliminate the possibility of inefficient ethanol extraction, separate analyses were performed on four sequential extracts. The fourth extraction yielded 5% SECO and 7% ANHSECO of the first extraction (specific data not shown). Thus, four extractions showed to be sufficient for this type of flaxseed. In a previous work by Popova *et al.* (2009), the

Table 2. Concentrations (μg/g) of SECO (as a sum of and ANHSECO and SECO) of fresh edible flaxseed obtained by different methods with related average coefficients of variations (*CV*%) on specific number of samples (*N*) – The results are presented as mean ± standard deviation

	EE DDS[a]	EE FS[b]	DH DDS[c]	DH FS[d]
SECO (*N* = 16)	5172 ± 49	5159 ± 83	8566 ± 169	8571 ± 192
Inter-day variations (*CV*%) (*N* = 4)	1.40	5.10	8.16	9.23
Intra-day variations (*CV*%) (*N* = 4)	4.08	4.41	3.50	3.93
Repeatibility of measurement (*CV*%) (*N* = 4)	1.10	0.75	0.99	1.33

[a]ethanol extraction of the defatted-dried seed; [b]ethanol extraction of the fresh seed; [c]direct hydrolysis of the defatted-dried seed; [d]direct hydrolysis of the fresh seed

extraction efficiency was evaluated after 4 h, and additional 4 hours. After 8 h extraction with the solution of 80% (v/v) methanol in water at 55°C, the recovery of the analysed lignans was 80–97%. The difference between the extraction times can be explained by utilising new solvent solution every hour of extraction which minimises the possibility of saturation of the solvent, and by the type of flaxseed which could contain a lower hull/embryo ratio. Nevertheless, it is important to note that the extraction efficiency should be examined prior to each analysis to ensure complete extraction.

The remaining oil average content in flaxseed defatted with hexane under magnetic stir mixing was high (13.8 ± 1.2 g/100 g), therefore for further analysis flaxseed defatted by Soxhlet extraction was used.

To eliminate errors that could have been caused by degradation of the derivatised compounds, all analyses were performed in two hours blocks. The peak eluting at 20.7 min was assigned to ANHSECO and that eluting at 21.4 min to SECO. The ions (mass to charge ratio *m/z*) selected for ANHSECO were 488.3 and 179.1, and for SECO 560.3 and 470.3 (Figure 3). Good linearity was observed with both standards, R^2 being 0.998 for ANHSECO and 0.999 for SECO. Each chromatogram was manually investigated to eliminate computer miscalculations.

The results of the four sample preparations applied were expressed as a sum of the concentrations of SECO and ANHSECO (μg/g of fresh edible seed) (Table 2) because SECO is proven to dehydrate to ANHSECO under acid conditions (Mazur *et al.* 1996; Meagher *et al.* 1999; Charlet *et al.* 2002), while only a negligible amount of ANHSECO is present in the free form in flaxseed (Popova *et al.* 2009). The defatted and dried extracts were corrected to fresh weight based on the results of the oil (41.7 ± 0.9 g/100 g) and water (5.5 ± 0.2 g/100 g) contents determined. Single factor ANOVA analysis confirmed a significant difference between the concentration levels obtained by ethanol extraction applied prior to acid hydrolysis and direct hydrolysis of flaxseed, at a level of $P < 0.01$. The direct hydrolysis method was proven to result in around 40% higher concentrations of the extracted SECO and ANHSECO, indicating that the introduction of the step of the ethanol preextraction resulted in a loss of the target compound. No significant difference was established between the defatted dried and fresh edible flaxseed (Figure 4). The use of the non-defatted seeds did not influence the identification and quantification of SECO and ANHSECO, since other compounds extracted from flaxseed did not interfere with the eluting peaks of SECO and ANHSECO (Figure 3). The method precision was determined by analysis of four samples prepared in the same way on a single assay day for intra-day precision, and on each of the four consecutive days to determine the inter-day variation. The repeatability of the measurement was done by four injections into GC for each sample

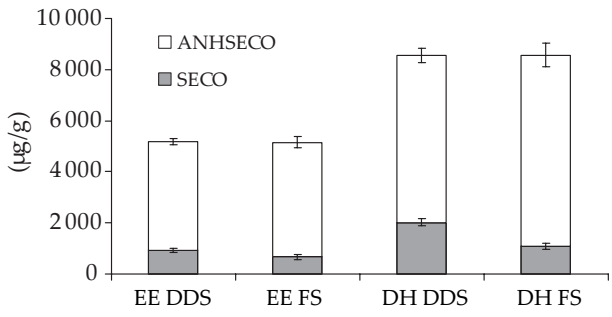


Figure 4. Concentrations (μg/g) of anhydrosecoisolariciresinol – ANHSECO and secoisolariciresinol – SECO (with one standard deviation) in fresh and defatted flaxseed prepared with and without ethanol preextraction

and proved to be acceptable for all four methods, contributing the least to the standard deviation of the results. Intra-day variations were similar for all the methods. The differences between the samples analysed on four different days contributed the most to the standard deviation, except for the EE DDS method. Inter- and intra-day variations can be partially explained by sampling the milled flaxseed, and by the proportion of embryo and hull in the 0.5 g of the analysed sample since the flaxseed hull contains on average 7.5 times more SDG than the embryo (Popova *et al.* 2009).

If the obtained values of SECO and ANHSECO are separately observed (Figure 4), it can be seen that the degradation of SECO is significantly higher ($P > 0.05$) if non-defatted flaxseed is analysed, as compared to defatted and dried flaxseed, indicating that the presence of oil could accelerate SECO dehydration. It was shown previously that the matrix in which lignans occur affects the time and extent of hydrolysis (Liggins *et al.* 2000; Popova *et al.* 2009). The ratios of ANHSECO/SECO concentrations obtained by Ethanol Extraction of the Defatted-Dried Seed (EE DDS), Ethanol Extraction of the Fresh Seed (EE FS), Direct Hydrolysis of the Defatted-Dried Seed (DH DDS), and Direct Hydrolysis of the Fresh Seed (DH FS) were 4.7, 6.7, 3.2, and 6.8, respectively. A possibility exists of incomplete hydrolysis of SDG to SECO (ANHSECO) and their intermediate product secoisolariciresinol monoglucoside (SMG), as shown in a work by Popova *et al.* (2009), although it was reported previously that a longer reaction time would result in further hydrolysis and thus the loss of lignans (Charlet *et al.* 2002; Kraushofer & Sontag 2002).

CONCLUSION

The aim of the analytical methods development is to obtain the best recovery and repeatability by decreasing the number of the preparation steps, as well as the necessary resources. Direct acid hydrolysis proved to be sufficient in flaxseed secoisolariciresinol analysis, resulting in 40% higher yield of the detected secoisolariciresinol in comparison with the method where ethanol preextraction had been used. No significant difference was shown between the extracted amounts when using fresh or defatted flaxseed. Defatting and drying of flaxseed prior to lignan extraction prolongs the time and cost of analysis, but if deffating is done and not performed by standardised methods for the oil content determination, fat extraction efficiency should be verified. The degree of the sample homogeneity and separation of different structure parts of the food analysed could positively influence the accuracy of the results.

Acknowledgements. The authors wish to thank Thomas Holmbom from Oy Separation Research Ab (Turku, Finland) for supplying the standard of secoisolariciresinol and prof. Dr.Sc. Nives Galic for valuable advice on writing the article.

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Received for publication March 30, 2010
Accepted after corrections February 28, 2011

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