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Identification and quantification of lignans in wheat bran by gas chromatography-electron capture detection

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ABSTRACT

Whole grain cereals are an important source of bioavailable lignans, the group of compounds with potential anti-cancerogenic, antioxidant, anti-proliferative, pro-apoptotic, and antiangiogenic properties. The aim of this work was to develop a sensitive method for determination of wheat bran lignans. The analysis of lignans secoisolariciresinol, hydroxymatairesinol, lariciresinol, matairesinol, pinoresinol, syringaresinol is based on derivatization with pentafluoropropionic anhydride (PFPA) and gas chromatography-electron capture detection (GC-ECD), using styrene glycol as internal standard. To our knowledge, this is the first time that EC detection has been used for lignan analysis. The results show that the technique is reproducible and sensitive enough for detecting lignans in wheat at parts-per-billion (ppb) levels, except for hydroxymatairesinol. The method developed showed good recovery (85–105%) and precision (4–20%) for five types of lignans and thus represents a simpler and more affordable alternative to state-of-the-art wheat lignan liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis.

1. Introduction

Lignans are the group of plant compounds classified as phytoestrogens. Due to their diphenolic ring, they are structurally similar to endogenous estrogens, making them act as weak estrogen agonists or antagonists. Lignans may exhibit anti-cancerogenic, antioxidant, anti-proliferative, pro-apoptotic, and antiangiogenic properties, though the mechanisms of action remain unclear [1,2]. The most abundant food in lignans is flaxseed; thus, many studies have been conducted on flaxseed lignans [3]. Nevertheless cereals are a staple food in the Western diet and therefore an important source of bioavailable lignans [4–7].

The commonly used separation techniques in food lignan analysis are gas chromatography (GC) and high performance liquid chromatography (HPLC) coupled with different detectors [8,9]. The detector of choice depends on the food analysed and the amount of lignans present. While various types of detectors have been combined with HPLC, the detector coupled with GC is always the mass spectrometer (MS) [10–12]. Wheat lignan content has been analysed by LC–MS/MS [13–15], which seems to be the state-of-the-art technique in food lignan analysis. However, MS/MS is not yet widely used, as the instrument's cost is prohibitive. To our knowledge only one study has been published on GC analysis of wheat lignans using MS [12]. Although MS allows conclusive proof of compound identity by taking into account retention times and mass spectral data, GC coupled with electron capture detection (ECD) is known to be a highly sensitive technique that can detect picogram or even femtogram levels of specific substances in complex matrices. This makes the EC detector an excellent detector for environmental and biomedical studies [16].

To separate and analyse food lignans by GC, they need to be converted to chemical forms that are less polar and sufficiently volatile. This chemical conversion is done through the process of derivatization. Although three basic types of derivatization reactions are generally used (silylation, acylation, and alkylation) so far only silylation was used for the GC analysis of food lignanas. Liggins et al. [11], Meagher et al. [17], Sicilia et al. [18], Popova et al. [19], and Bonzanini et al. [20] used N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) while Thompson et al. [6], Mazur et al. [10], and Peñalvo et al. [12] used hexamethyldisilanaze (HMDS)/trimethylchlorosilane (TMCS) in pyridine. However, the drawback of such a derivatization procedure is a possibility of side reactions which can affect the stability of the derivatives formed [21].

In order to compensate for random and systematic errors of the method or detector internal standardization is used [22]. Variety

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of internal standards (IS) are available in analysis of food lignans, which can be compounds structurally related to the analyte or isotope-labelled analogues of the analyte. For example, Mazur et al. [10], Milder et al. [13], Peñalvo et al. [12], and Smeds et al. [15] used isotope-labelled lignans, while others used various structurally related compounds: anthraflavic acid [11], 5α-androstane-3β,17β-diol [6], *o*-terphenyl [19], and betulinol [20]. Nevertheless, choosing the most appropriate internal standard is still a chalenge in food analysis.

The aim of the present work was to develop a simple, sensitive, reproducible and affordable GC-ECD method for the simultaneous determination of some of the currently most important lignans in wheat bran: secoisolariciresinol (SECO), hydroxymatairesinol (HMR), lariciresinol (LARI), matairesiol (MATA), pinoresinol (PINO), and syringaresinol (SYR) (Fig. 1).

2. Materials and methods

2.1. Chemicals and materials

Standards of secoisolariciresinol (>95% purity), pinoresinol (>95%), lariciresinol (~92%), and hydroxymatairesinol (~90%) were a kind gift from Oy Separation Research Ab (Turku, Finland). Syringaresinol (>95%) was purchased from Plantech UK (Berkshire, England). Matairesinol (≥85%); the derivatizing agents pentafluoropropionic anhydride (PFPA) (≥96%) and trifluoroacetic acid (TFA) (≥96%); the internal standards styrene glycol (≥98.0%), anthraflavic acid (90%) and *trans*-resveratrol (>99%); and the enzyme *H. pomatia* β-glucuronidase/sulfatase type H-1 were purchased from Sigma–Aldrich (Taufkirchen, Germany). Methanol, sodium hydroxide, glacial acetic acid, ethyl-acetate, dichloromethane, pyridine and *n*-hexane were purchased from J.T. Baker (Griesheim, Germany).

The Institute for Seeds and Seedlings (Osijek, Croatia) provided wheat, Žitarka type. Wheat bran was obtained by milling wheat in a laboratory mill (Bühler grinding machine, Bühler, Germany), followed by powdering in a coffee grinder.

2.2. Analysis of lignans

2.2.1. Sample pretreatment

Prior to sample preparation, all glassware was silanized in a 5% solution of dimethyldichlorosilane in heptane, followed by deactivation of excess reagent in methanol.

Sample preparation was done according to previously described procedures [12,15], with modifications. Extraction and alkaline hydrolysis of 200 mg of wheat bran was performed with 5 mL of 70% methanol (MeOH) containing 0.3 M sodium hydroxide at 60 °C. The samples were shaken every 5 min for 10 s. After 60 min the samples were centrifuged at 750 *g* for 15 min, the supernatant was transferred to a fresh tube, and the extraction was repeated. The supernatants were pooled and pH was adjusted to ~5 using glacial acetic acid. The supernatants were again centrifuged at 750 × *g* for 15 min to precipitate salts, and an aliquot of 8.5 mL was evaporated to dryness under an N_2 stream. To perform enzymatic hydrolysis, 2300 U of *H. pomatia* β-glucuronidase/sulfatase was dissolved in 3 mL of 0.05 M Na-acetate buffer (pH 5). The freshly prepared enzyme solution was added to the dried supernant, and the mixture was incubated for 18 h at 37 °C with slow magnetic stirring. Subsequently the hydrolysate was applied to a preconditioned SPE cartridge containing C-8 sorbent (Varian, Bond Elut – Certify II, 50 mg, 3 mL) at a maximum rate of 1–2 mL/min. Prior to sample application, the SPE cartridge was preconditioned with 2 mL of methanol followed by 2 mL of 0.05 M Na-acetate buffer (pH 5). After washing the cartridge with 3 mL of Na-acetate buffer, the lignans were eluted with 3 mL of methanol into safe lock tubes.

To verify extraction efficiency, extraction of lignans with MeOH:0.3 M NaOH (7:3) was examined. The wheat bran sample was extracted four times, each time with 5 mL of extraction solvent at 60 °C for 60 min. The obtained extracts were analysed separately as described above. In addition, tests were performed to measure the loading capacity of the SPE cartridge, as well as the ability of the lignans to bind to the sorbent. Following enzymatic hydrolysis, the Na-acetate buffer containing lignan extracts was applied to an SPE cartridge. The passed Na-acetate buffer solution was then loaded on another SPE cartridge. The content from both SPE cartridges was further eluted with 3 mL of methanol, and the amount of analyte in each methanol extract was compared. To test whether the amount of methanol was sufficient to elute the lignans from the SPE, the tubes were eluted with an additional 3 mL of methanol.

2.2.2. Calibration curves

A stock solution was prepared by mixing the six lignan standards in methanol at the following concentrations (μg/mL): SECO, 3.77; HMR, 1.91; LARI, 5.09; MATA, 0.85; PINO, 2.64; and SYR, 1.82. The solution was stored at −20 °C until use.

Calibration curves were constructed by spiking 200 mg of wheat bran with 10, 25, 50, 100, 200, 350, and 550 μL of a stock solution of lignan standards. Standards were extracted with 5 mL of methanol. After 60 min of extraction at 60 °C, with manual shaking every 5 min, the sample was centrifuged at 750 *g* for 15 min. The supernatant was transferred to a separate tube, evaporated to dryness under stream of N_2, and redissolved in 3 mL of 0.05 M Na-acetate buffer (pH 5). The tubes were vortexed for 1 min and loaded on a preconditioned SPE cartridge. Elution was performed as described in Section 2.2.1.

For internal standardization 50 μL of styrene glycol (17.75 μg/mL in methanol) was added before derivatization. The concentration of lignans in wheat bran was calculated by internal standardization using peak height measurements.

2.2.3. Precision, recovery and accuracy

The precision, recovery, and accuracy of the GC-ECD method were determined at two concentration levels (50 μL or 350 μL of the lignan standard stock solution) for each analyte. Each concentration contained eight replicates of spiked wheat bran samples, prepared as described in Section 2.2.2.

Precision was calculated by using the relative standard deviation (R.S.D.). Within-run precision (%) was calculated by repeated injections ($n = 5$) of the same sample on a single day. Intra-day variation (%) was determined by analysing eight replicates at both concentration levels on a single day. The variations in the slope of the calibration curves run on 5 different days were used to assess inter-day variations. To determine recovery (%), lignan standards were added at two concentration levels to non-hydrolysed wheat bran methanolic eluent after SPE and before derivatization (defined as 100% recovery), and compared for the peak height ratios (lignan/internal standard) with the extracts of spiked wheat bran sample. Accuracy was calculated by the equation: mean measured concentration/nominal concentration × 100.

2.2.4. Derivatization

Fifty microliters of styrene glycol as internal standard (IS) was added to the methanol extracts (see Section 2.2.1.) and evaporated to dryness. To each sample 500 μL of dichloromethane, 30 μL of pyridine and 30 μL pentafluoropropionic anhydride (PFPA) were added. The reaction mixture was heated at 70 °C for 60 min in a dry block heater. After cooling down to room temperature and drying completely under stream of N_2, samples were reconstituted in 1 mL of *n*-hexane, vortexed for 1 min and centrifuged for

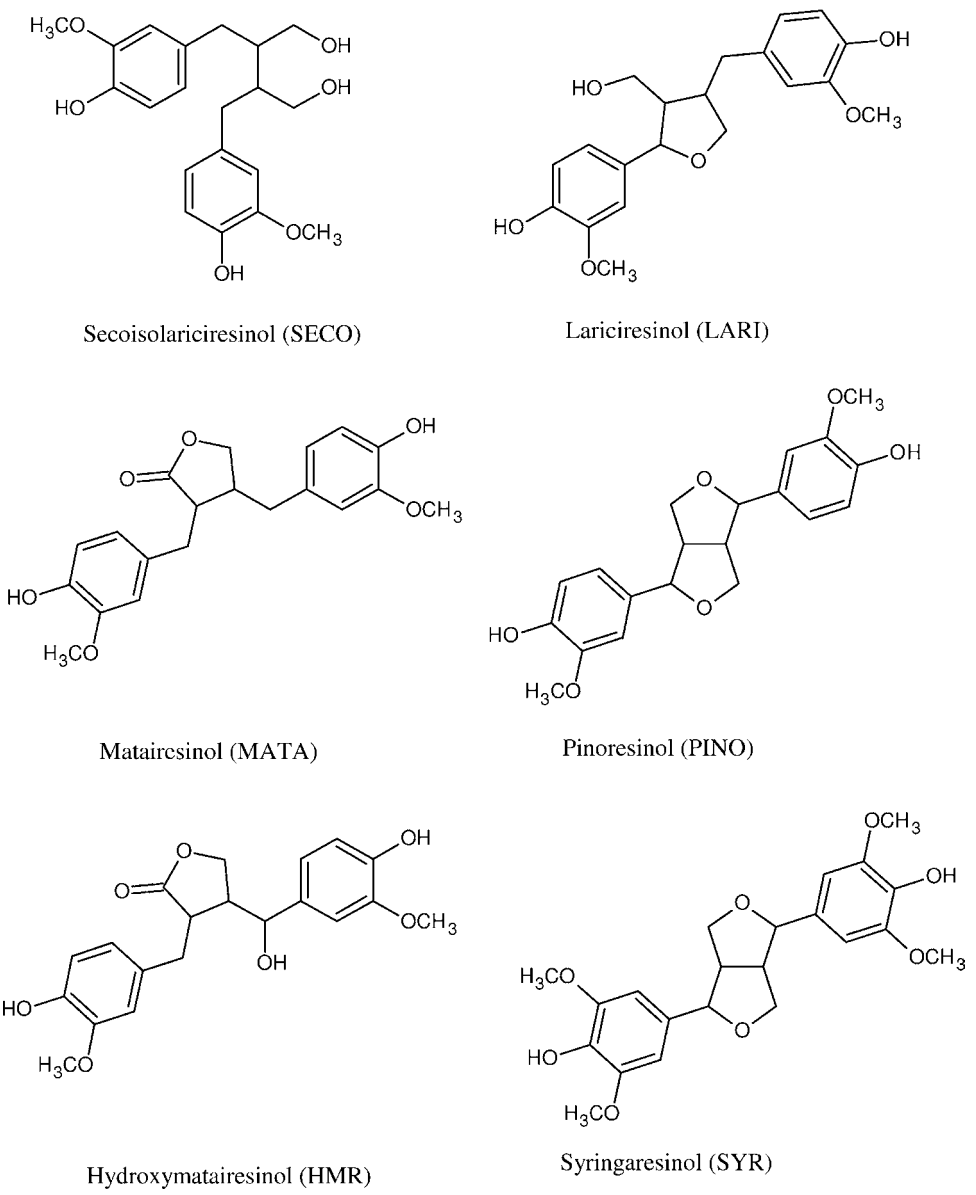

Fig. 1. Chemical structures of the analysed lignans.

3 min at 1000 × *g* [23]. The final *n*-hexane fraction was injected into the chromatographic system. Additionally, trifluoroacetic acid (TFA) has been examined as a potential derivatization reagent. TFA derivatization was performed in the following way: TFA (40 μL) and ethyl acetate (40 μL) were added to the samples and heated at 70 °C for 60 min in a dry block heater. After cooling down to room temperature, the samples were evaporated to dryness under stream of N_2, reconstituted in 1 mL of ethyl acetate, vortexed for 1 min and centrifuged for 3 min at 1000 × *g*.

2.2.5. Chromatographic conditions

The GC-ECD analysis was carried out with an Agilent 6890 GC equipped with a ^{63}Ni Micro-ECD. The column used was Rtx-5ms (30 m, 0.25 mm I.D., 0.25-μm film thickness; Restek, USA). The initial column temperature was 120 °C. After 2 min the temperature was increased to 160 °C at 10 °C/min, then it was increased to 200 °C at 20 °C/min, where it was held for 5 min, and finally it was increased to 290 °C at 20 °C/min, where it was held for 12 min. The injector temperature was 275 °C, detector temperature was 300 °C and the column head pressure was 88.0 kPa. Sample (1 μL) was injected by the split injection technique (split ratio, 20:1) using a deactivated cup inlet liner suitable for split injection (cat. no. 20510, Supelco, USA).

The sensitivity of the ECD was compared with the sensitivity of the MS detector (Agilent, Single Quadropole MS 5973) on the same Agilent 6890 GC instrument under the conditions described above, except that in the case of MS the sample was introduced by splitless injection and the analyses were performed in the full SCAN mode.

3. Results and discussion

3.1. Derivatization of lignan hydroxyl groups

To enable sensitive EC detection, PFPA and TFA were chosen as acylating agents to form fluoroacyl lignan derivatives. In food lignan analysis, the stability of derivatives is a major challenge for ensuring accurate results, particularly when analysing large numbers of samples and using autosampler. Lignans to be analysed by GC are usually silylated, but to our knowledge no information exists about the stability of these derivatives. Thus, the present study examined

Table 1
Analytical parameters of GC-ECD assays of PFPA derivatives of wheat bran lignans.

	N	SECO	LARI	MATA	PINO	SYR
Retention time		17.03	19.35	20.36	21.92	26.92
Linear range (μg/mL)	5	0.012–0.415	0.022–0.559	0.029–0.094	0.009–0.291	0.013–0.200
LOD (μg/mL)	3	0.012	0.022	0.029	0.009	0.013
LOQ (μg/mL)	3	0.038	0.065	0.090	0.027	0.038
Inter-day variations (CV, %)	5	8.5	9.1	19.4	13.9	8.5
Intra-day variation (CV, %)[a]	8	6.3	4.1	11.0	7.3	11.5
Intra-day variation (CV, %)[b]	8	4.0	4.9	7.7	5.7	8.2
Within-run precision (CV, %)[a]	5	3.0	4.5	7.7	2.5	5.3
Within-run precision (CV, %)[b]	5	2.9	2.6	4.9	2.5	3.0
Recovery (%)[a]	8	92	96	98	95	98
Recovery (%)[b]	8	96	92	85	87	85
Accuracy (%)[a]	8	85.2	93.8	30.6	83.0	73.2
Accuracy (%)[b]	8	104.6	102.4	101.04	102.5	94.7

LOD, limit of detection; LOQ, limit of quantitation; SECO, secoisolariciresinol; LARI, lariciresinol; MATA, matairesiol; PINO, pinoresinol; SYR, syringaresinol. Results are expressed as μg of lignan standards per mL of methanol extract (see Section 2.2.2)

[a] For the following concentrations: 0.038 μg/mL SECO; 0.051 μg/mL LARI; 0.009 μg/mL MATA; 0.026 μg/mL PINO; 0.018 μg/mL SYR.

[b] For the following concentrations: 0.264 μg/mL SECO; 0.356 μg/mL LARI; 0.060 μg/mL MATA; 0.185 μg/mL PINO; 0.128 μg/mL SYR.

the stability of PFPA and TFA derivatives of lignan standards. The deterioration of the PFPA derivatives during a period of 12 h was under 15% for SECO, PINO and SYR, while it was higer for LARI (20%) and MATA (28%) (Fig. 2). HMR showed to be the most unstable PFPA derivative starting almost immediately to deteriorate to only 26% of the initial concentration after 12 h. On the other hand the deterioration of TFA derivatives of SECO and SYR was also under 15%, but for PINO was more than 40%. Similar results were obtained for LARI and MATA. The deterioration of TFA derivatives of HMR (around 40%) was lower than for PFPA derivatives. It is obvious that neither of derivatization reagents is suitable for the determination of HMR. Although the stability of both PFPA and TFA derivatives were more or less similar, PFPA showed to increase the signal of the detector.

3.2. Internal standard

In MS, isotope-labelled analytes provide the best corrections for any losses during sample isolation, purification, derivatization, and separation, as well as for variations in instrument performance. Nevertheless they are not suitable for EC detection since their physicochemical properties are identical to those of the analyte, so no observable separation will occur during the procedure [22]. In the present study, styrene glycol, anthraflavic acid and *trans*-resveratrol were examined as potential IS. Anthraflavic acid failed to derivatize with both PFPA and TFA, while *trans*-resveratrol, although it exhibited a suitable retention time intermediate among those of the target lignans, showed extreme instability and degradation. Styrene glycol exhibited low recovery after SPE, but its PFPA derivative showed excellent stability even after 12 h. Thus, styrene glycol was used as IS in the present study; it was added to the lignan standards before derivatization, in order to correct for derivatization reactions and instrument response.

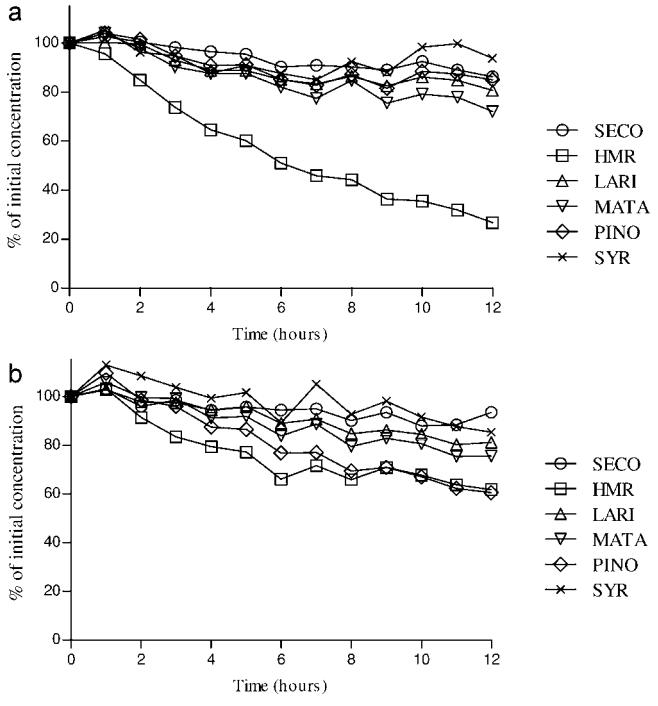

Fig. 2. Stability of (a) PFPA and (b) TFA lignan derivatives after 12 h, expressed as percentage of initial concentration.

3.3. Method development and validation

Food lignans separated by GC are normally analysed with the MS detector enabling confirmation of identity (Fig. 3). In the present study we compared the sensitivity of the MS and EC detectors coupled to the same Agilent 6890 GC instrument using the same GC column and working condition (see Section 2.2.5) except that for GC–MS analysis of the samples it was necessary to work in splitless mode. For the used instrument, the ECD response showed to be higher than that of MS detector for the same concentration of PFPA derivatives.

To verify the efficiency of extraction with MeOH:0.3 M NaOH (7:3), separate analyses were performed on four sequential extracts. A second extraction yielded 17% SECO, 13% LARI, 12% PINO and 22% SYR of the first extraction. The third extraction yielded only 2% LARI and 3% SYR of the first extraction, and no peaks at lignan standard retention times were detected in the fourth extract. Thus, two extractions were found to be sufficient to extract lignans from wheat bran. MATA was bellow LOD in all extracts. Our results also show good binding capacity and retention capability of used SPE sorbent. No lignans were observed in extracts purified twice on SPE, indicating that the chosen SPE cartridge has good binding capacity. To verify that the amount of methanol for elution from SPE was sufficient, tubes were eluted with an additional 3 mL of methanol. No lignans were observed in the second extract.

The results of GC-ECD method validation are shown in Table 1. To construct the calibration curve and determine precision, pure wheat bran and wheat bran spiked with lignan standards were analysed in the same way (Section 2.2.2). Pure and spiked wheat bran samples used for the construction of calibration curve were prepared without alkaline and enzymatic hydrolysis. One of the

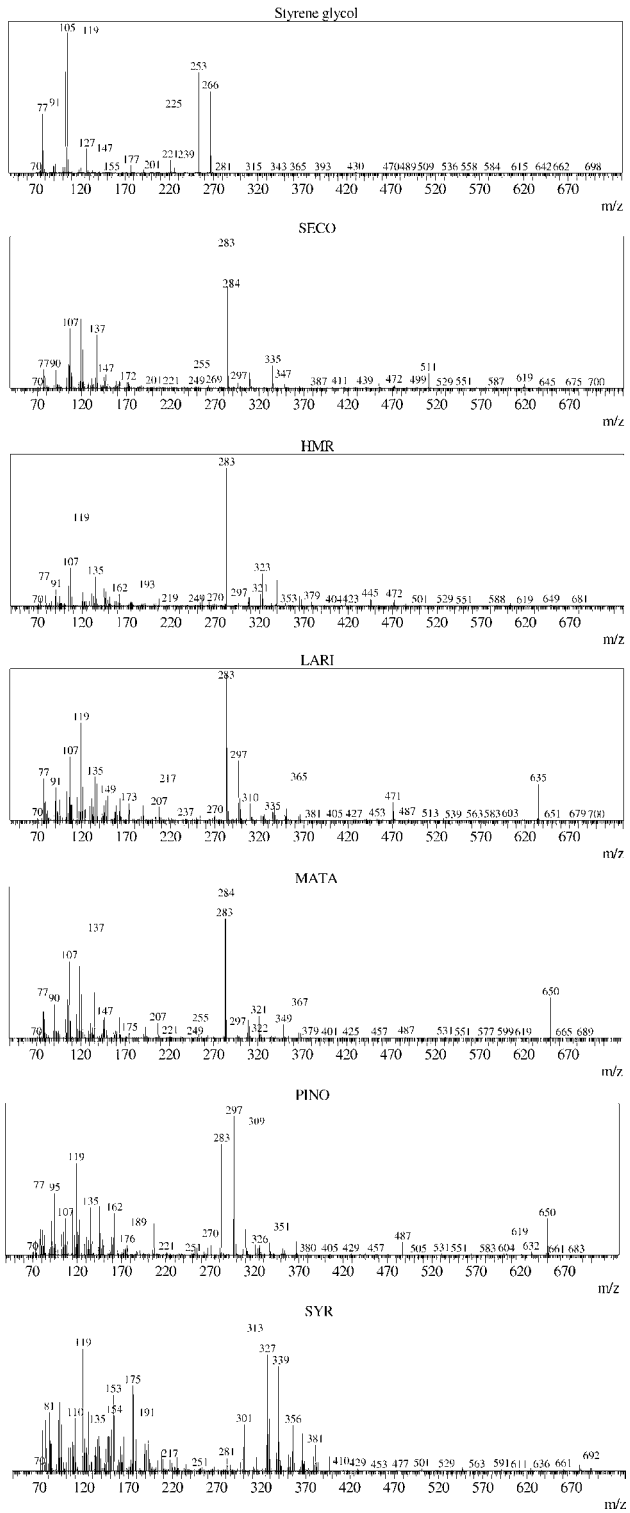

Fig. 3. Mass spectra of PFPA derivatives of styrene glycol and lignan standards: SECO, secoisolariciresinol; HMR, hydroxymatairesinol; LARI, lariciresinol; MATA, matairesiol; PINO, pinoresinol; SYR, syringaresinol.

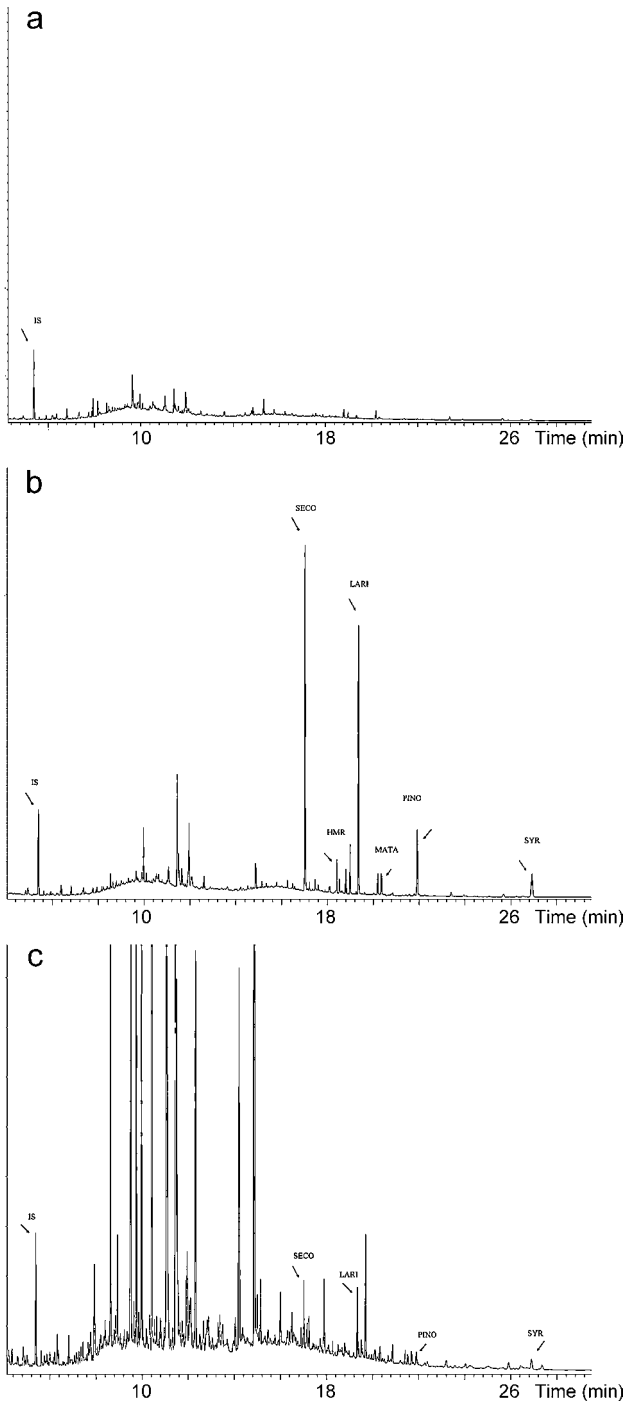

Fig. 4. GC-ECD chromatograms of (a) a non-hydrolysed wheat bran sample, (b) a non-hydrolysed wheat bran sample spiked with lignan standards, and (c) a hydrolysed wheat bran sample. IS, internal standard; SECO, secoisolariciresinol; HMR hydroxymatairesinol; LARI, lariciresinol; MATA, matairesiol; PINO, pinoresinol; SYR, syringaresinol.

main obstacles in food analysis is finding a representative blank sample. Food presents a complex matrix in which many non-target compounds interact with the analyte. The chromatograms of wheat lignan extracts show that other wheat bran matrix components are also extracted during the same procedure (Fig. 4) and may alter the detector response. Preliminary studies showed that calibration curves from spiked bran matrix had 1.5–2 times higher slope values than calibration curves from solvent (data not shown). Thus, a calibration curve directly from solvent would give incorrect results, as already observed by Milder et al. [13].

The non-hydrolysed wheat bran was also examined for free lignan content, to account for any free lignan forms that might contribute to the calibration curve (Fig. 4). In the present study, concentrations of the free forms of wheat bran lignans were below the limit of detection and therefore non-hydrolysed wheat bran was found to be a suitable blank sample for constructing calibration

curves and measuring precision. In previous studies the calibration curve was constructed without considering possible contributions of the food matrix [15] or other types of foods such as millet or rye bran were used to examine only the method precision [12].

The calibration curves prepared on 5 different days were linear within the range of concentration studied, with the correlation coefficients higher than 0.990 for all five lignan standards (Table 1). MATA exhibited irregular peak shapes at the two lowest concentration levels; therefore, these levels of MATA were not taken into account for calibration. On the basis of the standard deviation of the response (SD) and the slope of the five-point calibration curve (S) constructed around the expected limit of detection, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the following formulas: LOD = 3.3(SD/S) and LOQ = 10(SD/S) (Table 1).

LOD ranged from 0.009 to 0.029 μg of lignan standards per mL of methanol extract. For our purposes the determined LOD and LOQ were sufficiently low, however it is possible to increase sensitivity of the method by changing the split-ratio and injection volume. The sensitivity of the method enabled injection of the samples in split mode (20:1), whereas previous studies applied splitless injection mode [6,19,20].

The intra-day variation was determined by analysing eight replicate measurements at two concentration levels of spiked lignan standards (Table 1). The intra-day variations were higher for lower concentration levels of lignans but always below 12% for all five lignans. The inter-day variation was assessed on the basis of the calibration curve slopes run on 5 different days and was below 20% for all lignan standards. The within-run precision was determined by five consecutive injection of the same extract of non-hydrolysed spiked wheat bran (Table 1). Accuracy was in the range 94.7–104.6% at higher concentrations (350 μL of stock solution). At lower concentrations (50 μL of stock solution), accuracy was >70% for all lignan standards, except for MATA, which was only about 30%. The recoveries for both low and high concentration levels were higher than 85% for all five lignans. All the analysed lignans showed good retention time stability.

3.4. Wheat bran lignan quantification

Practical application of the method has been verified by analysing lignans in the bran of Žitarka wheat (Fig. 4). Quantification of lignans in wheat bran samples was achieved by direct comparison with calibration standards analysed under the same chromatographic conditions and during the same run. The wheat bran lignan extracts were prepared in three parallels. The lignan levels (μg/100 g) in the Žitarka wheat bran were as follows: SECO, 204; LARI, 385; PINO, 166; and SYR, 170. MATA and HMR were not detected in this wheat cultivar. Our preliminary results also show that most of the lignans investigated are located in the bran fraction which comprises only 13% of the whole wheat grain in average (data not shown). There is little information on the content of lignans in wheat. Few studies in which whole wheat grain was analysed by GC–MS [12] or LC–MS/MS [15,24] determined lignans in approximate levels of μg/100 g as follows: SECO, 20–42; LARI, 54–140; PINO, 37–83; and SYR, 372–1250. Although the lignan levels determined in our study may not be fully comparable with the available literature data, it has to be noted that there are known differences in lignan concentration levels between cultivars [15]. The results of the application of the developed method on other types of cereals will be published elsewhere.

4. Conclusions

In this paper a new method for lignan analysis in wheat bran using GC with electron capture detection is presented. The developed method is suitable for the simultaneous detection and quantification of five lignans in wheat bran at the ppb level. PFPA derivatization reagents and styrene glycol as internal standard have been used for lignan analysis for the first time to our knowledge, and they showed acceptable results. The method is relatively simple, requiring methanolysis and enzymatic hydrolysis, followed by solid-phase extraction as the sole technique for concentration and purification. Advantages of the method are its simplicity, high sensitivity, and repeatability, with the limit of quantitation in the ppb range. Future study will show whether the method can be extended to lignan analysis of other types of cereals.

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