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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