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Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (Part 1: non-volatile compounds)

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Abstract

Pumpkin seed oil is a common salad oil in Austria. It is not only of interest because of its typical taste but also because of its potential in curing prostate disease. Besides the fatty acids, the micronutrients, which comprise vitamin E, phytosterols and lignans, are of special interest. Since the seeds are roasted before pressing of the oil, changes occur in the composition of the fatty acids and micronutrients. The oxidation-sensitive linoleic acid decreases from 54.6 to 54.2% whereas the concentrations of the vitamin E isomers show a decrease during the first 40 min of about 30% followed by an increase during the last 20 min to about the same level as at the beginning of the roasting process. The concentrations of α-tocopherol and γ-tocopherol in the fresh dried seeds are 37.5 and 383 μg/g, respectively. The concentration of the tocotrienols is about one third of the corresponding tocopherols. The initial concentration of the total sterols (1710 μg/g) increases to 1930 μg/g. The increases of the sterols and vitamin E during the roasting process could be attributed to the changes of the seed meal, since at the end of the roasting the oil emerges from the seeds resulting in altered chemical behaviour of the extraction process. Secoisolariciresinol, which is only detectable at the beginning with a concentration of 3.8 μg/g, is destroyed after 20 min.
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Keywords: Cucurbita pepo; Pumpkin; Vitamin E; Phytosterols; Lignans; Secoisolariciresinol; Fatty acids

1. Introduction

Pumpkin (Cucurbita pepo L.) seed oil is a common salad oil in Austria. Due to its colour, the foaming and the strong aroma, it has only limited application for cooking. The seeds themselves are eaten and show good results in curing several prostate diseases (Nitsch-Fitz, Egger, Wutzel, & Maruna, 1979).

The oil is produced in the southern parts of Austria (Styria) by traditional very labour intensive methods in small mills that process lots of 50–100 kg of pumpkin seeds. After crushing, the seeds are roasted and pressed at elevated temperatures. The main characteristic of the Styrian Oil Pumpkin is the dark green colour of the thin-coated seeds (Teppner, 2000). The colour of the oil, which is pressed from the seeds, is dark green to red ochre and has a strong red fluorescence. The oil content of the pumpkin seed varies from 42–54% and the composition of fatty acids is dependent on several factors (variety, area in which the plants are grown, climate, state of ripeness). The dominant fatty acids comprise palmitic acid ($C_{16:0}$, 9.5–14.5%), stearic acid ($C_{18:0}$, 3.1–7.4%), oleic acid ($C_{18:1}$, 21.0–46.9%) and linoleic acid ($C_{18:2}$, 35.6–60.8%). These four fatty acids make up 98 ± 0.13% of the total. Other fatty acids are well below 0.5% (Murkovic, Hillebrand, Winkler, Leitner, & Pfannhauser, 1996).

The content of vitamin E in pumpkin seeds is very high (Murkovic, Hillebrand, Winkler, & Pfannhauser, 1996). The main vitamin E isomers occurring in pumpkin seeds

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are α- and γ-tocopherols with concentration of n.d.–91 mg/kg and 41–620 mg/kg, respectively. The other two tocopherols occur at very low concentrations. Additionally, α- and γ-tocotrienol are found in significant amounts but have not yet been quantified.

Pumpkin seeds are also rich in plant sterols which have recently become of great interest because of their serum cholesterol-lowering effect (e.g. Miettinen, Puska, Gylling, Vanhanen, & Vartiainen, 1995; Jones, Raeini-Sarjaz, Ntanios, Vanstone, Feng, & Parsons, 2000). They may also be beneficial against colon cancer (Awad, von Holtz, Cone, Fink, & Chen, 1998; Rao & Janezic, 1992). Plant sterols of pumpkin seeds are of special interest because of their highly characteristic composition (Breinhölder, 2001; Mandl, Reich, & Lindner, 1999). In general, the distribution of sterols in the family *Cucurbitaceae* constitutes one of the most complex patterns known in the plant kingdom (Garg & Nes, 1986). Some of their sterols are characteristic of organisms much lower on the evolutionary scale. In the case of *Cucurbita maxima*, it was shown that marked changes in sterol composition occur during germination and seedling developments (Garg & Nes, 1984, 1985). Pumpkin seeds mainly contain various Δ-7-sterols, rare in other plant seeds (Akihisha, Ghosh, Thakur, Rosenstein, & Matsumoto, 1986; Mandlet al., 1999; Garg & Nes, 1986). Therefore, the plant sterol profile of pumpkin seed oil can be used to detect adulteration (Mandl et al., 1999). On the other hand, properties of sterols typical of pumpkin seeds and oils are much less known than those of common sterols in other oils. Stability of sterols during food processing and storage is one of the questions which has created much recent research interest (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000). However, no studies on stability of pumpkin seed sterols are available.

Polyphenols of the isoflavone type, such as daidzein and genistein (Adlercreutz & Mazur, 1977) and lignan-type like secoisolariciresinol (Kraushofer, 2002), occur in pumpkin seeds. The content of these isoflavones in pumpkin seeds is in the low ppb range (5.6–15.3 ng/g) in contrast to the higher concentrations (210 μg/g) found for the lignan secoisolariciresinol. These naturally occurring compounds are reported to possess estrogenic, antiestrogenic, antioxidative, antiviral, antibacterial, insecticidal or fungistatic properties and have been shown to be proliferative in relation to many types of tumors in cell culture (Mazur & Adlercreutz, 1998). Secoisolariciresinol diglucoside is known for an antitumor effect when provided in the early promotion stage of tumorigenesis (Thompson, Seidl, Rickard, Orcheson, & Fong, 1996). Therefore the levels of human exposure and the concentration responsible for these effects have to be investigated further. From that point of view, the stability of secoisolariciresinol during the roasting process of pumpkin seeds is of interest.

This work describes the chemical changes that occur in the pumpkin seeds during the roasting process. The changes in fatty acids and the micronutrients vitamin E, phytosterols and secosisolariciresinol are investigated from a typical production lot.

2. Materials and methods

2.1. Materials

Secoisolariciresinol was bought from K. Wähälä (Department of Chemistry, University of Helsinki), cellulase 1.5 U/mg (*Trichoderma viride*) and other chemicals were from Merck (Darmstadt, Germany). Dihydrocholesterol (>99%) and the other plant sterols (sitosterol 95%, campesterol 98% and stigmasterol 95%) were purchased from Sigma Chem. Co., St. Louis, MO, USA. Ethanol and glacial acetic acid, were of analytical grade; methanol, *n*-hexane, dioxane, and acetonitrile were of LiChrosolv quality from Merck (Darmstadt, Germany). Bidistilled water was prepared with an Easypure® LF system (Barnstead/Thermolyne, Dubique, IO, USA). Silica cartridges were obtained from Varian (Harbor City, CA, USA).

2.2. Roasting process

Pumpkin seeds (60 kg) were milled in a stone mill and 8 l water and 300 g sodium chloride were added. After milling and homogenisation the first sample (ca. 30 g) was withdrawn. The milled seeds were heated in a pan with a jacket heater set to a temperature of 150 °C and stirred continuously. Every 10 min, a sample of ca. 30 g was taken and cooled to 0 °C with ice immediately. After bringing the samples to the lab, which took about 1 h, the samples were frozen and kept at −70 °C until analysis. The temperature measurement was carried out using a thermometer Quicktemp 826 T4 (Testo, Vienna, Austria) that allows hygienic measurement without contact of the food. The temperature of the milled seeds during the roasting process is shown in Fig. 1.

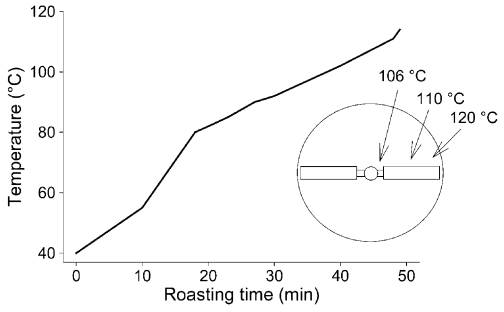

Fig. 1. Temperature gradient in the pan during the roasting process and radial temperature gradient at the end of the roasting process.

2.3. Analysis of fatty acids

2.3.1. Derivatisation of fatty acids and GC analysis

The oil was transesterified by using a boron trifluoride methanol complex. The methylated fatty acids were analysed by GC-FID, as described previously (Murkovic, Hillebrand, Winkler, Leitner, & Pfannhauser, 1996).

2.4. Vitamin E analysis

2.4.1. Sample preparation

About 1 g of the milled/roasted pumpkin seeds was extracted with 30 ml of *n*-hexane after addition of 5 g Na_2SO_4 for removing the water for 30 min at room temperature, in a 250 ml Erlmeyer flask closed with a screw-cap in a supersonic bath. For protection of the tocopherols against oxidation, 8 mg of butylated hydroxytoluene were added to the extraction solvent. The solvent was evaporated and the residue dissolved in 10 ml of hexane.

2.4.2. HPLC analysis

20 μl of the extracts were injected onto a normal phase column. The HPLC equipment used was a Merck/Hitachi liquid chromatograph (LaChrom, pump L 7100, autosampler L 7200) with a fluorescence detector (L 7480), equipped with a LiChrospher Si60 (5 μm, 250×4 mm) and a precolumn LiChroCART 4-4 (LiChrospher Si60, 5 μm). The columns were eluted with 96% *n*-hexane and 4% dioxane (v:v). The flow rate was 2.0 ml/min and the effluent was monitored at $\lambda_{ex} = 292$ nm, $\lambda_{em} = 335$ nm for determination of the tocopherols.

The tocopherols were identified by comparing their retention times with that of authentic standards. The quantification was based on an external standard method. The concentrations of stock solutions were determined spectrophotometrically and corrected with the area percent of a chromatogram with UV-detection. The molar extinction coefficients (ε) used were for α-tocopherol 3260 at 292 nm, β-tocopherol 3720 at 296 nm, γ-tocopherol 3810 at 298 nm and for δ-tocopherol 3510 at 298 nm (Dawson, Elliott, Elliott, & Jones, 1986). All standard solutions were prepared daily from a stock solution which was stored in the dark at −20 °C. The means and standard deviations were calculated from quadruplicate analyses for α- and γ-tocopherol. The tocotrienols were quantified using the fluorescence signal of the corresponding tocopherols standards (Thompson & Hatina, 1979).

2.5. Sterols

2.5.1. Sample preparation

About 1.0 g of the seeds was weighed into a test tube. The internal standards dihydrocholesterol (0.4 mg in 1 ml of ethanol) and ethanol (7 ml), were added before homogenizing the sample for 1.5 min (Ultra Turrax T25, Janke & Kunkel, Ika-Labortechnic GmbH&Co, Staufen, Germany). The samples were saponified, after which the unsaponifiable lipids were extracted with 20 ml of cyclohexane (Mattila, Lampi, Ronkainen, Toivo, & Piironen, 2002). A portion (15 ml) of the extract was evaporated to dryness and the residue was dissolved in 2 ml hexane for SPE purification with a silica cartridge. SPE purification was performed according to (Piironen, Toivo, & Lampi, 2001), except for applying only 1 ml of the hexane extract on the cartridge using hexane and hexane–diethyl ether mixtures as solvents. The sterols were converted to their trimethylsilyl (TMS) ether derivatives prior to analysis by gas chromatography (Toivo, Phillips, Lampi, & Piironen, 2001). Each sample was analysed in triplicate. For oil samples, the sample size was smaller (0.25 g) and no SPE purification was needed.

2.5.2. GC analysis

Sterols were analysed as their TMS ethers by capillary gas chromatography with flame ionization detection. The equipment used was a Hewlett-Packard gas chromatograph 5890 Ser II with an autosampler 7673 in on-column mode and controlled by ChemStation software. The column was an RTX-5 fused silica capillary column (5% diphenyl-95% dimethyl polysiloxane; 60 m×0.32 mm i.d., 0.1 μm film with 10 m Integra-Guard column; Restek Corp.). The GC parameters were as described earlier (Piironen et al., 2001).

A gas chromatograph with mass a spectrometer (Varian 3400; FinniganMat Incos 50) was used to further identify the sterols (Mattila et al., 2002). For mass spectroscopy, ionization energy of 70 eV in the electron impact mode was used and spectra were scanned within the range of m/z 100–600.

Identification of sterols was based on relative retention times of commercially available sterols, comparison with literature data (Mattilaet al., 2002; Akihisha, Kokke, & Tamura, 1991; Kamal-Eldin, Appelqvist, Yosif, & Iskander, 1992) and mass spectral analyses. Quantitation was based on an internal standard method. Calibration curves were calculated for the three sterols at six levels (1–200 mg/l) with a constant dihydrocholesterol level (20 μg). Contents of other sterols were calculated from the sitosterol curve.

2.6. Analysis of secoisolariciresinol

2.6.1. Sample preparation

About 1 g of the milled sample was refluxed with 40 ml *n*-hexane for 1 h. The liquid was decanted and centrifuged for 15 min at 1200 rpm/min. The clean liquid was discarded and the whole solid residue was again treated as described above. Then the residue was dried under vacuum at 40 °C for 30 min yielding a fine and homogeneous, fat-free pumpkin seed powder.

2.6.2. Methanolysis

To 0.1 g of the above powder, 5 ml sodium methoxide solution (400 mg sodium in 100 ml methanol abs.) were added. The mixture was sonicated for 3.5 h in a closed 20 ml scintillation vial. During this process, hot water was removed from the ultrasonic bath after one hour and exchanged against icewater to avoid evaporation of methanol. The mixture was cooled to room temperature, solid carbon dioxide was added and the sample was dried under nitrogen to remove methanol.

2.6.3. Enzymatic hydrolysis

Five millilitres sodium acetate solution (0.1 M) were added to the dry sample and the pH was adjusted to 5 with a few drops of glacial acetic acid. Then, 100 mg cellulase (5.4 U/mg) were added and the mixture was shaken for 24 h at 37 °C. After that, the solution was sonicated for 10 min, centrifuged 15 min, at 5000 rpm/min, decanted and made up to 10 ml with mobile phase.

2.6.4. HPLC analysis

Twenty microlitres of the extract were injected in the HPLC system, consisting of a L-6200 gradient pump and a AS 2000A autosampler from Merck. The compounds were separated on a Spherisorb-ODS column (250×4.6 mm, 5μm) using 60 mM sodium acetate/ethanol/acetonitrile/glacial acetic acid (81/9/9/1, v/v/v/v) as mobile phase with a flow rate of 0.9 ml/min. For detection, a coulometric electrode array system (ESA, Chelmsford, MA, USA), adapted with eight electrodes, was used. The potentials of the electrodes were set from +220 to +340 mV in 20 mV increments from the first to the seventh electrode and +750 mV for the eighth electrode against modified palladium electrodes. For data processing, the "CoulArray for Windows" software from the ESA Company was applied. To identify secoisolariciresinol, the retention time and the current/voltage curve of a secoisolariciresinol standard are compared with those of the corresponding compound in the sample. The calibration curve was obtained by plotting the peak heights in the optimal channel (+ 280 mV for secoisolariciresinol) against the concentration of secoisolariciresinol (25–50 000 μg/l). The linear range and the detection limit for secoisolariciresinol were determined. The repeatability was tested by injecting a mixture of 500 μg/l secoisolariciresinol six times. The recovery was determined by analysing standard mixtures containing 20, 50 and 500 μg/l secoisolariciresinol. The detection limit (LOD) for the analyte was calculated from diluted standards by using a signal to noise ratio of S/N = 3 and the limit of quantification (LOQ) from diluted samples at S/N = 5.

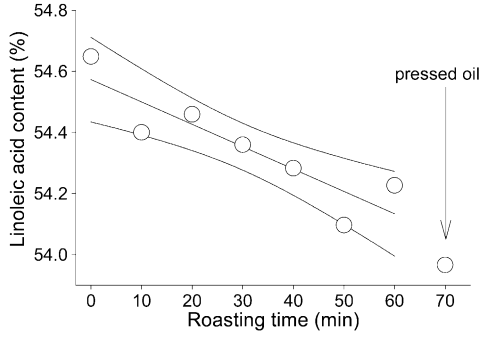

Fig. 2. Decrease of linoleic acid content in pumpkin seeds with 95% confidence interval.

3. Results and discussion

3.1. Fatty acids

The composition of the oil fraction of the pumpkin seeds before the roasting process was as follows: stearic acid 12.4%, palmitic acid 5.43%, oleic acid 27.6%, linoleic acid 54.6%. No change was found during the roasting process for three of the main fatty acids. The contents of palmitic acid, stearic acid, and oleic acid remained stable. Only linoleic acid decreased, from 54.6 to 54.2% (Fig. 2), a small but significant change. This small reduction led to oxidation products that were found by headspace GC (data not shown). During the time that was needed to transfer the roasted pumpkin seeds to the oil press, a further reduction of the linoleic acid to a final level of 54.0% occurred.

Since the temperature at the end of the roasting process was not very high (110–120 °C) the saturated and monounsaturated fatty acids were not expected to be oxidised.

3.2. Vitamin E

The analysis of vitamin E showed satisfying reproducibility with a coefficient of variation of 2.5% for α-tocopherol with a mean of 37.5 μg/g and a standard deviation 0.94 μg/g ($n = 5$). For γ-tocopherol, the coefficient of variation was 3.5%, with a mean of 383 μg/g and the standard deviation was 13 μg/g ($n = 5$). The content of vitamin E did not remain constant during the roasting process. The initial concentration of the vitamin E isomers was 37.5 μg/g for α-tocopherol, 15.9 μg/g for α-tocotrienol, 383 μg/g for γ-tocopherol and 128 μg/g for γ-tocotrienol. The changes of the observed concentrations of the four analysed vitamin E forms were similar. The initial value decreased, until about 40 min of heating and increased then again to about the initial value (Fig. 3). The underlying mechanism could have been twofold. At the beginning, the vitamin E concentration decreased due to oxidation reactions. After 40 min, when the oil emerges from the cellular structure, the measured vitamin E concentration increased,

because of an increased extraction efficiency. Looking at the ratios of α-tocopherol to α-tocotrienol and γ-tocopherol, to γ-tocotrienol which reduce the variation to a very small value, it can be seen that, with the γ-isomers, no change occurs but the α-isomers show a significant increase of the ratio. This means that the content of α-tocotrienol is reduced during the roasting process, indicating a lower stability of this substance.

3.3. Plant sterols

The determination limit was 10 μg/g and repeatability of the quantitation of the major sterols in triplicate analyses 5% when expressed as CV%. Repeatability of sterol analysis was monitored by analysing a rapeseed sample as an in-house reference standard, daily. The total sterol content of the rapeseed sample was 4640±210 μg/g (CV% = 4.5, $n = 14$).

In accordance with the previous studies (Piironen et al., 2002; Toivo et al., 2001), the plant sterols mainly occurred as various Δ7-sterols. Using these chromatographic conditions, they could not be separated as individual compounds but some of them coeluted, as shown by the GC-MS analysis. Furthermore, small amounts of sterols widely distributed in plants, i.e. sitosterol, campesterol and stigmasterol, as well as dimethyl sterols (2,4-dimethyl cycloartanol) were found. In Table 1, the total sterol contents and those of the main sterols are shown. Based on the results, no clear destruction of plant sterols was seen in the roasting process. The variation in the sterol contents of the seeds in the process (total sterol contents 1600–1930 μg/g FW) was probably caused by changes in the water content (early process stages) or extractability of plant sterols (later process stages). Before the addition of water, the total sterol content of the seeds was 2100 μg/g. At the beginning of the process, water was added and the first three measurements after that gave plant sterol contents of 1600–1710 μg/g, after which they started to increase toward the level measured in the seeds, reaching 1900 μg/g. The plant sterol content of the oil was 4030 μg/g which is in accordance of the results of Homberg and Bielefeld, (1989). The sterol profiles of the samples did not change during the process, indicating that there were no significant differences in the stability of the various sterols (Fig. 4).

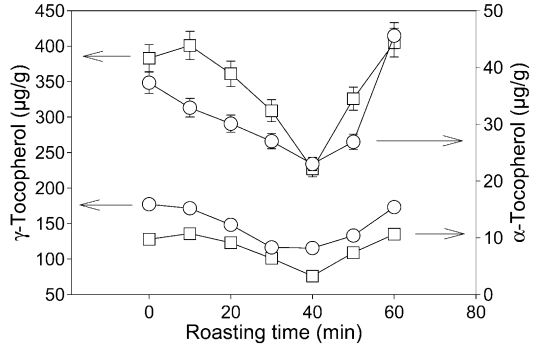

Fig. 3. Concentration profile of vitamin E during the roasting process (error bars = standard deviation).

Table 1
Summary of sterol and secoisolariciresionol (SECO) contents in pumpkin seeds and oil (given values are means of triplicate analyses with standard deviation)

Roasting time (min)	24-ethylcholest-7-enol, 24-ethylcholest-7, 22-dienol and coeluting sterols (μg/ g FW)	Total amount of sterols[a] (μg/g FW)	SECO (μg/g FW)
0	860±30	1710±80	3.8
10	810±100	1600±190	2.8
20	820±30	1620±80	n.d
30	980±30	1920±50	n.d.
40	940±30	1880±60	n.d.
56	980±30	1930±40	n.d.
oil	2060±10	4030±110	

n.d.: below limit of quantification.

[a] Includes e.g. Δ7-mono- and diunsaturated sterols with 29 C-atoms, dimethylsterols, campesterol and stigmasterol.

3.4. Secoisolariciresinol

Unroasted and roasted pumpkin seed samples were defatted with *n*-hexane, yielding a decrease of weight between 42.7 and 53.6% (w/w). To release secoisolariciresinol from its conjugates, the sample was hydrolysed in two steps with sodium methoxide and cellulase. The diluted extract was injected into the chromatographic system and the separation was performed isocratically on the reversed phase column. Figs. 5 and 6 show the electrode array chromatograms of an unroasted and a roasted pumpkin seed hydrolysate. Secoisolariciresinol was identified by retention time and its current voltage curve obtained by plotting the response of several working electrodes against their potentials.

Under the same chromatographic conditions, standard solutions in the range 25–50,000 μg/l secoisolar-

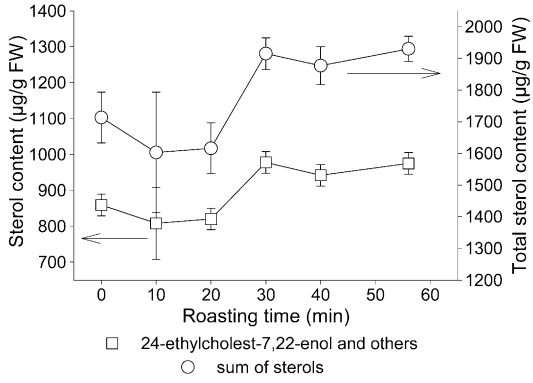

Fig. 4. Concentration profile of sterols during the roasting process (error bars = standard deviation).

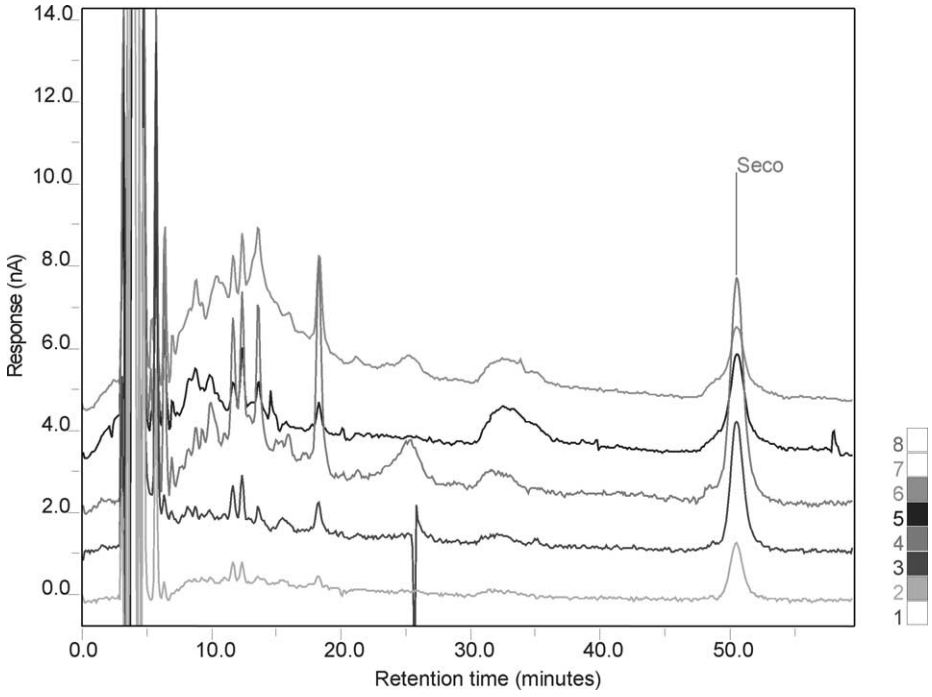

Fig. 5. Electrode array (channel 2–6) chromatograms of an unroasted pumpkin seed extract (sample number 0) after two step hydrolysis.

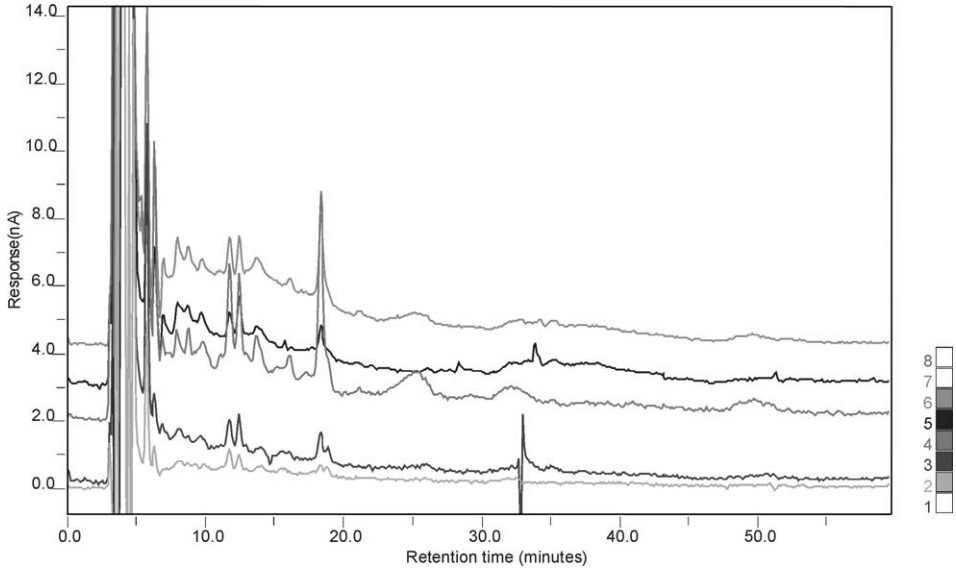

Fig. 6. Electrode array chromatograms (channel 2–6) of a roasted pumpkin seed extract (sample number 4) after two step hydrolysis.

iciresinol were injected. The response was linear ($R = 0.9998$) in the investigated range and its detection limit was found to be 20 μg/l (S/N = 3). The intraday repeatability for a standard mixture injected six times containing 500 μg/l secoisolariciresinol was 2.6%.

The recovery of the whole method, determined for standard solutions of different concentrations, was between 71.2 and 79.8% ($n = 4$).

Each unroasted and roasted sample was analysed three times and the results are summarized in Table 1. The limit of quantitation was found to be 2 μg secosisolariciresinol/g.

Neither in commercial nor in self prepared pumpkin seed extracts (ethanol/water = 1:1, v/v) was free secoisolariciresinol detected. Preliminary investigations showed that it was present in form of glycosides and small amounts were esterified (Kraushofer, 2002). Therefore, a two step hydrolysis method was necessary, first to cleave the ester bonds with sodium methoxide and then to hydrolyze the enzymatically-formed and the natu-

rally-occurring glycosides. Only in unroasted pumpkin seeds were small amounts secoisolariciresinol found after the two step hydrolysis. The results presented here for secoisolariciresinol (Table 1) were significantly lower than those published by Adlercreutz and Mazur (1997). This could be due to different sample preparation methods.

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