RESEARCH ARTICLE

Pumpkin Seed Oil

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Tocopherols and Polyphenols in Pumpkin Seed Oil are Moderately Affected by Industrially Relevant Roasting Conditions

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Traditionally, pumpkin seed oil is obtained by pressing the seeds after a roasting pretreatment, at temperatures up to 150 °C. However, the appropriate temperatures and roasting times are under discussion. In this study, oils from seeds roasted at different temperatures (60–150 °C) are compared with oil from non-roasted seeds. At higher roasting temperatures, lower roasting times are required to release the oil. Both, for tocopherols and phenolic compounds, no decreasing trend with the increasing roasting temperature are observed. In contrast, the oil from non-roasted seeds have relatively low levels of tocopherols and phenolics and lacked the typical aroma. Levels of polyaromatic hydrocarbons (PAHs) are very low, ranging from not detected in oil from non-roasted seeds to 13.8 μg kg^{-1} in the oil from seeds roasted at 150 °C. Therefore, the choice between the studied roasting conditions may depend rather on sensory evaluations than on the content of antioxidants or of PAHs.

***Practical Applications*: The process of the production of virgin pumpkin oil is based on a thermal treatment of the ground seeds, favoring the separation of the lipid fraction, and giving a typical aroma of the roasted oil. Results of this research provide important information regarding the influence of roasting conditions on the quality and safety of the oil. Roasting pumpkin seeds increased the tocopherol and phenols content in the pumpkin oil, with no significant formation of PAHs. The information will be valuable and important for not only for the pumpkin oil production, but also for all the seeds submitted to a roasting treatment before extraction.**

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1. Introduction

The roasting of the pumpkin seeds is on the one hand necessary to obtain the desired color, flavor, and taste characteristics.[1] On the other hand, it is expected that the heat treatment will adversely affect the content of poly-unsaturated fatty acids and of minor compounds. In general, among the fatty acids, the most abundant are palmitic (9.5–14.5%), stearic (3.1–7.4%), oleic (21.0–46.9%), and linoleic (35.6–60.8%).[2]

It has been reported that the roasting time and temperature (increase of paste temperature to 115 °C in 50 min) have important effects on the formation of volatile compounds in the seeds, which are responsible for the characteristic flavor of the oil.[3] In another related study, the compositional changes in the pumpkin seeds were monitored during the roasting process.[4] The content of linoleic acid decreased slightly but significantly (from 54.6 to 54.2%), as expected due to the temperature treatment. Tocopherol content in the seeds degraded initially but increased due to a better release and extraction after breaking the cell structures. Phytosterols seemed not to be affected. Finally, the lignan secoisolariciresinol disappeared from the seeds during the roasting.

In the above mentioned studies, the changes were monitored in the seeds during different stages of one roasting process (till 115 °C during 50 min), and only sterols and secoisolariciresinol were analyzed in the oil which was pressed from the seeds after the total roasting time.

Choosing a higher final roasting temperature, however, will decrease the time needed to extract the oil, and on the other hand, lower roasting temperatures will increase the required heating time. From the reports concerning the phytosterols in the seeds,[4] it is expected that the sterol content in the oils will not be much affected. However, more important differences may be expected in the content of less stable compounds such as tocopherols and phenolic alcohols and acids. It has been reported that roasting under different conditions increased the tocopherol content as well as the total phenolic compounds, leading to a higher oxidative stability.[5] Whereas tocopherols

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were identified individually, the phenolic alcohols and acids were determined as total phenolic acids using the Folin–Ciocalteu colorimetric method. No study to date describes the influence of roasting conditions on individual phenolic acids and alcohols.

In the study concerning the effect of roasting conditions on minor components in pumpkin seed oil,[5] pre-set combinations of temperature and time were used (90 vs. 110 vs. 130 °C and 30 vs. 60 min roasting). The advantage is that it allows comparing different roasting times for the same temperature. However, in practice, the roasting time is adapted in order to obtain a good oil release. In general, higher temperatures require a shorter roasting time. Further, the roasting step can induce the formation of polyaromatic hydrocarbons (PAHs). PAHs belong to a group of aromatic compounds composed from aromatic rings that contain only hydrocarbon and carbon. Many PAHs have genotoxic and carcinogenic effects, and some work as synergists.[6–7] Nevertheless, the effect of increasing roasting temperature on the PAHs formation has been exposed only recently.[8] The authors observed that at 150 °C light PAHs such as phenanthrene prevailed. However, at temperatures lower than 150 °C no PAHs were detected in the samples. Increasing roasting temperature also significantly changed the composition of the volatile compounds. Among the aldehydes, 3-methylbutanal prevailed and reached the highest concentration at 150 °C. It must be remarked that in this study the roasting time was kept constant at 60 min.

The objective of our study was to investigate the influence of roasting conditions, on the antioxidant content as well as the individual antioxidants in pumpkin seed oil, utilizing optimized temperature-time combinations for a good oil release. In addition, even though roasting temperatures did not exceed 150 °C, the levels of contaminants were determined to ensure that the levels were acceptable for human consumption.

2. Experimental Section

All reagents were HPLC grade or higher purity. Tocopherol standards were obtained from Calbiochem (San Diego, USA). Standards of the phenolic compounds (gallic acid [GA], caffeic acid [CA], protocatechuic acid, tyrosol, vanillic acid, vanillin, *p*-coumaric acid, *o*-coumaric acid, ferulic acid, sinapic acid, luteolin, and apigenin) were procured from ACROS (Geel, Belgium).

2.1. Pumpkin Seed Oil Samples From Differently Roasted Seeds

Oil samples were obtained from a Slovenian farm, where the oils were isolated from the seeds (variety Gleisdorfer Ölkürbis) according to following procedure. Oil processing based on the roasting process was performed in an oil-mill with a long tradition of pumpkin oil production. Cold pressing of oil was performed on an organic farm, where this kind of oil is also produced for commercial purposes. The seeds for samples were taken from one lot of seeds, which were produced mainly by integrated farmers from Prekmurje and Podravje regions (Slovenia).

For each roasting treatment, 40 kg milled seeds were used in a stone mill. Salt (NaCl, 200 g) and water (5.3 l) were added. The addition of water and NaCl has the main goal of facilitating the release of oil from the seeds, hence increasing the oil extraction efficiency. After milling and homogenization the 40 kg of seeds were heated in the pan to the temperature provided by the treatments. The time of the roasting process differs according to the possibility of separation of oils from the milled seeds, which is affected by treatment temperatures (**Table 1**). The oils were not refined, and no chemicals, except salt (NaCl) were added during or after the production.

All samples for this study were produced in the same farm and stored in dark bottles, under nitrogen, in the freezer until analysis. In order to avoid repeated frosting and defrosting of the samples, 50 ml portions of each of the oils were withdrawn and stored in smaller dark bottles, at 4 °C. After each opening for sampling, the nitrogen headspace was restored in the bottles.

2.2. Tocopherol Analysis

The tocopherols and tocotrienols were determined by normal phase HPLC following the AOCS Official Method Ce 8–89.[9] A normal phase Alltima Silica column (250 × 4.6 mm id, ps 5 μm) was used. Samples were filtered through a microfilter. Tocopherols were eluted in 20 min with n-hexane/isopropanol /99.5/0.5 v/v) at a flow rate of 1.5 ml min^{-1} and a column pressure of 50 bar. For detection, a fluorescence detector Hewlett Packard 1050 Series was used with the excitation wavelength at 290 nm and the emission wavelength at 330 nm.

2.3. Solid Phase Extraction of Phenolic Compounds

The method was based on the phenol extraction method developed for other vegetable oils,[10] and on the methods described by Siger et al.[19] and Andjelkovic.[11,20] Obtained extracts were analyzed for the individual (High Performance Liquid Chromatography – HPLC) and total content of phenolic compounds (Total Phenolic Compounds, TPC, Folin–Ciocalteu method).

Table 1. Roasting time and temperature of seeds for pumpkin seed oil production.

Treatment (roasting temperature –°C, combinations)	Required time for roasting (min)	No. of oil sample
60–70	100	PSO 11
90	75	PSO 6
95	70	PSO 1
100	65	PSO 12
105	65	PSO 2
110	60	PSO 10
115	60	PSO 9
120	55	PSO 3
125	55	PSO 8
130	50	PSO 4
150	45	PSO 7
90 and 130	30 and 30	PSO 5
0 – cold press (CP)		PSO 13
Oil mixtures: ½ cold press and ½ 150		PSO 14 (13 + 7)

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2.3.1. Sample Preparation

The oil was measured in quadruplet (2.5 g for each sample), where 0.5 ml methanol solution of internal standard (*o*-coumaric acid, concentration 0.01 mg ml^{-1}) was added to three repetitions of the oil sample. The solvent from each sample was evaporated in a rotary evaporator at 30 °C. Next, all repetitions were dissolved in 6 ml of hexane and the turbid samples (11, 13, and 14) were filtered over a paper filter.

The samples intended for total phenolic content analysis (Folin–Ciocalteu method) after the SPE separation were measured in triplicate without adding the internal standard.

2.3.2. Extraction Procedure

The samples (2.5 g of oil dissolved in 6 ml of hexane) were applied to a diol-bonded phase column (Grace, ps 50 µm, pore size 60 Å, 500 mg bed-size and 4 ml column-size) at a controlled flow-rate after conditioning those columns by consecutive passing of 6 ml of methanol and then 6 ml of hexane. Next, the sorbent in the columns was rinsed by the consecutive passing of twice 3 ml of hexane and then 4 ml of hexane/ethyl acetate solution (90:10, v:v).

Finally, the phenolic compounds were eluted from the sorbent with 5 ml of methanol. The eluents were collected in test tubes and combined in conical flasks, after which the solvent (methanol) was removed under reduced pressure in a rotary evaporator at 20 °C, with intermediate re-dissolving and mixing, until the dry phenolic compounds were concentrated on the bottom of the conical flask. The residue was re-dissolved in 0.6 ml (6 times 0.1 ml) of methanol for LC-MS analysis and 1 ml of methanol for the colorimetric determination.

2.4. Phenolic Compounds Identification and Quantification by HPLC

2.4.1. Separation of Phenolic Compounds by HPLC

Individual phenolic compounds were separated by reversed-phase high performance liquid chromatography (HPLC) and detected by both a diode array (DAD) detector and Mass Spectroscopy (MS) based on a chromatographic method developed for olive oil polyphenols.[10] The Agilent 1100 LC-MSD (Agilent software v A.09.03) chromatographic system equipped with a quaternary pump, C18 column (Phenomenex-Luna 4.6 × 250 mm, Ø 5 µm, 100 Å pore size), security guard column (Phenomenex C18), vacuum degasser, autosampler, 1100 6-port autoinjector valve, and quaternary pump were used. UV-detection was performed at wavelengths of 280 and 320 nm. The elution solvents used were A (0.2% acetic acid in LC-MS grade water), B (methanol), and C (acetonitrile) whereby B and C solvents were mixed in a 50:50 (v/v) ratio. Flow rate was 1 ml min^{-1} and run time 72 min. The column oven was set at 35 °C. The sample injection volume was 20 µl.

2.4.2. Identification of Phenolic Compounds by HPLC-UV-MS

Identification of compounds was (partly) achieved by comparing their retention time, UV-absorbance spectra, and m/z ratios to the retention times, UV- and m/z ratios of the available standards. The mass spectra were recorded by Agilent G1946D (SL) quadrupole mass spectrometer (Agilent Technologies) equipped with an electrospray ionization (ESI) system and controlled by Agilent Software v. A. 09.03. Nitrogen was used as nebulizing gas at a pressure of 50 psi and the flow was adjusted to 13 l min^{-1}. The heated capillary temperature and voltage were maintained at 350 °C and 3.5 kV, respectively. The full scan mass spectra of the phenolic compounds were measured from m/z 100 up to m/z 1000 and acquired in the negative ionization mode, at the fragmentary voltage of 70 eV.

2.4.3. Quantification of Phenolic Compounds by HPLC-UV

For each of the identified phenolic compounds the calibration curve (area = slope * concentration (µg ml^{-1}) + intercept) was prepared using increasing concentration of standards (protocatechuic acid, tyrosol, vanillic acid, vanillin, *p*-coumaric acid, ferulic acid, sinapic acid, luteolin, and apigenin). Molecular mass, retention time, and calibration curve properties are presented in **Table 2**.

Table 2. Molecular mass, retention time, and calibration curve properties of phenolic reference compounds.

Compound	Molecular weight	Retention time (min)	Calibration intercept	Curve slope	Correlation factor
Protocatechuic acid	154.12	8.38	−0.056	21.56	0.99
Tyrosol	138.17	9.97	0.006	12.20	0.99
Vanillic acid	168.15	11.82	−0.065	32.20	0.99
Vanillin	152.15	13.4	0.038	91.59	0.99
p-coumaric acid	164.16	14.49	−0.094	94.03	0.99
Ferulic acid	194.18	15.42	0.002	36.28	1.00
Sinapic acid	224.21	15.71	0.024	13.29	0.99
Luteolin	286.24	28.17	0.006	21.07	0.99
Apigenin	270.23	32.5	−0.010	39.86	1.00

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2.5. Phenolic Compounds Quantification by Folin-Ciocalteu Method

2.5.1. Calibration Curve

To prepare the calibration curves, a series of solutions of the standards GA and CA with increasing concentration (50; 100; 150; 200; 250; 400 $\mu g\,ml^{-1}$) was prepared. The standards were diluted with a solvent mixture methanol/water (1:1, v/v).

2.5.2. Sample Analysis

One mililiter of methanol phenolic extract was added to a 100 ml volumetric flask containing 65 ml of deionized water. Next, 5 ml of Folin–Ciocalteu reagent (10 times diluted) was added and mixed. After 5 min, 15 ml of 20% sodium carbonate solution was added and then the volume in the flask was adjusted with deionized water. The solution was left for 2 h covered and protected from light. Each sample was prepared and analyzed in triplicate. After this time the absorbance of each solution was measured by UV-Visible Spectrophotometer Cary 50 at 760 nm against the blank (water).

2.5.3. Quantification of Phenolic Compounds by Folin–Ciocalteu

Quantification of the total content of phenolic compounds was based on the calibration curves. The relation between the standards concentration and the absorbance was plotted resulting in linear equations with y = area and x = concentration ($\mu g\,ml^{-1}$) (GA: $Y = 0.001x - 0.0140$ ($R^2 = 0.9996$); CA: $Y = 0.011x + 0.0055$ ($R^2 = 0.9994$).

2.6. Determination of PAHs in Pumpkin Seed Oils

The levels of PAHs in the pumpkin seed oil samples were determined in a specialized laboratory (Faculté de Medicine Veterinaire, Dpt. Sciences denrées alimentaires, Université de Liège, Belgium). One gram of oil was extracted and purified,[12] and analyzed using HPLC-FLD according to published methods.[13–14]

2.7. Statistical Evaluation

Statistical analysis was performed using SAS version 9.2 for Windows. Statistical differences between the oil samples were estimated by applying one-way ANOVA and using the Duncan's test at a significance level of 5% ($p < 0.05$). All analyses were carried out in triplicate. Standard deviations were calculated using Microsoft Excel 2007 software. The One-Way Anova is used to evaluate if the means are significantly different from one another or if they are relatively the same.

3. Results and Discussion

3.1. Influence of Processing on Tocopherols Content

3.1.1. Total Tocopherol Content

The tocopherol composition of the oils obtained from differently processed seeds is represented in **Table 3**. By comparing the data in Table 3 it can be seen that there are significant differences ($p < 0.05$) between the cold pressed oil and the oils obtained from the roasted seeds, both in respect to the content and composition of tocopherols. The total tocopherol content varied between 584 and 687 $mg\,kg^{-1}$ (samples 12 and 1, respectively), which are intermediate values compared to those reported for Styrian

Table 3. Tocopherol content of pumpkin seed oils after with different seed roasting conditions. Values are means of three repetitions.

	Roasting		α-tocopherol		γ-tocopherol		α-T + γ-T
Sample	T (°C) or combination	Time (min)	mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}
PSO 11	60–70	100	45.8 ± 2.3bdc	7.3	580.4 ± 10.4fbecd	92.7	626.2 ± 12.7bcd
PSO 6	90	75	37.7 ± 0.9de	6.3	558.4 ± 3.2fed	93.7	596.2 ± 4.1cd
PSO 1	95	70	55.4 ± 8.7a	8.1	631.6 ± 34.2a	91.9	687.0 ± 42.9a
PSO 12	100	65	32.6 ± 6.2fe	5.6	551.9 ± 27.6fe	94.4	584.5 ± 33.8d
PSO 2	105	65	48.1 ± 8.6bac	7.8	571.3 ± 33.2fbecd	92.2	619.4 ± 41.8bcd
PSO 10	110	60	27.8 ± 1.7f	4.5	588.4 ± 0.9bcd	95.5	616.2 ± 2.6bcd
PSO 9	115	60	51.5 ± 6.4ba	7.9	603.9 ± 7.4ba	92.1	655.4 ± 13.8ba
PSO 3	120	55	46.4 ± 0.7bdac	7.4	582.9 ± 8.3becd	92.6	629.2 ± 9.0bc
PSO 8	125	55	37.2 ± 2.7de	6.4	547.2 ± 20.0f	93.6	584.4 ± 22.7d
PSO 4	130	50	41.9 ± 7.8dec	6.7	582.2 ± 7.7becd	93.3	624.2 ± 15.5bcd
PSO 7	150	45	39.6 ± 0.9dec	6.6	560.7 ± 2.0fed	93.4	600.3 ± 2.9cd
PSO 5	90 + 130	60	37.9 ± 1.0de	6.3	567.2 ± 15.8fecd	93.7	605.1 ± 16.8cd
PSO 13	CP		32.9 ± 4.5fe	5.6	553.5 ± 14.7fed	94.4	586.4 ± 19.2cd
PSO 14 (13 + 7)	CP + 150	45	52.4 ± 5.5ba	8.1	597.5 ± 18.6bc	91.9	649.9 ± 24.1ba

Different letters within columns indicate significant differences ($p < 0.05$) among the samples.

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pumpkin seed oils[15] (up to 282 mg kg^{-1} α-tocopherol and 800 mg/kg γ-tocopherol) and higher than reported for Serbian cultivars [5] (269.79–350.98 mg kg^{-1} total tocopherols). It is known that tocopherol content in vegetable oils is dependent on variety, geographical origin, and processing conditions.[16]

3.1.2. Tocopherol Composition

In all samples γ-tocopherol (547–632 mg kg^{-1}) was most abundant (approx. 92–95%). In normal phase HPLC, the β- and γ-tocopherol peaks are well separated, if both are present. However, β-tocopherol was detected in none of the samples, as confirmed by comparing with a 4-tocopherol standard mix.[17] The peak of α-tocopherol co-eluted in many cases with an unknown compound, which could not be quantified separately (approximate concentration 5–8% of the peak). The shoulder peak could be attributed to the presence of α-tocomonoenol, which was previously identified in crude palm oil samples.[18] In the Table 2, the quantification is done for the total peak, with shoulder (27–55 mg kg^{-1}). Finally, δ-tocopherol was detected as well but only in trace amounts. This pumpkin seed oil was obtained from the variety Gleisdorfer Ölkürbis in Slovenia. In the oil from the Olinka pumpkin seed variety (Serbia), lower values were reported of (β + γ)-tocopherol (197–267 mg kg^{-1}), together with 48–77 mg kg^{-1} α-tocopherol and minor but quantifiable amounts of δ-tocopherol (18–21 mg kg^{-1}).[5]

3.1.3. Influence of Roasting Conditions on γ-Tocopherol Content

The quantification of γ-tocopherol was most reliable because of its lower relative standard deviation and because it did not suffer from co-elution. Therefore, the influence of processing is interpreted only for this tocopherol. By comparing all the treatments, mathematically significant differences ($p < 0.05$) can be seen in the content of γ-tocopherol. It has been reported that during a roasting process the tocopherols content first decreases but afterwards increases again, probably due to breaking the cell structures.[4] In the present study, focusing on the industrially relevant conditions, necessary time for the release of oil was decreased with increasing roasting temperature, which is most probably the reason for the similar effects of all treatments on the tocopherol content. Further, other components which were not assessed, may have varied between the treatments, such as phospholipids, carotenoids, or oxidation products, which can have an influence on the oxidative stability of the oil, and cause small variation in the final content of tocopherols.

3.1.4. Extraction Method for Phenolic Compounds From Pumpkin Seed Oil

The extraction protocol was based on the phenol extraction method developed for other vegetable oils,[10] and on the method described by Siger et al.[19] and Andjelkovic.[20] This original SPE method did not give satisfactory results with pumpkin seed oil, yielding turbid final solutions of phenolic compounds, a low yield, and poor detection of phenolic compounds during the HPLC analysis of the extract. By adapting the conditions for solid phase extraction to the pumpkin seed oil matrix it was possible to obtain clear samples and, at the same time, to avoid losses of phenols, and obtain a good detection in HPLC.

3.2. Identification of Phenolic Compounds in Pumpkin Seed Oil by HPLC-MS

Reversed-phase high performance liquid chromatography (with UV and MS) was used for the separation and quantification of individual phenolic compounds. The identification was done on the basis of retention times, molecular masses, and UV-spectra (Table 3). Eight peaks could be identified based on the UV absorbance, retention time, and mass spectrum obtained with the LC-MS system (**Table 4**).

Regarding their molecular masses, two peaks at 57.47 and 60.42 min were most probably flavonols, but their exact structure could not be defined and they were therefore not included in the quantification based on HPLC results. Unidentified peaks were not taken under consideration in the following quantification based on HPLC results.

However, in this study the major goal was not the full profiling but to assess the influence of the roasting conditions on the overall antioxidants content (tocopherols, phenolic compounds). Total phenolic contents from HPLC quantification will be compared with the TPC from the spectrophotometric method.

3.3. Quantification of Phenolic Compounds in Pumpkin Seed Oil by HPLC

In **Table 5**, the individual and total phenolic contents of the pumpkin seed oils are presented. Phenolic profiles were collected by HPLC at 280 and 320 nm. Identification was done by combining recording UV and mass spectra with standard compounds and literature data. All samples of pumpkin seed oil contained tyrosol, *p*-coumaric acid, and ferulic acid. Most of the samples contained vanillic acid whereas vanillin was present in three samples. Apigenin was identified in four samples and protocatechuic acid in only two samples, at high concentration but with very high standard deviation. The treatment of PSO 2

Table 4. Phenolic compounds in pumpkin seed oils (PSO 3 sample).

Retention time (min)	UV max	MS (M^-)	Identification
8.38	287	153	Protocatechuic acid
9.93	290	137	Tyrosol
11.67	278	167	Vanillic acid
13.39	278	151	Vanillin
14.47	310	163	*p*-coumaric acid
15.41	325	193	Ferulic acid
32.07	287	269	Apigenin

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Table 5. Identified and quantified phenolic compounds in pumpkin seed oils (μg of phenol g^{-1} oil). Values are means of three repetitions.

	Roasting T (°C)	Roasting time (min)	Proto-catechuic acid	Tyrosol	Vanillic acid	Vanillin	*p*-coumaric acid	Ferulic acid	Apigenin	Total
PSO 11	60–70	100		2.4 ± 0.5d	0.9 ± 0.1cb		0.1 ± 0.0f	0.3 ± 0.0dc		3.7 ± 0.5dfe
PSO 6	90	75		2.3 ± 0.4 cd		0.1 ± 0.0a	0.2 ± 0.0de	0.5 ± 0.1c		3.2 ± 0.4fe
PSO 1	95	70		0.6 ± 0.3e			0.2 ± 0.0fe	0.1 ± 0.0e		0.8 ± 0.3g
PSO 12	100	65		2.3 ± 0.7d	0.1 ± 0.0d		0.2 ± 0.0fe	0.1 ± 0.0f		2.7 ± 0.7f
PSO 2	105	65	6.0 ± 2.6a	3.9 ± 1.4b	0.8 ± 0.6cb		0.5 ± 0.0ba	0.4 ± 0.2c	0.2 ± 0.0a	11.8 ± 3.0a
PSO 10	110	60		3.7 ± 0.1cb	0.7 ± 0.2cb		0.2 ± 0.0de	0.1 ± 0.0e		4.8 ± 0.3dc
PSO 9	115	60		3.7 ± 0.5cb	2.2 ± 0.0a		0.3 ± 0.0dc	0.2 ± 0.0de		6.3 ± 0.5bc
PSO 3	120	55	6.2 ± 3.6a	4.7 ± 1.3b	0.5 ± 0.2cb	0.5 ± 0.3a	0.4 ± 0.0bc	0.4 ± 0.2c	0.2 ± 0.1a	12.9 ± 3.9a
PSO 8	125	55		4.7 ± 1.1b			0.3 ± 0.0dc	0.3 ± 0.0dc		5.3 ± 1.1dc
PSO 4	130	50		6.3 ± 0.8a	1.1 ± 0.5b		0.5 ± 0.1a	1.8 ± 0.2a	0.2 ± 0.0a	10.0 ± 1.0ba
PSO 7	150	45		7.3 ± 0.7a	0.5 ± 0.0c		0.6 ± 0.1a	0.9 ± 0.1b	0.2 ± 0.0a	9.4 ± 0.7ba
PSO 5	90 + 130	60		3.4 ± 0.2cbd		0.3 ± 0.2a	0.4 ± 0.1dc	0.5 ± 0.0c		4.6 ± 0.6dce

Different letters within columns indicate significant differences ($p < 0.05$) among the samples.

and 3 did not differ so much from the others to explain the unique presence of protocatechuic acid or absence of the interference. It seems more probable that the selected extraction method has a good repeatability for most identified phenolic compounds but not for protocatechuic acid (and possible interference). Moreover, for those two samples PSO 2 and 3, high standard deviations were observed as well for the other phenolic compounds. All bottles were placed 14 h in advance at room temperature in the dark and thoroughly shaken before sampling, but the variability in those samples was still higher.

No phenols were detected in cold-pressed pumpkin seed oil (PSO 13), neither in the mixture of this oil with PSO 7 (= PSO 14). It is unlikely that the cold pressed sample did not contain any polyphenols but the failure to detect them can be caused by the necessity to filter the hexane solution of the heavily turbid oils before the application on the SPE columns and HPLC analysis.

PSO 5, heated during 30 min at 90 °C and 30 min at 130 °C, has an intermediate content of tyrosol, *p*-coumaric acid, and ferulic acid, compared to samples 6 and 4, which have been treated at 90 and 130 °C, respectively. However, for the other phenolic compounds, this relationship was not confirmed. The samples were obtained by roasting at 90 °C (30 min) followed by 130 °C (30 min) due to two main reasons: (1) the two roasting temperatures are generally used in the industrial reality, in order to facilitate oil release and improve taste and (2) the mixture of roasted and cold pressed oil allows an intermediate taste profile.

At first sight surprisingly, no decreasing trend can be observed with increasing roasting temperature. Taking into account all identified phenolic compounds; the highest total phenol content quantified by HPLC was found for sample PSO 3, heated at 120 °C, and sample PSO 2, heated at 105 °C. Based on the sum all compounds except protocatechuic acid, the highest phenolic content was seen for the sample heated at 130 °C during 50 min (PSO 4), followed by the samples heated at 150 °C (PSO 7), 120 °C (PSO 3), 115 °C (PSO 9), and 105 °C (PSO 2). In addition, for the samples with different roasting temperature but the same roasting time, the levels of phenolic compounds seemed to increase with roasting temperature, which is observed by comparing sample PSO 12 with PSO 2, and PSO 10 with PSO 9. This could be linked to a better extraction of the polyphenols at higher temperatures. However, this evaluation is not valid for PSO 3 compared to PSO 8. Thus, it was not possible to correlate all differences in polyphenol profile to the roasting temperature/time combinations. Therefore, other factors must have caused part of the differences. It is known that the polyphenols are hydrophilic compounds, possibly they did undergo an incomplete transfer from the seeds during the different processes. Further, other components may have influenced the final polyphenol content as in the case of tocopherols.

Overall, there is much more variation in the identified and quantified phenolic compounds than in the tocopherols content. While a clear trend cannot be seen, it seems that the higher temperature combined with shorter roasting time (samples PSO 4, 7) favors the extraction of tyrosol, *p*-coumaric acid, and ferulic acid, three compounds which were clearly more abundant in PSO 4 and 7 than in the other samples.

3.4. Quantification of Total Phenolic Content by Folin–Ciocalteu Method

The spectrophotometric method using the Folin-Ciocalteu reagent is widely used for the determination of the total phenol content.[10] The phenols were isolated by solid phase extraction as described above.

Total phenolic concentrations (TPC) measured in 14 pumpkin seed oil samples ranged from 72.8 to 326.6 μg of

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GA equivalents and from 70.5 to 301.2 μg of CA equivalents per g of oil (**Table 6**).

Sample PSO 10 had the highest TPC value, whereas the lowest level of phenolic compounds by spectrometry was found in sample PSO 13, the cold pressed pumpkin seed oil. In contrast to the HPLC method, where no peaks were detected at all for the cold pressed oil, the Folin–Ciocalteu method permitted to calculate a concentration of phenolic compounds, although for both methods the cold pressed samples had been filtrated before SPE analysis. Either the filtration selectively removed all (phenolic and possibly other) compounds detected in HPLC, and/or, more probably, the result is overestimated in the Folin–Ciocalteu method.

No correlation between extraction temperature and TPC could be observed. Furthermore, the sum of quantified phenolic compounds as determined by HPLC did not correlate with the colorimetrically determined total phenol content. This was not unexpected, because in HPLC only the identified simple phenolic compounds were quantified, which is an underestimation, not calculating possible phenols bound to sugars while the colorimetric method can suffer of overestimation as it measures all compounds which absorb at the wavelength of determination. The quantification via Folin–Ciocalteu was higher compared to the HPLC results. Total Phenolic Compounds were compared with the levels found by other researchers. In literature, widely different values are reported for the spectrophotometric determined TPC of pumpkin seed oil: 25–51 mg GAE kg^{-1} for pumpkin seed oil from different origins [11,19]; and up to 20 mg kg^{-1} in roasted versus only 4 mg kg^{-1} in cold pressed pumpkin seed oil.[5] On the other hand, Total Phenolic Compounds quantified by another research team[21] were considerably higher (980 μg GAE g^{-1}) than in the current study. Natural variations in phenolic contents occur without doubt, but an important problem in comparing these results, may be the different methodologies used by the authors for the phenol extraction and analytical method, due to the lack of a standardized method.

3.5. Influence of Processing on the Content of Contaminants (PAHs)

From the results presented above, an increasing roasting temperature of the pumpkin seeds did not have detrimental effects on the antioxidant contents in the oil. However, it is known that the roasting process can induce the presence of contaminants such as PAHs in the oil. The roasting process may give rise to levels of PAHs above the acceptable limits, as has been reported before.[15] In a set of Styrian (Austrian) pumpkin seed oils that were roasted under unfavorable conditions (too high temperature), PAH concentrations of up to 120 μg kg^{-1} were reported for the light fraction, and up to 3 μg kg^{-1} for the heavy-fraction PAHs. Optimization of the roasting conditions has led to a decrease of the heavy-fraction PAH to under the limits of detection and a decrease of the remaining light-fraction PAHs to below 40 μg kg^{-1}, which is a level frequently found in edible oil.[15]

However, the European Commission has set lower limits.[22] A limit of 2 μg kg^{-1} applies for benzo(a)pyrene in oils and fats for direct human consumption. Further, a maximum limit was set to 10 μg kg^{-1} for the sum of four marker compounds, being benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene, and chrysene. Therefore, roasting conditions should not only be based on sensory properties and differences in composition, but the levels of PAHs in the oil should be monitored as well.

Table 6. Total phenolic content (TPC) in pumpkin seed oils, as μg gallic acid equivalents (gae) g^{-1} oil and μg caffeic acid equivalents g^{-1} oil. Values are means of three repetitions.

	Roasting T (°C)	Roasting time (min)	GAE (μg g^{-1})	CAE (μg g^{-1})
PSO 11	60–70	100	83.2 ± 4.3dfe	77.2 ± 3.9dfe
PSO 6	90	75	156.3 ± 8.7b	143.6 ± 7.9b
PSO 1	95	70	89.1 ± 2.2dc	82.5 ± 2.0dc
PSO 12	100	65	80.9 ± 4.2gfe	75.0 ± 3.8gfe
PSO 2	105	65	76.3 ± 2.9hg	70.9 ± 2.6hg
PSO 10	110	60	326.6 ± 7.8a	298.4 ± 7.1a
PSO 9	115	60	272.9 ± 10.3ba	249.6 ± 9.4ba
PSO 3	120	55	84.7 ± 5.3dfe	78.5 ± 4.8dfe
PSO 8	125	55	130.5 ± 17.8bc	120.1 ± 16.1bc
PSO 4	130	50	79.4 ± 3.1hgf	73.7 ± 2.8hgf
PSO 7	150	45	136.3 ± 5.9bc	125.4 ± 5.3bc
PSO 5	90 + 130	60	88.6 ± 2.4dce	82.1 ± 2.2dce
PSO 13	CP	–	72.8 ± 8.9h	67.7 ± 8.1h
PSO 14	CP + 150		81.3 ± 4.5dgfe	75.4 ± 4.1dgfe

Different letters within columns indicate significant differences ($p < 0.05$) among the samples. CP = cold pressed (cfr Table 1).

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The samples from Slovenia, used in the current study, were produced under varying roasting conditions, and therefore the levels of PAHs were important quality parameters. Samples were analyzed in a specialized laboratory for the analysis of PAHs levels (Faculté de Medicine Veterinaire, Dpt. Sciences denrées alimentaires, Université de Liège, Belgium). In all samples, the total PAH content was far below the 40 μg kg^{-1} found by other researchers, ranging from not detectable/not quantifiable for all tested compounds in the nonroasted sample PSO 13, to 13.8 μg kg^{-1} for the sample roasted at 150 °C, nr. PSO 7. This PSO 7 was the only sample where benzo(a)pyrene was detected, to a level of 1 μg kg^{-1}, below the limit of 2 μg kg^{-1} set by the European Commission. Further, the sum of the four marker compounds was always below 10 μg kg^{-1}, with a max of 8,1 μg kg^{-1} for the PSO 7 sample roasted at 150 °C. However, as was the case for the tocopherols and phenolic compounds, a real correlation of PAH concentration with roasting temperature could not be observed, probably linked with the decreasing roasting time for the range of temperatures studied (90–150 °C). Overall, all samples contained very low levels of PAHs, which can be attributed to the continuous search for optimized roasting conditions. These findings are confirmed by Potocnik and Kosir,[8] who observed that up to 150 °C no PAHs were detected in the samples, and only after roasting the pumpkin seeds for 1 h at 150 °C some formation occurred, all at very low concentrations.

3.6. Conclusions

From the analyses conducted in this study, it appeared that the tocopherol content varied very little between the different temperature-time combinations, while larger variations were observed for the phenolic content. However, the expected decrease with higher temperatures was not seen. The less specific Folin–Ciocalteu method showed large and apparently uncorrelated variations in TPC, while the specific HPLC analysis suggested that certain individual phenolic compounds were present in higher levels after a shorter treatment at higher temperatures. Further, the roasting did not lead to high levels of PAHs, which were all well below the levels usually reported for vegetable oils.

These observations suggest that it is not useful to omit the roasting step for the sake of the preservation of antioxidants, and that the right roasting conditions do not lead to excessive formation of PAHs. In contrast, roasting is necessary to obtain the characteristic oil appearance and flavor. Within the tested conditions, the choice of roasting temperatures and time might depend therefore rather on sensory characteristics of the produced pumpkin seed oil.

Abbreviations

CA(E), caffeic acid (equivalents); FLD, fluorescence detector; GA (E), gallic acid (equivalents); HPLC, high performance liquid chromatography; MSD, mass spectrometric detector; PAHs, polyaromatic hydrocarbons; PSO, pumpkin seed oil; SPE, solid phase extraction; TPC, total phenolic compounds.

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Conflicts of Interest

The authors declare no conflict of interest.

Keywords

pumpkin seed oil, roasting, tocopherols, phenolic compounds, HPLC-MS

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