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PHYSICOCHEMICAL CHARACTERISTICS, PESTICIDE RESIDUE AND AFLATOXIN CONTAMINATION OF COLD PRESSED PUMPKIN SEED (*Cucurbita pepo L.*) OILS FROM CENTRAL ANATOLIA REGION OF TURKEY

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ABSTRACT

In this study, physicochemical characteristics, pesticide residues and aflatoxin contaminations of cold pressed pumpkin seed (*Cucurbita pepo L.*) oils cultivated in four different central Anatolia regions of Turkey, were investigated. Lab-scale screw press machine was used to produce cold pressed pumpkin seed oils and the oil contents were found between 42.8%−47.4% for naked seeds. The physicochemical characteristic (*refractive index, viscosity, color value, triglyceride profile analysis, peroxide value, iodine value, free fatty acid, saponification number, unsaponified matter, specific extinction values at 232 and 270 nm*) of cold pressed oils were determined by using different analytical techniques. The results showed that there was a non-significant difference between cold pressed pumpkin seed oils from different regions, in terms of physicochemical characteristics. The contents of pesticide residue and aflatoxin B_1, B_2, G_1 and G_2 contamination were determined by using validated UHPLC-MS/MS method. The chlorpyrifos pesticide residue was detected under the limit value declared by official authorities for the quality assessment of edible oils. Aflatoxins weren't detected in any of studied pumpkin seed oils. Therefore, the study provides important data for the cold pressed pumpkinseed oils, and proves that screw-pressed pumpkinseed oils are high quality, nutritious and valuable oils with higher levels of consumer acceptability.

Keywords: *Cucurbita pepo L.*, Cold pressed oil, Physicochemical characteristic, UHPLC-MS/MS

1. INTRODUCTION

Pumpkins (*Cucurbita spp*.) are familiar plants widely cultivated and used as foodstuff since ancient times. A wide variety of *Cucurbita* species are cultivated in mild and subtropical regions, with the studied species being *C. pepo*, *C. moschata*, *C. maxima*, and *C. mixta*[1]. Among these species, *Cucurbita pepo L.* is an economically important member of *Cucurbitaceae* familyand among the ten leading vegetable crops worldwide with an annual product growing in temperate and subtropical regions [2]. For many years, the *Cucurbita pepo L.* seeds have been commonly used in the food industry, are utilized in the pharmaceutical and alternative medicine applications but also in the production of cold pressed pumpkin seed oil (PSO) [3, 4].

Cold pressed PSO is mostly obtained by pressing of untreated-dried, naked (hull-less) pumpkin seeds, with continuous mechanical screw-presses and outlet oil temperature below 50°C [5]. During screw-presses, neither the untreated oily seed nor the outlet oil is exposed to higher temperatures and chemical application consistent with the Codex Alimentarius and legislations for cold pressed oils [6, 7]. The screw-processing technology of cold pressed PSO is performed by the following applications: harvesting, washing and drying to residual moisture content, and then storing of the pumpkin seeds. Before the screw-processing, the seeds pass throughout the magnetic cleaner, followed by elimination of organic impurities. The clean dried oily seeds are continuously reinforced to the screw-press and squeezed oil is then collected in dark-brown glass bottles. Undesired turbid matters in oil are removed by sedimentation or filtration, and then filtered oil is filled into dark glass bottles. Due to this traditional screw-process, the cold pressed PSOs maintain their natural composition with regard to the bioactive

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constituents and flavour. The main argument for the advantages of these oils is that during oil extraction, bioactive compounds present in the seed do not deteriorate due to the low processing temperatures. Thus, the consumption of cold pressed pumpkin seed oils is currently increasing [5].

Cold pressed PSO is an extraordinarily rich source of various bioactive compounds having functional properties used as edible oil or a potential nutraceutical. PSO belongs to the group of oils of high nutritive value due to its favourable bioactive compounds which have certain positive effects on the human health. Several health benefits from its regular dietary intake have been also reported, including cancer prevention, anti-inflammation, anti-diabetic and lowering of cholesterolemia [4, 8–10]. Besides the nutritive-pharmacological properties, there are several studies on PSOs that are characterized by their specific physicochemical properties which are considerably different from other kind of oils [5, 11]. Rabrenovic et al. (2014) evaluated the most important bioactive compounds of cold pressed oils from different pumpkin (*Cucurbita pepoL.*) seeds, and reported that the total tocopherol content ranged from 38.03±0.25 to 64.11±0.07 mg/100 g of oil and phyterol content ranged from 718.1±6.1 to 897.8±6.8 mg/100 g of oil [5]. In addition to tocopherols, pumpkin seed oil (*Cucurbita pepoL.*) has a simple fatty acid profile with the dominant fatty acid being the nutritionally linoleic acid (35.6–60.8 %), as well as palmitic (9.5–14.5%), stearic (3.1–7.4%) and oleic acids (21.0–46.9%) [12, 13]. Andjelkovic et al. (2010) also reported that the total polyphenol content in PSOs ranged from 25 to 51 mg/kg as gallic acid equivalents (GAEs) [12].

The researchers also reported that the content and amount of these bioactive compounds are differing among the varieties, dependent on climate and cultivation conditions. So, investigation of the bioactive contents of cold pressed oils from different varieties is very important to define the quality and shelf life of products. These quality parameters are also requested for labelling by the market [13]. The determination of the stability and physicochemical characteristics is a very sensitive issue; hence, studies on the cold pressed PSOs from different regions highlighted its richness in bioactive compounds and its physicochemical characteristics in respect to the edible oil quality. To our best knowledge, no data is available on the quality of pumpkin seed (*Cucurbita pepo L.*) oils cultivated in central Anatolia regions in detail and no investigation has been focused on the contamination of pesticide and aflatoxin in PSO by UHPLC-MS/MS technique. Therefore, this study was aimed (*i*) to determine the basic physicochemical characteristic (*refractive index, viscosity, color value, triglyceride profile analysis, peroxide value, iodine value, free fatty acid, saponification number, unsaponified matter, specific extinction values at 232 nm and 270 nm*) by using different analytical techniques and (*ii*) to determine the contents of pesticide and aflatoxin contamination by using validated UHPLC-MS/MS techniques, for cold pressed pumpkin (*Cucurbita pepo L.*) oils cultivated in four different central Anatolia regions of Turkey. The findings of present study are expected to increase the knowledge on the characteristics of these valuable edible oils and provide as valuable contributions to better assess their potential as a source of functional edible oils in the industry.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Reagents

All chemicals, reagents and solvents used were of analytical and HPLC grade and obtained from VWR International (Poole, UK), Sigma-Aldrich (St. Louis, USA) and Merck (Darmstadt, Germany). Deionised water (>18 MΩ .cm) was obtained from a Milli-Q system (Millipore, Brussels, Belgium) in our laboratory. Dispersive-solid phase extraction (SPE) sorbents for cleanup process included primary secondary amine (PSA) were obtained from Varian (Harbor City, CA, USA). The AflaTestP® immuno-affinity SPE cartridge columns were obtained from Sigma-Aldrich (St. Louis, USA). The blank pumpkinseed oil was obtained from Sigma-Aldrich (St. Louis, USA) certified organic (NOP)

Higher purity pesticide standards (95% or higher purity) were obtained from Wako (Osaka, Japan), Chemservice (West Chester, PA, USA), Sigma-Aldrich (St. Louis, USA) and Dr. Ehrenstorfer (Augsburg, Germany). Pesticide stock solutions were prepared in methanol at a concentration of 1000−2500 $\mu g.kg^{-1}$ (ppb). The individual stock solutions were diluted with methanol to prepare mixed stock solutions for UHPLC-MS/MS analysis containing: carbaryl, chlorpyrifos, dimethoate, diuron, malathion, methidathion, omethoate, phosmet and simazine at a concentration of 1−50 $\mu g.kg^{-1}$, 10−250 $\mu g.kg^{-1}$ and 10−500 $\mu g.kg^{-1}$. Additional pesticide solutions of relative concentrations for UHPLC-MS/MS were diluted appropriately from these stock solutions and all solutions were stored at -20°C before analyses. Identification of pesticides were based on the criteria specified in commission decision 2002/657/EC [14];(*i*) the t_R of the analytes in the extract should correspond to that of the calibration standard (*ii*) the occurrence of three identification points, precursor ion and two fragments (*iii*) the relative ion intensities had to match with the permitted tolerances. Quantification of pesticides was based on peak areas. The aflatoxin mix analytical standard, in methanol (AF-B_1, AF-B_2, AF-$G_1$and AF-G_2) was purchased from Sigma-Aldrich (St. Louis, USA). Pure triglycerides as standards including LLnLn, LLLn, LLL, OLLn, PLLn, OLL, PLL, LnPO, OOL, POL, StLL, PPL, OOO, StOL and PLSt were purchased from Sigma-Aldrich (St. Louis, USA) [triglyceride abbreviations are used in this study: P, palmitic acid (C16:0); St, stearic acid (C18:0); O, oleic acid (C18:1, Δ^9); L, linoleic acid (C18:2, $\Delta^{9, 12}$) and Ln, linolenic acid (C18:3, $\Delta^{9, 12, 15}$)].

2.2. Cold Pressed Pumpkin Seed Oil Production By Screw-Pressing

Four commercially available pumpkin seeds (*Cucurbita pepo L.*) were harvested in region of Celtik/Konya, Cumra/Konya, Iceri Cumra/Konya and Polatlı/Ankara, central Anatolia regions of Turkey. Naked pumpkin seeds were of domestic origin and harvested in mid autumn of 2015, were purchased from local suppliers for laboratory-scale screw-pressing process. To determine the moisture and oil contents of pumpkin seeds, ISO-665 [15] and ISO-734-1[16] standard methods were used, respectively.

A lab-scale screw press machine (15 kg $seed.h^{-1}$ capacity, single head, 2hp, 1.5 kw power) in our laboratory was used for pressing of pumpkin seed samples. 40 rpm screw rotation speed and 40°C temperatures were selected as process parameters. During the screw-pressing process, the temperature –did not exceed 40°C to prevent an increase of the content of oxidation products. The naked pumpkin seeds for screw-pressing were used as follows: Celtik region (10 kg), Cumra region (7 kg), Iceri Cumra region (10 kg) and Polatlı region (8 kg). Following each set, oil and meal with oily cake were collected and weighed, and the oil was filtered to remove suspended materials. The cold pressed oils were centrifuged in a refrigerated centrifuge system (Sigma 2-16K, Germany) for 20 min at 10°C. The oil phases were separated, weighed, flushed with inert gas and packed into dark-green glass bottles closed with screw caps, at the ambient temperature until analysis.

2.3. Determination of Physical Characteristics

The color values of cold-pressed oils were directly determined by a Lovibond tintometer (Model-F, Tintometer Ltd., Salisbury, U.K.) and were expressed as Lovibond units (LU). The color values were determined by using Cc 13e-92 AOCS official method [17] and expressed in yellow (Y), and red (R) units. Refractive index (RI) values were determined using Abbe Refactometer (Model NAR-3 T, ATAGO Co., Ltd., Tokyo, Japan) at a temperature of 25°C. The viscosities of cold-pressed oils were determined by Brookfield viscometer (Brookfield Eng. Lab. Inc., USA). Of the PSO, 1 mL was placed on the plate of the viscometer with a LV-SC4-18 spindle and maintained at 40°C by a circulating water bath.

2.4. Determination of Chemical Characteristics

The free fatty acid (FFA) content, peroxide value (PV), iodine value (IV), saponification value (SV) and unsaponified matter (USM) content of seed oils were determined according to Ca 5a-40, Cd 8b-90, Cd 1-25, Cd 3c-91 and Ca 6a-40 AOCS official methods [17], respectively. Conjugated dien and trien contents of seed oils were determined by measuring the specific extinction coefficients (K_{232} and K_{270}) as well as their relation or *R*-values (K_{232}/K_{270}), according to the AOCS method [17]. The determinations were performed at specified wavelengths and the absorption values were recorded to calculate the conjugated diene and trien contents. The oxidative stability index (OSI) values were determined according to the Cd 12b-92 AOCS official method [17], by using a 743 Rancimat system (Methrom AG, Herisau, Switzerland). In the rancimat test, 3 g of oil was weighed into the reaction vessel placed into the heating block kept at 120°C, air flow was set at 20 $L.h^{-1}$. Volatile secondary oxidation compounds (short-chain acids, epoxides, aldehydes, and ketones) released during the heating process were collected in a receiving flask filled with 60 mL ultra-pure water, and then the conductivity of this water was measured and recorded. The induction times were automatically determined as the inflection point of the generated plot of conductivity ($\mu S.cm^{-1}$) of the ultra-pure water versus time.

2.5. RP-HPLC/DAD Analysis of Triglycerides

Triglyceride profiles of cold pressed PSOs were determined by reverse-phase high performance liquid chromatography (RP-HPLC) technique according to the Ce 5b-89 AOCS official method [17]. Agilent 1200 series HPLC system (Agilent Technologies Inc., USA) was equipped with a G4214A model diode-array detector and ACE-5 C18 column (250×4mm i.d., 5µm) system at 30°C. 5 µL of the samples was injected and a mixture of acetone: acetonitrile (50:50, v/v) was used as a mobile phase under isocratic conditions with a 1.5 $mL.min^{-1}$ flow rate. Triglycerides were detected at wavelength of 205 nm and identified based upon comparison of their retention times with those of pure triglyceride standards, according to the equivalent carbon number (ECN).

2.6. UHPLC-MS/MS Analysis of Pesticide Residue

The extraction procedure for pesticide residue analyses was carried out according to the original QuEChERS method (AOAC Official Method 2007.01) [18]. The UHPLC-MS/MS method parameters were optimized and validated onto our analysis system, based on QuEChERS method.

Extraction procedure: In brief, 15 g oil sample was weighed into a 50 mL fluoroethylenepropylene centrifugation tube. 15 mL of 1% acetic acid (HOAc) in MeCN, 6 g anhydrous $MgSO_4$ and 1.5 g NaOAc were added to a centrifuge tube. Then, the tubes were shaken vigorously for 1 min by hand; and centrifuged at 4000 rpm for 5 min. 4 mL of the supernatant was then transferred to centrifuge tube and 1.2 g anhydrous $MgSO_4$/ 0.4 g primary secondary amine (PSA) sorbent were added. The mixture was centrifuged again for 5 min (4000 rpm). The extracts were put into auto-sampler vials for analysis by UHPLC-MS/MS to identify and determine a wide range of pesticide residues.

UHPLC-MS/MS method parameters: The chromatographic analyses were performed on Thermo Quantum AccMax UHPLC-MS/MS (Thermo, San Jose, CA, USA) system coupled to triple quadrupole analyzeroperated in the ESI positive mode. For the chromatographic separation, a Hypersil Gold C_{18} reversed phase column (50×2.1 mm; 1.9 µm) was used. The parameters of the UHPLC-MS/MS system were as follows: column temperature, 25°C; injection volume, 3 µL; flow rate, 300 $\mu L.min^{-1}$; eluent A: methanol; eluent B: 5 mM formic acid in water. The chromatographic gradient program started from 25% eluent A, then went up linearly to 90% A in 5 min, held this composition for 25 min. Nitrogen was applied in the ion source gas at 12 $L.min^{-1}$ and ion source temperature at 350°C. The N_2 supply pressure for the instrument was 40 psi, and the final MS/MS conditions included: MS_1/MS_2, 100°C/100°C; 400

V; 2.3 Torr- 8.79×10^{-6} Torr. Data acquisition and processing was carried out with the Thermo LC Quan software.

Method validation: The validation of method was carried out in accordance with ISO/IEC 17025 [19]and considering European guidelines [14] in relation to selectivity, linearity, recovery, repeatability, within laboratory reproducibility, limits of detection (LOD) and quantification (LOQ).

The validation experiments were performed on five days ($n=5$) and recovery studies were performed at 3 different mass fraction levels. The selectivity of proposed method was calculated by analysing blank pumpkin seed oils certified organic and checking for the lack of snooping peaks in the retention time section of analytes. The target analytes were differentiated from potential co-eluting matrix interferences by comparing the relative intensity of the ratio between quantifying and qualifying multiple reaction monitoring mode (MRM) transition obtained in the sample to that related to pure calibration standards. The calibration was carried out by injecting the set of pesticide standards in triplicate, on each day ($n=5$). Calibration curves were created by plotting the peak area versus the mass fraction ratios for all pesticides. At five different target mass fraction levels in the range; 10−250 $\mu g.kg^{-1}$ for carbaryl, chlorpyrifos, methidathion and simazine; 10−500 $\mu g.kg^{-1}$ for dimethoate and diuron; 1−50 $\mu g.kg^{-1}$ for omethoate, phosmet and malathion. The values were selected taking into account both the UHPLC-MS/MS method sensitivity and different maximum residue limits for each pesticide. Information about the regulation of pesticides was obtained from Codex Alimentarius and EU pesticide database [7, 20]. The linearity of each calibration curve was estimated by the residual plots and calculation of the correlation coefficients. To calculate the LOD and LOQ of proposed method, on each of the five days of the validation three matrix blanks were spiked at a level related to the lowest point of the calibration curve. The samples were processed as described and injected to the system in triplicate. LOD and LOQ values were calculated as 3 and 10 times, and the standard deviation of the signal declared in mass units. To determine the repeatability of method and within-laboratory intermediate precision; five blank samples were spiked at a mass fraction point corresponding to the middle point of the calibration curves (100 $\mu g.kg^{-1}$ for carbaryl, chlorpyrifos, methidathion and simazine; 200 $\mu g.kg^{-1}$ for dimethoate and diuron; 20 $\mu g.kg^{-1}$ for omethoate, phosmet and malathion), and injected in triplicate. The same procedure was repeated for five days. The repeatability and within-laboratory intermediate precision were estimated by using the obtained results and the single-factor analysis of variance, ANOVA. The recovery values were estimated by spiking at three different mass fraction levels on five samples each and calculated by comparing both the measured fractions and theoretical. The sum of spiked mass fractions for each pesticide was considered as the theoretical, and the measured fraction was the sum of the calculated mass fractions.

2.7. UHPLC-MS/MS Analysis of Aflatoxin Contamination

The extraction procedure for aflatoxin analyses was performed according to the Yang et al. (2011) [20]. The UHPLC-MS/MS analyses were carried out according to the proposed method by Shi et al. (2011) [21].

Extraction procedure: 5 g oil samples were accurately weighed and 5g NaCl and methanol/ultra-pure water (7:3, v/v) were added to give a final volume of 20 mL in centrifuge tube. The mixtures were shaken vigorously for 2 min by a mechanical shaker. Then, 15 mL extract was collected and diluted with 30 mL ultra-pure water and the mixture was re-filtered before SPE purification. The AflaTestP® immuno-affinity SPE cartridge columns were connected to the Vac-Elut-20 manifold system (Agilent Technologies Inc., USA). Then, 15 mL of the sample extract was eluted through the immuno-affinity SPE cartridge at a rate of $6mL.min^{-1}$. The cartridge was cleaned with 10 mL ultra-pure water. Aflatoxins were eluted with 1mL methanol and the mobile phase was kept in contact with the cartridge for 2 min to guarantee complete elution. Then, eluent were dried and diluted with 1mL of mixture of methanol: water (98:2, v/v) before UHPLC-MS/MS analysis.

UHPLC-MS/MS Method Parameters: The UHPLC–MS/MS system used for analysis was a Thermo Quantum AccMax UHPLC-MS/MS (Thermo, San Jose, CA, USA) in combination with a coupled to triple quadrupole mass spectrometer. A Hypersil Gold C_{18} reversed phase column (50×2.1 mm; 1.9 µm) was used as a chromatographic column. The mobile phase system consisted of (A) ultra-pure water and (B) methanol at a flow rate of 0.4 $mL.min^{-1}$. The mobile phase programme varied linearly as follows: 0.00 min, 2% B; 1.45 min, 80% B; 2.30 min, 90% B; 3.15 min, 98% B; 4.45 min, 2% B and held this composition for 8 min **[21]**. The injection volume was 20 µL and the column temperature was maintained at 25°C. The parameters used for the mass spectrometer with ESI positive mode were set as follows: spray voltage 3.5 kV, sheath gas pressure 50 arbitrary units, auxiliary gas pressure 20 arbitrary units, capillary temperature 250°C, Collision gas pressure 1.5 mTorr. Data acquisition and processing was carried out with the Thermo LC Quan software.

2.8. Statistical Analysis

All extractions and analyses were conducted in triplicates. Data were expressed as the mean ±standard deviation (SD). Results were analyzed with one-way analysis of variance, ANOVA and differences between individual means were deemed to be significant at $P<0.05$. All statistical analyses were performed by means of Excel (Microsoft, 2007) and OriginPro8 (OriginLab, USA) databases.

3. RESULTS AND DISCUSSION

3.1. Composition of Pumpkin Seeds (*Cucurbita pepo L.*) from Central Anatolia Region of Turkey

The moisture and oil contents of naked pumpkin seed samples (*Cucurbita pepo L.*) were analyzed before the screw-pressing process. The moisture contents of seeds were quite low and detected as 4.1% for Celtik, 3.9% for Cumra, 4.2% for Iceri Cumra, and 3.8% for Polatlı pumpkin seeds. Non-significant differences in moisture content were observed among the pumpkin seeds from the four different regions. Higher moisture content could lead to oily seed spoilage through rising microbial and enzymatic actions. Due to this reason, these pumpkin seed samples could be stored for a long time without any decomposition [22]. The oil contents of pumpkin seeds, as important nutritional factors, are calculated as the percent from the total amount of oil from a known amount of seeds. With screw-pressing process, the oil contents were detected as 45.2% for Celtik, 43.7% for Cumra, 47.4% for Iceri Cumra, and 42.8% for Polatlı pumpkin seeds. The results of oil contents obtained from our analysis were lower than 60% value that reported for the European variety of *Cucurbita pepo L* [23]. However, the results of our research are in good agreement with some reported studies. Vasconcellos et al. (1981) [24], Idouraine et al. (2009) [25], Jafari et al. (2012) [9], and Young Kim et al. (2012) [26]reported that the oil contents of *Cucurbita pepo L*. cultivars were determined between 21.0–43.0%, 34.5–43.6%, 36.9–47.8%, and 43.99–52.43% values, respectively. These researches also declared that varieties in the oil contents could be attributed to the differences in genetic diversity, climate conditions, ripening stage and extraction methods. These oil contents are higher than several commercial oilseeds, so studied pumpkin seed could be introduced as a novel source of vegetable oil for industry applications [9].

3.2. Physicochemical Characteristics aed Oxidative Stability of Cold Pressed Pumpkin Seed Oils

The results of basic physicochemical characteristics and oxidative stabilities of four naked cold pressed PSOs are presented in Table 1. The chemical properties; peroxide value (PV), iodine value (IV), free fatty acid (FFA), saponification number (SN), unsaponified matter (USM), specific extinctions at 232 nm (K_{232}) and 270 nm (K_{270}) were determined to characterize the cold pressed PSOs.

Peroxide value has been commonly used as one of the most important parameters to determine the quality and oxidative stability of edible oils [27]. The quite low PVs were detected as 3.10±0.10 meq O_2/kg oil for Celtik, 3.40±0.10 meq O_2/kg oil for Cumra, 3.16±0.05 meq O_2/kg oil for Iceri Cumra, and

3.60±0.50 meq O_2/kg oil for Polatlı PSOs, respectively. Even though PSOs were crude, none of the studied oils exceeded the limit of 15 meq O_2/kg oil offered by the Codex standard cold pressed and virgin edible oils [7]. The FFA contents, which indicate enzymatic activity, were detected as 0.270±0.010% oleic acid, 0.260±0.005% oleic acid, 0.270±0.010% oleic acid, and 0.260±0.005% oleic acid for Celtik, Cumra, Iceri Cumra and Polatlı PSOs, respectively. The FFA contents of all the studied cold pressed oils were also well within the Codex limit of up to 2% oleic acid of virgin and cold pressed oils [7, 27]. Another important parameter saponification number, indicating relatively molecular weight of fatty acids esterified in triacylglycerol structure, was determined. The saponification numbers were in the range of 201.91±1.49 to 290.78±1.26 mg KOH/g oil. The highest SV of 290.78 ±1.26 mg KOH/g oil was detected for Iceri Cumra PSO, while the lowest value of 201.91±1.49 mg KOH/g oil was detected for Cumra PSO. The saponification numbers in the present study were in good agreement with previous reports for cold pressed PSOs [9, 28]. The content of unsaponified matters (USM) provides useful data for identification of adulterants, detected as 0.84±0.01 g/kg oil for Celtik, 0.65±0.01 g/kg oil for Cumra, 0.70±0.02 g/kg oil for Iceri Cumra, and 0.60±0.03 g/kg oil for Polatlı PSOs. Since waxes, hydrocarbons, and valuable bioactive compounds such as sterols, tocochromanols, essensial fatty acids and vitamins considered as USM, determination of USM content in cold pressed oils is much significant [22]. The other important oil quality parameter, iodine value (IV) is a measure of the average amount of unsaturation and defined as a number of centigrams of iodine absorbed per gram of oil [27]. The iodine values indicating a relatively high degree of unsaturation were detected as 117.09 ±0.50 g I_2/100 g oil for Celtik, 117.23 ±0.40 g I_2/100 g oil for Cumra, 117.29 ±0.50g I_2/100 g oil for Iceri Cumra, and 117.09 ±0.30 g I_2/100 g oil for Polatlı PSOs. The obtained results indicate that, the cold pressed PSOs are intensely unsaturated and therefore responsible forthe oxidative degradation reactions.

Table 1. Physicochemical characteristics of the cold pressed pumpkin seed (*Cucurbita pepo L.*) oils cultivated in central Anatolia regions of Turkey

	Physicochemical characteristics of the cold-pressed pumpkin seed oils from central Anatolia region of Turkey (*Cucurbita pepo L.*)			
Physicochemical analysis	**Celtik/Konya**	**Cumra/Konya**	**Iceri C./Konya**	**Polatlı/Ankara**
peroxide value, (meq O_2/kg oil)	3.10±0.10	3.40±0.10	3.16±0.05	3.60±0.50
iodine value, IV, (g I_2/100 g oil)	117.09 ±0.50	117.23 ±0.40	117.29 ±0.50	117.09 ±0.30
free fatty acid, FFA, (% oleic acid)	0.270±0.010	0.260±0.005	0.270±0.010	0.260±0.005
saponification value, SV (mg KOH/ g oil)	235.14±1.00	201.91±1.49	290.78±1.26	290.68±1.09
unsaponified matter, (g/ kg oil)	0.84±0.01	0.65±0.01	0.70±0.02	0.60±0.03
specific extinction at 232 nm	4.10 ±0.02	3.59 ±0.01	3.59 ±0.01	4.02 ±0.01
specific extinction at 270 nm	2.15 ±0.01	2.07 ±0.01	2.07 ±0.02	2.41±0.02
R-value, K_{232}/ K_{270}	1.91	1.73	1.73	1.67
oxidative stability index, OSI, h (120°C, 20 L/h)	7.52 ±0.20	7.71 ±0.20	6.46 ±0.10	6.58 ±0.10
refractive index, RI, (25°C)	1.480 ±0.050	1.475 ±0.050	1.479 ±0.050	1.477 ±0.050
viscosity, 40°C, $mm^2.s^{-1}$	35.49 ±0.10	35.21 ±0.20	35.42 ±0.10	35.51 ±0.30
color	35 Y + 1.6 R	34 Y + 1.4 R	37 Y + 1.9 R	35 Y + 1.5 R

Results are reported as means ± SD of three replicate analyses (*n = 3*)

The specific extinction at wavelengths of 232 and 270 nm and *R*-values (K_{232}/ K_{270}) of studied oils are also presented in Table 1. K_{270} and K_{232} indices are used to give information on the quality of oil and content of primary and secondary oxidation products [29]. The contents of conjugated dien and trien of seed oils were detected by measuring the specific extinction coefficients at K_{232} and K_{270}, respectively. The *R*-values (K_{232}/ K_{270}) were determined as 1.91 for Celtik, for 1.73 Cumra, 1.73 for Iceri Cumra, and 1.67 for Polatlı PSOs. Thus, the *R*-values determined for cold pressed PSOs, were considerably lower

than the 10−15 for natural oils, and3 for refined oils, recommended by the codex standard for edible fats and oils [7]. The oxidative stability index (OSI) as an important criterion for quality control with respect to the stability and shelf life of oils [30].It wasdetected by rancimat measurements at 120°C with air flow of 20 $L.h^{-1}$. The oxidative stabilities of cold pressed PSOs, expressed by the induction period (IP), were detected as 7.52 ±0.20 h for Celtik, 7.71 ±0.20 h for Cumra, 6.46 ±0.10 h for Iceri Cumra, and 6.58 ±0.10 h for Polatlı PSOs. Therefore, Fruhwirth and Hermetter (2008) suggested the antioxidant capacity of seed oil as a novel and significant criterion for quality-control with regard to the oxidative stability index and shelf life [31].

The physical properties; refractive index (RI), viscosity, and color values were also determined to characterize the cold pressed PSOs (Table 1). RI value of oils depends on their molecular weight, chain length of fatty acids, degree of unsaturation, and conjugation, so the degree of difference of characteristic oil from its real values may be a sign of its purity. In the study, RI values were determined as 1.480 ±0.050 for Celtik, 1.475 ±0.050 for Cumra, 1.479 ±0.050 for Iceri Cumra, and 1.477 ±0.050 for Polatlı region PSOs. Previous studies on PSOs from different origins, also reported similar RI values with our data [6, 12]. The viscosity values obtained in our study were also similar to the findings of Andjelkovic et al. (2010) [12]. The viscosity values were measured as 35.49 ±0.10 $mm^2.s^{-1}$ for Celtik, 35.21 ±0.20 $mm^2.s^{-1}$ for Cumra, 35.42 ±0.10 $mm^2.s^{-1}$ for Iceri Cumra, and 35.51 ±0.30 $mm^2.s^{-1}$ for Polatlı PSOs. As is known, the color of a vegetable oil is one of the most important characteristic affecting its preference by consumers and for determining the acceptance of edible oil. This characteristic is generally attributed to the amount of pigments, carotenoids, and phenolic pigments and also morphological factors. The color values presented in the Lovibond tintometer scale are expressed in terms of Lovibond Red (R) and Yellow (Y) units. All PSO samples weredark in color and measured as 35 Y + 1.6 R value, 34 Y + 1.4 R value, 37 Y + 1.9 R value, and 35 Y + 1.5 R value for Celtik, Cumra, Iceri Cumra and Polatlı PSOs, respectively. As expected, the obtained color values were also in good agreement with Andjelkovic et al. (2010) [12]. Thus, there was a non-significant difference between the PSO samples from the four different regions, in terms of physicochemical characteristics. The results showed that the studied PSOs harvested in central Anatolia regions are high quality and stable cold pressed oils. Thus, the results proved that these cold pressed oils could be stored for a long time without any additional purification process.

3.3. Triglyceride Profiling by RP-HPLC/DAD Technique

In the study, the triglyceride analyses were performed according to the AOCS Ce5b-89 official method for triglyceride profiling in vegetable oils [17]. The results of triglyceride analysis are given in Table 2 and the chromatogram of Cumra cold pressed PSO is presented in Figure 1.

As can be seen from Table 2., the determined triglyceride isomers with different ECNs were; linoleinodilinolenin (LLnLn; 38:8), dilinoleinolinolenin (LLLn; 40:7), trilinolein (LLL; 42:6), oleolinoleolinolenin (OLLn; 42:6), palmitolinoleolinolenin (PLLn; 42:5), oleodilinolein (OLL; 44:5), linoleninopalmitooleoin (LnPO; 42:4), dioleolinolein (OOL; 46:4), palmitooleolinolein (POL; 46:3), dipalmitolinolein (PPL; 46:7), triolein (OOO; 48:3), stearooleolinolein (StOL; 48:3), and palmitolinoleostearin (PLSt; 48:2) (ECN: number of DBs). The percentages of main triglyceride isomer, OLL were detected as 23.703 ±0.013%, 23.895 ±0.018%, 23.155 ±0.017% and 24.022 ±0.016% for Celtik, Cumra, Iceri Cumra and Polatlı PSOs, respectively. Cold pressed PSOs have mainly triglycerides with equivalent chain numbers of 38, 40, 42, 44, 46 and 48. The major triglycerides with ECN of 42 values were detected as 33.98% for Celtik, 30.97% for Cumra, 36.54% for Iceri Cumra, and 35.65% for Polatlı PSOs. The triglycerides with ECN of 44 values were detected as 23.70% for Celtik, 23.90% for Cumra, 23.16% for Iceri Cumra, and 24.02% for Polatlı PSOs. The triglyceride analysis results are in good agreement with previous studies for PSOs. Butinar et al. (2010) and Nederal et al. (2012) reported that OLL was the dominant triacylglycerol in PSOs obtained, followed by LLL, LOO and PLO isomers [6, 32].

Table 2. Triglyceride profile of the cold pressed pumpkin seed (*Cucurbita pepo L.*) oils cultivated in central Anatolia regions of Turkey

	Triglyceride analysis results of cold-pressed pumpkin seed oils from central Anatolia region of Turkey (*Cucurbita pepo L.*)			
TG molecules	**Celtik/Konya**	**Cumra/Konya**	**Iceri Cumra/Konya**	**Polatlı/Ankara**
LLnLn	0.158 ±0.001	0.187 ±0.001	0.255 ±0.001	0.213 ±0.001
LLLn	0.605 ±0.002	0.640 ±0.003	0.646 ±0.001	0.656 ±0.002
LLL	18.168 ±0.012	19.553 ±0.014	16.020 ±0.10	19.269 ±0.013
OLLn	0.168 ±0.001	0.193 ±0.002	0.161 ±0.003	0.164 ±0.001
PLLn	0.319 ±0.001	0.313 ±0.001	0.239 ±0.001	0.289 ±0.001
OLL	23.703 ±0.013	23.895 ±0.018	23.155 ±0.017	24.022 ±0.016
LnPO	15.321 ±0.011	16.483 ±0.010	14.552 ±0.014	15.926 ±0.012
OOL	13.415 ±0.015	11.824 ±0.012	15.377 ±0.011	12.411 ±0.011
POL	16.078 ±0.012	15.842 ±0.011	16.088 ±0.015	15.537 ±0.013
PPL	2.077 ±0.004	2.277 ±0.007	2.281 ±0.003	2.273 ±0.008
OOO	1.641 ±0.002	1.287 ±0.003	2.290 ±0.005	1.431 ±0.002
StOL	6.390 ±0.006	5.587 ±0.008	6.857 ±0.009	5.726 ±0.004
PLSt	1.955 ±0.003	1.914 ±0.002	2.078 ±0.004	2.076 ±0.006

P; palmitic acid, St; stearic acid, O;oleic acid, L;linoleic acid, Ln; linolenic acid
Values are reported as means ± SD of three replicate analyses (n = 3)

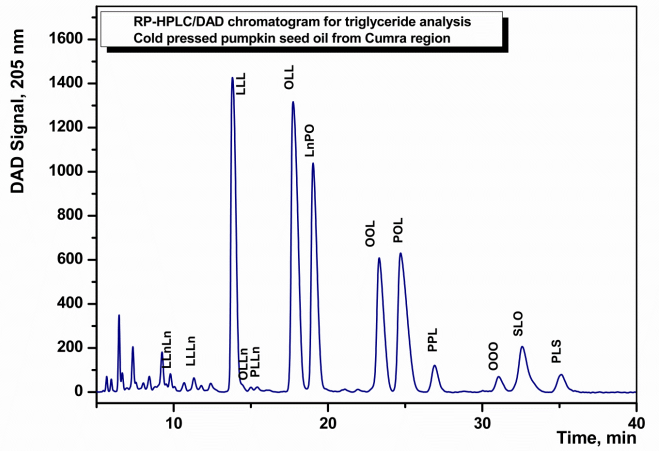

Figure 1. RP-HPLC/DAD chromatogram of cold pressed pumpkin seed oil from Cumra region for triglyceride analysis

3.4. Pesticide Residue and Aflatoxin Contamination Analyses by UHPLC-MS/MS

Due to common use of pesticides in cultivation, their residues in foodstuffs are practically unavoidable. Residues of pesticides, which were used in the past for plant/crop protection, in foods are mainly related to the environment, into which these chemicals are brought chiefly by human activities [11]. A sensitive, accurate and simple UHPLC-MS/MS method for the determination of 9 selected pesticides in PSOs, has been developed and validated. The procedure is based on sample extraction by the use of a QuEChERS AOAC-2007.01official method extraction [18], and following clean-up of the extract with PSA. The LC-MS/MS conditions of QuEChERS were also used with some modifications. The studied pesticide standards were separated on a Hypersil Gold C_{18} reversed phase column (50×2.1 mm; 1.9 µm) using gradient elution with methanol−water/5 mM formic acid in mobile phase, and finally identified and quantified by triple quadrupole mass spectrometry in the multiple reaction monitoring mode (MRM). In literature, different C_{18} analytical columns were reported using different aqueous-organic mobile phase

gradients, with either acetonitrile or methanol with 0.1% (v/v) of formic acid. When comparing aqueous-methanol versus aqueous-acetonitrile mobile phase systems, better response and separation of the pesticides were also achieved with the methanol mobile phase gradient of QuEChERS official method [18, 33].

To achieve separation of studied pesticides and to maximize both sensitivity and precision of the method, the validation of proposed method was performed. The specificity of method was evaluated by the analysis of blank PSO samples. Pesticides were identified in the oil samples according to their t_R, and differentiated from any possible matrix interference by comparing the relative intensity of the ratio between quantification & qualifying MRM transition obtained calibration standards, with that in relation to sample. The MRM ratios in samples were well within the acceptable levels for relative ion intensities as specified in the European guidelines [14], also no significant interferences from the PSO matrix were determined. The statistical parameters calculated from least-square regression are presented in Table 3. As can be seen from Table 3, the correlation coefficients (r) were determined higher than 0.99 value.

The LODs and LOQs of each analytes were calculated by spiking three blank PSO samples at the lowest level of the working range for each pesticide (Table 3). The LOD was theoretically estimated as 3 times the standard deviation, while the LOQ was estimated as 10 times the standard deviation obtained from analyses of independent samples at the lowest calibrated level. The LODs were confirmed experimentally, and the lowest spike mass fraction levels used in the recovery experiments were considered as validated the LOQs [14]. As given in Table 3, LOQs for studied pesticides were below the respective maximum residue limits. The precision of the method, estimated through the repeatability and within-laboratory intermediate precision is presented in Table 3.

The repeatability and intermediate precision values in the study stated as RSD were below 10%. The recovery studies performed at three mass fraction levels of each individual pesticide and obtained results are given in Table 4. The obtained values for studied pesticides were higher than 95%, with the exception of phosmet at the highest mass fraction level, for which recoveries of 93% was obtained, with RSD ranging from 4.2%. The obtained results present a good accuracy of the proposed methodology and so a suitability for the characterization of reference material.

Table 3. Working range and calibration equations of the selected pesticides (*n=5*) and performance characteristics of the proposed UHPLC-MS/MS method

				mass fraction linear range						**LOQ**	
Pesticide	**limits for oils, EU (µg.kg-1)**	**retention time (tR)**	**m/z parent**	**matrix (µg.kg-1)**	**standard (µg.kg-1)**	**calibration equation**	**r**	**regression std. dev.**	**LOD (µg.kg^{-1})**	**estimated (µg.kg-1)**	**validated (µg.kg-1)**
carbaryl	50	10.46	202.2	10−500	10−250	y = 16840x + 94918	0.9953	579	3	10	10
chlorpyrifos	50	14.05	351.9	10−500	10−250	y =16488x+105464	0.99832	338	2	10	10
dimethoate	200	8.82	230.0	10−1000	10−500	y = 8245x + 157856	0.9993	110	5	10	30
diuron	200	10.65	233.1	10−1000	10−500	y = 8350x + 175798	0.9979	192	8	10	30
malathion	20	11.30	331.0	10−200	1−50	y = 83468x + 156354	0.9994	1008	0.5	10	20
methidathion	50	11.26	303.0	10−500	10−250	y = 16452x + 125259	0.9962	506	1	10	20
omethoate	-	4.68	214.1	10−200	1−50	y = 84107x + 158154	0.9986	1581	0.4	10	10
phosmet	-	11.24	318.0	10−200	1−50	y = 84269x + 171374	0.9984	1680	0.7	10	15
simazine	100	9.21	202.0	10−500	10−250	y = 16573x + 118524	0.9961	519	6	10	25

r; correlation coefficient, LOD; limit of detection, LOQ; limit of quantification

Table 4. Mean recovery and RSD (n=5) values calculated for the studied pesticides spiked at three different concentration levels

Pesticides	spiked (µg.kg^{-1})	recovery (%)	RSD (%)	spiked (µg.kg^{-1})	recovery (%)	RSD (%)	spiked (µg.kg^{-1})	recovery (%)	RSD (%)
carbaryl	50	102	3.7	100	99	3.9	200	105	4.6
chlorpyrifos	50	99	4.2	100	104	4.1	200	97	4.1
dimethoate	100	97	5.1	200	98	6.1	400	99	6.3
diuron	100	103	4.3	200	97	4.2	400	101	5.2
malathion	10	102	4.1	20	106	5.1	40	97	5.3
methidathion	50	98	5.3	100	95	5.2	200	101	4.7
omethoate	10	99	3.9	20	98	4.8	40	97	4.9
phosmet	10	96	5.3	20	93	4.2	40	98	3.2
simazine	50	101	4.1	100	103	5.5	200	105	5.2

The validated method was applied to the analysis of pumpkin seed oil samples. Among the studied pesticides, only chlorpyrifos pesticide residues were detected as18.050 ±0.470 ppb (µg.kg^{-1}) for Celtik PSO, 10.430 ±0.620 ppb for Cumra PSO and 12.340 ±0.820 ppb for Polatlı PSO (Figure 2). For Iceri Cumra PSO sample, no pesticide residue was detected by using proposed method (Table 5). The contents of chlorpyrifos for studied oils were considerably under the limit value (50 µg.kg^{-1}) stated by official authorities for the quality assessment of edible oils [7, 14].

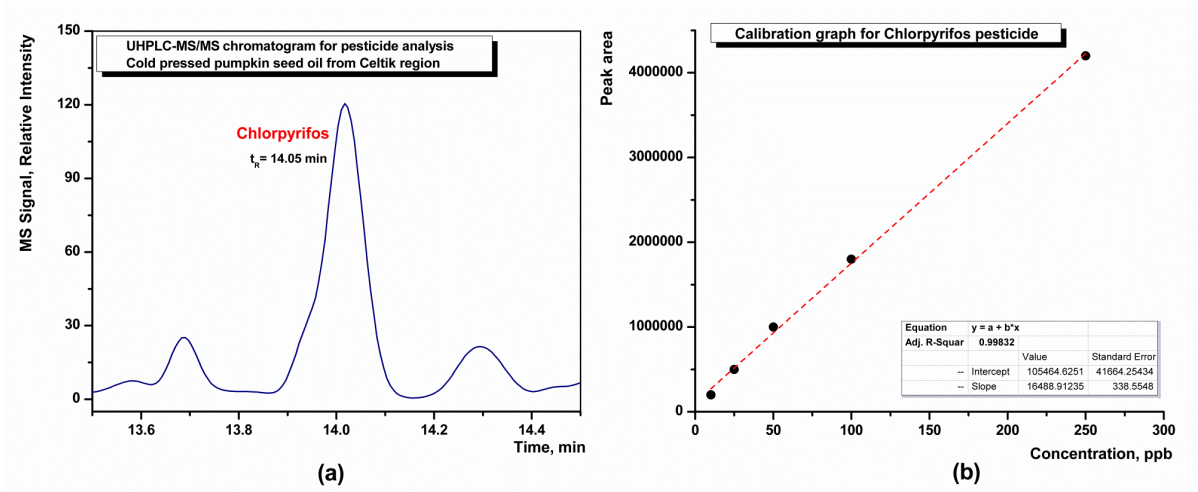

Figure 2. (a) UHPLC-MS/MS chromatogram of cold pressed pumpkin seed oil from Celtik region for pesticide analysis **(b)** calibration graph for chlorpyrifos pesticide

Table 5. Pesticide analysis results of cold-pressed pumpkin seed oils from central Anatolia region of Turkey (*Cucurbita pepo L.*)

Pesticide analysis results of cold-pressed pumpkin seed oils from central Anatolia region of Turkey (*Cucurbita pepo L.*)				
	Celtik/Konya	**Cumra/Konya**	**Iceri Cumra/Konya**	**Polatlı/Ankara**
content of chlorpyrifos (µg.kg^{-1})	18.050 ±0.470	10.430 ±0.620	nd	12.340 ±0.820

Results are reported as means ± SD of three replicate analyses (*n = 3*)

The contaminations of aflatoxin were determined by the LC-MS/MS method described by Shi et al. (2011)[21]. The stock mix aflatoxin solutions were prepared in methanol and four aflatoxins were analyzed under electrospray ionization (ESI^+) mode. The chromatogram of aflatoxin standards (AF-B_1, AF-B_2, AF-G_1 and AF-G_2) is presented in Figure 3.

Aflatoxin standard concentrations of 1µg.kg^{-1}, 5µg.kg^{-1}, 10µg.kg^{-1} and 25µg.kg^{-1} in methanol, were used for calibration curves by using UHPLC-MS/MS system. The correlation coefficients (linear regressions) were obtained higher than 0.9900 for each aflatoxin standards. Aflatoxin B_1, B_2, G_1 and G_2 contaminations were not detected in any of studied pumpkin seed oil sample. The maximum acceptable level of aflatoxin in food materials has been regulated in many countries and the legal limits may vary from one country to another, depending on the degree of development and economic consideration. The food and drug administration (FDA) has set a maximum acceptable level of 20 µg.$kg^{-1}$for total aflatoxin in all foods for human consumption [34]. Thus, the obtained data indicate that the cold pressed pumpkinseed oils from central Anatolia region of Turkey have high quality properties and physicochemical characteristics of edible oil and may be considered a good source of bioactive compounds. Also, the study is the first data on the presence of pesticides in cold pressed pumpkinseed oils.

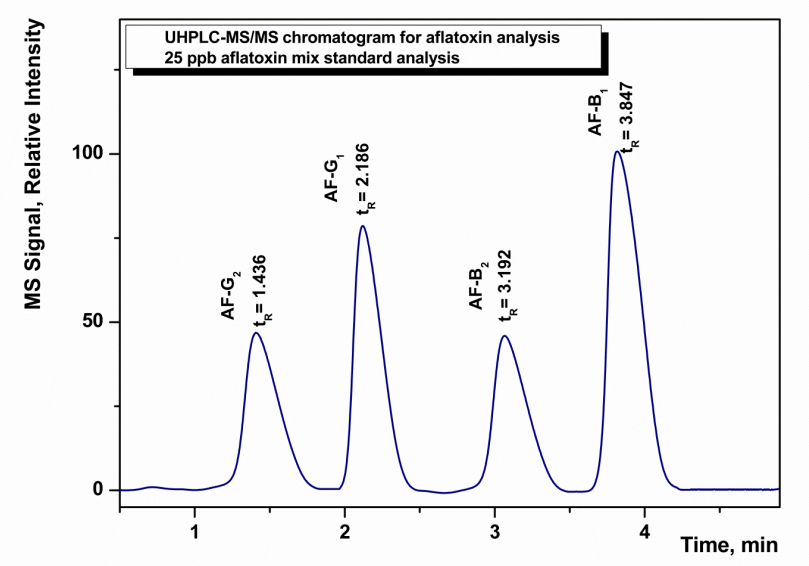

Figure 3. UHPLC-MS/MS chromatogram of aflatoxin mixture standard (AF-G_2, AF-G_1, AF-B_2 and AF-B_1)

4. CONCLUSION

Production of cold pressed pumpkin seed oils has been an important issue for edible oil industry and studied for different purposes. Cold pressed pumpkin seed oils are an extraordinary rich source of bioactive compounds. In this study, cold pressed pumpkin seed oils produced by screw-pressing the seeds, were characterised by determining their basic physicochemical characteristic and evaluating the contents of pesticide and aflatoxin contaminations. Physicochemical characteristic of the studied oils, cultivated in four different central Anatolia regions of Turkey, showed that the oils have high quality and potential to be used as nutrient rich edible oil. Besides, it was found that studied oils were well-accepted cold pressed oils in terms of toxic pesticide and aflatoxin contaminations. Therefore, pumpkin seed oils can be utilized as functional ingredients for the development and formulation of new functional food products. Moreover, the results of the study are expected to increase the knowledge on the characteristics of edible pumpkin seed oils and provide as valuable contributions to the literature by this study.

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ABBREVIATIONS

AF	aflatoxin
ANOVA	analysis of variance
AOCS	american oil chemical society
DAD	diode array detector
ECN	equivalent chain numbers
ESI	electrospray ionization
FDA	food and drug administration
FFA	free fatty acid
GAE	gallic acid equivalent
GC	gas chromatography
GC-MS	gas chromatography-mass spectroscopy
IV	iodine value
LC-MS/MS	liquid chromatography- mass spectroscopy/ mass spectroscopy
LLL	trilinolein
LLLn	dilinoleinolinolenin
LLnLn	linoleinodilinolenin
LnPO	linoleninopalmitooleoin
LOD	limit of detection
LOQ	limit of quantification
LU	lovibond unit
MRM	multiple reaction monitoring
MS	mass spectroscopy
NOP	national organic program
OLL	oleodilinolein
OLLn	oleolinoleolinolenin
OOL	dioleolinolein
OOO	triolein
OSI	oxidative stability index
PLLn	palmitolinoleolinolenin
PLSt	palmitolinoleostearin
POL	palmitooleolinolein
PPL	dipalmitolinolein
PSA	primary secondary amine
PSO	pumpkin seed oil
PV	peroxide value
RI	refractive index
RP-HPLC	reverse phase-high performance liquid chromatograph

SM	saponified matters
SPE	solid phase extraction
StOL	stearooleolinolein
SV	saponification value
UHPLC	ultra fast high performance liquid chromatograph
UHPLC-MS/MS	ultra fast high performance liquid chromatography-mass spectroscopy/ mass spectroscopy
USM	unsaponified matters

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