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CHARACTERIZATION OF THE SEED AND SEED EXTRACTS OF THE PUMPKINS *CUCURBITA MAXIMA* D. AND *CUCURBITA PEPO* L. FROM MACEDONIA

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Chemical composition of seeds of *C. maxima* D. and *C. pepo* L. cultivated in the Republic of Macedonia and physico-chemical characteristics, fatty acid profiles, and sterol and tocopherol contents in pumpkin seed extracts were determined. Higher kernel yield and content of moisture, ash, total nitrogen, proteins and carbohydrates were measured in the *C. pepo* than in *C. maxima* seed. The highest extract yield of 487.4 g/kg dry matter was obtained from *C. pepo* kernel, while 388.2 g/kg dry matter was extracted from *C. maxima* kernel, when *n*-hexane was used as solvent. In all extracts, the palmitic, stearic, oleic and linoleic acids were predominant. The linoleic/oleic acid ratio was higher in *C. maxima* extracts. Δ7-Sterols were predominant in all extracts, while Δ5-sterols content was higher in the whole seed than in the kernel extracts. Higher tocopherol content was determined in the extracts of *C. pepo* whole seed and kernel (153.79 mg/kg and 117.81 mg/kg, respectively), than in those of *C. maxima* (121.24 mg/kg and 117.55 mg/kg, respectively). In all extracts γ-tocopherol content was higher than α-tocopherol.

Keywords: *Cucurbita* sp.; seed chemical composition; extract; fatty acid composition; sterols; tocopherols

КАРАКТЕРИЗАЦИЈА НА СЕМЕТО И ЕКСТРАКТИТЕ ОД СЕМЕ ОД ТИКВА *CUCURBITA MAXIMA* D. И *CUCURBITA PEPO* L. ОД МАКЕДОНИЈА

Утврдени се хемискиот состав на семето од *C. maxima* D. и *C. pepo* L. одгледувани во Република Македонија, како и физичко-хемиските својства, профилите на масните киселини и содржината на стеролите и токоферолите во екстракти од семето од тиква. Повисок принос на јатката од семето, како и поголема содржина на влага, пепел, вкупен азот, протеини и јаглехидрати беа измерени во семето на *C. pepo* отколку во семето на *C. maxima*. Највисок принос на екстракт од 487,4 g/kg сува материја беше добиен од јатката на *C. pepo*, додека од јатката на *C. maxima* е екстрахирано 388,2 g/kg сува материја, кога како растворувач е користен *n*-хексан. Во сите екстракти доминантни беа палмитинската, олеинската и линолеинската киселина. Односот на линолеинската со олеинската киселина беше повисок во екстрактите од *C. maxima*. Δ7-стеролите беа доминантни во сите екстракти, додека содржината на Δ5-стеролите во екстрактите од целото семе беше повисока отколку во екстрактите од јатката на семето. Повисока содржина на токофероли беше утврдена во екстрактите од целото семе и од јатката од *C. pepo* (153,79 mg/kg и 117,81 mg/kg, соодветно) отколку во оние од *C. maxima* (121,24 mg/kg и 117,55 mg/kg, соодветно). Во сите екстракти содржината на γ-токоферол беше повисока од содржината на α-токоферол.

Клучни зборови: *Cucurbita* sp.; хемиски состав на семе; екстракт; маснокиселински состав; стероли; токофероли

1. INTRODUCTION

Pumpkin (*Cucurbita* sp.) has been known since the dawn of time. Today, pumpkins are widely cultivated as food and for decorative purposes. Pumpkin seed contribute significantly to the nutrition of human population in many parts of the world. The main nutritionally relevant components of pumpkin seed are proteins (30–51 %) and oil (up to 40 %). They are also rich in carbohydrates (up to 10 %) and microelements as representatives of micronutrients (between 4 and 5 %). Differences in the chemical composition of pumpkin seed between *Cucurbita* species and cultivars from different parts of the world might be related to growth and fertilization conditions, and also to the harvest time [1–4].

Over time, the application of the pumpkin seed extracts has been increasing. The pumpkin oil is greenish in color, with typical nutty and roast flavor. Mainly, it contains triglycerides with palmitic, stearic, oleic, and linoleic acid as the dominant fatty acids. The oxidative stability of pumpkin seed oil is influenced primarily by the ratio of linolenic to oleic acid. Other important components present in the pumpkin oil are tocopherols, sterols, phospholipids and hydrocarbons. In pumpkin oil, α- and γ-tocopherol are present in higher concentration than β- and δ-tocopherol. The antioxidant activity of tocopherols (vitamin E) has been studied extensively. It was found that γ- and δ-forms possess a much higher antioxidant activity than α- and β-forms, however, α-tocopherol is considered to have a higher vitamin potency than any other tocopherol isomer [5–9]. When α-tocopherol was added to the oil a strong pro-oxidative effect was observed [9]. Most plant oils predominantly contain Δ5-sterols, whereas Δ7-sterols are tipical for only a few plant families, e.g. *Cucurbitaceae*. Generally accepted technology of the pumpkin seed oil production includes cold pressing of row or roasted seeds. If roasted seeds are pressed, the highest "extra virgin" quality oil is obtained. Pumpkin seed oil, a local specialty produced mainly in South-Eastern Austria, is extracted by physical means from naked roasted seed [10–13].

Historically, pumpkin seed and oil have been used all around the world for healing purposes. For many years, in Europe particularly, extracts from *C. pepo* pumpkin seed have been used in folk medicine as a nutritional remedy for disorders caused by benign prostatic hyperplasia. Water extracts of pumpkin seed are used in the treatment of heterophyiasis. Nowadays, pumpkin seed oil is used successfully in preventing and alleviating prostate and bladder problems. Phytosterols present in the pumpkin seed oil are also being studied for their role in lowering cholesterol levels. In addition, together with the high content of linoleic acid, sterols can help in the treatment of lipid-associated disorders such as atherosclerosis. Pumpkin seed oil has been found to provide a significant source in tocopherols (vitamin E) in diets. Diets high in pumpkin seed oil have also been associated with lower level of gastric, breast, lung and colorectal cancer [13, 14–21].

Currently, pumpkin seed oil is not widely used commercially even though it has characteristics that are well suited for industrial application and can contribute to healthy human diets. Since the content of particular nutrients in the pumpkin seed may vary considerably, depending on soil conditions, climate and genetic factors, it would be of interest to analyze pumpkin seed from the Republic of Macedonia where predominantly *Cucurbita maxima* D. and *Cucurbita pepo* L are cultivated. The physical characteristics and chemical composition of the seed of pumpkins *Cucurbita maxima* D. and *Cucurbita pepo* L. from the Republic of Macedonia have not been reported yet. It is for this reason that this work is focused on determining the contents of moisture, crude proteins, total lipids, ash, crude fibre and carbohydrates in the whole seed, kernel and shell of Macedonian local pumpkins *Cucurbita maxima* D. and *Cucurbita pepo* L. Subjects of this research are also the extract yields obtained from the whole seed, kernel and shell of *C. maxima* and *C. pepo* by applying different solvents, as well as fatty acid composition of the extracts and sterol and tocopherol content.

2. EXPERIMENTAL

2.1. *Materials*

2.1.1. *Plant material*

Pumpkins, *Cucurbita maxima* D. (convar. Stambolka, cv. 1) and *Cucurbita pepo* L. (convar. Koskarka, cv. 2) were grown in Prilep (geographical location: +41º21' 36" N latitude, +21º33'36" E longitude and 640 m altitude), Republic of Macedonia, in the year 2009. Pumpkin seed was planted in April 20th and pumpkins were harvested in September 15th (116 days ripening period). The vegetative surface area for *C. maxima* was 100 × 100 cm (10000 plants/ha) and for *C. pepo* 80 × 80 cm (15625 plants/ha). The pumpkins were grown on colluvial-diluvial soil, the most common type of soil in the Republic of Macedonia. It has a poor supply of humus and total nitrogen, good supply of available phosphorus in the surface layer and high supply of potassium throughout the profile. The soil reaction (pH) is low. The results of the agrochemical analysis were interpreted in compliance with soil classification presented by Filiposki [22]. In autumn, the soil was plowed and fertilized with 600 kg/ha NPK mineral fertilizer (nitrogen : phosphorus : potassium = 8 : 22 : 20), and in spring with 300 kg/ha CAN (27 % calcium ammonium nitrate). The average temperature from April to September was 17.7 ºC. The total amount of precipitations in 2009 was 235 mm/m^2. Irrigation was applied several times, when necessary. Pumpkin seeds were hand-collected from the gourd and washed with tap water, then air-dried for two weeks. Dry seeds were shelled by cracking with a small iron rod and manually peeled to separate kernels and shells. The count showed 236 seeds of cv. 1 and 691 seeds of cv. 2 in 100 g of collected seed. The average weight per seed after six measurements was 0.486 g for cv. 1 and 0.153 g for cv. 2. The cv.1 kernels were thick and green, and cv. 2 kernels were flat and pale. The determined weigh fractions of kernel and shell seed were 58.1 % and 41.9 % for cv. 1 and 80.1% and 19.9 % for cv. 2, respectively. The dried seed were grounded using Retsch ZM1 mill (Germany), 0.25 mm sieve.

2.1.2. *Chemicals*

For the extraction of pumpkin seeds, pro-analysis-grade solvents *n*-hexane, diethyl ether, benzene, dichloromethane, ethyl acetate, methanol and ethanol were purchased from Merck (Germany). Preparation of the fatty acid methyl esters (FAMEs) was done with analytical grade methanol, diethyl ether and cyclohexane purchased from JT Baker (Deventer, Holland), toluene and anhydrous sodium sulphate from Fluka (Buchs, Switzerland) and *n*-hexane and ethyl acetate from Carlo Erba (Rodano, Italy). Silica gel 60 was supplied from Macherey-Nagel (Düren, Germany). Fatty acid methyl ester standard FAME MIX 18910 1-AMP, containing methyl esters of caprylic (C8:0), capric (C10:0), lauric (C12:0), tridecanoic (C13:0), myristic (C14:0), myristoleic (C14:1), pentadecanoic (C15:0), palmitic (C16:0), palmitoleic (C16:1), margaric (C17:0), stearic (C18:0), elaidic (C18:1 trans), oleic (C18:1 cis), linoleic (C18:2), linolenic (C18:3), arachidic (C20:0), gondoic (C20:1), behenic (C22:0) and erucic (C22:1) acids was purchased from Supelco (Bellefonte, USA). Trimethylsilyl ethers were prepared with analytical grade methanol, *tert*-butyl methyl ether and dichloromethane purchased from JT Baker (Deventer, Holland) and ethyl acetate from Carlo Erba (Rodano, Italy). Anhydrous pyridine and anhydrous sodium sulphate were supplied from Fluka Chemie AG (Buchs, Switzerland) and derivatizing agent *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) from Macherey-Nagel (Düren, Gemany). Silica gel, 75-150 micron particle size, was produced by Analtech (Newark, USA). Cholesterol (cholest-5-en-3β-ol) standard was supplied from ABCR GmbH&Co. (Karlsruhe, Germany), stigmasterol (24α-ethylcholesta-5,22-dien-3β-ol) standard from MP Biomedicals (Eschwege, Germany), β-sitosterol (24α-ethylcholest-5-en-3β-ol) standard from Calbiochem (Darmstadt, Germany), betulin (lup-20[29]-ene-3β,28-diol) internal standard from Sigma-Aldrich (Steinheim, Germany), α-tocopherol standard from Fluka Chemie AG

(Buchs, Switzerland) and γ-tocopherol standard from Supelco (Bellefonte, USA).

2. 2. *Extraction of pumpkin seed*

For the extraction of pumpkin seed Soxhlet procedure was used (AOAC 1995, 920.85). A 10 g of seed (0.0001 g accurately weighed, 0.25 mm particle size) was extracted in the presence of 2–3 boiling glass regulators by using following pro-analysis-grade solvents: *n*-hexane, diethyl ether, benzene, dichloromethane, ethyl acetate, methanol and ethanol. After 4 h extraction, the solvent was released from the product into rotary vacuum evaporator (35 °C, 100 mPa). The solvent traces were removed by drying at 40 ºC and 105 mPa followed by cooling in a dessicator and weighed. The steps of drying, cooling and weighing were repeated until the difference between two consecutive weights was smaller than 2 mg. The yield of extract was estimated based on both pumpkin seed weight and dry matter (DM) weight in pumpkin seed used for extraction.

2.3. *Characterization of pumpkin seed and seed extract*

2.3.1. *Pumpkin seed analysis*

The dry matter content was determined by drying at 105 ºC till constant mass (AOAC, 925.10), and the ash content by burning at 900 ºC till constant mass (AOAC, 923.03) [23]. The proteins content was determined from the nitrogen content by Kjeldahl method (AOAC, 978.04) using factor 6.25, and calculated as N × 6.25 [23]. The content of crude fibres was determined according to the gravimetric procedure of AOAC (920.860) [23]. Total and reductive sugars were determined by Bertrand method [24].

2.3.2. *Physico-chemical characterization of pumpkin seed extracts*

The specific gravity (920.212), refractive index (921.08), acid value (940.28), peroxide value (965.33), saponification value (920.160) and iodine value (993.20) of the oil samples were determined according to the AOAC [23].

2.3.3. *Determination of the fatty acid composition*

Prior to GC/MS analysis, the samples were transesterified to FAMEs with sodium metoxide [25]. 100 mg of extract was transesterified with freshly prepared 0.28 mol/L solution of sodium methoxide in methanol. Reaction mixture was stirred with magnetic stirrer and heated using water bath at 75 °C, for 20 min. After transesterification, saturated sodium chloride solution was added and esters were extracted with diethyl ether and distilled water. Prepared sample was dried with anhydrous Na_2SO_4 and filtered. The solvent was evaporated using rotary vacuum evaporator (35 °C). The clean-up was done on the silica gel column. It was prepared in Pasteur pipette by placing the plug of glass wool, then adding silica gel activated at 120 °C and anhydrous sodium sulfate at the top. It was conditioned with cyclohexane and then the sample was transferred to the top of the column. FAMEs were eluted from the column with the solution of cyclohexane/ethyl acetate mixture (2:1, v/v). Toluene was added to the sample and then solvents were evaporated in the rotary vacuum evaporator (50 °C, 150 mPa) to the volume of approximately 1 ml. The sample was transferred to the 2 ml vial, evaporated in the stream of nitrogen to the dry residue and additionally dried in heating cabinet at 40 °C for 30 min. For the GC/MS analysis, the sample was diluted with *n*-hexane to obtain a concentration of 1 mg/ml sample solution. The fatty acid composition of the extract was determined using Thermo Finnigan Trace GC unit furnished with an Optima 240 capillary column (60 m × 0.25 mm i.d. × 0.25 μm film thickness). Oven temperature was programmed as follows: 80 ºC at the start, 20 ºC/min to 120 ºC, 3 ºC/min to 240 ºC that held for 10 min. 1.5 ml/min He constant flow was applied. 1 μl of

the sample was injected by Thermo Finnigan AS 2000 autosampler. A PTV injector was used with 10 : 1 split ratio, at initial temperature of 60 ºC and heated up to 280 ºC. The Finnigan Trace mass selective (MS) detector coupled to GC *via* transfer line set at 250 ºC worked with ion source temperature of 220 ºC and electron impact mode of 70 eV during the full scan mode run. Each sample was analyzed in triplicate. The response factors were obtained using standard FAME solution as external standard.

2.3.4. *Sterol and tocopherol content determination*

A slightly modified procedures proposed by Mandl *et al*. [8] and Butinar *et al*. [26] were applied for sterol and tocopherol content determination, respectively.

Saponification and clean-up procedure. Pumpkin seed extract, enriched with known amounts of betulin (5 mg/ml, used as an internal standard) and cholesterol (5 mg/ml, used for recovery determination), was dissolved in dichloromethane, saponified with 1 ml potassium hydroxide solution (20 g KOH in 88 ml methanol with 12 ml deionized water to limit the transesterification to methyl esters) for 45 min at 70 °C in a 10 ml screw-capped reaction vial. The end of the reaction was indicated by the clearing of the two-phase oil-methanol/water system. A glass column with glass-wool frit at the bottom was dry-filled with anhydrous sodium sulfate as the lowest layer and silica gel as a second layer. The total saponified oil mixture, adsorbed on silica gel, was packed on top to form the third layer of this sandwich-type sample preparation column. The unsaponifiable fraction was eluted from the silica column with mixture of *tert*-butyl methyl ether and ethyl acetate (1 : 1, *v*/*v*), while the potassium salts of the fatty acids were retained on the column. An aliquot of eluted unsaponifiable fraction was submitted to derivatization/silylation.

Derivatization to trimethylsilyl (TMS) ethers. The aliquot, transfered to a 2 ml vial, was evaporated in a stream of nitrogen. The dry residue was treated with 50 µl of a mixture of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and dry pyridine (2 : 1, *v*/*v*) and 400 µl of *tert*-butyl methyl ether. The vial was heated at 70 °C for 2 h and 1 µl of this mixture was analyzed by GC/MS system.

Preparation of standard solutions. For quantitative determination of sterols in extract samples by internal standard method, five standard solutions of cholesterol, stigmasterol and *β*-sitosterol that ranged in concentrations 0.008–0.260 mg/ml, were prepared by transferring appropriate volumes of the stock solutions (in pyridine) of cholesterol, stigmasterol and *β*-sitosterol, together with the constant volume of the internal standard stock solution (betulin), to 2 ml screw cap vials. For external method calibration of tocopherols, five standard solutions, concentrations between 0.010 and 0.250 mg/ml, were also prepared by using appropriate volumes of the stock solutions of α- and γ-tocopherols.

After addition of 40 µl of MSTFA, the solutions were heated at 70 °C for 2 h, diluted with appropriate amount of *tert*-butyl methyl ether and analyzed by GC/MS.

GC/MS analysis. The sterol and tocopherol content in the extracts were determined by GC/MS, using a Thermo Finnigan Trace GC unit furnished with TR-50MS capillary column: 30 m × 0.25 mm i.d. × 0.25 µm film thickness. The oven working temperature was programmed as follows: 70 °C at the start, held for 1.5 min, raised at 40 °C/min to 245 °C, held for 1.5 min, increased to 280 °C at 2 °C/min and held for 10 min. The constant He flow rate was 1.5 ml/min. 1 µl of the sample was injected by the Thermo Finnigan AS 2000 autosampler. A PTV injector was used with the splitless mode, at an initial temperature of 55 °C and heated up to 250 °C. The Finnigan Trace mass selective (MS) detector, coupled to GC *via* transfer line set at 290 °C, was operated with an ion source temperature of 220 °C. Electron impact (EI) mass spectra were obtained at acceleration energy of 70 eV and a scan time of

1 s. Spectrum acquisition was performed in the full scan mode (in the range *m/z* 50–600), to confirm the retention times of target compounds, and in the SIM scan mode for their quantitative analysis. The response factors were determined using mixtures of sterol standards with betulin as internal standard and tocopherol standards. Data were collected and analyzed by Excalibur software (Thermo Finnigan). Each peak was analyzed *via* detection of the parent molecular ion and the fragmentation pattern of the TMS derivative. In addition to the presence of specific ion fragments, the relative intensity of the ion fragments was considered. Some sterol TMS ethers were identified by application of the NIST mass spectra library. The MS library search was performed by a PBM (Probability–Based Matching) algorithm.

Sterol and tocopherol quantitation. Sterols were quantified using an internal standard method. Calibration curves for cholesterol, stigmasterol and *β*-sitosterol were plotted. In the absence of suitable standards, campesterol, desmosterol and Δ7-sterols were quantified using the calibration plot of the nearest eluted sterol. External standard method was used for α- and γ-tocopherol quantification.

Method validation. The reliability of the method was verified by determination of accuracy and precision. The accuracy was determined by measuring the recovery; pumpkin seed extract samples were spiked with 300 mg cholesterol/100 g pumpkin seed extracts before the saponification. The precision was determined by means of replicate tests; each pumpkin seed extract sample was analyzed in triplicate.

3. RESULTS AND DISCUSSION

3.1. *Pumpkin seed composition*

Chemical composition of *C. maxima* and *C. pepo* whole seed, kernel and shell is presented in Table 1. Generally, it can be observed that content of ash, total nitrogen, proteins, total sugars and reductive sugars expressed in relation to the corresponding dry matter was higher in the *C. pepo* seed. In both of the investigated varieties of pumpkin seed, the highest content of proteins as the main nutritional relevant component of seed was determined in the kernel (375.9 g/kg in *C. maxima* and 395.4 g/kg in *C. pepo*). The high protein content is suitable for fortification of food. The literature data of higher proteins and ash contents, and smaller concentration of crude fibres, compared with the results obtained in our investigation of *C. maxima* [1, 3]. The results of nutritive status of *C. pepo* correspond to the determination results reported by Al-Khalifa [4], Younis *et al*. [2] and El-Adawy *et al*. [27]. However, Glew *et al*. reported 120 g/kg proteins content in *Cucurbita* spp. [28].

T a b l e 1

Chemical composition (g/kg) of pumpkin seed

Properties	*C. maxima*			*C. pepo*		
	Whole seed	Kernel	Shell	Whole seed	Kernel	Shell
Dry matter	948	974	947	940	955	945
Moisture	52	23	53	60	45	55
Ash*	37.1	10.0	10.5	46.9	63.5	32.1
Total nitrogen*	45.2	58.6	31.3	50.9	55.6	34.7
Proteins*	247.8	375.9	191.6	244.8	395.4	211.6
Crude fibres*	151.2	25.0	69.6	128.8	34.5	55.4
Total sugars*	24.9	27.7	4.1	26.7	32.1	5.0
Reductive sugars*	11.0	13.3	5.0	16.5	21.8	3.3

*Calculated to the corresponding dry matter weight (DM).

3.2. *Pumpkin seed extracts*

3.2.1. *Extract yields*

The color of the extracts obtained from *C. maxima* and *C. pepo* whole seed was brownish yellow and olive green, respectively. In Table 2 are given the yields of extracts obtained using different extraction solvents. Extract yields obtained from *C. pepo* seed were higher than those from *C. maxima*, for all solvents used. The highest extract yields of 358.6 g/kg DM and 429.2 g/kg DM for *C. maxima* and *C. pepo*, respectively, were obtained using n-hexane as non polar solvent. The extraction of whole pumpkin seed with solvents of high polar index such as methanol, ethanol and ethyl acetate gave lower extract yields in comparison with the solvents of lower polarity.

T a b l e 2

Extract yields (g/kg DM) obtained from C. maxima and C. pepo whole seeds*

Solvent	Extract yield	
	C. maxima	*C. pepo*
n-Hexane	358.6	429.2
Diethyl ether	310.0	354.2
Benzene	211.1	293.7
Dichloromethane	213.1	270.8
Methanol	80.2	325.4
Ethyl acetate	88.6	104.1
Ethanol	57.1	59.2

*DM – dry matter weight

The yields of extracts obtained by extraction of different parts of the seed, using *n*-hexane, diethyl ether and benzene, are shown in Table 3. Generally, yields obtained from *C. pepo* were higher than those from *C. maxima*, regardless of the part of the seed subjected to extraction and the solvent used. For *C. maxima*, the highest yield of extract of 388.2 g/kg DM was obtained from kernel when *n*-hexane was used. That yield is, however, lower than the results reported in the literature [1, 3]. And conversely, for *C. pepo* investigated in this work, the extract yield obtained from kernel with *n*-hexane (487.4 g/kg DM) was higher than the yield of 219 g/kg DM reported by Younis *et al*. [2] for *C. pepo* grown in low land (600–700 m). Our results are comparable with the results obtained by the quoted authors, but for pumpkins grown on higher altitudes (2100–2400 m) and under lower maximum and minimum average temperatures. The extract yields obtained in this work from *C. pepo* whole seed and kernel, when *n*-hexane was used, were also higher than the yields (417.4 g/kg DM and 368.9 g/kg DM, respectively) reported by Nakić-Neđeral *et al*. [29]. Murkovic *et al*. [6] and El-Adawy *et al*. [27], however, reported around 500 g/kg DM and 510.1 g/kg DM crude oil, respectively, obtained from *C. pepo* whole seed.

T a b l e 3

Extract yields (g/kg DM) obtained from C. maxima and C. pepo seeds*

Pumpkin seed		Extract yield obtained by		
		n-Hexane	Diethyl ether	Benzene
C. maxima	Whole seed	358.6	310.0	211.1
	Kernel	388.2	280.6	250.6
	Shell seed	31.7	42.4	33.8
C. pepo	Whole seed	429.2	354.2	293.7
	Kernel	487.4	387.4	376.6
	Shell seed	65.5	49.2	44.4

*DM – dry matter weight

3.2.2. *Physico-chemical characteristics of the extracts*

Chemical and physical properties of *C. maxima* and *C. pepo* whole seed and kernel extracts obtained with *n*-hexane are presented in Table 4. The refractive index for *C. maxima* and *C. pepo* extracts varied from 1.470 to 1.473. Specific gravity was around 0.917. The iodine, saponification and peroxide values were higher for the extracts of *C. pepo* seed.

T a b l e 4

Physico-chemical characteristics of the pumpkin seed extracts obtained with n-hexane

Property	Extract			
	C. maxima		*C. pepo*	
	Whole seed	Kernel	Whole seed	Kernel
Refractive index at 25 ºC	1.471	1.472	1.470	1.473
Specific gravity at 25 ºC	0.916	0.918	0.917	0.918
Iodine value (g I_2/kg extract)	119.3	101.5	150.6	139.0
Saponification value (mg KOH/g extract)	187.97	189.40	191.34	201.20
Acid value (mg KOH/g extract)	4.07	3.82	4.71	4.02
Peroxide value (meq O_2/kg extract)	4.93	4.26	6.06	5.70

For *C. maxima* oil, Alfawaz determined refractive index of 1.4656, specific gravity of 0.913, iodine value of 105.12 g I_2/kg oil and saponification value of 185.20 mg KOH/g oil [1]. For *C. pepo* oil extracted by mixture of chloroform-methanol, Al-Kalifa [4] reported the following physico-chemical characteristics: 1.4710 refractive index of 1.4710 at 30 ºC, 0.9280 specific gravity of 0.9280 at 60 ºC, acid value of 6.5, saponification value of 215.0 and iodine value of 111.5. Tsaknis *et al*. [7] reported peroxide values of 9.20 and 9.04 meq for *C. pepo* crude and purified seed oil, respectively. The data given in Table 4 are in good agreement with the values for physico-chemical characteristics of *C. pepo* oil reported by Younis *et al*. [2] and El-Adawy *et al*. [27], as well as with limitations legislated by the Codex Alimentarius Commission [32]. For some kinds of oils, the permitted maximum peroxide level is 10 meq peroxide oxygen per kg of oil and maximum acid value is 10 mg KOH/g oil.

3.2.3. *Fatty acid composition of extracts*

The dominant fatty acids (FA) identified in *C. maxima* and *C. pepo* seed extracts are palmitic (C16:0), stearic (C18:0), oleic (C18:1 cis), linoleic (C18:2) and linolenic (C18:3) (Table 5).

In almost all cases, higher contents of palmitic, stearic, linoleic and linolenic acids were determined in the extracts of *C. maxima* than in *C. pepo* extracts, while the oleic acid content was higher in *C. pepo* extracts. The ratio of the linoleic and oleic acid was almost two times higher in *C. maxima* seed extracts. Elaidic acid (C18:1 trans) was not determined in any extract. The linoleic acid content in *C. maxima* whole seed extract depends on the solvent used and is 51.82, 51.13, 49.06 and 52.13 % for *n*-hexane, diethyl ether, benzene and dichloromethane, respectively. In the *C. pepo* whole seed extracts the linoleic acid content ranged from 40.22 to 45.05 %, depending on the solvent used. As for the fatty acid content, our data are in agreement with the literature data. Applequist *et al*. [30] reported that linoleic acid is 40.4–57.2 % of the total lipophylic extract weight in *C. pepo* and 43.1–50.3 % in *C. maxima*. In *C. maxima* seed oil, Alfawaz [1] determined 18.14 % oleic acid, 53 % linoleic acid and 1.27 % linolenic acid. The fatty acid composition may vary depending on the climatic conditions. When the temperature is lower during the last weeks of seed filling, there will be a shift in content from oleic to linoleic acid. Linoleic acid content is always higher in localities where lower average temperature prevails [2, 5]. Younis *et al*. [2] reported that in the *C. pepo* extract content of oleic and palmitic acid decreased as the average growing temperature decreased. Also, it is confirmed that increased linoleic acid content is followed by decreased

Table 5

Total fatty acid content (g/kg extract) and fatty acid composition (% of total fatty acid content) in C. maxima (cv. 1) and C. pepo (cv. 2) seed extracts

Extract			Total FA content* (g/kg)	Fatty acid composition (%)														
				12:0	14:0	14:1	16:0	17:0	18:0	18:1 cis	18:2	18:3	20:0	22:0	18:2/ 18:1	SFA	MUFA	PUFA
n-Hexane	cv 1	whole seed	533.72	0.34	0.3	0.04	10.48	0.34	7.86	23.47	51.82	4.22	1.01	0.12	2.21	20.46	23.51	56.03
		kernel	596.29	0.31	0.33	0.04	12.02	0.32	7.15	22.04	52.77	4.01	0.87	0.13	2.39	21.14	22.08	56.78
	cv 2	whole seed	585.26	0.33	0.31	0.11	10.88	0.32	5.03	36.77	41.42	3.81	0.90	0.11	1.13	17.89	36.88	45.23
		kernel	678.09	0.22	0.22	0.04	10.31	0.22	6.11	41.44	38.15	2.51	0.68	0.10	0.92	17.86	41.47	40.64
Diethyl ether	cv 1	whole seed	481.88	0.40	0.38	00.07	11.25	0.37	6.92	22.49	51.13	5.83	1.00	0.15	2.28	20.49	22.55	56.96
	cv 2	whole seed	558.24	0.20	0.20	0.04	9.94	0.23	5.00	35.55	45.05	2.88	0.74	0.17	1.27	16.48	35.59	47.93
Benzene	cv 1	whole seed	358.28	0.53	0.44	0.08	11.06	0.48	6.60	24.94	49.06	5.39	1.33	0.07	1.97	20.52	25.02	54.46
	cv 2	whole seed	406.83	0.38	0.34	0.09	9.65	0.34	4.68	35.77	42.41	5.22	0.95	0.18	1.19	16.51	35.86	47.63
Dichloromethane	cv 1	whole seed	536.66	0.35	0.33	0.06	12.51	0.33	7.67	21.49	52.13	3.99	0.95	0.19	2.43	22.33	21.55	56.12
	cv 2	whole seed	568.79	0.32	0.31	0.07	10.67	0.33	5.40	37.42	40.22	4.13	0.90	0.25	1.07	18.16	37.49	44.35

*Values are given as the mean of each sample individually analyzed in triplicates.

oleic acid content [2, 5]. Data for linoleic and oleic acid given in Table 5 are comparable with the data obtained for extracts of pumpkin seed cultivated under lower average temperature conditions reported by Younis *et al*. [2]. Nakić-Neđeral *et al*. [29] reported, for *C. pepo*, that content of linoleic acid was higher in whole seed oil than in kernel oil, what was also confirmed in our work. The content of oleic acid in our *n*-hexane lipophylic extracts of whole seed and kernel of *C. pepo* (Table 5) was higher than content determined by Nakić-Neđeral *et al.* [29]. Murkovic *et al.* [5] determined higher linoleic acid content (around 60 %) in the extract of the European variety of *C. pepo* obtained by petrol ether (40–60 ºC), which corresponds to the linoleic content results (55.6 %) reported by El-Adawy *et al*. [27]. Al-Khalifa [4], using chloroform-methanol mixture during extraction, determined 43.1 % of linoleic acid in *C. pepo* seed lipophylic extract. In our work, the linolenic acid content in *C. maxima* and *C. pepo* whole seed extracts obtained with *n*-hexane was determined as 4.22 and 3.81 %, respectively. In the available literature smaller linolenic acid contents were reported [1, 2, 31].

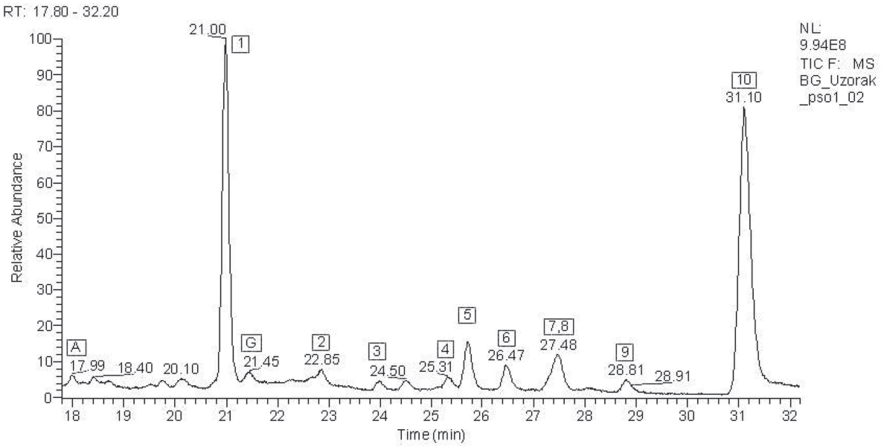
The content of unsaturated fatty acids (UFA), shown in Table 5 as monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), was higher in *C. pepo* extracts than in *C. maxima* extracts. Depending on the solvent used for the extraction of whole seed, UFA contents ranged between 82.11 and 83.52 % for *C. pepo* extracts, and between 77.67 and 79.54 % for *C. maxima* extracts. The content of MUFA was higher in *C. pepo* extracts obtained from whole seed. So, the ratio of MUFA to PUFA was higher in *C. pepo* whole seed extracts. As a measure of nutritional value, the ratio of PUFA to SFA in *C. maxima* seed extracts varied from 2.51 to 2.78, depending on the extraction solvent used. In the *C. pepo* whole seed extracts the PUFA/SFA ratio was higher when diethyl ether and benzene were used (Table 5). Our results for the SFA and UFA content in the extracts are within the range reported in the literature [1, 2, 30]. Murkovic *et al.* reported 2.81 PUFA/SFA ratio [5].

3.2.4. *Sterol and tocopherol content in extracts*

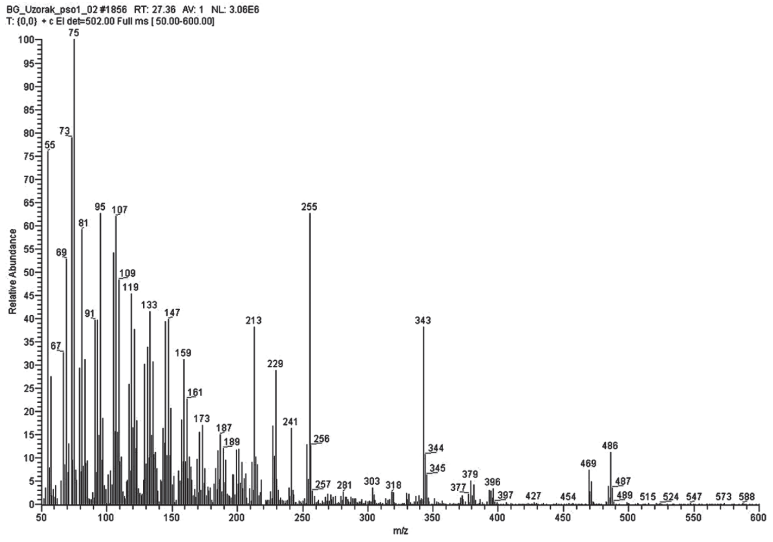
For sterol quantification, calibration with the reference substances stigmasterol and *β*-sitosterol with betulin as internal standard was performed. Chromatogram of *C. maxima* seed extract spiked with standard solution of cholesterol and betulin is shown in Figure 1(a). Each peak was evaluated via detection of the parent molecular ion and fragmentation pattern of the TMS derivatives. Peak 7,8 was identified as overlapped peaks of Δ7-stigmastenol and Δ7,25-stigmastadienol. The Δ7-stigmastenol spectrum, given in Figure 1(b), and Δ7,25-stigmastadienol spectrum, given in Figure 1(c), were distinguished by the molecular ion of *m/z* 484 for Δ7,25-stigmastadienol and *m/z* 486 for Δ7-stigmastenol, while the fragmentations of $[M\text{-}ROH]^+$ (*m/z* 394), $[M\text{-}Me\text{-}ROH]^+$ (*m/z* 379), $[M\text{-}SC]^+$ (*m/z* 345) and $[M\text{-}SC\text{-}ROH]^+$ (*m/z* 255), where R, Me and SC refers to the $(CH_3)_3Si$, CH_3 and side chain, respectively, were the same for both compounds.

Total sterol content of the analyzed pumpkin seed extracts is summarized in Table 6 where the contents of Δ5- and Δ7-sterols are also given. The predominant sterols in these extracts were Δ7-sterols. In *C. maxima* extracts, spinasterol and Δ7-stigmastenol with Δ7,25-stigmastadienol constituted around 60 % of the total sterols. In the *C. pepo* whole seed and kernel extracts, however, the quantity of spinasterol, Δ7,22,25-stigmastatrienol, Δ7-stigmastenol and Δ7,25-stigmastadienol, expressed to the total sterol amount, is 64 % and 70 %, respectively. In regard to the Δ7-avenasterol content, it is about 1.8 times higher in *C. pepo* than in *C. maxima* extracts. The amount of Δ5-sterols in whole seed extracts is almost the same for *C. maxima* and *C. pepo*, and about 2 times higher than Δ5-sterols content in the kernel extracts of both investigated varieties. From all Δ5-sterols determined in all pumpkin seed extracts, *β*-sitosterol is present in the highest quantity. Extracts obtained from *C. maxima* seed had higher total sterol content than the extracts ob-

a)



b)



c)

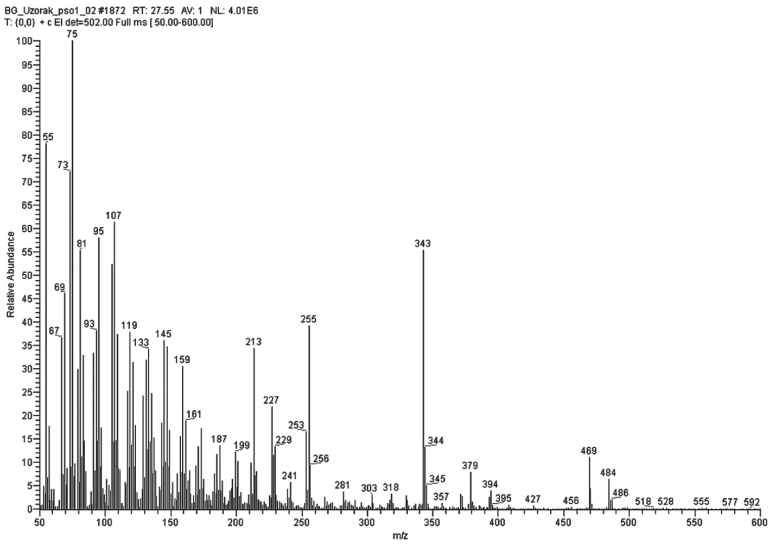


Fig. 1. Chromatogram of unsaponifiable fraction of *C. maxima* seed extract
(a): *G* γ-tocopherol, *1* cholesterol, *A* α-tocopherol, *2* desmosterol, *3* stigmasterol, *4* β-sitosterol, *5* spinasterol, *6* Δ7,22,25-stigmastatrienol, *7,8* Δ7-stigmastenol and Δ7,25-stigmastadienol, *9* Δ7-avenasterol, 10 betulin.
(b): Recorded EI mass spectrum of derivatized Δ7-stigmastenol.
(c): EI mass spectrum of derivatized Δ7,25-stigmastadienol

Table 6

Sterol content (mg/kg extract) in pumpkin seed oil extracted with n-hexane*

Sterol	C. *maxima*		C. *pepo*	
	Whole seed	Kernel	Whole seed	Kernel
Desmosterol	84.47 ± 1.68	89.10 ± 0.59	99.10 ± 2.06	90.19 ± 1.28
Stigmasterol	144.93 ± 4.51	49.85 ± 0.85	159.26 ± 2.63	52.04 ± 0.88
β-Sitosterol	382.77 ± 6.11	121.91 ± 2.44	360.31 ± 4.95	117.54 ± 3.11
Spinasterol	809.34 ± 2.41	705.32 ± 7.86	618.52 ± 5.57	521.99 ± 7.54
Δ7,22,25-stigmastatrienol	474.54 ± 2.33	353.29 ± 3.05	579.16 ± 4.45	493.96 ± 7.28
ΣΔ7-stigmastenol + Δ7,25-stigmastadienol	922.81 ± 1.17	749.20 ± 5.87	568.76 ± 4.44	538.81 ± 7.56
Δ7-avenasterol	227.91 ± 5.93	209.29 ± 1.20	368.53 ± 2.37	379.04 ± 5.84
Δ5-sterols	612.17 ± 11.23	260.86 ± 2.87	618.67 ± 10.65	259.77 ± 6.28
Δ7-sterols	2434.60 ± 6.34	2017.10 ± 17.93	2134.97 ± 16.82	1933.80 ± 28.21
Total	3046.77 ± 16.03	2277.96 ± 15.61	2753.64 ± 6.18	2193.57 ± 21.83

*Values are given as the mean ± standard deviation of each sample individually analyzed in triplicates

tained from *C. pepo*. The total sterol content in extracts of both varieties was higher in whole seed than in kernel extracts; precisely, total sterol content of 3046.77 mg/kg was measured in *C. maxima* whole seed extract, and 2277.96 mg/kg in kernel extract, while in *C. pepo* it was 2753.64 and 2193.57 mg/kg in whole seed and in kernel extract, respectively. Recovery of cholesterol was in the range from 93.03 to 99.38 %. Nakić-Neđeral *et al*. [29] determined total sterol content of 3852 mg/kg and 3172 mg/kg for *C. pepo* whole seed oil and kernel oil, respectively. Murkovic *et al*. [11] determined sterol content of 4030 mg/kg in *C. pepo* whole seed oil.

Table 7

Tocopherol content (mg/kg extract) in pumpkin seed oil extracted with n-hexane*

Tocopherol	C. *maxima*.		C. *pepo*	
	Whole seed	Kernel	Whole seed	Kernel
α-Tocopherol	38.04	35.73	38.59	27.91
γ-Tocopherol	83.20	81.82	115.20	89.90
Total	121.24	117.55	153.79	117.81

*Values are given as the mean of each sample analyzed in triplicate.

Peaks of γ- and α-tocopherol with retention times 17.99 and 21.45 min, respectively, are obvious in Figure 1(a). Both peaks were detected by standards and confirmed by NIST mass spectra library with the relative match of around 95 %. For both investigated varieties tocopherol content was higher in the extracts obtained with *n*-hexane from whole pumpkin seed than from kernel (Table 7). Slightly higher content of tocopherols was determined in *C. pepo* whole seed extract compared to *C. maxima*. The determined amounts of tocopherols in oil extracted from 100 breading lines of *C. pepo* investigated by Murkovic *et al*. [6] varied from 0 to 91 mg/kg and from 41 to 620 mg/kg for α- and γ-tocopherol, respectively. It is also reported that the content of α- and γ-tocopherol in the *C. pepo* oil was 37.5 and 383 mg/kg, respectively [11]. François *et al*. [12] reported concentration of α- and γ-tocopherol in raw *C. pepo* seed oil of 76.9 and 964 mg/kg, respectively. In the seed of African *C. pepo*, however, the α-tocopherol content was determined as around 30 mg/kg [2]. Murkovic and Pfannhauser [9] reported content of α-tocopherol in the range of 19.9 to 78.7 mg/kg, and γ-tocopherol content from 52.3 to 644 mg/kg. The total to-

copherol content in the analyzed samples of *C. pepo* was significantly higher in the oils obtained from husk than in those obtained from naked seed [29]. The mean values of total tocopherols in industrial oils obtained from *C. pepo* whole seed and kernel were reported as 709 and 520 mg/kg, respectively [29]. The tocopherol content in the oil obtained from twelve pumpkin cultivars ranged from 21.1 to 75.1 mg/kg for α-tocopherol and from 74.0 to 492.8 mg/kg for γ-tocopherol [31]. Although the data in the literature regarding tocopherol content in pumpkin seed oils varied depending on the authors, our results are mostly within the reported ranges.

4. CONCLUSIONS

The results of this study show presence of chemical constituents with positive nutritional and health properties in the seed and in the seed extracts obtained from *C. maxima* and *C. pepo* cultivated in the Republic of Macedonia. Both cultivars have a fairly high oil content with high levels of unsaturated fatty acids, mainly oleic and linoleic, and absence of the elaidic (C18:1 trans) acid. The potential health benefit of pumpkin seed oil consumption is thus confirmed. Moreover, the high content of tocopherols and sterols in both of the investigated pumpkin varieties is very important. Owing to the effect of these compounds on pumpkin seed oil quality, their concentrations and the varietal differences ought to be taken into consideration when planning cultivar choice for cultivation in the Republic of Macedonia. The results can be exploited in industrial edible oil production. It is also hoped that the information gathered will be a valuable contribution in the determination of the genotypic and phenotypic variations of pumpkins from different regions in the world.

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