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THE INFLUENCE OF EXTRACTION PROCESS PARAMETERS OF SOME BIOMATERIALS PRECURSORS FROM *HELIANTHUS ANNUUS*

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Solvent extraction, microwave-assisted extraction (MAE) and ultrasonic extraction (UE) was used for to extract phenolic compounds and amino acid from sunflower seeds. The influence of extraction process parameters (time, temperature, and the type of solvent (water and methanol) on the isolation of biological compounds was studied. The obtained extracts were qualitatively evaluated by chromatographic methods: HPLC, GC-MS, and spectroscopic methods: FT-IR, UV-VIS spectroscopy. It was shown that by microwave extraction were isolated free amino acids and phenolic compounds.

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Keywords: Ultrasonic and microwave assisted extraction technique, Sunflower seeds, Antioxidant activity

1. Introduction

Sunflower (*Helianthus annuus*) is an annual plant intensively cultivated as traditional oil crop being considered one of largest sources of vegetable oils. However, sunflower is represent a well-known herb used as antiinflammatory, cathartic, diuretic, emollient, expectorant, stimulant, vermifuge, antimalarial, anti-asthmatic, anti-oxidant, anti-tumor and antimicrobial agent and for cosmetics. In folk medicine, the sunflower seed are used in therapy of pulmonary disorders (sinusitis, bronchitis, pleuritis, laryngitis), different other types of infection (eye infections, whitlow, etc), thrombophlebitis, abscess, catarrh, blindness, diarrhea, dysentery, dysuria, rheumatism, hemorrhoids, fever, toothache, menorrhagia, scorpion stings, snakebite, inflammation, urogenital ailments, splenitis, aroma therapy, leg ulcer, etc. [1].

Plant represents an important source of oleic acid and linoleic acid, vitamins (E and B_1 vitamin B_5 and folate), alkaloids, glycosides, saponins, cardiac glycosides, tannins, phenolic, quercimeritrin, anthocyanin, cholin and betain and minerals (manganese, magnesium, copper, selenium, phosphorus) [2]. Phenolic compounds, the most important bioactive compounds from plant sources, are among the most potent and therapeutically useful bioactive substances, providing health benefits associated with reduced risk of chronic and degenerative disease. Recent research was focused on extraction of antioxidants from plants. The phenolic acids (chlorogenic acid or 5-*O*-caffeoyl-quinic acid, caffeic (4-hydroxycinnamic acid), gallic and p-coumaric acid are well known as bioactive classes of molecules with highly antitumoral, antibacterian, anti-inflammatory, antipyretic, antifungal and analgesic activity due to their antioxidant effect [3]. Another important property of these remarkable compounds consists in the prevention against categories of pathologies with high mortality rates such as cancer, cardiovascular diseases [3, 7-9]. Previous studies have shown that sunflower represent an important source not only of fats but also amino acids such as glutamic acid, methionine, tyrosine, histidine, cysteine, threonine, glycine, isoleucine, phenylanine, valine, proline aspartic acid, serine, alanine, leucine and lysine and different polyphenols especially chlorogenic acid, caffeic acid and quinic acid [10-11]. It is very

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important to develop selective extraction methods because the low amount of bioactive compounds is in herbs [12].

Extraction is one of the most imperative steps in the evaluation of phenolic compounds from plant. The capability of a number of extraction techniques have been investigated, such as solvent extraction [13] and enzyme-assisted extraction [14]. However, these extraction methods have drawbacks to some degree [15]. For example, solvent extraction is time consuming and enzyme in enzyme assisted extraction is easy to denature [16]. Moreover, the solvent extraction may induce the thermal degradation of a majority of plant bioactive constituents [17-20]. Depending on the extraction procedure, proteinaceous products from sunflower retain different amounts of phenolic compounds, especially chlorogenic acid, which are virtually impossible to be totally removed due to their strong interactions with proteins [21].

Ultrasonic is one of the most industrially used methods to enhance mass transfer phenomena [22-23]. Ultrasound assisted extraction is very efficient extraction procedure. Sonication induces cavitation, the process in which bubbles with a negative pressure are formed, grown, oscillated, and may split and implode. By this process different chemical compounds and particles can be removed from the matrix surface by the shock waves generated when the cavitation bubbles collapse. The implosion of the cavities creates microenvironments with high temperatures and pressures. Shock waves and powerful liquid micro jets generated by collapsing cavitation bubbles near or at the surface of the sample accelerate the extraction [24]. Meanwhile, microwave assisted extraction heats the extracts quickly and significantly accelerates the extraction process [25].

MAE is a rapid and effective extraction technique compared with traditional extraction techniques and has been applied to extract biological active compounds from different matrices [26]. Comparing with other modern extraction techniques such as supercritical fluid extraction and pressurized liquid extraction, MAE is easy to use and the systems are cheaper [27]. However, abundant organic solvents used are problematic in the extraction/separation of biological active compounds from the herb because of their toxicity, volatility and flammability [26]. This paper explores the possibility of using microwave and ultrasonic assisted extraction compared with a classical technique for separation of polyphenolic compounds and amino acid from sunflower seeds. These compounds with biological activity can be further used for the development of multifunctional biomaterials.

The present study investigates the influence of the solvent type and extraction methods on the separation efficiency of amino acids and polyphenolic compounds from the sunflower seeds, aiming to establish the most efficient way to obtain these bioactive molecules. The chemical elucidation of the extracted compounds was performed through chromatographic and spectrometric analysis.

2. Experimental

All used reagents are analytical grade and were acquired from VWR (Austria).

Plant material: Sunflower seeds (*Helianthus annuus L.*) was collected in August 2012 in Jiana, Mehedinti and identified by Dr. Dana Bobit (vicepresident of Romanian Ethnopharmacology Society, Dacia Plant SRL Brasov, Romania). A voucher sample (No. IdO 016) has been deposited in in the herbarium of the Cluj-Napoca Botanical Garden, Romania.

Materials Sunflower seeds (*Helianthus annuus L.*) were used for the comparative study of some the extraction methods (conventional, MAE and UAE). All chemicals used were of HPLC grade and were acquired from VWR (Austria).

Solvent extraction. The extraction of the sunflower seeds sample (2 g) was carried out at room temperature in 100 ml methanol.

Ultrasonic assisted extraction (UAE). Two samples, each of (2g) subjected to ultrasound were extracted in 45 mL methanol and respectively 45 mL water. The immersed seeds in extraction solvent were subjected to ultrasonic waves for 30 min at 60°C with a frequency of 50 KHz.

Microwave assisted extraction (MAE). MAE was performed using a Multiwave 3000, produced by Anton Paar at 2.45 GHz for a continuous power of 1000W. Two samples each of 500 mg was placed in a 15 mL methanol and respective 15 mL water. The power of the system was 600 W, the temperature 100°C, and the extraction time 3 min.

Chromatographic techniques

RP-HPLC analysis

The content of phenolic compounds from the sunflower samples was analyzed by high performance liquid chromatography (HPLC 3000 Ultimate, Germany) using photodiode array detector and EZ: faast 4u AAA-MS Column (250 x 3 mm ID).

RP-HPLC-DAD separation conditions: the separation was performed by gradient elution at Flow Rate: 0.8 mL/min., Col. Temp.: 35°C and with UV detection (λ=280 nm). Eluent **A**: acetic acid (0.2 %) H_2O, Eluent **B**: acetonitrile. Gradient elution program was: 0-10 minute 100% A and 0% B; 11-30 minute: 82% A and 18% B; 31-55 minute: 64% A and 36% B.

GC-MS Analysis

Qualitative analysis of free amino acids and peptides from sunflower extracts was performed on a GC-MS 7890A-5975C system (Agilent Germany) using the EZ: faast GC-MS free amino acids kit and ZB-AAA GC column (Phenomenex, USA). The used analysis conditions were the standard conditions written on the kit.

GC-MS separation conditions: the standard analysis conditions were following as the instructions from kit: Oven: 30ºC (hold 1 min) to 40ºC at 30ºC/min (hold 10 min) to 360ºC (hold 1 min). Equilibration time: 1 min. Injection: split 1: 15; 250ºC; 2µL. Carrier Gas: Helium 1.1mL/min; 110ºC. Inlet pressure: 5.824 kPa/min. Detector: MS; Mode: Scan Transfer Line Temperature: 250ºC. Analyzer Type: Electron Energy: 70eV.

Spectroscopy techniques

UV-Vis spectroscopy. To fingerprint the UV-Vis spectra, a Jasco V530 spectrophotometer was used and the spectra were recorded from 200 to 400 nm. The samples were properly diluted with different quantities of methanol (5-15 mL) and were analyzed in quartz cuvettes.

FT-IR Spectroscopy: the FT-IR spectra were recorded in KBr pellet on a Bruker FT/IR-Vertex 70 instrument (resolution 4 cm^{-1}) in spectral range 400-4000 cm^{-1}.

3. Results and discussion

Phenolic compounds and amino acids from sunflower seeds have been reported to possess many biologic activities. The extracts from sunflower seeds containing these two kinds of components should be very useful for further investigation. For this purpose, the extraction efficiency and the contents of each compound as evaluation index become very important.

In this study was investigated the influence of the solvent type and the extraction technique on the separation of biologically active compounds from sunflower seeds. Three processes: solvent extraction (E), ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE) for extraction of phenolic acids and amino acids from sunflower, Asteraceae family, was comparatively evaluated.

Chemical screening of phytochemicals recovered from extracts was achieved by spectrometry determinations (UV-Vis and FTIR spectrometry) and to identify individual molecules was used in parallel RP-HPLC and GC-MS analysis.

UV-VIS Analysis

The UV-Vis spectrophotometer method represents a simple, fast and inexpensive for determination of specific bioactive classes of molecules. The UV-Vis spectra for methanolic and water extracts are shown in Figures 1 and Figure 2. The UV scanning of the extracts showed a strong absorbance with a first maximum in region at approximate 285 nm and another maximum in region 330 nm.

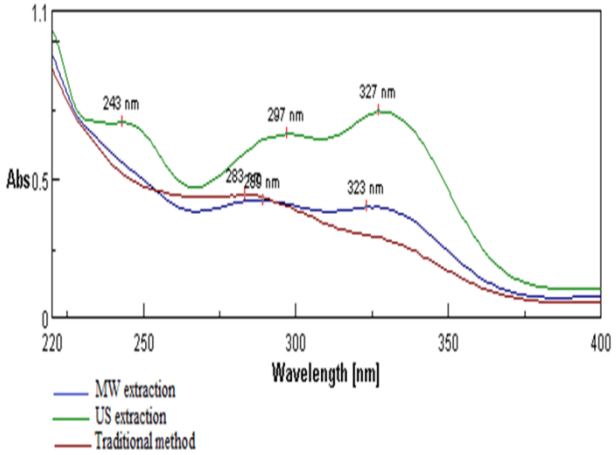

Fig. 1. UV-Vis spectra for methanolic extracts

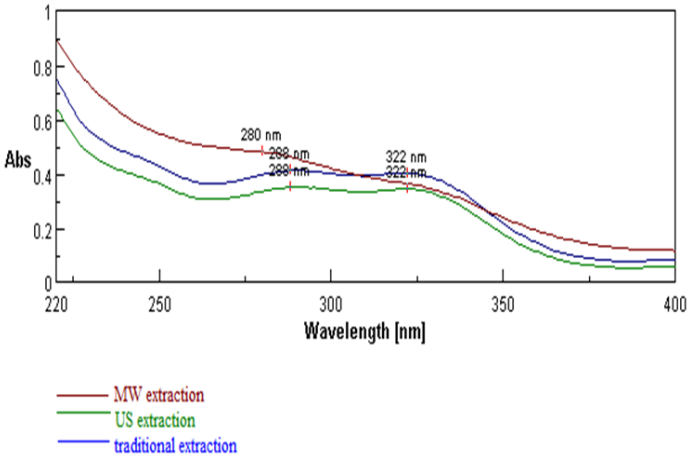

Fig. 2. UV-Vis spectra for water extracts.

UV scanning of the methanolic extracts at ultrasonic-assisted extraction (UAE) (Figure 1) showed the first maximum being at (~0.75) with λmax at 240-245 nm, the second peak was at 285 - 290 nm and a good absorbance (~0.75) with λ_{max} at 325 nm. Obtained results proved that methanolic extract showed absorption peaks attributed to phenolic acids and: chlorogenic acid (240 and 324 nm) and acid gallic (288 nm) (Abebe 2009).

The MAE and also the traditional methanolic extracts (Figure 1) showed only good absorption at 285 nm and 324 nm. By, comparison, the water extracts, in similar extraction condition, (Figure 2) were founded the same specific bioactive classes of molecules. UV scanning of these showed moderate absorbance at 285 nm and 323 nm. These extracts prove that phenolic compounds concentrations are much lower in this case. Evaluation of the solvent and extraction techniques through UV-Vis analysis conduct to the conclusion that the best results were obtained in US alcoholic extraction, followed by the MAE method. The lowest yielding was found by

maceration technique for both solvents. Identification of the specific bioactive classes of molecules found by UV-Vis analysis lead to the conclusion that from the phenolic compounds was extracted mainly chlorogenic acids (240 and 324 nm) and gallic acid (285nm) [28].

FT-IR SPECTROSCOPY showed the presence of wavelength numbers characteristic to phenolic compounds, according the next table (Table 1).

Table 1. Characteristic IR band for phenolic acids

Acids	**IR (KBr) (cm^{-1})**
Gallic acid	bands at 1651, 1634 cm^{-1} and 1555 cm^{-1} representing the C=C bond, O-H bonds at 3445 cm^{-1} (3700-2500 cm^{-1}), C-C bond at 1420 and 1384 cm^{-1}
Chlorogenic acid	The presence of a hydroxyl group (3327 cm^{-1}), the presence of α, β unsaturated carboxyl group (2928 cm^{-1}): 3421, 2929, 1697, 1635, 1456, 1398, 1268, 1182, 812.
p-Coumaric acid	3397, 1634, 1509, 1460, 1245, 1186, 1122, 977, 672, 657 and 519
Caffeic acid	3435, 1656, 1651, 1620, 1452, 1437, 1276, 1217, 1122, 1113, 974, 590 and 578.

In Fig. 3 are shown FT-IR spectra for the methanolic (a) and water (b) extract using conventional, MAE and UAE methods. Investigation of the IR spectra dates release the fact that extraction of the biologically active compounds depends both on the technique but also by the solvent polarity. According to the results, the most efficient process seems to be methanolic microwave assisted extraction since the noticeably the presence of the wavelength numbers of FTIR spectra of gallic acid, chlorogenic acid, p-coumaric acid and caffeic acid were observed, followed by conventional process, which allowed detection of three compounds: chlorogenic acid, *p*-coumaric acid and caffeic acid. Wavelength FTIR spectra corresponding for the UAE showed the presence mainly of caffeic and chlorogenic acids in methanolic extracts. By, comparison, in the water extracts were detected gallic, chlorogenic and caffeic acids for UAE process and only chlorogenic acid for MAE.

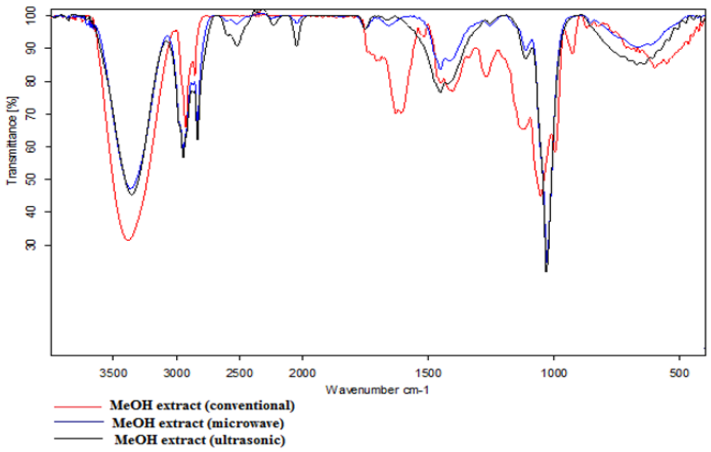

Fig. 3a. FT-IR spectra for methanolic extracts.

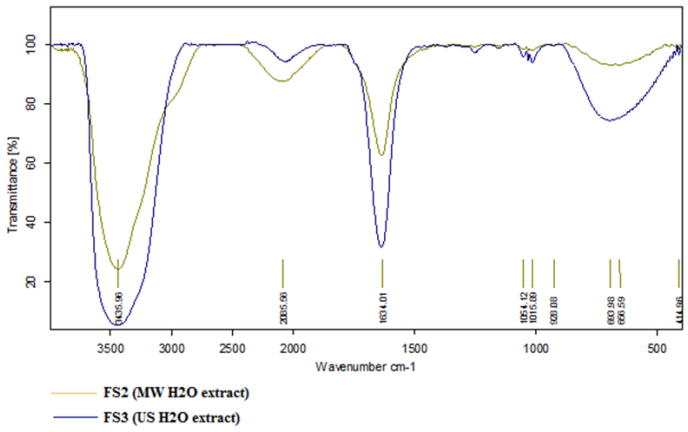

Fig. 3b. FT-IR spectra for water extract.

HPLC analysis

The presence of polyphenols in sunflower extracts was evaluated using HPLC. The influence of the solvent (polarity), extraction method parameters (time, temperature and energy type) on the content of phenolic acids in the analyzed samples (SF_1-SF_5) was also taken under consideration. Hence, a qualitative reverse phase HPLC analysis (RP-HPLC) for the phenolic acids was investigated. Comparing the results of the standard phenolic acids and the literature [29] with those of the RP-HPLC analysis of the sunflowers extract, we could identify the compounds from extracts and evaluate the efficiency of the extraction techniques. On this regard, it was investigated a comparative qualitative reverse phase HPLC analysis for phenolic compounds from the extract samples in the same chromatographic condition. The proposed RP-HPLC method enabled the identification of the phenolic acids existing in the sunflower fractions S_1-S_5 (see Table 2).

Table 2. RP-HPLC results for the phenolic acids identified through three methods (MAE, UAE, conventional)

Extraction technique/solvent	Phenolic acids	Retention Time (Rt) (min)	Peak area (mAu·min)	Peak high (mAu)	Relative area
MAE/MeOH (SF_1)	*Gallic acid*	4.657	0.8540	2.571	4.39
	Chlorogenic acid	11.297	52.599	60.11	65.06
	Caffeic acid	16.410	5.932	15.49	11.26
	p-coumaric acid	21.443	2.0119	1.47	19.29
MAE/H_2O (SF_2)	*Chlorogenic acid*	11.300	9.4362	55.178	99.07
UAE/H_2O (SF_3)	*Gallic acid*	4.657	0.5052	3.655	43.48
	Chlorogenic acid	11.329	5.3355	51.373	36.29
	Caffeic acid	16.422	2.9739	7.958	20.23
UAE/MeOH (SF_4)	*Chlorogenic acid*	11.323	5.1732	51.113	10.34
	Caffeic acid	16.358	26.5627	65.368	53.10
Conventional/ MeOH (SF_5)	*Chlorogenic acid*	11.320	43.832	289.034	46.19
	Caffeic acid	16.424	10.0910	4.646	16.74
	p-coumaric acid	21.442	100.3787	140.944	37.07

The developed RP-HPLC method that allows the separation and identification of the phenolic acids in the sunflower extracts (SF_1- SF_5) is based on the retention times of the used standards. The investigation of the phenolic acids composition from the sunflower fractions SF_1-SF_5 highlighted the same compounds identified by previous analytical methods. The analysis of the results obtained led to the conclusion that the acquisition of the number of biologically active compounds from crude plant depends not only on the type of extraction procedures which have been used but also on the solvent polarity. Chlorogenic acid was detected in methanolic and water extracts regardless of the used procedures, even the amount are completely different. However it was found to be present in high yield in microwave methanolic extract, SF_1, (Table 2). The recovery of the caffeic acid was obtained in MAE, UAE and conventional methanolic extracts. In terms of efficiency, in similar condition, MAE using water as solvent showed a considerably loss of phenolic constituents and occurs only the recovery of chlorogenic acid. This can be explained by the fact that, in polar solvent with high dielectric constant such as water, in the microwave heating process increases temperature which leads to the degradation of thermo labile compounds [30-35].

Although, conventional process (maceration) was considered disadvantageous because of the long duration and bulk amount of solvent, results showed that a single compound (gallic acid) was not detected in the final mixture.

The study was shown that a particular importance has the choice of the solvent type for an efficient recovery of the constituent biomolecules from sunflower. Thus, methanol was proved to be more efficient for the extraction of phenolic compounds than water.

GC-MS Analysis

Amino acids and peptides content of sunflower extracts depending on the solvent type and extraction techniques were investigated by GC-MS analysis. The obtained chromatograms are shown in the Figs. 1 - 5.

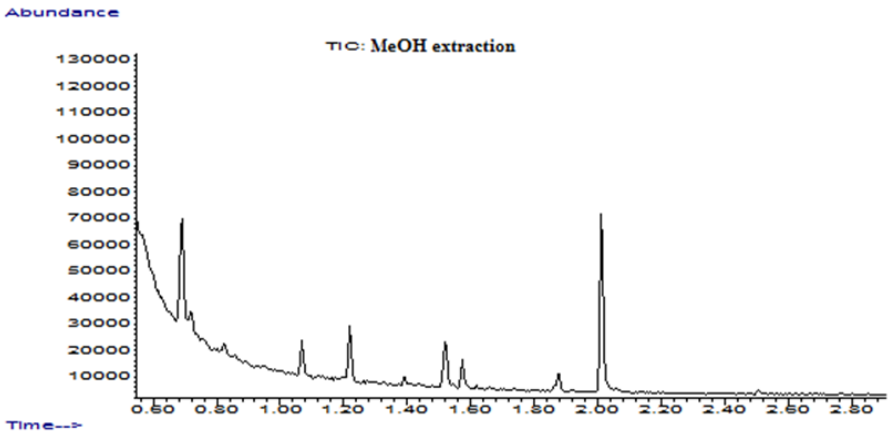

Fig. 4. GC-MS chromatogram for conventional methanolic extract

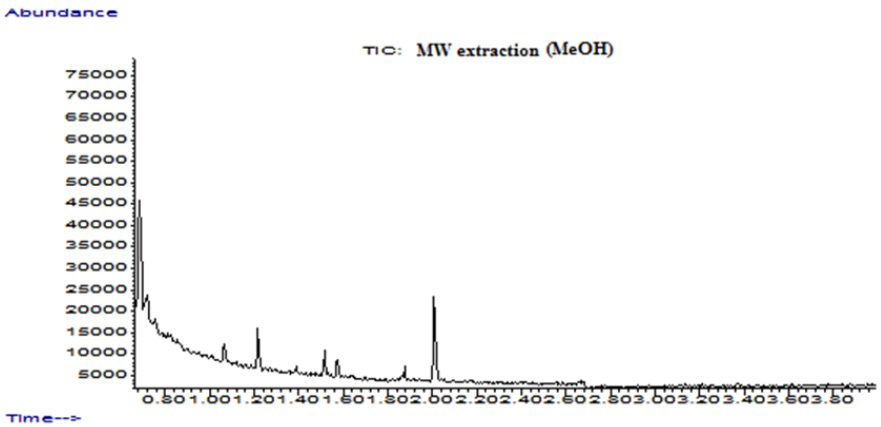

Fig. 5. GC-MS chromatogram for methanolic extract

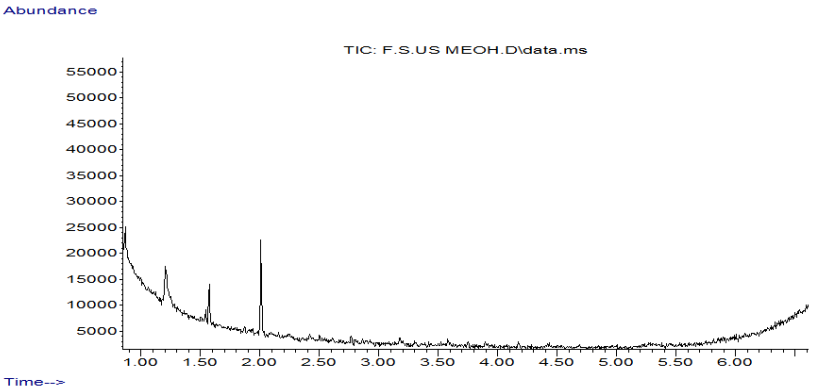

Fig. 6. GC-MS chromatogram for US methanolic extract

The mass spectrum of constituents from GC-MS chromatograms was compared with the spectra from NIST/NBS spectral database and the identified amino acids are showed in the next tables (table 3).

Table 3. Amino acids identified in conventional extract (SF_1)

Proposed structure	Abbreviation	SIM
Lysine	Lys	128, 170
Phenylglycine	Phe	58 (56-60)
Norvaline	Norv	72, 158
Valine	Val	158, 116
Glycine	Gly	74, 116
Proline-hydroxyproline (dipeptide)	PHP	156, 114
Glutamine	Glu	41
Serine	Ser	142
Cystine (C-C)	Cys	41, 42
Ornitine	Orn	58, 61 (156, 70)

Table 4. Amino acids identified in MAE extract (SF_4)

Proposed structure	Abbreviation	SIM
Lysine	Lys	128, 170
Phenylglycine	Phe	58 (56-60)
Norvaline	Norv	72, 158
Valine	Val	158, 116
Glycine	Gly	74, 116
Glutamine	Glu	41
Pyroglutamic	pGlu	84
Serine	Ser	142
Cystine (C-C)	Cys	41, 42
Ornitine	Orn	58, 61 (156, 70)
Hystidine	Hys	84 (78-89)

Table 5. Amino acids identified in UAE sunflower extract (SF_5)

Proposed structure	Abbreviation	SIM
Lysine	Lys	128, 170
Phenylglycine	Phe	58 (56-60)
Norvaline	Norv	72, 158
Valine	Val	158, 116
Glycine	Gly	74, 116
Glutamine	Glu	41
Pyroglutamic	pGlu	84
Serine	Ser	142
Cystine (C-C)	Cys	41, 42
Ornitine	Orn	58, 61 (156, 70)
Hystidine	Hys	84 (78-89)

The information gained from this investigation corroborates with the existing data from literature [31-34].

The comparative analysis of the GC-MS results led to the conclusion that in conventional method was found fewer amino acids and even small changes occur compared with the results obtained by the other two proposed alternative methods (Tables 3-5). This difference means that the extraction time influence the efficiency of amino acids separation from sunflower seeds.

The chromatographic techniques results revealed that the proposed methods proved to be useful tools for the separation and identification of single compounds from natural extracts. The reverse phase HPLC was developed for the separation of polar compounds (phenolic acids) with high variation of partition coefficients and GC for the analysis of amino acids and small dipeptides.

Sunflower polyphenols with high antioxidative potential and amino acids can be used for the development of new biomaterials.

4. Conclusion

This study was designed to investigate the influence of extraction process parameters on bioactive compounds (phenolic acids and amino acids) from sunflower. The data presented of the

comparative study of the extraction process parameters indicates that alternative methods: methanol MAE and respective UAE for aqueous extraction are most suitable for these two classes of highly important biomolecules than conventional extraction. The developed characterization methodology showed feasibility and a good potential concerning efficient identification of the target compounds.

Further investigation will be focused on developed new biomaterials through functionalization of magnetic nanomaterials with these bioactive compounds.

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References

[1] R. Subashini, U.S. Rakshitha, L. Che Sci Rev Lett, , **1**(1), 30 **(**2012).
[2] S. Saini, S. Sharma, Helianthus Annuus (Asteracea): A Review. International Journal of Pharma Professional's Research, **2** (4). (2011).
[3] P. M. Kris-Etherton, C. L. Keen, Current Opinion in Lipidology, **13**(1), 41 (2002).
[4] Y. Kadoma, S. Fujisawa, Molecules. **13**(10), 2488 (2008).
[5] J. C. Cheng, F. Dai, B. Zhou, L. Yang, , Z. L. Liu, Food Chemistry., **104**(1), 132 (2007).
[6] D. Gumul, J. Korus, Electronic Journal of Polish Agricultural Universities., **9**(4), 1 (2006)
[7] M.D. Santos, M.C. Almeida, N.P. Lopes, G.E.P., Souza, Biol. Pharm. Bull., **29**, 2236 (2006)
[8] T.T. Wang, X.H. Jiang, L. Yang, J. Chroma. A., **1180**(1–2), 53 (2008).
[9] T. Nakamura, Y. Nakazawa, S. Onizuka, Mutat. Res., **338**, 7 (1997).
[10] M. Yoshimoto, S. Yahara, S. Okuno, M.S. Islam, K. Ishiguro, O. Yamakawa, Leaf. Biosci. Biotech. Biochem., **66**, 2336 (2002).
[11] S. Ingale, S.K. Shrivastava, Agric. Biol. J. N. Am., **2**(8), 1171 (2011).
[12] G. Sripad, V. Prakash, M. S. Narasinga, RAO. Extractability of polyphenols of sunflower seed invarious solvents. J. Biosci., **4**(2), 145 (1982).
[13] J.A. Saunders, K. A. Rosentrater, Biomass and Bioenergy**,** , **33**(10) 1486 (2009).
[14] B.B. Li, Smith B., Hossain M.M. Separation and Purification Technology, **48**, 189 (2006).
[15] I. Ignat, Volf I., Popa V.I. Food Chemistry.,**126**, 1821 (2011).
[16] Y. G. Tu, , Y. Z. Sun, , Y. G. Tian, Xie, M. Y., Chen, Food Chemistry, **114**, 1345 (2009)
[17] M. Naczk, F. Shahidi, J. Chromatogr. A, **1054**, 95 (2004).
[18] U. Vrhovsek, A. Rigo, Tonon D., Mattivi F., Quantitation J. Agric. Food Chem. **52,** 6532 (2004)
[19] I. Parejo, Jauregui O., Sanchez-Rabaneda F., Viladomat F., Bastida J., Codina C., J. Agric. Castro M.D. Food Chem. **52**, 3679 (2004).
[20] F.S. Taha, Mohamed G.F., Mohamed S.H., Mohamed S.S., Kamil M.M. American Journal of Food Technology, **6**(12), 1002 (2011).
[21] S. Gonzalez-Perez, Vereijken J.M., Journal of the Science of Food and Agriculture. **87**(12), 2173 **(**2008).
[22] M.-H. Lee, C.-C. Lin, Food Chemistry, **105**, 223 (2007).
[23] T. Jerman, P. Trebše, Mozetič Vodopivec B. Food Chemistry. **123**(1), 175 (2010).
[24] I. Rezic, D. Krstic, Lj. Bokic, Ultrasonics Sonochemistry, **15,** 21 (2008).
[25] T. S. Ballard, P. Mallikarjunan, K. Zhou, O'Keefe S. Food Chemistry **120**, 1185 (2010).
[25] F.Y. Du, X.H. Xiao, X.J. Luo, G.K. Li, Talanta., **78**, 1177 (2009).
[26] J.A. Pérez-Serradilla, Luque de. Food Chemistry, **124**, 1652 (2011)
[27], A. V. Abebe Belay, African Journal of Pure and Applied Chemistry., **3**(11), 234 (2009)
[28] J. Kamal, African Journal of Biotechnology., **10**(16), 3149 (2011).
[29] P.C. Veggi, J. Martinez, M.A.A. Meireles, Microwave-assisted Extraction for Bioactive 15,

Compounds: Theory and Practice, Eds. Chemat F., Cravotto G. (eds.), Food Engineering Series 4, Springer Science, Business Media New York, 2013, 15-52.
[30] Z. Mohammedi, F. Atik, International Journal of Pharma and Bio Sciences, 2(1), (2011)
[31] A. Villanueva, J. Vioque, R. Sanchez-Vioque, A. Clemente, J. Pedroche, J. Bautista, F. Millan, JAOCS, , **76**, 12 (1999).
[32] M. Nadeem, F.M. Anjum, S. Hussain, M.R., Khan M.A. Shabbir Pak. J. Food SCI., **21**(1-4): 7 (2011).
[33] C. Socaciu, F. Ranga, F. Fetea, L. Leopold, F. Dulf, R. Parlog, Czech J. Food Sci., **27**, Special Issue; 2009
[34] M. Hadi Meshkatalsadat, A. H. Papzan, A. Abdollahi, Digest Journal of Nanomaterials and Biostructure, **6**, 319 (2010).
[35] D. Benedec, L. Vlase, D. Hanganu, I. Oniga, Digest Journal of Nanomaterials and Biostructures, **7**(3), 1263 (2012).