LC-DAD/ESI-MS/MS CHARACTERIZATION OF PHENOLIC COMPOUNDS OF SUNFLOWER OIL

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ABSTRACT

The study investigates the phenolic contents and antioxidant potential of sunflower oils from commercial markets in Turkey were extracted with methanol/water. Extracts were used for the phenolic and antioxidant studies. A simple and reproducible method for qualitative and quantitative analysis of phenolic compounds in sunflower oils, high performance liquid chromatography with diode array detector (HPLC-DAD), and HPLC-mass spectrometry (MS) in tandem mode was developed. Detection and quantification were performed at 280, 320 and 360 nm. For identification purposes, HPLC-MS/MS was equipped with electrospray ion source in the negative and positive-ion mode. Most of the compounds detected were mainly hydroxycinnamic acids. Chlorogenic acid was found as the major compound in the group of phenolic acids followed by vanillic acid, while rutin was determined as the most abundant compound in the overall phenolics of sunflower oil. Rutin has an average concentration of 2.70 mg/kg oil whereas chlorogenic acid has an amount of 1.66 mg/kg oil as the second most dominant phenolic compound. Antioxidant activities of sunflower oils were measured as a comparison of two methods; the DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis- (3-ethyl-benzothiazoline-6-sulphonic acid) assays. Our results showed strong correlations between antioxidative capacity and total phenolic content of sunflower oils.

Keywords: Sunflower oil phenolics, phenolic characterization, LC-MS/MS analysis, antioxidant assays.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is a crucial crop producing annually and native to North America (Amakura *et al.,* 2013). It is widely used in oil production and sunflower oil ranks fourth in world vegetable oil production, after palm oil, soybean oil and canola oil (Weisz *et*

al., 2009). Sunflower oil is also popularly used in Turkey and the seed production in 2013 was 1523000 tons (FAO-STAT,2013). Sunflowers date back to 26th century, are known and used till then (Pope *et al*., 2001).Sunflower oil (sunflower seed oil) is an abundant source of unsaturated fat, vitamin E, and some phenolic compounds (Fiori, 2009). Sunflower seeds are affluent in oil and with this oil having high ratio of polyunsaturated/saturated fatty acids and high linoleic acid content, sunflower oil is considered to be good for human consumption (Salgın *et al*., 2006).

Sunflower seeds have been shown to have antioxidant activities and are a very good source of vitamin E and several B-vitamins. Moreover, sunflower seeds contain a number of phenolic compounds largely responsible for the modifications occurring during the processing of sunflower seeds (Weisz *et al*., 2009). Phenolic compounds have been proposed to be the potent and important contributors in reducing oxidative stress due to their antioxidant activity, which are of great importance. Therefore, food industry is concentrating on foods containing various bioactive compounds for health promotion and disease prevention (Kelebek *et al*., 2015a).The major phenolic constituents of sunflower seeds are chlorogenic acid, smaller quantities of caffeic acid, cinnamic, coumaric, ferulic, sinapic and hydroxy-cinnamic and finally traces of vanillic, syringic and hydroxy-benzoic acids (Pedrosa *et al.,* 2000), but the phenols are present only in traces in sunflower seed oils due to the oil production process (Leung *et al.,* 1981).

In this research, the determination of phenolic content and their antioxidant activity was aimed. As regarding the lack of studies in these terms of sunflower oil, this paper will be helpful in understanding the characterization of sunflower oils.

MATERIAL AND METHODS

Chemicals

Methanol, acetonitrile, formic acid, and cyclohexane HPLC-grade solvents were purchased from Riedel-deHaen (Switzerland). All other reagents used were of analytical grade. Ultrapure water generated by the MilliQ system (Millipore, Bedford, MA) was used. Phenolic compounds (p-hydroxybenzoic, vanillic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, qurcetin-3-galactoside, kaempferol-3-glucoside and rutin) were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

Sunflower oil samples were collected from domestic markets in Adana, Turkey. 5 different commercial brands of oils were analysed.

Extraction of the Phenolic Fraction

According to Rotondi *et al*. (2004), 4 g of the oil sample was added to 2 mL of n-hexane and 4 mL of a methanol/water (70/30; v/v) solution in a 10 mL centrifuge tube. After vigorous mixing, they were centrifuged for 15 min at 5500 rpm. The hydro-alcoholic phase was collected, and the hexane phase was re-extracted twice with 2 mL of methanol/water (70/30; v/v) solution each time. Finally, the hydro-alcoholic fractions were combined, washed with 2 mL of n-hexane to remove the residual oil, then concentrated and evaporated in vacuum at 35 °C. The dry extracts were re-suspended in 0.5 mL of a methanol/water (50:50, v/v) solution and filtered through a 0.2 μm nylon filter (Whatman Inc., Clifton, NJ) before being analyzed by LC-ESI-DAD-MS/MS.

LC-DAD-ESI-MS/MS analysis of phenolic compounds

An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, California, USA) operated by Windows NT-based ChemStation software was utilized; the HPLC equipment was used along with a diode array detector (DAD). The system comprised a binary pump, degasser, and auto sampler. The column used was a Phenomenex reversed-phase C-18 column (4.6 mm × 250 mm, 5 μm) (Torrance, California, USA). The mobile phase consisted of two solvents: Solvent A, water/formic acid (99.5:0.5; v/v) and Solvent B, acetonitrile/solvent A (60:40; v/v). Phenolic compounds were eluted under the following conditions: 0.5 ml min^{-1} flow rate with temperature set at 25 °C; isocratic conditions from 0 to 5 min with 0% B; gradient conditions from 0% to 5% B in 20 min; from 5% to 15% B in 18 min; from 15% to 25% B in 14 min; from 25% to 50% B in 31 min; from 50% to 100% B in 3 min; followed by washing and reconditioning of the column. The ultra-violet-visible spectra (scanning from 200 nm to 600 nm) were recorded for all peaks. Triplicate analyses were performed for each sample. The identification and assignation of each compound was performed by comparing retention times and UV spectra to authentic standards; and confirmed by an Agilent 6430 LC-MS/MS spectrometer equipped with an electrospray ionization source. The electrospray ionization mass spectrometry detection was performed in negative ion mode with the following optimized parameters: capillary temperature 400°C, N_2 12 L/min; nebulizer pressure, 45 psi (Kelebek *et al*., 2015a). Data gaining was performed using the Multiple Reactions Monitoring (MRM) method that solely monitors specific mass transitions during preset retention times. The curves were obtained using the commercial standards of the concentrations normally present in sunflower oils (approximately 1-100 mg kg^{-1}), obtaining regression coefficients (r^2) above 0.995 in all cases.

Measurement of antioxidant activity

DPPH Assay: 0.1 mL of diluted sunflower oil extract was mixed with 3.9 mL of DPPH solution (2.36 mg/100 mL methanol) and vigorously vortexed. The solution was held in the dark at ambient conditions for 15 min. The absorbance was measured at 517 nm by a UV-Visible spectrophotometer (Shimadzu UV-1201, Kyoto-Japan). Trolox calibration curve was used to calculate the antioxidant activity of the oil extracts and to express the antioxidant capacity in mM Trolox equivalent per kg of sunflower oil. The mean and standard deviation were calculated for the three replicates (Kelebek *et al*., 2015a; Kesen *et al*., 2013).

ABTS Assay: The ABTS solution was created at a concentration of 7 mM and mixed with 2.5 mM of potassium persulphate, and stored after incubation at 23 °C in the dark for 12–16 h. The ready-made solution was diluted with 80 % methanol to measure an absorbance of 0.7±0.01 at 734 nm. Then, 3.9 mL of ABTS solution was added to 0.1 mL of the oil samples and mixed vigorously. Finally 10 min. were waited to ensure reaction and the absorbance was monitored at 734 nm [13, 14]. The calibration curve equations related to the Trolox standard were y=0.0004x + 0.0089 with R2= 0.9996 for ABTS and y=0.0004x + 0.0082 with R2= 0.9995 for DPPH within a concentration range from 5 to 150 μmol/L.

RESULTS AND DISCUSSION

Phenolic Compounds of Sunflower Oil

Table 1 lists the compounds identified according to different families, including the information provided by HPLC-DAD-ESI-MS/MS analysis: retention time, λ_{max} in the ultraviolet region, molecular ion, main fragment ions in MS/MS, and tentative identification. A total of 10 phenolic compounds were identified and quantified in oils, including p-hydroxybenzoic acid, vanillic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, quercetin-3-galactoside, kaempferol-3-glucoside and rutin. Rutin is the

major phenolic compound in sunflower oils followed by chlorogenic acid as they constitute the large proportion of the total phenolic content (Table 2). These two compounds were reported to present in the seed and kernel of the sunflowers in addition to caffeic and ferulic acid with chlorogenic acid being the most abundant compound that is one of the natural phenols includes one molecule of caffeic acid and one of quinic acid. Caffeic acid is reported to found more in the kernels of the sunflower than the chlorogenic acid Žilić *et al*., 2010. Phenolic acids have higher proportion in phenolic compounds of sunflower oils. Rutin is a flavonol formed by a quercetin which is a flavonol and a rutinoside, a dissaccharide (Kelebek *et al*., 2015b). This compoundhas the majority having a concentration between 2.23 and 2.99 mg/kg in flavonoids of sunflower oil Chlorogenic acid has a varying concentration between 1.40 and 1.80 mg/kg followed by vanillic acid (1.14-1.35 mg/kg) in phenolic acids. The other phenolic acids present in traces due to the refining process of the oil (Leung *et al*., 1981). Sunflower oil phenolic acids show similarity with phenolics of olive oil including vanilic, caffeic, ferulic and p-coumaric acid (Godoy-Cabarello *et al*., 2012; Kelebek *et al*., 2012) while olive oil having total phenol content approximately two or three times more than sunflower oil (Guzel *et al*., 2009). Also the amounts of phenolic compounds is known to vary according to the conditions of the region where in the crop grows, the extraction methods and the conditions of storage (Kelebek *et al*., 2012).

Antioxidant Activity of Sunflower Oil

Antioxidant capacity was measured by two methods namely, ABTS and DPPH assays. Table 3 presents the results of the antioxidant activities obtained by the sunflower oils. As it can be seen from the results, ABTS assay stated better the antioxidant activity of phenolic compounds than the DPPH assay as the method gave higher values.

DPPH is a free radical scavenging method, being simple, rapid and repeatable, preferably used in determining the antioxidant activity of compounds (Kelebek *et al*., 2015b). On the other hand, ABTS is used more in the food and agriculture industry which is clearly the better method for evaluating the antioxidant capacity of sunflower oils (Kelebek and Selli, 2011). Antioxidant capacities were found as 7.16 µM Trolox/kg oil using DPPH assay and 11.76 µM Trolox/kg oil by ABTS assay in average while the maximum values were 7.71 and 12.76 respectively. Rutin is known to have antioxidant, antiinflamatory activities and can be used in preventing cancer diseases (Kelebek *et al*., 2015b). In addition to the antioxidant behavior, chlorogenic acid shows antiviral, hypoglycaemic and hepatoprotective activities yet caffeic acid is reported to have higher antioxidant capacity (Dixon *et al*., 1995; Chen and Ho, 1997).

CONCLUSION

A total of ten phenolic compounds were isolated from sunflower oil samples and identified by HPLC-DAD-ESI-MS/MS analysis. Rutin is found as the most dominant phenolic compound with a concentration of 2.70 mg/kg oil followed by chlorogenic acid (1.66 mg/kg) and vanillic acid (1.35 mg/kg). The other phenolic acids present in sunflower oil are determined as p-hydroxybenzoic acid, caffeic acid, p-coumaric acid, ferulic acid and sinapic acid while flavonoids include quercetin-3-galactoside and kaempferol-3-glucoside. Antioxidant capacity of these phenolic compounds was determined as a comparison of DPPH and ABTS methods. ABTS assay is found as more appropriate in determining antioxidant activity of sunflower oil. By using DPPH assay antioxidant capacity is determined as 7.16 µM Trolox/kg oil while ABTS had a result of 11.76 µM Trolox/kg oil. In conclusion, the phenolic content and antioxidant activity supply a beneficial contribution to the characterization of sunflower oils. Regarding this matter, further investigation is advised.

Table 1. HPLC-DAD-ESI-MS/MS identification of phenolic compounds

Peak	Compounds	MF	λ (nm)	Fragmentor (v)	Precursorion	Collision energy (v)	Quantitative transition (m/z)
	Phenolic acids (PA)						
1	p-hydroxybenzoic acid	$HOC_6H_4CO_2H$	256	90	137	15	137>93
2	Vanillic acid*	$C_8H_8O_4$	258, 293	90	167	15	167>123
3	Chlorogenic acid*	$C_{16}H_{18}O_9$	326	90	353	15	353>191
4	Caffeic acid*	$C_9H_8O_4$	325	90	179	15	179>135
5	*p*-coumaric acid*	$C_9H_8O_3$	236, 310	90	163	15	163>119
6	Ferulic acid*	$C_{10}H_{10}O_4$	323, 293	90	193	15	193>134
7	Sinapic acid*	$C_{11}H_{12}O_5$	324	90	223	15	223 >149
	Flavonoids (FLA)						
8	Quercetin-3-galactoside*	$C_{21}H_{20}O_{12}$	353	90	463	15	463>301
9	Kaempferol-3-glucoside*	$C_{21}H_{20}O_{11}$	348	90	447	15	447>285
10	Rutin*	$C_{27}H_{30}O_{16}$	360	90	609	15	609>301

Table 2. Phenolic content of sunflower oil extracts (mg/kg oil)

Phenolic Compounds	p-hydroxybenzoic acid	Vanillic acid	Chlorogenic acid	Caffeic acid	p-coumaric acid	Ferulic acid	Sinapic acid	Qurcetin-3-galactoside	Kaempferol-3-glucoside	Rutin	*Total*
Sample 1	0.61±0.01	1.46±0.00	1.80±0.03	1.04±0.01	0.52±0.01	0.38±0.01	0.33±0.00	0.09±0.00	0.13±0.00	2.75±0.04	***9.10±0.10***
Sample 2	0.54±0.01	1.40±0.01	1.72±0.02	0.98±0.01	0.57±0.00	0.44±0.01	0.21±0.01	0.11±0.00	0.15±0.00	2.99±0.01	***9.10±0.13***
Sample 3	0.58±0.01	1.43±0.02	1.76±0.03	1.01±0.02	0.55±0.01	0.41±0.01	0.27±0.01	0.10±0.01	0.14±0.00	2.87±0.03	***9.10±0.10***
Sample 4	0.53±0.02	1.31±0.01	1.62±0.02	0.93±0.01	0.50±0.03	0.37±0.01	0.25±0.02	0.09±0.01	0.13±0.00	2.64±0.02	***8.38±0.10***
Sample 5	0.47±0.01	1.14±0.03	1.40±0.02	0.80±0.01	0.42±0.01	0.31±0.00	0.23±0.01	0.08±0.00	0.11±0.00	2.23±0.01	***7.18±0.08***
Min	***0.47***	***1.14***	***1.40***	***0.80***	***0.42***	***0.31***	***0.21***	***0.08***	***0.11***	***2.23***	***7.18***
Max	***0.61***	***1.46***	***1.80***	***1.04***	***0.57***	***0.44***	***0.33***	***0.11***	***0.15***	***2.99***	***9.10***
Mean	***0.55***	***1.35***	***1.66***	***0.95***	***0.51***	***0.38***	***0.26***	***0.09***	***0.13***	***2.70***	***8.57***

Table 3. Antioxidant capacities of sunflower oil extracts (μM Trolox/kg oil)

	DPPH	ABTS
Sample 1	7.05±0.54	11.58±0.88
Sample 2	7.45±0.26	12.23±0.43
Sample 3	7.08±0.96	11.61±1.57
Sample 4	7.71±0.41	12.66±0.68
Sample 5	6.52±0.60	10.70±0.99
Min	6.52±0.26	10.70±0.43
Max	7.71±0.96	12.66±1.57
Mean	7.16±0.56	11.76±0.91

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