PHENOLIC COMPOUNDS IN POMEGRANATE VINEGARS

Serkan SELLİ

Cukurova University, Faculty of Agriculture, Department of Food Engineering, 01330, Adana, Turkey

sselli@cu.edu.tr

Abstract: Pomegranates are widely researched fruits for their nutritional and health benefits as well as their organoleptic properties. Pomegranate juices are rich in vitamins, minerals and phytochemicals. In addition, pomegranate juices contain potentially bioactive compounds and they are a great source of phenolic compounds such as flavonoids and phenolic acids. The use of second quality pomegranate for vinegar production can be a proper method to reduce losses due to discard of fruits. There has been growing interest in the beneficial health effects of certain fruits, wines and their by-products, like vinegars. In this study, the main phenolic compounds of pomegranate vinegars were determined by HPLC–DAD–ESI-MS. Diode-array detection (DAD) has been used for screening of the different classes of phenolic compounds, whereas MS and MS^n fragmentation data were employed for their structural characterization. Most of the compounds detected were mainly anthocyanins such as delphinidin, cyanidine and pelargonidine, and ellagitannins such as ellagic acid, punicalagin and punicaline. Additionally, phenolic acids were detected. Antioxidant activities of pomegranate vinegars were measured by using the DPPH (2,2-diphenyl-1-picryl hydrazyl) and ABTS (2,2′-azino-bis-3-ethyl-benzothiazoline-6-sulphonic acid) assays. The results showed strong correlations between antioxidative capacities and total phenolic content of pomegranate vinegars.

Keywords: Pomegranate vinegars; phenolic compounds; antioxidant capacity; DPPH.

Introduction

Modern life styles have driven consumers away from healthy dietary routines. In point of fact, their increasing concern about their health has prompted the industry to become involved in the need for food products which contribute to the prevention of illness. Pomegranate fruits are widely consumed fresh and in other processed forms as juice, jam, jelly, vinegar, wine, oil and in extract supplement. Actually, pomegranate fruits are a source of carbohydrates, minerals, crude fibres, and various biologically active compounds, such as vitamin C, and certain phenolic compounds as punicalagin, ellagic acid, gallotannins, anthocyanins (Zaouay et al., 2012). Brewed vinegar, a commonly used condiment of food, also has medicinal uses by virtue of its physiological effects, such as promoting recovery from exhaustion, regulating blood glucose, blood pressure, aiding digestion, stimulating the appetite, and promoting calcium absorption (Xu et al., 2007). Vinegar is traditionally produced from grapes by means of double fermentation (alcoholic and acetic acid). There are several advantages of using fruits for vinegar production, such as their health and organoleptic properties. The quality of vinegars changes according to the raw material, acidification system and ageing procedure (Tesfaye et al., 2002). The consumer demands for natural and high quality food products have increased the characterization and quality control these products (Cerezo et al., 2008). Nonetheless, there is no detailed information on phenolic and antioxidant properties of pomegranate vinegar. Therefore, the objectives of the present study were to evaluate phenolic profiles and antioxidant capacities of pomegranate vinegar. In addition, antioxidant capacity was measured using two common antioxidant activity methods (DPPH and ABTS).

Material and Methods

Samples and Chemicals

Pomegranate vinegar sample was obtained from domestic markets in Adana, Turkey. HPLC-grade solvents; methanol, acetonitrile, formic acid, and cyclohexane 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 were obtained from Sigma-Aldrich (Steinheim, Germany).

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

Samples were filtered through a 0.45-µm pore size membrane filter before injection. 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.

Individual compounds were quantified using a calibration curve of the corresponding standard compound. When reference compounds were not available, the calibration of structurally related substances was used, including a molecular weight correction factor. The stock solution was diluted to a series of appropriate concentrations with the same solvent, and an aliquot of the diluted solution was injected into the HPLC apparatus for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

Measurement of antioxidant activity

DPPH Assay: 0.1 mL of diluted vinegar 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 vinegar extracts and to express the antioxidant capacity in mM Trolox equivalent per l of vinegar. The mean and standard deviation were calculated for the three replicates.

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 vinegar samples and mixed vigorously. Finally 10 min. were waited to ensure reaction and the absorbance was monitored at 734 nm. The calibration curve equations related to the Trolox standard were $y=0.0004x + 0.0089$ with $R^2= 0.9996$ for ABTS and $y=0.0004x + 0.0082$ with $R^2= 0.9995$ for DPPH within a concentration range from 5 to 150 µmol/L.

Results and Discussion

Phenolic compounds of pomegranate vinegar

The phenolic compounds belonging to different families were identified according to 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 as listed in Table 1. A total of 23 compounds were identified, including six anthocyanins (delphinidin-3,5-glycosides, cyanidin-3,5-glycoside, pelargonidin-3,5-diglucoside, cyanidin-3-glycoside, pelargonidin-3-glycoside and cyanidin pentoxide), two hydrolysable tannins (galloyl hexose and digalloyl hexose), ten ellagic tannins (ellagic acid, HHDP-hexoside, ellagic acid-hexose, ellagic acid pentose, ellagic acid-deoxyhexose, galloyl-HHDP-hexose, bis-HHDP-hexose, digalloyl-HHDP-hexose, ellagic acid derivative and galloyl-HHDP-glycoside), two gallagyl esters (gallagyl-hexose and HHDP-gallagyl-hexose) and three hydroxycinnamic acids (caffeic acid-hexose, caffeic acid-hexose derivative and caffeic acid derivative). Composition of phenolic compounds in pomegranate vinegar was similar to fruit and juice of the fruit.

Anthocyanins are natural colorants belonging to the flavonoid family. LC-ESI-MS/MS MRM chromatograms of some of the identified anthocyanins in pomegranate vinegar were shown in Figure 1. As displayed in Table 1, the molecular ion and its fragments were used to confirm the identity of the anthocyanins isolated. The retention time 26.5 minutes, showed a molecular ion m/z 449, suggesting the presence of cyanidin-3-O-glucoside, which was confirmed by the fragment ion m/z 287, which corresponds to aglycone cyanidin.

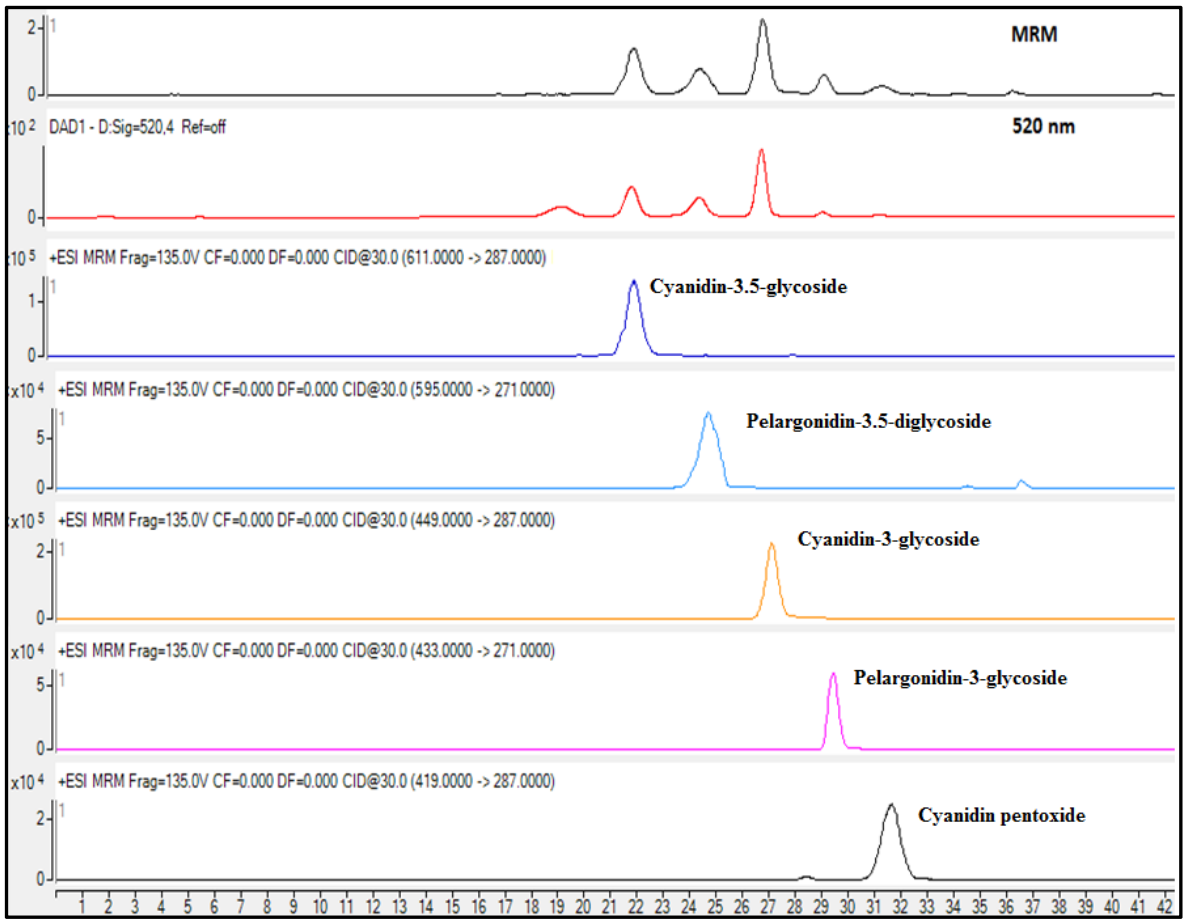

Figure 1. LC-DAD and LC-ESI-MS/MS MRM chromatograms of the identified anthocyanins. Peaks correspond to compounds in Table 1.

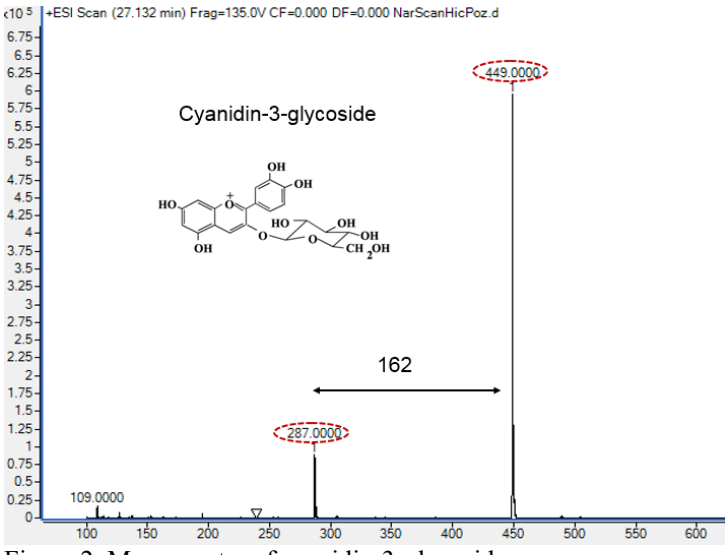

Figure 2. Mass spectra of cyanidin-3-glycoside

Anthocyanins are widely distributed among flowers, fruits (particularly in berries), and vegetables (Wang et al., 1997). Total amount of anthocyanins was found as 11.86 mg l^{-1} in pomegranate vinegar. In the study about functional constituents comparison of pomegranate juices after alcoholic and acetic acid fermentation by Ordoudi et al. (2011), total anthocyanin content of pomegranate vinegar was found close to fresh juice composition. This result displays that anthocyanins penetrate from the pomegranate to vinegar. Among the identified anthocyanins, cyanidin-3-glycoside was the major phenolic compound in vinegar followed by cyanidin-3,5-glycoside and pelargonidin-3,5-diglucoside as they constituted the large proportion of total anthocyanin content. These

compounds have already been identified by previous studies in different parts of pomegranate. Cyanidin-3-glycoside (C3G), also known as kuromanin, is probably the most notable compound among the investigated cyanidin-glycosides. Generally, this compound exists as a free form that would be of sure benefit to its antioxidant activity. Due to their strong antioxidant properties, anthocyanins are of considerable interest to the scientific community and consumer market. The naturally electron- deficient chemical structure of anthocyanins makes them highly reactive toward free radicals and, consequently, makes them powerful natural antioxidants (Nasr et al., 1996; Gómez-Caravaca et al.,2013).

Ellagitannins were the most abundant group of the pomegranate vinegar phenolic composition. Ellagitannins and hydrolysable ellagitannins are both implicated in protection against atherogenesis, along with their potent antioxidant capacity. It has been suggested that ellagitannins may prevent chronic diseases such as cancer and cardiovascular diseases. These protective activities are attributed to both classes of phytochemicals, which are thought to provide antiproliferative, anti-inflammatory, and antioxidant activities and to function as glycemic regulators (Aviram and Rosenblat, 2013). Similar to our study, ellagitannins have been found as the major phenolic group of the pomegranate juice (Gómez-Caravaca et al., 2013). Among this group galloyl-HHDP-hexose was overwhelmingly the major compound having a concentration 11.10 mg l^{-1}. The compound followed by an ellagic acid derivative, bis-HHDP-hexose and galloyl-HHDP-glycoside with the concentration 8.18 mg l^{-1}, 6.90 mg l^{-1} and 6.23 mg l^{-1} respectively.

Gallagyl-hexose (punicaline) and HHDP-gallagyl-hexose (punicalagin) compounds were identified as gallagyl esters. Between these compounds punicalagin has the majority having a concentration of 13.90 mg l^{-1} (Fig 3). Among the phenolic compounds detected in the pomegranate, highest antioxidant activity was observed for punicalagin. This compound is also abundant in the fruit husk and during processing is extracted into pomegranate juice in significant quantities reaching levels of > 2 g/L juice (Gil et al., 2000).

Antioxidant activity of pomegranate vinegar

Antioxidant capacity was measured by two methods namely, ABTS and DPPH assays. The results were found as 9.78 mM Trolox/l vinegar using DPPH assay and 12.87 mM Trolox/l vinegar by ABTS assay in average while the maximum values were 9.84 mM Trolox/l and 13.01 mM Trolox/l respectively. As it can be seen from the results, ABTS assay stated better the antioxidant activity of phenolic compounds than 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. 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 pomegranate vinegar (Kelebek et al, 2015b). Zaouay et al. (2012), studied antioxidant activity of different cultivars of pomegranates. In this research antioxidant activity of the fruits were detected between 11.24-21.52 μM Trolox/l with ABTS and 11.91-22.50 with DPPH methods. Likewise, a strong correlation was observed between phenolic contents and antioxidant activities. Ordoudi et al. (2014) determined the phenolic content and antioxidant activity of pomegranate juice, wine and vinegar in comparison of two methods (DPPH and CUPRAC). Reported results displayed that antiradical potential of pomegranate vinegar showed a decrease about 55% from the fruit juice. This loss can imply that radical scavengers may degrade during fermentation. This information also shows parallelism with our results. In the very same study, punicalagin and cyanidin-3-glycoside were reported to be the main contributors in antioxidant activity. Pomegranate vinegars are stated to have higher antiradical potential from apple vinegar whereas persimmon vinegars have the highest activity among this group (Sakanaka et al., 2007; Ordoudi et al., 2014).

Conclusion

In this study, the main phenolic compounds and antioxidant activities of pomegranate vinegars were determined by HPLC–DAD–ESI-MS. Various phenolics were determined in the sample and ellagitannins were responsible for the majority with a concentration of 44.42 mg l^{-1}.Ellagitannins are known to have beneficial effects to human health due their antioxidant activities. Punicalagin were the most abundant compound in overall phenolics, hence the highest antioxidant activity was observed for this compound. Antioxidant activities of pomegranate vinegars were measured by using the DPPH and ABTS assays. ABTS method was found to evaluate better the antioxidant activity of pomegranate vinegars. The results showed correlations between antioxidative capacities and total phenolic content of pomegranate vinegars. It is deducible that the change in antioxidant activity of pomegranate vinegars depends on the phenolic content and concentration of pomegranates. However, further researches are advised to have a better understanding in pomegranate vinegars.

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

Peak	Compounds	λ (nm)	Precursor ion	Quantitative transition (m/z)	Amount (mg/l)
	Anthocyanins				
1	Delphinidin-3.5-glycoside	519, 277	627	627>303	0.74±0.01
2	Cyanidin-3.5-glycoside	513, 277	611	611>287	3.24±0.00
3	Pelargonidin-3.5-diglycoside	499, 274	595	595>271	1.00±0.00
4	Cyanidin-3-glycoside	516, 280	449	449>287	6.05±0.01
5	Pelargonidin-3-glycoside	503, 274	433	433>271	0.57±0.00
6	Cyanidin pentoxide	513, 277	419	419>287	0.24±0.00
	Total				**11.86±0.01**
	Hydrolysable tannins				
7	Galloyl hexose	375, 266	331	331>169	1.84±0.01
8	Digalloyl hexose	363, 264	483	483>331	0.81±0.01
	Total				**2.65±0.02**
	Ellagitannins				
9	Ellagic acid	367, 275	301	301>229	1.69±0.01
10	HHDP-hexoside	267	481	481>301	1.72±0.01
11	Ellagic acid-hexose	363, 252	463	463>301	1.59±0.01
12	Ellagic acid pentose	359, 255	433	463>301	0.66±0.01
13	Ellagic acid-deoxyhexose	360	447	447>300	1.31±0.01
14	Galloyl-HHDP-hexose	365	633	633>301	11.10±0.40
15	bis-HHDP-hexose	377, 253	783	783>481	6.90±0.07
16	Digalloyl-HHDP-hexose	272	785	785>633	5.04±0.03
17	Ellagic acid derivative	265	799	799>479	8.18±0.02
18	Galloyl-HHDP-glycoside	250	649	649>301	6.23±0.01
	Total				**44.42±0.37**
	Gallagyl esters				
19	Gallagyl-hexose (punicaline)	371, 262	781	781>601	3.05±0.01
20	HHDP-gallagyl-hexose (punicalagin)	378, 258	1083	1083>601	13.90±0.02
	Total				**16.95±0.02**
	Hydroxycinnamic acids				
21	Caffeic acid-hexose	293	341	341>179	6.76±0.55
22	Caffeic acid-hexose derivative	293	451	451>341	1.40±0.02
23	Caffeic acid derivative	260	299	299>137	1.26±0.01
	Total				**10.43±0.03**

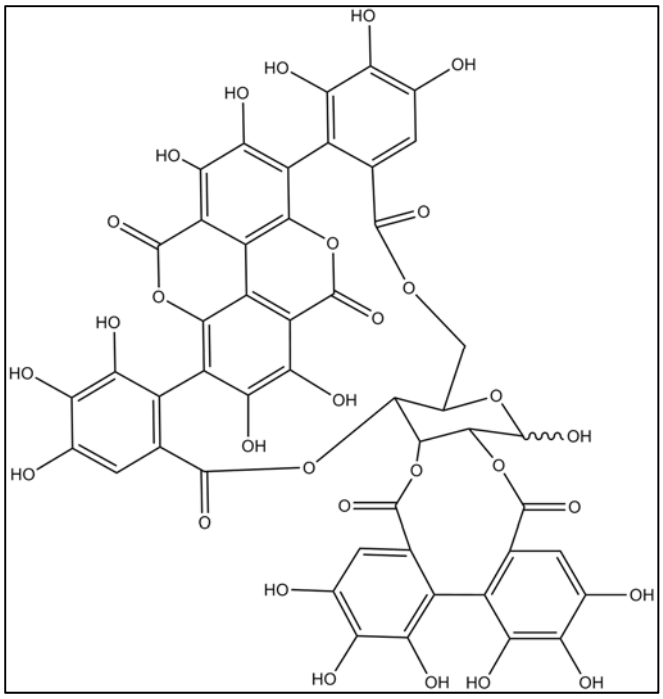

Fig 3. Structure of punicalagin, the major phenolic compound in pomegranate vinegar

References

Aviram, M., & Rosenblat, M. (2013). Pomegranate for your cardiovascular health. *Rambam Maimonides Medical Journal*, *4*, e0013.

Cerezo, A. B., Tesfaye, W, Torija, M. J., Mateo, E., Garcia-Parrilla, M. C. & Troncoso, A. M. (2008). The phenolic composition of red wine vinegar produced in barrels made from different woods. *Food Chemistry*, 109, 606–615.

Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M., & Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry, 48*(10), 4581-4589.

Gómez-Caravaca, A. M., Verardo, V., Toselli, M., Segura-Carretero, A., Fernández-Gutiérrez, A., & Caboni, M. F. (2013). Determination of the major phenolic compounds in pomegranate juices by HPLC–DAD–ESI-MS. *Journal of Agricultural and Food Chemistry, 61*(22), 5328-5337.

Kelebek, H., Kesen, S., & Selli, S. (2015a). Comparative Study of Bioactive Constituents in Turkish Olive Oils by LC-ESI/MS/MS. *International Journal of Food Properties, 18*(10), 2231-2245.

Kelebek, H., Selli, S., Gubbuk, H., & Gunes, E. (2015b). Comparative evaluation of volatiles, phenolics, sugars, organic acids and antioxidant properties of Sel-42 and Tainung papaya varieties. *Food Chemistry, 173*, 912-919.

Nasr, C. B., Ayed, N., & Metche, M. (1996). Quantitative determination of the polyphenolic content of pomegranate peel. *Zeitschrift für Lebensmittel-Untersuchung und Forschung, 203*(4), 374-378.

Ordoudi, S. A., Mantzouridou, F., Daftsiou, E., Malo, C., Hatzidimitriou, E., Nenadis, N. & Tsimidou, M. Z. (2014). Pomegranate juice functional constituents after alcoholic and acetic acid fermentation. *Journal of Functional Foods*, *8*, 161–168.

Sakanaka, S. & Ishihara, Y. (2007). Comparison of antioxidant properties of persimmon vinegar and some other commercial vinegars in radical-scavenging assays and on lipid oxidation in tuna homogenates. *Food Chemistry, 107*, 739–744.

Tesfaye, W., Morales, M.L., Garcia-Parrilla, M.C. & Troncoso, A.M. (2002). Wine vinegar: technology, authenticity and quality evaluation. *Trends in Food Science & Technology*, *13*, 12–21.

Wang, H., Cao, G., & Prior, R. L. (1997). Oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry, 45*(2), 304-309.

Xu, Q., Tao, W., & Ao, Z. (2007). Antioxidant activity of vinegar melanoidins. *Food Chemistry, 102*(3), 841-849.

Zaouay, F., Mena, P., Garcia-Viguera, C., & Mars, M. (2012). Antioxidant activity and physico-chemical properties of Tunisian grown pomegranate (Punica granatum L.) cultivars. *Industrial Crops and Products, 40*, 81-89.

