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Analytical Methods

Determination of phenolic composition and antioxidant capacity of blood orange juices obtained from cvs. Moro and Sanguinello (*Citrus sinensis* (L.) Osbeck) grown in Turkey

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Abstract

The phenolic compounds and antioxidant capacities of orange juices obtained from cvs. Moro and Sanguinello (*Citrus sinensis* (L.) Osbeck) were determined. A high-performance liquid chromatographic method coupled with diode-array detection was used to identify and quantify phenolic compounds of the orange juices. A total of 18 phenolic compounds were identified and quantified in Moro and Sanguinello orange juices, including hydroxybenzoic acids (2), hydroxycinnamic acids (5), flavanones (5), and anthocyanins (6). It was observed that total phenolic content of Moro juice was higher than that of Sanguinello juice. Ferulic acid was the most dominant hydroxycinnamic acid and cyanidin 3-(6″-malonyl glucoside) and cyanidin 3-glucoside were the most dominant anthocyanins in both cultivars. Antioxidant activities of orange juices were measured using the DPPH· (2,2-diphenyl-1-picrylhydrazyl) method. According to DPPH· assays, the antioxidant capacity of Moro juice was found to be higher than that of Sanguinello juice.

Keywords: Blood orange; Moro; Sanguinello; Phenolic compounds; Anthocyanins; Antioxidant activity

1. Introduction

Moro and Sanguinello (*Citrus sinensis* (L.) Osbeck) are blood orange varieties originating from Malta and Sicilia islands and have been cultivated, for many centuries, in Mediterranean areas (Maccarone, Maccarone, & Rapisarda, 1985; Mouly, Gaydou, Faure, & Estienne, 1997). Moro variety is the most colourful of all the blood oranges (Maccarone et al., 1985; Mondello, Cotroneo, Errente, Dugo, & Dupo, 2000). The rind shows a bright red blush, and the juice colour is deep red. It is excellent as an early-season variety. Sanguinello is small to medium sized with a thin skin and few or no seeds midseason orange variety. Its flesh is orange with multiple blood-coloured streaks (Klein, Moore, & Sweet, 1985). These cultivars are characterized by their unique flesh and rind colour due to red pigments belonging to the anthocyanin class (Maccarone et al., 1985; Mondello et al., 2000; Rapisarda, Pannuzzo, Romano, & Russo, 2003).

Cyanidin 3-glucoside and cyanidin 3-(6″-malonyl glucoside) are the main anthocyanins present in juice of pigmented oranges and responsible for their red brilliant colour (Hillebrand, Schwarz, & Winterhalter, 2004; Maccarone, Rapisarda, Fanella, Arena, & Mondello, 1998). Dugo, Mondello, Morabito, and Dugo (2003) characterized delphinidin 3-glucoside, peonidin 3-glucoside, cyanidin 3-rutinoside, and the 6″-malonyl glucose esters of delphinidin, peonidin, and petunidin, respectively, as minor anthocyanins of Sicilian blood orange juice by ESI-MS. The content of anthocyanin pigments in the juice depends on the season and varies from 96 to 166 mg/l of juice obtained from California oranges (Lee, Carter, Barros, Dezman, & Castle, 1990) and from 1.2 to 278 mg/l of juice obtained from Italian oranges (Rapisarda, Carollo, Fallico, Tomaselli, & Maccarone, 1998; Rapisarda, Fanella, & Maccarone, 2000). Comprising over 90% of total anthocyanin pigments, cyanidin

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is the predominant substance, followed by delphinidin with about 6%, peonidin at 1.2–1.7%, and traces of petunidin and pelargonidin (Lee et al., 1990).

Another peculiar characteristic of blood oranges is the higher concentration of hydroxycinnamic acids and flavanones than in blond orange varieties (Rapisarda et al., 1998). As well as affecting fruit-sensory influencing qualities, these substances have an important biological role owing to their antioxidant activity (Rapisarda, Bellomo, & Intelisano, 2001). Peleg, Naim, Rouseff, and Zehavi (1991) determined the distribution of bound and free caffeic, *p*-coumaric, ferulic, and sinapic acids in the Shamuti orange fruit and confirmed ferulic acid predominance over the other hydroxycinnamic acids. Rapisarda et al. (1999) reported that hesperidin and narirutin were the most abundant flavanones in orange juices. Depending on season, blood oranges contain from 457 to 657 mg of hesperidin per litre of juice. Narirutin is the other major flavanone glycoside found in orange juices (Lee et al., 1990).

Several studies exist on the potential role of blood oranges juices (Jayaprakasha & Bhimanagouda, 2007; Klimczak, Malecka, Szlachta, & Gliszczynska-Swiglo, 2007; Tarozzi et al., 2006; Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007). In vitro studies demonstrated the high antioxidant capacity of these orange juices, due to their phenolic contents (Scalzo, Iannoccari, Summa, Morelli, & Rapisarda, 2004). Rapisarda et al. (1999) showed that anthocyanin content is the main factor influencing the antioxidant activity of pigmented orange fruit. Furthermore, a considerable body of evidence indicates that the anthocyanins present in blood orange can play a key role in the prevention of human pathologies related to oxidative damage of biomolecules, including heart diseases and cancer (Duthie, Duthie, & Kyle, 2000). Arena, Fallico, and Maccarone (2001a) studied the evaluation of antioxidant capacity of blood orange juice as influenced by constituents, concentration process and storage. They reported that blood orange juices have higher total antioxidant activity than blond juices, and freshly-squeezed juices are higher than processed.

Orange is the third largest fruit crop grown in Turkey after grape and apple with an annual production of 1,250,000 ton in 2005 (FAO, 2005). It is produced large amounts in the Cukurova region of the southern part of Turkey. There has been no detailed research on the phenolic components and antioxidant capacity of orange juices obtained from cvs. Moro and Sanguinello grown in Turkey. This research was undertaken to determine the phenolic components and their antioxidant capacity of the orange juices obtained from cvs. Moro and Sanguinello grown in Turkey.

2. Materials and methods

2.1. Chemicals

Milli-Q water (Millipore, Bedford, MA) was used in all work. HPLC-grade acetonitrile and formic acid (Merck, Darmstadt, Germany) were used after filtration through a 0.45-μm pore size membrane filter. 2,2-diphenyl-1-picryl hydrazyl ($DPPH^{\cdot}$) was obtained from Sigma–Aldrich (St. Louis, MO) and ascorbic acid was purchased from Merck (Darmstadt, Germany). Phenolic acids (gallic, protocatechuic, caffeic, chlorogenic, *p*-coumaric, ferulic, and sinapic acids) and flavanones (narirutin, naringin, hesperidin, neohesperidin, and didymin) were purchased from Sigma–Aldrich (Steinheim, Germany). Cyanidin 3-glucoside and delphinidin 3-glucoside were purchased from Extrasynthese (Lyon, Genay-France).

2.2. Preparation of juice sample

The blood oranges of Moro and Sanguinello (*Citrus sinensis* L. Osbeck) were harvested at optimum maturity from the Cukurova University experimental orchard and transported to the biotechnology laboratory of the Department of Food Engineering, University of Cukurova, Adana, Turkey. Harvest involved a random sampling from 12 trees. Researches were carried out with 50 kg oranges of each variety.

The oranges were extracted by cutting the fruit in half and careful hand-squeezing in a kitchen juicer (Arcelik K-1775, Turkey). The juices were passed through a strainer to remove pulp and seeds. The freshly squeezed juices were centrifuged at 4000 rpm in a centrifuge (Eppendorf 3810R, Hamburg, Germany) for 20 min, the supernatant were then filtered through 0.45-μm pore size membrane filters and were kept at temperature of −18 °C until analysis.

2.3. Standard chemical analysis

The total titratable acidity was assessed by titration with sodium hydroxide (0.1 N) and expressed as citric acid %. The pH value was measured using a digital pH meter (WTW Inolab pH-L1, Germany). Total soluble solids were measured as Brix using a refractometer (Carl Zeiss, Jena, Germany) (AOAC, 1990; Ting & Rouseff, 1986). Ascorbic acid was determined by visual titration, using 2,6-dichlorophenolindophenol (Rapisarda & Intelisano, 1996). The contents of total phenolic compounds were determined by measuring the absorbance value at 280 nm. Sample was diluted with water in a 1:100 ratio and the absorbance was directly measured at 280 nm. The value of the total phenolics (280 index) was calculated by multiplying the absorbance × 100 (Ribereau-Gayon, Glories, Maujean, & Dubourdieu, 2000).

2.4. Spectrophotometric colour analysis

A direct measurement of absorbance (Abs) of the juices at 420, 520, and 620 nm was carried out using Shimadzu UV-1201 model spectrophotometer (Kyoto-Japan). The following variables were calculated; colour intensity (CI) as the sum of 420 nm, 520 nm, and 620 nm absorbances; tint was calculated by dividing the absorbance of 420–

520 nm; proportion of yellow (Ye %), red (Rd %) and blue colour (Bl %) were calculated by dividing the absorbance of 420, 520 and 620, to the colour intensity (CI), respectively (Glories, 1984).

2.5. Antioxidant activity determination

The free radical scavenging activity of orange juices was measured according to the DPPH· method reported by Brand-Williams, Cuvelier, and Berset (1995) with modifications (Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998). Five different dilution of each orange juice (30/100, 20/100, 15/100, 10/100, and 5/100) were prepared in ethanol/water (v/v). An aliquot of 0.1 ml of diluted orange juice was added to 3.9 ml of DPPH· solution in methanol (6×10^{-5} M) and vortexed. The decrease in absorbance of DPPH· at 515 nm was measured at different time intervals by a Shimadzu UV-1700 spectrophotometer (Kyoto-Japan) until the reaction reached plateau (time at the steady state). The DPPH· concentration (C_{DPPH} as mg/ml) in the reaction medium was calculated from the following equation:

$$A515\ nm = 31.275 C_{DPPH}\ (R^2 = 0.9998).$$

The percentage of remaining DPPH· (% $DPPH_{REM}$) was calculated as follows:

$$\%DPPH_{REM} = 100 \times C_{DPPH}/C_{DPPH,t=0}$$

where $C_{DPPH,t=0}$ is the initial DPPH· concentration and C_{DPPH} is the DPPH· concentration at the steady state. The percentage of remaining DPPH· at the steady state for the five dilution was plotted versus the ratio mg DPPH·/ml orange juice. The parameters EC_{50} and $T_{EC_{50}}$ were calculated graphically, and the AE was determined with the following equation:

$$AE = 1/EC_{50} T_{EC_{50}}$$

All tests were performed in triplicate.

2.6. Liquid chromatographic 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, CA, USA) operated by Windows NT based ChemStation software was used. The HPLC equipment was used with a diode array detector (DAD). System consisted of a binary pump, degasser and auto sampler. The column used was a Beckman Ultrasphere ODS (Roissy CDG, France): 4.6 mm × 250 mm, 5 μm equipped with a precolumn 4.6 mm × 10 mm (same granulometry). The mobile phase consisted of two solvents: Solvent A, water/formic acid (95:5; v/v) and Solvent B, acetonitrile/solvent A (60:40; v/v). Phenolic compounds were eluted under the following conditions: 1 ml/min flow rate and the temperature was set at 25 °C, isocratic conditions from 0 to 10 min with 0% B, gradient conditions from 0% to 5% B in 30 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 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 of phenolic compounds were obtained out by using authentic standards and by comparing the retention times and ultra-violet–visible spectra with those found in the literature (Anagnostopoulou, Kefalas, Kokkalou, Assimopoulou, & Papageorgiou, 2005; Dugo et al., 2003; Hillebrand et al., 2004; Lee, 2002; Mata-Bilbao, Andrés-Lacueva, Jauregui, & Lamuela-Raventos, 2007; Merken & Beecher, 2000), while quantification was performed by external calibration with standards.

3. Results and discussion

3.1. Chemical composition of the orange juices

The chemical compositions of Moro and Sanguinello orange juices are given in Table 1. As can be seen, Sanguinello juice showed a higher concentration of total acidity, total sugar, tint, and ascorbic acid compared to Moro juice. Orange juices are a rich source of ascorbic acid, which is an important antioxidant in these juices (Arena, Fallico, & Maccarone, 2001b). Concentration of ascorbic acid in cvs. Moro and Sanguinello were found by 506.5 and 534.2 mg/l, respectively (Table 1). Generally, the orange juices composition were in accordance with previous studies carried out on Moro and Sanguinello orange juices (Mondello et al., 2000; Rapisarda et al., 2001; Selli, 2007).

3.2. Phenolic composition of orange juices

Table 2 shows the phenolic compounds of the Moro and Sanguinello orange juices, expressed by mean (mg/l), which correspond to the three analytical replicates. A total of 18 phenolic compounds were identified and quantified in Moro orange juice (Fig. 1), including hydroxybenzoic acids

Table 1
Chemical composition of Moro and Sanguinello orange juices

Analysis	Moro	Sanguinello
Juice yield (%)	35.1 ± 2.25	38.9 ± 3.35
Total acidity[a] (g/l)	11.3 ± 1.10	13.4 ± 1.80
pH	3.35 ± 0.02	3.05 ± 0.02
Brix	12.0 ± 0.20	12.6 ± 0.20
Total sugar (g/l)	103.7 ± 1.20	109.5 ± 0.80
Total phenolics (280 index)	55.4 ± 0.50	28.0 ± 0.20
Colour intensity	1.40 ± 0.12	0.84 ± 0.10
Tint	0.59 ± 0.01	1.23 ± 0.02
% Ye	32.6 ± 0.04	41.9 ± 0.12
% Rd	55.0 ± 0.16	34.1 ± 0.08
% Bl	12.4 ± 0.04	24.0 ± 0.02
Ascorbic acid (mg/l)	506.5 ± 21.3	534.2 ± 24.7
Extract (g/l)	116.7 ± 0.08	119.4 ± 0.10

[a] As citric acid.

Table 2
Phenolic content (mg/l ± standard deviation) of Moro and Sanguinello orange juices

Compounds	Peak no.	Moro	Sanguinello
Hydroxybenzoic acids			
Gallic acid	1	7.54 ± 0.24	5.82 ± 0.63
Protocatechuic acid	2	1.71 ± 0.03	0.92 ± 0.03
Total		9.25	6.74
Hydroxycinnamic acids			
Caffeic acid	3	6.79 ± 0.33	5.23 ± 0.52
Chlorogenic acid	4	15.08 ± 0.21	12.87 ± 0.26
p-Coumaric acid	5	8.29 ± 0.85	6.27 ± 0.14
Ferulic acid	6	26.89 ± 1.10	19.84 ± 1.02
Sinapic acid	7	17.3 ± 1.41	13.55 ± 1.19
Total		74.35	57.76
Flavanones			
Narirutin	8	29.8 ± 1.47	32.59 ± 1.80
Naringin	9	2.6 ± 0.18	1.7 ± 0.12
Hesperidin	10	143.20 ± 6.38	112.98 ± 0.99
Neohesperidin	11	0.51 ± 0.14	0.22 ± 0.08
Didymin	12	4.89 ± 0.21	3.77 ± 0.18
Total		181.0	151.26
Anthocyanins			
Delphinidin 3-glucoside	13	12.5 ± 0.84	2.60 ± 0.19
Cyanidin 3-glucoside	14	110.8 ± 5.19	15.83 ± 0.54
Delphinidin 3-(6″-malonyl glucoside)	15	7.6 ± 1.42	1.48 ± 0.29
Cyanidin 3-(6″-malonyl glucoside)	16	132.6 ± 4.64	16.95 ± 0.10
Cyanidin 3-*O*-(6″-dioxalyl glucoside)	17	16.1 ± 1.08	4.64 ± 0.18
Peonidin 3-(6″-malonyl glucoside)	18	11.7 ± 0.33	1.57 ± 0.06
Total		291.3	43.07

Results are the means of three repetitions.

(2), hydroxycinnamic acids (5), flavanones (5), and anthocyanin (6) compounds. Moro juice had 9.25 mg/l of hydroxybenzoic acids, 74.35 mg/l of hydroxycinnamic acids, 181.0 mg/l of flavanones, 291.3 mg/l of anthocyanins. Sanguinello juice, having the same number of total phenolic compounds, contained 6.74, 57.76, 151.26 and 43.07 mg/l of these compounds, respectively. As can be seen, phenolic content of Moro juice was clearly higher than Sanguinello juice (Table 2). These findings are in accordance with previous studies on blood orange juices made on Moro and Sanguinello (Rapisarda et al., 1998, 2000).

Two hydroxybenzoic acids; gallic and protocatechuic acid, were detected in both orange juices (Table 2). As can be seen in Table 2, Moro orange juice contained more hydroxybenzoic acids than Sanguinello juices. The major hydroxybenzoic acid in orange juices is gallic acid (3,4,5-trihydroxybenzoic acid). Gallic acid is a naturally abundant plant phenolic compound. It is present in food of plant origin, and since it was found to exhibit antioxidative properties, it has attracted considerable interest (Strlic, Radovic, Kolar, & Pihlar, 2002).

The five hydroxycinnamic acids identified in the analysis were caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, and sinapic acid. Ferulic acid was the most dominant hydroxycinnamic acids in Moro and Sanguinello juices, as it accounted for the largest proportion of the total hydroxycinnamic acids contents (Table 2). Ferulic acid is a precursor for 4-vinyl guaiacol, the transformation catalysed by the enzyme ferulic acid decarboxylase. Objectionable off-flavours resulting from 4-vinyl guaiacol have been reported for improperly stored orange juice (Peleg et al., 1991; Fallico, Lanza, Maccarone, Asmundo, & Rapisarda, 1996). The amount of ferulic acid in Moro juice was higher than Sanguinello juice (Table 2). Sinapic acid was the second most abundant hydroxycinnamic acid and followed by chlorogenic, coumaric, caffeic acids in both juices. The levels of ferulic and sinapic acids in the both varieties were slightly lower than those reported by Rapisarda et al. (1998). Recent studies have shown that hydroxycinnamic acids were more abundant in Moro orange than in other blood varieties as well as in blond orange fruit juices, and the distribution of four acids (caffeic, sinapic, *p*-coumaric, and ferulic acids) was typical in each variety (Maccarone et al., 1985; Rapisarda et al., 1998).

Flavanone is the major flavonoids in orange varieties. Five flavanones; narirutin, naringin, hesperidin, neohesperidin and didymin were identified in Moro and Sanguinello juices. Table 2 shows that, of the five flavanones, hesperidin was the most abundant in both Moro and Sanguinello juices. Neohesperidin was the least abundant flavanone in the samples. Hesperidin is tasteless and therefore does not contribute to the taste of orange juice (Horowitz & Gentili, 1977). The concentration of hesperidin was higher in Moro juice than Sanguinello juice (Table 2). Narirutin was the second most abundant flavanone in Moro and Sanguinello juices. In the literature narirutin-to-hesperidin ratio has been proposed for quality control of orange juices (Aturki, Braudi, & Sinibaldi, 2004). The ratio obtained for Moro and Sanguinello juices were 0.208 and 0.288, respectively. According to earlier work on orange juices, the narirutin-to-hesperidin ratio ranged from 0.151 to 0.262 (Aturki et al., 2004). Rouseff (1988) reported that this ratio has to be <0.339 for authentic orange juices. Our findings were below 0.339 value. Narirutin and didymin content were in agreement, while hesperidin content was found to be lower than that previously reported by Mouly et al. (1997) and Proteggentea, Saija, Pasquale, and Rice-Evansa (2003).

A total of six anthocyanin compounds were identified and quantified in Moro and Sanguinello juices. It was observed that the anthocyanin profiles of both varieties were similar. However, the total anthocyanin content in Moro juice was clearly greater than Sanguinello juice (Table 2). As previously reported, cyanidin 3-(6″-malonyl glucoside) and cyanidin 3-glucoside were the most dominant anthocyanins in Moro and Sanguinello juices, as it accounted for the largest proportion of the total anthocya-

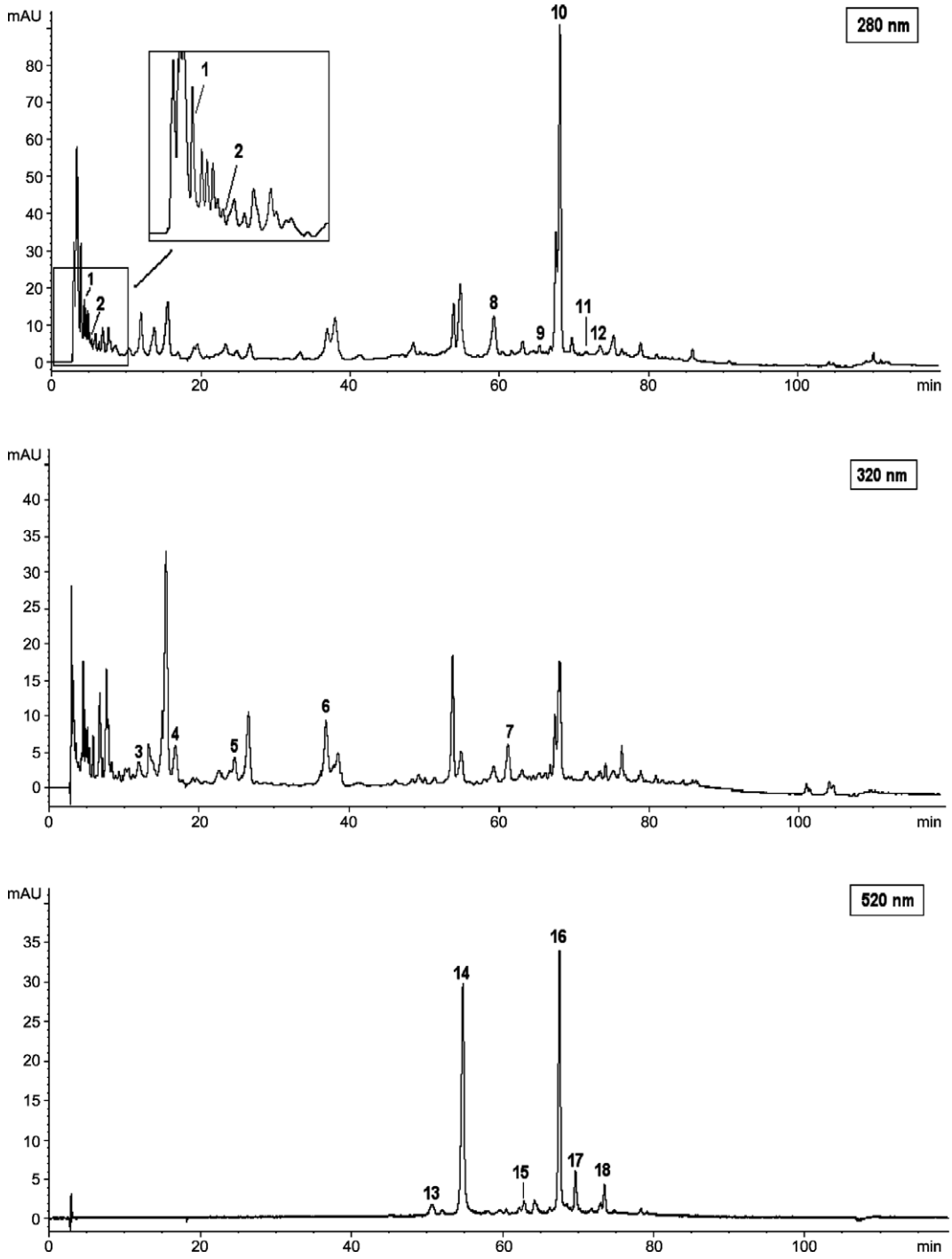

Fig. 1. HPLC-DAD chromatogram at 280, 320 and 520 nm of the Moro orange juice (identification of the peaks can be found in Table 2).

nin compounds (Table 2). The total amount of cyanidin 3-(6″-malonyl glucoside) and cyanidin 3-glucoside were 132.6 and 110.8 mg/l in Moro juice and 16.95 and 15.83 mg/l in Sanguinello juice, respectively. Lee (2002) reported that cyanidin 3-(6″-malonyl glucoside) was the most dominant anthocyanin in Moro juice, comprising more than 44% of total anthocyanin content, followed by cyanidin 3-glucoside. The contents of cyanidin 3-(6″-malonyl glucoside) and cyanidin 3-glucoside reported here for Moro juice were in agreement, while for Sanguinello juice was found to be slightly lower than reported by Lee (2002).

3.3. Antioxidant activity of orange juices

In our study, the antioxidant activities of orange juices were evaluated using $DPPH^{\cdot}$ free radical-scavenging assays. This method is recommended by many authors (Klimczak et al., 2007; Sanchez-Moreno et al., 1998; Shah-

idi, Liyana-Pathirana, & Wall, 2006; Villaño et al., 2007) as easy and accurate assays for measuring the antioxidant activity of orange juices and other fruits. EC_{50} is inversely related to the antioxidant capacity of a compound, as it expresses the amount of antioxidant needed to decrease the radical concentration by 50%. The lower EC_{50} value the higher the antioxidant activity of a compound (Villaño et al., 2007). The EC_{50} value of Moro juice (0.18 mg/ml) was found lower than Sanguinello juice (0.29 mg/ml). Moro juice has a higher antioxidant activity contained higher concentration of phenolic compounds. $T_{EC_{50}}$ is the time need to reach the steady state to EC_{50} concentration. Time at steady state depends on the reactivity of antioxidants and the concentrations used. The antiradical efficiency (AE) is a new parameter for the measurement the free radical scavenging of samples, and it combines the EC_{50} and $T_{EC_{50}}$ (Sanchez-Moreno et al., 1998). AE value of Moro juice (50.5×10^{-3}) was found higher than Sanguinello juice (28.1×10^{-3}). In the literature, antioxidant activity of citrus juices depends on the type (position and number of hydroxyl in the molecule) and the concentration of the phenolic compounds, as well on that of the transition metal (Jayaprakasha & Bhimanagouda, 2007; Sendra, Sentandreu, & Navarro, 2006).

4. Conclusions

According to the data obtained in the present study, the total phenolic and anthocyanin contents of Moro juice were higher than those of Sanguinello juice. Ferulic acid was the most dominant hydroxycinnamic acids and cyanidin 3-(6″-malonyl glucoside) and cyanidin 3-glucoside were the most dominant anthocyanins in Moro and Sanguinello juices. The antioxidant capacity of Moro juice was found higher than Sanguinello juice.

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