Research Article

Qualitative and Quantitative Estimation of Hesperidin in Peel and Juice of Citrus Fruits by RP-HPLC Method Growing in Kurdistan Region/Iraq

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ABSTRACT

The present investigation aimed to identify and quantify of hesperidin content in peel and juice of different citrus fruits and compare between them by high performance liquid chromatography. The identification and quantification of hesperidin content in peel and juice of different citrus fruits were investigated. This identification was performed by using high performance liquid chromatography and then quantified by using C18 column, isocratic mobile phase (Methanol: water (0.1% orthophosphoric acid) 50:50) and UV/Visible detector at 280 nm. Identification and quantification of hesperidin in different citrus fruits by high performance liquid chromatography revealed the presence of hesperidin in peel and juice extracts. The results of comparative quantitative analysis indicated that hesperidin content highest concentration in peel of *Citrus medica* and minimum in *Citrus sinensis* while in juice highest quantity determined in *Citrus sinensis* and lowest in *Citrus limon*, this method showed stability and accuracy for identification and quantification of hesperidin in citrus fruits. The developed method gave a good linearity over concentration 20-100 µg/ml with correlation coefficients 0.988 and limit of detection 12.49 µg/ml. The relative standard deviation of the method was 0.44-1.80% and the average recoveries were between 89.7-107%.

Keywords: Citrus fruits, Hesperidin, Qualitative, Quantitative analysis, RP-HPLC

INTRODUCTION

Flavonoids are aromatic secondary plant metabolites which is derived from the shikimate pathway and the phenylpropanoid metabolism, the compounds of these groups sharing the common feature of phenol moieties and providing much of the flavor and color to fruits and vegetables[1]. Flavonoids are mostly found in fruits, cocoa, teas, vegetables, fruit juices and wines. Among these flavonoids the flavanone analog hesperidin are common constituents in many citrus species such as Lemon (*Citrus limon*), orange (*Citrus sinensis*), citron (*Citrus medica*), tangerines (*Citrus reticulata*) and grapefruit (*Citrus paradisi*). Hesperidin consist of hesperitin and rutinose, the highest level of hesperidin is detected in very young tissues of the fruit[1,2]. Now a day there is more attention in the healthfulness of citrus fruits because increase consumption appears to be associated with decrease risk of certain chronic diseases and increased survival[3-5]. Hesperidin enhance microcirculation, possess antioxidant effect, improve venous tone, anti-inflammatory, analgesic, blood lipid lowering, assist healing of venous ulcers, also used for the treatment of hemorrhoids, chronic venous insufficiency and exhibit pronounced anticancer activities[6,7]. From literature survey several analytical methods used for the determination of hesperidin either alone or in mixture with other flavonoids in citrus fruits. Therefore the aim of present investigation was to develop a simple, precise and accurate high performance liquid chromatography (HPLC) method for the identification and determination quantity of hesperidin in five citrus fruits and compare between amount of them from the same harvest season grown under the same geographical and climatic conditions in Iraq.

MATERIALS AND METHODS

Reagents and chemicals

Hesperidin 80% pure was bought from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany), ethanol from Scharlab S.L. (Spain), methanol HPLC grade was purchased from Scharlau Chemie S.A. (Europian Union) and ortho-phosphoric acid from SDFCL s d fine-chem limited. Deionised water was obtained by means of a Milli-Q Water Purification system supplied by Millipore (Bedford, UK).

Sample preparation

Different type fresh citrus fruits such as lemons, oranges, citron, tangerines and grapefruits were collected from garden in Iraq. Citrus fruits then washed, peeled and squeezed to extract the juice during 2014-2015. One gm of peel and juice of citrus fruits were extracted with ethanol 80% using ordinary reflex for 1hr then filtered through a filter paper and the analyte samples were obtained by evaporation solvent to dryness at 40°C under vacuum. After the residues were filtered through 0.45 µm filters, they were dissolved in 1 mL of methanol: water before HPLC detection.

Preparation of stock and standard solutions

Hesperidin (1 mg) were accurately weighed and dissolved in methanol: water (1:1) filled up to volume for preparing stock solutions. Standard solutions were prepared for each compound at three different concentration levels

(100, 60 and 20µg) in 5 ml volumetric flasks for the establishment of calibration curves.

Chromatographic condition

The qualitative and quantitative analysis of hesperidin was performed on Knauer HPLC instrument equipped with Chrom Gate HPLC software provided by Knauer was use with Eurospher 100 and a thermostated column oven was used with a reverse-phase C18 column (4.6 mm i.d. x 250 mm, 5 mm) and temperature of column was controlled at 30 °C. Compounds were monitored at 280 nm using a UV/Visible detector. An isocratic mobile phase condition consisting of methanol (Solvent A) and water containing (0.1% O-phosphoric acid) (Solvent B) were used as 50% A and 50% B, the total run time was 10 min at flow rate of the mobile phase 1 ml/min and injection volume was 20 µl.

Method validation

The following parameters for developed HPLC method for hesperidin determined: linearity, precision, accuracy, and limits of detection (LOD) and quantification (LOQ). Calibration graphs for linearity determination were established with standard solutions of hesperidin were prepared at three different concentration (20, 60, and 100 µg/ml). The prepared dilutions were injected in series, peak area was calculated for each dilution and concentration was plotted against peak area. Accuracy was determined by standard adding for three concentration (20, 60 and 100µg/ml) and percentage of recovery was calculated for each concentration. System precision was performed by injecting hesperidin three times on the same day and on three different days was determined as intra-day and inter day variation. In both cases relative standard deviation (RSD) consider as a measure for precision. Sensitivity was evaluated by determining the LOD and LOQ. LOD was defined as the amount of analyte that gives a peak with a signal-to-noise ratio of 3, whereas LOQ was the lowest amount of analyte with a signal-to-noise ratio of 10. Validation of the method was performed as recommended by the International Conference on Harmonization (ICH)[8,9].

Quantification of hesperidin in 80% ethanolic extract of citrus fruits

The ethanolic extract of five citrus fruits were applied and chromatograms were obtained under the same conditions as for analysis of standard hesperidin. The area of the peak corresponding to the retention time (R_t) value of hesperidin standard was recorded and the amount present was calculated from the regression equation obtained from the calibration plot.

RESULTS AND DISCUSSION

Identification and quantification of hesperidin in peel and juice of citrus fruits

Fruit and vegetables are considered to be important natural products as a disease-preventing diet[10]. A validated HPLC has been developed for identification and quantification of hesperidin in 80% ethanolic extract of peel and juice of citrus fruits by using C18 column and an isocratic mobile phase condition consisting of methanol and water containing (0.1% O-phosphoric acid) (50%:50%) and detected at 280nm. Hesperidin was detected in peel and juice of analyzed citrus fruits by comparing their retention times and UV spectra with standards the results shown in (Figure 1 and 2).

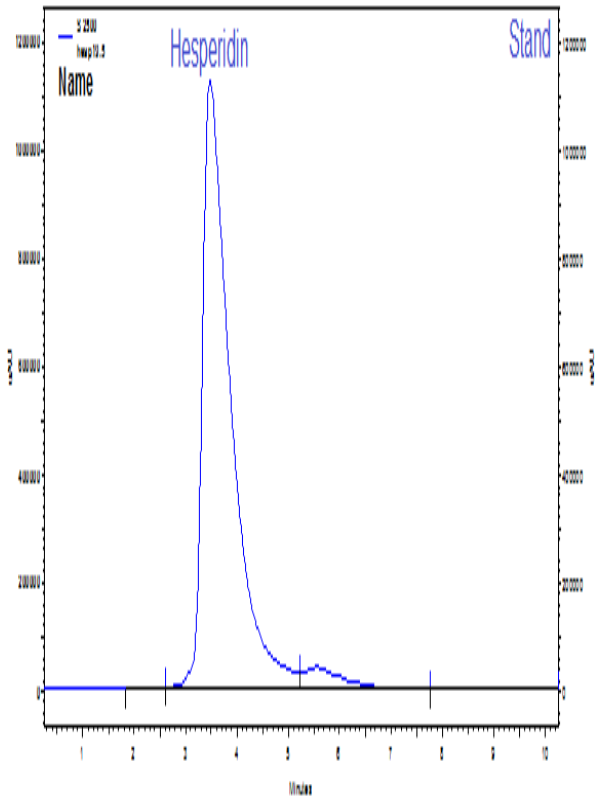
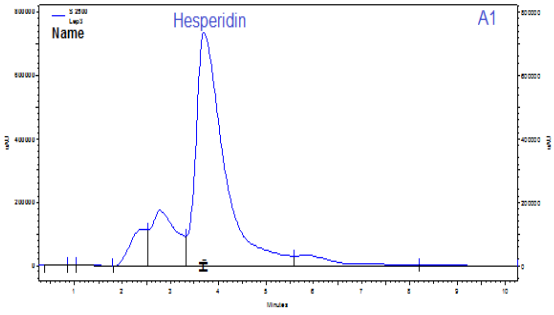
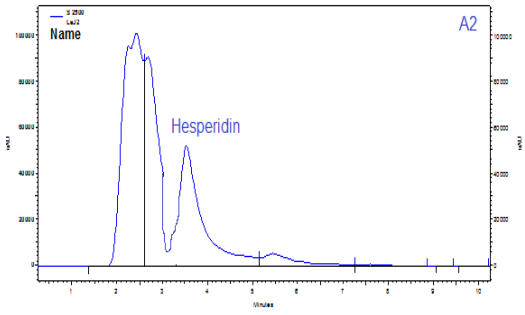


Figure 1: HPLC chromatogram of standard hesperidin





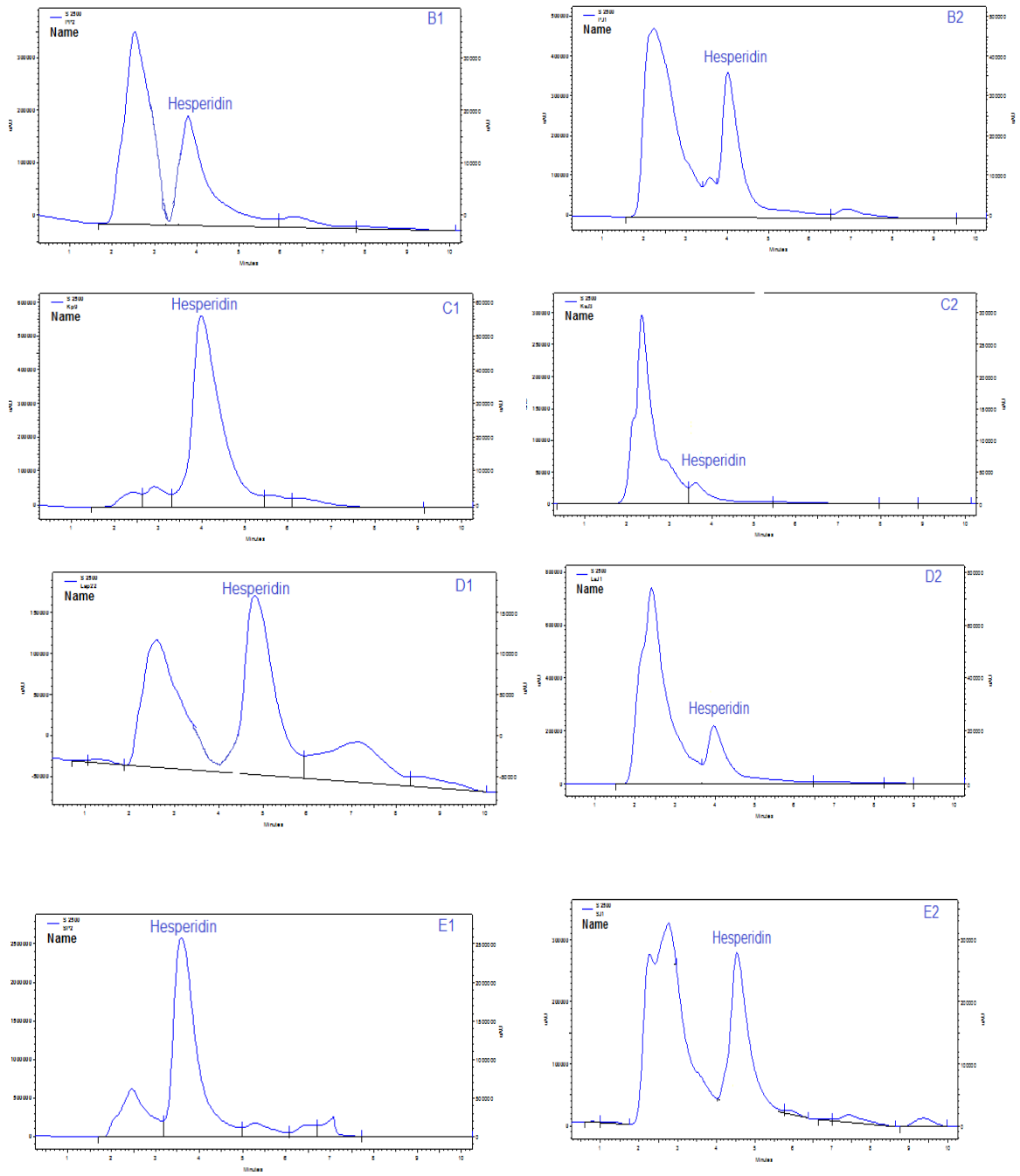


Figure 2: The HPLC chromatograms of the Peel (1) and juice (2) of citrus fruits. (A) *Citrus lemon* (B) *Citrus sinensis* (C) *Citrus medica* (D) *Citrus reticulata* (E) *Citrus paradisi*

Table 1: Amount of hesperidin content in peel and juice of citrus fruits

Compound name	*Citrus limon*		*Citrus sinensis*		*Citrus* medica		*Citrus reticulata*		*Citrus paradisi*	
	Peel	Juice	Peel	Juice	Peel	Juice	Peel	Juice	Peel	Juice
Hesperidin (µg/gm of peel and juice)	125.3	9.4	19.5	20.6	261.9	17.8	184.2	14.8	204.2	18.7

Also the developed method was applied for the determination amount of hesperidin in different citrus fruit peel and juices the results summarized in (Table 1). But on comparison of their quantity vary in different citrus fruit peel and juice. In peel the highest content hesperidin was found in *Citrus medica* 261.9 µg/ml, followed by *Citrus paradisi*, *Citrus reticulata* then *Citrus lemon* and very small concentrations in peel of *Citrus*

cinensis 19.5 µg/ml. While their quantity in juice found in highest amount in *Citrus sinensis* 20.6 µg/ml followed by *Citrus paradisi, Citrus medica* then *Citrus reticulata* and minimum amount in *Citrus lemon* 9.4 µg/ml. Presence of hesperidin in citrus fruits supported by previously recorded data but the results obtained from these study differ from previous work due to influence of several factors such as environmental and climatic condition during plant growth, geographical area in which plant growth because concentration and presence of constituents vary according to those condition and choice of extraction method, also use of different HPLC conditions might explain why our results for hesperidin content in citrus fruits were differ from other reports. Presence of hesperidin in *Citrus sinensis* and *Citrus paradisi* fruit supported by previously recorded data on citrus were grown in Israel and harvested in the season 2004–2005 compounds were separated on a Spherisorb ODS1 column, the mobile phase was a gradient prepared from 2% aqueous acetic acid, pH 2.58 (A) and acetonitrile (B) at 40°C and peaks are detected at 285 nm, the Rt hesperidin (33.2 min) in which concentration of hesperidin 0.879 mg/ml in *Citrus sinensis* from edible total weight 286 g/L[10]. Other study reported results for hesperidin in fresh, hand-squeezed juice from different cultivars of *Citrus sinensis* were 12.2–25.4 mg/100 g[11].

The content of hesperidin in *Citrus paradisi* averaged 3 mg aglycone/100 g edible fruit or juice according to Peterson[12] and hesperidin 23 mg/100ml[13,14]. Also presence of hesperidin in *Citrus reticulata* juice quantified by other workers which is (23, 24.3 mg/100 ml)[14,15].

A HPLC-photodiode array detection system was used to analyze five flavonoids namely naringin, hesperidin, didymin, tangeretin and nobiletin in different parts of *Citrus reticulata* fruit. The chromatographic analysis was performed on a C18 column with a gradient elution of acetonitrile and water at a flow rate of 1.0 ml/min. Detection was carried out using a photodiode array detector at 280 nm the quantity of hesperidin in peel 55260.4 and pulp 8369.4 µg/g[16].

The content of hesperidin in *Citrus lemon* juice (23, 20.5 mg/100 mL)[17], and16 mg aglycone/100 g edible fruit or juice[12]. Hesperidin content in *Citrus medica* reported by other workers[18] but quantitative determination of hesperidin content in *Citrus medica* by HPLC method was conducted for the first time.

Method Validation

Linearity, LOD and LOQ

The linearity of the method was confirmed for concentrations ranging from 20 to 100 µg/ml for hesperidin glycosides.

Calibration curves exhibited good linear regressions (Y= 0.59X+ 0.688, R^2 = 0.988) results shown in (Table 2 and Figure 3).

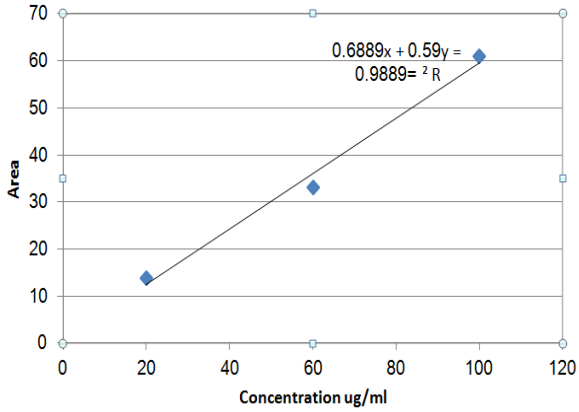


Figure 3: Linearity of hesperidin standard

Table 2: Calibration data for the proposed HPLC methods

Compound	Concentration (µg/ml)	Peak area 10^5 (mAU)
Hesperidin	20	23.03
	60	47.86
	100	66.03

LOD used for detection of minimum amount of analyte in plant extract and LOQ used for determination quantity of minimum amount of analyte in plant extract. LOD and LOQ of hesperidin for the proposed method was found to be 12.49 and 37.86 µg/ml which indicated that the method can be used in wide range for detection and quantification of hesperidin effectively in (Table 3).

Table 3: Linearity range, limit of detection and quantification of hesperidin by HPLC method

Parameters	Hesperidin
Linearity range (µg/ml)	20-100
Correlation coefficient(r)	0.988
Slope	0.59
Intercept	0.688
SE of intercept	1.29
SD of intercept	2.23
LOD (µg/ml)	12.49
LOQ (µg/ml)	37.86
Retention time (min)	3.7

Precision and accuracy

The precision and accuracy tests performed by injecting sample three time within same and over three consecutive days the results shown in (Table 4).

The repeatability of intra-day and inter-day precision which expressed by RSD of the peak areas of hesperidin in which ranged between 0.44-1.80 which is under limit 2.5% as per recommendations of ICH guidelines[8,9].

Accuracy was determined by calculating recovery values for hesperidin ranged between 89.7-107%, the results revealed that the developed method an accurate method.

Table 4: Recovery and relative standard deviation data of hesperidin by HPLC method

Compound	Amount added (µg/ml)	Amount recovered (µg/ml)[a]	Recovery (%)[a]	RSD (%)	
				Intra day[b]	Inter day[c]
Hesperidin	20	17.95 ± 0.28	89.7 ± 0.28	0.87	1.58
	60	64.20 ± 0.28	107.0 ± 0.28	1.62	0.44
	100	98.03 ± 1.76	98.0 ±1.76	0.53	1.80

[a]Mean ± SD (n=3) mean the sample analyzed three times; [b]Samples were analyzed three times a day; [c]Sample were analyzed once a day over three consecutive days.

CONCLUSION

A simple, accurate and reliable analytical method for the qualitative and quantitative determination of hesperidin in five different citrus fruits by HPLC has been developed.

Hesperidin were determined in peel and juice of citrus fruits at 3.7 min.

The results of the quantification indicated that hesperidin content highest concentration in peel of *Citrus medica* and juice of *Citrus sinensis* and this method showed good linearity, accuracy, sensitivity and sufficient limit of detection.

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REFERENCES

1. Kanaze FI, Gabrieli C, Kokkalou E, Georgarakis M, Niopas I, Simultaneous reversed-phase high-performance liquid chromatographic method for the determination of diosmin, hesperidin and naringin in different citrus fruit juices and pharmaceutical formulations, J Pharm Biomed Anal, 33, 2003, 243–9.
2. Tsai TH, Liu MC, Determination of extracellular hesperidin in blood and bile of anaesthetized rats by microdialysis with high performance liquid chromatography, a pharmacokinetic application, J Chromatogr B, 806, 2004, 161–166.
3. Chen H, Ward MH, Graubard BI, Heineman EF, Markin RM, Potischman NA, Dietary patterns and adenocarcinoma of the esophagus and distal stomach, Am J Clin Nutr, 75, 2002, 137–144.
4. Palli D, Russo A, Ottini L, Masala G, Saieva C, Amorosi A, Red meat, family history, and increased risk of gastric cancer with microsatellite instability, Cancer Res, 61, 2001, 5415–5419.
5. Fortes C, Forastiere F, Farchi S, Rapiti E, Pastori G, Perucci CA, Diet and overall survival in a cohort of very elderly people, Epidem, 11, 2000, 440–445.
6. El-Shafae AM, El-Domiaty MM, Improved LC methods for the determination of diosmin and/or hesperidin in plant extracts and pharmaceutical formulations, J Pharm Biomed Anal, 26, 2001, 539–545.
7. Kanaze FI, Kokkalou E, Georgarakis M, Niopas I, A validated solid-phase extraction HPLC method for the simultaneous determination of the citrus flavanone aglycones hesperedin and naringenin in urine, J Pharm Biomed Anal, 36, 2004, 175–181.
8. ICH, Guideline Q2A, text on validation of analytical procedures: federal register, rockville, MD, USA, 60(40), 1995, 11260-11262.
9. ICH, Guideline Q2B, validation of analytical procedures: methodology, federal register, rockville, MD, USA, 62(96), 1997, 27463–27467.
10. Gorinstein S, Huang D, Leontowicz H, Leontowicz M, Yamamoto K, Soliva-Fortuny R, Determination of naringin and hesperidin in citrus fruit by high-performance liquid chromatography, The antioxidant potential of citrus fruit, Acta Chromatogr, 17, 2006, 108-124.
11. Belajova E, Suhaj M, Determination of phenolic constituents in citrus juices, Food Chem, 86, 2004, 339-343.
12. Petersona JJ, Beecherb RG, Bhagwatc AS, Dwyera TJ, Gebhardtc ES, Haytowitzc BD, Flavanones in grapefruit, lemons, and limes: A compilation and review of the data from the analytical literature, J Food Compos Anal, 19, 2006, 74–80.
13. De Castro WV, Mertens-Talcott S, Rubner A, Butterweck V, Derendorf H, Variation of flavonoids and furanocoumarins in grapefruit juices: a potential source of variability in grapefruit juice–drug interaction studies, J Agric Food Chem, 54, 2006, 249–55.
14. Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M, HL-60 differentiating activity and flavonoid content of the readily extractable fraction prepared from Citrus juices, J Agric Food Chem, 47, 1999, 128–35.
15. Mouly PP, Gaydou EM, Auffray A, Simultaneous separation of flavanones glycosides and polymethoxylated flavones in *Citrus* juices using liquid chromatography, J Chrom A, 800, 1998, 171–179.
16. Sun Y, Wang J, Gu S, Liu Z, Zhang Y, Zhang X, Simultaneous determination of flavonoids in different parts of *Citrus reticulata* 'Chachi' fruit by high performance liquid chromatography—photodiode array detection, Molecules, 15, 2010, 5378-5388.
17. Caristi C, Bellocco E, Panzera V, Toscano G, Vadalà R, Leuzzi U, Flavonoids detection by HPLC-DAD-MS-MS in lemon juices from sicilian cultivars, J Agric Food Chem, 51, 2003, 3528–34.
18. Berhow M, Tisserat B, Kanes K, Vandercook C, Survey of phenolic compounds produced in Citrus, USDA ARS Technical Bull, 1856, 1998, 1–154.

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