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Determination of total polyphenol index and flavonoids profile combined with chemometric analysis in Iranian commercial juices

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ABSTRACT

Food fraud, defined as the deliberate alteration or misrepresentation of food products for economic gain, has long posed significant challenges for consumers and the food industry. Fruit juices are among the ten commodities most vulnerable to fraudulent practices, as identified by the European Committee on the Environment, Public Health and Food Safety. Therefore, the development of robust, sensitive, and economically viable analytical techniques is essential to ensure the authenticity, quality, and safety of fruit juice products. The main objective of the present study was to develop and apply an analytical method for the quality control of commercial juice products. For this purpose, 73 Iranian commercial juices from 11 brands were analyzed. Based on their labels, the samples were classified into two categories: still fruit drinks and nectars, including orange, pineapple, peach, and sour cherry flavors. Physicochemical parameters, including pH and Brix, as well as total polyphenol content and flavonoid profiles, were determined. Total polyphenol content was measured using a spectrophotometric method, while catechin, eriocitrin, naringin, hesperidin, and quercetin were quantified by reverse-phase high-performance liquid chromatography with UV detection at 280 nm. The chromatographic separation was performed on a C8 column using gradient elution with water, acetic acid, and acetonitrile, and was completed within 30 min. The method showed acceptable analytical performance, with the highest limit of detection being 1.39 ppm for eriocitrin and spike recovery values of at least 82.81% for naringin. Statistical analysis revealed significant differences in total polyphenol and flavonoid contents among different types of fruit juices. Overall, the results indicated that flavonoid profiling is a valuable tool for the quality control and authenticity assessment of commercial fruit juices, whereas physicochemical parameters such as pH, Brix, and total polyphenol content alone are not sufficient for this purpose.

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1. Introduction

Dietary consumption of fruits and vegetables helps to the reduction of the common human cancers and cardiovascular disease risk (1, 2).

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Likewise, fruits have some health benefits such as protection against coronary heart disease, high blood pressure or high level of cholesterol. An imbalance between antioxidants and free radicals causes oxidative stress, leading to cellular damage (3). Fruits and vegetables with antioxidant and radical scavenging properties may help limitation of this imbalance and promote the body health (4). Additionally, the antiviral and antimicrobial properties of fruits and vegetables could be considered as a significant characteristic. Flavonoids, a group of compounds with effective role in reducing carcinogenesis by controlling cell cycle and apoptosis, are present in both fruits and vegetables. It is assumed that the consumption of their juices may be as effective as whole fresh fruits and vegetables consumption (5). Flavonoids are categorized into flavone, flavanone, anthocyanin and isoflavone. Quercetin, as a flavone present in fruits and vegetables, is thought to have potent antioxidant, anti-diabetic, anti-tumoral, antiviral and anti-inflammatory characteristics. Furthermore, Hesperidin, a flavone glycoside abundant in citrus fruits, has indicated antioxidant, anti-inflammatory, and anti-cancer effects, based on some *in vitro* studies. Another flavanone, naringenin, acts as a free radical scavenger and antioxidant. It also reduces total cholesterol and inhibits selected cytochrome p-450 enzymes, such as CYP1A2 and CYP3A4 (6-11).

Several significant effects, including antioxidant, anti-inflammatory and antitumor activities, have been reported for catechin in prior studies. Its antioxidant mechanism occurs through the inhibition of lipid peroxidation by alkoxyl and peroxyl radicals' elimination (12). Moreover, eriocitrin, a flavanone glycoside found in citrus plants, exhibits antioxidant, anti-inflammatory, anti-obesity, and anti-nociceptive effects. According to some researches, its beneficial effects have been shown on hepatocellular carcinoma, hepatic steatosis, osteoarthritis, oral carcinogenesis, and oxidative damage (13).

According to Iranian national standards, fruit juices are categorized into two main classifications regarding the percentage of juice contents: 1) Non-carbonated fruit drinks (Standard No. 2837) which should contain at least 20% fruit juice (14), 2) nectars (Standard No. 10526 for sour cherry, 3137 for orange nectar, 10498 for pineapple nectar and 3414 for peach nectar) which should contain at least 40% fruit juice (15-17).

Since the flavonoids determination is notably considered in recent years because of their beneficial effects on promoting human health, the evaluation of their levels in food products has become increasingly important (18). Several methods such as HPLC with diode array detection, UV spectrophotometry and electrochemical detection were applied for determination of flavonoids. In the current study, the HPLC with UV detection was developed to investigate the flavonoids (19).

The purpose of this study was to design methods for detection, separation, identification and quantification of flavonoids in optimized time in commercial juice by HPLC. According to the validation procedure, the method produces accurate results. Another goal of this study was to measure physicochemical parameters such as total polyphenol, pH and Brix in the samples. Finally, the method was applied to approximately 73 commercial fruit juice samples to validate its suitability for quality control purposes.

2. Materials and Methods

2.1. Sampling

From supermarkets in Tehran, 73 commercial juice samples from 11 brands were collected and analyzed in the present study. According to their labels, the samples were classified into two different types: non-carbonated fruit drinks and nectar. The samples comprised four kinds of fruits: orange, pineapple, peach and sour cherry. Samples were compared to the relevant standards based on their type and kind. The samples were transported and stored to the laboratory under the conditions mentioned on their labels.

2.2. Chemicals and reagents

All analytical grade solvents and chemicals were purchased from Merck Company (Darmstadt, Germany). Catechin (CAT), Eriocitrin (ERIO), Narengin (NAR), Hesperidin (HES) and Quercetin (QUE) (Sigma, St Louis) were used as standards. De-ionized water was generated using the Thermo Scientific Barnstead Easy pure II system.

2.3. Determination of physicochemical parameters

Based on Iranian national standard (ISIRI) No. 2685 for fruit juice test methods, Brix measurements was carried out using an Atago Digital Refractometer, and pH was determined by 827 model Metrohm pH meter. Total polyphenol content was also measured using the Folin - Ciocalteu method, with gallic acid as the standard. Working standard solutions were prepared by diluting of stock solutions to achieve concentrations ranging from 10 mg/L to 50 mg/L.

For sample preparation, 300 µL of each sample was diluted with ethanol (1:1 v/v). Then, 300 µL of the diluted solution was mixed with 750 µL of Folin-Ciocalteu reagent (previously diluted 1:10 with distilled water). The prepared solution was allowed to stand at room temperature for 5 min. Subsequently, 750 µL of sodium bicarbonate solution (6% w/v) was added, and the mixture was kept in a dark place for 60 min. Eventually, the absorbance of standards and samples was measured at 780 nm using a UV/Visible spectrophotometer (Varian Carry 100scan, Australia) (5).

2.4. Determination of flavonoids

2.4.1. Chromatographic method

Chromatographic analysis was carried out using an Agilent 1200 series liquid chromatograph equipped with a quaternary gradient pump, a vacuum membrane degasser, a 20 µL loop injector, and a UV Detector. Analysis was performed on an Eclipse-XDB C_8 column (250×4.6 mm, 5µm) and the UV detector was set to a wavelength of 280 nm. The mobile phase consisted of two solvents A (water /acetic acid, 98/2 v/v) and B (acetonitrile). In order to achieve optimal resolution, different gradient elution programs were tested while maintaining a constant flow rate of 1 mL/min. The optimized gradient program began with 15% solvent B, then it was increased linearly to 30% over 30 min. The column was kept at room temperature and was initially conditioned for 5 min before each analysis.

2.4.2. Preparation of standards and samples

Each flavonoid reference standard was dissolved in methanol to produce a stock solution of 100 mg/L. In the following working standard solutions for each flavonoid were prepared in the mobile phase at concentrations ranging from 0.2 mg/L to 20 mg/L. Fruit juice samples were centrifuged at 4000 rpm for 10 min at 20˚C. The supernatants were then filtered through a 0.45 µm PVDF syringe filter, and 20 µL of

each one was injected into the chromatographic column.

2.4.3. Method validation

The calibration curves of each flavonoid were prepared over the concentration range of 0.2–20 mg/L. The linearity between concentration and peak area was obtained, and the correlation coefficient for each standard calibration curve was determined.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using the following equations: LOD = $3.3\times S_y/S$ and LOQ = $10\times S_y/S$, where S_y and S represent the intercept standard deviation and slope of the calibration curve, respectively. The value of S_y was calculated using the data of calibration curve. In order to verify the feasibility of the method, sample recovery was calculated by analyzing samples before and after the addition of known quantities of flavonoids. As detailed in the "Sample Preparation" section, triplicate preparations of each sample were performed to evaluate recovery and intra-day precision (RSD), with each preparation being measured three times on the same day.

2.5. Statistical analysis

The acquired data were analyzed using Matlab software (version 7.12) and the SPSS statistical package (version 21; SPSS Inc. Chicago, IL, USA). One-way analysis of variance (ANOVA) was employed to evaluate differences in distribution among the different brands. For multiple comparisons, Tukey's post hoc test was used. Results were expressed as mean ± standard deviation (SD) for all samples in each table. Statistical significance was set at a threshold of $p<0.05$.

3. Results

3.1. Method of chromatography

In this study, the flavonoid profile-comprising CAT, ERIO, NAR, HES and QUE- was monitored in fruit juice samples using HPLC. Complete separation was performed within 30 min. The retention times for CAT, ERIO, NAR, HES and QUE were 4.6, 8.6, 13.1, 13.9 and 24.3 min, respectively. The chromatograms of the standard solution, a randomly selected juice sample and corresponding spiked sample are shown in Fig. 1.

The calibration data, LODs, LOQs, and recovery percentages for the individual flavonoids are summarized in Table 1.

3.2. Analysis of fruit juices

Several kinds of commercially available fruit juices were analyzed using the developed method. The samples were categorized by kind and brand, as described in the sampling section. The results of pH, Brix, total polyphenol contents for different kinds of juices are presented in Table 2.

The flavonoid contents in different kinds of juices are shown in Table 3.

According to One-way Anova analysis, there was a significant difference in total polyphenol content among the various kinds of fruit juices ($p<0.05$). Tukey analysis showed that pineapple juice differed significantly from the other juice kinds in terms of total polyphenol content.

HES was detected in all orange samples, with concentrations ranging from 41.480 to 495.549 mg/L. NAR was also detected in most of orange juices, with a mean concentration of 1.158 mg/L. As it can be seen, orange, pineapple and sour cherry exhibited the highest values of NAR, CAT and QUE, respectively, with significant differences observed among them ($p<0.05$, Tukey analysis).

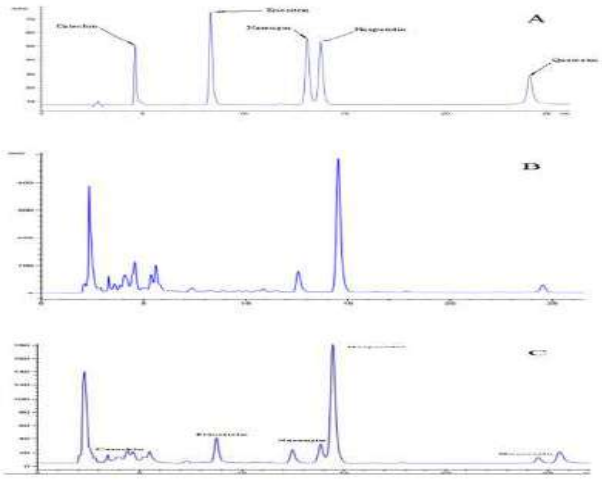

Figure 1. HPLC chromatogram for CAT, ERIO, NAR, HES and QUE: A. standard solution, B. a randomly selected sample, C. corresponding spiked sample.

Table.1 The calibration data, LOD, LOQ, and recovery rates of each flavonoid

Analyte	LOD mg/L	LOQ mg/L	Calibration equation	R^2	Recovery (%)
Catechin	0.619	1.877	y=17.755x-2.471	0.999	103.980
Eriocitrin	1.391	4.218	y=40.871x-17.721	0.997	89.911
Narengin	0.570	1.727	y=35.428x-6.926	0.999	96.495
Hesperidin	0.787	2.385	y=35.50x-3.94	0.999	82.814
Quercetin	0.587	1.780	y=28.57x1.19	0.999	85.172

Table 2. The amounts of pH, Brix and total polyphenol in different kinds of fruit juices.

Kind of fruit juices	pH	Brix %	Total polyphenol mg/L
Sour cherry	3.076±0.181	12.690±0.741	289.200±52.174
Orange	3.166±0.132	12.5±0.468	216.967±68.621
Peach	3.610±0.173	12.680±0.848	205.717±57.185
Pineapple	3.606±0.287	12.550±0.554	155.954±101.627

Table 3. The flavonoid contents in different kinds of fruit juices

Flavonoid	Catechin mg/L	Eriocitrin mg/L	Narengin mg/L	Hesperidin mg/L	Quercetin mg/L
Sour cherry	ND	3.258±2.342	1.941±1.311	1.595±0.685	0.654±0.954
Orange	ND	1.784±1.322	1.158±0.768	116.296±119.618	ND
Peach	0.342±1.677	0.843±2.600	2.236±7.461	1.550±1.505	0.153±0.376
Pineapple	2.170±3.304	0.281±0.540	0.173±0.364	1.036±1.914	ND

***ND: not detected**

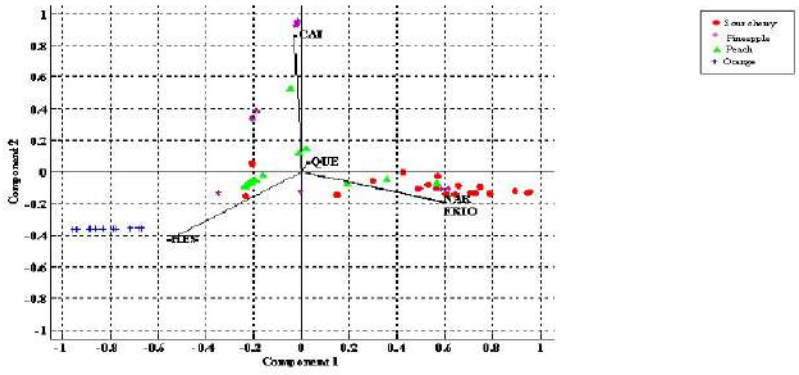

Figure 2. PCA was performed on the percentage of flavonoids data set then shown biplot.

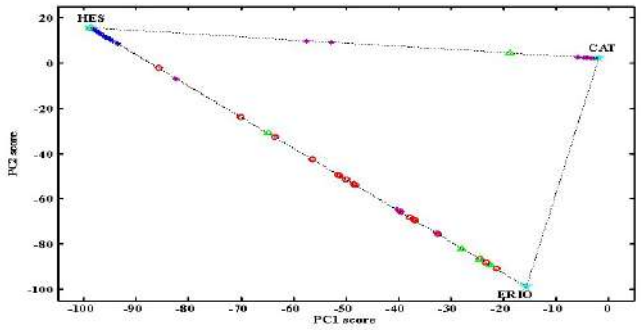

Figure 3. PCA performed on the new data set, ERIO, NAR and HES ternary set, after variable selection and calculation the new percentages of flavonoids

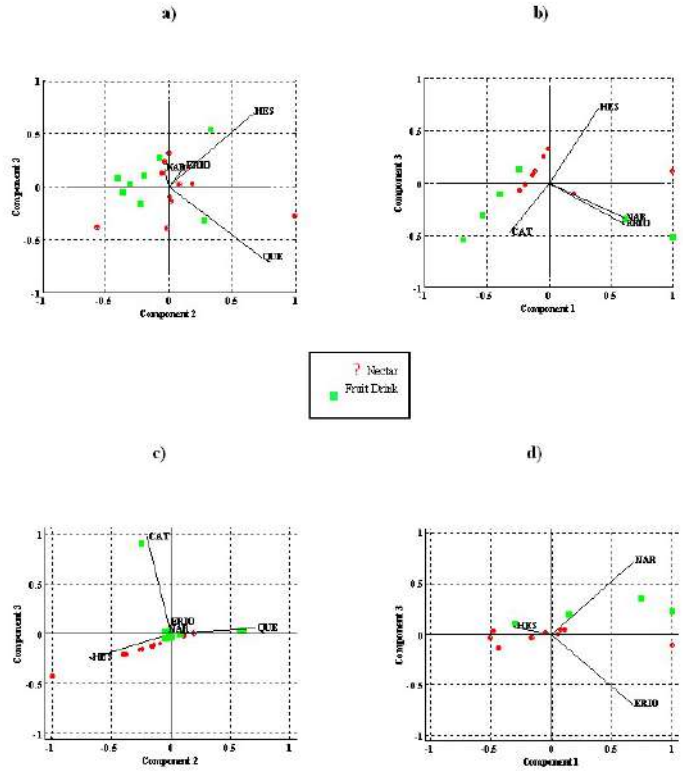

Figure 4. Clustering of different fruit juices according to the percentage of juice contents. a) Sour cherry b) pineapple c) peach d) orange.

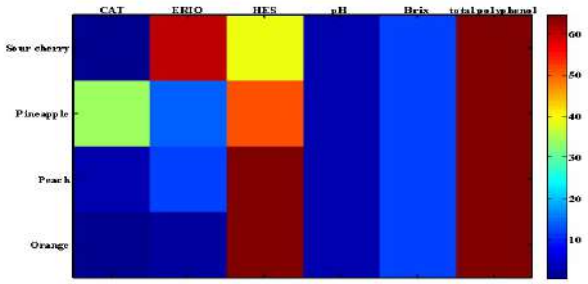

Figure 5. Display all of the selected flavonoids and physicochemical parameters that examined in this survey for each kind of fruit juices.

The percentage of each flavonoid relative to the total flavonoid concentration was calculated for each sample, and these values were used for principal component analysis (PCA).

The biplot of these data sets is demonstrated in Fig. 2

Among the five measured flavonoids -ERIO, CAT and HES- were selected for pattern recognition. As shown in Fig. 2, NAR and ERIO are dependent variables, as they are placed in the same direction on the biplot. Between these two, ERIO was selected because of its greater contribution. QUE was excluded due to its minimum contribution in the flavonoid content based on vector length in biplot.

The percentage of the effective selected flavonoids was recalculated, and principal component analysis was performed again on this dataset. The resulting score plot is presented in Fig. 3.

The corners of the obtained triangle correspond to the pure flavonoids. Juice samples positioned at the corners contain only one flavonoid, while those positioned on one of the sides contain two flavonoids. Samples which contain all three flavonoids can be found within the interior of the triangle. Based on this figure, it can be concluded that in orange juice, contribution of HES is more than the other flavonoids, whereas in pineapple juice, CAT has a significant impact.

Additionally, flavonoid content is a valuable discriminating factor between nectars and fruit drinks among different kinds of fruit juices (Fig. 4).

The results showed that the samples containing pulp had more flavonoid contents compared to the same samples without pulp. In 2018, Pavun et al. reported that although many factors may influence the total phenolic content of commercial fruit products, juices with lower fruit nectar content may still offer comparable health benefits to those with higher fruit nectar content (20).

In similar studies, the flavonoids NAR and HES were determined in orange juices. Hesperidin was detected in all orange samples, with mean concentrations of

246.6 mg/L in natural orange juice and 37.3 mg/L in commercial orange. NAR was only found in some natural orange juices (21-23).

Among all the parameters examined in this study, the physicochemical parameters such as pH, Brix and total polyphenol alone were insufficient for the quality control of fruit juices. In contrast, the kind and content of flavonoids proved to be suitable parameters. These findings are clearly illustrated in Fig. 5.

4. Discussion

Flavonoids can be quantified using a range of analytical techniques, such as electrochemical determination, spectrophotometric techniques, high-performance liquid chromatography (HPLC), gas chromatography (GC), thin-layer chromatography (TLC), capillary electrophoresis (CE), paper chromatography (PC) (24, 25). Among these, HPLC is a user-friendly technique with high sensitivity and selectivity for quantification of nonvolatile compounds without the need for prior derivatization. So, it is the most common analytical technique for the detection and quantification of flavonoids in different matrices and food products (25). In this study, the development of a rapid, sensitive and simple method for the simultaneous determination of common flavonoids occurred naturally in fruits was investigated.

In 2014, Hajimahmoodi et al. studied lemon juices using a mobile phase consisting of water, acetonitrile and acetic acid (77:21:2 v/v/v) on a C_8 column. The separation and determination of diosmin (DIOS), eriocitrin (ERIO), hesperidin (HES), and quercetin (QUE) were performed at 280 nm, with a total run time of 21 min (26).

In 2011, Sa'ed et al. carried out an HPLC method to measure the amount of three flavonoids -diosmin (DIOS), hesperidin (HES), and eriocitrin (ERIO)- in lemon juice. In their research, the solid phase extraction (SPE) was used for sample preparation. (19)

In 2003, an HPLC method was developed in Greece for the determination of Dios, HES and NAR in citrus fruits. The mobile phase consisted of 21 % tetrahydrofuran (THF), a solvent that is not environmentally friendly (21).

In 2011, a study quantified flavonoids in pomegranate using a C_{18} column with a mobile phase consisting of water, formic acid and methanol under gradient elution. However, the method had a run time of approximately 48 min, which is too lengthy for routine quality control applications (27).

In this study, flavonoid profile consisting of CAT, ERIO, NAR, HES and QUE was monitored in fruit juices. The respective retention times for these compounds were 4.6, 8.6, 13.1, 13.9 and 24.3 min with separation obtained within 30 min. The mobile phase was a mixture of water, acetonitrile and acetic acid, which is greener than that used in a previous study (21). This method enabled the simultaneous analysis of a greater number of flavonoids, with a shorter run time and suitable resolution (21, 26-29).

Peaks were identified by matching retention times with standards, and concentrations were determined via linear regression calibration curves.

Based on Table 1, recoveries ranged from 82.81% to 103.98%, indicating that the method possesses suitable accuracy for the determination of the five flavonoids.

Brix and pH are physicochemical criteria for juice quality control according to Iranian national standards. The pH and Brix values of all fruit drinks and nectars (sour cherry, orange and peach) were in compliance

with the relevant standards: pH ranged from 3.079 to 3.610, and Brix ranged from 12.408 to 12.693. However, two pineapple samples from two different brands did not comply with Iranian national standard No.10498 (17).

The total polyphenol content of four fruit juice kinds was compared. It should be noted that, according to Iranian national standards, quantification of total polyphenol content is mandatory only for the quality control of lemon juice.

The mean total polyphenol content of orange samples was 216.967 mg/L, ranging from 38.000 to 291.455mg/L. This value is considerably lower than the mean total polyphenol content measured in natural orange juice in England in 2000 (755 mg/L). The lowest mean total polyphenol concentration was observed in pineapple samples (155.957 mg/L). Considering the percentage of fruit juice content, this value appears consistent when compared with the mean total polyphenol content of natural pineapple juice in England (358 mg/L) (30).

The results show that samples containing pulp and pineapple pieces exhibited higher total polyphenol levels (22).

In 2020, Ashari et al. reported that the total phenolic concentration in fruit juice ranged from 28.39 to 114.20 mg GAE/L (5).

In 2005, Scalzo et al. reported total polyphenols concentrations in natural peach varieties ranging from 210 to 346 mg/L (31). In the present study, total polyphenol concentrations in peach samples ranged from 124.000 to 327.030 mg/L. Furthermore, peach juices containing pulp also exhibited higher total polyphenol levels.

Sour cherry juices had the highest mean total polyphenol content (289.200 mg/L), with values ranging from 136.33 to 337.39 mg/L.

5. Conclusion

In this study, the concentrations of five flavonoids—catechin, eriocitrin, naringin, hesperidin, and quercetin—were evaluated in 73 commercial juice samples from 11 brands. The proposed RP-HPLC method demonstrated simplicity, reliability, sensitivity, rapidity and selectivity for detection at very low concentrations. For each sample, pH, Brix, total polyphenol content and flavonoid levels were determined. Most physicochemical analyses were found to comply with the relevant national standards. The results showed that samples containing pulp and pineapple pieces exhibited higher total polyphenol levels. Additionally, pineapple juice differed significantly from other fruit juice types in terms of total polyphenol content. Regarding flavonoid concentrations, orange, pineapple, and sour cherry juices exhibited the highest levels of naringin (NAR), catechin (CAT), and quercetin (QUE), respectively, with significant differences observed among them. Based on the findings of this study, it appears that the determination of flavonoid and total polyphenol content, in addition to standard physicochemical parameters, is suitable for the quality control of commercial fruit juices. Therefore, it is recommended that maximum permissible limits for these parameters be defined for fruit juices in Iranian national standards.

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Authorship contribution

Conceptualization: Mannan Hajimahmoodi, Fatemeh Zamani Mazdeh

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Funding acquisition: Mannan Hajimahmoodi

Investigation: Naficeh Sadeghi, Mohsen Amini

Methodology: Fatemeh Zamani Mazdeh, Mohsen Amini

Project administration: Mannan Hajimahmoodi

Supervision: Mannan Hajimahmoodi

Validation: Naficeh Sadeghi, Mohsen Amini, Shaghayegh Keikhosravy

Writing – original draft: Anita Chalipour, Fatemeh Zamani Mazdeh

Writing – review and editing: Mannan Hajimahmoodi, Mohsen Amini

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data are available on demand.

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