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Research Article

Measurement of Phenolic Compounds by Spectrometry and Chromatography in Lime Juices

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Juice demand has been increasing at a rapid rate in recent years, and one of the major efforts underway to meet this demand is to minimize changes during the juice process. Due to the high consumption of juice and the carcinogenicity of synthetic and chemical substances, this research uses high performance liquid chromatography (HPLC) and spectrophotometry to detect fake juices. For the detection of fraud, the tests of sodium and potassium content along with determining the amount of flavonoids (hesperidin and eriocitrin) were carried out using spectrophotometry and HPLC. The results showed the average amount of total polyphenol was from 32.4 to 42.6 mg L-1. The total polyphenol content in all samples conformed to the standard, and there was no significant difference between the samples and the standard. The amounts of flavonoids (hesperidin and eriocitrin) in the juice samples were below the standard level (a minimum of 90 and 20 g/mL, respectively). Also, there was a significant difference between the mean sodium and potassium content of standard versus feigned juices. Generally, the amount of hesperidin, eriocitrin, Na+, and *K*+ as diagnostic biomarkers of natural juice in all samples was below the standard level. All the analysed samples in the experiment were nonstandard. There is a lot of fraud in the juice business, so it has been suggested that the government should have more control over how manufacturing companies make juice.

1. Introduction

Flavonoids are polyphenolic substances isolated from a wide range of plants with many health-related properties due to their high antioxidant capacity [1, 2]. They exhibit various physiological properties such as anti-inflammatory, antiallergic, anticarcinogenic, antihypertensive, and antiarthritic activities [3–7]. Because of the commercial value and importance of flavonoids as contributors to the beneficial health effects of citrus fruit, much effort has been invested in the isolation and characterization of flavonoid components in industrially processed juices [8]. In recent years, citrus juice demand has been expanding at a rapid rate. There is a shift in consumer preferences from

commercial products to fresh juice; therefore, one of the major efforts underway is to minimize changes during orange juice processing. Juice is characterized by the presence of a significant amount of polyphenolic compounds such as hesperidin, eriocitrin, naringin, rutin, quercetin, luteolin, and kaempferol [9, 10]. However, adulteration is one of the major problems faced in juice production that poses health risks for consumers. Hence, various analytical methods have so far been utilized to authenticate commercial products [11].

Besides analytical methods involving colorimetric reaction, chromatographic techniques allow simultaneous analysis of most of the components in citrus. High-performance liquid chromatography (HPLC) is one of the promising and more commonly used techniques [12, 13]. Saeidi et al. applied solid phase extraction (SPE) and HPLC for the determination of hesperidin, diosmin, and eriocitrin in Iranian juice samples [7]. Caristi et al. used HPLC coupled with electron spray mass spectrometry to detect the flavonoid compounds in lime juices. They demonstrated that the different levels of eriocitrin, hesperidin, diosmin, and some of the abovementioned minor components in the cultivars allow juices to be readily differentiated [14]. Evaluation of fruit juice quality and authenticity is an important applied research area, with a relevant impact on the industry, food science, and consumer protection [15]. Taghizadeh et al. used new methods to detect the fraud of lime juice in Iran. Their results showed that 51.11% of the lime juice samples were counterfeit. The fake lime juices enjoy a higher sodium content and lower potassium than those of normal lime juices [16]. Lime juice is one of the products that has been rigged in its production since ancient times. At present, cheating is somewhat more advanced in juice production, including the addition of completely synthetic compounds like sodium citrate, citric acid, glycerin, and numerous essential oils. Due to the carcinogenicity of synthetic and chemical substances and because of the high consumption of juice, this research attempts to identify fake juices in Bandar Abbas, Iran, using HPLC and spectrophotometry.

2. Materials and Methods

2.1. Materials. In 2021, the juice samples were obtained from seven best-selling brands of Iranian juice. An investigation was carried out on three samples obtained from each of the brands.

2.2. Reagents and Standard Solutions. Standards of hesperidin and eriocitrin were purchased from Sigma Aldrich. HPLC grade methanol and acetonitrile were from Fluka. Glacial acetic acid and HCl were purchased from Merck (Darmstadt, Germany). The water used was double-distilled and deionized. Stock solutions of hesperidin and eriocitrin were prepared separately by dissolving appropriate amounts of the compounds in methanol/dimethyl sulfoxide (1 : 1) to achieve concentrations of 400 μg mL^{-1} for each compound. These solutions were stored in the dark at 4°C and were observed to be stable at least for three months. All solutions were filtered through 0.45 μm membrane filters before use. The juice samples were provided from different stores in Bandar Abbas, Iran.

2.3. HPLC Analysis of Flavonoids. The HPLC analysis of flavonoids was performed according to Saeidi et al. with some modifications [7]. Stock solutions of hesperidin and eriocitrin were prepared by dissolving appropriate amounts of the compounds in methanol/dimethyl sulfoxide (1 : 1) to achieve concentrations of 400 μg/mL for each compound. All solutions were filtered through 0.45 μm membrane filters before use. Juice samples (5 mL) were centrifuged (4000 rpm for 15 min) and mixed with 25 mL of double-distilled deionized water and adjusted at pH = 3 with concentrated HCl. The solution was filtered through 0.45 μm membrane and injected into the HPLC system. The sample injection volume was 50 μL, and the analytes were monitored at 280 nm.

2.3.1. Determination of the Amount of Polyphenolic Constituents of Lime Juice by Spectrophotometric Method. The total phenolic contents of samples were estimated using the Folin–Ciocalteu reagent as described by Matic et al. [17]. The calibration curve (Figure 1) was plotted by mixing 1 mL aliquots of different gallic acid solutions with 5.0 mL of Folin–Ciocalteu reagent and 4.0 mL of sodium carbonate solution (75 g L^{-1}). 10 mL of the juice samples were mixed with 10 mL of ethanol to dilute the samples with 50% ethanol. For the extracts, 1 mL was mixed with the same reagents, as performed for constructing the calibration curve (Figure 1). After 1 h, the absorbance was measured to determine the total phenolic contents using the following formula:

$$TP = A2 \times \frac{C1}{A1}, \tag{1}$$

where T_P = total phenolic content in mg L^{-1}, A_1 = absorbance of the standard sample (gallic acid), A_2 = absorbance of juice sample, and $C1$ = concentration of gallic acid in mg L^{-1}.

2.3.2. Determination of Na^+ and K^+ Contents in Lime Juice. The levels of Na+ and K+ in studied juice samples were directly measured by applying flame atomic emission spectroscopy with a flame photometer (Drawell DW-AA320 N, Shanghai, China). The measurements were conducted at wavelengths of λ = 589.0 and λ = 766.5 nm for Na+ and K+, respectively.

2.4. Statistical Analysis. The chemical data were analysed using SPSS (version 19.0 Inc, Chicago, IL, USA) software. The significance of differences in the means at the 5% level was determined using a one-way analysis of variance (ANOVA).

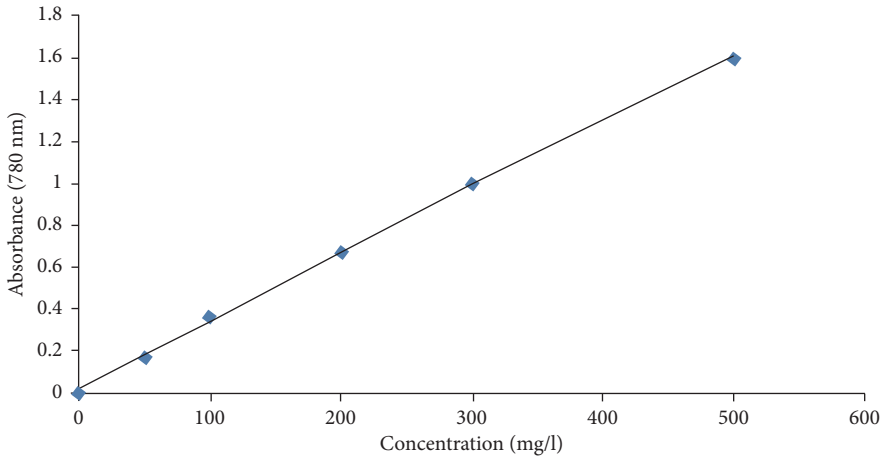

Figure 1: Standard (calibration) curve of gallic acid.

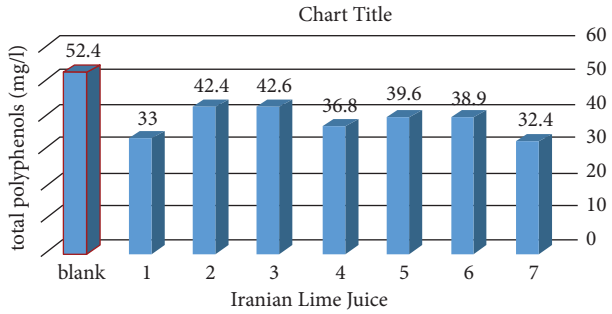

Figure 2: Comparison of the total amount of polyphenols of Iranian juice manufacturing products.

3. Results and Discussion

3.1. Amount of Polyphenolic Constituents in Lime Juice. Analytical methods are improving in their ability to detect and qualify simultaneously the largest possible number of compounds, especially the health-beneficial phenolic [3]. It has been demonstrated that citrus juices, in particular, lime juice are rich sources of phenolic compounds, predominantly flavanones [18]. In the last few decades, numerous pieces of research have been conducted on evaluating the total phenolic content in juice, as a consequence of its usefulness in monitoring lime juice authenticity [19, 20]. Therefore in this investigation, we have evaluated the amount of the total polyphenol in various Iranian lime juice samples. Figure 2 represents the difference in the mean value of the total polyphenols in samples. The average amount of total polyphenol was from 32.4 to 42.6 mg L^{-1}.

According to the results of the experiment, the total polyphenol content in all samples conformed to the standard, and there was no significant difference between the samples and the standard (Table 1).

3.1.1. Contents of Characteristic Flavonoids in Lime Juice. Although controlling the amount of polyphenolic compounds is an appropriate technique, by adding chemical additives such as peel and chemical dyes, the manufacturers can reach the standard level of total polyphenol in their produced juices. Therefore, by identifying the determination of some compounds such as hesperidin and eriocitrin levels in produced juice in combination with total polyphenol content, fraud in the sample can be detected. It has been illustrated that the most abundant flavonoids in lime juice are hesperidin, followed by eriocitrin, so the amounts of hesperidin and eriocitrin could be utilized as an effective tool for recognition of adulteration [3, 17]. Since due to the high cost of purchasing these compounds (hesperidin and eriocitrin), factories cannot add these compounds to lime juice.

Xi et al. have also claimed that the total polyphenol content, eriocitrin, and hesperidin are important parameters for the authority of juice, and this assay is very necessary for the quality control of juice [21]. High-performance liquid chromatography combined with different detectors is the commonly used analytical method for separation and identification of flavonoids [11] as was utilized in this study. Figures 3 and 4 show the amount of eriocitrin and hesperidin in different samples. Figures 5 and 6 illustrate the chromatograms corresponding to a standard mixture of hesperidin and eriocitrin and a juice sample containing hesperidin, respectively. Hesperidin in the juice sample was identified by comparing the retention time with a known standard solution (Figures 5 and 6). The peak corresponded to hesperidin which appeared in the analysis of the juice sample showed a remarkable reduction compared to the

Table 1: Total polyphenols (mg/l), hesperidin, eriocitrin, Na^+, and K^+ contents of Iranian lime juice manufacturing products.

Juice	Variable	Mean[a]	RSD[a] (%)	Max[a]	Min[a]
1	Total polyphenols (mg/l)	33.00	8.25	39.48	23.71
	Hesperidin content**	29.81	1.91	32.02	28.54
	Eriocitrin content*	3.90	5.05	9.74	0.93
	Na^+ content**	1295.29	73.40	1378.35	1293.13
	K^+ content**	157.98	25.22	186.97	140.96
2	Total polyphenols** (mg/l)	42.48	2.24	44.81	40.34
	Hesperidin content**	41.59	6.45	46.87	34.40
	Eriocitrin content**	4.11	0.24	4.37	3.89
	Na^+ content*	832.88	893.99	1865.18	314.73
	K^+ content*	1078.07	721.10	1529.47	246.47
3	Total polyphenols** (mg/l)	42.63	3.68	44.77	38.38
	Hesperidin content**	41.9	4.34	45.01	36.94
	Eriocitrin content**	3.88	0.81	4.82	3.31
	Na^+ content*	580.48	46.15	617.22	528.68
	K^+ content*	964.0	86.07	1039.09	870.07
4	Total polyphenols** (mg/l)	36.86	2.78	38.82	33.68
	Hesperidin content**	29.18	0.34	29.58	28.92
	Eriocitrin content*	1.12	1.94	3.37	0.00
	Na^+ content*	1319.97	232.63	1612.93	972.57
	K^+ content**	192.19	23.50	219.22	176.49
5	Total polyphenols* (mg/l)	39.66	4.67	44.84	35.75
	Hesperidin content*	47.85	4.32	56.13	39.72
	Eriocitrin content*	5.69	5.32	10.55	0
	Na^+ content*	370.63	41.08	411.56	329.39
	K^+ content**	1988.86	34.65	2026.42	1958.13
6	Total polyphenols** (mg/l)	38.90	5.23	44.18	33.71
	Hesperidin content**	51.32	1.76	53.07	49.55
	Eriocitrin content*	3.35	2.90	5.17	0
	Na^+ content*	395.32	40.29	436.46	355.92
	K^+ content**	1295.02	3.26	1297.89	1291.47
7	Total polyphenol** (mg/l)	32.48	5.24	38.54	29.32
	Hesperidin content*	34.81	15.48	52.63	24.59
	Eriocitrin content**	1.43	2.48	4.31	0
	Na^+ content**	1043.30	1043.30	1106.54	982.47
	K^+ content*	49.48	49.48	619.41	522.14

[a]Means, maximum (max) and minimum (min), and relative standard deviation (RSD). Significant differences between lime juice manufacturing products and blank at $^*P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$. $P > 0.05$ is considered as nonsignificant (ns).

standard. These findings were in good accordance with the results of the study conducted by Saeidi et al. They have reported that the amounts of hesperidin and eriocitrin in the studied juice samples were below the standard level [7]. Supplementary experiments showed that the amount of hesperidin and eriocitrin conflicted with the standard

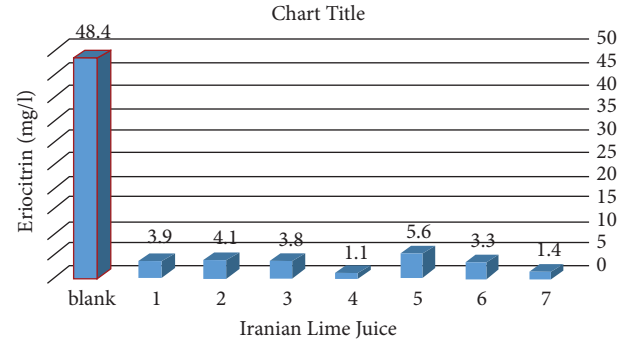

Figure 3: Comparison of the amount of eriocitrin of Iranian juice manufacturing products.

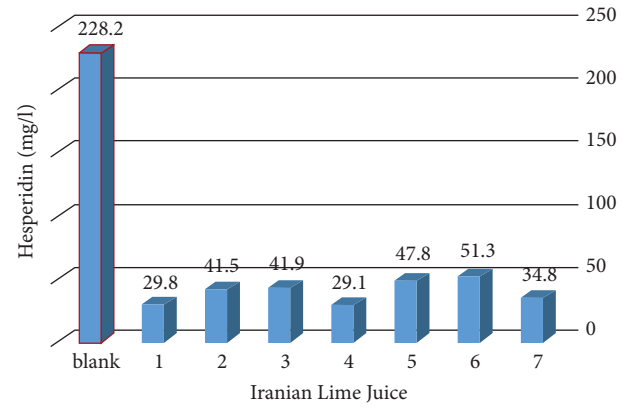

Figure 4: Comparison of the amount of hesperidin of Iranian juice manufacturing products.

(Table 1). According to Iranian national standards, the approved amounts of hesperidin and eriocitrin in juice are a minimum of 90 and 20 μg/mL, respectively [22]. A statistical analysis by ANOVA also revealed significant differences between juice-manufacturing products and standards for hesperidin (Table 1).

Jandric et al. have also revealed that the determination of hesperidin content can be applied as an effective approach for evaluating citrus juice authenticity. In the beginning, they identified characteristic markers by ultrahigh performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS), which had the potential to be employed for monitoring citrus juice quality. One of the most abundant markers was hesperidin, which was used to detect adulteration down to 2% in studied citrus juices [23]. In a study by Xue et al., five flavonoid glycosides (eriocitrin, narirutin, hesperidin, rutin, and diosmin) were determined by UPLC-MS in Limeade to authenticate the commercial products. It was stated that the level of rutin in some samples was unreasonably high, which was an indication of adulteration in Limeade by adding rutin [24].

3.1.2. Amounts of Na^+ and K^+ in Lime Juice. There was also a significant difference between the amounts of Na^+ and K^+ of all samples and the standard (Table 1), which indicated that

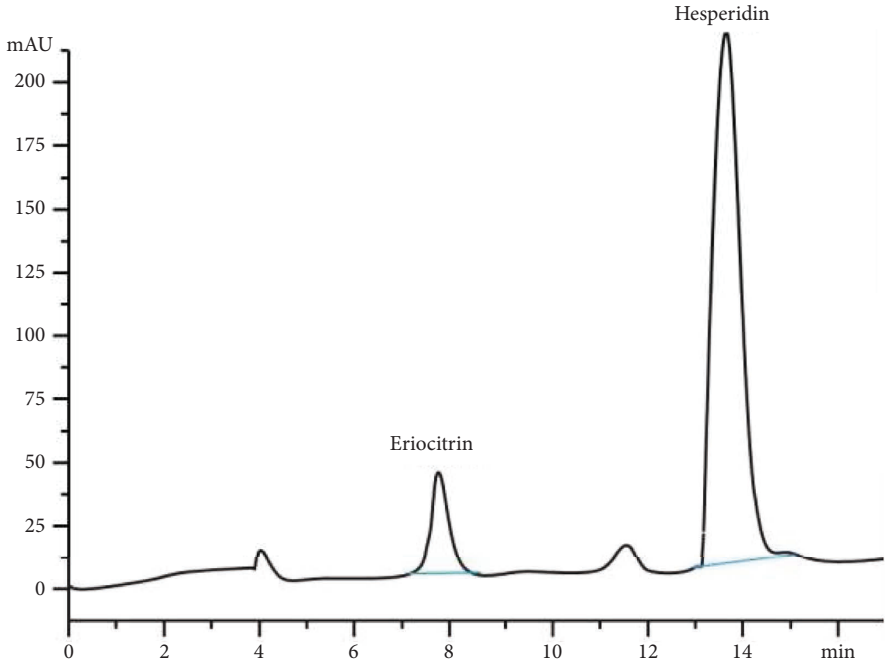

Figure 5: Typical chromatogram of standard (5 μg/ml).

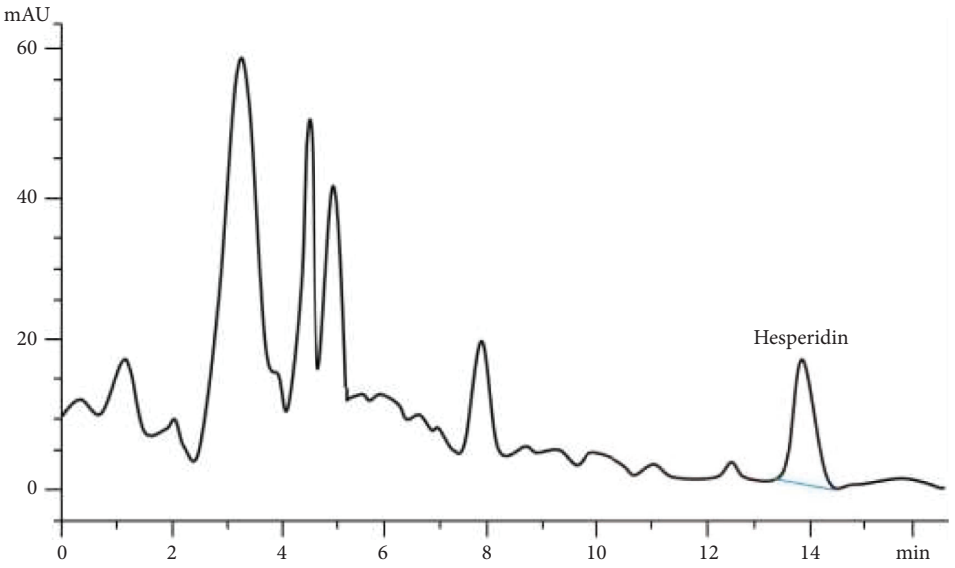

Figure 6: Chromatogram of juice sample containing hesperidin.

all samples were fake. The significant difference in the sodium (Na^+) content of natural juice (standard) and samples of lime juice produced in the factory in the current experiment could be due to the addition of methionine sodium sulphite and sodium citrate [13]. Natural juice has higher potassium and lower sodium content than industrial juices, and factories that produce fraudulent juice cannot use potassium citrate due to high cost and inappropriate in terms of emulsifier strength. The results obtained from samples made from juice agree with those found by Taghizadeh et al. [13]. However, among all samples of juice, sample 5 showed the highest contents of eriocitrin (Figure 3) and hesperidin (Figure 4).

4. Conclusion

The present research results indicated that the total polyphenol content in all the juice samples conformed to the standard; however, it is important to note that the factories can reach the standard level of total polyphenol in their produced juices by adding chemical additives including peel and chemical dyes. Therefore, by identifying some compounds such as hesperidin and eriocitrin, fraud in the produced juice samples can be detected. The amounts of hesperidin, eriocitrin, Na^+, and K^+ in all samples were below the standard level. Therefore, considering that these indices are diagnostic biomarkers of natural juice, all samples used

in the present experiment were found to be nonstandard. Due to the widespread fraud in the juice industry, it is suggested that relevant authorities have more control over the production of fruit juices in manufacturing companies. To detect these frauds, the use of reference books and new laboratory methods seems to be necessary. Meanwhile, it is crucial to inform the public through the media.

Data Availability

Data are available upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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