



Facile and Accelerated Method for Detection of Adulteration in Commercially Available Lime Juice Products in Iranian Market

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ABSTRACT

Background: The present study was aimed to introduce a rapid, simple and cost-effective method to detect adulterated lime juice products commercially available in the Iranian market.

Methods: Twelve different brands of marketed lime juice products were randomly purchased from the local stores in Tabriz, Iran. Fresh lime juice, as a control, was prepared by squeezing lime fruits using a plain hand juicer. Ehrlich's reagent was used for a colorimetric analysis; solid phase extraction-thin layer chromatography (SPE-TLC) technique was utilized for fingerprinting of methanol/water fractions of samples on the silica gel plates at two wavelengths of 366 and 254 nm, before and after spraying aluminum chloride reagent; and attenuated total reflectance-infrared spectroscopy (ATR-IR) was used for further studies.

Results: The obtained results from colorimetric analysis indicated appearance of orange-brown color indicating presence of limonoids in fresh lime juice and only five commercially available products. Regarding SPE-TLC fingerprinting, except for five products with identical or comparable TLC patterns to the fresh lime juice, other samples represented completely different TLC patterns, suggesting the presence of adulterants in these products. Therefore, the developed colorimetric and SPE-TLC techniques revealed some extents of adulteration in half of the studied products (58.3%). However, in the case of ATR-IR analysis, the obtained spectra were not illustrating enough to be applied for the detection of adulterated samples.

Conclusion: It can be concluded that the described colorimetric method in combination with the developed SPE-TLC technique could be utilized for the preliminary screening of adulterated lime juice products.

Introduction

Safety of food and beverage is one of the most important concerns in food industry. Nowadays, adulteration that reduces the quality and safety of food products and poses some serious health problems to the consumers such as cancer, diarrhea and asthma, is a growing problem attracted the attention of governments and regulatory agencies.¹⁻³ Adulteration can be defined as the intentional substitution or addition of substances in food products in order to decrease the manufacturing costs, increase economical profit and even for deceptive or malicious purposes.^{4,5}

Citrus-based products play a great role in human diet since they contain a large number of health promoting compounds such as secondary metabolites such as polyphenolic compounds, carbohydrates, minerals, proteins, vitamins, folate, and many other fundamental nutrient and non-nutrient substances.^{6,7} It has been

demonstrated that citrus juices could express anti-inflammatory, anti-tumor and antifungal activities and their consumption could reduce risk of coronary disease and chronic asthma.⁸

Lemons and limes are of the commonly used members of citrus family that are commercialized as fresh fruits and juices.⁹ It is worth mentioning that, adulteration in fruit juices is a widespread problem that has become a significant issue in food industry since the 1980s.^{10,11} This adulteration covers a wide range of practices such as diluting with water, addition of sugars, pulp wash, substitution of cheaper ingredients and addition of non-recommended minor compounds.¹⁰⁻¹² Therefore, in order to be able to commercialize fruit-derived products such as citrus-based juices, strict guidelines should be met to ensure their safety for consumers.⁶⁻¹⁰

Since presence of special sugars, organic acids, polyphenolic compounds and/or minerals in fruit and

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fruit-derived products could point to their quality, access to the techniques that could detect and quantify these characteristic compounds, would help regulatory bodies to evaluate the authenticity of fruit juice products and inspect the probable adulterations.^{13,14}

Numerous analytical techniques based on chromatography,^{9,10,12-14} spectroscopy¹¹ and electrophoresis^{15,16} have been reported for the adulteration inspection in fruit juices. Thin layer chromatography (TLC) is a rapid, simple, versatile and low-cost method applicable as the preliminary technique to identify phytochemical profile of samples; indeed, TLC is a classical fingerprinting technique,^{16,17-19} since the color of appeared spots on TLC plates in addition to their retention factors (R_f) could be utilized as biochemical markers applicable to quality control and adulteration inspection. With respect to the complexity of citrus juices' matrices, some clean-up and extraction procedures are required prior to TLC analysis. Solid phase extraction (SPE) as a fast, high throughput and selective technique is an applicable clean-up procedure²⁰ and aqueous methanol is a suitable solvent system for the extraction of polyphenolic compounds.²¹

Flavonoid and limonoid glucosides are the most abundant constituents in citrus juices,²²⁻²⁴ moreover lime juice is a rich source of citric acid (45.5 g/Kg).²⁵ The presence of these compounds and their concentration range can affect the quality (especially taste) of the citrus juices and could point to their naturalness.

In spite of a considerable number of researches done for the analysis of flavonoids and limonoids in citrus juice,^{24,26,27} there is still no general method for detection of possible adulteration in the lime juice.²⁸ Therefore, the main objective of the present work was to introduce a rapid, easy to use and low cost SPE-TLC method in combination with a primitive colorimetric analysis applicable to the preliminary adulteration screening of commercially available lime juice products based on the detection of the presence of characteristic compounds such as polyphenols and limonoids. Moreover, the applicability of attenuated total reflectance - infrared spectroscopy (ATR-IR) analysis was evaluated for the detection of adulterated samples based on the presence of citric acid and sugars in lime juice products.

Materials and Methods

Chemicals

Methanol and ethyl acetate were purchased from Duksan (Kowloon, South Korea) and Samchun (Gyeonggi-do, South Korea), respectively. Formic acid, glacial acetic acid, perchloric acid, chloroform, dichloromethane, aluminum chloride ($AlCl_3$) and Ehrlich's reagent were purchased from Merck (Darmstadt, Germany). All solvents used for extraction and chromatographic purposes were of HPLC grade.

Preparation of samples

In order to prepare fresh lime juice, lime fruit (*Citrus*

Latifolia var. persa) was purchased from a local market in Tabriz, Iran, cut in half and squeezed using a plain hand juicer.

For evaluating the marketed lime juice products, twelve different brands of commercially available products were collected randomly from local stores in Tabriz, Iran. In order to evaluate inter-batch consistency, three batch numbers of each brand were purchased. The codes of products and their production dates are shown in Table 1.

Table 1. The codes of the commercial products and their production dates.

Product code	Production date
P	2016-Feb-08
T	2015-Nov-23
B	2016-Mar-10
CH	2015-Aug-24
E	2015-Aug-19
SB	2016-May-30
SH	2016-Apr-17
M	2015-Sep-05
H	2016-May-30
A	2015-Dec-31
MN	2016-May-30
HN	2015-Sep-14

Preparation of fractions

With the purpose of fractionating of lime juices, SPE was carried out using Sep-Pak C18 cartridges (Waters, Ireland), where cartridge was pre-washed with 20 mL of methanol and 100 mL of distilled water, respectively. The lime juice (5 mL of either fresh or commercially available juice) was loaded on the cartridge and washed using a step gradient of methanol-water mixture (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0) followed by 50 mL of dichloromethane. The solvents of obtained fractions were dried off using a rotary evaporator (Heidolph, UK) at a temperature of 50 °C and pressure of 100 mbar, and finally the dried residues were utilized for TLC analysis.

Colorimetric Method

Preparation of Ehrlich's reagent

The Ehrlich's reagent was freshly prepared prior to use by dissolving 1.11 g of 4-dimethylamino benzaldehyde in 30 mL of stock acid solution which was prepared by mixing perchloric acid 70% and glacial acetic acid (44:56 % v/v).

Procedure

In order to detect the presence of substances such as limonoids and flavonoids in lime juice samples, 5 mL of each sample was transferred into a suitable test tube and 1 mL of Ehrlich's reagent was added and the appeared color in each test tube was visually evaluated after 15 min.

TLC analysis

SPE fractions were analyzed using a TLC analysis, that was performed on Silica gel 60 F₂₅₄ (Merck, Germany) plates. Two solvent systems were developed to conduct TLC analyses.



Figure 1. Results of colorimetric analysis of fresh lime juice (N) and products P, T, B, CH, E, A, SH, MN, SB and H using Ehrlich's reagent.

Formic acid-acetic acid-distilled water-ethyl acetate (7.4:7.4:17.6:67.6 % v/v) was utilized for the analyses of the 10 to 60% methanol/water fractions and chloroform-ethyl acetate (74:26 % v/v) was applied to the analyses of 80% methanol/water and 100% methanol and dichloromethane fractions. After development, the plates were air-dried and studied under UV lamp at two wavelengths of 366 and 254 nm before and after spraying aluminum chloride reagent (AlCl_3) (10% w/v in methanol). The SPE fractions of fresh lime juice were used as a standard for comparison.

ATR-IR analysis

In order to evaluate the presence of citric acid and sugars (such as fructose, glucose, lactose, maltose and galactose) in the prepared fractions, ATR-IR experiments were carried out using a IR spectrophotometer (Bruker-Tensor27, Germany) with a detection range of 20,000-650 cm^{-1} and resolution of 1 cm^{-1} , where small amounts of dried residues of 10 and 20% methanol/water fractions were placed in a cell made of zinc selenide crystals, and then the ATR spectra of samples were scanned and recorded.

Results and Discussion

Colorimetric analysis

Limonoid glucosides are known to form specific red-colored reaction products with Ehrlich's reagent. This reaction is used as a colorimetric method for the identification and quantification of limonoid glucosides in solutions or by TLC-densitometry analysis.^{26,27,29}

As it can be seen in Figure 1, the experiments indicated appearance of orange-brown color in fresh lime juice and some of commercially available products including P, T, B, CH, E, whereas the other products produced a light yellow color, comparable to the color of Ehrlich's reagent.

A closer look at the obtained results revealed that application of Ehrlich's reagent could be utilized as an easy to use and primitive screening step to detect the adulterated lime juice products. However, it should be mentioned that the complimentary studies are required to confirm the obtained results.

SPE-TLC fingerprinting

TLC fingerprinting is one of the easiest, inexpensive and yet more reliable methods for analysis of flavonoid and limonoid glycosides. Actually, visual evaluation of TLC plates especially after application of a suitable reagent or UV lamp allows convenient comparison of many samples abreast, where similarities and differences can clearly be noticed. According to the oxidation and substitution patterns of phenylchromane skeleton, the flavonoids display different characteristic colors under the UV light. They can also represent a different color, with spray reagents.^{30,31}

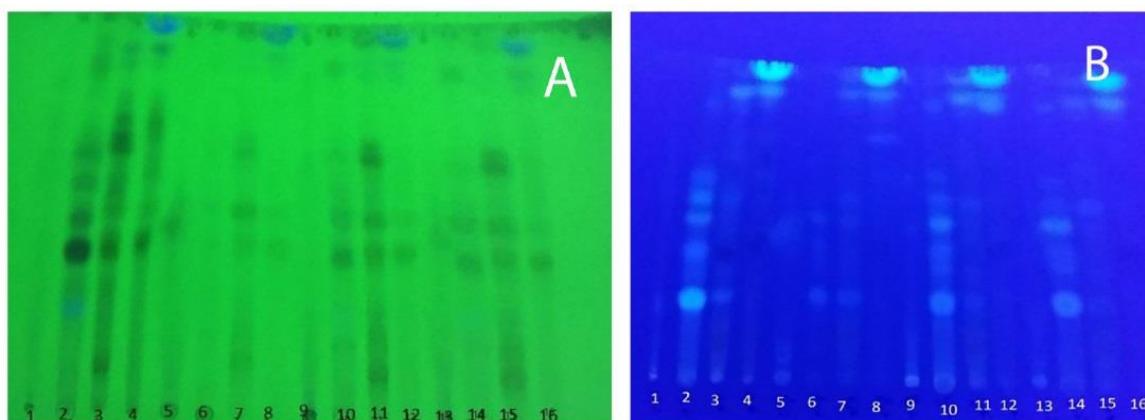
Therefore, the resulting SPE fractions of test samples were analyzed using TLC and the R_f values of all the observed spots were calculated and compared to those of a fresh lime juice. The TLC analysis of fresh lime juice indicated that five spots with R_f values of 0.38, 0.42, 0.54, 0.62 and 0.64 were observed in 20% methanol/water fractions, seven spots with R_f values of 0.38, 0.42, 0.46, 0.51, 0.61, 0.64 and 0.68 were detected in 40% methanol/water fractions, six spots with R_f values of 0.42, 0.45, 0.63, 0.71, 0.75 and 0.85 were appeared in 60% methanol/water fractions, 7 spots with R_f values of 0.34, 0.42, 0.53, 0.65, 0.73, 0.76 and 0.8 were observed in 80% methanol/water fractions and a spot with R_f value of 0.53 was detected in 100% methanol fraction.

The R_f values of the observed spots in the TLC analysis of test samples are shown in Table 2. As it can be seen from this table, test samples with codes of P and T showed the identical TLC patterns to the different fractions of natural lime juice samples, whereas in test samples with codes of B, E and CH, the obtained TLC patterns were somehow comparable to the natural samples since majority of the observed spots were similar to those detected in natural samples (Figure 2). However, only one spot with R_f value of 0.16 was observed in the TLC plates of samples MN, SB, SH and no spot was observed in samples H, HN, M and A suggesting presence of adulterants in these samples.

Additionally, appearance of colored spots visualized at 254 and 366 nm after spraying AlCl_3 on the plates, could be attributed to the presence of flavonoids (yellow spots) and chlorophyll (green spots) in methanol/water fractions and the subsequently naturalness of the analyzed juice.

Table 2. The R_f values of the observed spots in the TLC analysis of methanol/water fractions of fresh lime juice and the test samples.

Sample	R_f 1	R_f 2	R_f 3	R_f 4	R_f 5	R_f 6	R_f 7
Fresh lime juice 20%	0.38	0.42	0.54	0.62	0.64		
Fresh lime juice 40%	0.38	0.42	0.46	0.51	0.61	0.64	0.68
Fresh lime juice 60%	0.42	0.45	0.63	0.71	0.75	0.85	
Fresh lime juice 80%	0.34	0.43	0.53	0.65	0.73	0.76	0.8
Fresh lime juice 100%	0.53						
P 20%	0.38	0.42	0.54	0.62	0.64		
P 40%	0.38	0.42	0.46	0.51	0.61	0.64	0.68
P 60%	0.42	0.45	0.63	0.71	0.75	0.85	
P 80%	0.34	0.43	0.53	0.65	0.73	0.76	0.8
P 100%	0.53						
T 20%	0.38	0.42	0.54	0.62	0.64		
T 40%	0.38	0.42	0.46	0.51	0.61	0.64	0.68
T 60%	0.42	0.45	0.63	0.71	0.75	0.85	
T 80%	0.34	0.43	0.53	0.65	0.73	0.76	0.8
T 100%	0.53						
E 20%	0.38	0.42	0.56	0.61	-		
E 40%	0.38	0.42	-	0.49	-		
E 60%	0.42	-	0.63	-	0.75	0.88	
E 80%	-	-	0.53	0.65	-	-	
E 100%	-						
CH 20%	0.38	0.42	0.54	-	-		
CH 40%	0.38	0.42	-	-	-		0.69
CH 60%	0.42	0.45	-	-	-		
CH 80%	-	-	0.53	0.65	-	0.76	0.8
CH 100%	-						
B 20%	0.38	0.42	-	-			
B 40%	0.38	0.42	0.46	-	-	0.65	0.68
B 60%	0.42	0.48	0.63	-	-	-	
B 80%	-	-	0.53	0.65	0.73	-	0.8
B 100%	0.53						

**Figure 2.** Results from the TLC analysis of the methanol/water fractions (10%-20%-40% and 60% from left to right) of fresh lime juice (1-2-3-4), product CH (5-6-7-8), product E (9-10-11-12) and product B (13-14-15-16) with solvent system of formic acid-acetic acid-distilled water-ethyl acetate (7.4:7.4:17.6:67.6 % v/v) under UV light at 254 nm (A) and 366 nm (B).

It has been illustrated that flavonoids could produce spots with yellow or yellowish-blue fluorescence at 366 nm after addition of AlCl_3 reagent owing to the complex formation between Al (III) ions and carbonyl and hydroxyl groups of flavones and flavonols.³¹⁻³³

Therefore, it can be claimed that both the elution behavior of the detected spots and their color could be utilized as a marker to detect adulterated commercially available lime juice products.

The obtained results from the TLC analysis revealed that expect for the samples P and T representing the identical pattern to the natural lime juice and samples B, E and CH representing comparable pattern to the natural lime juice, adulteration was observed in the other samples due to the absence of the markers discussed above.

It is worth mentioning that since the elution behavior of adulterated samples was somehow similar and the production dates of all the products were close together (between September 2015 and May 2016); an underlying assumption could form in our minds regarding the similarity of the utilized raw materials in the production of these products. On the better words, it cannot be farfetched that these products' manufacturers have provided their raw materials from the same source and commercialized their lime juice products after addition of some flavoring and coloring agents. Nevertheless, it should be noted that further studies using more reliable analytical techniques are required to confirm this assumption.

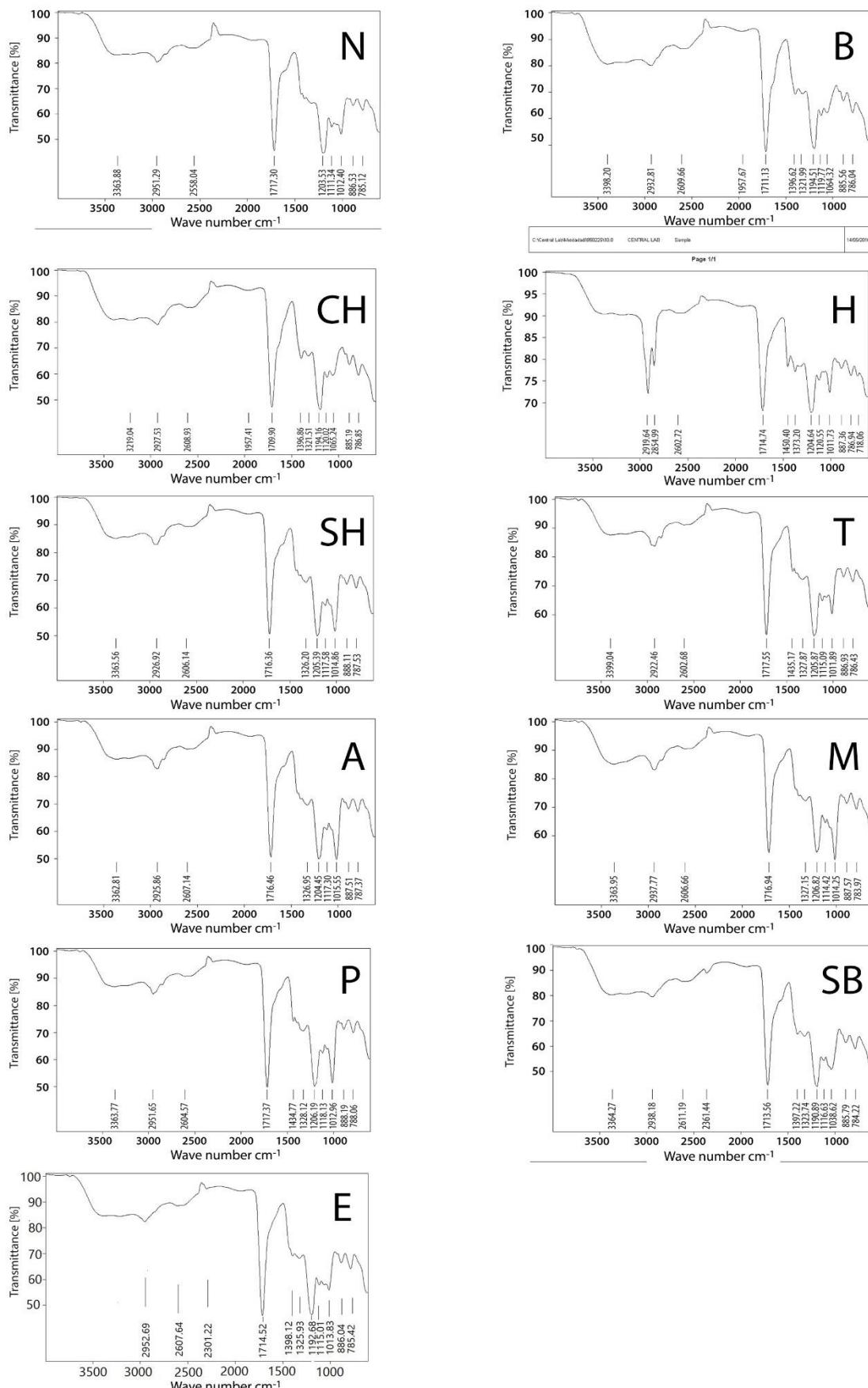


Figure 3. ATR-IR spectra obtained from the analysis of 10% methanol/water fractions of fresh lime juice (N) and products P, T, B, CH, E, A, SH , MN, SB and H.

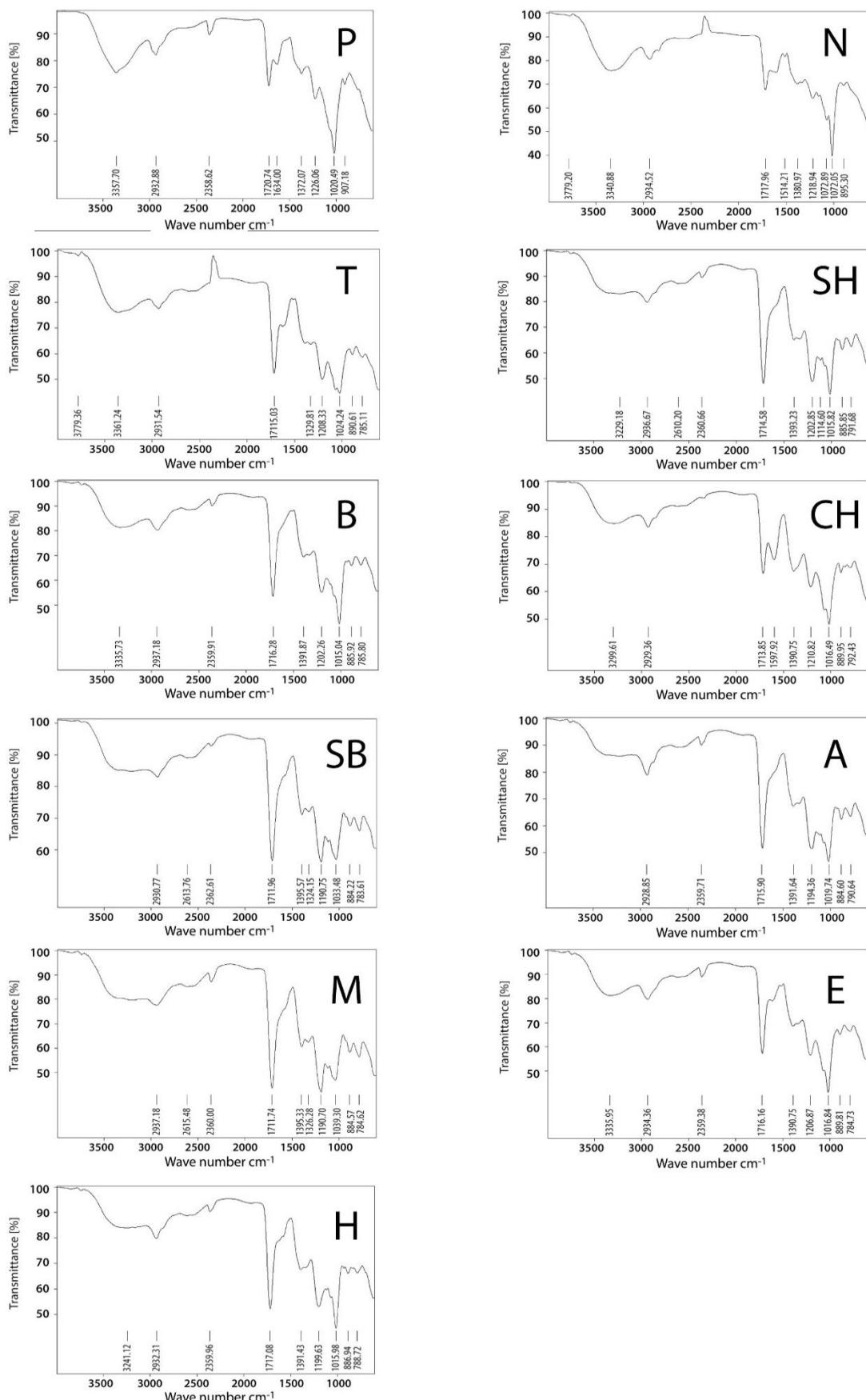


Figure 4. ATR-IR spectra obtained from the analysis of 20% methanol/water fractions of fresh lime juice (N) and products P, T, B, CH, E, A, SH, MN, SB and H.

ATR-IR analysis

ATR-IR analyses were utilized to study the presence of citric acid and sugars in lime juice samples and to compare the observed peaks in different samples to be able to detect adulterated ones. It is worth saying that since the water content of 10 and 20% methanol/water fractions were high, it was expected that the amount of citric acid and sugars would be higher in these fractions; therefore, 10 and 20% methanol/water fractions of different samples were utilized for ATR-IR analyses. The obtained spectra from the analyses of methanol/water fractions of natural and commercially available samples can be followed in Figure 3 and 4.

The obtained spectra from the analysis of 10% methanol/water fraction of fresh lime juice, indicated the appearance of four predominant peaks at 3363.88, 2951.29, 1717.30, 1203.53 cm^{-1} possibly pointing to the H-bonded OH stretch, sp^3 C-H stretch, C=O stretch and C–O stretch of carboxylic acid moieties of citric acid, respectively, and another major peak at 1012.40 cm^{-1} probably related to the C–O stretch of 1° alcohol moiety of sugars; in the case of 20% methanol/water fraction of fresh lime juice, the same major peaks were observed. These findings were in a good conformity with those obtained from the analysis of citrus juices in previously published work.³⁵ A closer look at the spectra reported in Figure 3, revealed that the 10% methanol/water fraction could not be utilized to distinguish among natural and adulterated samples due to the fact that apart from product H with additional peaks at 2919 and 2854 cm^{-1} , the spectra of other samples were comparable to that of fresh lime juice.

As shown in Figure 4, spectra of 20% methanol/water fraction were more informative; regarding product P, the main peaks and the overall pattern were somehow comparable with those of fresh lime juice; in the case of products B, CH and E, the main peaks at wavenumbers of around 3300, 2900, 1700, 1200 and 1000 cm^{-1} were observed indicating the presence of citric acid and sugars in these products, however the intensity of the mentioned peaks and their shape were not completely resembling those observed in the spectra of fresh lime juice, probably due to the matrix effect; the spectra of products SH, A, SB, M and H were close together and different from the spectrum of fresh lime juice showing the probability of adulteration in these products. These observations were somewhat in compliance with the results obtained from colorimetric and TLC analyses. However, it should be mentioned that the observed differences in the reported spectra utilized for the detection purposes might be due to the factors affecting the obtained results from ATR analysis such as the amount of the markers available in the dried fractions influencing the intensity of the observed peaks and/or matrix effect resulting in the appearance of additional peaks. Therefore, it seems that in the present study, the obtained results from ATR analyses were not informative enough to be able to distinguish among natural and adulterated lime juice products.

Comparing the developed colorimetric-SPE-TLC technique to the previous methods reported for adulteration detection in lime juice

Different techniques have been described for the adulteration detection in lime juice products available in the Iranian market.³⁶⁻³⁸ For instance, Asemi et al.³⁶ utilized the polarimetric technique together with the spectrophotometry to determine the optical rotation and polyphenolic compounds' content in lime juice products; Hajimahmoudi et al.³⁷ applied the combination of an HPLC method and an enzymatic kit to evaluate the ratio of citric acid to D-isocitric acid in lime juice products; and Saiedi et al.³⁸ used a SPE-HPLC technique to quantify the amount of hesperidin, diosmin and eriocitrin in lime juice. A precise look at the above discussed studies reveals that although the employed techniques in these studies are more expensive, labour intensive and difficult to operate compared to the colorimetric-SPE-TLC method presented in the current work, they are quantitative methods working based on the detection and quantification of known compounds such as polyphenolic compounds, citric acid, D-isocitric acid and limonoids. These compounds are valuable markers that their presence in the lime juice together with their concentrations could directly point to the naturalness of the commercial products.

Therefore, despite the fact that the developed colorimetric-SPE-TLC technique benefits from the low cost and simple, rapid and easy to operate procedure, it is a qualitative technique which could not provide quantitative data regarding these markers. Accordingly, it should be stated that the developed colorimetric-SPE-TLC technique could only be utilized as an early screening test in adulteration inspection.

Conclusion

Due to the increasing concerns regarding the presence of adulteration in food and beverage, there is a growing demand to introduce easy and quick techniques applicable to the adulteration inspection in food products.

In present work a simple, easy to use and time and cost effective method based on the preliminary colorimetric analysis followed by the SPE-TLC fingerprinting has been introduced to screen the adulterated lime juice products via detecting some markers available in natural lime juice; where Ehrlich's reagent was utilized as a colorimetric indicator to detect the presence of limonoids and SPE-TLC analysis was used to provide chemical fingerprints for studied samples through separating secondary metabolites of lime including polyphenols and limonoids. In the following, ATR-FTIR analyses of methanol/water fractions were carried out; however, the recorded spectra were not illuminating enough to be utilized for detection of adulterated samples.

The obtained results revealed some extents of adulteration in half of the studied products (58.3%), a finding which could raise the awareness about the need to consider stricter assessments of lime juice products' manufacturers in Iran. Since the developed technique could successfully

distinguish between natural and adulterated samples, it can be claimed that the newly developed colorimetric-SPE-TLC technique could be utilized by the food quality control laboratories as a first step toward adulteration detection in lime juice products. It is obvious that, the developed method could only be employed as a fast adulteration screening technique, due to the fact that it could not provide any complimentary information regarding the type of adulterations; therefore, further studies using high throughput methods are required to be able to point out to the exact kind of possible adulterations.

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Conflict of interests

The authors claim that there is no conflict of interest.

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