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Sunset yellow, tartrazine and sodium benzoate in orange juice distributed in Iranian market and subsequent exposure assessment

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Abstract

The usage of coloring agent and preservative including sunset yellow, tartrazine and sodium benzoate in orange juice is forbidden in Iran because of bad effects on public health. The present study included 30 samples of orange juice purchased from local market of Tehran in Iran for simultaneous identification and quantification of sunset yellow, tartrazine and sodium benzoate through high performance liquid chromatography (HPLC) with UV detection and ACE C18, 5µm (4.6 × 250 mm) column. The additives were eluted with a mixture of methanol and ammonium acetate as mobile phase at flow rate of 1.0 ml/min and monitored at 230 nm. Exposure assessment (estimated daily intakes) of consumers calculated through official reports on annual orange juice production in Iran. Among the samples analyzed, at least one additive detected in all orange juices. The amount of sunset yellow, tartrazine and sodium benzoate were ranging from 5.84 ± 0.05 to 23.12 ± 0.19; 0.70 ± 0.01 to 2.32 ± 0.01 and 12.23 ± 1.50 to 56.80 ± 2.13 µg/ml, respectively. Satisfactory repeatability with relative standard deviation < 2.03, excellent sensitivity (Limit of detection: 0.20, 0.25, 0.22 µg/ml; Limit of quantification: 0.70, 0.90, 0.80 µg/ml) with recovery more than 96.3% were obtained. Estimated daily intake of sunset yellow, tartrazine and sodium benzoate were 0.85, 0.96 and 1.11mg/kg body weight through orang juice, respectively which was lower than acceptable daily intakes in society of Iran. We concluded that the level of additives found in orange juice does not affect adversely on consumer's health.

Introduction

Creation attractive appearance in drinks is a way to increase the consumer's demand. For this purpose food colorant agents such as sunset yellow (FD and C yellow no. 6; E110) and tartrazine (FD and C yellow no.5; E102) are added to alter or confer final drink's color (Belitz and Grosch, 1999). These colorants belonged to azo compounds with functional group R-N=N-R' (R and R' can be either aryl or alkyl) (Stolz, 2001). Furthermore, drinks are prone to spoil by different factors. Preservatives are employed in these products to control natural spoilage and avoid contamination by microorganisms (Tajkarimi *et al*., 2010). Benzoic acid (E210) and its salt sodium benzoate (E211) are extensively used as antimicrobial agents in order to retard or eliminate the yeast and mold activity, less bacteria, through membrane disturbance and inhibition of citrate cycle enzymes and oxidative phosphorylation (Belitz and Grosch, 1999; Saad *et al*., 2005). Notwithstanding the common use of food colorants and sodium benzoate, unpleasant effects of these additives are reported in some surveys. Sunset yellow and tartrazine has been implicated as contributor to the increase of cholestasis in combination with other criteria for developing of primary biliary cirrhosis (Axon *et al*., 2012). The ability of sunset yellow and tartrazine in DNA damage, learning and memory deficits is indicated in some surveys (Mpountoukas *et al*., 2010; Gao *et al*., 2011; Sayed *et al*., 2012; Ceyhan *et al*., 2013). Sodium benzoate was regarded as nontoxic but some researchers found mutagenic, clastogenic and cytotoxic effects in human peripheral blood lymphocyte (Yilmaz *et al*., 2009; Zengin *et al*., 2011).

The acceptable daily intake of sunset yellow, tartrazine and sodium benzoate have been approved at 4, 7.5 and 5 mg/kg body weight, respectively (WHO, 2006; EFSA, 2009; EFSA, 2014). Specific

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regulations are approved in different countries for food additives. In Iran, the usage of colorants and preservatives is prohibited in drinks and its occurrence must be stated on label (Hosseini *et al*., 2008; Esfandiari *et al.*, 2016). However, in some researches, Iranian authorities have detected sodium benzoate in dairy products (Akbari *et al*., 2013; Esfandiari *et al.*, 2013; Zamani mazdeh *et al*., 2014; Amirpour *et al.*, 2015; Esfandiari *et al*., 2016). With the importance of simultaneous quantification of preservative and food colorants in beverages, high performance liquid chromatography has appeared as a powerful tool to determine the additives at low concentration (Tfouni and Toledo, 2002; Yoshioka and Ichihashi, 2008; Scherer *et al.*, 2012).

To author's knowledge, no research has been previously performed on analysis of preservative and colorants agents in Iranian orange juice. Therefore the present paper describes a simple method for simultaneous determination of sunset yellow, tartrazine and sodium benzoate by HPLC in orange juice that can be used for quality control in supervisor authorities. In the second part of study, estimated daily intake of aforementioned quantified additives examined in consumed orange juice in Iranian population.

Material and Methods

Sample preparation

Thirty packages of orange juice manufactured from ten different brands (coded from A to J; for each 3 samples) with apparent adaption to national standard for orange juice specification and without any physical damage were purchased from local retail market in Tehran, center of Iran. A 50 ml of homogenized sample was degassed thoroughly in an ultrasonic bath (Model 55743, Fritsch, Germany) for 10 min. A 10 ml aliquot transferred to polypropylene tube for centrifugation (Heraeus Biofuge, USA) in 8000 rpm for 15 min. The supernatant was passed through a PTFE 0.45 µm membrane filter (Sartarius, Germany) and collected in the HPLC vial. The analysis was performed in two replicates to determine the mean of the measurements of additives.

Standard preparation

Standards of sodium benzoate and both sunset yellow and tartrazine were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich (Steinheim, Germany), respectively. Standard stock solutions of sodium benzoate, tartrazine and sunset yellow were prepared at 1.0 mg/mL level in ultra-pure water. Intermediate standard solutions (200 and 400 µg/ml) were obtained from dilution of stock solutions by 30:70 methanol/water in volumetric flask as well as working standard solutions in different concentrations (2.5-40 µg/mL) by diluting appropriate volumes of the intermediate standard solution with 20 mM acetate buffer (pH=6.0): methanol (1:2 V/V).

Chromatographic analysis

The chromatographic analysis was performed in Waters HPLC system equipped with a vacuum degasser, Waters 515 pump to deliver solvent in isocratic mode, a 486 Waters UV-Vis detector and a 7725i model Rehodyne injector with a 25 µl sample loop. Chromatographic separation was achieved on an ACE C18, 5µm (4.6 mm × 250 mm) HPLC column. For the mobile phase, a degassed mixture of methanol and 20 mM ammonium acetate (pH=6.0) (30:70 V/V) was prepared and delivered at flow rate of 1.0 ml/min. All of the analyses were carried out at an operation wavelength of 230 nm. The HPLC data were acquired and processed using a PC and Millennium 2010 chromatography manager software (Waters, Version 2.1). In all solutions the pH was controlled and adjusted by digital Metrohm pH meter (model 744, Switzerland) equipped with a combined glass-calomel electrode. Ultra-pure water was prepared by Pure Lab option equipment from ELGA LabWater (High Wycombe, UK).

Qualitative and quantitative analysis

Identification of each additive in orange juice samples was performed by comparing their retention times with those of the corresponding standard in the HPLC chromatogram. Quantification was calculated by referring analyte signal to its equation in calibration curve. The measurement was in accordance with procedure described previously (Shekarchi *et al*., 2010).

Exposure estimation

The data for per capita orange juice consumption was obtained from the latest report of Agri- Jahad administration official announcement (Agri-Jahad Ministry, 2014). Estimated daily intake was calculated using the sum of additives amounts in the total analyzed samples (Lino and Pena, 2010).

Statistical analysis

Analysis of variance (ANOVA) was used to determine the differences of distribution between ten brands in SPSS (Version 20.0 for windows, SPSS Inc., Chicago, IL, USA). The results were expressed as mean ± SD for samples. Statistical significance was set at $p<0.05$.

Results and Discussion

Monitoring the safety of food stuff after distribution in the market is an important part of controlling in food chain. This control is of great importance in health policy which can protect public health and preserve consumer confidence. In some studies we reported post market surveillance results as a legal strategy for quality and safety control of honey (Akbari, *et al*., 2012) and tuna fish (Akbari, *et al*., 2012) samples in retail market. Moreover, we designed a post-market surveillance study to measure some artificial additives in orange juice at this study.

A typical HPLC chromatogram for mix standard solutions containing 5.0 µg/mL of sodium benzoate, tartrazine and sunset yellow is shown in Figure 1. Three peaks observed in the chromatogram at mean retention time 13.56, 16.41 and 19.02 min are corresponding to sodium benzoate, tartrazine and sunset yellow, respectively. In all chromatograms, especially orange juice samples with complex matrix, the baseline was flat and clean and there were a good resolution between artificial color additives. In Figure 1-b the chromatogram of orange juice sample containing high levels of tatrazine and sodium benzoate was illustrated. There were no interfering peaks in chromatograms and there was not any sunset yellow in this sample however the concentration of two other additives were considerable. In Figure 1-c the chromatogram of orange juice sample containing considerable amount of tartrazine with trace amount of sodium benzoate is shown.

The reliability of the HPLC method for simultaneous analysis of measured additives was validated through its selectivity/specificity, linearity, calibration curve, detection limits, precision and accuracy. Selectivity is the ability of method to measure accurately the analyte response in the presence of all interferences. The UV scan of the additives was compared with that observed from UV full scan (200-370 nm) of the peaks in the standard solutions. There was no significant difference between these results and so this method has the requisite degree of selectivity when it is applied to orange juice samples. Linearity was evaluated through the relationship between the concentration of additive and the signal obtained from the UV-HPLC detector. Regression coefficient (r^2) for each additive was calculated by means of the least-square analysis (Norouzi *et al.*, 2006). The calibration curves were achieved through two replicates from each concentration of additive (2.5-40 µg/mL), to identify the extent of the total variability of the response that could be explained by the linear regression model.

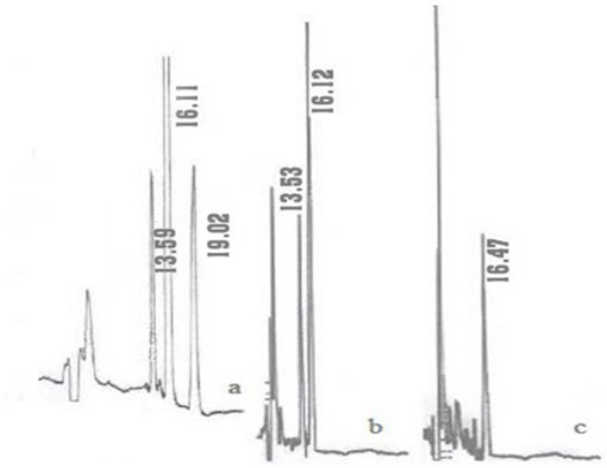

Figure 1. Typical HPLC chromatogram of (a) A mix standard solution containing 5.0 µg/mL of sodium benzoate (retention time=13.56), tartrazin (retention time=16.41) and sunset yellow (retention time=19.02), (b) Orange juice sample containing high levels of tartrazine and sodium benzoate without any sunset yellow, (c) Orange juice sample containing tartrazine, trace amount of sodium benzoate

The calibration curves constructed for sodium benzoate, tartrazine and sunset yellow were linear over the concentration range of 2.5-40.0 µg/mL. Peak area of these additives in the chromatogram were plotted versus its concentration and linear regression analysis performed on the resultant curve. A correlation coefficient of more than 0.9998 with relative standard deviation values ranging from 0.11 – 2.03% across the concentration range studied were obtained (Table 1). The limit of detection and limit of quantification were determined based on a signal-to-noise ratios calculated by chromatographic software and were based on analytical responses of 10 and 3 times the background noise, respectively (Shekarchi *et al*., 2010). The limit of detection was found to be 0.20, 0.25, 0.22 µg/mL and the limit of quantification 0.70, 0.90, 0.80 µg/mL for sodium benzoate, tartrazine and sunset yellow, respectively. The sensitivity and analysis time of the method is superior to all previously reported methods. The sensitivity was improved with better limit of detection up to equal or lowers values. Furthermore, the simultaneous detection of three additives can help to reduce the analysis time.

The precision of each method indicates the degree of dispersion within a series on the determination of the same sample. Three additive standard solution in three levels (2.5, 10.0, 40.0 µg/mL) were analyzed on the same day (intra-day) and three for consecutive days (inter-day), then the relative standard deviations were calculated. The precision results show that mean concentrations for sodium benzoate were found to be

Table 1. Regression analysis results for the preservative and color additives

Additive	Regression Equation	Correlation Coefficient	Mean RSD%
Sodium benzoate	y = 838.39x + 208.63	0.9999	2.03
Tartrazine	y = 2410.1x - 438.97	0.9998	0.19
Sunset yellow	y = 797.9x +124.91	0.9999	0.11

Table 2. Accuracy results for determination of additives in orange juice samples

Additive	Mean found values in the spiked sample (µg/mL)			Mean Recovery (%)
	2.5	10.0	40.0	
Sodium benzoate	2.6	9.9	39.9	100.6
Tartrazine	2.6	9.4	39.9	96.3
Sunset yellow	2.4	10.1	40.0	99.0

2.3, 9.8 and 39.9 with associated relative standard deviation of 3.60, 2.11 and 1.34%, respectively. These values were found to be 2.4, 9.9 and 40.1 with relative standard deviation of 2.88, 2.44 and 1.48% for tartrazine and 2.3, 9.7 and 40.4 with relative standard deviation of 2.11, 1.92 and 2.39% for sunset yellow, respectively. Due to the lack of certified reference material, accuracy evaluation was performed in terms of recovery percent and it was carried out on blank orange juice samples spiked with known amount of additive. Two replicate amounts of orange juice (3×1.0 mL) were collected and each of them was divided into three equal portions (0.2 g). One part was used as the real sample and others had been spiked with the additive standard solution (2.5, 10.0 and 40.0 µg/mL) in three levels. In each additional level, three measurements were carried out and the recovery percentage was calculated in each case. All of the samples were injected three times into the HPLC system. Accuracy of the assay was determined by interpolation of replicate peak areas of three accuracy standards from a calibration curve prepared as previously described. The resultant concentrations were shown in Table 2.

The mean concentration of sunset yellow, tartrazine and sodium benzoate are given in Table 3. At least one additive involved in the current study detected in all orange juices. The detection of sunset yellow in orange juices showed that 80% of 30 samples were positive with the range of 5.84-23.12 µg/mL (Table 3). Some surveys on the presence of sunset yellow in fruit drinks conducted in different countries revealed the ranges found were lower than those reported in the present study. It was reported the amount of 3.5-3.74 mg/L for sunset yellow in orange drinks analyzed in Spain (Capitan-Vallvey *et al*., 1998). It was analyzed many types of fruit flavored drink for presence of sunset yellow in Greece (Miniote *et al.*, 2007). Their results showed the amount of 0.35 mg/L of sunset yellow. In another study, sunset yellow was found in five out of thirty types of imported commercial products to Japan including soft drinks, syrups, candies, gelatin candies and marshmallows in the range of 3.1–17.3 µg/g (Yoshioka and Ichihashi, 2008). The quantity of 2.95-42.6 µg/L was detected for sunset yellow in soft drinks in China (Chen *et al*., 2014). In other study in China the amount of 2.08 to 6.09 µM was found in orange juice samples (Qiu *et al*., 2016). In Contrast, the mean concentration of sunset yellow in Iranian orange juice purchased from two brands (Zam zam and Hamineh) reported in the range of 23.25 to 30 mg/L being higher than the results of our study (Ghoreishi *et al*., 2012). Furthermore, in Brazil, Argentina and two reports from China, the amount of sunset yellow was similar to those found in our study ranging from 6.4- 20.9, 9.2 - 30.2, 6.37-11.35 mg/L and 11.71 µg/mL in analyzed soft drinks, respectively (Ma *et al.*, 2006; Llamas *et al*., 2009; Andrade *et al*., 2014; Songyang *et al*., 2015).

Table 3. The amount of measured additives (µg/mL) in orange juice*

Code of Orange Juice processing plants	Sunset Yellow		Tartrazine		Sodium benzoate	
	Mean** ±SD	Range	Mean ±SD	Range	Mean ±SD	Range
A	15.91±0.15	15.74-16.02	1.52±0.01	1.51-1.53	44.24±2.89	41.12-46.83
B	14.54±0.07	14.49-14.62	0.96±0.01	0.95-0.97	32.59±3.28	28.85-34.98
C	1.83±0.04	11.79-11.86	1.26±0.01	1.25-1.27	N.D	N.D
D	23.12±0.19	22.91-23.26	1.98±0.02	1.96-2.00	49.29±1.52	48.11-51.00
E	9.12±0.11	9.01-9.22	1.63±0.01	1.62-1.64	28.44±1.35	27.14-29.83
F	11.33±0.08	11.27-11.42	0.70±0.01	0.70-0.72	56.80±2.13	54.62-58.88
G	N.D	N.D	N.D	N.D	38.03±2.24	35.84-40.31
H	19.08±0.08	19.01-19.5	2.32±0.01	2.31-2.34	23.93±1.17	22.92-25.21
I	N.D	N.D	0.98±0.01	0.97-0.99	42.73±1.47	41.31-44.49
J	5.84±5.05	ND-8.78	2.12±0.43	1.63-2.39	12.23±1.50	1.26-7.79

* Results related to mean of three analyses
* N.D: Not Detected

Results of this study revealed that the amount of 0.7-2.32 µg/mL for tartrazine (Table 3). Moreover, only three samples of one brand (G) did not contain any tartrazine which is 10% of the orange juice samples. Chen *et al.*, (2014) and Andrade *et al.*, (2014) reported the lack of tartrazine in Chinese soft drink and Brazilian orange juice, respectively. The amount obtained for tartrazine in present study is much lower than that of some other studies, e.g., 6.4-8.2 mg/L; 9.3-14.36 mg/L, 10.52 µg/mL, of soft drink in Argentina, Spain and China as well as 23.25 to 30 mg/L of Iranian orange juices (Capitan-Vallvey *et al.*, 1998; Ma *et al.*, 2006; Llamas *et al.*, 2009; Ghoreishi *et al.*, 2012).

According to Table 3, nine brands contained sodium benzoate which is 90% of the orange juice samples with concentration ranging from 12.23 ± 1.50 to 56.80 ± 2.13 µg/mL. These results show that most producing factories do not follow the Iranian supervision rules (ISIRI, 2011). To author knowledge, there is one report of the determination of sodium benzoate in orange juice mixed with honey as "orange and honey sports drink and RIOTM) with HPLC in the amount of 151.73 ± 4.61 mg/kg showing much higher than the result of present study (Ren *et al.*, 2014). Totally, no correlation was observed between additives amount and different brands of orange juice in the present study ($p>0.05$).

Using the methods with more accuracy, sensitivity and simplicity such as Ultrasensitive Immunoassay (Li *et al.*, 2013), enhanced electrochemical platform based on graphene oxide and multi-walled carbon nanotubes nanocomposite (Qiu *et al.*, 2016) and gold nanodumbbells as surface-enhanced Raman spectroscopy (SERS) sub-strates (Meng *et al.*, 2016) is devoted to some studies to determine the food additives. Therefore, further research is needed to compare the different methods to quantify the food colorants and preservatives.

Table 4. Estimated daily intake (mg/kg b.w./day) of sunset yellow, tartrazine and sodium benzoate through orange juice consumption in society of Iran

Additives	Acceptable daily intake	Estimated daily intake
Sunset yellow	4.0	0.85
Tartrazine	7.5	0.96
Sodium benzoate	5.0	1.11

The estimated daily intake of three additives through orange juice by general Iranian population was below the acceptable daily intake. Although the examination for estimated daily intake in drink is limited, it observes the real utilization of sunset yellow, tartrazine and sodium benzoate is significantly less than the acceptable daily intake levels in our study (Table 4). No information existed on estimated daily intake of sunset yellow and tartrazine in orange juice or soft drinks. However, the estimated daily intake of sodium benzoated reported below the acceptable daily intake in soft drinks consumed in Portugal and Brazil (Tfouni and Toledo, 2002; Lino and Pena, 2010).

This study is subjected to some limitations. First, examination of sunset yellow, tartrazine and sodium benzoate in other sources of food is necessary to calculate the estimated daily intake for Iranian society. Second limitation of this study is the synergistic effect of aforementioned additives that required the examination *in vivo* test. Additionally, it suggests further investigations on food matrices with high sample size to determine the different food

additives in Iran.

Conclusion

The proposed method by HPLC is simple and useful for simultaneous quantification of sodium benzoate, tartrazine and sodium benzoate in routine monitoring of orange juice at very low concentration (µg/mL range). Albeit the usage of food additives is indicative of adulteration, the results of present study shows that sunset yellow, tartrazine and sodium benzoate are common additives applied in orange juice. Furthermore, all analyzed samples of our study did not comply with the criteria of supervision organization. The levels of additive found in samples were not of concern for consumer health since the estimated daily intake of additive was below the acceptable daily intake. However the orange juice manufacturers have taken procedures to avoid reoccurrence of the issues related to levels above legislative limit.

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