

Rapid determination of main constituents of packed juices by reverse phase-high performance liquid chromatography: an insight in to commercial fruit drinks

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Abstract The present work reports the compositional analysis of thirteen different packed fruit juices using high performance liquid chromatography (HPLC). Vitamin C, organic acids (citric and malic) and sugars (fructose, glucose and sucrose) were separated, analyzed and quantified using different reverse phase methods. A new rapid reverse phase HPLC method was developed for routine analysis of vitamin C in fruit juices. The precision results of the methods showed that the relative standard deviations of the repeatability and reproducibility were <0.05 and <0.1 respectively. Correlation coefficient of the calibration models developed was found to be higher than 0.99 in each case. It has been found that the content of Vitamin C was less variable amongst different varieties involved in the study. It is also observed that in comparison to fresh juices, the packed juices contain lesser amounts of vitamin C. Citric acid was found as the major organic acids present in packed juices while maximum portion of sugars was of sucrose. Comparison of the amount of vitamin C, organic acids and sugars in same fruit juice of different commercial brands is also reported.

Keywords HPLC · Packed fruit juice · Sugars · Organic acid · Vitamin C

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Introduction

Fruit juice is considered to be one of the healthiest foods in human diet. Commercially prepared juices claim to retain their nutritional effects. Juices contain water, sugars, acids and minerals. Sugars are the most commonly found compounds in all beverages. They are present naturally and as additives to impart sweetness and texture. Sugars are also used as preservatives. Sucrose, glucose and fructose are main sugars found in fruit juices. Organic acids are another important component of fruit juices. Their presence and concentration determine tartness and flavor. Malic acid (MA) and citric acid (CA) are the major organic acids of fruit juices. The MA is predominant in apple, pear and stone fruits (Eisele and Drake 2004) while CA is most abundant in citrus and tropical fruits (Vera et al. 2006). The principal vitamin available in fruit juices is vitamin C, chemically known as ascorbic acid (AA). It is a powerful antioxidant naturally present in food and food products. It plays an important role in the prevention of infectious diseases.

Different kinds of fruits are processed into juices. Due to the loss of some nutrients in the processing of juice, cheaper juices, sugars, acids, colorants, water and other additives are often added into the pure juice. The taste and health benefits of the processed juices are influenced by variations in the main ingredients, particularly the composition of the sugars, organic acids and vitamins. Vitamin loss during processing is also of great concern for nutritionists, processors and consumers. Since vitamin C is an important indicator and used as a parameter for the determination of fruit quality as well as deterioration of juices due to storage, the quantitative determination of vitamin C becomes vital. It is a labile substance, which is

easily degraded by atmospheric oxygen and enzymes. It also undergoes oxidation under the influence of light and heat. This makes it important to monitor its variation during manufacturing and storage.

Quantification of sugars can give an estimation of the amount of artificial sugar present in packed juice (Jahren et al. 2006). Organic acids including citric and malic acids can be used as “fingerprints” representing characteristics of individual fruit juice (Shui and Leong 2002; Soyer et al. 2003). This allows detection of the addition of a juice of different origin and fermentation in fruit juices. All these factors require the need for reliable techniques to detect the quality of juices.

Several methods have been reported for the determination of above-mentioned components in food items (Castellari et al. 2000; Zeppa et al. 2001). The most traditional method to detect the organic acids was developed by enzymatic procedures, where precision and time consumption were considered as the main defaults (Han and Cui 1996). The analysis of sugars, acids and vitamins by traditional methods is often complicated (Timpa and Burke 1986). In view of these disadvantages some techniques using high precision instruments came in use. In the field of food chemistry some commonly employed techniques are: fluorescence analysis (Chen et al. 2008), photospectrometric analysis (Fish et al. 2002), electroanalysis (Kapor et al. 2001), and capillary electrophoresis (Blatny et al. 1995). Gas chromatographic methods are also reported for food component analysis (Silva and Ferraz 2004; Lehotay and Hajslova 2002; Ikeda et al. 2006). However most of these methods have some limitations, viz in case of gas chromatography, in spite of good separation and sensitivity, tedious and time-consuming derivatization steps are required. Moreover, toxic derivatization agents are used in the analysis. High temperature required by some analyzing techniques also lead to sample decomposition.

High performance liquid chromatography (HPLC) is a powerful technique for the analysis of carbohydrates, organic acids, preservatives and various other components in food items (Muntean et al. 2003; Gattuso et al. 2007; Belajova and Suhaj 2004; Cunha et al. 2002; Nour et al. 2010; Bhattacharjee et al. 2010; Chen et al. 2011; Venkateswaran and Vijayalakshmi 2010). It is a faster and easier technique for the analysis of sugars and acids in food (Kafkas et al. 2006; Versari et al. 2008; Chinnici et al. 2005; Arora et al. 2011; George et al. 2010). It does not require derivatization and provides accuracy. The resolving power of HPLC is invaluable for accurately quantifying many food components. It has been used to analyze acids in beverages like tomato juice (Marconi et al. 2007) and wine (Fang et al. 2007; Sergio et al. 2007)

To the best of our knowledge, no article related to the compositional analysis of commercially available fruit juices is reported in literature. Packing of commercial juices also does not provide information about the individual sugar and acid content. Considering the prevalence of these juices and frequent use by all age groups, knowledge of their contents need attention. In the present work, the occurrence and quantity of vitamin C, organic acids and sugars in commercial fruit juices from different brands is investigated using reverse phase high performance liquid chromatography technique.

Experimental

Chemicals and reagents

All the chemicals used were of analytical grade. D-fructose, D-glucose and sucrose were procured from Qualigens fine chemicals, India. Citric and malic acid were procured from Merck Ltd., India and ascorbic acid was brought from Sisco Research Laboratories Pvt. Ltd., India. Acetonitrile, ammonium di-hydrogen phosphate buffer and potassium di-hydrogen orthophosphate buffer used were of HPLC grade procured from Qualigens fine chemicals, India. Water used for dilutions and making solutions was obtained through Milli Pore water purification system. Rankem 0.45 μm nylon membrane filters and 0.22 μm syringe filters were used for filtration of mobile phase and samples. Tropicana apple, pineapple, Appy classic apple, Frooti mango, Real guava, litchi, pineapple, mixed fruit, mango, mausami, orange, Onjus mixed fruit, apple juices were purchased from local stores.

Instrument

Analysis was carried out using Shimadzu HPLC (UFLC Prominence) equipped with a binary pump (LC-20 AD), a variable wavelength UV-VIS (SPD-20A) detector, a refractive index detector (RID-10A), a column oven (CTO-20A), a degasser and a manual injection valve fitted with 20 μl sample loop. The instrument was controlled by LC solution software.

Chromatographic conditions

Vitamin C (Ascorbic acid)

Vitamin C was analyzed using Phenomenex Luna C18 column with a mobile phase of acetonitrile and 10 mM potassium di-hydrogen ortho phosphate buffer mixed in a

ratio of 40:60 (pH=2.1) at a wavelength of 268 nm with UV detector. The flow of the mobile phase was maintained at a speed of 1 ml/min and the temperature of the column oven was set at 50°C.

Organic acids

The acids were isocratically separated using a Phenomenex Luna C18 column at a flow rate of 1 ml/min. The mobile phase used was 2% (w/v) ammonium di-hydrogen ortho phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) buffer (pH 2.18). Column oven temperature was set at 32°C. UV-VIS detector at wavelength 214 nm was used for detection.

Sugars

For the analysis of sugars, binary gradient elution was used for separation with a flow rate of 1 ml/min. The mobile phase used consisted of acetonitrile and water (75:25,v/v). The separating column for sugars was Phenomenex Luna NH_2 . Column oven temperature was maintained at 40°C. A refractive Index detector was used for detection.

For all the components analyzed, the conditions like column equilibration and mobile phase flow rate were standardized according to the method used.

Standard solutions

Standard solutions of vitamin C, citric acid, malic acid, fructose, glucose and sucrose were prepared in volumetric flask by dissolving the required amount of reagent (weighed by Mettler Analytical Balance) in 100 ml of water. Flanking ranges of concentration commonly found in commercial fruit juices, were chosen. The standard ranges of concentration were 0.01%–0.1% for vitamin C, 0.05%–1.6% for citric acid and malic acid, 1%–10% for fructose and 0.5%–10% for glucose and sucrose. Twelve standard solutions of each component were prepared and 20 μl of each standard solution was subjected to chromatographic run and their chromatograms were recorded.

Analysis of commercial juices

Juice samples were analyzed under the same conditions as of standards. Chromatographic peaks were identified by comparing retention times of separated components in chromatograms of juice samples against known standards and also by spiking samples with pure compounds. Data acquisition and all computation were done with Shimadzu LC solution software. Calculation of concentration (expressed in %) of vitamin C, organic

acids and individual sugars of various juices was carried out by using external standard method. Quantitation values were obtained by applying their peak areas to the calibration curve.

Three different samples of same fruit juice were analyzed. Good repeatability was obtained in terms of retention times of different constituents under study. For quantification the values were averaged for the three values obtained for each juice.

Results and discussion

Method development

Different methods are available for the analysis of vitamins by reversed phase HPLC (Anyakora et al. 2008; Almagro et al. 2002). Many of these methods utilize silica bonded columns, a mobile phase consisting of a phosphate buffer with acidic pH and some percentage of acetonitrile or methanol (Maia et al. 2006). These conditions were used as the base for method development for vitamin C. As the mobile phase was highly aqueous, columns that were stable under those conditions were investigated. Phenomenex Luna 5 μ (250 \times 4.6 mm) C8, C18 and NH_2 column were tried. The NH_2 column did not provide sufficient retention of vitamin C, while the peak with C8 column was not sharp and clear. C18 column provided acceptable retention of vitamin C at 12 min with 100% phosphate buffer as mobile phase at a flow rate 0.5 ml/min. However the peak, while symmetric, was broad. After several trials, the mobile phase consisted of a mixture of acetonitrile and phosphate buffer (40:60) with C18 column at a wavelength of 268 nm with UV detector gave excellent peak shape and retention time was also reduced significantly. The method using these conditions was then optimized: the flow rate was increased to reduce run time and column oven temperature was also increased. The pH of the mobile phase was reduced to 2.1. The final conditions for the analysis of vitamin C are presented in Table 1. The chromatographic conditions achieved satisfactory resolution, reasonable retention and symmetric peak shape for the separation and determination of vitamin C.

Precision of the method developed for vitamin C is assessed by performing replicate analysis of a standard vitamin C solution. For this purpose 0.02% solution is injected to the HPLC five times at intra and inter-day intervals. The results showing the slope, correlation coefficient, mean response factor, percent relative standard deviation of response factor of calibration made at intra and interday intervals are tabulated in Table 2.

Table 1 Chromatographic conditions for determination of vitamin C in fruit juices

Method parameter	Proposed conditions
Buffer	10 mM potassium di-hydrogen ortho phosphate buffer pH=2.1 with Hydrochloric acid
Mobile phase composition	Buffer: ACN (60:40)
Column	Phenomenex Luna 5 μ C18 (250 \times 4.6 mm)
Column temperature	50 $^{\circ}$ C
Flow rate	1.0 ml/min
Detector	UV, 268 nm
Injection volume	20 μ l
Test concentration	0.01%
Retention time	3.41 min

Calibration and validation

Figure 1a shows the chromatogram of standard vitamin C (ascorbic acid) under the chromatographic conditions described in previous section. Vitamin C elutes at 3.4 min. Calibration graphs for standard vitamin C is obtained by plotting peak area against concentration. A straight-line relationship between concentration and intensity is observed as evident from Fig. 1D. Some outliers (points lying outside of the curve) are detected, which are not discarded. These outliers can be attributed to the experimental error due to inaccuracy in weight measurement or standard solution preparation. Figure 1b and c depicts the chromatogram of standard organic acids: citric and malic acid respectively. Citric acid elutes at 7.5 min and malic acid elutes at 4.0 min. Figure 2a, b and c shows the chromatograms of standard sugars: fructose, glucose and sucrose

respectively. Fructose eluted at 6.3 min while the retention times of glucose and sucrose were 7.8 and 9.4 min respectively. Calibration of organic acids and sugars is achieved in the same manner as of vitamin C. Calibration graphs for citric acid, malic acid, fructose, glucose and sucrose are shown in Fig. 1b and c and Fig. 2d, e and f respectively.

Table 3 shows the results of the analysis of linearity of the calibration curves and their regression equations. The correlation coefficients obtained are found to be higher than 0.99, showing a good relationship between peak area and concentrations in the studied ranges. To verify the linearity, the response factor (RF) is computed based on the linear regression of a plot of peak area vs. concentration. Validation of all the calibration curves is achieved by subjecting standard samples of vitamin C, organic acids and sugars. The results obtained after the concentration determination using calibration curve are in good agreement with the standard values.

The repeatability of all the methods used is established by injecting the standard vitamin C, organic acids and sugar samples five times in a day. The reproducibility is established by analyzing each standard sample on three different days for over 2 weeks. The standard deviation for repeatability is in the range of 0.01 to 0.04 while that of reproducibility is of 0.06 to 0.1.

Traceability and recovery

To check the traceability of the HPLC methods developed for sugars, organic acids and ascorbic acids, an analyte was quantified in the juice sample. The sample was then spiked with five increasing concentrations of the same analyte. A calibration model was then constructed with six calibration points including the unspiked juice sample. A linear calibration curve accounted for the traceability of

Table 2 Statistical evaluation of the calibration studies for Vitamin C determination

Analyte		Intraday ($N=5$)	Interday ($N=5$)		
			First day	Second day	Third day
Vitamin C	Slope	7.677823e-009	7.677823e-009	7.658152e-009	7.676868e-009
	R	0.9997	0.9997	0.9996	0.9997
	Mean RF	7.567236e-009	7.567236e-009	7.541913e-009	7.548070e-009
	RF%RSD	2.4519	2.4519	2.5132	2.59023

R Correlation coefficient

RF Response Factor

RF%RSD Percent relative standard deviation of response factor

N Number of samples

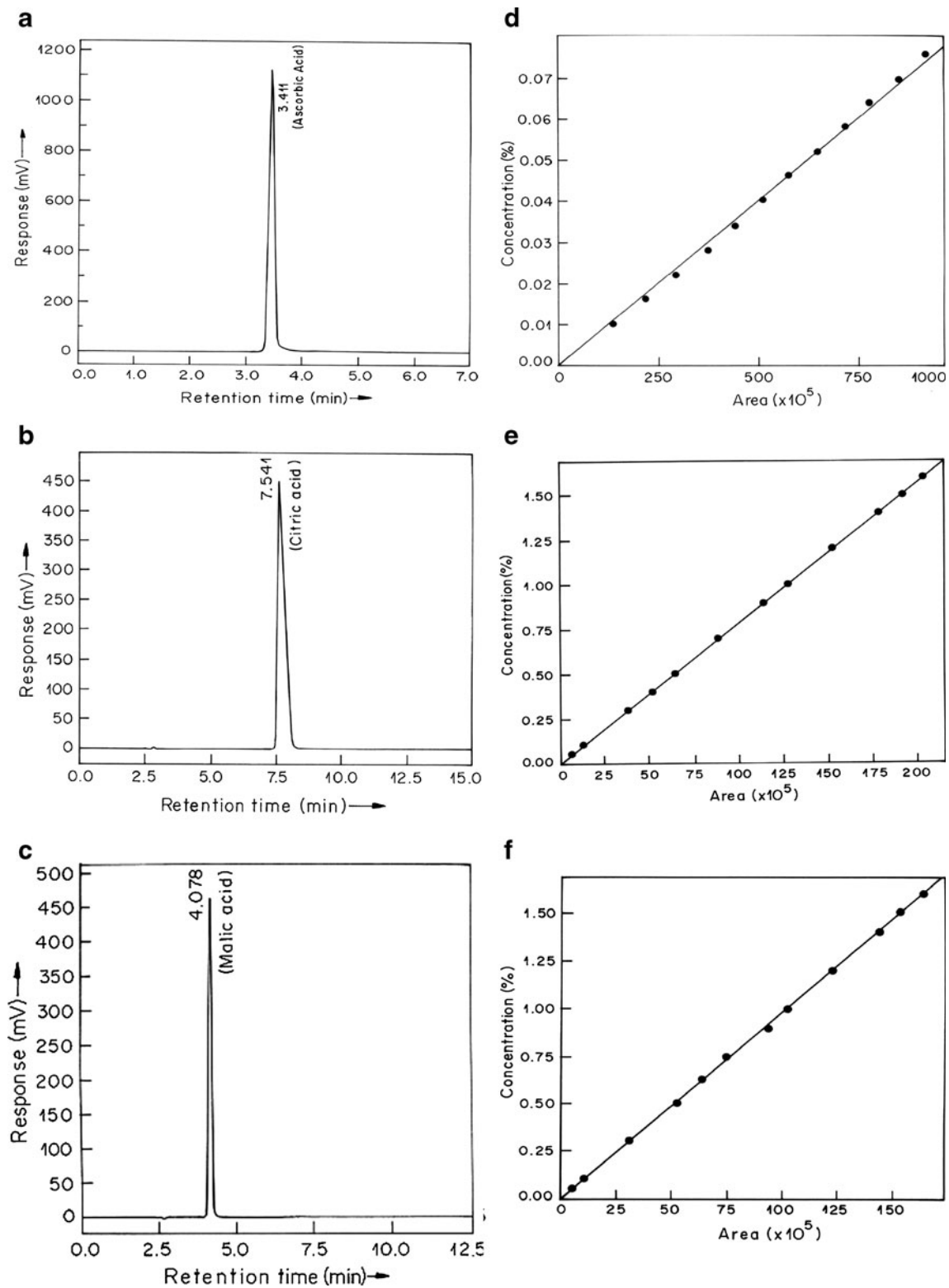


Fig. 1 Chromatogram and calibration curve of standard vitamin C [a and d] citric acid [b and e] and malic acid [c and f]

the method used in the study. Three injections of each sample were given over a period of 3 days. Good repeatability and reproducibility in terms of retention

time, peak area, peak height and peak asymmetry was found in the pure real samples and the samples spiked with analytes.

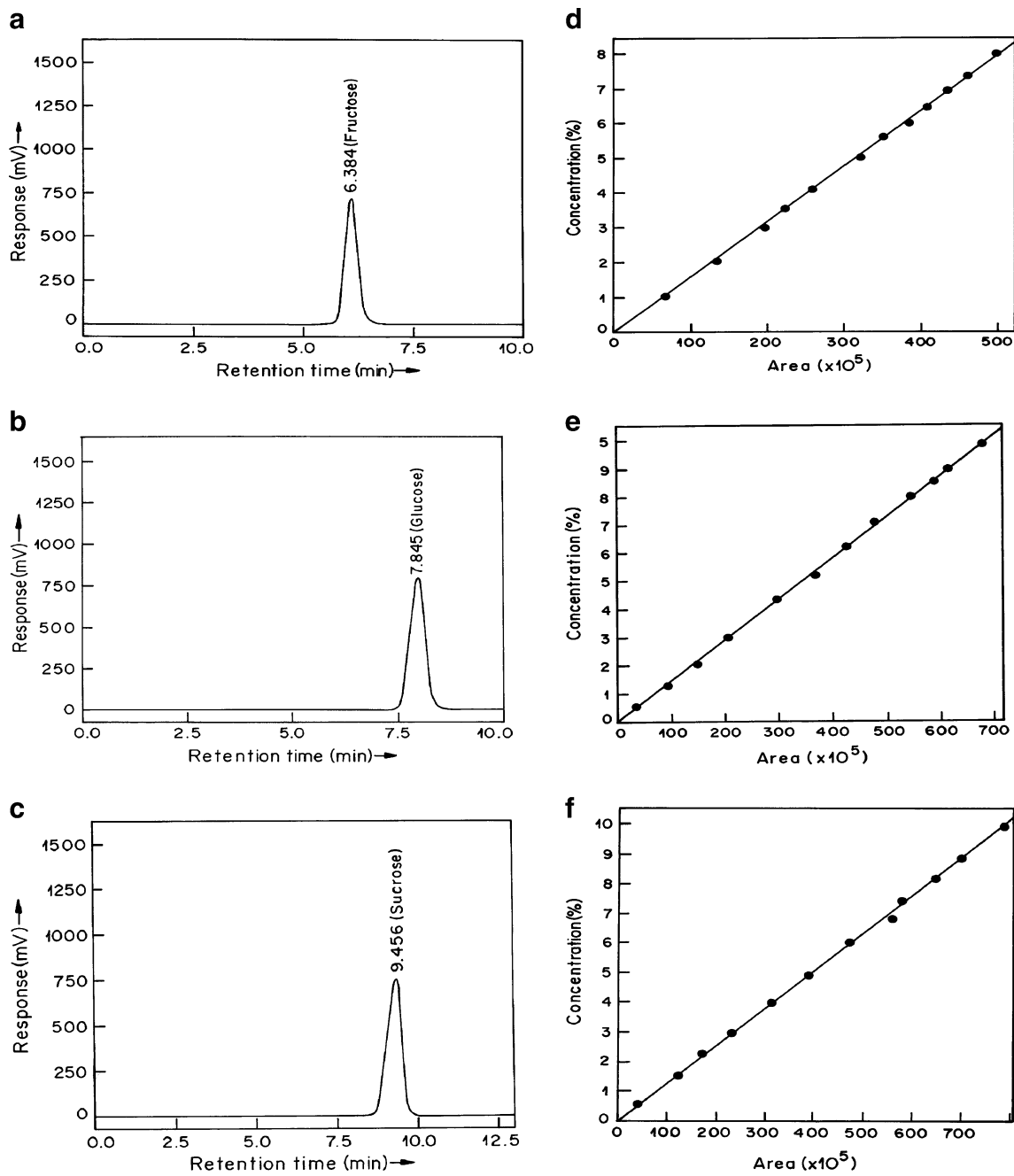


Fig. 2 Chromatogram and calibration curve of standard fructose [a and d] glucose [b and e] and sucrose [c and f]

Table 3 Regression equations of the calibration curves and their analysis of linearity

Analyte	Range (%)	Regression Equation	Correlation Coefficient	RF%RSD
Ascorbic acid	0.01–0.1	$Y=7.67E-07x$	0.9997	2.4519
Citric acid	0.05–1.6	$Y=7.83E-08x$	0.9999	0.6447
Malic acid	0.05–1.6	$Y=1.48E-07x$	0.9992	1.7015
Fructose	1.0–10	$Y=1.35E-07x$	0.9997	1.0326
Glucose	0.5–10	$Y=1.44E-07x$	0.9992	1.7420
Sucrose	0.5–10	$Y=1.22E-07x$	0.9999	0.9140

RF%RSD Percent relative standard deviation of response factor

Recovery studies were conducted by calculating the concentration of an analyte in spiked samples of real juices by developed calibration model. The obtained values were then compared with the expected values. Percent recovery obtained for each spiked sample was found to be between 97–100%.

Determination of different components in commercial fruit juices

Vitamin C (Ascorbic acid)

Chromatograms of ten different varieties of juices are recorded for vitamin C determination. Figure 3a shows the chromatogram of Real orange juice showing a significant peak of vitamin C at 3.4 min. Less variation is observed in the content of vitamin C when same fruit juice from different brands is analyzed (Table 4). Although all juices are found to contain not more than 0.03% of vitamin C, Real litchi has maximum amount, i.e. 0.025% amongst the varieties under study, while Tropicana apple contains the minimum amount (0.002%). The lesser amount of vitamin C found in packed juices as compared to fresh juices (Kabasakalis et al. 2000) can be attributed to the processing and storage conditions and its oxidation.

Organic acids

Chromatograms of eleven different juice samples are recorded and efficient separation of organic acids found in packed fruit juices are achieved using the reversed-phase C18 column. Figure 3b shows the chromatogram depicting organic acids separation in Real orange juice. The peak at 4.0 min is identified as malic acid while the one at 7.5 min is ascribed to citric acid. Various concentrations of organic acids determined by external standard method in juice samples are given in Table 4. It has been observed that distinct brands of same fruit juice are composed of different concentrations of acids. The malic acid content of apple juice from Tropicana is 0.30%. Apple juice from Apy Classic contains 0.20% of malic acid, while its content in Onjus apple juice is 0.07%. The amount of citric acid in the above labels is found to be 0.004%, 0.24% and 0.50% respectively. Maximum concentration of malic acid is found in Real mausami (0.61%), while Onjus mixed fruit juice is found to contain maximum concentration of citric acid (0.61%).

Sugars

Ten different juice samples are subjected to chromatographic analysis and a good resolution is observed

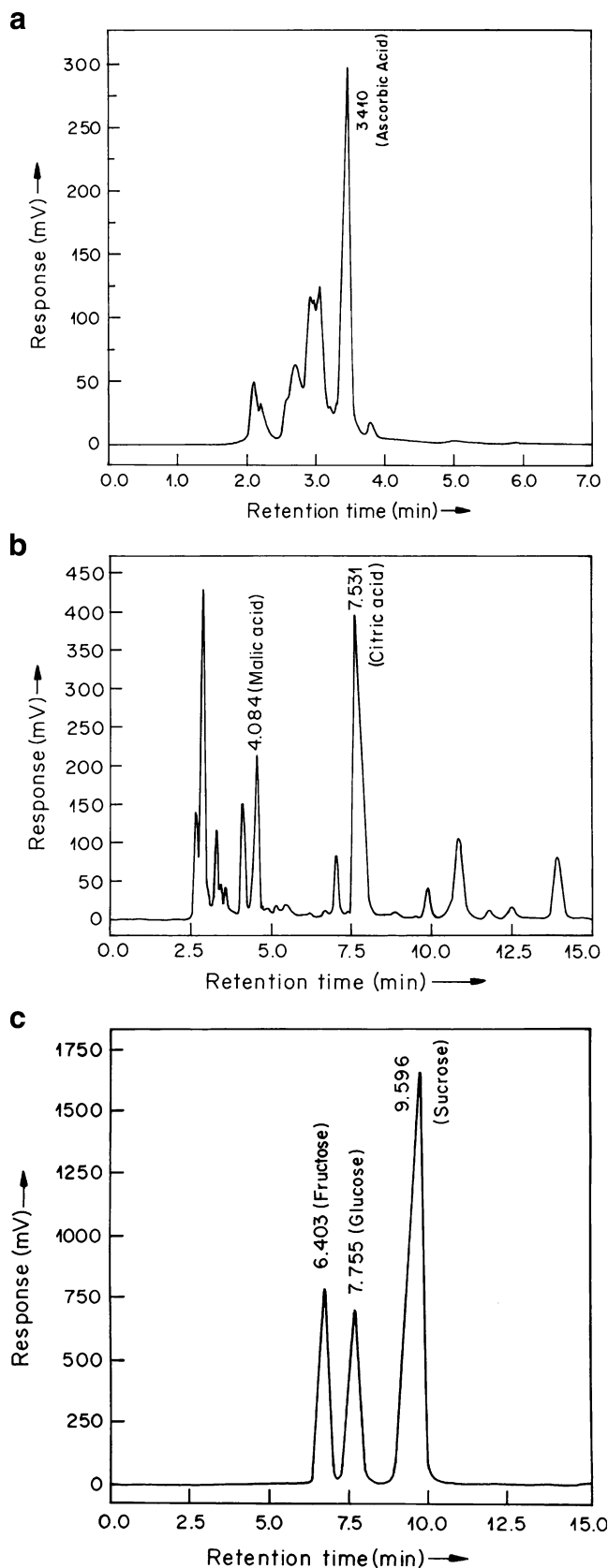


Fig. 3 Chromatogram of Real orange juice showing vitamin C **a** organic acids **b** and various sugars **c**

Table 4 Concentration of various sugars, organic acids and vitamin C in different commercial fruit juices

Juices	Vitamin C	Citric acid	Malic acid	Fructose	Glucose	Sucrose
Concentration in %						
Tropicana apple	0.002	0.004	0.301	5.885	3.025	0.937
Appy classic apple	0.006	0.238	0.205	6.131	5.162	1.948
Real litchi	0.025	0.204	0.213	3.173	2.806	6.662
Real mixed fruit	0.008	0.414	0.155	2.850	2.155	5.934
Real mango	0.006	0.312	0.023	3.136	3.500	3.852
Real orange	0.018	0.596	0.121	2.130	2.013	6.879
Real pineapple	0.009	0.561	0.204	3.037	3.101	6.702
Onjus apple	0.020	0.073	0.503	4.231	3.621	1.374
Real guava	0.007	–	–	3.353	2.986	4.474
Real mausami	–	0.443	0.067	–	–	–
Frooti	0.006	–	–	2.757	2.311	4.519
Onjus mixed	–	0.608	0.098	–	–	–
Tropicana pineapple	–	0.465	0.117	–	–	–

– This particular brand was not available on the day of experiment

under the method used for sugar separation. The reverse phase method employed in this study succeeded in eluting all sugars in a single chromatographic run. All sugars are separated and detected within 10 min under the described conditions after column equilibration. The order of sugar elution and peak identity in juice samples is confirmed when peak retention times are found identical to those of pure standards of each sugar. Figure 3c depicts the HPLC chromatogram of Real orange juice with major sugars separation. The peak at 6.4 min is identified as of fructose and peak at 7.7 min is of glucose, while the peak at 9.5 min is assigned to sucrose. It is quiet evident from the chromatographic study that major sugars in all packed fruit juices are fructose, glucose and sucrose as their peaks are significantly observed.

Table 4 reports different concentrations of sugars obtained after applying external standard quantification method. It is evident from the results that different commercial brands of even same fruit juice show characteristic variation in their sugar content. Tropicana apple juice contains 5.89% of fructose, 3.03% of glucose and 0.94% of sucrose, while the apple juice from Appy classic is composed of 6.13% of fructose, 5.16% of glucose and 1.95% of sucrose. Apple juice from another brand Onjus contains 4.23% of fructose, 3.62% of glucose and 1.37% of sucrose. Amongst the commercial labels under study Appy classic apple juice is found to contain maximum amount of fructose (6.13%) and glucose (5.16%), while

maximum amount of sucrose (6.87%) is found in Real orange juice.

Conclusions

Vitamin C, the major vitamin, malic and citric acid, the main organic acids and fructose, glucose and sucrose, the main sugar markers of fruit juices can be efficiently analyzed, compared and quantified by presented RP-HPLC methods. The results obtained in terms of retention times of sugars and acids agree well with earlier reported work (Karadeniz 2003; Sanchez-mata Maria et al. 2002). A novel and rapid reverse phase method is developed for the analysis of vitamin C, which significantly reduced its retention time. Difference is observed in the content of organic acids and sugars and vitamin C of same fruit juice belonging to different brands. The present procedures can be easily applied for routine analysis in the fruit juice industry for determining composition of packed fruit juices. Moreover, information on wholesomeness of juices and exogenous addition can be provided. The information provided in this study can be useful for the knowledge of individual components of commercially available packed fruit juices.

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