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Separation and determination of organic acids and phenolic compounds in fruit juices and drinks by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic (HPLC) separation method with photo-diode array detection has been developed for the simultaneous determination of organic acids and phenolic compounds in juices and drinks. The chromatographic analysis of organic acids and phenolic compounds was carried out after their elution with sulphuric acid solution (pH 2.5) and methanol from C_{18} stationary phase. The mobile phase employed was sulphuric acid solution working at a flow-rate of 0.35 ml min^{-1} for the whole run, while methanol was linearly increased to 0.45 ml min^{-1} from 15 to 75 min followed by a 5-min isocratic elution. Ten organic acid acids were eluted in 30 min and 21 phenolic compounds, which include phenolic acids and flavonoids, were eluted in the following 50 min. Target compounds were detected at 215 nm. The repeatability ($n=3$) and between day precision of peak area ($n=3$) were all within 5.0% RSD. The within-day repeatability ($n=3$) and between-day precision ($n=10$) of retention times were within 0.3 and 1.6% relative standard deviation (RSD), respectively. The accuracy of the method was confirmed with an average recovery ranging between 85 and 106%. The method was successfully used to measure a variety of organic acids and phenolic compounds in juices and beverages. This method could also be used to evaluate the authenticity, spoilage or micronutrient contents of juices.

Keywords: Fruit juices; Food analysis; Organic acids; Phenolic compounds

1. Introduction

Organic acids are widely distributed in fruits and vegetables. They are also used extensively as food acidulants in the manufacturing of beverages, fruit and vegetable drinks or juices. The principal acids used to enhance beverage flavours are citric, tartaric, fumaric and phosphoric acids. Citric acid is the most widely used acid while malic and tartaric acid are important natural compounds of fruits that are used along with fumaric acid in fruit-flavoured drinks. In addition, benzoic acid is widely used as a preservative in fruit drinks and juices because the pH imparted by natural and added acids is not sufficient to ensure long-term microbial stability. The content of organic acids in fruit juices not only influences their flavour but also their stability, nutrition, acceptability and keeping quality. Therefore, it is important to be able to precisely determine food acids

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present for quality control purposes, as well as for meeting various laws and regulations and for labelling purposes.

Phenolic compounds widely exist in fruits and vegetables and are reported to have multiple biological effects, including antioxidant activity [1], antitumor [2], antimutagenic [3], and antibacterial and angioprotective properties [4]. Lately, more interest has been shown in these compounds due to their possible additional health benefits. This group of compounds mainly include hydroxybenzoic acids, hydroxycinnamic acids, flavonoids and isoflavonoids. The determination of some phenolic acids using chromatographic methods include gas chromatography (GC) [5,6], capillary electrophoresis (CE) [7] and HPLC [8–12]. For the analysis of flavonoids in foods by HPLC, as reviewed by Merken and Beecher [13], columns are almost exclusively reversed-phase, and elution systems are usually binary, with an aqueous acidified polar solvent and a less polar organic solvent. The aqueous acidified polar solvent included acetic acid, phosphoric acid, perchloric acid, or formic acid. The elution may be isocratic or gradient. Further chromatographic separation and quantification of phenolic acids and flavonoids in natural fruits and beverages has been reported by Zuo and coworkers [14,15].

In most of the previously developed methods, significant restrictions are placed on the compounds to be analysed, either for carboxylic acids or one or two subclasses of phenolic compounds. In addition, chemical derivatizations and solid-phase extraction (SPE) are usually involved in the assay of these target compounds. The main difficulty arises from low resolution among the organic acids and phenolic compounds. In addition, large differences in the levels of phenolic compounds in a juice or beverage usually complicate the simultaneous analysis of different classes of phenolic compounds. The purpose of this study is to separate, identify and quantify common organic acids and phenolic compounds in a variety of juices and beverages using HPLC with photodiode array detection (DAD), which will identify compounds not only by their retention times but also their individual spectra.

This will not only provide more nutritional data for a juice or beverage but also an indicator of microbial spoilage such as an increase in lactic acid content indicating lactic acid bacteria spoilage [7] and the authenticity of the juices.

2. Experimental

2.1. Chemicals and standards

L-Ascorbic acid, butylated hydroxytoluene (BHT), (−)-quinic acid, malonic acid, lactic acid, fumaric acid, *p*-coumaric acid, vanillic acid, myricertin, quercetin, (−)-epicatechin, (−)-gallocatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate (EGCG), (−)-catechin gallate, kaempferol, and eugenol were purchased from Sigma (St Louis, MO, USA); (+)-catechin hydrate, ferulic acid, chlorogenic acid from Aldrich (Milwaukee, WI, USA); *trans*-cinnamic acid, salicyclic acid, syringic acid, caffeic acid, gallic acid from Acros Organics (Fairlawn, NJ, USA); ethanol, acetic acid, benzoic acid, malic acid, oxalic acid, L-(+)-tartaric acid, hydrochloric acid and HPLC-grade methanol were purchased from Merck (Darmstadt, Germany); citric acid and sulfuric acid from BDH (Poole, UK).

The individual standards were dissolved in 0.2% BHT methanol or pH 2.5 sulfuric acid and injected to determine individual retention times. Stock solutions of 500 μg/ml of tartaric acid, 2000 μg/ml quinic acid, 150 μg/ml oxalic acid, 1000 μg/ml malic acid, 100 μg/ml L-ascorbic acid, 1000 μg/ml malonic acid, 1800 μg/ml citric acid, 2000 μg/ml acetic acid, 1000 μg/ml citric acid, and 20 μg/ml fumaric acid were prepared by dissolving pure standards into pH 2.5 sulphuric acid. The stock solutions and the four diluted standards up to 10 times dilution from stock solutions were injected for linearity range and detection limit tests. Similarly, stock solutions of other individual standards were prepared by dissolving 2 mg pure standard in 0.2% BHT methanol. The stock solutions and the four diluted standards up to 20 times dilution from stock solutions were injected for linearity range and detection limit tests.

(−)-Gallocatechin, (−)-epigallocatechin, (+)-catechin, chlorogenic acid, (−)-epigallocatechin gallate, (−)-epicatechin, caffeic acid, (−)-catechin gallate, ellagic acid, myricetin and quercetin, eugenol and kaempferol were dissolved in about 30 ml of

0.2% BHT methanol. This solution was mixed with 30 ml of sonicated water. As methanol will interfere with peak shape (see Section 3.3.3), the mixed solution was further evaporated to remove most of the methanol at room temperature under vacuum. Other compounds were dissolved in 50 ml of H_2SO_4 solution (pH 2.5), and mixed with the above solution. The final volume of this solution was made up to 100 ml. The solution was kept at 4 °C and used to optimize the HPLC separation conditions. The solution was also applied to observe the within-day and between-day precisions of retention times and peak areas.

All solutions were filtered through a 0.45-μm membrane filter (Iwaki Glass) before HPLC analysis, and the mobile phase solvents were degassed before use.

2.2. Samples

Fruit juices and drinks were purchased from convenient stores and local supermarkets. Brand A apple juices (Australia) are 100% fresh apple juice without addition of preservative and sugar. Percentage of juice in Brand B apple juice drink (Malaysia) is unknown. Brand C juice drink (USA) contains 18% fruit juice, which includes raspberry, cranberry, apple and grape juice.

2.3. Apparatus

The HPLC analyses were carried out using a Shimadzu class LC-VP HPLC system with class LC-VP software, a pump (LC-10ATvp), an autosampler (SIL-10ADvp), and a diode-array detector (SPD-M10Avp) (Shimadzu, Kyoto, Japan). The separation was carried out on a Shim-Pack VP-ODS column (250×4.6 mm I.D.) (Shimadzu, Kyoto, Japan) with a guard column (GVP-ODS, 10×4.6 mm I.D.).

2.4. Sample preparation

Direct analysis. Juices and beverages were kept at 4 °C before analysis. Fruit juice was centrifuged at 5000 rpm for 10 min. The supernatant was filtered through a 0.45-μm membrane filter before injection. Fruit drinks were filtered through a 0.45-μm membrane filter (Iwaki Glass) and injected directly into the HPLC.

2.5. Chromatographic conditions

Optimum efficiency of separation was obtained using 0.35 ml/min of pH 2.5 sulfuric acid solution (solvent A), and the flow-rate of methanol (solvent B) was increased from 0 to 0.45 ml/min from 15 to 75 min and kept at 0.45 min/l for a period of 5 min and then reduced to initial conditions in another 5 min. Ten minutes of equilibration is required before the next injection. Other parameters adopted were as follows: injection volume, 20 μl; column temperature, 40 °C; detection wavelength, 215 nm and UV spectra, 200–600 nm range.

3. Results and discussion

3.1. Method development

After multiple preliminary assays, an elution program using methanol–H_2SO_4–water as solvent was chosen. This program allowed carboxylic acids and phosphoric acid to be eluted in 30 min and then phenolic compounds. Fig. 1 illustrates the separation of a standard mixture of 29 acids and phenolic compounds.

As shown in Fig. 1, a good separation can be achieved in 80 min. It is essential to keep the flow-rate of solvent A at 0.35 ml/min during the whole run and increase the flow-rate of solvent B to 0.45 ml/min from 15 to 75 min to achieve good resolution of the target compounds since both solvent polarity and pH are very important for separation of target compounds. Table 1 lists the retention times of individual carboxylic acids and phenolic compounds.

As can be seen from the chromatogram and Table 1, most target compounds are separated well. However, some compounds such as (−)-epicatechin and *p*-coumaric acid may be eluted with similar retention times, and thus it is difficult to identify and quantify them by simply using retention time and spiking test. In this experiment, HPLC–DAD was used to obtain the spectrum of individual compounds, which could be used to further confirm the unknown peaks in samples.

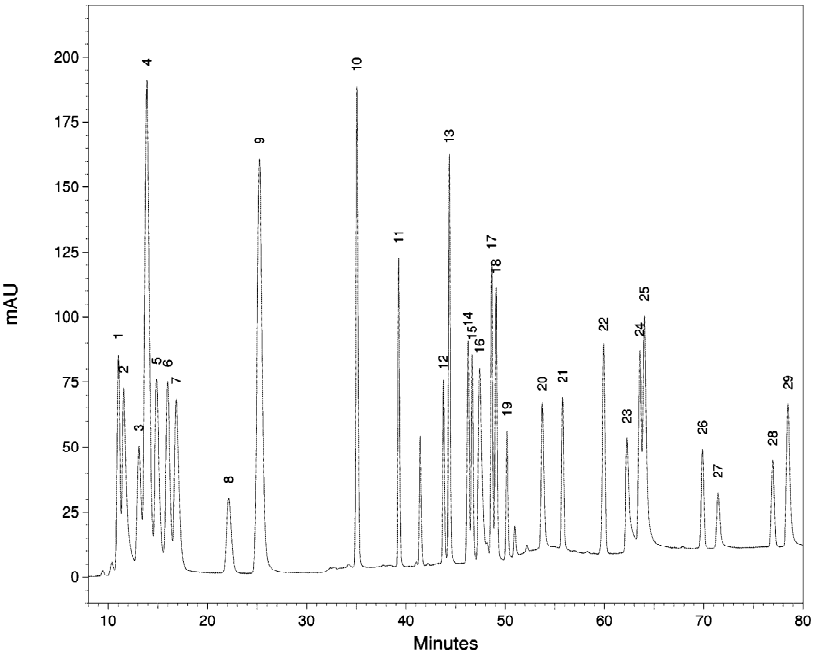

Fig. 1. HPLC of the mixture of standards. Detection at 215 nm. 1, Tartaric acid; 2, oxalic acid; 3, malic acid; 4, L-ascorbic acid; 5, malonic acid; 6, lactic acid; 7, acetic acid; 8, citric acid; 9, fumaric acid; 10, gallic acid; 11, (−)-catechin gallate; 12, (−)-epigallocatechin; 13, (+)-catechin; 14, *p*-hydroxybenzoic acid; 15, chlorogenic acid; 16, (−)-EGCG; 17, (−)-epicatechin; 18, caffeic acid; 19, syringic acid; 20, (−)-catechin gallate; 21, ferulic acid; 22, benzoic acid; 23, ellagic acid; 24, salicyclic acid; 25, myricetin; 26, *trans*-cinnamic acid; 27, quercetin; 28, eugenol; 29, kaempferol.

3.2. Method validation

The within-day repeatability ($n=3$) and between-day precision ($n=10$) of retention times were within 0.3 and 1.6% relative standard deviation (RSD), respectively.

The repeatability ($n=3$) and between day precision ($n=3$) of peak area except L-ascorbic acid, which was not very stable, were all within 5.0% RSD.

The accuracy of the method was confirmed by analyzing the mixture prepared by adding suitable amounts of standard mixture to juices with known contents of these target compounds. The recoveries of target compounds were between 85 and 106%.

The limit of detection (LOD, $S/N=3$) of individual compounds at 215 nm is given in Table 1. Compounds, such as L-ascorbic acid, benzoic acid and *p*-hydroxybenzoic acid, will have lower LOD at their maximum absorbance wavelength than those at 215 nm.

3.3. Applications

3.3.1. Determination of organic acids and phenolic compounds in pure apple juice and apple juice drink

Apple juice contains a variety of organic acids and phenolic compounds such as malic acid, ascorbic acid, chlorogenic acid and flavonoids. Fig. 2 gives the chromatographic profile of Brand A apple juice.

Measurements of organic acids and phenolic compounds are useful for labelling purpose as well as for the determination of the authenticity of the juice. For example, the levels of fumaric acid in apple juice could be important indicators of microbial spoilage of juices such as fumaric acid produced by moulds (significantly by *Rhizopus stolonifer*) [16], processing of decayed fruits, or addition of synthetic malic acid, which contains fumaric acid as a minor contaminant [17]. The natural content of fumaric acid in freshly prepared clarified apple juices without heat treatment varies from 0 to 1.7 mg/l [18]. During heat

Table 1
Linear range and limit of detection of carboxylic acid and phenolic compounds

Common name	Retention time, t_R (min)	Concentration range (mg/l)	a	b	r^2	LOD (mg/l)
Tartaric acid	11.24	50–500	4109	48 457	0.9987	2.20
(−)-Quinic acid	11.37	200–2000	1073	514	0.9992	12.50
Oxalic acid	11.92	15–150	20 333	10 319	0.9999	0.94
DL-Malic acid	13.58	100–1000	1964	24 849	0.9902	5.17
L-Ascorbic acid	14.58	10–100	53 183	102 130	0.9957	0.25
Malonic acid	15.30	100–1000	2478	−63 204	0.9988	6.00
Lactic acid	16.26	180–1800	1417	3499	0.9999	9.64
Acetic acid	17.06	200–2000	1224	−20 151	0.9993	10.91
Citric acid	22.88	100–1000	2424	13 039	0.9993	5.93
Fumaric acid	25.61	2–20	263 676	64 360	0.9987	0.12
Gallic acid	35.28	1.0–20	340 231	57 743	0.9903	0.02
(−)-Gallocatechin	39.32	1.0–20	169 183	35 273	0.9999	0.04
(−)-Epigallocatechin	43.73	1.0–20	117 270	−19 483	1.0000	0.06
(+)-Catechin	44.47	1.0–20	163 184	146 362	0.9998	0.04
p-Hydroxybenzoic acid	46.25	1.0–20	105 184	83 096	0.9972	0.09
Chlorogenic acid	46.61	1.0–20	79 339	88 640	0.9922	0.12
(−)-EGCG	47.70	1.0–20	220 713	73 747	1.0000	0.09
(−)-Epicatechin	48.56	1.0–20	171 884	95 802	0.9977	0.04
p-Coumaric acid	48.79	1.0–20	189 394	43 283	0.9989	0.04
Caffeic acid	49.15	1.0–20	147 866	−94 063	0.9919	0.05
Syringic acid	50.20	1.0–20	217 760	92 128	0.9991	0.03
(−)-Catechin gallate	53.76	1.0–20	198 614	−112 171	0.9912	0.05
Ferulic acid	55.51	1.0–20	102 981	−34 506	0.9972	0.08
Benzoic acid	59.61	1.0–20	62 388	47 227	0.9997	0.18
Ellagic acid	62.04	1.0–20	44 703	8268	0.9992	0.29
Salicyclic acid	63.50	1.0–20	108 380	18 898	0.9965	0.13
Myricetin	64.05	1.0–20	139 015	186 640	0.9960	0.06
trans-Cinnamic acid	69.57	1.0–20	130 278	−7697	0.9974	0.06
Quercetin	71.39	1.0–20	114 287	91 952	0.9999	0.03
Eugenol	76.67	1.0–20	66 253	55 598	0.9999	0.16
Kaempferol	78.26	1.0–20	90 207	8556	1.0000	0.15

processing (evaporation, pasteurisation, and sterilisation) of apple juices, the content of fumaric acid increases slightly due to malic acid dehydration. The content of fumaric acid of well-prepared, authentic and fresh apple juice normally does not exceed 3 mg/l [19].

As shown in Fig. 2(A), malic acid and L-ascorbic acid were probably the main organic acids in Brand A apple juice. The content of fumaric acid in Brand A apple juice was found to be 1.1 mg/l, which is within the normal range of freshly prepared apple juice.

In addition, the phenolics profile can be used as an indicator of adulteration of apple juice [20]. Fig. 2(B) shows the chromatographic profile of phenolic compounds in Brand A juice. Chlorogenic acid, (−)-epicatechin, (−)-EGCG and (−)-epigallocatechin were identified in Brand A apple juice. In addition, several other peaks, named unidentified compounds (UC), have similar spectra to phenolic compounds or benzoic acid. UC1 and UC 4 have similar spectra to that of benzoic acid. UC 2 has a similar spectrum to that of (+)-catechin while UC 3, UC 5 and UC 6 have similar spectra to that of cinnamic acid or syringic acid. In addition, UC2, 3, 5 and 6 all have a maximum absorbance wavelength of 280 nm, indicating their possibilities as phenolic compounds.

The low content of fumaric acid and a variety of phenolic compounds in Brand A apple juice indicates its authenticity and good quality.

Fig. 3 shows chromatogram of Brand B apple juice drink. Fumaric acid content in this apple juice

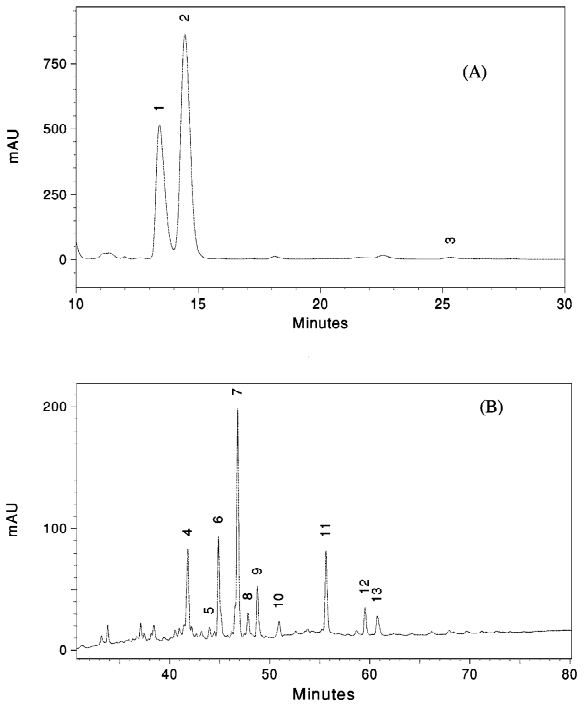

Fig. 2. Chromatogram of Brand A apple juice showing carboxylic acid and phenolic compounds profiles: (A) 0–30 min; (B) 30–80 min. 1, Malic acid; 2, L-ascorbic acid; 3, fumaric acid; 4, UC 1; 5, (−)-epigallocatechin; 6, UC 2; 7, chlorogenic acid; 8, (−)-EGCG; 9, (−)-epicatechin; 10, UC 3; 11, UC 4; 12, UC 5; 13, UC 6.

drink was found to be 10 mg/l. This could be due to the addition of synthetic malic acid. Chlorogenic acids, (−)-epicatechin and other phenolic compounds of pure apple juice were higher than in the juice drink.

3.3.2. Determination of organic acids and phenolic compounds in Brand C juice drink and other applications

Bottled Brand C juice drink was found to contain quinic acid, malic acid, L-ascorbic acid, citric acid, fumaric acid and gallic acid (Fig. 4). High content of fumaric acid is likely an indication of microbial spoilage due to the presence of *Rhizopus stolonifer*. In this study, the contents of phenolic compounds were low. No aglycones were identified in Brand C juice drink, this could be due to a low content of pure juice (18%) that includes cranberry, raspberry and apple juice, and flavonols in fruit juice mainly existed as glycosides [20].

The present method could also be used directly to analyse organic acids and free phenolic acids and flavonoids in fruits, vegetables and other plants. This method was successfully used by the authors to identify organic acid and flavonoids in star fruit juice and residue extract, and the result will be reported soon. This method is also being used for analysis of other fruits and vegetables by members of the research group. It should be stated that, as flavonoids and phenolic acids usually occur as glycosides or simple esters or are bound to the cell wall, hydrolysis before analysis is required to liberate them from their bound forms. Acid, base or enzyme catalysed hy-

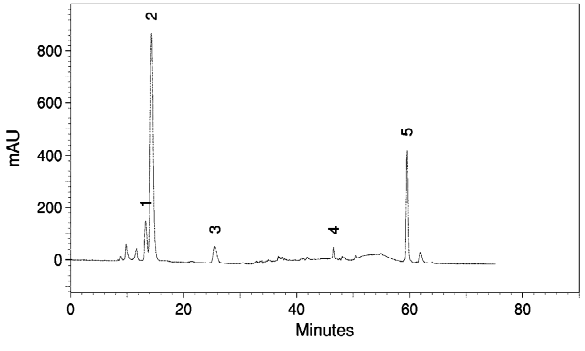

Fig. 3. Chromatogram of Brand B apple juice drink showing carboxylic acids and phenolic profiles at 215 nm. 1, Malic acid; 2, L-ascorbic acid; 3, fumaric acid; 4, chlorogenic acid; 5, benzoic acid.

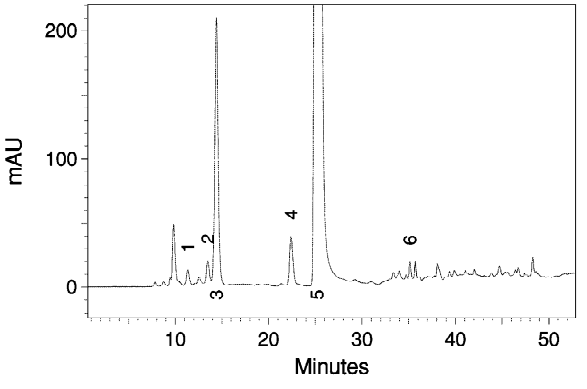

Fig. 4. Chromatogram of Brand C juice drink showing carboxylic acids and phenolic contents at 215 nm. 1, Quinic acid; 2, malic acid; 3, L-ascorbic acid; 4, citric acid; 5, fumaric acid; 6, gallic acid.

drolysis processes are usually employed as an aid to structural elucidation and characterisation of glycosides [12]. However, when solvent extraction or solvent-involved hydrolysis is used, care should be taken as solvent might influence the resolution of target compounds. In addition, t_R for phosphoric acid is 9.3 min, which indicates that this method could also be applied in the analysis of other non-fruit beverages.

3.3.3. Negative effects on chromatographic profiles by sample solvents

It was found that when organic solvent such as methanol was used in sample treatment, the resolution of quinic acid, oxalic acid, malic acid, L-ascorbic acid and malonic acid peaks was decreased (Fig. 5). It was found that if methanol is less than 10%, the resolution is still acceptable although the t_R values were slightly reduced. This might be due to close distribution coefficients and the competitive adsorption behaviour of methanol and those components between mobile phase and stationary phase.

Ethanol and acetone, two other frequently-used extraction solvents, may reduce the retention time of organic acids or influence their chromatogram profiles before 30 min, but not phenolic compounds. Therefore, it is necessary to remove most of these organic solvents before HPLC assay if the peaks of interest fall within these time frames.

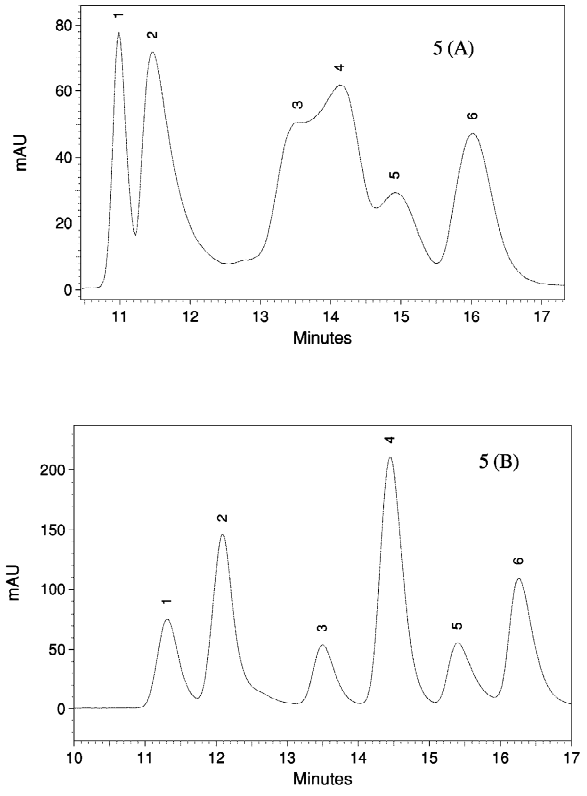

Fig. 5. Negative effects on chromatographic profile by methanol. (A) Standards dissolved in methanol–water (25:75, v/v); (B) Standards dissolved in water. 1, Tartaric acid; 2, oxalic acid; 3, malic acid; 4, L-ascorbic acid; 5, malonic acid; 6, lactic acid.

4. Conclusions

A simple method was developed for simultaneous determination of organic acids and phenolic compounds in juices and drinks by HPLC with photodiode array UV detector. Ten non-phenolic acids and 21 phenolic compounds were eluted in 80 min. The established method was successfully used to measure a variety of organic acids and phenolic compounds in fruit juices and drinks. This method could also be used to evaluate the authenticity, spoilage or nutrient contents of juices.

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