Simultaneous Analysis on Phenolic Substance in Cloudy Apple Juice by HPLC

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Abstract. Phenolic substances are important bioactive substances in apple juice. The phenolic substance extraction methods in ‘Ralls’ cloudy apple juice and the simultaneously detected conditions using high-performance liquid chromatography (HPLC) were investigated. The results showed that the extraction method for mixed phenolic substance by ethyl acetate was better than that extraction for neutral phenols and acidic phenols respectively. Most phenolic substances in apple cloudy juice could be detected at 280 nm, and which was the primary detection wavelength for simultaneous analysis on phenolic substance by HPLC. Chlorogenic acid, epicatechin and gallic acid were the main phenolic substance in ‘Ralls’ apple juice. Quantitative analysis showed that the content of chlorogenic acid was the highest, and it was the characteristic phenolic substance in ‘Ralls’ apple juice.

1. Introduction

Apple is one of the most widely cultivated fruits in the world. Nowadays, more than 40% of apples in the world are produced in China, which plays an indispensable role in the world apple industry. In recent years, the world's apple production had exceeded 80 million tons, showing an ever-growing trend [1]. Except for some apples used for fresh-eating, approximately 35% of them are used for processed food (juice, cider, vinegar and jam), of which apple juice accounts for 65%, ranking second for global consumption and production after orange juice[2, 3]. It is commonly served as clarify juice for better stability. However, the market for cloudy apple juice is growing at an unprecedented rate for the abundant nutrition. Cloudy apple juice is well known to be rich in polyphenols, which has potential effects on anti-cancer, inflammation, hyperglycaemia, lipids metabolism, anti-oxidation, antocardiac and vascular function[4, 5].

Some studies had shown that the number of polyphenols exceeds 5000, which was a secondary plant metabolite with diverse structures and extensive phylogenetic distribution[6]. At present, the detections of polyphenols mainly include spectrophotometry, chemiluminescence and high performance liquid chromatography (HPLC). Among these methods, HPLC has become the most common and accurate method for the separation and quantification of polyphenols in plants[6, 7]. However, the proper extraction method and accurate detection parameters depend on the properties of raw material. In this study, the effects of two common methods on the extraction of phenolic substance from cloudy apple juice were evaluated, and the effects of detection wavelengths on simultaneous analysis of phenolic substance using HPLC were also studied. Then, three characteristic phenolic compounds in apple juice were quantified after the analysis on limit of detection (LOD) and recovery rate of HPLC.
2. Materials and Methods

2.1 Materials and reagents

Materials. Mature apples (Malus pumila) cultivar ‘Ralls’ were hand-harvested from commercial maturity trees in local farm of Jinzhou city, Liaoning Province, China. Cored apples with ascorbic acid (0.1%, g/kg) as antioxidant were crushed with a crusher (DS-1, Wanhua Experimental Instrument Factory, Jiangsu, China). Then juice was hydrolyzed by enzyme, and then filtered by a filter cloth of 300 mesh to remove the fruit residue. After sterilization, the cloudy apple juice was stored at 4 °C for subsequent analysis.

Standards and reagents. All standards, including rutin, anthocyanin, gallic acid, tannic acid, phlorin, chlorogenic acid and epicatechin were chromatographic pure, Sigma-aldrich.

2.2 Comparison of extraction methods of phenolic substance

Extraction method 1: Extraction for mixed phenolic substance. Cloudy apple juice (50 mL) were added in 25 mL ethyl acetate, and the mixtures were oscillated at room temperature for 10 min before separated in a separatory funnel. Then, repeated extraction twice with ethyl acetate. The supernatant extracted for the three times were combine collected, and frozen at -18ºC for 2 hours to remove moisture. After that, the sample was filtered, and then concentrated using a rotary evaporator (RE-2000B, Chengdu Kangyu Instrument Co., Ltd. Sichuan, China) at 55 ºC for 10 minutes. The concentrate was dissolved in methanol and diluted to 10 mL. The dilution was stored at 4ºC, and filtered through a filter of 0.45 μm before HPLC analysis.

Extraction method 2: Extraction for neutral phenol and acid phenol. Cloudy apple juice was diluted with ultra-pure water until 11.5 °Brix. After adjusting the pH of the dilution (25 mL) to 7.0 with NaOH (1 mol/L), the dilution was mixed with 25 mL ethyl acetate. The mixtures were oscillate extracted at room temperature for 10 min, and then, the ester phase and the aqueous phase in the mixture were separated in a separatory funnel. The aqueous phase was re-extracted once with ethyl acetate, and the twice extracted ester phases were combined in a dark-colored flask. The ester phase was concentrated in a rotary evaporator, and the residue was dissolved in 25 mL of ultra-pure water to obtain neutral phenol. The pH of the aqueous phase was adjusted to 2.0 with HCl (6 mol/L), and repeat extracted twice with ethyl acetate, then, combined ester phases. The ester phase was concentrated, and the residue was dissolved in 25 mL of ultra-pure water to obtain acid phenol. The neutral phenol and the acid phenol were stored at 4 ºC, and filtered through 0.45 μm filters respectively before HPLC analysis.

Preparation of standard solutions. A mixed standard reserve solution was prepared in methanol and stored in the dark at 4ºC for further analysis. The standard solutions were filtered through a 0.45 μm filter prior to injection.

HPLC analysis method. The HPLC analysis was refer to the research of Gaurav Rajauria [6] and Adina Frum [8] with slight modifications using an HPLC system, 1206 series provided by Agilent Technologies. The column employed was the Zorbax Eclipse Plus C18 with the following dimensions: Analytical 250 mm×4.6 mm, 5-Micron. The mobile phase finally adopted was (A) ultra-pure water, (B) acetonitrile, (C) methanol and (D) purified water / formic acid (98/2, v/v) with flow rate at 0.6 mL/min and column temperature of 25ºC. The gradient schedule was: 0-30 min, 5% B and 95% D; 30-45 min, 25% B and 75% D; 45-50 min, 40% B and 60% D; 50-51 min, 40% B and 60% D; 51-60 min, 5% B and 95% D; 60-65 min, 5% B and 95% D. The injection volume was 10 µL. UV absorbance at 280nm, 320nm and 360nm were recorded respectively.

Qualitative and quantitative analysis of phenolic substance in cloudy apple juice. The phenolic substances extracted in cloudy apple juices were detected by HPLC, and the main phenolic substances were quantified. Standard solutions (1mg/mL) of chlorogenic acid, epicatechin and gallic acid were prepared by methanol solution, respectively. For quantitative analysis, standard solutions are diluted to different concentrations with methanol. The calibration curve was drawn by plotting the peak area against the concentration of each standard solution, and then, the contents of three main phenolic compounds in cloudy apple juice were calculated.
3. Results and discussions

HPLC analysis method optimization. In HPLC analysis, elution conditions were established by testing several gradients of two different phases containing ultra-pure water/formic acid and acetonitrile. Phenolic substances, especially phenolic acids, are prone to decomposition, resulting in tailing, broadening and asymmetry of the chromatographic peaks. The mobile phase is acidified with an appropriate amount of acid inhibitor (formic acid and acetic acid, etc.) to avoid tailing of the chromatographic peak and improve peak shape[6]. Inappropriate pH value will damage the chromatographic column and reduce the efficiency, resulting in severe baseline drift [9]. Initially, various combinations of mobile phases were applied using total gradient system, which showed that mobile phase of (B) acetonitrile and (D) ultra-pure water/formic acid (98/2, v/v) showed better eluting profile than (A) ultra-pure water and (C) methanol for polyphenol extract in cloudy apple juice. In addition, the mobile phase of (B) and (D) also showed a clear separation of more peaks in gradient system. Therefore, acetonitrile and ultra-pure water/formic acid (98/2, v/v) were used as mobile phases. The qualitative analysis of phenolic substance was applied by comparing the RT with the standard of phenolic substance (Fig.1) detected by HPLC.

![Fig.1. Mixed standards of phenolic substance detected by HPLC. Peaks: 1. Gallic acid; 2. Chlorogenic acid; 3. Epicatechin; 4. Rutin; 5. Phlorin.](image)

Comparison of extract methods of phenolic compounds. Phenolic compounds by different extract methods in cloudy apple juice were showed in Fig.2. From “Extraction method 1” (Fig.2-A), the detected peaks were better separated, the peaks shape were more symmetrical, and no tailing occurred. By comparing their retention times (RT) with phenolic substance standard (Fig.1) in HPLC analysis, the higher content phenolic compounds in ‘Ralls’ apple juice were chlorogenic acid and epicatechin. By “Extraction method 2”, acidic phenol and neutral phenol were extracted. As shown in Fig.2-B, the extracted acid phenol has only one peak with good peak shape and strength, which was identified as chlorogenic acid. From Fig.2-C, there were three peaks as neutral phenols, however, their signal intensity were weaker. Therefore, “Extraction method 1” was used to extract mix phenolic substance from cloudy apple juice.

![Fig.2. Phenolic compounds by different extract methods in cloudy apple juice detected using HPLC: extraction for mixed phenolic substance (A) by “Extraction method 1”, extraction for acid phenol (B) and neutral phenol (C) by “Extraction method 2”](image)
maximum absorption wavelengths. Research showed that the maximum absorption wavelength of phenolic substance is as follows: gallic acid (280 nm), catechin (280 nm), chlorogenic acid (320 nm), epicatechin (280 nm), rutin (360 nm), phlorizin (280 nm) and phloretin (280 nm) [10]. So, in this study, the three different wavelengths of 280 nm, 320 nm and 360 nm were chosen for sample analysis using HPLC (Fig.3) for evaluating effects of simultaneously analysis on phenolic substance. From Fig.3-A, the detected peaks were sharper, and the signal intensity was stronger. The phenolic substance in cloudy juice were mainly identified as chlorogenic acid (RT=19.599 min), epicatechin (RT=24.083 min), tannic acid (RT=35.340 min) and possibly isomer of chlorogenic acid (RT=21.505 min). Fewer phenolic compounds were detected at 320 nm (Fig.3-B), which were mainly chlorogenic acid (RT=19.863 min), rutin (RT=31.741 min) and tannic acid (RT=35.849 min). The signal intensity of chlorogenic acid was relative stronger. At 360 nm (Fig.3-C), the signal intensity was the weakest, and the peaks were more interfered by baseline. Therefore, the detection wavelength of 280 nm was better adopted as the final detection wavelength of HPLC for simultaneously analysis on phenolic substance in cloudy apple juice.

Fig.3. Phenolic compounds in cloudy apple juice analyzed by HPLC with different detection wavelength: A. 280nm, B. 320nm, C. 360nm.

Quantification of characteristic phenolic substance in cloudy apple juice. It was shown in Fig.3-A, phenolic substance in 'Ralls' apple juice were chlorogenic acid, epicatechin and gallic acid, therefore, the three compounds were quantified. Calibration curves (Table 1) were plot using standard phenolic compounds, and the limit of detection (LOD) was also determined. In addition, the extraction of juice both added and didn’t add standard substance were determined by HPLC, and the recovery rates of phenolic compounds were detected (Table 1). It was shown that, the recovery rates were above 90%, indicating that this method was reliable and relatively accurate.

| Phenolic substance | Calibration curves Y=mx+b | \(R^2\) | LOD [μg/mL] | Recovery rate [%] |
|--------------------|--------------------------|--------|-------------|------------------|
| Chlorogenic acid   | Y=4471.5x-1419.9         | 0.9934 | 0.0638      | 102.6            |
| Epicatechin        | Y=11.305x-34.539         | 0.9995 | 0.0688      | 94.3             |
| Gallic acid        | Y=38.403x-18.251         | 0.9997 | 0.0235      | 95.4             |

Table 2. Contents of main phenolic substance in cloudy apple juice

| Phenolic substance | Content [mg/L] |
|--------------------|----------------|
| Chlorogenic acid   | 174.21±1.04    |
| Epicatechin        | 92.76±1.84     |
| Gallic acid        | 4.74±0.20      |

The values were calculated as means ± standard deviation of three replicates (\(p<0.05\)).

The contents of main phenolic substance in cloudy apple juice were determined under the optimized chromatographic conditions, and the results were shown in Table 2. The content of chlorogenic acid was the highest phenolic compound in 'Ralls' cloudy apple juice, which reached 174.21 mg/L, and was much higher than epicatechin and gallic acid.

4. Conclusion

Phenolic substance extraction method and HPLC detect conditions were important for simultaneous...
analysis on the phenolic substance in cloudy apple juice. In general, extraction for mixed phenolic substance of ‘Extraction method 1’ was more fit for cloudy apple juice. Most phenolic substances in apple cloudy juice could be detected at 280 nm, and which was the primary detection wavelength for simultaneous analysis on phenolic substances. Quantitative analysis showed that the content of chlorogenic acid was the highest, and it was the characteristic phenolic compound in ‘Ralls’ apple juice.

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