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EXTRACTION OF PROCYANIDIN B2 FROM APPLE PEEL USING SUBCRITICAL WATER

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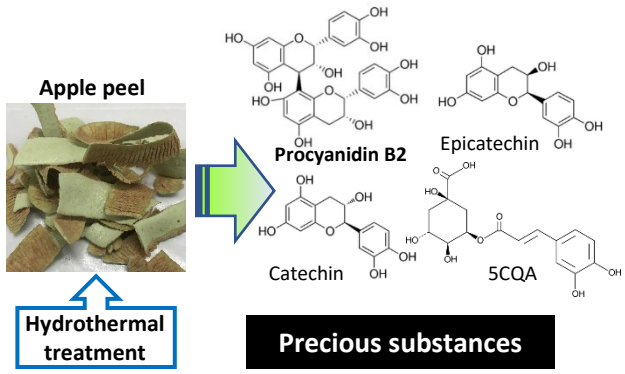
Graphical abstract



Abstract

Subcritical water, which is an effective solvent for polar and nonpolar substances, has been used to extract numerous natural ingredients. In this study, subcritical water was used to extract bioactive substances from apple peel in a batch process in the temperature range of 100–175 °C for 5–60 min. The Fourier-transform infrared spectroscopy results revealed that phenolic compounds were released from apple peel in the aforementioned temperature range. The ultraviolet–visible spectra of the liquid products at 280 nm revealed the high content of phenolic compounds in the extracts. The high-performance liquid chromatography results demonstrated that the yield of procyanidin B2 was approximately 2.28 mg/g of dried apple peel when extraction was performed at 150 °C for 15 min.

Keywords: Subcritical water, Extraction, Liquefaction, Apple fruit, Procyanidin

1.0 INTRODUCTION

Water is a universal solvent because of its ability to dissolve many substances. Typically, water has a high dielectric constant (ε) and high polarity because of the hydrogen bonds between its molecules [1, 2]. However, under ordinary conditions, water cannot dissolve nonpolar or organic substances. Consequently, water is not an appropriate solvent for extracting phytochemical substances from plant biomass at room temperature. Water possesses unique properties owing to the strong hydrogen bonds between its molecules. However, the hydrogen bonds can be destroyed under high temperature or pressure conditions, causing changes in water properties. Subcritical water can be obtained by increasing water temperature from 100 °C (boiling point of water) to 374 °C (critical point of water) and adjusting the pressure to ensure that water retains a liquid state. Under such conditions, the physical and chemical properties of water are significantly different [3–8]. For example, the ε value of water at 250 °C is approximately 27, which is similar to those of methanol (ε = 33) and ethanol (ε = 24) at 25 °C. Therefore, subcritical water can substitute methanol or ethanol as a solvent for numerous phytochemical substances in plant biomass.

Apples (*Malus domestica*) are one of the most consumed and popular edible fruits worldwide. Apple trees are cultivated worldwide under a wide range of climatic conditions; hence, the chemical composition of apples can vary. Moreover, the chemical composition of apples can be affected by horticultural and cultivar practices. Apples are typically used as a source of phytochemical compounds owing to the high quality and quantity of polyphenols they contain, such as procyanidins, flavanols, phlorizin, chlorogenic acid, anthocyanins, and flavonols [9–13]. In addition to apples being

affluent sources of minerals [14] and vitamins [15], they are also a good source of water-soluble carbohydrates, such as fiber pectin, starches, and sugars, which can help decrease blood glucose and cholesterol levels in humans [10, 13]. Considering their chemical composition, apples present many health advantages for treating asthma, cardiovascular disease, Alzheimer's disease, colon cancer, and other types of cancer [9, 13, 16].

Because apple peel is a superior source of bioactive substances, in this study, we used it as a raw material for the extraction of procyanidin B2 utilizing subcritical water in a batch system via liquefaction. Apple peel is a valuable source of bioactive substances, especially polyphenol compounds, because such compounds accumulate more in the peel than in other parts of apples [11, 17]. Procyanidin B2 is the main procyanidin in numerous natural products, such as fruits and other crops. The antitumor and antiinflammatory effects of procyanidin B2 are stronger than those of other procyanidins, that is procyanidin B1, B4, and B5, at similar concentrations. Moreover, procyanidin B2 is beneficial for diabetes complications and presents a protective effect against diabetic nephropathy by ameliorating podocyte injuries and preventing mesangial cell apoptosis [18, 19]. Plant biomass, including apple peel, liquefies upon treating it with subcritical water. Typically, plant biomass liquefaction using subcritical water is a two-step process. First, plant biomass components dissolve in water, and then the main liquefaction product is converted into a light product [3–8]. Several methods have been used to extract bioactive substances from plant biomass, and the advantages and disadvantages of each method have been described in the literature [18, 19, 22]. For example, the Soxhlet extraction method is simple; however, it requires long extraction times, and organic solvents are typically used as extraction media. Even though microwave-assisted extraction methods are environmentally friendly, the equipment required is moderately expensive. Dhanani et al. [22] extracted phenolic compounds, including procyanidin B2 from Saraca asoca using a methanol–water mixture as a solvent at room temperature. Although the recovery of procyanidin B2 reached approximately 100%, a long extraction time (3 days) was required. In addition, because methanol is a toxic solvent, separation of methanol from the extraction product was also required. To mitigate such drawbacks, in this study, subcritical water was used to extract bioactive substances from apple peel.

2.0 METHODOLOGY

2.1 Materials

The apples used as the raw material were picked at the end of July from the Nakahira Farm, Nagano, Japan. Typically, apples reach maturity and are harvested at the beginning of December. Water, the extraction solvent, was distilled using a distillation apparatus (Auto Still WS 200, Yamato Scientific Co., Ltd., Japan). Procyanidin B2 (ASB–00016231–005, Fuji Film Wako Junyaku Co., Ltd., Osaka, Japan), epicatechin (E582260, Hayashi Kasei Co., Ltd., Nagoya Group, Aichi, Japan), catechin (C217500, Toronto Research Chemicals, Inc., Toronto, Canada), and 5-caffeoylquinic acid (5CQA; catalog number 10924-1A, Kanto Kagaku Co., Ltd., Tokyo, Japan) were used as chemical reference standards for chromatographic analysis. Other chemicals, such as acetonitrile (99.8% purity, catalog number 015-08633), ethanol (99.5% purity, catalog number 05400-461), and acetic acid (99.5% purity, catalog number 010-19112) were purchased from Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan.

2.2 Sample Preparation

The flowchart of the sample preparation process is illustrated in Figure 1. Apples were washed with tap water and peeled manually using an apple peeler.

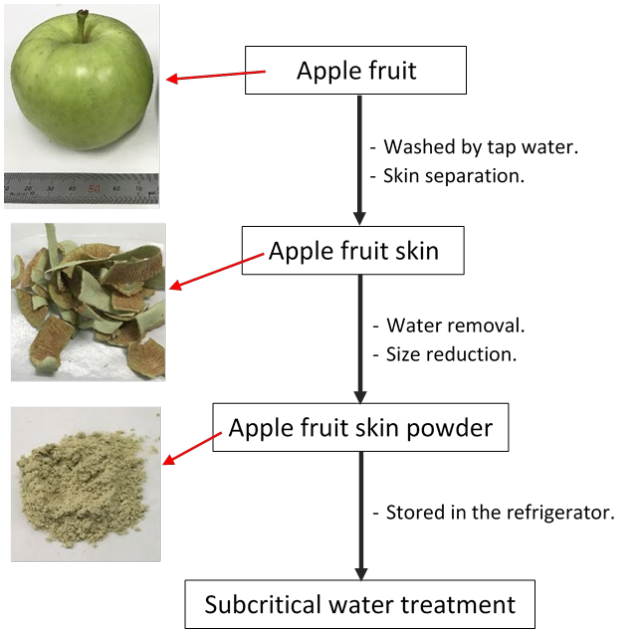


Figure 1 Separation procedure of the apple fruit skin

The water in the apple peel was removed using a freeze-drying instrument (Eyela FDU-1200, Rikakikai Co., Ltd., Tokyo, Japan) through dehydration via ice sublimation. Consequently, most of the degradation reactions were stopped, which can increase the quality of the final product. To decrease the size of the apple peel samples, the dried apple peel was crushed using a laboratory mill (IFM-800, Iwatani Corp., Tokyo, Japan), followed by passing the powder through a stainless-steel sieve (125−250 µm, Sanpo, Tokyo, Japan). Next, the apple peel powder was stored in a refrigerator at < 6 °C until further use.

2.3 Subcritical Water Extraction

The flowchart of the extraction of bioactive substances from apple peel using subcritical water is presented in Figure 2. The extraction was performed in a SUS-316 batch-type reactor (AKICO Co., Ltd., Tokyo, Japan) that consisted of an 8 mL tube fitted with a cap. The maximum temperature and pressure in the reactor were 300 °C and 30 MPa, respectively. Apple peel powder (0.1 g) and distilled water (4 mL) were added to the reactor. Thereafter, the reactor was sealed tightly, loaded into an electric furnace (NMF-13AD, ISUZU Co., Ltd., Tokyo, Japan)

and quickly heated to the desired temperature in the range of 100–175 °C. A digital pressure gauge (NS NPG-500A, Nihon Seimitsu Kagaku Co., Ltd., Japan) was used to measure the pressure inside the reactor (4–7 bar). During extraction, the reactor was shaken at 60 shakes/min using a mechanical device. After 5–60 min (including the heating time), the reactor was removed from the electric furnace and quickly quenched in a cool water bath under ambient conditions. The time required to increase the reactor temperature from ambient temperature to the desired temperature was approximately 3 min. Thereafter, the reactor temperature was the same as the furnace temperature. After cooling, the reactor was removed from the water bath and opened. Next, the solid and liquid products were collected in a screw bottle. The residue inside of the reactor was washed with distilled water, such that the total volume of the collected products was 6 mL. Next, the screw bottles containing the collected products were wrapped with aluminum foil and stored in a refrigerator at < 6 °C until analysis. Each experiment was performed in duplicate/triplicate and stated as the mean ± standard deviation.

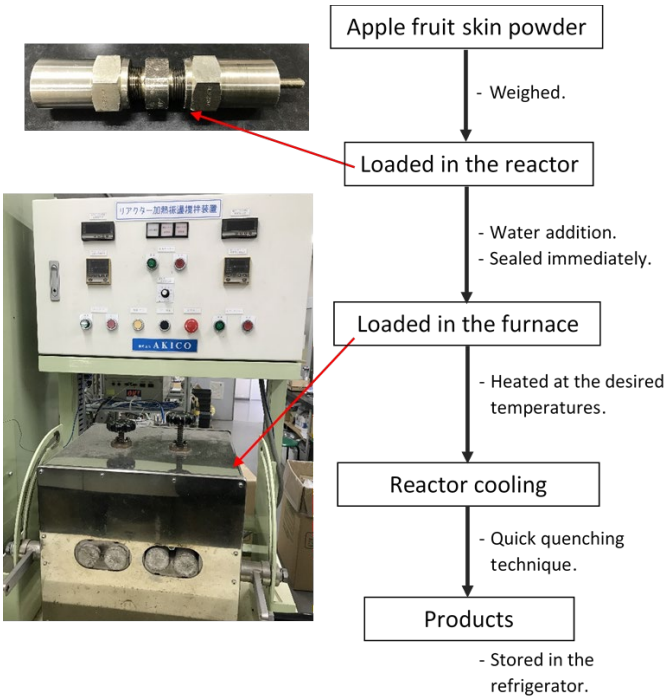


Figure 2 Extraction procedure by subcritical water

2.4 Analytical Methods

The water-soluble substances in the liquid product were analyzed using an ultraviolet–visible instrument (UV–Vis; V-550 Jasco Corp., Tokyo, Japan) with quartz cuvettes with a path length of 1 cm. The solid residue was analyzed using a Spectrum Two Fourier-transform infrared (FT-IR) spectrophotometer (PerkinElmer Ltd., Buckinghamshire, UK) in the wavenumber range of 400–4000 cm^{-1}. The amounts of procyanidin B2, epicatechin, catechin, and 5CQA in the liquid fraction were determined using a LC-10AD high-performance liquid chromatography (HPLC) gradient system equipped with a SDP-M10A diode array detector (Shimadzu Corp., Kyoto, Japan). A STR ODS-II (Shinwa Chemical Industry, Kyoto, Japan) column (4.6 mm × 250 mm) was used as the separation column at 40 °C. The mobile phase consisted of 2.5% acetic acid in water (solvent A), and acetonitrile (solvent B). The flow rate was 1.0 mL/min and the wavelength was set at 280 nm. Gradient elution was performed as follows: 0 min A–B (97:3), 5 min A–B (91:9), 15 min A–B (84:16), 33 min A–B (64:36), 38 min A–B (0:100), 48 min A–B (97:3), and 60 min A–B (97:3). An auto sampler (SIL-10 AF, Shimadzu Corp., Kyoto, Japan) was used to inject 10 µL samples in the HPLC system. The yields of procyanidin B2, epicatechin, catechin, and 5CQA were defined as the weight of the product recovered to the initial weight of apple peel loaded into the HPLC system.

2.5 Statistical Analysis

Statistical analysis of the experimental data was used to determine the variance and significance of the experimental parameters using analysis of variance. The experimental parameters were considered significant at $P < 0.05$.

3.0 RESULTS AND DISCUSSION

As mentioned above, when apple peel was treated with subcritical water, its chemical structure underwent a transformation process owing to the autohydrolysis reaction of the cell wall components. The decomposition reaction of the cell wall components generated backbone units and numerous radicals as thermal cleavage products. To elucidate the chemical structure of the water-insoluble products in apple peel after subcritical water treatment, the solid residue was analyzed using the Spectrum Two FT-IR spectrometer. This is a simple method for identifying unknown compounds and chemical bond types in compounds. The FT-IR spectra of apple peel and its solid residue after subcritical water treatment at 150 °C are presented in Figure 3. As a reference, some distinct bands of the FT–IR spectrum and the typical functional groups for plant biomass were assigned as follows [3–8]: the O–H stretching groups (3600–3000 cm^{-1}), the C–Hn stretching (2860–2970 cm^{-1}), the C=O stretching groups (1700–1730, 1510–1560 cm^{-1}), the C=C stretching groups (1613, 1450 cm^{-1}), the aromatic C=C groups (1632 cm^{-1}), the O–CH_3 groups (1470–1430 cm^{-1}), the O–H bending groups (1440–1400 cm^{-1}), the C–H bending groups (1402 cm^{-1}), the C–O–C stretching groups (1232 cm^{-1}), the C–O stretching groups (1215 cm^{-1}), the C–O–C stretching vibration groups (1170, 1082 cm^{-1}), the O–H association groups (1108 cm^{-1}), the C–O stretching and C–O deformation (1060 cm^{-1}), the aromatic C–H groups (700–900 cm^{-1}), and the C–C stretching groups (700–650 cm^{-1}). The main apple peel constituents, namely lignin, hemicellulose, and cellulose are similar to those found in other plant species and are connected via intra- and intermolecular bonds. Lignin is bound and strongly conjugated with hemicellulose and cellulose via hydrogen and covalent bonds. Nevertheless, these bonds can be broken when apple peel is treated with subcritical water.

The intensity of the FT-IR peaks of raw apple peel was stronger than that of the FT-IR peaks of its residue after subcritical water treatment (Figure 3). The intensity of the peaks in the wavenumber range of 3330.19–3323.19 cm^{-1}

(stretching of the O–H bonds) decreased after the subcritical water treatment owing to the consumption of alcohol groups during treatment. The intensity of the peaks in the wavenumber range of 2924.08–2915.89 cm^{-1} (stretching of the aromatic and aliphatic C–H bonds) also decreased significantly after the subcritical water treatment. This indicated that aromatic and aliphatic compounds were extracted using subcritical water. A similar behavior was observed for the peaks in the wavenumber ranges of 1736.52–1715.32, 1637.11–1610.12, 1446.1–1373.8, 1161.7–1148.30, and 1029.94–1016.13 cm^{-1} (stretching of the aromatic C=O bonds, stretching of the C–N and N–H bonds, stretching of the methoxyl–O–CH_3 bonds, stretching of the C–O–C bonds, and vibration of the aromatic skeletal groups, respectively). These results indicated that the apple peel constituents were extracted by subcritical water [3–8].

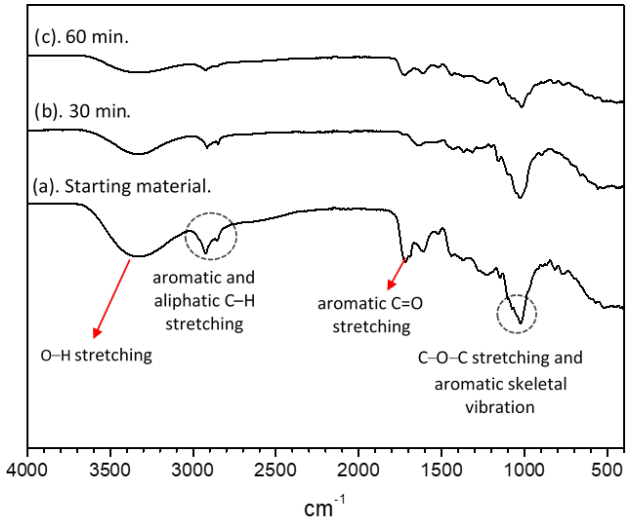


Figure 3 FT–IR spectrum of apple fruit skin (starting material and its solid residue)

To identify the substances extracted from apple peel, the liquid product was subjected to UV–Vis spectrophotometry analysis. The UV–Vis spectra of the liquid products extracted at 150 °C at different times are presented in Figure 4. It is well known that the cellular structure of plants, including apple peel, can be destroyed at subcritical water conditions. The intra- and intermolecular bonds in and/or between cellulose, hemicellulose, and lignin were cleaved through autohydrolysis. Next, the constituents were separated and allowed to dissolve in hot water. The predominant peak in the UV–Vis spectra of all the water-soluble products was observed at approximately 280 nm. We hypothesized that the intramolecular hydrogen bonds of the phenolic groups of the lignin component of the apple peel were destroyed during subcritical water treatment [23–25]. Ersan et al. [23] extracted the components of *Pistacia vera* L. hull, including phenolic compounds, using subcritical water in the temperature range of 110–190 °C. Lachos-Perez et al. [24] also used subcritical water to extract phenolic compounds from defatted orange peel in the temperature range of 110–150 °C. Furthermore, Niazmand et al. [25] optimized the extraction of phenolic substances from Ziziphus jujuba using the subcritical water technique. Using a response surface method, they evaluated the effect of temperature on the extraction process and confirmed that the extraction yield of phenolic substances increased significantly upon increasing the temperature from 110 to 170 °C. Therefore, subcritical water can extract the constituents of apple peel, especially the phenolic substances in lignin, via an autohydrolysis reaction, which can destroy the cell wall of apple peel. The total amount of phenolic compounds in the liquid products was not determined.

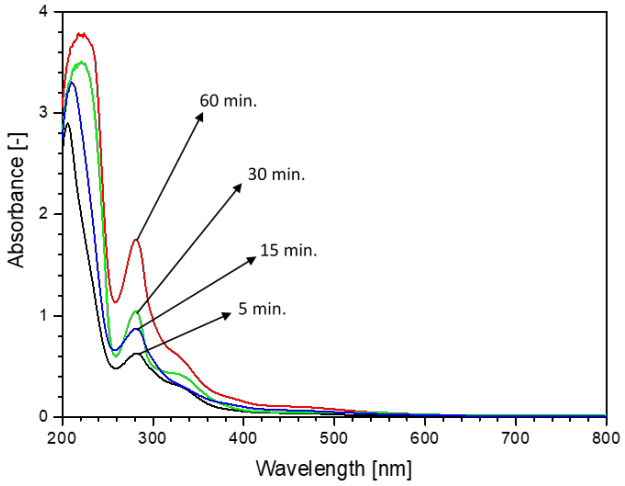


Figure 4 UV–Vis spectra of water-soluble products

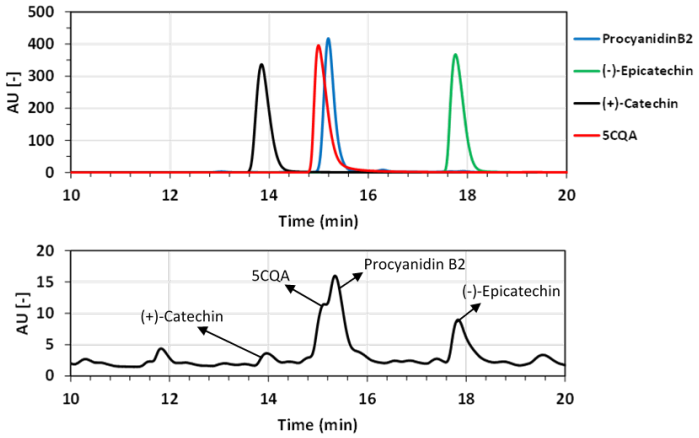


Figure 5 HPLC chromatograms of extracted compounds

Liquid solvent extraction is defined as the removal of soluble components from an insoluble solid or liquid substance using a liquid solvent. This can occur, particularly by force, when the liquid solvent comes in contact with plant matter under certain conditions. Therefore, the constituents of apple peel can be extracted under subcritical water conditions. To analyze the extracted compounds, the liquid product recovered from the subcritical water extraction of the apple peel was injected into the HPLC system. The HPLC profile of the liquid product extracted at 150 °C after 15 min is presented in Figure 5. Some compounds extracted from apple peel, especially phenolic compounds, were identified using UV–Vis spectroscopy at a wavelength of 280 nm. Nevertheless, in this study, procyanidin B2, epicatechin, catechin, and 5CQA were subjected to target compound recovery and quantitatively analyzed using the HPLC system. Such phenolic compounds present several health benefits owing to their antioxidant activity [9–13, 26]. First, pure procyanidin B2, epicatechin, catechin, or 5CQA standard

compounds were injected into the HPLC system. The calibration curve for each compound was created using four to five points. Thereafter, the amounts of procyanidin B2, epicatechin, catechin, and 5CQA in the liquid product were determined using their respective calibration curves.

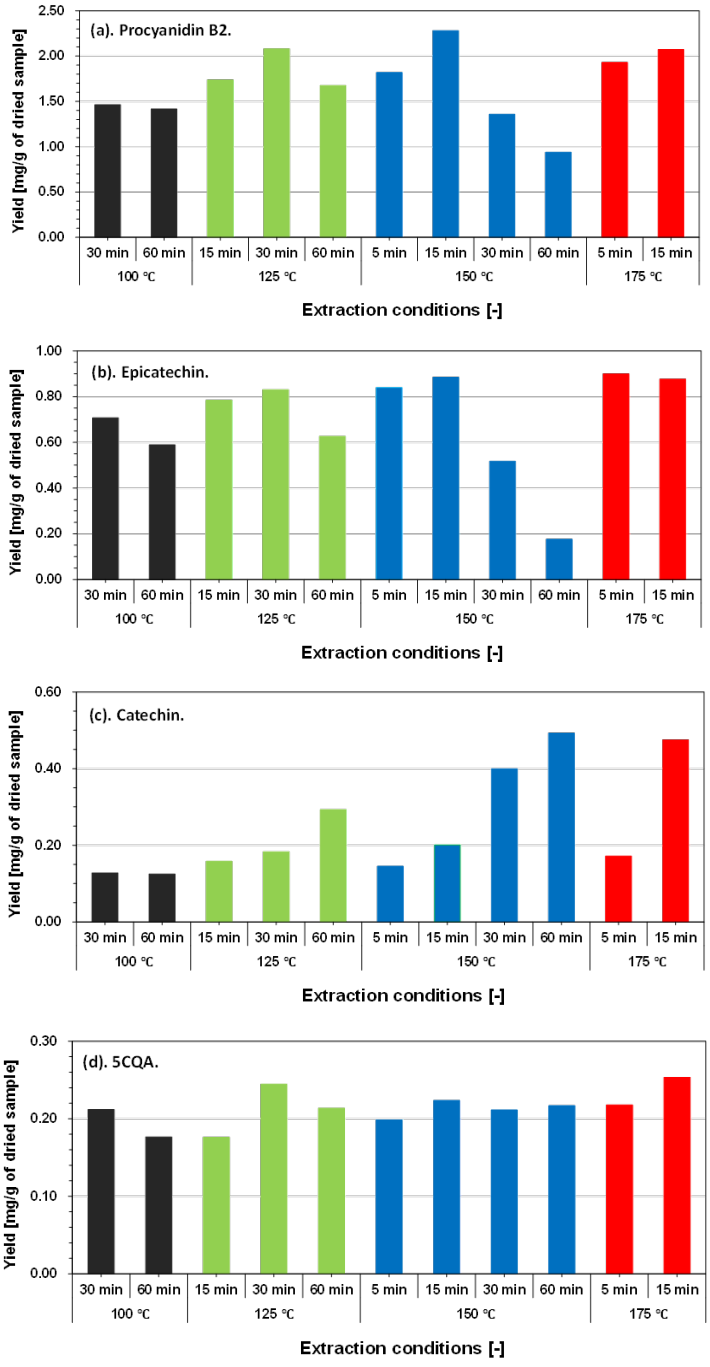


Figure 6 Yield of extract: (a). Procyanidin B2; (b) Epicatechin; (c). Catechin; (d). 5CQA

The yields of procyanidin B2, epicatechin, catechin, and 5CQA under various extraction conditions are illustrated in Figure 6. At the same extraction time, the yields increased with increasing extraction temperature. Typically, increasing the temperature during subcritical water extraction significantly affects the extraction yield [24, 25, 27, 28]. Extraction temperature is a critical factor that can destroy the cell wall of apple peel and promote component secretion, affecting the efficiency and selectivity of the subcritical water extraction process. Moreover, temperature can affect the physicochemical properties of water and promote the hydrolysis or decomposition of thermally labile substances. The physical properties of water at high temperature are advantageous; that is, water exhibits low surface tension, low viscosity, and high diffusivity. Under these conditions, the polarity of liquid water is similar to that of nonpolar compounds. Therefore, the solubility of less polar compounds in liquid water under these conditions is improved. In addition, the efficiency of subcritical water extraction can be enhanced by increasing temperature because rapid thermal desorption is favored and the vapor pressure of the target compounds is higher at high temperatures [1, 2, 25].

In addition to the extraction temperature, the extraction time significantly affects the subcritical water extraction process [24, 25, 28]. Herein, the extraction time was the time the liquid water came in contact with the apple peel under the predetermined experimental conditions. Prolonging the extraction time can improve the exposure of target substances to the solvent, resulting in an effective extraction process. This was followed by liquid water penetrating the apple peel matrix, which further promoted the diffusion and dissolution of the substances in the apple peel matrix. Consequently, the yields of procyanidin B2, epicatechin, catechin, and 5CQA increased with increasing extraction time at the same extraction temperature (Figure 6). Nevertheless, the extraction time in subcritical water extraction systems is usually affected by the extraction temperature and target substances, and prolonging the extraction time can promote the degradation of bioactive substances. At 150 °C, the yields of procyanidin B2 and epicatechin increased upon increasing the extraction time from 5 to 15 min. However, the yields decreased upon further increasing the extraction time. This indicated that a longer extraction time led to a decrease in the yields of procyanidin B2 or epicatechin by increasing the consumption of these phenolic substances via oxidation during subcritical water treatment. Conversely, the yields of catechin and 5CQA increased or remained unchanged with increasing the extraction time at the same extraction temperature. This suggested that prolonging the extraction time promoted the decomposition of the extracted substances, which affected the extraction yield. When extraction was performed at 110 and 125 °C for up to 60 min, the highest extraction yield of procyanidin B2 was approximately 2.08 mg/g of dried sample at 125 °C at an extraction time of 30 min. It appeared that extraction reached its solubility limit; therefore, increasing the extraction time beyond 30 min promoted the degradation of extracted procyanidin B2 because the extraction of phenolic substances, including procyanidin B2, is typically affected by the equilibrium concentration during extraction [2]. The procyanidin B2 extracted at 175 °C and at extraction times of 5 and 15 min also underwent degradation (Figure 6). Hence, we concluded that a longer extraction time and/or a higher extraction temperature were not appropriate for the extraction of phenolic compounds using subcritical water. In this study, the optimal conditions for the subcritical water extraction of procyanidin B2 from apple peel were an extraction time of 15 min and an extraction temperature of 150 °C.

A simple procedure for the subcritical water extraction of apple peel was proposed based on the FT-IR and UV–Vis spectra (Figures 3 and 4, respectively). As the main components of plant biomass, including apple peel, are mixed in diverse proportions and depend on the nature of each type of biomass, the subcritical water extraction of apple peel constituents is a complex process. Hence, a detailed extraction procedure was not included herein. When the extraction temperature was increased, water diffusivity also increased, which improved the mass transfer properties of water and increased extraction yields. Therefore, the extraction of the desired substances using subcritical water is typically described as a series of mass transfer steps [29, 30]. Extraction was initiated by the desorption of the solute from the active sites of the sample matrix. Next subcritical water diffused into the sample matrix. Thereafter, the substances from the sample matrix dissolved in subcritical water. Although evaluating the effects of the aforementioned steps on extraction efficiency was difficult, we determined that high extraction temperatures can increase extraction efficiency. However, the extraction conditions, including temperature, during subcritical water extraction, can facilitate extraction and cause the degradation of the extracted substances. This is the primary reason the yield of extracted substances decreases with increasing extraction temperature and/or time in subcritical water extraction systems.

To evaluate the effects of the extraction parameters on the yield of extracted substances, the F-test and P-value were determined, and the results are summarized in Table 1. For all parameters, a large F-test and a small P-value indicated a significant effect on the yield of the extracted substances. Although all parameters were insignificant ($P > 0.05$), it appeared that subcritical water extraction was effective for extracting polyphenol compounds from dried apple peel.

Table 1 Analysis of variance (ANOVA) for experimental parameters

Parameter	Procyanidin B2		Epicatechin	
	F	P-value	F	P-value
T (°C)	0.6175	0.6209	0.3162	0.8135
t (min)	0.2810	0.8379	0.3001	0.8246

Parameter	Catechin		5CQA	
	F	P-value	F	P-value
T (°C)	1.5628	0.2650	0.7749	0.5368
t (min)	0.6567	0.5988	0.2518	0.8582

4.0 CONCLUSION

The subcritical water extraction of bioactive substances from apple peel was analyzed in the temperatures range of 100–175 °C and extraction time range of 5–60 min in a batch-type reactor. During liquefaction, the apple peel constituents were dissolved in liquid water. The FT-IR spectra indicated that the intermolecular bonds between apple peel constituents were cleaved, especially the labile ether bonds between phenylpropane units, and the individual compounds were extracted. The UV–Vis spectra of the liquid products at 280 nm revealed the high content of phenolic compounds of the extracts. The maximum yields of procyanidin B2, epicatechin, catechin, and 5CQA were 2.28 (150 °C, 15 min), 0.90 (175 °C, 5 min), 0.49 (150 °C, 60 min), and 0.25 mg/g of dried apple peel (175 °C, 15 min), respectively. Based on these results, we believe that subcritical water extraction can be used to extract bioactive substances from other types of plant biomass because it is a simple and environmentally friendly extraction method.

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