博士論文（要約）

Characterization of *Citrus natsudaidai* peel and its application as a natural food additive

（柑橘類ナツミカンの皮の特性評価と天然食品添加物としての応用に関する研究）

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SUMMARY

Chapter 1. Introduction

Reducing food waste is a global challenge that significantly impacts the environment, society, and economics. At the United Nations Summit in 2015, the Sustainable Development Goals (SDGs) are adapted. SDGs Goal number 12 contains 11 targets. A target of 12.3 is set to “By 2030, halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses”. The concept of this study is to use up food processing waste using food-industrially available methods. It can estimate that the amount of plant food waste is about 8.36 million tons per year in Japan, and utilization of this waste effectively may greatly contribute to the economy and the environment.

In this study, citrus was selected as a sample because it is widely cultivated worldwide, its waste part ratio is high, and its processing waste can be eaten without heating. Among various citrus species, Citrus natsudaidai (CN) was used as a sample, assuming utilization of the residue after processing into food products such as juice and jelly. CN has a singular characteristic which is a very thick and hard peel. Thus, it is challenging to manage effectively; however, alternatives for its utilization should be considered to contribute to a reduction in waste.

The aim of this study was the utilization of food processing waste. The study was divided into three steps as follows: (1) investigation of nutritional and health-promoting components in the target food processing waste; (2) the establishment of extraction and separation method for components to be used; (3) a trial test assuming the actual utilization way.

Chapter 2 Proximate composition and profiles of free amino acids, fatty acids, minerals, and aroma compounds in *Citrus natsudaidai* peel

The color, proximate composition, free amino acids, fatty acids, minerals, and aroma compounds of three cultivars of CN peels were determined. Regarding the proximate composition and minerals, CN peels included approximately 18% of carbohydrate, which may consist of pectin, and the most predominant mineral was potassium. The color of peels varied, probably due to the difference in cultivars. The contents of free amino acids were not enough to affect the taste of extracts. The proportion of individual fatty acids was not comparable to other plant oils; however, typical aroma compounds with citrusy smell were identified. It was estimated that CN peel would be useful as an ingredient for pectin and natural flavor due to the carbohydrates and aromatic compounds.

Chapter 3 Comparison of antioxidant properties of different crude extracts of *Citrus natsudaidai* peel

The purposes of this chapter were obtaining health-beneficial compounds from CN peels by simple and food-use extraction way; investigating the antioxidant capacities of extracts of different polarities without overestimating the results. CN peels were extracted with water-ethanol and hexane-ethanol mixture solutions to obtain five different soluble fractions. The highest total phenolic content (TPC) was obtained from a water-soluble fraction (WSF), and the lowest content was observed in hexane soluble fraction (HSF). WSF also showed the most active antioxidant activities, and minimum activities were found in HSF. A positive correlation was detected between TPC and antioxidant activities.

Chapter 4 Identification of the antioxidant active phenolic compounds in *Citrus natsudaidai* peel

In Chapter 3, CN peel extracts revealed the presence of phenolic compounds. There

was a positive correlation between the TPC and antioxidant activity, meaning that the polar phenolic compounds are responsible for the antioxidant capacity of CN peels. Some of the flavonoids were identified in CN peel extract using the on-line HPLC-ABTS system, yet their antioxidant properties were not detected. In addition, there was a considerable difference between total antioxidant activity in the on-line HPLC-ABTS and ABTS recorded by spectrophotometer without compound separation in Chapter 3. Although many studies have used an on-line HPLC-antioxidant assay, the antioxidant capacity differs depending on the experimental conditions. Therefore, this chapter aimed to identify phenolic compounds acting as a radical scavenger in CN peel and improve an on-line HPLC-ABTS method simultaneously.

The results verified that neoeriocitrin, narirutin, naringin, hesperidin, and neohesperidin were included in CN peel. Regarding the optimized experimental conditions to improve the ABTS reactivity of the on-line HPLC-ABTS assay, the high temperature was recommended to enhance the chemical kinetics between target compounds and ABTS radical. Moreover, 0.30 mM ABTS concentration and acetonitrile-based mobile phase were suitable to stabilize the ABTS absorbance during the measurement. Preparing ABTS solution with 100 mM PB was suggested to adjust pH during the chemical reaction. These findings could contribute to the development of the utility and enhancement of the value of the on-line HPLC-ABTS assay.

Chapter 5 Utilization and evaluation of *Citrus natsudaidai* peel waste as a source of natural food additive

The objective of this study was to obtain beneficial ingredients from CN peels to benefit the food industry and contribute ecologically and economically. The yields of ingredients were 26.2-31.6 mg/g dried material (DM) in pectin, 9.22-11.3 g/100 g fresh material (FM) in insoluble dietary fiber, 5.56-7.15 g/kg FM in peel essence, 23.8-27.0 mg/g

DM in naringin, and 8.30-10.2 mg/g DM in neohesperidin. Naringin and neohesperidin were predominant flavonoids in CN peel. Although CN peel pectin had lower viscosity than commercial pectin, it could be applied to smooth texture products. A sensory evaluation by consumer panelists (n=31) and instrumental assays were performed to evaluate aqueous solutions flavored with 0.02 % CN peel essence. The CN-flavored solutions had a preferred smell over commercial citrus-flavored water and were classified into the same group with commercial citrus beverages in the electronic nose assay. The CN-flavored solution had a sourness, bitterness, and orange-like taste, and the overall acceptance of the CN-flavored solution did not significantly differ from commercial flavored water. Given that 1000 tons/year of CN peels are obtained, it provides 5.56-7.15 tons/year of peel essence, equivalent to 55.6-71.5 million bottles/year when 500 ml bottle beverages containing 0.02% CN peel essence are produced. CN peels have the potential as additive agents and ingredients for food, pharmaceutical, and cosmetic products.

Chapter 6 Conclusions and recommendations

In this present study, a model case for food processing waste utilization was established using CN. In the utilization flow of CN peel, 5.56-7.15 g of peel essence, 5.05-6.12 g of water-soluble pectin, 4.71-5.22 g of naringin, 1.61-1.96 g of neohesperidin, and 92.2-113 g of insoluble dietary fiber were obtained from 1 kg of CN peel. Since the extraction methods used in this study were simple and available for food use, they can be employed as a CN processing waste utilization way in the food industry field. The model case of CN processing waste utilization can also be applied to other plant food processing wastes. These may contribute to solving and reducing the problem of food processing waste discharged at 8.36 million tons annually in Japan.

Another outcome of this study was the possibility of improving analytical methods. In Chapter 4, consideration of experimental conditions to improve the reactivity of the on-line

HPLC-ABTS assay was performed. The optimized method found in this study could contribute to developing the utility and enhancement of the value of the on-line HPLC-ABTS assay in the academic field.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Reducing food waste is a global challenge that significantly impacts the environment, society, and economics. According to the Food and Agriculture Organization (FAO), approximately 1.3 billion tons of food are wasted annually worldwide (FAO, 2019). In Japan, about 25.5 million tons of food waste was generated in 2017 (Ministry of Agriculture, Forestry and Fisheries (MAFF), 2020). At the United Nations Summit in September 2015, all Member States, including Japan, adopted the 2030 Agenda for Sustainable Development. The Sustainable Development Goals (SDGs) are international goals aimed at a sustainable and better world by 2030, consisting of 17 goals and 169 targets (Ministry of Foreign Affairs of Japan (MOFA)). SDGs Goal number 12, which refers to “Responsible Production and Consumption,” contains 11 targets. A target of 12.3 is set to “By 2030, halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post-harvest losses” (UN Department of Economic and Social Affairs). Besides, reducing food waste also interlinks to the other SDGs targets such as food security, climate variability, and environmental sustainability (FAO, 2019).

Currently, there are no international definitions of “food waste.” In Japan, “materials that are derived as by-products in food manufacturing, and during processing and cooking” are included in addition to the leftover foods (Liu et al., 2016). This thesis focuses on only the by-products generated by food processing. Thus, to distinguish between “leftover food,” the term “processing waste” is used in the text.

The concept of this study is to use up food processing waste. Many articles have studied beneficial compounds in food waste, focusing on only a specific compound (Lou et al., 2014; Park et al., 2014), although residues remain after obtaining a specific compound. Moreover, the methods used in the articles are not based on the premise of food use. For instance, MeOH, acetone, chloroform, acids, and alkalis are commonly employed as extraction solvents (Azmir et al., 2013) since they are suitable for obtaining high-yield compounds. However, their extracts cannot be applied as ingredients to food products due to the toxicity to humans. Using food-industrially available methods is necessary to apply beneficial ingredients obtained from food processing waste to food production.

In Japan, in 2017, it was estimated that approximately 14.4 million tons of food processing waste were generated in food manufacturing (Ministry of the Environment (MOE), 2020). The global food waste per year of vegetables and fruit was approximately 45%. It comprises the category generating a lot of food waste than cereals, meat, and dairy products (FAO, 2019). According to MAFF, the production of crops for food manufacturing was 9.57 million tons in vegetables (in 2019) and 9.0 million tons in fruits (in 2009) in Japan. It can estimate that the amount of plant food waste is about 8.36 million tons per year, and utilization of this waste effectively may greatly contribute to the economy and the environment.

There are many types of ingredients in plant foods (Takebayashi et al., 2013; Zhang et al., 2014). Extraction and measurement methods have already been studied and established for each ingredient, and they could be applied to various plant foods changing the conditions. Therefore, if a food-industrially available method for utilization of plant-food processing waste is established, it can also be applied to various plant foods. In this study, citrus was selected as a sample because it is widely cultivated worldwide, its waste part ratio is high, and its processing waste can be eaten without heating.

Among various citrus species, *Citrus natsudaidai* (CN), which has a high ratio of

waste part among citrus fruits and has not been well studied scientifically, was used as a sample. CN is famous citrus in Japan. In 2017, about 32 thousand tons of CN were harvested, and about 28 thousand tons were shipped, among which about 3 thousand tons were for food processing (MOE, 2020). The processing waste of citrus fruits is mainly a peel part divided into an outer layer called flavedo and a middle layer called albedo. In this study, flavedo and albedo were not separated and used as a “peel sample,” assuming utilization of the residue after processing into food products such as juice and jelly. CN has a singular characteristic which is a very thick and hard peel. For that reason, it is challenging to manage effectively; however, alternatives for its utilization should be considered to contribute to a reduction in waste.

1.2 AIMS AND OBJECTIVES

The aim of this study was the utilization of food processing waste. The study was divided into three steps as follows: at first, investigation of nutritional and health-promoting components in the target food processing waste; secondly, the establishment of extraction and separation method for components to be used; finally, a trial test assuming the actual utilization way. To use up the food processing waste, the acquisition of the target components should be carried out in a series of operations, and no residue should remain after the acquisition. In addition, the acquisition method should be simple and available for food use. These were set as prerequisites for the study.

In this study, the objective was to establish a model case for food processing waste utilization using CN. Nutritional compounds and health-promoting compounds in CN peel were investigated in Chapters 2 and 4. A simple and food-industrially available extraction method was considered in Chapter 3. The potential and possible use of CN peel as a natural food ingredient were evaluated in Chapter 5. Lastly, in Chapter 6, conclusions and

recommendations for future work are presented.

CHAPTER 2

PROXIMATE COMPOSITION AND PROFILES OF FREE AMINO ACIDS, FATTY ACIDS, MINERALS, AND AROMA COMPOUNDS IN *CITRUS NATSUDAIDAI* PEEL

2.1 INTRODUCTION

Many studies have demonstrated that citrus peel contains some components related to the health-promoting effect, which are generally more remarkable than the pulp itself (Nair et al., 2017; Assefa et al., 2017; de Moraes Barros et al., 2012). Moreover, citrus peel is an important source of essential oils used as ingredients for cosmetics and perfumes. Its unique smell is due to several odor compounds including terpenes (Cuevas et al., 2017a; Cuevas et al., 2017b). Besides, compounds with health-related potential can be obtained from citrus peel by extraction, separation, and purification. The nutritional composition and other physical characteristics of citrus can vary depending on the species and cultivars. For instance, differences in size, the whole piece's weight, peel yield, moisture, and mineral profiles have been reported (de Moraes Barros et al., 2012). Also, there are some differences in smell among citrus species or varieties (Zhang et al., 2017) due to their diversity in the volatile compounds profile. Limonene is frequently the most predominant volatile compound of citrus. However, other significant compounds, such as linalool, β-myrcene, and valencene can also be present (Cuevas et al., 2017a; Cuevas et al., 2017b; Cheong et al., 2012). The investigation of nutrients and volatile compounds will provide valuable information for the utilization of citrus peel.

Some citrus species, such as *C. sinensis*, *C. unshiu*, *C. reticulate*, and *C. limon* have

been mainly selected for citrus peel research, and their health-promoting effects were already certificated (Nair et al., 2017; Assefa et al., 2017; de Moraes Barros et al., 2012). On the other hand, *C. natsudaidai* (CN) has been studied to a lesser extent. One of the reasons is that it has a very thick and hard peel compared to other citrus species. CN is a widely cultivated and popular citrus fruit in Japan. However, some wild CN species are not usually harvested. Fundamental information is necessary to consider other research options, such as extraction, separation, and purification of health-related compounds. Therefore, the present work focused on the proximate composition and profile of mineral, free amino acid, fatty acid, and aroma compounds in CN peel.

The solvent extraction method is frequently used to obtain free amino acids, fatty acids, and aroma compounds in plant-food. Methanol and dichloromethane are commonly applied for extraction; however, these are toxic and harsh organic solvents. On the other hand, ethanol and hexane are allowed solvents for food use in Japanese regulation. The drying procedure is also frequently used before extraction; however, it requires energy and cost. Moreover, volatile compounds can be removed after the drying procedure. Thus, citrus peels were extracted with ethanol and hexane without any drying procedure for this study.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

Chloroform, ethanol, hexane, nitric acid, mineral standard solutions (calcium, magnesium, iron, zinc, copper, and manganese), sodium sulfate, sodium sulfite, hydroquinone, sulfuric acid, bromocresol green, and methyl red were purchased from KANTO CHEMICAL CO., INC. (Tokyo, Japan). Milli-Q water was prepared by Milli-Q® Integral A10® System (Molsheim, France). Methanolic HCl 3 N and ammonium molybdate were obtained

from Sigma Aldrich (St. Louis, MO, USA). Amino acids mixture standard solution type H, decanoic acid, sodium chloride, sodium hydroxide, potassium chloride, potassium dihydrogen phosphate, potassium sulfate, copper sulfate, and titanium oxide were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan).

2.2.2 Samples

Three different cultivars of CN were obtained from Chiba Prefecture, Japan, namely CN1, CN2, and CN3 in this study. CN1 cultivar is newly developed by a farmer; CN2 and CN3 are commercial CN fruits from the same farmer. All of the fruits were cultivated for three years without pesticides and annually harvested from May to July. Upon arrival at Toyo University Food Science Laboratory, the fruits were immediately washed and manually peeled. Approximately 500 g of peels per cultivar were crushed using a hand grinder (Click & Mix Plus T-fal, Tokyo, Japan) and stored at -30°C before any other treatment.

2.2.3 Hydrophilic extract

The detail of the hydrophilic extraction method is explained in Chapter 3, section 3.2.3.1. Ground-frozen citrus peel (5 g) was extracted with 80% aqueous ethanol (v/v) with a 1:10 (w/v) at the sample-solvent ratio of 1:10 (w/v) under a magnetic twist for 24 h at room temperature (25°C). The extract was centrifuged at 8000 rpm (7090 × g) for 5 min at 4°C (Suprema 21, TOMY SEIKO CO., LTD., Tokyo, Japan), and the supernatant was collected in a round-bottomed flask through filtration with Advantec® 5B 150 mm filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). Residues were re-extracted twice by hand-shaking (1 min) following the same procedure. Further, the filtrated supernatant was evaporated in a rotary evaporator (Eyela N-1100, Rotary evaporator N-1110, TOKYO RIKAKIKAI CO., LTD., Tokyo,

Japan) at 37°C until the volume became less than 5 ml. The remaining extract was then quantitatively transferred in a separatory funnel by adding Milli-Q water, ethanol, and hexane (3 times, 10 ml of each solvent) before being submitted to the liquid-liquid partition. After clear separation, the hydrophilic phase was transferred to a second separatory funnel and was defatted with 20 ml of hexane another two times. The hydrophilic phase was concentrated in a second round-bottomed flask, followed by evaporation at 37°C until the volume became less than 5 ml. The remaining extract was dissolved with Milli-Q water and transferred to a 25 ml volumetric flask. The extract was filtered on with a hydrophilic nylon 0.22 µm syringe filter (Starlab Scientific Co., Ltd, Shaanxi, China) and stored at -30°C until further analysis.

2.2.4 Hydrophobic extract

The detail of the hydrophilic extraction method is explained in Chapter 3, section 3.2.3.2. Ground-frozen citrus peel (5 g) was extracted with the ethanol-hexane solvent mixture (2:1, v/v) at the sample-solvent ratio of 1:10 (w/v) under a magnetic twist for 24 h at room temperature (25°C). Further, the sample was centrifuged at 8000 rpm (7090 × g) for 5 min at 4°C, and the supernatant was transferred to a separatory funnel after filtration. Residues were re-extracted with 50 ml of the ethanol-hexane mixture by hand-shaking (1 min) twice following the same procedure. All the re-extraction solvents were pooled with the extracted supernatant in the separatory funnel, and 30 ml of Milli-Q water was added. After clear separation, the hydrophilic phase was discarded, and the extract was partitioned again with 30 ml of Milli-Q water another two times following the same procedure. The hydrophobic phase was collected in a conical flask in which sodium sulfate was added to withdraw any remaining water content. The extract was then filtrated to a round-bottomed flask and evaporated to dryness using a rotary evaporator. It was solubilized with 10 ml of hexane and transferred in a glass tube. The extract was then evaporated at 37°C to dryness using a tube

evaporator (Eyela TVE-1100, Rotary evaporator N-1110, TOKYO RIKAKIKAI CO., LTD., Tokyo, Japan) and stored at -30°C until further analysis.

2.2.5 Color analysis

The CN peel color was measured with a color meter (Color Meter ZE6000, NIPPON DENSHOKU INDUSTRIES CO., LTD., Tokyo, Japan). Color parameter were expressed as L* (L*= brightness, 100 means white; 0 means black), a* (+a* means red; a* means -a* means green), b* (+b* means yellow; -b* means blue). The difference between the two colors is given by the ΔE*Lab value: ΔE*Lab = (ΔL*) 2 + (Δa*) 2 + (Δb*) 2. According to Mokrzycki & Tatol (2012), the results are interpreted as follows. 0 < ∆E < 1: Observer does not notice the difference, 1 < ∆E < 2: Only experienced observer can notice the difference, 2 < ∆E < 3.5: Unexperienced observer also notices the difference, 3.5 < ∆E < 5: Clear difference in color is noticed, 5 < ∆E: Observer notices two different colors.

2.2.6 Moisture

The moisture content of CN peel was measured using a moisture analyzer (Moisture Analyzer MX-50, A&D Company, Limited, Tokyo, Japan). Approximately 0.5 g of Ground-frozen CN peel were subjected to the moisture analyzer, and moisture content was expressed as g per 100 g of fresh matter basis.

2.2.7 Protein

Protein was analyzed by the Kjeldahl method (AOAC, 2002). Each Ground-frozen

CN peel was weighed 0.5 g on a paraffin paper (Hakuaisha Co., Ltd., Tokyo, Japan), which was then inserted into a Kjeldahl flask. Potassium sulfate, copper sulfate, and titanium oxide mixture (100:6:4) were added to promote decomposition, and concentrated sulfuric acid was put into each flask and left to stand overnight. The flasks were carefully heated in a Micro Kjeldahl digester (Semimicro Kjeldahl Digesting Apparatus, Model SE-6, 6-unit, electric heating, SIBATA SCIENTIFIC TECHNOLOGY LTD., Saitama, Japan). Sulfuric acid was added when necessary during the sample decomposition. This procedure occurred until obtaining a colorless solution. After cooling, the remaining solution was collected into a 100 ml volumetric flask and fill up with MilliQ-water. Next, part of this diluted solution was dropped into the Micro Kjeldahl Distillation Apparatus (Parnas-Wagner type, SIBATA SCIENTIFIC TECHNOLOGY LTD., Saitama, Japan) followed by 10 ml of 30% sodium hydroxide. The distillate produced was collected into a receiving Erlenmeyer flask containing 10 ml of 0.01 N sulfuric acid solution. Drops of bromocresol green-methyl red composite indicator were added, and then this mixture was titrated with 0.01 N sodium hydroxide, and the volume required to reach the endpoint (change of color) was recorded. Nitrogen-protein conversion factor (6.25) was used for the calculation to obtain the content of crude protein. The results were expressed as g per 100 g of fresh matter basis.

2.2.8 Ash

The determination of ash content was performed according to AOAC (2005). Weighed Ground-frozen CN peel (approximately 1 g) were incinerated in muffle (KDF-S90, DENKEN-HIGHDENTAL Co., Ltd., Kyoto, Japan) for 5 h at 550°C. The difference in weight of the crucible before and after incineration was used to determine the ash content. Ash contents were expressed as g per 100 g of fresh matter basis.

2.2.9 Lipid

Lipids were obtained based on the method established by Bligh & Dyer (1959) with some modifications. Each Ground-frozen CN peel was weighed 5 g into the 50 ml centrifuge tube (Nalgene® Teflon® Oak Ridge Centrifuge Tube, FEP with ETFE Screw Cap, Thermo Scientific, NY, USA), and a solution of chloroform and methanol (1:2, v/v) was added on a 1:6 ratio (w/v). The mixture was hand shaken, and then it was centrifuged at 8000 rpm (7090 × g) for 3min at 4°C (High speed refrigerated centrifuge Suprema 21, TOMY DIGITAL BIOLOGY CO., LTD., Tokyo, Japan). The supernatant was filtrated with filter paper (Quantitative ashless 5B, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The procedure was repeated another two times. The combined extracts were collected into a separation funnel, and 0.9% potassium chloride (w/v) was added. This solution was mixed and kept overnight protected from light. After clear separation, the chloroform layer was collected, dehydrated with sodium sulfate, and evaporated using a rotary evaporator (Rotary evaporator N-1110, TOKYO RIKAKIKAI CO., LTD., Tokyo, Japan) below 37°C. The remaining extract was then transferred in a 50 ml pear flask, which was previously weighted, by the addition of chloroform and evaporated at 37°C using a rotary evaporator until dry. The flask was heated at 110 °C for 2 h, and once it cooled down, the weight was measured. The difference in weight of the flask was used to determine the lipid content of citrus peels. The results were expressed as g per 100 g of fresh matter basis.

2.2.10 Carbohydrate

The value of carbohydrate was determined by calculation from moisture, protein, ash, and lipid content. The results were expressed as g per 100 g of fresh matter basis. Carbohydrate content = 100 - (Moisture + crude protein + ash + lipid)

2.2.11 Minerals

The mineral content was analyzed using wet ashed samples prepared by the following method: from 1 to 3 g of Ground-frozen CN peel were accurately weighed in a 50 ml Teflon tube (Teflon Digestion Tube, SCP science, Quebec, Canada), and nitric acid was added. Samples were heated by Graphite digestion block (DigiPREP mini, SCP science, Quebec, Canada) at 90°C until obtaining a clear color solution. Nitric acid was added when necessary during the decomposition of samples. Phosphorous (P) contents of citrus peels were measured by Molybdenum blue method based on Briggs (1924). Briefly, 1 ml of wet ashed sample solution was placed in a 25 ml volumetric flask. Next, 2 ml of an ammonium molybdate solution consisted of 25 g ammonium molybdate was dissolved in 300 ml MilliQ-water and then mixed with 200 ml of sulfuric acid aqueous solution (75 ml of concentrated sulfuric acid diluted with 125 ml of MilliQ-water). After 3 min, 2 ml of 0.5% hydroquinone solution and 2 ml of 10% sodium sulfite solution were added, and the volumetric flask was filled up with MiiliQ-water. The mixture was shaken and left to stand for 30 min at room temperature (25°C). Absorbance was recorded at 823 nm by a Spectrophotometer (UV-VIS Spectrophotometer UV-1800, SHIMADZU CORPORATION, Kyoto, Japan). Blank was prepared using MilliQ-water replacing the sample solution.

The other minerals were analyzed using an atomic absorption system (atomic absorption spectrophotometer AA-7000 series, SHIMADZU CORPORATION, Kyoto, Japan). Sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) contents were measured using an AA-7000 flame atomic absorption with an air-acetylene flame. Iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) were determined by graphite furnace atomic absorption, which was consisted of an AA-7000 spectrophotometer, GFA-7000 graphite furnace atomizer, and ASC-7000 autosampler. As a graphite tube, a pyro-coated graphite tube (SHIMADZU

CORPORATION, Kyoto, Japan) was used for analysis.

Standard calibration curves were built for all minerals. Na, K, and P standard solutions were prepared on the same day of analysis due to their instability using sodium chloride, potassium chloride, and potassium dihydrogen phosphate, respectively; the other standards were commercial solutions.

2.2.12 Free amino acids

The free amino acid profile was obtained using an amino acid analyzer (High-speed Amino Acid Analyzer L-8900, Hitachi High-Technologies Corporation, Tokyo, Japan) attached with Hitachi custom ion-exchange column (Hitachi High-Technologies Corporation, Tokyo, Japan). The equipment was applied to “the hydrolysates analysis mode.” The hydrophilic extracts (section 2.2.3) were filtrated with a 0.45 µm cellulose acetate syringe filter (Disposable membrane filter unit DISMIC-13CP, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and then 20 µl was injected into the amino acid analyzer. Results were expressed as mg per 100 g of fresh matter basis, referring to an amino acid mixture standard.

2.2.13 Fatty acid composition

The hydrophobic extracts (section 2.2.4) were transferred to a glass tube containing decanoic acid as an internal standard, which was previously measured. Samples were transesterified using the methanolic hydrochloric acid protocol (Laurens et al., 2012) to quantify the fatty acids methyl esters (FAMEs). The mixture was evaporated until drying by using a tube evaporator (Eyela TVE-1100, Rotary evaporator N-1110, TOKYO RIKAKIKAI CO., LTD., Tokyo, Japan) at 37°C. Next, methanolic HCl was added, and the tube was heated

in a dry brock bath (Eyela MG-2300, Rotary evaporator N-1110, TOKYO RIKAKIKAI CO., LTD., Tokyo, Japan) for 2 h at 110°C to transesterification of the fatty acids. The FAMEs produced were recovered with hexane, dehydrated with sodium sulfate, and transferred to a vial for identification and quantification by a gas chromatography-mass spectrometry (GC-MS) system (Thermo Fisher Scientific, MA, USA). The GC-MS consisted of a Trace GC ultra, DSQⅡMS, AS3000 autosampler, capillary column (InertCap Pure-WAX, 0.25 μm, 0.25 mm × 30 m, GL Sciences Inc., Tokyo, Japan) and a split injector. The injector, the MS transfer line, and the ion source temperature were set at 250°C. The oven temperature was programmed from 50°C to 250°C at 4°C /min with an initial holding time of 1 min and a final holding time of 5 min. Helium gas was the carrier gas with a 1.0 ml/min flow rate. The injection mode in MS was the electron impact (EI) mode as the ionization energy of 70 eV. 2 μl of the FAMEs was directly injected by an autosampler. Results were calculated based on the relative MS peak area of each fatty acid detected, referred to as the internal standard, and they were expressed as mg per 100 g of fresh matter basis.

2.2.14 Aroma compounds

Headspace Solid Phase Microextraction (HS-SPME) analysis of CN peel was carried out based on the method described by Cuevas et al. (2017b) with some modifications. Small pieces of ground-fresh CN peel (approximately 2 mm × 2 mm), 1 ml of hydrophilic extracts (section 2.3.3), and a portion of hydrophobic extracts (section 2.3.4) were each placed in a 15 ml SPME vial (Sigma Aldrich, St. Louis, MO, USA). Hydrophobic extracts were wiped with a small size (1 cm × 2 cm) of paper (KimWipes, NIPPON PAPER CRECIA Co., Ltd., Tokyo, Japan) and then placed in the vial. For Ground-frozen citrus peels and hydrophobic extracts, 1 ml of Milli-Q water was added to keep the air inside under an amount of saturated vapor. The vials were left to stand for 30 min, and then the volatiles were sampled by HS-SPME with

a 50/30 mm CAR/PDMS/DVB fiber (StableFlex, Sigma Aldrich, St. Louis, MO, USA). SPME fiber was conditioned 1 h at 250°C before analysis, and the extraction occurred for 30 min at room temperature (25°C). Desorption was carried out at 250°C for 30 min with manual injection. The GC-MS system (Thermo Fisher Scientific, MA, USA) consisted of a Focus GC ultra, DSQⅡMS, and capillary column (VOCOL fused silica capillary column, 1.5 μm, 0.25 mm × 60 m, GL Sciences Inc., Tokyo, Japan). The injection part was assembled with GC signet septa (EC septa 17mm, SGE Analytical Science, Melbourne, Australia) and an SPME liner glass insert (TQ straight through 0.8 mm ID). The injection mode was splitless for 1 min, snd the ion source and MS transfer line temperatures were 200°C and 230°C, respectively. Electronic ionization mode was electron impact (EI) using ionization energy of 70 eV, and helium gas was the carrier gas with a 1.0 ml/min flow rate. The oven temperature conditions were 45°C for 1 min, at 6°C/min, going to 90°C at 3°C/min, increasing to 210°C at 6°C/min, then reaching 250°C held for 16 min. Results were analyzed using Xcalibur software (v. 2.2; Thermo Fisher Scientific, MA, USA). Detected compounds were identified using mass spectral matching against NIST Standard Reference Database Number 69 (NIST National Institution of Standards and Technology, U.S. Department of Commerce) and Restek Searchable Chromatogram Library (RESTEK Corporation, U.S.).

2.2.15 Statistical analysis

All experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD). The data were analyzed using the Statistical Package for Social Sciences (IBM® SPSS® Statistics for Windows version 22). Statistical significance was declared at $p < 0.05$.

2.3 RESULTS AND DISCUSSION

2.3.1 Color and proximate composition

The color, moisture, protein, ash, lipid, and carbohydrate content of CN peel are shown in **Table 2.1**. The whole weight average of a piece of CN1 was 337 g with a peel yield of 34.2%; CN2 weighted 350 g obtaining 28.8% of peel yield; a whole piece of CN3 weighted 394 g with peel yield of 33.4%. Color is an important parameter of quality in food processing. L* value of CN2 and CN3 peels were significantly higher than CN1. On the other hand, CN1 peel exhibited the highest a* and b* values, followed by CN2 and CN3, which means that CN1 has a more red and yellow color when compared to the other two cultivars. ΔE*Lab value between CN1 and the other two cultivars was higher than 5 (CN1 - CN2: 23.5 and CN1 - CN3: 26.6, respectively), meaning that “observer notices two different colors.” Between CN2 and CN3, ΔE*Lab value was 4.09, which indicates that a “clear difference in color is noticed” (Mokrzycki et al., 2012).

Table 2.1 Color and proximate composition of peels from three *C. natsudaidai* (CN) cultivars (mean ± SD, n=3).

	CN cultivar		
	CN1	CN2	CN3
Color			
L*	59.5 ± 0.66^{b}	72.0 ± 0.28^{a}	71.5 ± 0.47^{a}
a*	9.97 ± 0.38^{a}	1.13 ± 0.09^{b}	0.50 ± 0.09^{c}
b*	77.5 ± 0.42^{a}	59.6 ± 0.91^{b}	55.6 ± 1.50^{c}
Proximate composition			
Moisture (g/100 g FM)	80.6 ± 0.85	80.8 ± 0.63	80.2 ± 0.88
Protein (g/100 g FM)	0.70 ± 0.18^{b}	1.07 ± 0.11^{a}	0.37 ± 0.02^{c}
Ash (g/100 g FM)	0.61 ± 0.06^{a}	0.43 ± 0.04^{b}	0.28 ± 0.03^{c}
Lipid (g/100 g FM)	0.47 ± 0.01^{a}	0.39 ± 0.03^{b}	0.33 ± 0.02^{c}
Carbohydrate (g/100 g FM)	17.6 ± 0.77	17.3 ± 0.71	18.8 ± 0.88

g/100 g FM: g per 100 g of fresh matter basis. L* = brightness, range of whiteness (100) to darkness (0); a* = represent red (+a) to green (-a); b* = represent yellow (+b) to blue (-b). Means with different superscripts in the same row are significantly different ($p<0.05$).

The moisture contents were approximately 80% in all cultivars. The high moisture content in citrus peels makes them perishable; thus, in the case of immediate use, removing moisture or sterilization is necessary to avoid spoiling. The highest protein content was 1.07 ± 0.11 g/100 g FM in CN2, followed by CN1 and CN3. Ash and lipid contents ranged from 0.28 ± 0.03 to 0.61 ± 0.06 and 0.33 ± 0.02 to 0.47 ± 0.01 g/100 g FM, respectively. CN1 showed the highest ash and lipid contents, followed by CN2 and CN3. There was no significant difference in the carbohydrate contents (17.3 ± 0.71 to 18.8 ± 0.88 g/100 g FM). M'hiri et al. (2015a) reported the proximate composition of Citrus sinensis peel (moisture: 76 g; proteins: 1.9 g; ash: 0.76 g; Fat (lipid): 0.19 g/100 g FM), showing similar results from the ones obtained in this study. Some variations could be due to the difference in species. According to the Ministry of Education, Culture, Sports, Science & Technology (MEXT) (2015), the pulp of CN contains 88.6 g of moisture, 0.9 g of protein, 0.4 g of ash, 0.1 g of lipid, and 10.0 g of carbohydrate in 100 g of fruit on fresh matter basis. When comparing CN peel and pulp, peel revealed around 2 times higher content of carbohydrate. It can be explained by the existence of pectin in peel parts (Liu et al., 2006; Wang et al., 2008).

2.3.2 Minerals

As shown in **Table 2.2**, nine minerals were quantified in citrus peels, including five macro minerals (Na, K, Ca, Mg, and P) and four trace minerals (Fe, Zn, Cu, and Mn). K was the principal mineral present in citrus peels, followed by Ca and Mg. CN1 showed significantly higher K and Ca content (377 ± 8.34 and 97.9 ± 4.14 mg/100 g FM), followed by CN2 and CN3. The highest Mg content was observed in CN2 (32.6 ± 0.24 mg/100 g FM), followed by CN3 and CN1.

Table 2.2 Mineral composition of peels from three *C. natsudaidai* (CN) cultivars (mean ± SD, n=3).

	CN cultivar		
	CN1	CN2	CN3
Na (mg/100 g FM)	14.0 ± 1.59^{a}	10.6 ± 0.86^{b}	10.5 ± 0.97^{b}
K (mg/100 g FM)	377 ± 8.34^{a}	268 ± 18.6^{b}	246 ± 7.02^{b}
Ca (mg/100 g FM)	97.9 ± 4.14^{a}	87.9 ± 2.43^{b}	58.9 ± 1.68^{c}
Mg (mg/100 g FM)	24.2 ± 0.30^{b}	32.6 ± 0.24^{a}	24.6 ± 0.83^{b}
P (mg/100 g FM)	10.9 ± 0.39^{ab}	12.4 ± 0.57^{a}	10.0 ± 1.11^{b}
Fe (µg/100 g FM)	254 ± 8.08^{b}	237 ± 7.98^{b}	283 ± 8.97^{a}
Zn (µg/100 g FM)	176 ± 32.3^{a}	119 ± 10.0^{b}	140 ± 6.85^{ab}
Cu (µg/100 g FM)	33.7 ± 1.73^{b}	31.0 ± 1.74^{b}	40.2 ± 2.26^{a}
Mn (µg/100 g FM)	59.1 ± 0.97^{c}	148 ± 2.85^{b}	182 ± 17.5^{a}
Total (mg/100 g FM)	0.52 ± 0.01^{a}	0.41 ± 0.02^{b}	0.35 ± 0.01^{c}

mg/100 g FM: mg per 100 g of fresh matter basis; µg/100 g FM: µg per 100 g of fresh matter basis. Means with different superscripts in the same row are significantly different (p<0.05).

de Moraes Barros et al. (2012) determined eight minerals in citrus peels from five different cultivars. They reported that citrus peel contains a higher amount of minerals than pulp, being K the most predominant mineral in citrus peels, followed by Ca and Mg, a similar trend in this study. Also, CN peel has higher K, Ca, and Mg contents than the pulp compared to the food database (MEXT, 2015). According to the Ministry of Health, Labour and Welfare (MHLW) (2015), mineral contents of CN peel is much smaller than recommended daily allowance (RDA) for adult (18 to 29 years old male and female). Thus, this result showed that CN peel could not be used as a mineral source in the diet. Nevertheless, small mineral content is suitable for extraction because salting-out will not occur.

2.3.3 Free amino acids

The free amino acid profile of CN peel is presented in **Table 2.3**. The amount of most individual free amino acids was significantly different among the three cultivars. Considering the total free amino acid content, CN3 showed significantly the highest amount (121 ± 4.96 mg/100 g FM), followed by CN1 and CN2.

Table 2.3 Free amino acid profile of peels from three *C. natsudaidai* (CN) cultivars (mean ± SD, n=3, mg/100 g FM).

	CN cultivar		
Amino acid	CN1	CN2	CN3
Asp	11.7 ± 1.57^{b}	8.61 ± 0.58^{c}	21.6 ± 0.64^{a}
Thr	11.9 ± 1.48^{b}	7.28 ± 0.43^{c}	25.3 ± 0.92^{a}
Ser	3.11 ± 0.40^{c}	4.48 ± 0.33^{b}	7.68 ± 0.38^{a}
Glu	0.25 ± 0.01^{a}	0.21 ± 0.08^{ab}	0.13 ± 0.004^{b}
Gly	0.27 ± 0.03^{c}	0.36 ± 0.01^{b}	0.82 ± 0.03^{a}
Ala	6.97 ± 0.80^{c}	9.67 ± 0.57^{b}	12.9 ± 0.57^{a}
Cys	0.69 ± 0.02	0.76 ± 0.09	0.79 ± 0.01
Val	0.35 ± 0.03^{b}	0.63 ± 0.02^{a}	0.69 ± 0.03^{a}
Met	0.26 ± 0.01^{a}	0.16 ± 0.01^{b}	0.04 ± 0.01^{c}
Ile	0.10 ± 0.02^{c}	0.14 ± 0.01^{b}	0.19 ± 0.01^{a}
Leu	0.54 ± 0.06^{c}	0.74 ± 0.02^{b}	1.09 ± 0.05^{a}
Tyr	1.06 ± 0.07^{b}	1.06 ± 0.03^{b}	1.34 ± 0.04^{a}
Phe	26.0 ± 3.34^{a}	19.3 ± 0.95^{b}	28.6 ± 1.53^{a}
Lys	3.06 ± 0.34^{ab}	2.59 ± 0.10^{b}	3.32 ± 0.19^{a}
His	0.30 ± 0.01^{c}	0.37 ± 0.02^{b}	0.54 ± 0.01^{a}
Arg	0.91 ± 0.12^{b}	0.22 ± 0.004^{c}	1.52 ± 0.02^{a}
Pro	12.9 ± 1.85^{a}	5.54 ± 0.24^{b}	14.1 ± 0.59^{a}
Total	80.4 ± 10.1^{b}	62.1 ± 3.44^{c}	121 ± 4.69^{a}

mg/100 g FM: mg per 100 g of fresh matter basis. Means with different superscripts in the same row are significantly different ($p<0.05$).

According to MEXT (2015) data, CN pulp usually contains more amino acids than the

peel. For instance, the content of Asp in peel was 10 to 20 times lower than those of pulp (from the MEXT database) among three cultivars. Total amino acid contents were much smaller likewise. In this study, eight essential amino acids (Thr, Val, Met, Ile, Leu, Phe, Lys, and His) were detected, and the total amount of essential amino acids ranged from 31.2 to 59.8 mg/100 g FM). These amounts were deficient compared to the amino acid requirements for adults and infants (WHO, FAO, and United Nations University (UNU), 2007). Results showed that CN peel is not suitable as a source of amino acids.

Besides, amino acids are responsible for the taste quality of foods (Schiffman & Sennewald, 1981). The quantity of detected free amino acids from CN peel extracts was much smaller than their taste threshold. Phe was the most predominant free amino acid among the three cultivars (from 19.3 ± 0.95 to 28.6 ± 1.53 mg/100 g FM). According to Schiffman et al. (1981), the threshold value of Phe is 6.61 mM = 1.09 g/l (adjusted the unit by calculation). It means that CN peel needs to be extracted at a ratio of more than 4 kg/l (about 20 times) to feel the taste. Therefore, considering the utilization of CN peel extracts as food additives, it is logical that amino acids would not affect the taste of the extracts. Besides, citrus peel hydrophilic extracts frequently contain natural antioxidant components (de Moraes Barros et al., 2012; Ramful et al, 2010). Thus, it could be used as some food additives which have natural antioxidant property.

2.3.4 Fatty acid composition

The fatty acid composition of CN is summarized in **Table 2.4**, expressed as mg/100 g. The fatty acids detected in this analysis were: lauric acid (C12:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1, cis-9), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1, cis-9), linoleic acid (C18:2, cis-9, 12), α-linolenic acid (C18:3, cis-9, 12, 15), arachidic acid (C20:0), behenic acid (C22:0),

lignoceric acid (C24:0). The major fatty acid present in all three cultivars was linoleic acid (from 42.8 to 48.5%), followed by palmitic acid (from 20.2 to 20.8%), α-linolenic acid (from 12.1 to 14.6%), and oleic acid (from 8.60 to 10.1%). These results indicate that most of the fatty acids present in all three cultivars are unsaturated, representing approximately 70% of the total fatty acid content. Among the cultivars, CN1 contained the highest total fatty acid amount (111 ± 11.4 mg/100 g FM) among the other cultivars. The fatty acid amounts were low; thus, CN peel is not suitable as a fatty acid source.

Table 2.4 Fatty acid profile of total lipid of peels from three *C. natsudaidai* (CN) cultivars (mean ± SD, n=3, mg/100 g FM).

	CN cultivar		
Fatty acid	CN1	CN2	CN3
C12:0	2.03 ± 0.16^{a}	1.03 ± 0.14^{b}	0.73 ± 0.07^{b}
C14:0	2.58 ± 0.31^{a}	1.31 ± 0.10^{b}	1.05 ± 0.09^{b}
C15:0	0.24 ± 0.02^{a}	0.18 ± 0.01^{b}	0.15 ± 0.01^{c}
C16:0	22.9 ± 3.07^{a}	9.99 ± 0.68^{b}	9.05 ± 0.64^{b}
C16:1, cis-9	0.65 ± 0.05^{a}	0.42 ± 0.04^{b}	0.18 ± 0.01^{c}
C17:0	0.80 ± 0.11^{a}	0.44 ± 0.04^{b}	0.44 ± 0.04^{b}
C18:0	1.68 ± 0.17^{a}	1.17 ± 0.13^{b}	1.09 ± 0.08^{b}
C18:1, cis-9	9.53 ± 1.31^{a}	4.84 ± 0.13^{b}	4.35 ± 0.39^{b}
C18:2, cis-9, 12	53.6 ± 4.91^{a}	20.6 ± 1.01^{b}	19.2 ± 1.16^{b}
C18:3, cis-9, 12, 15	13.3 ± 1.18^{a}	6.52 ± 0.31^{b}	6.55 ± 0.46^{b}
C20:0	0.41 ± 0.04^{a}	0.16 ± 0.01^{b}	0.21 ± 0.00^{b}
C22:0	1.11 ± 0.11^{a}	0.51 ± 0.02^{c}	0.69 ± 0.04^{b}
C24:0	1.66 ± 0.12^{a}	0.94 ± 0.06^{b}	1.16 ± 0.11^{b}
SFA	33.4 ± 3.96^{a}	15.7 ± 0.67^{b}	14.6 ± 1.07^{b}
MFA	10.2 ± 1.34^{a}	5.26 ± 0.13^{b}	4.54 ± 0.40^{c}
PUFA	66.9 ± 6.09^{a}	27.1 ± 1.32^{b}	25.8 ± 1.63^{b}
Total	111 ± 11.4^{a}	48.1 ± 1.86^{b}	44.9 ± 3.08^{b}

mg/100 g FM: mg per 100 g of fresh matter basis. Means with different superscripts in the same row are significantly different ($p<0.05$).

2.3.5 Aroma compounds

The proportion of volatile compounds from fresh CN peel and hydrophobic extracts were summarized in **Table 2.6**. The hydrophilic extracts did not show remarkable peaks (data not shown). A total of 28 volatile compounds were detected using HS-SMPE-GC-MS analysis, and 23 of them were identified. Among 23 identified volatile compounds: hexanal, thujene, α-pinene, δ-3-carene, myrcene, β-pinene, octanal, α-terpinene, ocimene, limonene, p-cymene, γ-terpinene, 2-nonen-1-ol, terpinolene, nonanal, citronellal, decanal, neryl acetate, geranyl acetate, α-copaene, α-selinene, valencene were reported in previous researches of volatile compounds in citrus (Cuevas et al., 2017a; Cuevas et al., 2017b; Högnadóttir & Rouseff, 2003). Concerning fresh peel samples, terpenes accounted for over 98% of the total area% of peaks. In all fresh samples, limonene was the most major terpenes, and it ranged from 79.8 ± 2.00 to 85.4 ± 1.39 % of the total area. γ-terpinene was the second major terpenes, and the proportion of it ranged from 6.67 ± 0.60 to 9.68 ± 0.37 %. For limonene and γ-terpinene, there was no significant difference among the three cultivars. p-cymene and myrcene were also major terpenes in fresh peel samples, ranged from 1.45 ± 0.08 to 3.86 ± 0.70 and from 1.55 ± 0.34 to 2.18 ± 0.47 %, respectively. The majority of volatile compounds detected by this study have aroma activity, like fruity, citrusy, lemon, or green (Cuevas et al., 2017a; Cuevas et al., 2017b; Kesen et al. 2013; Högnadóttir & Rouseff, 2003), as shown in **Table 2.5**.

Hexanal, thujene, α-pinene, and δ-3-carene were not detected in the hydrophobic extracts. Similar to what happened with fresh peel samples, terpenes compounds corresponded to most volatile compounds in the hydrophobic extracts, accounting for over 78% of the total area%. Limonene was the most predominant compound, ranging from 46.5 ± 7.08 to 70.2 ± 6.12 %, in which CN1 and CN3 showed significantly higher amounts than CN2. p-cymene, γ-terpinene, α-selinene, and valencene were also major terpenes in hydrophobic extracts. Two ester compounds, neryl acetate and geranyl acetate were also showed peaks

appreciably in hydrophobic extracts (from 1.50 ± 0.14 to 4.76 ± 1.48 and from 1.70 ± 0.61 to 5.01 ± 1.85 %, respectively). CN2 presented a significantly higher ratio of these esters compare to CN1 and CN3.

Hydrophobic extracts showed a higher percentage of neryl acetate, geranyl acetate, α-copaene, α-selinene, and valencene when compared to fresh peels. In contrast, the percentage of myrcene, octanal, α-terpinene, limonene, and terpinolene were lower than those of fresh peels. Extraction with hexane may have caused profile changes of these terpenes groups; however, hydrophobic extracts still had citrusy aroma characteristics. Thus, CN peels hydrophobic extracts may have the ability to be used as a flavoring of other food products such as citrus flavor beverages.

Table 2.5 Retention index (RI), retention time (RT), ion model, and descriptor of volatile compounds of peels from three *C. natsudaidai* (CN) cultivars determined by HS-SPME-GC-MS analysis.

No.	Compounds	RT (min)	Ion model (m/z) main peak/others	Descriptors	References
1	Hexanal	21.66	44/41/56	Green, cut grass	Cuevas et al., 2017a; Cuevas et al., 2017b; Kesen et al., 2013; Högnadóttir & Rouseff, 2003
2	Thujene	27.51	93/91/77		
3	α-pinene	28.38	93/91/92	Fruity, piney, green	Cuevas et al., 2017a; Cuevas et al., 2017b; Högnadóttir & Rouseff, 2003
4	unknown	29.86	41/69/93		
5	unknown	30.22	41/69/95		
6	δ-3-carene	30.33	93/43/91		
7	Myrcene	30.92	41/93/69	Musty, geranium, green, metallic	Cuevas et al., 2017a; Cuevas et al., 2017b; Högnadóttir & Rouseff, 2003
8	β-pinene	31.64	93/41/91	Musty, green, piney, fruity	Cuevas et al., 2017a
9	Octanal	32.82	41/43/44	Citrus-like, lemon, green, fruity, herbal	Cuevas et al., 2017a; Cuevas et al., 2017b; Kesen et al., 2013; Högnadóttir & Rouseff, 2003
10	α-terpinene	33.38	121/93/91		
11	Ocimene	33.77	93/91/41		
12	Limonene	34.22	68/93/67	Citrusy, lemon, licorice, fruity	Cuevas et al., 2017a; Cuevas et al., 2017b; Högnadóttir et al., 2003
13	p-cymene	34.52	119/91/134	Lemon, fruity	Högnadóttir & Rouseff, 2003
14	γ-terpinene	35.89	93/91/136	Citrusy, terpeny, minty, piney, fruity	Cuevas et al., 2017a; Cuevas et al., 2017b; Högnadóttir & Rouseff, 2003
15	2-nonen-1-ol	36.78	57/41/95	Fruity, waxy	Kesen et al., 2013
16	Terpinolene	37.76	93/121/91	Fruity, green	Cuevas et al., 2017a
17	unknown	37.95	121/93/91		
18	Nonanal	38.24	41/57/43	Sweet, melon, grassy	Kesen et al., 2013; Högnadóttir & Rouseff, 2003
19	p-cymenene	38.66	117/132/115		
20	Citronellal	41.19	41/69/95	Lemon, citronella	Högnadóttir & Rouseff, 2003
21	unknown	42.24	59/43/73		
22	unknown	42.92	43/73/56		
23	Decanal	43.44	43/41/57	Lemon, fatty, soapy	Kesen et al., 2013; Högnadóttir & Rouseff, 2003
24	Neryl acetate	51.20	69/41/41	Green, citrus-like	Cuevas et al., 2017a; Cuevas et al., 2017b
25	Geranyl acetate	52.04	69/43/41	Green, citrus-like	Cuevas et al., 2017a; Cuevas et al., 2017b
26	α-copaene	52.24	119/161/105	Sweet, fruity	Kesen et al., 2013
27	α-selinene	57.58	189/91/93		
28	Valencene	58.13	161/119/91	Citrus-like, mint, orange blossom	Cuevas et al., 2017b; Kesen et al., 2013

Table 2.6 Relative content of volatile compounds of peels from three *C. natsudaidai* (CN) cultivars determined by HS-SPME-GC-MS analysis (mean ± SD, n=3, area%).

No	Compounds	Fresh peel			Hydrophobic extract		
		CN1	CN2	CN3	CN1	CN2	CN3
1	Hexanal	0.02 ± 0.01^{a}	0.00 ± 0.002^{a}	0.01 ± 0.01^{a}	N.D.	N.D.	N.D.
2	Thujene	0.11 ± 0.02^{a}	0.09 ± 0.01^{a}	0.09 ± 0.00^{a}	N.D.	N.D.	N.D.
3	α-pinene	0.38 ± 0.08^{a}	0.31 ± 0.04^{a}	0.28 ± 0.01^{a}	N.D.	N.D.	N.D.
4	unknown	0.01 ± 0.00^{ab}	0.01 ± 0.00^{a}	0.01 ± 0.00^{b}	N.D.	N.D.	N.D.
5	unknown	N.D.	0.00 ± 0.00^{b}	0.01 ± 0.00^{a}	N.D.	N.D.	N.D.
6	δ-3-carene	0.01 ± 0.00^{a}	0.01 ± 0.00^{b}	0.01 ± 0.00^{ab}	N.D.	N.D.	N.D.
7	Myrcene	1.60 ± 0.08^{a}	2.18 ± 0.47^{a}	1.55 ± 0.34^{a}	0.57 ± 0.02^{a}	0.07 ± 0.02^{b}	0.82 ± 0.24^{a}
8	β-pinene	0.25 ± 0.03^{a}	0.30 ± 0.03^{a}	0.25 ± 0.02^{a}	N.D.	0.19 ± 0.03^{a}	0.17 ± 0.06^{a}
9	Octanal	0.26 ± 0.08^{a}	0.32 ± 0.09^{a}	0.38 ± 0.10^{a}	0.10 ± 0.00^{a}	0.05 ± 0.01^{b}	0.03 ± 0.01^{b}
10	α-terpinene	0.39 ± 0.00^{b}	0.46 ± 0.02^{a}	0.42 ± 0.02^{ab}	0.19 ± 0.03^{b}	0.41 ± 0.03^{a}	0.33 ± 0.05^{a}
11	Ocimene	0.02 ± 0.00^{a}	0.02 ± 0.002^{a}	0.02 ± 0.00^{a}	N.D.	0.07 ± 0.02^{a}	0.03 ± 0.01^{a}
12	Limonene	85.4 ± 1.39^{a}	79.8 ± 2.00^{b}	83.8 ± 3.07^{ab}	69.5 ± 7.38^{a}	46.5 ± 7.08^{b}	70.2 ± 6.12^{a}
13	p-cymene	2.93 ± 0.44^{b}	3.86 ± 0.70^{a}	1.45 ± 0.08^{c}	4.39 ± 1.04^{b}	8.02 ± 1.04^{a}	6.20 ± 4.35^{ab}
14	γ-terpinene	6.67 ± 0.60^{b}	9.68 ± 0.37^{a}	9.20 ± 1.97^{ab}	1.28 ± 0.34^{b}	6.61 ± 2.46^{a}	6.52 ± 0.48^{a}
15	2-nonen-1-ol	N.D.	N.D.	0.02 ± 0.01	N.D.	N.D.	0.80 ± 0.16
16	Terpinolene	0.79 ± 0.06^{b}	1.00 ± 0.08^{a}	0.84 ± 0.13^{ab}	0.37 ± 0.05^{b}	0.98 ± 0.21^{a}	0.11 ± 0.03^{c}
17	unknown	0.09 ± 0.00^{a}	0.10 ± 0.02^{a}	0.08 ± 0.01^{a}	0.08 ± 0.01^{b}	0.13 ± 0.03^{a}	ND.
18	Nonanal	0.06 ± 0.03^{a}	0.10 ± 0.03^{a}	0.11 ± 0.05^{a}	0.15 ± 0.04^{b}	0.47 ± 0.18^{a}	0.17 ± 0.01^{b}
19	p-cymenene	0.19 ± 0.01^{ab}	0.22 ± 0.01^{a}	0.17 ± 0.01^{b}	0.23 ± 0.07^{b}	0.36 ± 0.01^{a}	0.18 ± 0.14^{ab}
20	Citronellal	0.05 ± 0.02^{a}	0.06 ± 0.01^{a}	0.05 ± 0.02^{a}	0.14 ± 0.02^{a}	N.D.	0.06 ± 0.03^{b}
21	unknown	0.04 ± 0.00^{a}	0.04 ± 0.01^{a}	0.04 ± 0.00^{a}	0.22 ± 0.21^{ab}	0.35 ± 0.13^{a}	0.10 ± 0.02^{b}
22	unknown	0.10 ± 0.03^{a}	0.14 ± 0.03^{a}	0.15 ± 0.04^{a}	0.72 ± 0.16^{b}	2.01 ± 0.54^{a}	0.84 ± 0.42^{b}
23	Decanal	0.19 ± 0.10^{a}	0.38 ± 0.10^{a}	0.43 ± 0.23^{a}	0.76 ± 0.14^{b}	3.75 ± 1.15^{a}	0.96 ± 0.46^{b}
24	Neryl acetate	0.08 ± 0.04^{a}	0.12 ± 0.03^{a}	0.09 ± 0.03^{a}	2.22 ± 0.50^{b}	4.76 ± 1.48^{a}	1.50 ± 0.14^{b}
25	Geranyl acetate	0.06 ± 0.02^{a}	0.10 ± 0.03^{a}	0.10 ± 0.06^{a}	1.70 ± 0.61^{b}	5.01 ± 1.85^{a}	2.04 ± 0.38^{ab}
26	α-copaene	0.05 ± 0.00^{b}	0.10 ± 0.01^{a}	0.04 ± 0.01^{b}	1.57 ± 0.68^{b}	4.25 ± 1.43^{a}	0.95 ± 0.27^{b}
27	α-selinene	0.09 ± 0.01^{b}	0.27 ± 0.04^{a}	0.23 ± 0.02^{a}	6.39 ± 2.74^{a}	7.40 ± 1.96^{a}	5.17 ± 0.83^{a}
28	Valencene	0.14 ± 0.02^{b}	0.30 ± 0.03^{a}	0.16 ± 0.02^{b}	9.39 ± 3.62^{a}	7.99 ± 1.49^{a}	2.80 ± 0.34^{b}

N.D.: not detected. Different superscripts in the same row of each sample are significantly different ($p<0.05$).

2.4 CONCLUSION

The color, proximate composition, minerals, free amino acids, fatty acids, and volatile components in three cultivars of *C. natsudaidai* (CN) peels were determined. The results showed that CN peel contained approximately 18% of carbohydrates, and it could be used as a pectin source. Additionally, hydrophobic extracts could be applied as a natural flavor essence for adding to other products due to aroma components. Sensory evaluation with consumer panelists is recommended to verify if natural food additives from CN peel extracts are viable.

CHAPTER 3

COMPARISON OF ANTIOXIDANT PROPERTIES OF DIFFERENT CRUDE EXTRACTS OF *CITRUS NATSUDAIDAI* PEEL

3.1 INTRODUCTION

Plant-based foods and their processing wastes such as the peel, seeds, and leaves are well known to contain several antioxidants that may positively affect human health (Takebayashi et al., 2013). Citrus species are primary sources of vitamin C, flavonoids, carotenoids, and other phenolic compounds that have high antioxidant activity (Zhang et al., 2014; de Moraes Barros et al., 2012; Ramful et al., 2010). In most cases, the peel contains higher amounts of these compounds than the pulp (Assefa et al., 2017; de Moraes Barros et al., 2012). In Chapter 2, CN peel extract included beneficial compounds that can be used as natural additives for foods or cosmetics. Therefore, the next step of this work looks at evaluating health-promoting effects, such as antioxidant capacity.

Compounds present in plant-based foods and their processing wastes have different chemical features, such as molecular weight, an arrangement of branching, linkage-type, and degree, which can influence their physicochemical behavior (Zhang & Tsao, 2016). Considering the extraction conditions, polarity and solubility are important since these features have a considerable effect on extract yield and composition. For instance, hydrophilic compounds can be extracted with polar solvents such as water, ethanol, and methanol, whereas chloroform, ether, and hexane are commonly used for the extraction of hydrophobic compounds (Azmir et al., 2013).

To obtain compounds present in plant-based foods and their processing wastes,

conventional solvent extraction is usually performed since it can be conducted at room temperature (25°C) and at atmospheric pressure. Many researchers have shown interest in extraction methods of citrus peel. M'hiri et al. (2015b) compared various extraction techniques (conventional solvent extraction, microwave-assisted extraction, ultrasound-assisted extraction, high hydrostatic pressure extraction, and supercritical fluid extraction) to investigate the total phenolic content (TPC) and the antioxidant activity of *C. sinensis* peel. Chan et al. (2009) studied the effect of extraction condition (solvent type, concentration, temperature, and time) on the TPC from *C. hystrix* and determined the optimum conditions to be 52.9% aqueous ethanol, 48.3 °C, and 126.3 min of extraction time. Other reports compared the impact of various extraction solvents on citrus peel TPC and antioxidant activity using 2,2-diphenyl-1-pikrylhydrazyl (DPPH) radical. Lou et al. (2014) found the highest TPC and DPPH radical scavenging activity in water extract compared to ethanol, methanol, and ethyl acetate extracts. Park et al. (2014) found the highest TPC in acetone extract followed by methanol and ethanol extract, while acetone showed the lowest DPPH radical scavenging activity, and methanol revealed the highest activity. Hegazy & Ibrahium (2012) compared six extraction solvents: methanol, ethanol, dichloromethane, acetone, hexane, and ethyl acetate. TPC was higher in the order of ethanol > methanol > acetone > dichloromethane > ethyl acetate > hexane, and DPPH radical scavenging activity followed the order ethanol > methanol > ethyl acetate > acetone > dichloromethane > hexane.

Most of the studies that investigated different solvents for citrus peel extraction are focused on obtaining the best yield of phenolic compounds and antioxidant activities (Lou et al., 2014; Park et al., 2014; Hegazy & Ibrahium, 2012), regardless of the presence of overlaying components in extracts with similar polarity. For instance, the most commonly used solvents seem to be ethanol, methanol, and acetone, which are polar to intermediate polar solvents. Thus, some compounds that are soluble in ethanol could also be soluble in methanol and acetone. Therefore TPC or the antioxidant activity can be estimated

considering overlapping compounds and not compounds specifically extracted by only one solvent type. We propose here an original extraction procedure that avoids overlapping extracted components to determine the TPC combined with the estimation of antioxidant activity of CN peel based on DPPH and 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity.

To our knowledge, no study has been conducted on the antioxidant activity of CN peel extracts prepared with different polar solvents avoiding overlapping composition. Therefore, the objectives of this study were set to (1) obtain extracts of CN peel with food-use solvents and cost-effective methods; (2) investigate the TPC and antioxidant properties without overestimating; (3) compare the TPC and antioxidant activities of each extract of different polarity.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Ethanol, hexane, and sodium sulfate were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Milli-Q water was prepared by Milli-Q® Integral A10® System (Molsheim, France). Sodium carbonate, potassium persulfate, and ABTS were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). DPPH, Folin-Ciocalteu phenol reagent, and 6-Hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox) were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). Gallic acid hydrate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

3.2.2 Samples

Three different cultivars of CN were obtained from Chiba Prefecture, Japan, namely CN1, CN2, and CN3 in this study. CN1 cultivar is newly developed by a farmer; CN2 and CN3 are commercial CN fruits from the same farmer. All of the fruits were cultivated for three years without pesticides and annually harvested from May to July. Upon arrival at Toyo University Food Science Laboratory, the fruits were immediately washed and manually peeled. Approximately 500 g of peels per cultivar were crushed using a hand grinder (Click & Mix Plus T-fal, Tokyo, Japan) and stored at -30°C before any other treatment.

3.2.3 Conventional solvent extraction

Two solvent mixtures (water-ethanol and ethanol-hexane) were used as extraction solvents. From the perspective of yield and efficiency, the ethanol ratio of water-ethanol mixture and extraction time were set at 80% (v/v) and 24 h, respectively (Lou et al., 2014; Chan et al., 2009; Li et al., 2006). Ethanol-hexane mixture was prepared in a 2:1 (v/v) ratio (Bligh & Dyer, 1959), and extraction time was also set at 24 h. To prevent the thermal degradation of antioxidants, extraction was carried out at room temperature (25°C).

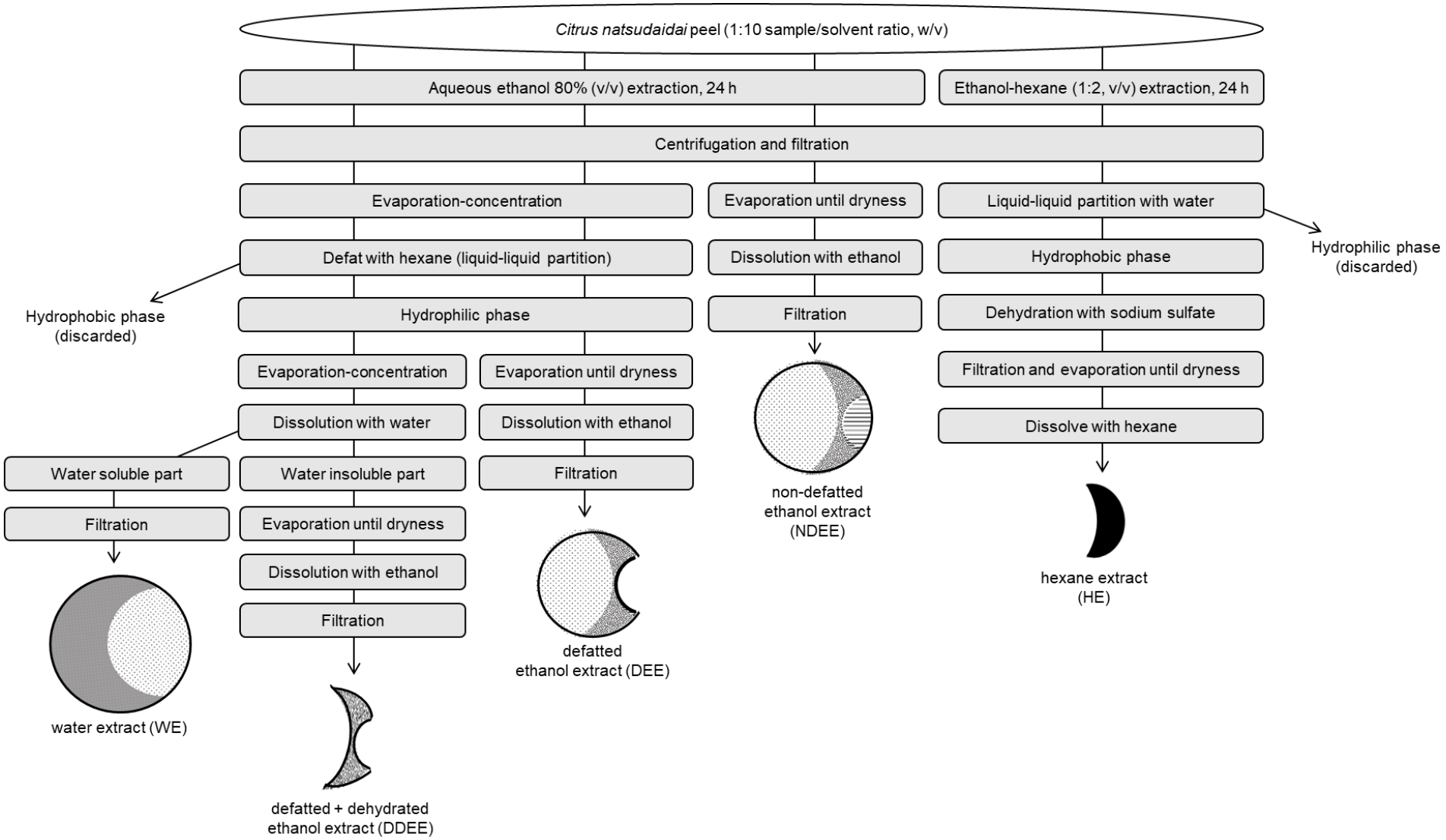

Figure 3.1 The extraction method used to obtain five different extracts.

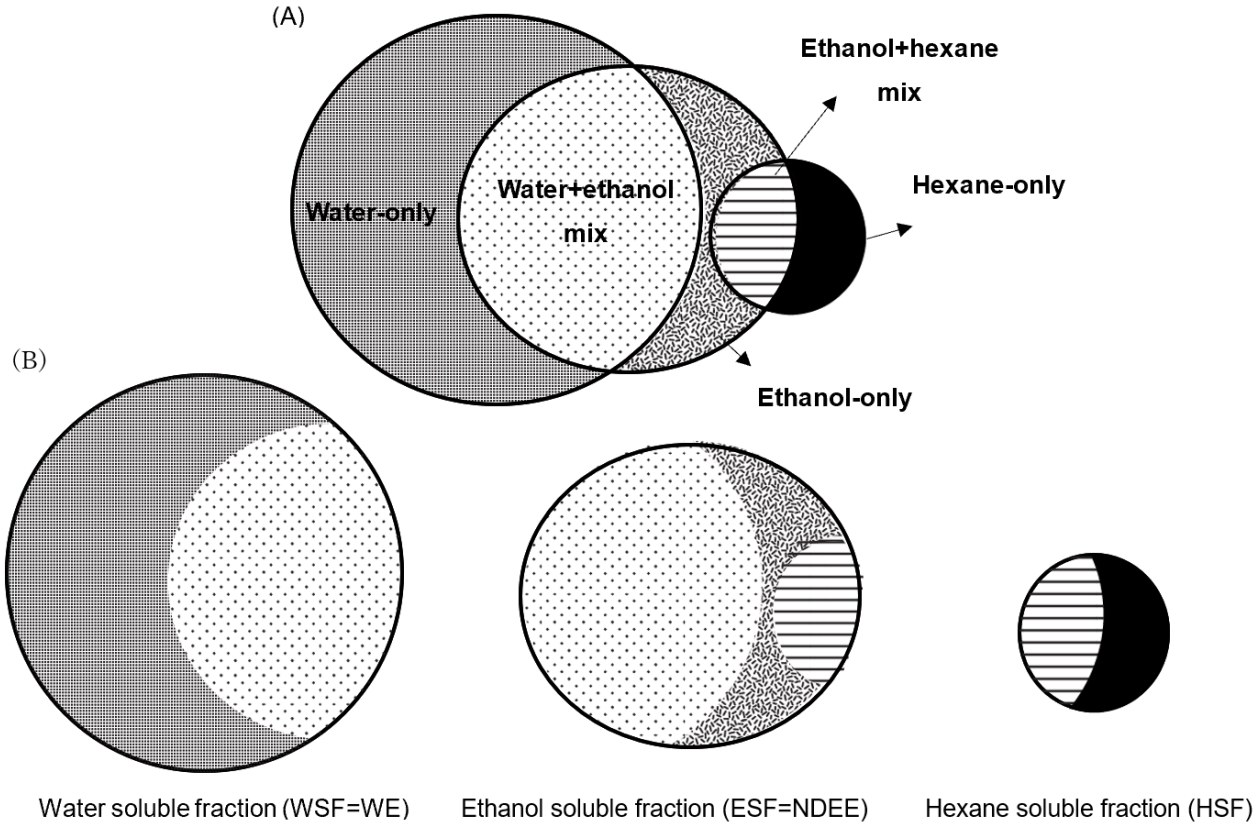

Figure 3.2 Representation of the five extracts obtained from the extraction method (Figure 3.1), showing that they have overlapping soluble parts (A); Illustration of the three main soluble fractions. WE: water extract; NDEE: non-defatted ethanol extract (B).

3.2.3.1 Extraction with water-ethanol mixture

Four polar extracts were obtained following the method described by Zhang et al. (2011) and Li et al. (2006) with some modifications. Ground-frozen citrus peel (5 g) was extracted with 80% aqueous ethanol (v/v) with a 1:10 (w/v) ratio of sample-solvent under magnetic stirring for 24 h at room temperature (25°C). A first extract, namely non-defatted ethanol extract (NDEE), was obtained by centrifuging the extract at 8000 rpm (7090 × g) for 5 min at 4 °C. Then, the supernatant was collected in a round-bottomed flask through filtration with Advantec® 5B 150 mm filter paper. Residues were re-extracted twice by hand-shaking (1 min) following the same procedure. Further, the filtrated supernatant was evaporated at 37°C

in a rotary evaporator to dryness, and then dissolved in 100% ethanol. The extract was then transferred to a volumetric flask and filled up to 25 ml. The extract was filtered on with a hydrophilic nylon 0.22 µm syringe filter. A second extract, named defatted ethanol extract (DEE), was obtained similarly as shown in **Figure 3.1**, except that the extract was evaporated at 37°C until the volume became less than 5 ml. The remaining extract was then quantitatively transferred in a separatory funnel by the addition of Milli-Q water, ethanol, and hexane (3 times, 10 ml of each solvent) before being submitted to the liquid-liquid partition. After clear separation, the hydrophilic phase was transferred to a second separatory funnel and was defatted with 20 ml of hexane another two times. The hydrophilic phase was concentrated in a second round-bottomed flask, followed by evaporation at 37°C until dryness, dissolved in 100% ethanol and then, transferred to a 25 ml volumetric flask. At last, filtration was performed. A third extract, namely the WE, was obtained following similar conditions as the DEE. The second evaporation step was ended when the remaining extract became less than 5 ml, and the remaining extract was dissolved with Milli-Q water and transferred to a 25 ml volumetric flask, followed by filtration. After this procedure, a water-insoluble residue (as shown in **Figure 3.2**) was formed on the wall of the round-bottomed flask. To collect the remaining part of the extract, the flask of WE was subjected again to evaporation at 37°C to dryness, and then the residue was dissolved in 100% ethanol in a 10 ml volumetric flask. The extract was filtered and was called defatted+dehydrated ethanol extract (DDEE). All the extracts were stored at -30°C until further analysis.

3.2.3.2 Extraction with ethanol-hexane mixture

The fifth extract, namely hexane extract (HE), was obtained based on the methods of Cheong et al. (2012) and Bligh & Dyer (1959). Ground-frozen citrus peels (5 g) were extracted with ethanol-hexane solvent mixture (2:1, v/v) at a sample-solvent ratio of 1:10 (w/v) under

magnetic twist for 24 h at room temperature (25°C). Further, the sample was centrifuged at 8000 rpm (7090 × g) for 5 min at 4 °C, and the supernatant was transferred to a separatory funnel after filtration. Residues were re-extracted with 50 ml of ethanol-hexane mixture by hand-shaking (1 min) twice following the same procedure. All the re-extraction solvents were pooled with the extracted supernatant in the separatory funnel, and 30 ml of Milli-Q water was added. After clear separation, the hydrophilic phase was discarded, and the extract was partitioned again with 30 ml of Milli-Q water another two times following the same procedure. The hydrophobic phase was collected in a conical flask in which sodium sulfate was added to withdraw any remaining water content. The extract was then filtrated to a round-bottomed flask and evaporated at 37°C to dryness using a rotary evaporator. It was solubilized with 10 ml of hexane and transferred in a glass tube. The extract was then evaporated to dryness using a tube evaporator and stored at -30°C until further analysis.

3.2.4 Determination of TPC

The TPC of citrus peel extracts was measured using the Folin-Ciocalteu method described by Sheikh et al. (2009) with minor modifications. Briefly, 0.5 ml aliquot of the sample or gallic acid standard was placed in a glass tube, mixed with 0.75 ml Milli-Q water and 0.25 ml of Folin-Ciocalteu reagent. Samples were diluted according to their solvent solution (Milli-Q water, ethanol, or hexane). After 1 min, 0.5 ml of saturated sodium carbonate solution was added and mixed again. Then, it was allowed to react for 90 min at room temperature (25°C), and the absorbance was measured at 765 nm using a UV-VIS spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan). A blank was prepared using Milli-Q water instead of the sample solution. Results were expressed as mg of gallic acid equivalent (GAE) per 100 g of citrus peel.

3.2.5 ABTS radical scavenging activity

The ABTS radical scavenging activity of citrus peel extracts was determined according to the method of Marathe et al. (2011) with some modifications. Briefly, 7 mM of ABTS was prepared with Milli-Q water and potassium persulfate was added with mixing to a 2.45 mM concentration. This solution was left overnight at 4 °C with protection from light exposure. Before analysis, the ABTS stock solution was diluted with ethanol at a ratio of 1:89 (v/v). The reaction mixture consisted of 0.8 ml of ABTS solution and 0.2 ml of citrus peel extract at different concentrations, which were diluted with ethanol. The mixture was shaken and left to react for 30 min at room temperature (25°C) in the dark. The absorbance at 734 nm was recorded by a UV-VIS spectrophotometer. Ethanol was used as the blank. A control was prepared using ethanol instead of the sample solution. Half maximal inhibitory concentration (IC50) values, which correspond to the concentrations required to reach 50% of the antioxidant effect, were obtained by calculation. Trolox was used as the reference antioxidant compound, and the results were expressed as mg of Trolox equivalent (TE) per 100 g of citrus peel.

3.2.6 DPPH radical scavenging activity

The DPPH radical scavenging activity of citrus peel extracts was determined using a modified method based on Sheikh et al. (2009). Briefly, 0.5 ml of 0.1 mM DPPH ethanolic solution was mixed with 0.5 ml of citrus peel extract at different concentrations diluted with ethanol. The mixture was shaken and left to stand in the dark for 30 min at room temperature (25°C). Absorbance was measured at 517 nm using a UV-VIS spectrophotometer. Ethanol was used as the blank. The control consisted of the DPPH solution and ethanol. DPPH radical scavenging activity was obtained by the same calculation as for the ABTS assay.

3.2.7 Statistical analysis

All experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD). The data were analyzed using the Statistical Package for Social Sciences (IBM® SPSS® Statistics for Windows version 22). Statistical significance was declared at $p < 0.05$.

3.3 RESULTS

As shown in **Figures 3.1** and **3.2**, five extracts were obtained using two solvent mixtures: water/ethanol and ethanol/hexane. Ethanol has an acceptable cost and is safe as it is categorized as generally recognized as safe (GRAS) (FDA, 21 CFR 184-Substances Affirmed as GRAS in Food as of 04 October 2019). Hexane is permissible for extracting fats or oils in the manufacturing of edible fats or oils in Japanese industry (MHLW, Standards for Use of Food Additives of 06 June 2019). Moreover, thermal processing and additional equipment(s) to control temperature and pressure were not necessary. Therefore, the extraction method used in this study is useable in foods and is an environmentally friendly and cost-effective process.

Water solubilizes hydrophilic compounds regardless of molecular weight. Since ethanol is an intermediate-polar solvent, it can extract hydrophilic to hydrophobic compounds. However, high molecular weight compounds such as polysaccharides and proteins are not solubilized. Moreover, ethanol can be well mixed with water and hexane. Hexane can extract hydrophobic compounds such as fat; therefore, it was also used to defat the aqueous solution. Extracted compounds that are water-soluble can be mixed with ones that are soluble in ethanol and vice-versa, thus creating an overlapping part, as shown in **Figure 3.2**. The extracts obtained in this study contain overlapping parts. However, through calculations, it

was possible to estimate the TPC and the total antioxidant activity without considering overlapping compounds in the results. To eliminate the overlapping parts, TPC and the antioxidant activity of CN peel extract were calculated and divided into five soluble parts (**Figure 3.2**): water-only soluble, water+ethanol mix soluble, ethanol-only soluble, ethanol+hexane mix soluble, and hexane-only soluble part. These were called “soluble parts” to distinguish them from extracts. Further, these data were combined to calculate the TPC and antioxidant activity of the three different soluble fractions, water-soluble fraction (WSF), ethanol-soluble fraction (ESF), and hexane-soluble fraction (HSF), as shown in **Figure 3.2**, and they were named the “soluble fraction.”

The WE fraction was extracted using 80% ethanol as the solvent (**Figure 3.1**). Therefore, it is reasonable to conclude that water-soluble and ethanol-soluble compounds were obtained in this process. It includes the water-only soluble, water+ethanol mix soluble, ethanol-only soluble, and ethanol+hexane mix soluble parts, which are represented in **Figure 3.2**. Then, continuing the extraction process, the extract was defatted with hexane; hence, the compounds that were in the ethanol+hexane mix soluble part were removed. After all solvents were evaporated (80% ethanol and hexane), water was added and water-soluble compounds (water-only soluble and water+ethanol mix soluble parts) were solubilized (named WE). Water-insoluble compounds (only soluble in ethanol) remained in the flask. Ethanol was added after drying the flask, and the remaining compounds were solubilized in ethanol (named defatted+dehydrated ethanol extract, DDEE). For defatted ethanol extract (DEE), the same process as for WE was conducted until the defatting step (using hexane). After evaporation, water-only soluble, water+ethanol mix soluble, and ethanol-only soluble parts were all present in the flask. Ethanol was added, dissolving the ethanol-soluble compounds (water+ethanol mix soluble and ethanol-only soluble parts). Non-defatted ethanol extract (NDEE) was obtained using the same process as for DEE but without the defatting step. Thus, NDEE includes the ethanol+hexane mix soluble part. The HE differed from the others in that

ethanol-hexane mixture was used for extraction; hence, ethanol-soluble and hexane soluble compounds (water+ethanol mix soluble, ethanol-only, ethanol+hexane mix soluble, and hexane-only soluble parts) were extracted first. Water was added, and the hydrophilic phase was discarded (water+ethanol mix soluble, ethanol-only soluble, and ethanol+hexane mix soluble parts). The “hydrophilic phase” includes water-soluble and ethanol soluble compounds. Since ethanol will mix with water more efficiently than hexane, ethanol was removed with water. Finally, the hexane-only soluble part remained in the flask, and it was solubilized with hexane.

3.3.1 Determination of TPC

TPC of CN peel extracts, soluble parts, and soluble fractions is summarized in **Table 3.1**. Concerning the extracts (WE, DDEE, DEE, NDEE, and HE), WE had the significantly highest TPC (320-369 mg GAE/100 g FM) followed by NDEE (149-185 mg GAE/100 g FM) and DEE (116-128 mg GAE/100 g FM). For the soluble parts (water-only, water+ethanol mix, ethanol-only, ethanol+hexane mix, and hexane-only), the water-only soluble parts exhibited the highest TPC (204-242 mg GAE/100 g FM), followed by the water+ethanol mix soluble parts (115-127 mg GAE/100 g FM) and the ethanol+hexane mix soluble parts (20.8-68.6 mg GAE/100 g FM). Regarding the soluble fractions (WSF, ESF, and HSF), the WSF(=WE) showed the highest TPC, followed by the ESF(=NDEE) and the HSF for all cultivars.

From the comparison of TPC in CN cultivars, CN1 and CN3 showed higher TPC in the WSF(=WE) than did CN2. While present in ESF(=NDEE) and HSF, CN2 had significantly higher TPC than CN1 and CN3. The total amount of phenolics (sum of all “soluble parts”) showed no significant differences.

Table 3.1 Total phenolic content of peel extracts from the three citrus cultivars (mg GAE/100 g FM; n=3; mean ± SD) soluble parts and soluble fractions.

Citrus natsudaidai (CN) cultivar	Water extract (WE)	Defatted + dehydrated ethanol extract (DDEE)	Hexane extract (HE)
CN1	369 ± 9.04[a]	0.63 ± 0.07[b]	1.43 ± 0.21[a]
CN2	320 ± 1.24[c]	0.54 ± 0.02[b]	0.48 ± 0.01[b]
CN3	345 ± 6.38[b]	0.95 ± 0.05[a]	0.50 ± 0.02[b]

	Defatted ethanol extract (DEE)
CN1	128 ± 1.89[a]
CN2	116 ± 6.08[b]
CN3	126 ± 0.60[a]

	Non-defatted ethanol extract (NDEE)
CN1	149 ± 7.45[b]
CN2	185 ± 2.69[a]
CN3	155 ± 10.2[b]

	Water-only	Water+ethanol mix	Ethanol-only	Ethanol+Hexane mix	Hexane-only	Total
CN1	242 ± 9.56[a]	127 ± 1.93[a]	same as DDEE	20.8 ± 7.07[b]	same as HE	392 ± 13.3[a]
CN2	204 ± 7.26[b]	115 ± 6.10[c]		68.6 ± 7.85[a]		389 ± 9.11[a]
CN3	220 ± 6.84[b]	125 ± 0.64[a]		29.2 ± 10.15[b]		375 ± 15.5[a]

	Water-soluble fraction (WSF)
	same as WE

	Ethanol-soluble fraction (ESF)
	same as NDEE

	Hexane-soluble fraction (HSF)
CN1	22.2 ± 7.30[b]
CN2	69.0 ± 7.86[a]
CN3	29.7 ± 10.1[b]

mg GAE/100 g FM: mg of gallic acid equivalent per 100 g of sample based on fresh matter weight; Water-only = WE – (Water+ethanol mix); Water+ethanol mix = DEE – DDEE; Ethanol+hexane mix = NDEE – DEE; Different superscripts in the same extract / soluble part / soluble fraction indicate significant differences among three cultivars ($p<0.05$). Partition was carried out following Figure 3.1.

3.3.2 ABTS radical scavenging activity

The ABTS radical scavenging activity of CN peel extract is reported in **Table 3.2**. The WE exhibited the highest antioxidant activity (132-159 mg TE/100 g FM) followed by the NDEE (51.7-56.5 mg TE/100 g FM) and the DEE (32.0-43.9 mg TE/100 g FM) for all cultivars. After removing the overlapping part in the same way as TPC (**Table 3.1**), the water-only soluble part represented the highest value (100-117 mg TE/100 g FM) significantly, followed by the water+ethanol mix soluble part (31.6-42.7 mg TE/100 g FM) and the ethanol+hexane mix soluble part (9.92-21.8 mg TE/100 g FM) in all of the three cultivars. The WSF(=WE) displayed significantly the highest antioxidant activity, followed by the ESF(=NDEE) and the HSF. When comparing the antioxidant activity among the cultivars, CN1 and CN3 showed significantly higher values than CN2 in the WSF(=WE), whereas there were no significant differences in the ESF(=NDEE) and the HSF. Besides, the total value (sum of each of all “soluble parts”) showed no significant difference. In this study, the ABTS value of CN extracts was higher in the water (polar) part and lower in the hexane part.

Table 3.2 ABTS radical scavenging activities of peel extracts from the three citrus cultivars (mg TE/100 g FM; n=3; mean ± SD) soluble parts and soluble fractions.

Citrus natsudaidai (CN) cultivar	Water extract (WE)	Defatted + dehydrated ethanol extract (DDEE)	Hexane extract (HE)
CN1	159 ± 3.83[a]	0.34 ± 0.07[b]	5.57 ± 0.85[a]
CN2	132 ± 3.93[b]	0.36 ± 0.06[b]	1.14 ± 0.12[b]
CN3	158 ± 6.60[a]	1.16 ± 0.19[a]	1.24 ± 0.03[b]

	Defatted ethanol extract (DEE)
CN1	41.8 ± 2.62[a]
CN2	32.0 ± 2.79[b]
CN3	43.9 ± 2.49[a]

	Non-defatted ethanol extract (NDEE)
CN1	51.7 ± 3.54[a]
CN2	53.7 ± 1.58[a]
CN3	56.5 ± 3.63[a]

	Water-only	Water+ethanol	Ethanol-only	Ethanol+Hexane	Hexane-only	Total
CN1	117 ± 5.20[a]	41.4 ± 2.57[a]		9.92 ± 6.04[a]		174 ± 6.57[a]
CN2	100 ± 6.74[b]	31.6 ± 2.81[b]	same as DDEE	21.8 ± 4.36[a]	same as HE	155 ± 8.20[a]
CN3	115 ± 6.16[ab]	42.7 ± 2.56[a]		12.6 ± 5.50[a]		173 ± 9.22[a]

	Water-soluble fraction (WSF)
	same as WE

	Ethanol-soluble fraction (ESF)
	same as NDEE

	Hexane-soluble fraction (HSF)
CN1	15.5 ± 6.52[a]
CN2	22.9 ± 4.25[a]
CN3	13.9 ± 5.47[a]

mg TE/100 g FM: mg of Trolox equivalent 100 g of sample based on fresh matter weight; Water-only = WE – (Water+ethanol mix); Water+ethanol mix = DEE – DDEE; Ethanol+hexane mix = NDEE – DEE; Different superscript in the same extract / soluble part / soluble fraction indicate significant differences among three cultivars ($p<0.05$). Partition was carried out following Figure 3.1.

3.3.3 DPPH radical scavenging activity

Table 3.3 shows the DPPH radical scavenging activity of CN peel extracts. The WE exhibited the highest DPPH value (34.2-37.8 mg TE/100 g FM) followed by the NDEE (30.2-33.0 mg TE/100 g FM) and the DEE (20.7-29.7 mg TE/100 g FM) for all cultivars. Considering the soluble parts, the water+ethanol mix soluble part clearly displayed the highest activity (20.6-29.1 mg TE/100 g FM) followed by the water-only soluble part (8.79-13.6 mg TE/100 g FM), and the ethanol-hexane mix soluble part (3.32-10.9 mg TE/100 g FM). Among the three cultivars, there were no significant differences in the WSF and the ESF, as well as for the total DPPH values (each of the “soluble parts” sums). From all the results, it was observed that the DPPH radical scavenging activity of CN peel extracts is higher in the polar and intermediate-polar parts and lower in the non-polar parts.

Table 3.3 DPPH radical scavenging activities of peel extracts from the three citrus cultivars (mg TE/100 g FM; n=3; mean ± SD) soluble parts and soluble fractions.

Citrus natsudaidai (CN) cultivar	Water extract (WE)	Defatted + dehydrated ethanol extract (DDEE)	Hexane extract (HE)
CN1	36.7 ± 2.19[a]	0.18 ± 0.03[b]	0.86 ± 0.01[a]
CN2	34.2 ± 1.59[a]	0.13 ± 0.01[b]	0.60 ± 0.04[b]
CN3	37.8 ± 0.89[a]	0.65 ± 0.02[a]	0.72 ± 0.09[ab]

	Defatted ethanol extract (DEE)
CN1	26.3 ± 1.99[a]
CN2	20.7 ± 1.11[b]
CN3	29.7 ± 1.84[a]

	Non-defatted ethanol extract (NDEE)
CN1	30.2 ± 0.27[a]
CN2	31.7 ± 0.82[a]
CN3	33.0 ± 2.23[a]

	Water-only	Water+ethanol	Ethanol-only	Ethanol+Hexane	Hexane-only	Total
CN1	10.6 ± 2.95[a]	26.1 ± 2.01[a]		3.88 ± 2.04[b]		41.6 ± 2.60[a]
CN2	13.6 ± 1.17[a]	20.6 ± 1.11[b]	same as DDEE	10.9 ± 0.95[a]	same as HE	45.9 ± 0.68[a]
CN3	8.79 ± 2.44[a]	29.1 ± 1.83[a]		3.32 ± 2.29[b]		42.5 ± 3.10[a]

	Water-soluble fraction (WSF)
	same as WE

	Ethanol-soluble fraction (ESF)
	same as NDEE

	Hexane-soluble fraction (HSF)
CN1	4.74 ± 2.06[b]
CN2	11.5 ± 0.91[a]
CN3	4.05 ± 2.21[b]

mg TE/100 g FM: mg of Trolox equivalent 100 g of sample based on fresh matter weight; Water-only = WE – (Water+ethanol mix); Water+ethanol mix = DEE – DDEE; Ethanol+hexane mix = NDEE – DEE; Different superscripts in the same extract / soluble part / soluble fraction indicate significant differences among three cultivars ($p<0.05$). Partition was carried out following Figure 3.1.

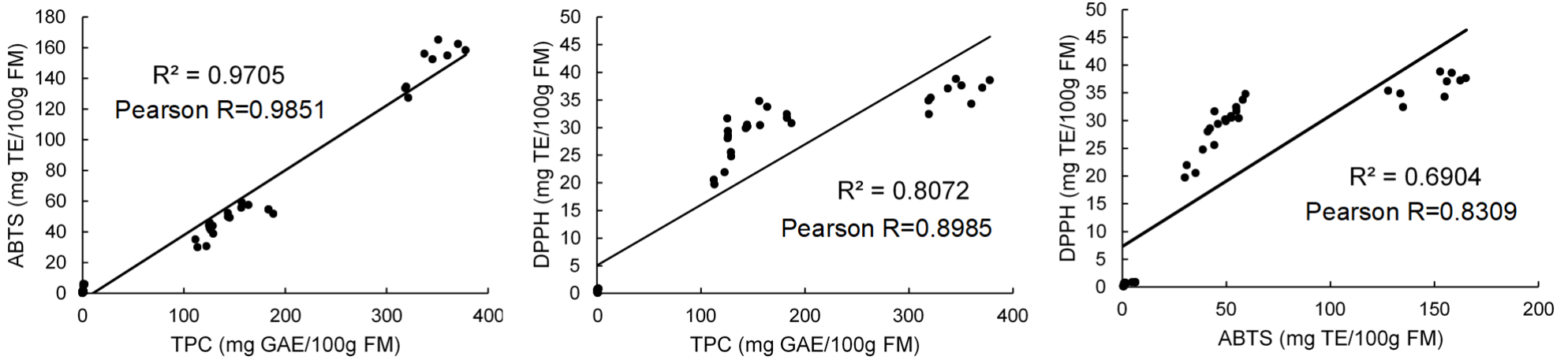

Figure 3.3 Phenolic content; ABTS: ABTS radical scavenging activity; DPPH: DPPH radical scavenging activity; mg GAE/100 g FM: mg of gallic acid equivalent per 100 g of sample based on fresh matter weight; mg TE/100 g FM: mg of Trolox equivalent of sample based on fresh matter weight. All correlations were significant at $p<0.01$.

3.3.4 Correlation between the TPC and the antioxidant activities

Pearson's correlation coefficients were calculated to determine the relationship between the TPC and the antioxidant activities (**Figure 3.3**). There were strong positive correlations among them (TPC × ABTS: r = 0.9851; TPC × DPPH: r = 0.8985; ABTS × DPPH: r = 0.8309).

3.4 DISCUSSION

The results of the TPC determination clarified that the phenolic compounds in CN peel are present mainly in the polar part. The TPC of citrus peel extract varies among citrus species. After adjusting the units to compare with our study, Ramful et al. (2010) found a TPC ranging from 188.2 to 766.7 mg GAE/100 g FM in citrus peel 80% methanol extracts from 36 varieties. Lagha-Benamrouche & Madani (2013) showed that seven kinds of citrus peel extracts, obtained with 80% methanol, contained from 241 to 765 mg GAE/100 g FM. These reports did not include the use of other extraction solvents. Comparing the TPC between different polar solvents, CN peel showed that the WSF(=WE) includes 1.7 to 2.5 times higher amounts than the ESF(=NDEE) and 4.7 to 16 times higher amounts than HSF. The ESF(=NDEE) comprised a 2.6 to 6.7 times higher TPC than the HSF. Some other previous reports showed a similar pattern. Hegazy & Ibrahium (2012) described that ethanol extract of the dried orange peel powder contained 2.7 times higher TPC than the HE. Emam & El-bassyouni (2015) also found that the TPC of dried orange peel ethanol extract had 1.3 times higher TPC than its HE. Although the amounts of phenolics vary, those results revealed that phenolic components in citrus peel distribute mainly on the polar to intermediate-polar parts.

Regarding antioxidant properties, Rekha & Bhaskar (2013) determined the DPPH radical scavenging activity of orange peel extract prepared with several solvents, including ethanol and hexane. They reported that the hexane extract showed higher antioxidant activity than the ethanol extract. Hegazy & Ibrahium (2012) and Lou et al. (2014) also reported the DPPH radical scavenging activity of the citrus peel extracted with different solvents. The authors found a similar trend to our results: DPPH radical scavenging activity is higher in the polar extracts of citrus peel. Jayaprakasha et al. (2008) reported the ABTS radical scavenging activity in various extracts of pomelo and navel orange “edible parts” and showed stronger activity of ABTS inhibition in the methanol extract and lower activity in the hexane extract. Sahreen et al. (2010) reported ABTS and DPPH radical scavenging activity of *Carissa opaca* fruit in various extracts. The authors indicated that the best antioxidant values of the extracts were aqueous > methanol > hexane for both radicals. Barbouchi et al. (2020) described the DPPH radical scavenging activity of *Pistacia lentiscus* twigs, leaves, and fruit extracted with different solvents, and they showed that the best antioxidant values of the fruit extract were aqueous > ethanol > hexane extract. These findings are consistent with the results presented here.

Several previous studies reported that the TPC of citrus peel showed a positive correlation with their antioxidant activity (Lou et al., 2014; Lagha-Benamrouche & Madani, 2013; Ramful et al., 2010). In contrast, de Moraes Barros et al. (2012) reported no correlation between the TPC and the antioxidant activity in citrus peel extract. Rahman et al. (2018) founded a negative correlation between TPC and DPPH values. The differences in the studied citrus species and the extraction procedure could explain this diversity. Citrus peel contains several phenolic compounds such as phenolic acids and flavonoids, and their composition varies among species (Lou et al., 2014; Zhang et al., 2011; Ramful et al., 2010). The majority of phenolic compounds act as good antioxidants, while some compounds have weak activity (Pannala et al., 2001). Besides, the presence of other bioactive compounds

such as vitamins, terpenoids, mineral elements, amino acids, fatty acids, and pectin probably could affect antioxidant capacities (Matsuo et al., 2019; Assefa et al., 2017; Zou et al., 2016; de Moraes Barros et al., 2012).

3.5 CONCLUSION

Extractions using solvents of various polarities compatible with the food industry process were conducted on CN peel to obtain antioxidant compounds. Then, the TPC and the antioxidant properties were determined. The TPC and the antioxidant properties were higher in the polar extracts, and there was a high correlation between the TPC and antioxidant activity. Polar extracts of CN peel with antioxidant activity have the potential to be used as a natural food additive or ingredients for cosmetic and pharmaceutical products. From this perspective, it would be of great interest to obtain information on the identification of phenolic compounds present in polar extracts and to investigate their respective antioxidant activities.

CHAPTER 4

IDENTIFICATION OF THE ANTIOXIDANT ACTIVE PHENOLIC COMPOUNDS IN *CITRUS NATSUDAIDAI* PEEL

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定。

CHAPTER 5

UTILIZATION AND EVALUATION OF CITRUS NATSUDAIDAI PEEL WASTE AS A SOURCE OF NATURAL FOOD ADDITIVE

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。

5 年以内に出版予定。

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

In this present study, a model case for food processing waste utilization was established using *C. natsudaidai* (CN). Chapter 2 was the investigation of color, proximate composition, free amino acids, fatty acids, minerals, and aroma compounds for obtaining general information of CN peel. From the results, it was estimated that CN peel would be useful as an ingredient for pectin and natural flavor due to the presence of carbohydrates and aromatic compounds. In Chapters 3 and 4, a simple and appropriate extraction method for getting antioxidant compounds from CN peel based on the premise of food use was established, and phenolic compounds in CN peel were revealed. Typical citrus flavonoids, neoeriocitrin, narirutin, naringin, hesperidin, and neohesperidin were detected in CN peel hydrophilic extract. In Chapter 5, the flow of utilization of CN processing waste was assumed and tried in the actual steps as follows: peel essence including aroma compounds, phenolic compounds, and water-soluble pectin were obtained in this order, and insoluble dietary fiber remained. Peel essence was applied to citrus-flavored solution as a natural odorant, and it was acceptable for consumer panelists by sensory evaluation. The predominant phenolic compounds obtained in this method were naringin and nephesperidin, which are typical flavonoids in citrus species, and they can be a source of pharmaceutical products.

In the utilization flow of CN peel, 5.56-7.15 g of peel essence, 5.05-6.12 g of water-soluble pectin, 4.71-5.22 g of naringin, 1.61-1.96 g of neohesperidin, and 92.2-113 g of insoluble dietary fiber were obtained from 1 kg of CN peel. Since the extraction methods used in this study were simple and available for food use, they can be employed as a CN

processing waste utilization way in the food industry field. The model case of CN processing waste utilization can also be applied to other plant food processing wastes. For other citrus species, fruits, and vegetables processing waste, the method flow can be used in the same way. These may contribute to solving and reducing the problem of food processing waste discharged at 8.36 million tons annually in Japan (estimated by the database from Ministry of Agriculture, Forestry and Fisheries (MAFF), 2020). In addition, the solvent can be used repeatedly by creating a closed system for extraction. It is helpful in terms of cost reduction and environmental protection.

Another outcome of this study was the possibility of improving analytical methods. In Chapter 4, consideration of experimental conditions to improve the reactivity of the on-line HPLC-ABTS assay was performed. The optimized method found in this study could contribute to developing the utility and enhancement of the value of the on-line HPLC-ABTS assay in the academic field.

6.2 RECOMMENDATIONS FOR FUTURE WORK

This study focused on the plant food processing waste, including an outer peel part of the plant food. Generally, pesticides are used to cultivate plant foods, and pesticides may remain on the rind. Methods for removing pesticide residues should also be considered.

Besides, the optimization of the on-line HPLC-ABTS method is expected to further improve by additional research. For example, it was considered that 45°C was the best under the conditions compared in this study, yet more sensitive reactivity is expected on the measurement at a higher temperature than 45°C. However, it should be attention to the material of the reaction loop and to avoid temperature above thermal degradation of molecules of the interior. Additionally, it is necessary to cool down before recording

absorbance because air bubbles may be generated in the solution due to heating.

In addition to the experimental conditions, it is also effective to improve the on-line HPLC-ABTS equipment itself. This method commonly uses two or more different mobile phases, and these are combined in the pump. However, sometimes different solution does not blend well and flow without mixing. This situation has an influence on compounds separation of sample extracts and also the reaction with ABTS solution. This may be solved by using specific highly efficient microfluidic mixers. It is also can be proposed to change the UV detector used for ABTS measurement to a mass spectrometer.

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