Discrimination of citrus fruits using FT-IR fingerprinting by quantitative prediction of bioactive compounds

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Abstract High throughput screening of citrus samples containing elevated concentrations of total carotenoids, flavonoids, and phenolic compounds was accomplished using ultraviolet–visible spectroscopy and Fourier transform infrared (FT-IR) spectroscopy, combined with multivariate analysis. Principal component analysis and partial least squares discriminant analysis using FT-IR spectra were able to differentiate seven citrus fruit groups into three distinct clusters corresponding to their taxonomic relationship. Quantitative prediction modeling of total carotenoids, flavonoids, and phenolic compounds in citrus fruit was established using a partial least squares regression algorithm from the FT-IR spectra. The regression coefficients (R^2) of predicted and estimated values of total carotenoids, flavonoids, and phenolic compounds were all 0.99. The results indicated that accurate quantitative predictions of total carotenoids, flavonoids, and phenolic compounds were possible from citrus fruit FT-IR spectra, and that the resulting quantitative prediction model might be useful as a rapid selection tool for citrus fruits containing elevated carotenoids, flavonoids, and phenolic compounds.

Keywords Citrus · Fourier transform infrared spectroscopy · Partial least square-discriminant analysis · Partial least squares regression · Principal component analysis

Introduction

Bioactive compounds and secondary metabolites contained in citrus fruits could be used as bioresources to improve the human health. Carotenoids in citrus fruits have several properties, including antioxidative, anti-carcinogenic, and anti-inflammatory activities [1]. Additionally, flavonoid compounds have activities that are similar to carotenoids [2]. However, studies of citrus cultivars and fruit development have revealed that the contents differed for carotenoids [3, 4], flavonoid [5, 6], phenolic compounds [5–8], and ascorbic acid [6]. Further, the flavonoid and phenolic contents were affected by environmental conditions [5].

Near infrared spectroscopy has been applied to monitor the fruit quality and classified the variety of citrus [9, 10]. However, although these approaches are non-destructive applicable to total soluble solids and overall features, there are some limitations on the quantitative and qualitative analysis of secondary metabolites among the samples. The bioactive compounds in citrus fruits have been qualified and quantified using gas chromatography (GC), and high performance liquid chromatography (HPLC) [11, 12]. However, although these fractionation methods offer several advantages such as high accuracy, there are several inherent limitations including complex and expensive sample preparation procedures, high extraction costs, and extended processing times for multiple samples [13]. To overcome the limitations, ultraviolet–visible (UV–VIS)

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spectrophotometry has been used for the rapid analysis of compounds [14–16].

Metabolomics is a research field that focuses on elucidating the quantitative and qualitative differences in secondary metabolites among samples. Metabolomics has been used to identify and develop metabolite profiles of samples using data obtained following proton nuclear magnetic resonance (HNMR) spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, and mass spectrometry [17–19]. The application of FT-IR spectroscopy is an accurate and effective when accompanied by multivariate statistical analysis [18]. Consequently, metabolomics has been used to investigate the mechanisms of metabolites, to discriminate crop cultivars, and to ensure food safety. Further, multivariate statistical analyses have been used to extract more useful data from spectroscopy results. Partial least squares (PLS) regression has been used in predictive modeling of various components using exact quantitative data from samples and as a method for predicting the contents of various compounds through analysis of the correlation of quantitative measures and spectral data [20–23].

Recently, PLS regression was applied to the prediction of the fatty acid contents of *Camellia* [13], the concentration of carotenoids in apples, oranges, and peaches [24], the monosaccharide and polysaccharide contents in mushrooms [25], and the bioactive compounds in African yams [26]. In the current study, a model for the rapid prediction of total carotenoids, flavonoids, and phenolic compounds for the differentiation of citrus fruits was established.

Materials and methods

Plant materials

Citrus samples used in this study were six of Satsuma mandarin (*Citrus unshiu* Marc. cv. Miyagawa Wase) under two cultivation conditions *Fortunella japonica*, *Citrus hybrid* (*C. hybrid*) ‘Setoka,’ *C. hybrid* ‘Kanpei,’ *C. hybrid* ‘Kiyomi,’ and *C. hybrid* ‘Shiranuhi’. *C. unshiu* Marc. cv. Miyagawa Wase plants were cultivated under rain shelter or in the field. The remaining cultivars were cultivated in the green house. For statistical analysis, three full ripened fruits from each citrus sample per one batch were collected. The whole fruit was freeze-dried and the dried citrus was ground and stored at -70°C . The contents of three compounds were measured from three individual replicates.

Determination of chlorophyll a and b and carotenoid contents

Total carotenoid content was determined by UV–VIS spectrophotometry (DU®730, Beckman Coulter, Brea, CA, USA) according to a previously reported method [27]. Citrus powder (20 mg) was mixed with 1 mL of 80% acetone and centrifuged at 13,000 rpm for 5 min. The absorbance of each sample was measured from 350 to 750 nm using a UV–VIS spectrophotometer with > 2 nm interval. The absorbance at 663, 647, and 470 nm are the major absorption peaks of chlorophyll a and b, and of carotenoids, respectively. Absorbance values were used to calculate the concentration of chlorophyll a and b, and total carotenoids according to a previously reported equation [28].

$$\begin{aligned} \text{Chlorophyll a } (\mu\text{g mL}^{-1}) (C_a) \\ = 12.25 \cdot A_{663} - 2.79 \cdot A_{647} \end{aligned} \quad (1)$$

$$\text{Chlorophyll b } (\mu\text{g mL}^{-1}) (C_b) = 21.50 \cdot A_{647} - 5.1 \cdot A_{663} \quad (2)$$

$$\begin{aligned} \text{Total carotenoids } (\mu\text{g mL}^{-1}) \\ = (1000 \cdot A_{470} - 1.82 \cdot C_a - 85.02 \cdot C_b) / 198 \end{aligned} \quad (3)$$

The total carotenoids value is the sum of xanthophylls and carotenes. A_{663} indicates absorbance at 663 nm. Statistical significance was determined using analysis of variance (ANOVA).

Determination of total flavonoids contents

Total flavonoid contents were investigated by UV–VIS spectrophotometry using a previously reported method [21]. Citrus powder (10 mg) was mixed with 1 mL of 100% ethanol and incubated at room temperature for 1 h. The mixture was centrifuged at 13,000 rpm for 5 min. The resulting supernatant (100 μL) was mixed with 400 μL of double distilled water (DDW) and 30 μL of 5% NaNO_2 , and incubated at room temperature for 10 min. Next, 30 μL of 10% AlCl_3 was added to the mixture, followed by incubation for 1 min. After incubation, 200 μL of 1 M NaOH and 240 μL DDW were added to the reaction mixture. The absorbance at 510 nm was measured by UV–VIS spectrophotometry. Catechin (100 mg L^{-1}) was used as a reference compound. Statistical significance was determined by ANOVA.

Determination of total phenolic compounds

The concentration of total phenolic compounds was determined by UV–VIS spectrophotometry according to a previously reported method [29]. Citrus powder (10 mg)

was mixed with 50 μL of 80% ethanol, and the mixture was incubated at 95 $^{\circ}\text{C}$ for 5 min. Following incubation, the mixture was centrifuged at 12,000 rpm for 15 min. Next, 50 μL of 2 N Folin–Ciocalteu reagent, 100 μL of 20% Na_2CO_3 , and 830 μL of DDW were added to 20 μL of the mixture. After incubation of the reaction mixture at room temperature for 20 min, the absorbance at 725 nm was measured by UV–VIS spectrophotometry. Statistical significance was determined by ANOVA.

FT-IR spectrum analysis and multivariate statistical analysis

Citrus powder (20 mg) was mixed with 200 μL of 20% methanol. The mixture was incubated at 50 $^{\circ}\text{C}$ for 20 min and then centrifuged at 13,000 rpm for 15 min. The supernatant was collected and stored at -20°C until analysis.

FT-IR spectra were measured using a Tensor 27 FT-IR spectrometer (Bruker Optics GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulfate detector and a high-efficiency HTS-XT automation device. A 5- μL sample of the extracted supernatant was dispensed into a 384-well zinc selenide (ZnSe) plate, and dried at 37 $^{\circ}\text{C}$ on a hot plate for 20 min before loading into a 384-well dried ZnSe plate. Each spectrum was investigated at range of 4000–400 cm^{-1} at 4 cm^{-1} intervals of spectral resolution by averaging 128 scans. Each sample was analyzed in triplicate prior to statistical analysis. OPUS Lab (ver. 6.5, Bruker Optics Inc.) was used for the evaluation of the spectra. For the multivariate FT-IR spectral data were preprocessed (linear baseline correction, normalization, and mean centering) using the R program (version 2.15.0, Auckland, New Zealand). For the baseline correction, the absorbances of the FT-IR spectral analysis at both 800 and 1800 cm^{-1} end points were adjusted to 0, and each spectrum was normalized to the same area to minimize experimental error. Next, a mean centering process was applied, and preprocessed spectral data were used as standardized data for multivariate statistical analysis after applying a differential quadratic equation. Processed FT-IR spectral data were analyzed by principal component analysis (PCA) or partial least square discriminant analysis (PLS-DA) using the R program (version 2.15.0, Auckland, New Zealand) using a NIPALS algorithm [30, 31].

Prediction modeling for total carotenoid, flavonoid, and phenolic compound concentrations in citrus

Prediction modeling for the concentration of total carotenoids, flavonoids, and phenolic compounds was established through a comparison of the measured levels of total carotenoids, flavonoids, and phenolic compounds and FT-

IR spectral data from seven citrus samples. The X-variable represented the FT-IR spectral data, while the three Y-variables represented the measured concentrations of total carotenoids, flavonoids, and phenolic compounds obtained following analysis by UV–VIS spectrophotometry. To improve the accuracy of prediction modeling, cross-validation by each X variable and the three Y variables was applied. The total dataset was divided into two parts, training set and test set. The training and test set data were obtained from nine samples, respectively. The prediction of total carotenoid, flavonoid, and phenolic compound concentrations were carried out using the developed prediction model. To measure the accuracy of the model for predicting the contents of each citrus sample, the actual measurement and predicted values of total carotenoids, flavonoids, and phenolic compound concentrations were validated by the calculation of correlation coefficients using linear regression.

Results and discussion

Contents of total carotenoids, flavonoids, and phenolic compounds in citrus fruits

The concentration of total carotenoids, flavonoids, and phenolic compounds in citrus fruits was measured using UV–VIS spectrophotometry (Table 1). Evaluation by ANOVA revealed significant differences ($P < 0.05$) in the concentration of each compound among the seven citrus samples analyzed. The highest concentration of total carotenoids was $3.53 \pm 0.34 \mu\text{g g}^{-1}$ dry weight (wt), in ‘Setoka’. The total carotenoid concentration in cv. Miyagawa Wase cultivated in the field was $1.93 \pm 0.13 \mu\text{g g}^{-1}$ dry wt, while ‘Shiranuhi’ contained $1.54 \pm 0.13 \mu\text{g g}^{-1}$ dry wt. *F. japonica* and cv. Miyagawa Wase cultivated in a greenhouse exhibited lower carotenoid levels than other citrus samples (Table 1). The total flavonoid content was the highest in ‘Setoka’ ($4.50 \pm 0.36 \mu\text{g g}^{-1}$ dry wt), followed by cv. Miyagawa Wase cultivated in the field and in a greenhouse, which exhibited similar flavonoid concentrations regardless of cultivation conditions (Table 1). *F. japonica* displayed the lowest level of total flavonoids, which corresponded to the pattern of total carotenoids. The citrus sample with the highest level of total phenolic compounds was ‘Setoka’ ($6.29 \pm 0.24 \text{ mg g}^{-1}$ dry wt). Similar to total carotenoids and flavonoids, the lowest concentration of total phenolic compounds was detected in *F. japonica* (Table 1). In summary, the highest concentration of total carotenoids, flavonoids, and phenolic compounds were found in ‘Setoka’ (Table 1), and the lowest levels in *F. japonica*. As well, the results indicated that total carotenoids, flavonoids, and phenolic compounds in

Table 1 Quantitative analysis of total carotenoids, flavonoids, and phenolic compounds in citrus using UV–VIS spectrophotometry. At the 0.05 level, the population means are significantly different ($P < 0.05$)

Samples	Line numbers ^a	Compound contents*		
		Total carotenoids (ug g ⁻¹ dry wt)	Total flavonoids (ug g ⁻¹ dry wt)	Phenolic compounds (mg g ⁻¹ dry wt)
FJ	9	0.75 ± 0.09	1.97 ± 0.09	1.26 ± 0.12
CK	9	0.90 ± 0.06	2.85 ± 0.07	6.16 ± 0.44
CMP	9	1.93 ± 0.13	3.02 ± 0.05	5.16 ± 0.15
CMB	9	0.85 ± 0.04	3.01 ± 0.23	4.20 ± 0.17
CP	9	1.02 ± 0.07	2.73 ± 0.24	4.44 ± 0.50
CS	9	3.53 ± 0.34	4.50 ± 0.36	6.29 ± 0.24
CR	9	1.54 ± 0.13	2.35 ± 0.06	3.67 ± 0.17

CMB, *Citrus unshiu* Marc. cv. Miyagawa (rain shelter); CBP, *C. unshiu* Marc. cv. Miyagawa (field); FJ, *Fortunella japonica*; CS, *C. hybrid* 'Setoka'; CP, *C. hybrid* 'Kanpei'; CK, *C. hybrid* 'Kiyomi'; CR, *C. hybrid* 'Shiranuhi'. ANOVA evaluation values are $R^2 = 0.80$, Coefficient Var = 0.558, Root MSE = 0.876, and Data Mean = 1.569

* Data represent the mean ± SD of measurements

^aNine replicates were performed for all samples

citrus cultivated under different conditions differed even in the same citrus cultivar. The results were in accord with other reports that showed large differences in the concentration of functional compounds such as total phenolic content in different citrus cultivars [3–7].

FT-IR spectrum analysis of citrus

When FT-IR spectral data from whole cell extracts of citrus samples were compared, the qualitative and quantitative changes in metabolomic patterns were observed in the following ranges: 1700–1500, 1500–1300, and 1100–950 cm⁻¹ (Fig. 1). These regions of the FT-IR spectra represent qualitative and quantitative information on the presence of phosphorus from nucleic acids and phospholipids, and amino acids and amide bonds I and II of protein; organic acids containing phosphodiester bonds; a carbohydrate family containing a monosaccharide complex polysaccharide, respectively [32–36]. Specifically, differences on the FT-IR spectra indicated qualitative and quantitative differences in amino acids and proteins, fatty acids, and carbohydrates in citrus fruits. Thus, FT-IR spectral analysis of different citrus fruit samples could be a useful tool for investigating levels of specific chemical compounds.

FT-IR spectrum data and multivariate statistical analysis in citrus

To better understand FT-IR spectra variability, PCA of FT-IR was performed by generating two-dimensional plots among citrus samples using two principal components, PC1 and PC2. PCA analysis of the FT-IR spectral data

Fig. 1 Representative FT-IR spectra from citrus fruit samples. FT-IR spectral ranges revealed quantitative information for protein/amides I and II (1500–1700 cm⁻¹, A), phosphodiester groups (1300–1500 cm⁻¹, B), and sugar compounds (950–1100 cm⁻¹, C). Solid or dotted lines and abbreviations represent each citrus fruit species. CMB, *C. unshiu* Marc. cv. Miyagawa (rain shelter); CBP, *C. unshiu* Marc. cv. Miyagawa (field); FJ, *Fortunella japonica*; CS, *C. hybrid* 'Setoka'; CP, *C. hybrid* 'Kanpei'; CK, *C. hybrid* 'Kiyomi'; CR, *C. hybrid* 'Shiranuhi'. Arrows indicate the FT-IR regions showing significant spectral variations between citrus fruit samples

revealed that the overall variation in principal component 1 (PC1) (R2X 0.37, Q2 0.37) and PC2 (R2X 0.093, Q2 0.46) scores were 37.0 and 9.3%, respectively. This result indicated that overall variability was 46.3% when PC1 and PC2 were combined [Fig. 2(A)]. The PCA score from the FT-IR spectra region coincided with the largely different regions observed in the representative fruit FT-IR spectra (Fig. 1). The coincident result is thought to play an important role at the metabolic scale level. Examination of a plot of citrus

Fig. 2 PCA scores and loading values plot of PCA scores from citrus sample FT-IR spectral data: (A) Dotted ellipses represent the clustering boundary of citrus fruit samples with high (CS), medium (CP) and low (FJ) contents of total phenolic compounds and flavonoids. Abbreviations in the PCA score plots represent each citrus fruit sample. Solid and dotted lines represent PC1 and PC2

Fig. 3 PLS-DA score plot of FT-IR data from citrus samples. Dotted ellipses and capitals represent the clustering boundary of citrus fruit samples with (A) low and (B) high contents of functional compounds. Abbreviations in the PLS-DA score plots represent each citrus fruit sample. CMB, *C. unshiu* Marc. cv. Miyagawa (rain shelter); CBP, *C. unshiu* Marc. cv. Miyagawa (field); FJ, *Fortunella japonica*; CS, *C. hybrid* 'Setoka'; CP, *C. hybrid* 'Kanpei'; CK, *C. hybrid* 'Kiyomi'; CR, *C. hybrid* 'Shiranuhi'

sample PCA scores revealed that the citrus samples were divided into two groups on the left and the right sides of the graph according to the PCA score. *F. japonica* and cv. Miyagawa Wase cultivated in the rain shelter were localized on the left side of the plot, while cv. Miyagawa Wase cultivated in the field, 'Kiyomi,' 'Kanpei,' 'Setoka,' and Shiranuhi' were found on the right side of the PCA score plot [Fig. 2(A)]. Other citrus samples containing middle range of contents of the three compounds were located in the upper of right quadrant of the plot (Fig. 3).

scores, respectively; (B) Arrows indicate the FT-IR regions that were important in citrus fruit sample clustering. The arrows were same factor of A, B and C from FT-IR spectra data. CMB, *C. unshiu* Marc. cv. Miyagawa (rain shelter); CBP, *C. unshiu* Marc. cv. Miyagawa (field); FJ, *Fortunella japonica*; CS, *C. hybrid* 'Setoka'; CP, *C. hybrid* 'Kanpei'; CK, *C. hybrid* 'Kiyomi'; CR, *C. hybrid* 'Shiranuhi'

The correlation between the differences in the contents of the three bioactive compounds among the citrus samples (Table 1) and the locations on the PCA score plot were investigated. The correlation coefficient (R^2) from the analysis was 0.80. *F. japonica* revealed the lowest level of total carotenoids, flavonoids, and phenolic compounds, and was localized to the left portion of the plot as an individual group. 'Setoka,' which contained the highest levels of total carotenoids, flavonoids, and phenolic compounds, was located on the right side of the plot. The location of citrus samples on the PCA score plot were located on the different portion according to the contents of three bioactive compounds. Thus the PCA score plot suggest a correlation between location on the PCA score plot and concentration of total carotenoids, flavonoids, and phenolic compounds was detected.

To search important regions on the FT-IR spectrum for the clustering of citrus samples on the PCA score plot according to content of the three compounds, the FT-IR spectral region important for the determination of PC1 and PC2 was investigated [Fig. 2(B)]. Loading value analysis revealed the 1700–1500 and 1500–1300 cm^{-1} regions of the FT-IR spectra were important for determining PC1 because it plays a critical role in the classification of the right and left sections of the PCA plot. The 1100–950 cm^{-1} region played a significant role in determining PC2 for discriminating between the top and bottom samples [Fig. 2(B)]. Further, these regions of the FT-IR spectra coincided with regions observed on the citrus fruit FT-IR spectra that differed markedly (Fig. 1). The differences in amides I and II and in the carbohydrate group compounds play an important role in the discrimination of

citrus fruits at the metabolomic level [Fig. 2(B)]. In particular, PC2 plays a key role in both the classification and discrimination of citrus samples containing different levels of bioactive compounds. The qualitative and quantitative differences in the carbohydrate compounds significantly affected PC2, which implied that quantitative changes in the primary metabolic components of sugar contents strongly correlated with qualitative and quantitative alterations of secondary metabolic factors.

The results indicated that PLS-DA (comp1: R2X 0.4665, Q2 0.4665, comp 2: R2X 0.1414, Q2 0.6079) analysis could more discriminate the contents of citrus samples than PCA analysis (Fig. 3). The cluster boundaries among citrus samples were narrower in the PLS-DA analysis than in the PCA analysis, and replicates belonging to the same sample clustered more tightly. Consequently, PLS-DA analysis was a more useful tool for improved discrimination of citrus fruits than PCA analysis. The results indicated that both PCA and PLS-DA analyses suggest a correlation between the contents of total carotenoids, flavonoids, and phenolic compounds and the spatial locations on the citrus sample score plots. Thus FT-IR spectra and both PCA and PLS analyses could be used to control the citrus fruit quality.

PLS regression modeling for the contents prediction of total carotenoids, flavonoids and phenolics

PLS regression analysis was performed for the prediction of total carotenoid, flavonoid, and phenolic compound content using FT-IR spectra and UV-VIS spectrophotometry (Fig. 4). For total carotenoids, the resulting correlation coefficients (R^2) were 0.99 using both the component values estimated from FT-IR spectra following PLS modeling, and the contents measured from the same samples [Fig. 4(A)]. For total flavonoids and phenolic compounds, the respective R^2 values were also 0.99 [Fig. 4(B, C)]. These results indicated that the quantitative predictions of total carotenoids, flavonoids, and phenolic compound contents could be estimated with approximately 90% accuracy from the citrus fruit FT-IR spectral data alone.

A model for predicting citrus fruit contents on the carotenoid has been reported [4]. According to carotenoid species, R^2 values were 0.73–0.91. For carrots with a high carotenoid content, the R^2 was 0.9 following analysis of the correlation between FT-IR spectral data and total carotenoid contents [37]. Additionally, R^2 values for the contents of both total flavonoids and phenolic compounds were 0.94 [38]. For the African yam, R^2 values for the concentration of total carotenoids, flavonoids, and phenolic compounds were 0.72–0.83 [26], which is very low compared to the results obtained for citrus in this study. The present results demonstrate that the correlation coefficient

Fig. 4 Linear regression analysis results of estimated and predicted values of total carotenoids, flavonoids, and phenolic compounds. Values of total (A) carotenoids, (B) flavonoids, and (C) phenolic compounds determined by the PLS regression model from FT-IR spectral data. The regression coefficient values (R^2) of the three compounds were 0.99, respectively

of bioactive compounds content in citrus was higher than that of component correlation coefficients in previous research of other plants.

Because the quantitative analysis of bioactive compounds requires an extended length of time and is difficult

when handling a large volume of samples, FT-IR spectra and PLS-DA analysis could be useful tools for the discrimination of citrus cultivars and cultivation conditions based on the concentration of total carotenoids, flavonoids, and phenolic compounds. Further, PLS regression modeling from FT-IR spectral data is easy to apply, cost effective, and enables the rapid prediction of three bioactive compounds when using the model described in this study. The bioactive compounds content prediction modeling technique described in this study enables the use of an evaluation tool for high-quality trait characteristics for a majority of unidentified citrus samples. This could be a useful rapid selection method for citrus samples or outstanding citrus lines. Nevertheless, further studies involving a larger number of citrus cultivars and citrus samples cultivated under varying conditions could improve the accuracy of the modeling, despite the successful results demonstrated in this study.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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