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Title
Effects of apple juice manufacturing processes on procyanidin concentration and nondestructive analysis by fluorescence fingerprint

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Abstract
Procyanidins are one of the main polyphenols in apple fruit. In this study, we aimed to increase the amount of extractable procyanidins by micro–wet milling (MWM), a novel milling process that can wet-mill foods to micrometer scale, in addition to commonly applied apple juice manufacturing processes. The effects of milling, pasteurization, centrifugation, and enzymatic treatment on extractable procyanidin concentration were investigated, and MWM was shown to increase the procyanidin concentration by 16.7% compared with mixer milling. Conversely, other processes such as pasteurization, centrifugation, and enzymatic treatment decreased the procyanidin concentration in apple juice. Since procyanidin concentrations in apple juice are also affected by the variability between individual apples, we attempted to nondestructively estimate the procyanidin concentration at each process of apple juice manufacturing by obtaining the fluorescence fingerprint (FF). The FFs are a set of fluorescence spectra acquired at consecutive excitation wavelengths. Partial least-squares regression was used to estimate the procyanidin concentrations of apple juice from the FFs, and the most accurate model was able to estimate procyanidin concentration at the quality control analysis level.

Keywords
excitation–emission matrix; nondestructive measurement; polyphenols; wet milling; pasteurization; enzymatic treatment

Abbreviations
MWM: micro–wet milling; FF: fluorescence fingerprint; EEM: excitation-emission matrix; CFU: colony-forming units; HPLC: high-performance liquid chromatography; VIP: variable importance in projection; RPD: residual predictive deviation; RMSEP: root-mean-square error of prediction

Declarations

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The authors declare no conflicts of interest or competing interests.

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Not applicable

Code availability
Not applicable

Authors' contributions
Okino, S. conceived of the presented idea, carried out the experiment, analyzed the data, and took a lead in writing the manuscript. Kokawa, M. conceived of the presented idea, analyzed the data, and wrote the manuscript. Islam, M.Z. conceived of the presented idea and aided in interpreting the results. Kitamura, Y. conceived of the presented idea and aided in interpreting the results. All authors discussed the results and commented on the manuscript.

Introduction

Apples are one of the most consumed fruits in the world, and approximately 2000 varieties of apples are grown in Japan. Apples contain high levels of polyphenols, which are composed of various chemical components, such as catechins, procyanidins, phenolic acid, dihydrochalcones, and flavanols (Renard et al. 2017). Among these, procyanidins account for 69–87% of apple polyphenols (Guyot et al. 2002). Procyanidins are oligomers obtained by the polymerization of polyphenols, such as catechin and epicatechin, and there are various isomers depending on the bonding degree. They are known to be poorly absorbable, but nonetheless exhibit a very strong antioxidant effect. In addition to antioxidant activity, they have various functions, such as suppression of blood glucose elevation, and antiallergic and anticancer activities (Yamashita and Ashida 2016).

Most polyphenols, including procyanidins, exist in the vacuoles of apple cells. Plants cells are protected by cell walls whose main component is cellulose, and since humans do not produce cellulolytic enzymes, ingestion of large amounts of intracellular polyphenols requires the destruction of cells by chewing or grinding. Therefore, it is very reasonable to ingest apple procyanidins in the form of apple juice, where the apple cells are crushed and ground. Apple juice is manufactured through various processes, such as crushing, squeezing, centrifugation, clarification, and sterilization, and these processes affect the content of procyanidin in the apple juice (Wojdylo et al. 2008).

In contrast, milling the apple flesh to micrometer order may increase the amount of extractable procyanidin in the juice. From this perspective, a new milling technique called micro–wet milling (MWM) was used to mill the apple fruit and produce juice with higher procyanidin concentration. MWM is a modified electric stone mill, where millstones with grooves can efficiently cleave materials into small particles (Li et al. 2018). MWM has been applied to develop rice slurry with fine rice particles (Koyama and Kitamura 2014), produce concentrated orange juice with higher nutritional values and antioxidant activity (Islam et al. 2017), and increase the amount of phosphatidic acid in Japanese mustard spinach (Li et al. 2017).

In addition to changes due to processing, the procyanidin contents in individual apples vary considerably depending on the variety, climate, ripeness, and cultivation and preservation methods. Therefore, to produce apple juice with added value such as higher procyanidin concentration, it is necessary to measure the procyanidin concentration in individual juices. Chemical analyses such as high-performance liquid chromatography (HPLC) may be used for this purpose, but these methods are time–consuming.

Consequently, many studies on nondestructive analysis using light sensing technology for component analysis in food have been reported, and application to beverages has also

been performed (Yu et al. 2009; L. J. Xie et al. 2008; L. Xie et al. 2009; Shen et al. 2010; Lorenzo et al. 2009; Liu and He 2009; Di Egidio et al. 2010; Castritius et al. 2010). Since procyanidins show fluorescence emission, fluorescence measurement is potentially effective for estimating their concentrations in apple juice (Karoui and Blecker 2011). Furthermore, the fluorescence fingerprint (FF), also known as the excitation-emission matrix (EEM), could provide more comprehensive information about the fruit juice. FFs are a set of fluorescence spectra acquired at consecutive excitation wavelengths (Jiang et al. 2010), giving a three-dimensional diagram consisting of fluorescence intensity values at different excitation and emission wavelengths. The advantage of the FF technique over conventional fluorescence spectroscopy is that it includes the signals of many fluorescent constituents existing in the sample. Many studies using the FF technique to evaluate various food stuffs, such as olive oil (Guimet et al. 2006), beer (Sikorska et al. 2008), cheese (M. Kokawa et al. 2015), soymilk (Mito Kokawa et al. 2017), and apples (Trivittayasil et al. 2018), have been reported. Trivittayasil et al. (2017) reported that a model for estimating the active oxygen-scavenging ability of peach extract was constructed from the fluorescence patterns of epicatechin and procyanidin (Trivittayasil et al. 2017).

In this study, the fluorescence characteristics of MWM and mixer-milled apple juice were examined, and models to estimate total procyanidin concentration from FF data were constructed. Samples of apple juice prepared by various processes, such as crushing, squeezing, centrifugation, enzyme treatment, clarification, and pasteurization, were targeted, and prediction models that can handle apple juice in various processing stages were constructed.

2. Materials and methods

2.1. Preparation of apple juice

Figure 1 shows a schematic diagram of apple juice production. In this study, apples of the ‘Fuji’ variety were purchased from York Benimaru Co., Ltd., between September 2019 and January 2020 and were used as raw materials for all experiments. After washing the apples with water, the cores were removed and the apples were divided into 12 equal parts using a fruit cutter having an inner diameter of 10 cm. L(+)-ascorbic acid (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to the apple pieces to prevent browning, to a final concentration of 1.0% (w/w). The mixture of apple pieces and L(+)-ascorbic acid was milled with a blender (Commercial Food Blender 990, Hamilton Beach Brands, Inc., Washington, D.C., Unites States) for 2 min to obtain a coarse slurry. Half of this slurry was directly supplied to the MWM system. Then the

lower mill was rotated at a rotation speed of 50 rpm to perform fine grinding of the remaining slurry. The milling conditions of the MWM were optimized on the basis of the results of pre-experiments to minimize the particle size of the slurry.

The slurry obtained by each milling treatment was filtered with a polyethylene mesh sieve (925 μm mesh, AS ONE, Japan) to separate the juice from the pomace. The juice obtained from the coarse slurry will hereafter be referred to as "mixer-milled apple juice", whereas that obtained from the MWM slurry will be referred to as "MWM apple juice". The following processes, namely, centrifugation, cellulase treatment, clarification, and pasteurization, were individually performed on both the mixer-milled and MWM juices.

For centrifugation, 40 mL of juice was dispensed into a 50 mL conical tube and centrifuged for 5 min at 3000 rpm or 5000 rpm using a centrifuge (CN-1050, AS-ONE). The supernatant was used as the centrifuged sample. Centrifugation conditions were decided on the basis of the results of a previous study (Iino and Watanabe 1986) and pre-experiments.

Cellulase treatment was performed following the method reported earlier (Center 2005). Both the MWM apple juice and mixer-milled apple juice (30 mL) were dispensed into 50 mL conical tubes and cellulase (Tokyo Chemical Industry Co., Ltd.) was added at a concentration of 2.0% (w/w), followed by vortexing. The sample was slowly shaken at 100 rpm for 3 h in a water bath at 55±2℃. A portion of the cellulase-treated juice was centrifuged at 3000 rpm for 5 min with a centrifuge (CN-1050, AS-ONE), and the supernatant was collected. This was referred to as the clarified juice.

For pasteurization, 5 mL of juice was dispensed into a test tube and heated at 65±2℃ for 10 min or at 95±2℃ for 1 min. The conditions were determined by pre-experiments, and it was verified that the number of colony-forming units (CFU) in 1 mL of the pasteurized juices was below 100. Pasteurization was performed in a water bath and the time was measured as soon as the center temperature of the sample reached the target temperature. The samples were cooled with cold water immediately after pasteurization. All apple juice samples were frozen and stored at –60℃ until FF measurement. HPLC analysis was carried out immediately after processing.

2.2. Measurement of total procyanidin concentration by HPLC

For both the mixer-milled and MWM apple juices, samples were obtained for procyanidin measurement after each process. Centrifugation, cellulase treatment and clarification, and pasteurization were performed on four batches of apples each, and for all experiments, the mixer-milled and MWM apple juices prepared by only milling and filtration were used as control samples. Extraction and HPLC analysis conditions

followed those of Obara et al. (2016). All reagents used were HPLC-analysis-grade products manufactured by FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Apple juice samples (9 mL) were mixed with 5 mL of acetone and shaken with a shaker for 15 min. The mixture was centrifuged at 3600 rpm for 10 min and the supernatant was recovered to obtain the first apple juice extract. Pure acetone (8 mL) was added to the precipitate and the mixture was shaken with a vortex and centrifuged under the same conditions as above to obtain the second extract. This operation was repeated, and the first to third extracts were mixed to obtain a total of 30 mL of apple juice extract. This extract was injected into a 2 mL syringe and filtered with a 0.45 μm PTFE syringe filter (AS ONE Corporation, Osaka, Japan).

The filtered apple juice extract was poured into vials (Shimadzu GLC Ltd., Osaka, Japan) of approximately 1 mL, and the target components were detected by HPLC (Shimadzu Corporation) with an RF-20AXS fluorescence detector (Shimadzu Corporation). A size exclusion column (Inertsil® WP300 Diol, GL Science Inc.) [i.d. 4.6 × 250 mm; 5 μm] was used at 30℃. Acetonitrile–acetic acid solution (98:2, v/v, hereinafter mobile phase A) and methanol–water–acetic acid solution (95:3:2, v/v/v, hereinafter mobile phase B) were used as mobile phases. Gradient elution was performed using the two types of mobile phases (from 0 to 3 min: 0–7% mobile phase B; from 3 to 60 min: 7–30% mobile phase B; 60 to 70 min: 30–100% mobile phase B; 70 to 80 min: 0% mobile phase B). The sample injection volume was 5 μL and the flow rate of the mobile phase was 1.0 mL/min. The procyanidins were detected by setting the fluorescence excitation and emission wavelengths to 230 and 321 nm, respectively. The gain of the photomultiplier was set to × 4 between 29 to 65 min. From the peak area of the obtained chromatogram, the total procyanidin concentration was calculated on the basis of a calibration curve prepared with a standard procyanidin B2 product (EXTRASYNTHESE). The total procyanidin concentration was represented by the sum of procyanidin dimers to octamers and represented as the amount of procyanidin B2. The total procyanidin concentration was expressed as weight (mg) in 100 mL of apple juice.

2.3. Statistical analysis

For both the mixer-milled and MWM apple juices, samples were obtained and analyzed after milling and filtration, centrifugation, cellulase treatment, clarification, and pasteurization. Four samples were made for each process, and the total procyanidin concentration was calculated as relative values against the control sample (mixer-milled apple juice undergoing milling and filtration). The paired-samples *t*-test was performed to compare the two corresponding groups (samples made from the same apples) using

statistical analysis software JMP13 (SAS Institute Inc.), and the significance was evaluated at three levels (*: p <0.05, **: p <0.01, ***: p <0.001) for the pairs in which significant differences were observed.

2.4. Fluorescent fingerprint measurement

The frozen apple juice samples were thawed at room temperature before measurement. The FFs were measured using a fluorescence spectrophotometer (F-7000, Hitachi High-Technologies Corporation) using data acquisition software (FL Solutions 4.0, Hitachi High-Technologies Corporation). A quartz cell was filled with 3 mL of apple juice sample, and set at a front-face angle (Becker et al. 2003). FFs were obtained in the excitation wavelength range of 200 to 500 nm and the emission wavelength range of 200 to 700 nm, at 10 nm intervals. The scan speed was 30000 nm/min, the slit width was 5.0 nm, the photomultiplier voltage was 700 V, and the measurement time was 78 s per sample. The measurement was performed three times for one sample, and a total of 216 FF datasets were obtained.

2.5 Models for predicting procyanidin concentration

To estimate total procyanidin concentration from the FFs, partial least-squares (PLS) regression was performed using numerical analysis software (MATLAB R2018a, MathWorks) and multivariate analysis add-on software (PLS_Toolbox, Eigenvector Research). In this experiment, the FF data of apple juice was used as the explanatory variable, and the total procyanidin concentration obtained by HPLC analysis was used as the objective variable.

The FF and chemical analysis data were divided into a calibration group for model construction and a validation group for model evaluation. Since four samples were made for each process, FF and total procyanidin concentration data from the first three samples were used for the calibration group, and data from the last samples were used for the validation group.

Eighteen models were made by combining three sample conditions (mixer-milled apple juice, MWM apple juice, and both types of juice) and six combinations of the following processes: filtration, centrifugation, and cellulase treatment with or without clarification. The pasteurized samples were always included in the models because pasteurization is an indispensable process when producing apple juice.

FF data were preprocessed by autoscaling (each wavelength was scaled to zero mean and unit variance), and the number of latent variables (LVs) was decided on the basis of the root-mean-square error of cross-validation (RMSECV). The venetian blinds method

(Lenhardt et al. 2015) was used to construct the datasets for cross-validation. Subsequently, verification was performed using the validation group in order to evaluate the performance of the calibration model. As a measure of the predictive power of the model, the residual predictive deviation (RPD) value, which is calculated as the ratio of the root-mean-square error of prediction (RMSEP) to the sample standard deviation, was calculated (Fearn 2002).

$$\mathrm{RPD} = \mathrm{SD} \,/\, \mathrm{RMSEP} = 1 \,/\, \sqrt{1 - \mathrm{R}^2}$$

RPD values between 2.5 and 3.0 enable rough screening, values between 3.1 and 4.9 enable firm screening, and values between 5.0 and 7.9 allow analysis at the quality control level (Williams and Sobering 1993).

To consider which wavelengths were involved in estimating the target component, variable importance in projection (VIP) values, which indicate the importance of variables, were determined (Kvalheim 2010; Rajalahti et al. 2009; Chong and Jun 2005). The VIP value summarizes the importance of each original variable (wavelength conditions), and since the average value VIP value is 1, if VIP> 1, the importance of the variable can be said to be above the average (Chong and Jun 2005).

Results and discussion

3.1. Change in total procyanidin concentration during processing

Figure 2 shows the total procyanidin concentration in the apple juice after each process. The procyanidin concentrations of the centrifuged, cellulase-treated, and pasteurized samples are shown in relative values, where the concentration of mixer-milled apple juice samples made from the same apples are taken to be 100%. This calculation allows us to cancel the difference in procyanidin concentration among individual apples.

The average procyanidin concentrations of the mixer-milled and MWM juices were 29.9 and 34.8 mg/100 mL of juice, respectively. Applying MWM to the apple juice increased the total procyanidin concentration by 16.7%. This increase is due to the incorporation of apple pomace in the MWM juice. The pomace that was removed from the mixer-milled apple juice by filtration contained a 23.7% higher concentration of procyanidins than the mixer-milled apple juice itself (data not shown). The MWM slurry was also filtered to produce MWM juice, but there was little pomace remaining on the filter owing to the small particle size of the MWM slurry. This indicates that the pomace removed from the mixer-milled slurry remained in the MWM juice, increasing the procyanidin concentration. A similar trend was observed when comparing processed MWM juice with their mixer-milled counterparts, and the procyanidin concentration was higher in the MWM juice. The difference was especially large for the pasteurized

samples; the MWM juice showed 23.4 and 20.0% higher procyanidin concentration than the mixer-milled juice for the 65℃ and 95℃ pasteurized samples, respectively. On the other hand, the centrifuged MWM and mixer-milled juices showed similar procyanidin concentrations. This was because the pomace remaining in the MWM was removed during centrifugation. A study on the effects of different milling methods on polyphenol contents of mulberry juice (Li et al. 2016) also showed that milling methods that decrease particle size produce juice with high polyphenol contents and very small amounts of pomace.

Next, we compare procyanidin concentrations of juice processed by centrifugation, enzymatic treatment, and pasteurization with those of the milled and filtrated juices. To evaluate the effects of each process on procyanidin concentration, MWM juices undergoing the three processes are compared with the filtered MWM juice, and the processed mixer-milled juices are compared with the filtered mixer-milled juice. Centrifugation significantly reduced the total procyanidin concentration in both juices. Reductions after centrifugation were 9.5 and 16.9% under low speed conditions (3000 rpm) and 17.0 and 21.6% under high speed conditions (5000 rpm) for the mixer-milled and MWM juices, respectively, compared with the filtrated juices. The procyanidin concentrations were reduced considerably for the MWM juice at a higher centrifugation rate. A possible reason for this result was stated by Renard et al. (2017), who mentioned that procyanidins bind spontaneously to plant cell wall polysaccharides upon the destruction of plant tissue that occurs during milling, chewing, and heat treatment. Also, it has been reported that orthoquinone produced by browning has high electrophilicity and promotes dimer formation between polyphenols and other substances (Renard et al. 2017). The MWM juice, which also contained the pomace that was filtered out from the mixer-milled juice, contained high concentrations of cell wall substances, and the procyanidin was presumed to be in an environment where it could easily interact with the precipitate. This is the reason for the large decrease in procyanidin concentration from the MWM juice. Higher rotational speed also reduced the cell wall substances in the juice, thereby precipitating the procyanidins with it.

The enzyme treatment reduced the total procyanidin concentration in the mixer-milled juice by 19.9%, and the clarification treatment showed a similar reduction ratio. On the other hand, the enzyme treatment reduced the procyanidin concentration in the MWM juice by 12.5%, and the clarification decreased it by 18.5%. Although cellulase treatment was expected to improve the extraction of procyanidins inside the cells through the degradation of cell wall cellulose, the results showed a reduction in the amount of extracted procyanidin. This decrease may be due to the reaction of enzymes that promoted

312 the oxidization of procyanidins during cellulase treatment. Prior to this study, several
313 enzymatic treatments using different enzymes (pectinase, cellulase, and a mixture of the
314 two) were applied to the apple juice, and this pre-experiment showed that cellulase was
315 most effective in increasing the concentration of procyanidins. However, enzymatic
316 activity is greatly affected by the concentration of the enzyme, as well as temperature and
317 reaction time, and further investigation is required to determine the optimal enzyme
318 conditions.
319 Pasteurization at 65±2℃ reduced the total procyanidin concentration by 2.6 and 5.7%
320 for the mixer-milled and MWM apple juices, respectively. Similarly, pasteurization at
321 95±2℃ reduced the procyanidin concentration by 4.7 and 10.1% for the mixer-milled and
322 MWM apple juices, respectively. It has been confirmed that the procyanidin
323 concentrations in general processed products decreases with time when heated and stored
324 at pH 4.0 or higher (Morifuji et al. 2013). Our findings were in accordance with the results
325 reported by De Paepe et al. (2014), where procyanidin concentrations decreased during
326 thermal treatment with degradation rate coefficients (k) between 0.39×10^{-2} and
327 0.90×10^{-2} s^{-1}, depending on the isomer. Since both pasteurization conditions were
328 effective for decreasing the number of CFU to below 100 in 1 mL of pasteurized juice,
329 pasteurization at 65℃ for 10 min was shown to be better for maintaining the procyanidin
330 concentration.
331
332 3.2. Estimation of fluorescent components
333 Figure 3 shows the average FFs of mixer-milled (108 samples) and MWM (107
334 samples, with one deleted sample due to measurement problems) juices after each of the
335 processes. The two FFs were similar to each other, and strong fluorescence peaks, which
336 are considered to indicate procyanidins, were observed at excitation (Ex)/emission (Em)
337 wavelengths of 210/310 nm and 280/310 nm. These peaks were also observed in the
338 procyanidin standard reagent. In addition to the fluorescence peak of procyanidins, broad
339 peaks were also observed at Ex/Em of around 320–350/420 nm and 400–440/670 nm.
340 These peaks can be related to chlorogenic acid and chlorophyll, respectively (Włodarska
341 et al. 2017). Another strong peak was observed at Ex/Em of 210/350 nm in both samples,
342 which might be related to L(+)-ascorbic acid.
343
344 3.3. Practicality evaluation of estimation model by PLS regression analysis
345 Various PLS regression models for estimating total procyanidin concentration from FF
346 data were constructed by varying the samples included in the models. Various processes
347 included in apple juice production affect not only the procyanidin concentration but also

348 the turbidity and fluidity of the juice. Since these differences also affect the optical
349 properties of the juice, including many samples in the model that have undergone
350 different processes may increase the error of the model. On the other hand, estimation
351 models that cover a large variation of apple juices are more versatile. Therefore, we aimed
352 to determine how the difference in the range of samples affects the performance of the
353 model and to create a standard method for estimating apple juice procyanidin. Table 1
354 shows the combination of processes included in the 18 models, as well as the indices of
355 model performance, RMSECV, RMSEP, coefficients of determination of cross-validation
356 (R^2CV) and prediction (R^2P), and RPD.
357 RPD values were used for evaluating the performance of the models. Model 17, which
358 was constructed on the basis of filtrated and pasteurized mixer-milled juice, showed the
359 highest RPD value of 5.20, which is fit for quality control. In addition, Model 14, which
360 was constructed on the basis of filtrated, cellulase-treated (not clarified), and pasteurized
361 mixer-milled juice, showed performance fit for rough screening (RPD=2.92). These
362 results suggest the possibility of estimating total procyanidin concentration in mixer-
363 milled juices that have undergone similar processes using the FF, although additional
364 samples may be needed to increase reliability of the models.
365 RPD values of the models constructed from mixer-milled juice decreased significantly
366 when centrifuged samples were included in the model and were lower than the RPD
367 values of models constructed from MWM samples. This is because of the large
368 differences between the samples that were centrifuged and those that were not. When cell
369 wall substances are present in the apple juice, some of the procyanidins are bound to these
370 substances and cannot be extracted, and therefore are not included in the HPLC
371 measurement results. However, these bound procyanidins also emit fluorescence during
372 FF measurement; thus, there is a difference between procyanidin concentrations
373 measured by HPLC and those measured with FF. On the other hand, in the centrifuged
374 sample, the bound procyanidins are transferred to the precipitate, and most of the
375 procyanidins remaining in the supernatant can be extracted. Therefore, in the centrifuged
376 samples, the procyanidin concentrations measured by HPLC are equal to those measured
377 with FF.
378 The RPD values of the models based on the MWM samples did not change
379 considerably with different processes and remained lower than the practical level. This
380 may be due to physical factors such as increased turbidity and decreased fluidity
381 (Lakowicz 2006) owing to the milling process as well as changes in chemical components
382 that contribute to the estimation model.
383 Finally, the RPD values of the models created using both mixer-milled and MWM

384 samples were generally low and did not change much with the addition of different
385 processes. The reason may be similar to the effect of centrifugation: mixer-milled and
386 MWM juice samples contained different ratios of cell-wall-bound procyanidins, which
387 affected the ratio of total procyanidins (measurable with FF) to extractable procyanidins
388 (measurable by HPLC). Considering these results, it can be concluded that it is
389 undesirable to evaluate fruit juices that have been milled by different methods using a
390 single model. Alternatively, the juices could be diluted and filtered before FF
391 measurement, to eliminate the effects of insoluble particles. Although the initial objective
392 of this study was to develop a non-destructive measurement method where the samples
393 can be measured directly, combining these preprocessing procedures with FF
394 measurement may lead to a simple and accurate method which could be applicable to a
395 large range of samples.
396 Scatterplots of estimated and measured values of total procyanidin concentration for
397 models 14 and 17 are shown in Fig. 4a and 4b. The RMSEP of model 17 was 2.50 mg/100
398 g juice, which is very small compared with the total concentration of procyanidins in
399 apple juice (approximately 30 mg/100 g juice). Pissard et al. (2013) reported that the RPD
400 of the model for estimating the concentration of apple polyphenols from near-infrared
401 spectra was 5.1, which is similar to that of model 17. This indicates that the prediction
402 performance of this model was very high.
403 To estimate important variables contributing to the estimation model, the VIP values
404 of each wavelength condition were shown in the form of FFs (Fig. 4c and 4d). In Model
405 14, peaks with high VIP values were observed at Ex/Em of 240/310 nm, 240/370 nm,
406 310/320 nm, and 490/690 nm. In Model 17, an additional peak was observed at 440/500
407 nm. The three peaks observed at 240 nm and 310 nm were located at slightly longer
408 wavelengths than the procyanidin fluorescence peak, and the peak observed at around
409 480 nm was located at slightly longer wavelengths than the chlorophyll fluorescence peak.
410 On the other hand, there was no strong fluorescence peak corresponding to the peak at
411 440/500 nm, suggesting that chemical components other than the estimated fluorescent
412 component may be used as important variables. The VIP values for the wavelengths
413 corresponding to the procyanidin fluorescence peak (Ex/Em of 210/310 nm and 280/310
414 nm were not high, which may be because multiple components fluoresce at this
415 wavelength, including catechins and epicatechins.
416 For both models, the highest VIP values were seen at 490/690 nm, which suggests that
417 the chlorophyll fluorescence may be related to the estimation model. According to Hagen
418 et al. (2006), flavonoids in apples are mainly concentrated in the chloroplast epidermis
419 where chlorophyll is present, and the absorbance calculated from chlorophyll

fluorescence correlates with the concentrations of anthocyanins and total flavonoids in apple fruits. In this experiment, a positive correlation was observed between the fluorescence peak of chlorophyll and the total procyanidin concentration, which is the reason for the high VIP value of wavelengths related to chlorophyll.

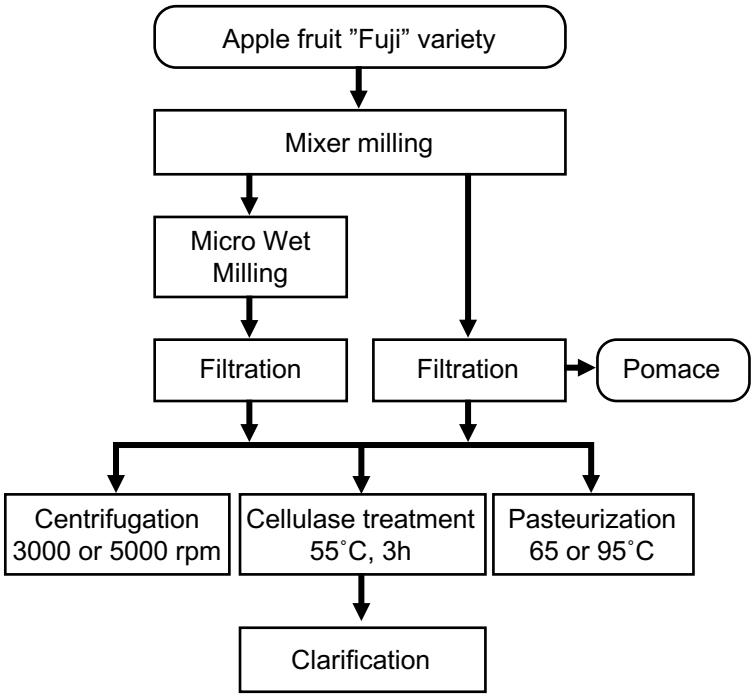
Conclusion

In this study, we investigated the changes in total procyanidin concentration in relation with apple juice manufacturing processes, such as milling, centrifugation, enzymatic treatment, and pasteurization. While most processes decreased the concentration of procyanidins, a novel milling method named micro–wet milling (MWM) increased the procyanidin concentration by 16.7% compared with mixer milling.

In addition, to establish a simple and quick method for estimating the total procyanidin concentration in apple juice, we measured all apple juice samples at various processing stages using the FF. The FFs of the apple juices showed autofluorescence that could be attributed to epicatechin, procyanidin, chlorogenic acid, and chlorophyll. To estimate the procyanidin concentration from FF data, PLS regression analysis models were constructed and their performance was evaluated. Various PLS regression models were constructed by varying the samples included in the models, since models applicable to a large variety of samples would be versatile but may have higher prediction error. The model with the highest performance included mixer-milled samples that were filtrated and pasteurized. Prediction error increased when centrifuged samples were added to the model, and models constructed with MWM apple juice resulted in poor performance, possibly due to physical factors, such as increased turbidity and reduced fluidity.

In this study, we showed that the procyanidin concentration can be estimated using fluorescence spectroscopy for samples prepared under certain conditions. It may be possible to construct models with increased versatility by increasing the number of samples and using techniques such as variable selection, or by adding a preprocessing step before measurement such as dilution and filtration.

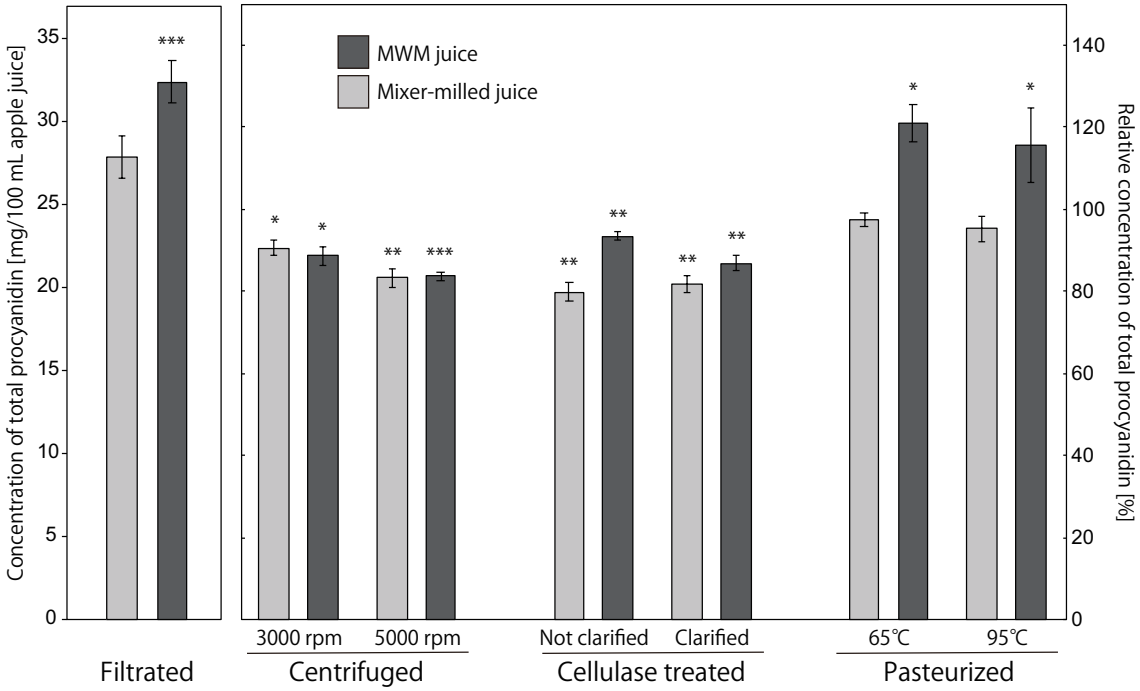
449 **Figures**



450

451 **Fig.1** Flowchart of method for producing apple juice.

452



453

454 **Fig.2** Average concentrations of milled and filtrated mixer-milled and MWM juices,
455 and effects of various processes (centrifugation, cellulase treatment, and pasteurization)
456 on total procyanidin concentration in apple juice. Error bars indicate standard error of
457 relative procyanidin concentration (calculated as the ratio to the filtrated mixer-milled
458 juice made from the same batch of apples). Asterisks indicate significant differences
459 between the sample and the filtrated mixer-milled juice (control, *: $p < 0.05$, **: $p < 0.01$,
460 ***: $p < 0.001$).

461

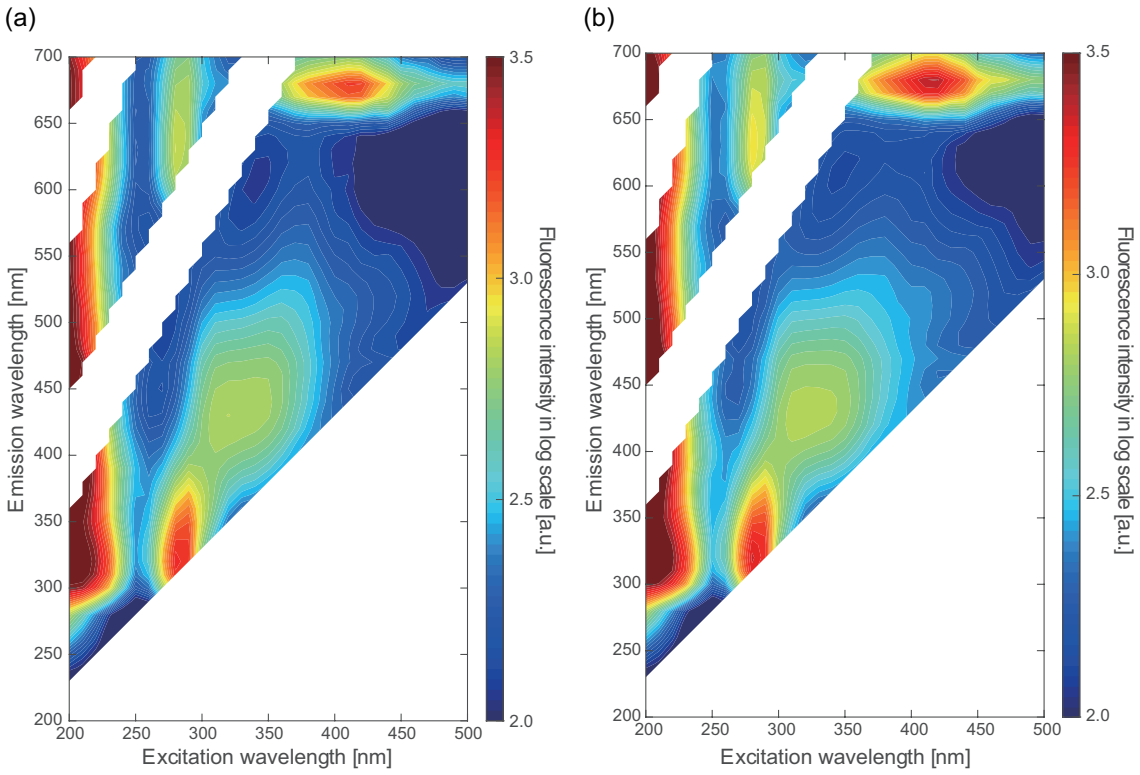


Fig.3 Average fluorescence fingerprints (FFs) of mixer-milled (a) and MWM (b) apple juices. The color axis indicates the fluorescence intensity in log scale.

Table 1 Results of PLS regression analyses of 18 models produced by combining various processed apple juices.

Model No.	Sample condition (Number of samples)	Processing	Number of LVs	RMSECV	RMSEP	R^2 CV	R^2 P	RPD
1	Both juices (215)	filtration / centrifugation / cellulase treatment / clarification / pasteurization	4	2.51	3.47	0.59	0.26	1.16
2	mixer-milled juice (108)		4	2.49	2.93	0.36	0.32	1.21
3	MWM-milled juice (107)		6	2.42	2.79	0.63	0.53	1.46
4	Both juices (191)	filtration / centrifugation / cellulase treatment / pasteurization	5	2.44	3.14	0.63	0.52	1.45
5	mixer-milled juice (96)		4	2.50	3.05	0.38	0.40	1.29
6	MWM-milled juice (95)		5	2.38	2.74	0.61	0.64	1.66
7	Both juices (167)	filtration / cellulase treatment / clarification / pasteurization	5	2.18	2.92	0.69	0.62	1.62
8	mixer-milled juice (84)		7	1.52	2.83	0.72	0.68	1.78
9	MWM-milled juice (83)		9	1.64	3.12	0.78	0.56	1.51
10	Both juices (167)	filtration / centrifugation / pasteurization	6	2.26	3.36	0.68	0.46	1.36
11	mixer-milled juice (84)		6	2.64	3.58	0.44	0.28	1.18
12	MWM-milled juice (83)		7	2.17	3.44	0.69	0.52	1.45
13	Both juices (143)	filtration / cellulase treatment / pasteurization	3	2.65	4.11	0.56	0.34	1.23
14	mixer-milled juice (72)		9	1.15	2.71	0.84	0.88	2.92
15	MWM-milled juice (71)		8	1.77	3.48	0.69	0.57	1.52
16	Both juices (119)	filtration / pasteurization	6	2.03	3.36	0.74	0.69	1.80
17	mixer-milled juice (60)		9	1.17	2.50	0.85	0.96	5.20
18	MWM-milled juice (59)		7	1.56	4.38	0.69	0.45	1.35

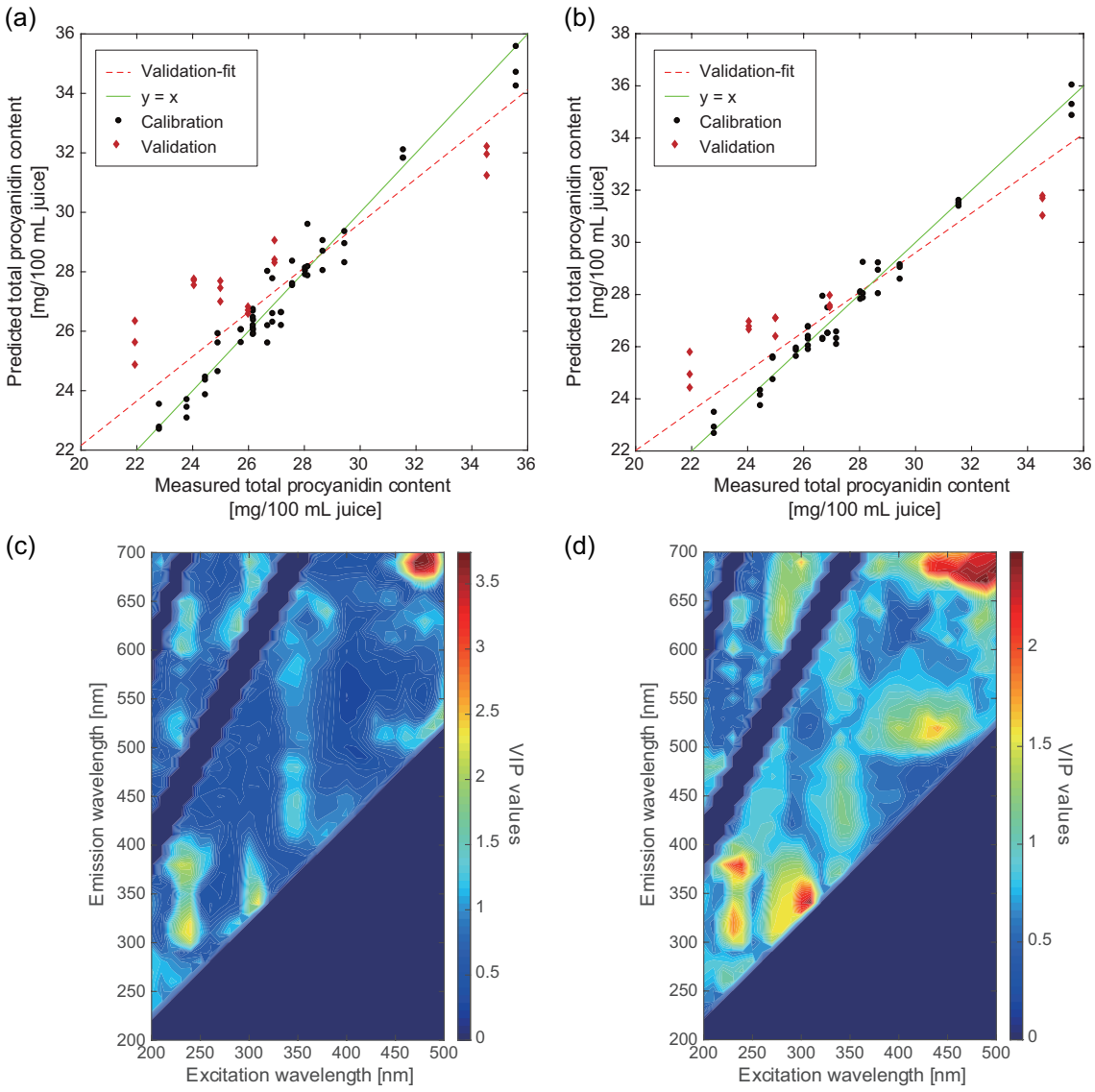


Fig.4 Scatter plots of estimated and measured values of two models created to estimate total procyanidin concentration from fluorescence fingerprint diagrams (a and b) and selected plots of key variables (c and d). Figures 4a and 4c, and 4b and 4d indicate the results of Models 14 and 17, respectively. The number of datasets for models 14 and 17 were calibration: 54, validation: 18, and calibration: 45, validation: 45, respectively.

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