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Comparison of the HPLC Method and FT-NIR Analysis for Quantification of Glucose, Fructose, and Sucrose in Intact Apple Fruits

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A rapid quantification method was developed and validated for simultaneous and nondestructive quantifying the constituent sugar concentrations of intact apples using Fourier transform near-infrared (FT-NIR) spectroscopy in diffuse reflectance mode. Multiplicative scatter correction (MSC), the second derivative of Savitsky−Golay, and mean centering were used as spectral preprocessing options. Calibration models were established by the partial least squares (PLS) regression analysis, and validation of the method was performed according to the high-performance liquid chromatography (HPLC) chromatographic method. Spectral range and the number of PLS factors were optimized for the lowest root-mean-square error of prediction (RMSEP) and correlation coefficient of determination (r). The best models showed satisfactory predictions as measured by the RMSEP and r values: glucose, 0.201 and 0.950; fructose, 0.298 and 0.968; sucrose, 0.335 and 0.969, respectively. FT-NIR analysis of constituent sugar concentrations in the intact apple form was found to be more flexible and much faster than performed with the HPLC method.

KEYWORDS: Constituent sugar concentrations; HPLC; FT-NIR spectroscopy; multivariate analysis; apple fruit

INTRODUCTION

The determination of individual sugar contents in fresh fruits and their juice products is an important food nutrition analysis means for evaluating quality and detecting adulteration or contamination (*1*). Several conventional methods for the determination of constituent sugar concentrations in food are based on refractive index methods, which provide information about the amount of dry matter present in a juice and give a quick appraisal about the total sugar. Alternatively, different volumetric procedures provide information about the total sugar content and the amount of glucose and fructose (*2*). To quantify the specific concentration of each carbohydrate, several modern instrumental methods can be employed, including enzymatic analysis, chromatographic methods, and electrochemical or spectrometric methods (*3*, *4*). While chromatographic techniques are very accurate, they are tedious and time-consuming and require extensive sample preparation.

The low molar absorptivity of near-infrared (NIR) bands permits the measurement of solid samples with little or no sample preparation, thus avoiding manipulation errors. Some papers have been published on the NIR analysis of constituent sugar concentrations in aqueous solutions of fruit juice. Measurements of inner components for growing Japanese pear fruit were reported byYamaki et al. (*5*−*7*) for sugars, hemicellulose, and cell-wall polysaccharides and monosaccharides, but these were complex chemical analyses and therefore impractical methods for use in the orchard.

Measurements of constituent sugars of agricultural products by NIR were reported by Kawano et al. (*8*) for sucrose content in sugar cane juice, by Lanza and Li (*9*) for sugar content in fruit juices, and by Giangiacomo and Dull (*10*) for individual sugars in aqueous mixtures. Tanaka and Takayuki (*11*) developed the NIR-monitoring techniques for the growth period of Japanese pear fruit based on the constituent sugars in the juice. However, the NIR spectra measurements were only for pear juices by diffuse transreflectance, and therefore, this method is impractical in the online system or in the orchard.

In this research, a comparison of an high-performance liquid chromatography (HPLC) method and Fourier transform near-infrared (FT-NIR) analysis for quantification of glucose, fructose, and sucrose in intact apple fruit were investigated.

Multiplicative scatter correction (MSC), the second derivative of Savitsky−Golay, and mean centering would be used as spectral preprocessing to find the robust calibration models for glucose, fructose, and sucrose. Validation of the method would be performed according to the HPLC chromatographic method.

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Table 1. Statistic Values of Calibration and Validation Sets of Apple Cultivated in 2004[a]

	calibration set				validation set			
property	n[b]	range	mean	SD[c]	n[b]	range	mean	SD[c]
glucose (%)	97	4.50–1.92	3.10	0.60	30	4.49–2.20	3.13	0.56
fructose (%)	97	10.41–4.68	7.24	1.21	30	9.51–5.10	7.52	1.12
sucrose (%)	98	4.78–0.13	1.49	0.97	30	4.05–0.28	1.48	1.02

[a] Unit used, % (g/100 g). [b] n = number of samples. [c] SD = standard deviation.

MATERIALS AND METHODS

Samples. A total of 130 "Fuji" apples cultivated in 2004 were used in this research. The "Fuji" apples came from 12−14-year-old tree orchards in Shandong and Shanxi provinces in the northeast region of China. These apple samples were stored for 2 days at 25 °C and 68% relative humidity to equilibrate before being examined by the FT-NIR measurement and presented a broad range of variation for the calibration purposes. Because apple tissue samples would deteriorate rapidly, all measurements including reference analyses and spectra acquisitions were carried out on the same or next day. **Table 1** summarizes the respective sugar concentrations of the calibration and validation samples.

Reagents. Anhydrous D(+)-glucose, D(−)-fructose, and sucrose, all of analytical grade, were purchased from Sigma−Aldrich (Sigma, St. Louis, MO), and standard stock solutions were prepared from 5 g of the appropriate sugar dissolved in 50 mL of Milli-Q distilled water. Pure solutions of each one of the standard constituent sugar concentrations were used to the calibration sets.

Spectroscopic Measurements and Software. Diffuse reflectance spectra of intact apples were measured using a Nexus FT-NIR spectrometer (Thermo Nicolet Corporation, Madison, WI) equipped with a NIR fiberoptic probe, an interferometer, a cooled InGaAs detector, and a wide-band quartz halogen light source (50 W). Apples were placed steadily upon the fruit holder, with the stem-calyx axis horizontal (*12*). On each apple, a diffuse reflectance spectrum was measured on three evenly spaced equatorial positions and only the averaged spectrum of three spectra per apple was used for analysis. In the head of the bifurcated cable, the source and detector fibers were arranged randomly. Light was guided to the sample by the source fibers, and the diffusely reflected light from the sample was detected and was then received by the detector fibers to the Nexus FT-NIR spectrometer, which has a spectral range of 4000−12 500 cm^{-1}. The mirror velocity was 0.9494 cm/s, and the resolution was 16 cm^{-1}. The total number of data points was 1228 for each spectrum. Prior to calibration, the FT-NIR reflectance data were mean-centered, smoothed with a 25-point polynomial-fit Savitsky−Golay function. The spectrometer was equipped with the software package from Thermo Nicolet, including OMNIC version 6.1a for spectral acquisition and TQ Analyst version 6.2.1 for spectral processing and chemometric analysis (*13*).

HPLC Analysis. Prior to the FT-NIR measurement, all samples were identified by the reversed-phase HPLC to avoid using mislabeled samples. Tissue samples of 50 g were then cut from each apple separately, from the marked areas close to the regions in which the FT-NIR readings had been taken, and were macerated with a manual fruit squeezer. Samples of the filtered juice were then measured for constituent sugar concentrations (glucose, sucrose, and fructose) by the HPLC method. A 10 mL aliquot of apple juice was diluted 6 times by adding 50 mL of water, centrifuged at 1000 rpm for 20 min to remove solids, and passed through a 0.45 μm porosity filter. The injected sample volume was 10 μL. The HPLC settings were as follows: Hypersil NH2 (150 × 4.6 mm i.d.) column, mobile phase of V(acetonitrile)/V(water) = 80:20, flow rate of 1.0 mL/min, run time of 10 min, column temperature of 35 °C, and refractive index detection (model 2414).To check the reproducibility of the HPLC measurements, each sample was measured twice (*14*). Three calibration curves ($R^2 \geq 0.9996$) were established for glucose, fructose, and sucrose respectively, from duplicate determinations of three standard samples at six different constituent sugar concentrations (**Table 2**). The HPLC method was developed using a computer-assisted optimization program, DryLab2000 (LC Resources). The standard errors of HPLC values (%, g/100 g) of each constituent sugar concentration were as follows: glucose, 0.042; fructose, 0.035; and sucrose, 0.032. The values measured by HPLC were of sufficient accuracy for the FT-NIR analysis.

Table 2. Regression Statistical Data of Standard Samples at Six Different Constituent Sugar Concentrations

sugars	regression	R^2
glucose	$y = 5704.5x - 3676.70$	0.9996
frucose	$y = 6160.2x - 2556.4$	0.9998
sucrose	$y = 6451.9x - 3146.67$	0.9999

Multivariate Data Analysis. Calibration models for the FT-NIR spectral data were constructed by using the partial least squares (PLS-1) algorithm. All calculations were performed in TQ analyst (version 6.21, Thermo Nicolet). All data matrices were mean-centered and further transformed with the multiplicative scatter correction (MSC) or second-derivative method according to the algorithm proposed by Savitsky and Golay.

The 130 original samples were split into two groups. A total of 30 samples were for external validation, and the rest of the 100 samples were used for calibration. The samples presenting extreme values for each property were included in the calibration set. Full cross-validation following the leave-one-out procedure was performed to determine the optimum number of factors for the model and to detect any outliers (*15*). External validation was performed, and the models with best predictive abilities were selected on the basis of the root-mean-square error of prediction (RMSEP) (eq 1), the lowest mean percent error (MPE) (eq 2), and the higher validation correlation coefficient (eq 3)

$$\mathrm{RMSEP} = \sqrt{\frac{1}{I_\mathrm{p}}\sum_{i=1}^{I_\mathrm{p}}(\hat{y}_i - y_i)^2} \quad (1)$$

$$\mathrm{MPE} = \frac{\sum(|\hat{y}_i - y_i|/y_i)}{I_\mathrm{p}} \times 100\% \quad (2)$$

$$r = \frac{\sum_i(\hat{y}_i - y_i)^2}{\sqrt{s^2(y)s^2(\hat{y})}} \quad (3)$$

where RMSEP is the root-mean-square error of prediction, r is the correlation coefficient between the estimated and predicted values; $s^2(y)$ and $s^2(\hat{y}_i)$ are, respectively, the variances for the measured and predicted values for the property y, $\hat{y}_i$ is the predicted value of the ith observation, y_i is the measured value of the ith observation, and I_p is the number of observations in the prediction set.

Variable Selection Techniques. Wavelength selection is about selecting a subset of spectral regions with which the established calibration model gives the minimum errors in prediction. Wavelength selection not only enhances the stability of the model resulting from the collinearity in multivariate spectra but also helps in interpreting the relationship between the model and the sample compositions. The selection of an appropriate portion of the spectra is also crucial to the performance of a calibration model.

In this research, according to our FT-NIR spectrometer, the spectra were divided into three regions and calibrations were performed for each spectral region as well as for combinations of them (see **Figure 2A**). Region one ranged from 4000 to 6000 cm^{-1}, region two from

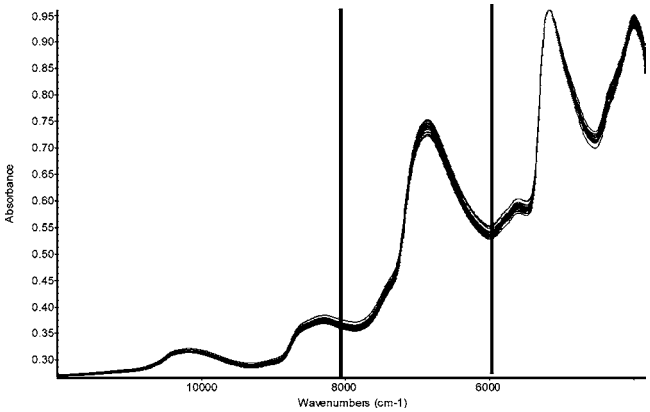

Figure 1. Splitting original spectra as a variable selection method for calibration.

6000 to 8000 cm^{-1}, and region three from 8000 to 12 000 cm^{-1}. **Figure 1** shows that each region has at least one peak and one shoulder. For each region, PLS models with 1−20 factors were investigated. The optimal spectral range and model size were then selected on the basis of the minimum prediction residual error sum of squares (PRESS) computed from the calibration sets with the following equation:

$$\text{PRESS} = \sum_i (\hat{y}_i - y_i)^2 \quad (4)$$

MPE corresponds to the prediction error obtained when the model is applied to an independent validation set of products, which are not employed for calibration. Once obtained, the optimal parameters were applied to the full calibration data set to obtain the final calibration model. Model performance was evaluated by comparing the standard error of calibration and standard error of prediction calculated.

RESULTS AND DISCUSSION

Spectral Features. Figure 2A shows the 130 original spectra collected for the 130 apple samples. The spectra of all samples were quite homogeneous, and no outliers were identified a priori by visual inspection. Consistent baseline offsets and bias were present. These are quite common features in NIR spectra acquired by diffuse reflectance techniques (*16*). In the original spectra, the prominent absorption bands around 6896 and 5154 cm^{-1} were attributed to water absorption. The third absorption band around 8300 cm^{-1} was also found in the original spectra. Vertical differences of absorption among each spectrum were detected around the water absorption bands (*11*). In the second-derivative spectra (**Figure 2D**), several absorption bands in addition to water absorption were observed from 1000 to 6896, 6250 to 5208, and 5000 to 4000 cm^{-1}. Noticeable spectral changes in in each spectrum were observed for the wavenumber region from 5000 to 4000 cm^{-1}, and the spectral differences in this regions in intact apple spectra depended upon sugar absorption.

To clarify the absorption difference between different orchard apples, the second-derivative difference was based on all of samples of calibration sets (**Figure 2D**).

Spectral Pretreatment and Calibration Models. Generally, noise and systematic behavior are undesirable features in the spectra. To solve these probrems, the original spectra were preprocessed by mean smoothing (noise and variable number reduction, **Figure 2B**), followed by MSC (offset reduction, **Figure 2C**) and the second derivative (offset and bias removal, **Figure 2D**). The smoothing window chosen reduced the number of variables and did not eliminate important features of the spectra. It is also noticeable from **Figure 2D** that the second-derivative preprocessing, despite removing bias in the baseline,

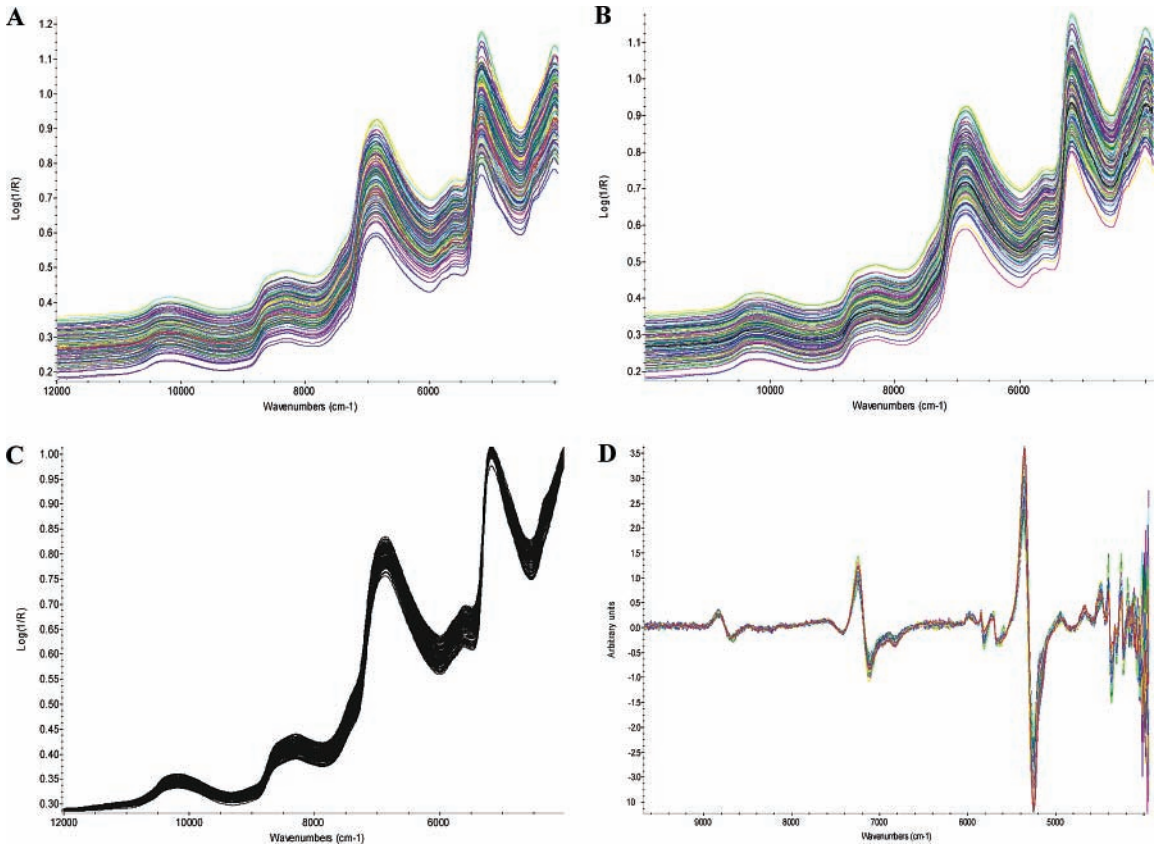

Figure 2. (A) Original spectra of the 130 intact apples. (B) Smoothed spectra by averaging, using a 21-point window. Preprocessed spectra by (C) MSC and (D) the second derivative after smoothing.

Table 3. Calibration Models for Apple Properties with Different Preprocessing Methods for Full Spectra

property	model number	pretreatment	factors	RMSEC	RMSEP	r_{val}	outliers
glucose (%)	1	MSC	14	0.185	0.185	0.954	3
	2	second derivative	11	0.141	0.211	0.930	3
fructose (%)	3	MSC	10	0.274	0.333	0.956	3
	4	second derivative	9	0.226	0.369	0.946	3
sucrose (%)	5	MSC	10	0.361	0.315	0.953	2
	6	second derivative	7	0.309	0.360	0.954	2

Table 4. Summary of Best Calibration Models for Apple Properties by Using the Selected Spectra Region of Full Spectra

property	model number	pretreatment	factors	RMSEC	RMSEP	r_{val}
glucose (%)	7	MSC	8	0.275	0.201	0.950
	8	second derivative	9	0.181	0.187	0.943
fructose (%)	9	MSC	7	0.290	0.298	0.968
	10	second derivative	8	0.166	0.322	0.960
sucrose (%)	11	MSC	10	0.315	0.323	0.953
	12	second derivative	6	0.254	0.335	0.969

has added any noise to the spectra, which, as shown later, resulted in relatively poor calibration models. The preprocessed full spectra (no variable selection) were submitted to the PLS-1 calibration for total sugar content, fructose content, glucose content, and sucrose content. **Table 3** shows the results from different spectral pretreatment to the calibration models.

Good calibration models were obtained by using the full spectra (4000−12 000 cm^{-1}; **Table 3**). The models (1, 3, and 5) from the MSC-processed spectra were consistenly better than those models (2, 4, and 6) from the second-derivative spectra, as measured by RMSEP and r_{val}, with the exception of model 1, whose r_{val} was not the same as that for model 2. However, models 1 and 5 used a higher number of factors than others. Second-derivative preprocessing presented no good models (models 2 and 6), with the only exception being model 1. For all three sugars, the models from the MSC-pretreated spectra had a greater number of factors than those from the second-derivative spectra.

Selection of the Optimum Calibration Models. Different calibration models were investigated to find the optimum model. The quality of these models was evaluated by the root-mean-square error of calibration (RMSEC) and the RMSEP. The *F* test ($\alpha = 0.01$) was applied to determine the statistical significance of outliers. Two or three outliers were found in the calibration sets (**Table 3**). The optimal spectral regions, number of factors, RMSEC, and RMSEP for each sugar are summarized in **Table 4**.

Results from fragments of the spectra presented better predictive abilities than their counterparts with full spectra (**Table 4**). For this property, models 1, 3, and 5 had shown better predictions for the validation samples than models 2, 4, and 6. Again, the second-derivative preprocessing did not result in good calibration models compared to those from MSC. **Figure 3** shows the HPLC-measured values versus FT-NIR-predicted values from the best calibration model for each property. The black solid circles represent calibration samples, and the open circles represent prediction or validation samples.

Calibration models for the glucose component are different to those for fructose, in that calibration models are obtained from both a combination band and short wavelength spectra, from 4000 to 6000 cm^{-1} and from 8000 to 12 000 cm^{-1}. Glucose was measured very well with the RMSEC and RMSEP values of 0.181 and 0.187%, respectively. The corresponding concentration correlation plots are presented in **Figure 3A**.

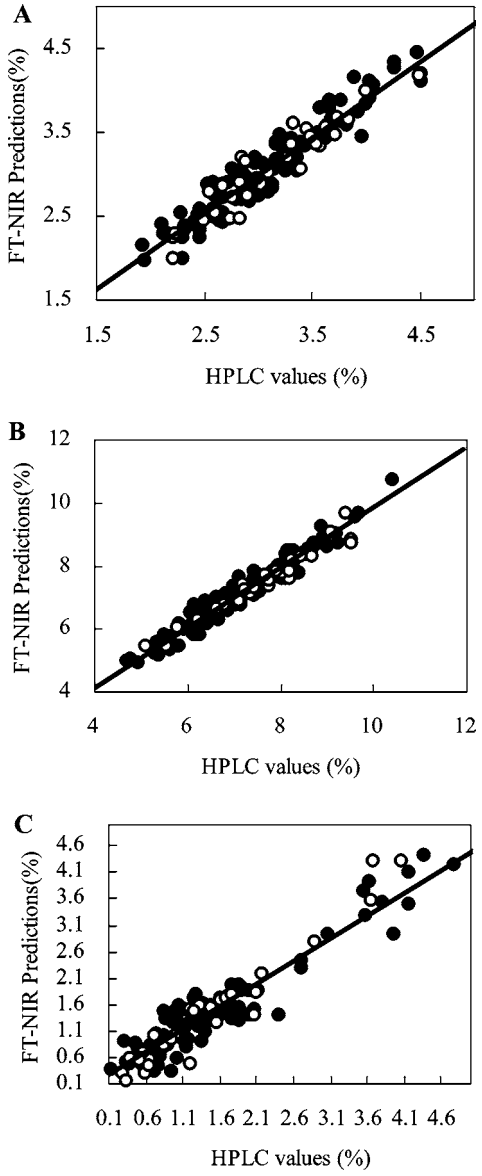

Figure 3. Correlation statistics between the measured data by the FT-NIR and HPLC methods. (A) Glucose (model 1), (B) fructose (model 9), and (C) sucrose (model 12). (●) Calibration samples, and (○) external validation samples.

Table 5. Comparison of the Data Obtained by the HPLC and FT-NIR Methods

sugars	glucose (g/100 g)			fructose (g/100 g)			sucrose (g/100 g)		
number	HPLC	FT-NIR	bias	HPLC	FT-NIR	bias	HPLC	FT-NIR	bias
1	2.55	2.53	–0.02	7.08	7.05	–0.03	2.07	1.58	–0.49
2	3.39	3.17	–0.22	7.14	6.82	–0.32	1.33	1.06	–0.27
3	2.49	2.80	0.31	7.72	7.44	–0.28	1.48	1.92	0.44
4	2.66	2.47	–0.19	6.77	6.64	–0.13	0.58	0.21	–0.37
5	3.71	3.64	–0.07	7.73	7.41	–0.32	0.83	0.98	0.15
6	3.31	3.31	0.00	5.76	5.88	0.12	1.20	0.91	–0.29
7	2.73	2.98	0.25	5.53	5.85	0.32	0.64	0.47	–0.17
8	2.76	3.11	0.35	6.52	6.58	0.06	1.19	1.69	0.50
9	3.99	3.67	–0.32	8.22	8.31	0.09	0.55	0.82	0.27
10	2.83	2.65	–0.18	6.60	6.67	0.07	0.71	1.10	0.39
11	2.89	2.78	–0.11	8.18	7.77	–0.41	1.24	1.16	–0.08
12	2.83	2.97	0.14	7.32	7.24	–0.08	1.60	1.85	0.25
13	4.49	4.14	–0.35	7.83	7.78	–0.05	2.11	1.63	–0.48
14	3.44	3.33	–0.11	5.10	5.15	0.05	0.28	0.49	0.21
15	3.83	3.74	–0.09	6.2	6.26	0.06	1.55	0.86	–0.69
16	2.84	3.13	0.29	8.02	7.53	–0.49	0.99	0.94	–0.05
17	3.56	3.58	0.02	8.17	7.68	–0.49	1.67	1.72	0.05
18	3.32	3.29	–0.03	8.65	8.42	–0.23	0.52	0.82	0.30
19	3.73	3.54	–0.19	6.6	6.68	0.08	2.16	1.91	–0.25
20	3.09	3.18	0.09	7.67	7.74	0.07	0.37	0.68	0.31
21	3.31	3.12	–0.19	7.08	6.66	–0.42	1.48	1.94	0.46
22	3.53	3.29	–0.24	7.4	7.21	–0.19	0.32	0.18	–0.14
23	3.63	3.35	–0.28	8.44	8.46	0.02	2.1	1.82	–0.28
24	2.88	2.9	0.02	7.18	7.62	0.44	1.75	2.08	0.33
25	3.48	3.69	0.21	7.21	7.4	0.19	0.51	0.43	–0.08
26	2.2	2.07	–0.13	9.51	8.82	–0.69	0.93	0.92	–0.01
27	3.26	3.62	0.36	9.49	8.8	–0.69	3.64	3.76	0.12
28	2.21	2.21	0.00	9.37	9.66	0.29	4.05	3.92	–0.13
29	2.23	2.10	–0.13	9.08	9.19	0.11	2.89	3.04	0.15

The calibration model 9 gave excellent fructose prediction results with the MPE of 2.90%. Fructose was measured very well over the 6000−12 000 cm^{-1} spectra region with the RMSEC and RMSEP values of 0.290 and 0.298%, respectively (**Figure 3B**). The optimal spectral range includes a unique absorption peak for fructose at 6318 cm^{-1}. Calibration models from the 6000−12 000 cm^{-1} spectra region generally outperform models from the 4000−6000 cm^{-1} spectra region.

Calibration models for the sucrose component are similar to those for fructose, in that the calibration models were obtained from both the first overtone and short wavelength region. For glucose calibration spectra, no systematic correlations are evident in the residual plot. The corresponding concentration correlation plots are presented in **Figure 3C**. Although not as drastic as for the total sugar calibration model, both first overtone and short wavelength spectra generally outperform models from first overtone spectra. This finding is evident from the listing of RMSEC and RMSEP values in **Table 4**. A comparison of the data obtained by both methods was given in **Table 5**.

Comparison to Previous Reports. Measurements of constituent sugars of fruit products by NIR spectroscopy have been reported in the literature [e.g, Rodriguez-Saona et al. (*1*) and Tanaka and Takayuki (*11*)]. In the paper by Rodriguez-Saona et al., a simple analytical procedure using FT-NIR and multivariate techniques for the rapid determination of individual constituent sugar concentrations in fruit juices was evaluated and different NIR detection devices and sample preparation methods were tested by using model solutions to determine their analytical performance. In the paper by Tanaka and Takayuki, multiple components, including sucrose, glucose, fructose, and sorbitol were quantified from Japanese pear juice by NIR spectroscopy. This research indicated that the sugar components changed with the growth period of products. Determinations of constituent sugar concentrations of fruit juices by NIR spectroscopy were reported by Kawano et al. (*8*) for sucrose in sugar cane juice, by Lanza and Li (*9*) for sugar content in fruit juices, by Giangiacomo and Dull (*10*) for individual constituent sugar concentrations in aqueous mixtures, and by Ramla et al. (*17*) for total sugar, glucose, fructose, and sucrose in aqueous solutions of fruit juices.

In the above-mentioned studies, NIR spectra were collected from juices samples held in a sample holder by using transmittance or diffuse transreflectance measurement. Aqueous solutions of sugar mixtures (glucose, fructose, and sucrose) were used to create calibration and validation sets. In the paper by Tanaka and Takayuki, NIR diffuse transreflectance spectra were collected from 9090 to 4000 cm^{-1} with an aluminum cell for liquid sample (0.1 mm thickness). Similarly, the Rodriguez-Saona paper involved collecting spectra over the 10 000−4000 cm^{-1} spectral range with a 0.5 mm optical path length. Limitations of the above-mentioned researches are different optical path length, NIR detection devices, sample preparation methods, and wavelength spectral regions. It is impossible to get the best analytical performance by using a single path length. Therefore, the performance of calibration models are different, and the calibration models lack robust and stability. Of all of the limitations, the major limitation is the fact that aqueous solutions or fruit juice of constituent sugar concentrations was used for developing the calibration models. Thus far, the rapid quantification of constituent sugar concentrations in intact apples by FT-NIR spectroscopy has not been reported in the literature.

Our results from intact apple fruit are comparable to those of the above papers from fruit juices (*1*, *10*, *17*). The performance of the FT-NIR method is comparable to that of the reference HPLC method, but the former is much faster and easier to carry out. The models presented here are already in use at the Zhejiang University with satisfactory performance.

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