Improved Method for Determination of Pectin Degree of Esterification by Diffuse Reflectance Fourier Transform Infrared Spectroscopy

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An improved method for the determination of pectin degree of esterification (DE) by diffuse reflectance Fourier transform infrared spectroscopy (DRIFTS) was developed. Pectin samples with a range of DE as determined by gas chromatography were used for developing a calibration curve by DRIFTS. A linear relationship between the DE of pectin standards and FTIR peak ratio for ester carboxyl peak area to total carboxyl peak area was found ($R^2 = 0.97$). Pectin DE of various samples was calculated from the linear fit equation developed by DRIFTS. Accuracy of the DRIFTS method was determined by comparing the DE values of four commercial pectins obtained by DRIFTS methods to the values obtained by the gas chromatography method. Greater precision was obtained for the FTIR measurement of test pectin samples when the ester peak ratio was used relative to the ester peak area.

Keywords: *Pectin; degree of esterification; DRIFTS; FTIR*

INTRODUCTION

Pectic substances are present in practically all fruits and vegetables. The main constituent of the pectin polysaccharides is poly(1−4)-α-D-galacturonan (*1*). The carboxyl group of the D-galacturonic acid units may be in the free acid form, a salt (carboxylate) form, or a methyl ester form. The ratio of esterified carboxylic acid units to total carboxylic acid units in pectin is termed the degree of esterification (DE) (*2*). The DE has a major influence on pectin properties, including solubility, gel-forming ability, conditions required for gelation, gelling temperature, and gel properties (*3*).

The most commonly used method of determining the DE is the titrimetric method as proposed by the *Food Chemical Codex* (*4*). Initial and saponification titer values are obtained by titrating free and de-esterified carboxylic acid with 0.1 N NaOH. Wood and Siddiqui (*5*) used a colorimetric method for the analysis of the methanol content of pectin ester following de-esterification. This procedure involves oxidation of methanol to formaldehyde with potassium permanganate to yield the final colored product 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine. An enzymatic method using alcohol oxidase was developed by Klavons and Bennett (*6*). Mangos and Haas (*7*) improved the procedure using peroxidase and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) for greater sensitivity (0.05−1.0 μg/mL of methanol). Walter et al. (*8*) described a gas chromatographic (GC) method to quantify methanol obtained by pectin de-esterification. A detailed procedure to analyze the total methanol in isolated plant cell wall materials by gas chromatography was described by McFeeters and Armstrong (*9*). Manes et al. (*10*) determined the pectin DE by selective reduction of esterified galacturonic acid to galactose.

Infrared spectroscopy has been used to determine the pectin content as uronic acids (*11*) and polygalacturonic acid (*12*). The mid-infrared region (4000−400 cm^{-1}) is a useful means of analysis because it involves the fundamental absorption of chemical groups. Gnanasambandam and Proctor (*13*) used diffuse reflectance Fourier transform infrared spectroscopy (DRIFTS) for the determination of pectin DE. They found that peaks at 1760−1745 and 1640−1620 cm^{-1} indicated the ester carboxyl and acid carboxyl groups, respectively (*14*). The ester carboxyl peak area increased and the acid carboxyl peak area decreased as the DE increased. They developed a DRIFTS method for the rapid determination of DE by collecting FTIR spectra of pectin samples of known DE and correlating the ester peak areas of pectins with their DE values to develop a linear fit equation for calculation of DE from FTIR spectra (*13*). By definition, DE is the ratio of ester carboxyl peak area to total carboxyl area. Use of this ratio, rather than ester carboxyl peak area, would be more appropriate for DE determination and may improve the DRIFTS procedure (*13*).

The objective of this study was initially to develop a DRIFTS DE method by correlating the ester carboxyl peak/total carboxyl peak of pectins with their corresponding DE values obtained by GC. Then pectin samples were used to validate the method by obtaining DE by GC and comparing the data with those obtained from the DRIFTS DE calibration curve.

MATERIALS AND METHODS

Calibration Curve Development. *Standard Pectin Samples.* A wide range of pectin samples with known DE values (26.2, 36.5, 44.3, 54.2, 63.0, and 76.2) were provided

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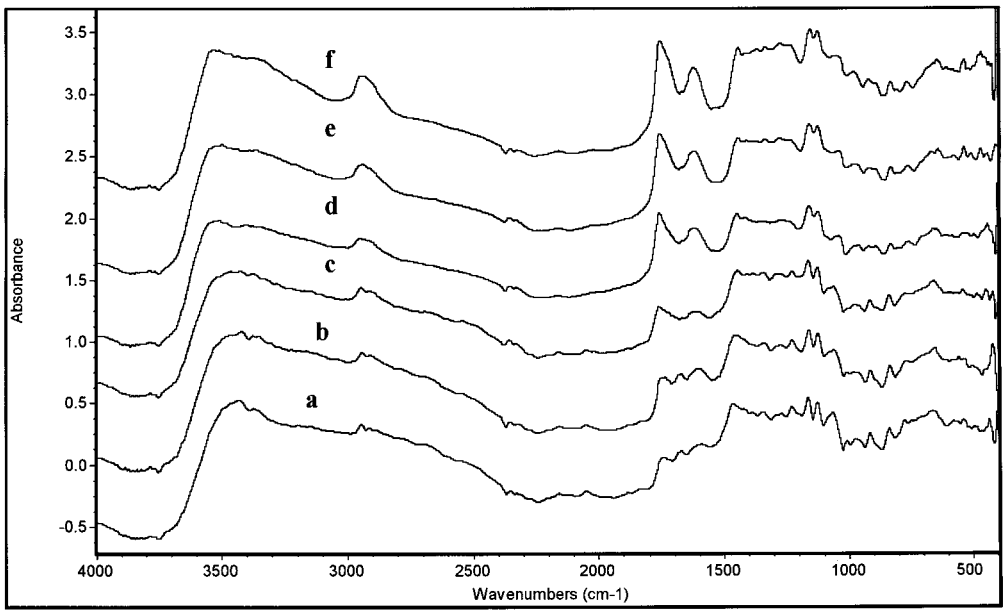

Figure 1. DRIFTS spectra obtained by co-adding 100 scans at a resolution of 8 cm^{-1} of pectin samples with various DE values [(a) 26.2%, (b) 36.5%, (c) 44.3%, (d) 54.2%, (e) 63.0%, and (f) 76.2%] as determined by GC (*9*).

by Danisco Ingredients USA Inc. (New Century, KS). These samples were used as standards in the FTIR analysis for developing a linear regression equation for a calibration curve.

Development of DRIFTS Calibration Curve for DE Determination. DRIFTS spectra of standard pectin samples were collected using a Nicolet model 410 FTIR instrument (Nicolet Analytical Instruments, Madison, WI). DRIFTS spectra were obtained by co-adding 100 scans at a resolution of 8.0 cm^{-1}. The spectra were collected using a blank disk, which was subtracted from each sample spectrum to compensate for absorption due to CO_2 and moisture in the air. Peak frequency, peak height, and peak area for esterified and nonesterified carboxyl peaks were obtained using the OMNIC 4.1 software package (Nicolet Analytical Instruments). Five replications of the FTIR analysis were performed and used to develop a calibration curve for the determination of DE in pectin samples. Because DE = area of esterified carboxyl group/(area of esterified carboxyl group + area of nonesterified carboxyl groups) × 100, it is inferred that this ratio should be proportional to the DE. The calibration curve was prepared by correlating the ratio of esterified carboxylic group to the total carboxyl groups of pectin standards with their corresponding known DE values. A linear fit equation was established for the determination of unknown test sample DE. For comparison with the previous DRIFTS method (*13*) an additional calibration curve was developed by correlating the ester carboxyl peak areas with their corresponding known DE values. A linear fit equation was also developed for the determination of unknown test sample DE by DRIFTS method using ester peak area.

Determination of DE by GC Method. The DE of pectin samples was measured using the GC method of McFeeters and Armstrong (*9*). A Hewlett-Packard gas chromatographic system (model 5890, Hewlett-Packard) with flame ionization detector was used to analyze the pectin samples for methanol liberation after de-esterification. A Hewlett-Packard HP-3392A integrator was used for analysis of the chromatograms.

Validation of Calibration Curve. *Test Pectin Samples.* The four test pectin samples used to validate the calibration curve were BB rapid set pectin (Hercules Inc., Wilmington, DE), TIC pretested pectin (lot 3433, TIC Gums, Inc., Belcamp, MD), citrus pectin (Sigma Chemical Co., St. Louis, MO; lot 96H0580), and Grindsted pectin (Danisco Ingredients USA).

Determination of DE by DRIFTS Method. DRIFTS spectra of test pectin samples were obtained using the method described to develop the calibration curve. The DE values of these samples were calculated from the linear fit equations developed from DRIFTS analysis. The values obtained by using the DRIFTS method using ester peak ratio and by the DRIFTS method using ester peak area were compared with DE values obtained by the GC method.

Determination of DE by GC Method. The DE values of test pectin samples were determined using the GC method of McFeeters and Armstrong (*9*).

Statistical Analysis. A linear regression model was developed by correlating the ratio of ester peak area to total carboxyl peak area of the pectin standards with their corresponding DE values obtained by GC. For comparison with the previous method (*13*), a second regression model was also developed by correlating the ester peak area of the pectin standards with their corresponding DE values. Results of three replicates were used to calculate the correlation coefficient (R^2) and regression line. Student's *t* test was used to analyze data, and least significant difference (LSD) values were obtained to differentiate mean values (*15*).

RESULTS AND DISCUSSION

Calibration Curve Development. DRIFTS spectra of pectin standards are presented in Figure 1. According to Wellner et al. (*16*), bands in the 1000−2000 cm^{-1} region are independent of pectin source and may be used to identify galacturonic acid. The mean frequencies were 1753 ± 5.62 and 1617 ± 8.59 cm^{-1} for esterified (COO−R) and nonesterified (COO−) carboxyl groups, respectively. Gnanasambandam and Proctor (*14*) found that peaks at 1760−1745 and 1640−1620 cm^{-1} indicated the ester carboxyl (COO−R) and nonester carboxyl (COO−) groups, respectively.

The mean values and standard deviations of the carboxyl absorption peak area with various DE values are presented in Table 1. The esterified carboxyl peak area was calculated as the area above the baseline between 1844 ± 29.8 and 1682 ± 12.3 cm^{-1}. The nonesterified carboxyl peak area was measured as the area above the baseline between 1682 ± 12.3 and 1532 ± 20.2 cm^{-1}. The area under the curve was used to calculate the esterified and nonesterified carboxyl peak areas. Nonesterified carboxyl groups included both acid and anionic forms, which absorb at 1600 and 1650−1550

Table 1. FTIR Ester Carboxyl Peak Area, Nonester Carboxyl Peak Area, Total Carboxyl Peak Area, and Ratio of Ester Carboxyl Peak Area to Total Carboxyl Area of Pectin Samples with Various DE Values

DE[a] values of pectin standard	ester carboxyl peak area[b]	nonester carboxyl peak area[b]	total carboxyl peak area[b]	ester carboxyl/total carboxyl peak area[b]
26.2 ± 0.12	$4.78^{d} \pm 0.02$	$6.66^{c} \pm 0.07$	$11.44^{d} \pm 0.08$	$41.80^{f} \pm 0.35$
36.5 ± 1.21	$8.73^{c} \pm 0.17$	$10.19^{b} \pm 0.22$	$18.92^{c} \pm 0.34$	$46.13^{e} \pm 0.50$
44.3 ± 1.37	$12.36^{b} \pm 0.23$	$10.46^{b} \pm 0.31$	$22.82^{b} \pm 0.52$	$54.16^{d} \pm 0.40$
54.2 ± 2.17	$22.30^{a} \pm 0.50$	$14.14^{a} \pm 0.24$	$36.46^{a} \pm 0.66$	$61.16^{c} \pm 0.47$
63.0 ± 2.25	$22.01^{a} \pm 0.87$	$11.26^{b} \pm 0.16$	$33.27^{a} \pm 1.03$	$66.13^{b} \pm 0.60$
76.2 ± 2.58	$25.09^{a} \pm 0.92$	$10.64^{b} \pm 0.15$	$35.74^{a} \pm 0.09$	$70.20^{a} \pm 0.36$
	$R^2 = 0.9175$	$R^2 = 0.3294$	$R^2 = 0.8357$	$R^2 = 0.9718$

[a] DE values obtained by GC method. [b] Values with different superscripts in each column are significantly ($P < 0.05$) different from each other. [c] R^2 values are calculated on the basis of the linear fit equation between DE values obtained by GC and FTIR parameters.

Table 2. FTIR Ester Carboxyl Peak Height, Nonester Carboxyl Peak Height, Total Carboxyl Peak Height, and Ratio of Ester Carboxyl Peak Height to Total Carboxyl Peak Height of Pectin Samples with Various DE Values

DE[a] values of pectin standard	ester carboxyl peak height[b]	nonester carboxyl peak height[b]	total carboxyl peak height[b]	tster carboxyl/total carboxyl peak height[b]
26.2 ± 0.12	$0.114^{e} \pm 0.002$	$0.045^{d} \pm 0.006$	$0.159^{f} \pm 0.005$	$71.75^{b} \pm 2.906$
36.5 ± 1.21	$0.193^{d} \pm 0.007$	$0.065^{c} \pm 0.004$	$0.258^{e} \pm 0.004$	$74.79^{ab} \pm 1.936$
44.3 ± 1.37	$0.204^{d} \pm 0.005$	$0.060^{c} \pm 0.001$	$0.265^{d} \pm 0.005$	$77.06^{a} \pm 0.805$
54.2 ± 2.17	$0.416^{c} \pm 0.006$	$0.138^{b} \pm 0.002$	$0.556^{c} \pm 0.005$	$75.08^{ab} \pm 0.528$
63.0 ± 2.25	$0.458^{b} \pm 0.003$	$0.195^{a} \pm 0.003$	$0.653^{b} \pm 0.006$	$70.13^{b} \pm 0.185$
76.2 ± 2.58	$0.584^{a} \pm 0.002$	$0.207^{a} \pm 0.008$	$0.790^{a} \pm 0.010$	$73.84^{ab} \pm 0.760$
	$R^2 = 0.9576$	$R^2 = 0.8884$	$R^2 = 0.9508$	$R^2 = 0.0170$

[a] DE values obtained by GC method. [b] Values with different superscripts in each column are significantly ($P < 0.05$) different from each other. [c] R^2 values are calculated on the basis of the linear fit equation between DE values obtained by GC and FTIR parameters.

cm^{-1}, respectively (*17*). In a similar study Chatjigakis et al. (*18*) used the pectin standard that was adjusted to pH 5.5 prior to FTIR analysis; this was done to convert nonesterified carboxyl groups into carboxylate ions. In this rapid determination method we used the pectin sample in its native form without any additional treatments to calculate the area under the curve for esterified and nonesterified carboxyl groups. The area under the curve for nonesterified carboxyl groups includes both ionic and nonionic forms of nonesterified carboxyl groups. The ratio of ester carboxyl peak area to total carboxyl area of the pectin standard was calculated by dividing the area under the curve for esterified carboxyl area by the sum of esterified and nonesterified carboxyl areas. The ester peak areas of pectin standards were not all significantly different from each other. In a similar study Gnanasambandam and Proctor (*13*) found a linear relationship between titrimetric DE values and ester carboxyl area with a correlation coefficient of 0.82. They used this relationship to develop a calibration curve for the determination of DE of pectin samples. The DE values for pectin samples obtained from a linear fit equation were comparable to the values obtained by a titrimetric method (*4*). Similarly, no statistically significant differences for all samples were found for nonester carboxyl and total carboxyl peak areas. However, the ratios of ester carboxyl peak area to total carboxyl area of the pectin standards with different DE values were significantly different from each other (Table 1).

The mean values and standard deviations of the carboxyl absorption peak heights with various DE values are presented in Table 2. Although ester peak height increased significantly with increased DE, the ratios of ester carboxyl peak height to total carboxyl peak height were not significantly different for all of the pectin standards used for analysis (Table 2).

Therefore, the ratio of ester peak area to total carboxyl area was selected for possible correlation with DE. The ratio of ester peak area to total carboxyl peak area showed a high correlation with DE values obtained by the GC method ($R^2 = 0.97$) (Figure 2). The DE values of pectin standards were 26.2, 36.5, 44.3, 54.2, 63.0, and 76.2 as determined by the GC method. The fitted model was represented by the equation ester carboxyl peak area/total carboxyl peak area = 26.123 + 0.6087 × DE. A second fitted model was also developed by correlating the ester peak area with the corresponding known DE values for comparison with the previous method (*13*). The fitted model using only ester peak area was represented by ester peak area = 0.4405 × DE − 6.1771.

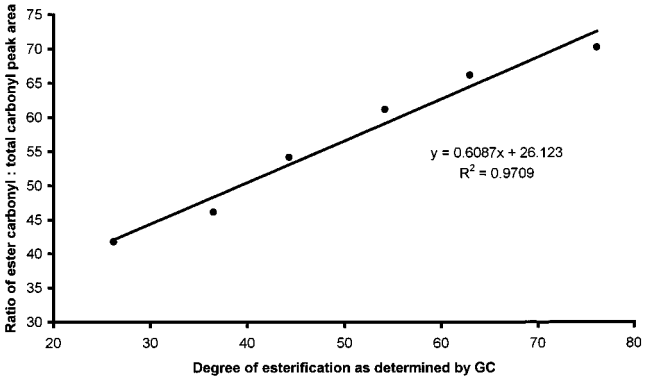

Figure 2. Regression analysis and linear fit equation for DE as determined by GC for ester carboxyl peak area/total carboxyl peak area of pectin samples using DRIFTS procedure.

Validation of Calibration Curve. DRIFTS spectra of pectin samples are shown in Figure 3. The ester carboxyl peak area and the ratio of ester carboxyl peak area to total carboxyl peak area were used to determine the DE of pectin samples from the fitted model developed by the DRIFTS method using only ester peak area and by the DRIFTS method using ester peak ratio.

Table 3 shows the pectin analysis by GC, DRIFTS ester carboxyl peak area/total carboxyl peak area method, and DRIFTS method of ester carboxyl peak area. Mean values obtained from the DRIFTS method using peak ratio were comparable to those obtained by the GC method. For all pectin samples tested DE values ob-

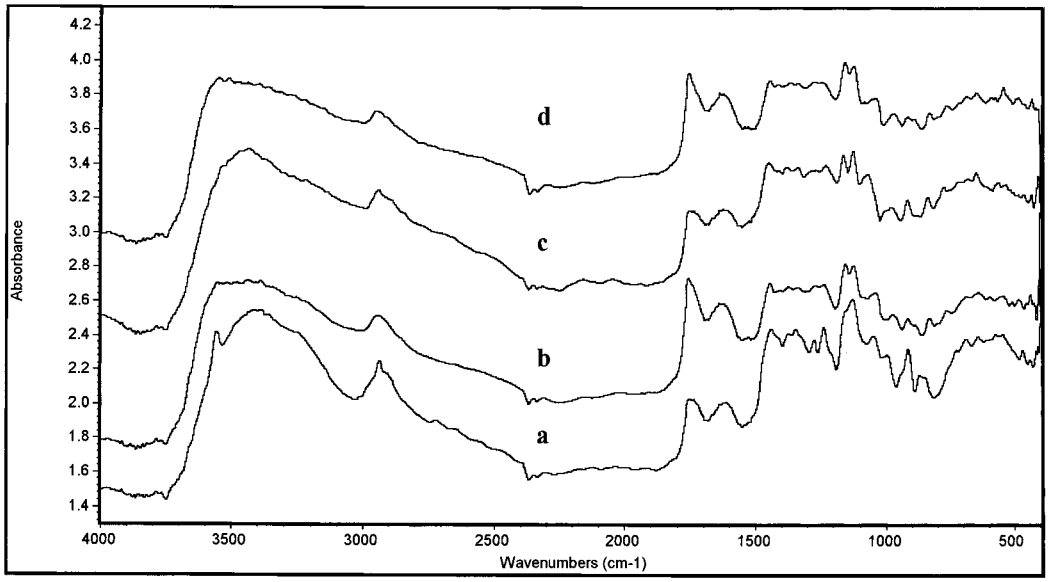

Figure 3. FTIR spectra of the 4000−400 cm^{-1} region of commercial pectins: (a) BB rapid set pectin (Hercules Inc.); (b) citrus pectin (Sigma Chemical Co., lot 96H0580); (c) TIC pretested pectin (lot 3433, TIC Gums, Inc.); (d) Grindsted pectin (Danisco Ingredients USA).

Table 3. Degree of Esterification of Commercial Pectin Samples by GC, DRIFTS (Ester Carboxyl/Total Carboxyl Peak Area), and DRIFTS (Ester Carboxyl Peak Area)

method[a]	Grindsted pectin[b]	pretested pectin[b]	citrus pectin[b]	BB rapid set pectin[b]
gas chromatograpy	$62.89^{a} \pm 4.59$	$24.97^{ab} \pm 2.00$	$67.73^{a} \pm 1.96$	$58.69^{a} \pm 2.23$
DRIFTS (ester carboxyl/total carboxyl peak area)	$60.58^{a} \pm 1.59$	$20.48^{b} \pm 2.53$	$65.51^{a} \pm 2.96$	$58.12^{a} \pm 4.87$
DRIFTS (ester carboxyl peak area)	$56.31^{a} \pm 4.66$	$27.76^{a} \pm 2.13$	$61.23^{a} \pm 4.52$	$46.88^{b} \pm 4.79$

[a] LSD values to differentiate analytical methods for Grindsted pectin, pretested pectin, citrus pectin, and BB rapid set pectin were 7.77, 4.46, 6.63, and 8.30, respectively. [b] Values with different superscripts in each column are significantly ($P < 0.05$) different from each other.

tained by GC and DRIFTS methods using peak ratio were similar, and the differences were not significant ($P < 0.05$). The DRIFTS method using ester peak area produced a significantly different DE value (46.88) from the GC and DRIFTS peak ratio method for BB rapid set pectin. The standard deviation values for DRIFTS method of ester peak area were large compared to those obtained by GC and DRIFTS peak ratio methods. Both DRIFTS methods were faster than conventional methods, but the DRIFTS method using ester peak ratio showed better precision and accuracy relative to the DRIFTS method using only ester peak area.

Regression analysis showed a linear relationship between the ratio of ester carboxyl peak area to total carboxyl peak area and DE values of pectin standards. Slope, intercept, and random scattering in the residual plot (figure not presented) indicated a linear model within the pectin range used. DRIFTS could be used as a rapid, economical, and simple alternative to previous methods developed for pectin DE analysis. The improved method unlike the previous DRIFTS method (*13*) can be used for any sample regardless of the pectin content.

Conventional DE determination by GC requires that the total galacturonic acid content of the pectin also be determined. Galacturonic acid determination by either HPLC or colorimetric methods is tedious and time-consuming, requiring complete hydrolysis of the pectin by acid or enzyme (*19*, *20*). FTIR determination is rapid and nondestructive and can be used for a variety of samples. However, accurate determination by FTIR does require homogeneous samples such as a powder.

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