

General Application of Raman Spectroscopy for the Determination of Level of Acetylation in Modified Starches¹

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ABSTRACT

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A method using Raman spectroscopy was recently developed for the determination of the degree of acetylation in modified wheat starch. In this article, we show that the method can be generalized to a wide range of starches of different botanical origin and amylose content. Calibration sets were used to develop regression equations for 11 types of acetylated starches, including cereal (rice, maize, wheat) and noncereal (potato and sweetpotato)

sources. The calibration lines were then used to predict the level of acetylation of starch samples with unknown level of acetylation using their Raman spectra. In each case, $R^2 > 0.98$ for linear regression of Raman vs. titrimetric determination of acetylation. The Raman-based calibration curves allow fast and nondestructive determination of the degree of acetylation for different types of starches.

Chemical modification of starches can enlarge the range of certain starch physical properties and enhance their use in a number of applications found in industrial processes and food manufacture. The acetylation of starches is a commonplace chemical modification used to significantly change the physicochemical properties of the parent starch (Rutenberg and Solarek 1984). The extent of physicochemical property changes in the acetylated starch compared to the parent starch is proportional to the degree of acetylation or degree of C=O substitution. Therefore, it is important to measure this quantity to control quantitatively the desired degree of acetylation of a starch for use in different processes. Traditionally, the degree of acetylation in chemically modified starches is found using wet chemistry methods that involve separation and titration procedures (Genung and Mallatt 1941, Wurzburg 1964). These wet chemistry methods are time consuming, labor intensive, and susceptible to interference from residual materials in the starch samples. The wet chemistry method developed by Wurzburg assumes that the modified starch samples have been purified and are free of any residual compounds that could interfere with the titration used to measure the degree of acetylation. We have recently developed a Raman spectroscopic method for fast determination of the degree of acetylation in modified wheat starches (Phillips et al 1998). We used purified acetylated starch samples and Raman spectra to prepare a calibration curve for the level of acetylation versus the intensity ratio of the C=O stretch Raman band to a C–C stretch Raman band. Then Raman spectra of the unknown wheat starch samples were obtained and the ratio of the C=O stretch to the C–C stretch Raman bands was compared to the calibration curve to find the level of acetylation in the unknown wheat samples. This new Raman spectroscopic method allows faster determination of the degree of acetylation, is nondestructive of the starch sample, and is less susceptible to interference from residual impurities than the traditional wet chemistry methods for finding the degree of acetylation.

Because each compound has a different vibrational spectrum, different compounds contributing to a Raman spectrum of a sample can be distinguished. This helps minimize interference from impurities in Raman spectroscopic measurements. The intensity of Raman peaks increases linearly with the amount of compound present in the sample (Long 1977, Hendra et al 1991). The pharmaceutical and polymer industries have long used Raman spectroscopy as a quantitative analytical technique (e.g., Hendra et al 1991), although only in the past decade has Raman spectroscopy been finding

increasing use as an analytical method in food science and in the food industry (Li-Chan 1996). The Raman peak positions and relative intensities can be very sensitive to the particular structure of the molecule (or compound) and the environment surrounding that molecule. The peak positions and relative intensities of C=O stretch vibrational features and C–C stretch vibrational features may vary significantly in different molecules that possess both C–C and C=O bonds. Thus, the Raman analytical method we have developed for wheat starches (Phillips et al 1998) may require different calibration curves for the degree of acetylation of different types of starches. In this article, we report the further development of Raman spectroscopy as an analytical method for the determination of the level of acetylation in a number of different types of starches including wheat, regular rice, glutinous rice, amylopectin from maize, high amylose maize, sweetpotato, waxy maize, regular maize, purified amylose from maize, and potato. The objectives were to: 1) demonstrate the generality of Raman spectroscopy for the determination of the degree of acetylation in many types of starches, and 2) indicate how much variation in the molecular structure or environment around the C=O and C–C bonds occurs in starches from different biological sources.

MATERIALS AND METHODS

Starch Samples

Regular rice (19% amylose) and glutinous rice (2.7% amylose) starch were supplied by Bangkok Starch Industrial Co., Bangkok, Thailand; regular (Maize Cornflour 3401C, 22.4% amylose), Gelose 50 (47% amylose), Hi-Maize (66% amylose), and Mazaca 3401X (3.3% amylose) maize starches were supplied by Starch Australasia Ltd., Lane Cove, Australia. Wheat starch (30% amylose), potato starch (23% amylose), amylopectin from maize (2.6% amylose), and amylose from maize (74% amylose) were from Sigma Chemical Co. (St. Louis, MO). Sweetpotato starch (19% amylose) was extracted in the laboratory from a Philippine experimental cultivar at the Institute of Food Science and Technology, University of the Philippines at Los Baños (Collado and Corke 1997). All amylose determinations were means of triplicate analysis using the Megazyme (Bray, Ireland) amylose-amylopectin assay kit, which uses the concanavalin-A lectin binding method.

Laboratory Acetylation of Starch

Starch samples were acetylated at laboratory scale as described by Phillips et al (1998) following the method of Wolff et al (1951) with minor modifications (Hoover and Sosulski 1985, Liu et al 1997). Starch (100 g) was dispersed in 225 mL of distilled water, and stirred for 60 min at 25°C. The suspension was adjusted to pH 8.0 with 3.0% NaOH. Acetic anhydride (using 2.55, 5.1, 7.65, and 10.2 g to give different degrees of acetylation) was added dropwise to the stirred slurry while maintaining the range within pH 8.0–8.4 using

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3.0% NaOH solution. The reaction was allowed to proceed for 10 min after completion of acetic anhydride addition. The slurry was then adjusted to pH 4.5 with 0.5N HCl and centrifuged for 3 min at 2,000 rpm. It was washed free of acid twice with distilled water and once with 95% ethanol and then oven dried at 40°C.

Determination of Percent Acetylation

The percent acetylation (acetyl %) was determined titrimetrically following Wurzburg (1964), which is based on Genung and Mallatt (1941). Acetylated starch (1.00 g) was placed in a 250-mL flask, and 50 mL of 75% ethanol in distilled water was added. The loosely stoppered flask was agitated, warmed to 50°C for 30 min, cooled, and 40 mL of 0.5M KOH was added. The excess alkali was back titrated with 0.5M HCl with phenolphthalein as an indicator. The solution was stood for 2 hr, then any additional alkali which may have leached from the sample was titrated. A blank using the original unmodified starch is also used. The calculation was: acetyl % = ([blank

– sample] × molarity of the HCl + 0.043 × 100)/sample weight. Blank and sample are titration volumes in milliliters, sample weight is in grams, dry weight basis. Degree of substitution (DS) = (162 × acetyl %)/(4,300 – [42 × acetyl %]).

TABLE I
Percent of Acetylation and Ratio of the Raman Intensity of the 1,732 cm⁻¹ C=O Stretch and 941 cm⁻¹ C–C Stretch Bands

Sample	Percent Acetylation ^a	Ratio
Rice		
A	4.19	0.090
B	2.48	0.050
C	2.13	0.046
D	1.05	0.020
E	0.00	0.000
Glutinous rice		
A	4.15	0.084
B	2.93	0.057
C	2.04	0.043
D	0.94	0.021
E	0.00	0.000
Waxy maize		
A	4.05	0.087
B	2.60	0.047
C	2.13	0.040
D	1.12	0.021
E	0.00	0.000
Regular maize		
A	2.41	0.057
B	1.46	0.035
C	1.05	0.027
D	0.45	0.016
E	0.00	0.000
Gelose 50 maize		
A	3.33	0.076
B	2.01	0.043
C	1.78	0.038
D	0.75	0.021
E	0.00	0.000
Hi-Maize		
A	3.49	0.074
B	2.08	0.041
C	1.41	0.033
D	0.68	0.018
E	0.00	0.000
Amylopectin from maize		
A	3.72	0.074
B	2.23	0.450
C	1.99	0.037
D	0.91	0.017
E	0.00	0.000
Amylose from maize		
A	3.19	0.075
B	1.80	0.045
C	1.69	0.037
D	0.61	0.016
E	0.00	0.000
Wheat		
A	3.38	0.077
B	2.18	0.045
C	1.63	0.038
D	0.95	0.018
E	0.00	0.000
Potato starch		
A	3.02	0.067
B	1.71	0.038
C	1.31	0.030
D	0.80	0.015
E	0.00	0.000
Sweetpotato		
A	2.58	0.067
B	1.62	0.039
C	1.36	0.034
D	0.00	0.000

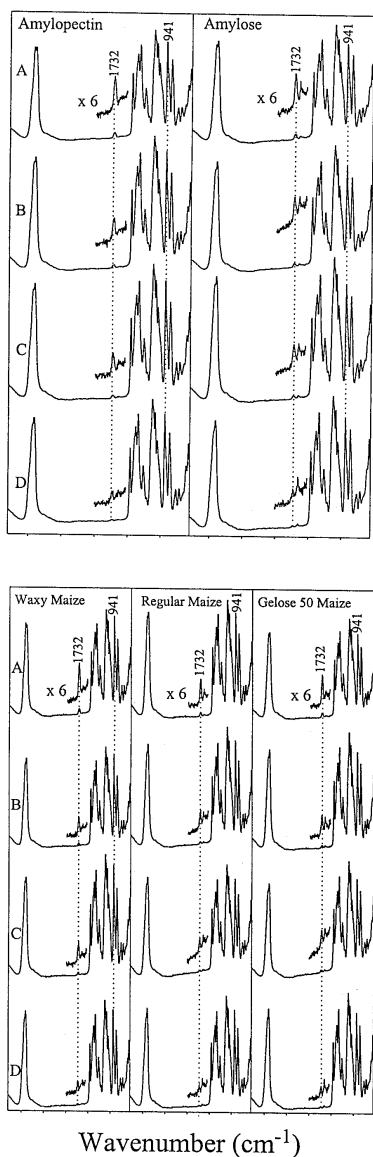


Fig. 1. Five representative FT-Raman spectra of acetylated maize starches and purified amylose and amylopectin, at varying levels of acetylation. The 1,732 cm⁻¹ Raman peak due to the C=O stretch vibration and the 941 cm⁻¹ Raman peak due to the C–C stretch vibration are labeled in the spectra. Intensity of the 1,732 cm⁻¹ C=O stretch Raman peak increases in intensity as level of acetylation increases. A–D indicate decreasing levels of acetylation as indicated in Table I. For unmodified starches, no peak is observed at 1,732 cm⁻¹

^a Percent acetylation determined from average of three replicates of the Wurzburg titrimetric method. Average standard deviation of replicates was 0.06% acetyl.

Method for Collection and Analysis of Raman Spectra

An FT-Raman spectrometer (Bio-Rad, Cambridge, MA) using 1,064 nm excitation was used to conduct all of the Raman spectroscopic measurements of the starches, as described in Phillips et al (1998). Starch samples were placed in glass capillary tubes and the FT-Raman spectra (100–3,000 cm^{-1}) were obtained for both the glass tube with the starch sample and the glass tube alone. The FT-Raman spectra of the glass tube was subtracted from the FT-Raman spectra of the glass capillary tube with starch sample to obtain the Raman spectrum of the starch samples. A white light source spectrum with known intensity as a function of wavelength was used to correct the intensities of the spectra. The FT-Raman spectra were collected in a backscattering geometry using $\approx 100\text{--}400$ mW of 1,064-nm laser light. FT-Raman spectra were taken with a resolution of 4 cm^{-1} and 3,680 data points for each spectrum. Approximately 200–600 scans were summed ($\approx 3\text{--}10$ min) to obtain each FT-Raman spectrum used in the analysis. The intensities of the $\approx 1,732$ cm^{-1} C=O stretch and ≈ 941 cm^{-1} C–C stretch Raman peaks were integrated (integrated band areas were found by fits to a Gaussian function plus a baseline) and the ratio of the 1,732 cm^{-1} C=O stretch peak area to the 941 cm^{-1} C–C stretch peak area was obtained. This ratio was plotted versus the acetyl % found from the titrimetric method to derive a calibration curve for each type of starch investigated.

RESULTS AND DISCUSSION

Similar Raman spectra for the 11 different sources of starch at varying levels of acetylation were obtained, despite the wide range of botanical sources and amylose content of the materials used. The examples shown (Fig. 1) indicate the development of a peak at 1,732 cm^{-1} due to the C=O stretch at increasing acetylation, which is not present in unmodified samples. The ratio of the Raman intensity of the 1,732 cm^{-1} C=O stretch and 941 cm^{-1} C–C stretch bands and the level of acetylation determined by the Wurzburg method for the starch samples was calculated (Table I). Using the ratio of the 1,732 cm^{-1} C=O stretch and 941 cm^{-1} C–C stretch Raman bands with the wet chemistry measurement of the level of acetylation, we obtained the calibration equations for the Raman intensity ratio versus level of acetylation (Table II). Representative plots of the intensity ratio of the 1,732–941 cm^{-1} peaks versus the level of acetylation for the starch samples (Fig. 2) indicate best fit linear regressions to the data plotted in each figure. Inspection of Fig. 1 and corresponding plots of Fig. 2 show that the 1,732 cm^{-1} Raman peak increases in intensity as the degree of acetylation

TABLE II
Parameters for Linear Regression Analysis
of the Calibration Curves for the Raman Intensity Ratio
vs. Level of Acetylation^{a,b}

Sample	B	A	r
Rice	0.02151 ± 0.00062	-0.00108 ± 0.00149	0.999
Glutinous rice	0.01978 ± 0.00063	0.00120 ± 0.00157	0.998
Waxy maize	0.02103 ± 0.00135	-0.00275 ± 0.00324	0.994
Regular maize	0.02274 ± 0.00127	0.00235 ± 0.00173	0.995
Gelose 50 maize	0.02205 ± 0.00123	0.00078 ± 0.00239	0.995
Hi-Maize	0.02061 ± 0.00105	0.00167 ± 0.00205	0.996
Amylopectin from maize	0.0199 ± 0.00053	-0.00058 ± 0.00115	0.999
Amylose from maize	0.0235 ± 0.00094	0.00059 ± 0.00171	0.998
Wheat	0.02277 ± 0.00114	-0.00162 ± 0.00225	0.996
Potato	0.02255 ± 0.00063	-0.00081 ± 0.00107	0.999
Sweetpotato	0.02568 ± 0.001	-0.00074 ± 0.00167	0.998
Combined (N = 44)	0.02076 ± 0.00061	0.0019 ± 0.00136	0.983

^a $Y = A + B \times X$, where Y = ratio of the Raman intensity of the 1,732 cm^{-1} C=O stretch and 941 cm^{-1} C–C stretch bands, B = slope of linear regression, A = intercept of linear regression, X = percent acetylation. $n = 5$ for all individual samples, except sweetpotato where $n = 4$. For combined samples, $N = 44$ (using only one zero point).

^b ± Standard deviation.

increases. The calibration curves (Fig. 2) have linear regression correlation coefficients of $r \geq 0.99392$ and their slopes varied from 0.01978 to 0.0235 (Table II). These results show that the Raman intensity ratios of the 1,732–941 cm^{-1} peaks versus the degree of acetylation have a very high degree of linearity and calibration curves (such as shown in Fig. 2) can be used with confidence to find the degree of acetylation from Raman spectra of the different types of acetylated starches.

As amylose and amylopectin have different chemical structures, the C=O bonds added to them by the acetylation process would experience a somewhat different molecular environment. The slopes of the calibration lines for the degree of acetylation for amylose (0.0235 ± 0.00094) and amylopectin (0.0199 ± 0.00053) are noticeably different from one another, and this is likely due to the different molecular environment about the C=O bonds added to them by acetylation. The intensity of the C=O 1732 cm^{-1} Raman peak to the C–C stretch 941 cm^{-1} peak appears to be larger for amylose than for amylopectin for the same degree of acetylation. The alpha C–C bands at $\approx 960\text{--}880$ cm^{-1} are sensitive to carbohydrate structure, and the 941 cm^{-1} band used for an internal standard varies a small amount with biological source, which causes the apparent ratio of the C=O 1,732 cm^{-1} band to the C–C stretch 941 cm^{-1} band to vary to a small extent. These results suggest that one should use a calibration curve for the particular starch of interest to obtain the most accurate results. For example, if one is interested in the degree of acetylation of modified rice starches then one should use a calibration curve for acetylated rice starch samples. This will give the most accurate determination of the degree of acetylation of the starch samples with unknown degrees of acetylation by using the

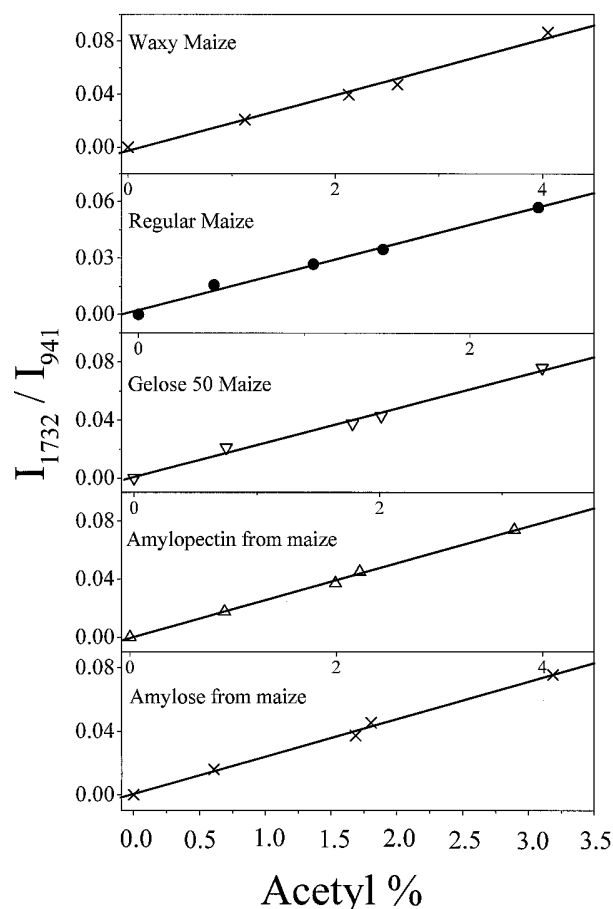


Fig. 2. Plot of the ratio of the 1,732 cm^{-1} Raman peak to the 941 cm^{-1} peak vs. level of acetylation determined using titrimetric method for acetylated maize starches and purified amylose and amylopectin. Lines show best fit. Regression parameters listed in Table II.

Raman intensity ratio method we have presented here. It would be desirable to develop a new calibration with a larger number of samples of the same type. However, we did calculate a combined regression equation (Table II, Fig. 3) using pooled data from all 11 sample sets. This appears to be quite robust and suitable for general purpose use where it is not feasible to develop a new calibration, or where many different types of starch may be analyzed. Certainly rice, potato, wheat, Gelose 50 maize, regular maize, and waxy maize could probably all use the same average calibration curve. As all of the acetylated starch samples have calibration curves that are very close to one another, this suggests that the C=O bonds added to them experience similar molecular environments. Besides the noticeable different calibration curves for acetylated amylose and amylopectin samples, the rice and glutinous rice appear to be somewhat different and may suggest that the two forms of rice starch have significantly different structural features about the C=O bonds added by acetylation. It may be possible to reduce the amount of variance in the slopes of the C=O stretch 1,732 cm⁻¹ band ratio to the internal standard band by choosing to use the C–C stretch doublet bands at ≈960–880 cm⁻¹ as the internal standard. This may allow the development of an improved single calibration for all or multiple biological starch sources, and this is being investigated.

We note that some minor peaks in the Raman spectra appear to be indicative of relative differences in amylose to amylopectin ratio, and further investigation of the analytical utility of such peaks will be reported elsewhere (*unpublished data*). The commercial procedure for starch acetylation may leave some residual salts such as sodium acetate that could possibly interfere with the determination of acetylation by Raman spectroscopy. However, comparison of results for commercial samples with our laboratory-prepared samples indicate that this is not likely to be a problem. In addition, we washed starch with 5% sodium acetate and dried it, and did not detect interference with the Raman method (results not shown). The C=O stretch vibrational frequency of sodium acetate salt is ≈1,731 cm⁻¹ compared to 1,732 cm⁻¹ for acetylated starch. However, sodium acetate also has other bands of comparable intensity at 1,560 cm⁻¹ and 1,644 cm⁻¹ which were not present in the starch samples we studied and are resolvable from the amylose band ≈1656 cm⁻¹. If sodium acetate impurity were present, these two new bands would appear and their intensities could be used to subtract the background sodium acetate impurity Raman spectrum

from the acetylated starch spectrum. However, with wet chemistry methods, the presence of sodium acetate may lead to overestimation of the degree of acetylation of the starch.

There is some concern as to whether the location of the acetyl substituents would affect the Raman spectra. For example, in granular starch the acetyl substituents are located mostly in the amorphous regions of the granule principally in the amylopectin region where there is dense branching or at the tips of A and B chains of amylopectin (Biliaderis 1982) whereas amylose is uniformly substituted. This different location of acetyl substitution would give different microenvironment interactions for the C=O group and shift the vibrational frequency which would affect both the bandshape and bandwidth of the C=O Raman band. Further research is needed to investigate the effect of acetyl substitution location on the Raman spectra, but since we integrate over the entire C=O band and there is only one C=O band in the spectrum, the location of acetyl substitution will not have any practical impact on the analytical Raman method presented here. The C=O stretch Raman band is substantially broader than the 4 cm⁻¹ resolution of the spectrometer, and this is probably due to some inhomogeneous broadening from acetyl substituent location in the modified starch. Additionally, the Raman method reported here could potentially be affected by starch type (e.g., cook-up granular type, pregelatinized nongranular type, and instant type). Although we believe the method would be fairly insensitive to these differences, further research is in progress to clarify the effect of starch type on Raman spectra. Ordinarily, it should be expected that a separate calibration should be developed for each type of starch, as least as a confirmation or validation before its use in routine analysis.

In conclusion, we have reported additional 1,732–941 cm⁻¹ Raman intensity ratios versus the degree of acetylation calibration curves for 11 types of starches. These calibration curves all show a very high degree of linearity and are suitable for determining the degree of acetylation of starch samples with unknown degrees of acetylation from their Raman spectra. The calibration curves presented here can be used to quickly determine the degree of acetylation of many starch samples from their Raman spectra as described in our earlier work on wheat starches. There are some differences in the slopes of the calibration curves for some starches (in particular amylose and amylopectin) which suggests that the C=O bonds added by acetylation may experience noticeably different molecular environments. However, a combined calibration curve derived from all

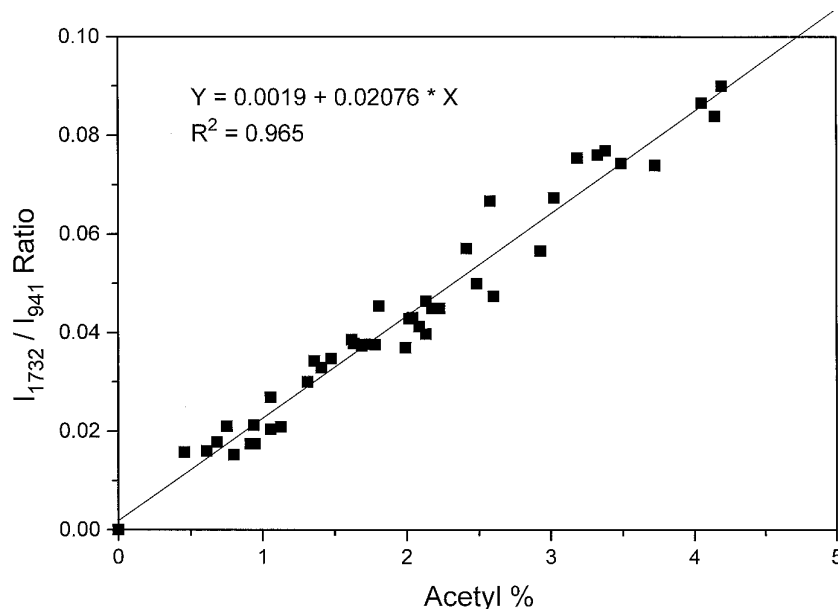


Fig. 3. Combined plot of the ratio of the 1,732 cm⁻¹ Raman peak to the 941 cm⁻¹ peak vs. level of acetylation determined using titrimetric method for all 11 acetylated maize starches and purified amylose and amylopectin sources. Lines show best fit. Regression parameters listed in Table II.

sample data may be more robust and suitable for general use if it is not feasible to develop an extensive calibration for a particular application.

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