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Comparison of Analytical Methods for Determining Methylesterification and Acetylation of Pectin

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Abstract: The esterification of galacturonic acid with methanol and/or acetic acid is important for the structural analysis of pectin. Although several methods have been reported for determining the degree of methylesterification (DM) and acetylation (DAc), the present study compares and optimizes three methods (titration, FT-IR and HPLC) using commercial citrus pectin (CP). Our results showed that the DM of CP was 47.0%, 47.6% and 48.0% as determined by titration, FT-IR and HPLC, respectively, suggesting that DM determination is nearly identical using any of these methods. However, the titration approach requires more sample than the other two. HPLC showed that the DAc of CP was 1.6%, an approach that can be used to determine the DM and DAc of pectin simultaneously. Here, we simplified and optimized sample treatment for HPLC analysis and compared it with the reported literature. Our results provide useful information for choosing appropriate methods for determining the DM and DAc of pectin based on various sample properties and experimental conditions.

Keywords: pectin; degree of methylesterification; degree of acetylation; titration; FT-IR; HPLC

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1. Introduction

Pectin is a complex polysaccharide with diverse structures in the primary cell wall and middle lamellae of plants [1]. Pectin plays a crucial role in regulating the mechanical properties of the cell wall and has several industrial applications related to its gelling properties [2,3]. Pectin can be esterified with methanol or acetic acid. Generally, methylesterification is widespread in pectin, whereas acetyl-esterification is less abundant [4]. The degree of methylesterification (DM) is defined as the molar percentage of carboxyl groups esterified with the methyl group. The degree of acetylation (DAc) is defined as the molar percentage of galacturonosyl residues esterified with the acetyl group [5]. DM and DAc have significant impacts on the properties and physiological activities of pectin and play an important role in the strength and flexibility of plant cell walls, such as fruit softening during storage which is caused by the de-esterification of pectin [6,7]. DM and DAc also influence the gelling, stabilization, and thickening of pectin. DM dictates the conditions and mechanisms of gel formation [8,9]. The pharmacological activities of pectin are also correlated to the degree of DM and DAc formation. It has been reported that low DM pectin can significantly reduce cholesterol concentration in hamster plasma and liver compared to high DM pectin [10]. Characterization of pectin includes not only analysis of sugar composition and molecular weight, but also of the DM and DAc.

Until now, various methods have been established for the determination of DM and DAc. The earliest approach used for determining the degree of DM was titration, including acid–base titration, colloid titration, and isoelectric spot determination [11–13], often used in industrial production. In recent years, FT-IR has often been used to determine the DM

of pectin, because this approach requires no pretreatment of the sample [7,14,15]. The kit manufactured by Megazyme (i.e., Acetic Acid Assay Kit) can be used to detect the content of acetic acid quantitatively following the saponification of pectin [16]. HPLC and GC methods have also been reported for simultaneously determining the DM and DAc of pectin. With these methods, pectin is saponified to produce methanol and acetic acid, and then detected by HPLC or GC [17–20]. Additionally, ^{1}H-NMR has been used in determining the DM and DAc of pectin, but this method is seldom used because of its higher cost [21,22]. There were some reports concentrated on the determination of DM by different methods and discussed advantages and limitations of the applied methods [17,23–25].

In this paper, we compared three frequently used approaches, including titration, FT-IR, and HPLC for determining the DM and DAc of citrus pectin, and listed the advantages and limitations of these methods. Furthermore, we improved the sample pro-treatment and neutralization process of HPLC analysis, which can simplify the operation.

2. Materials and Methods

2.1. Materials

Citrus pectin (CP), a typical homogalacturonan (HG) pectin, was purchased from Sigma-Aldrich and contains > 74% GalA. Methanol and acetic acid were purchased from Sigma-Aldrich (Milpitas, CA, USA). Other reagents were of analytical or HPLC grade, and made in China (Beijing chemical works, Beijing, China).

2.2. Monosaccharide Composition Analysis

Monosaccharide composition was determined by high-performance liquid chromatography (HPLC) following acid hydrolysis of pectin [26]. Briefly, 2 mg of pectin was first hydrolyzed using anhydrous methanol containing 2 M HCl at 80 °C for 16 h and then with 2 M TFA at 120 °C for 1 h. Released monosaccharides were derived by using 1-phenyl-3-methyl-5-pyrazolone (PMP) and analyzed by HPLC on a DIKMA Inertsil ODS-3 column (4.6 mm × 150 mm) connected to a Shimadzu HPLC system (LC-20ATvp pump and UV–VIS detector, Shimadzu, Tokyo, Japan).

2.3. Preparation of Pectin Standards with Different DM

CP (10 g) was saponified with 0.1 M NaOH (400 mL, PH ≈ 13) at 4 °C to remove methyl and acetyl groups. The de-esterified CP (5 g) was then hydrolyzed with 0.5 M HCl (500 mL, PH ≈ 0.3) at 80 °C for 5 h. After centrifugation (15,000× *g*, 20 min), the acid-soluble and acid-insoluble fractions were separated. The insoluble material, which mainly contained HG, was suspended in water and solubilized by gradually increasing the pH to ~7 with 0.2 M NaOH. The solution was desalted by hollow fiber membrane (MWCO 3 KDa) and freeze-dried to give the purified non-esterified HG domain (CP-HG). The CP-HG (5 g) was suspended in 2 M methanol–sulfate solution (200 mL). After stirring at 4 °C for 4 days, methylesterification of carboxyl group of GalA was complete. The pectin was collected and washed twice with methanol, and then with methanol–water (3:1) to remove H_2SO_4 [27]. This high methyl-esterified pectin was termed CP-HM-HG. Pectin standards with different DM levels were prepared by mixing CP-HG and CP-HM-HG in different proportions.

2.4. Determination of DM by Titration

The DM of pectin was determined using the titration method [28]. Pectin (500 mg) was refined with HCl–ethanol solution to remove organic acid impurities. It was then titrated with 0.1 M NaOH using phenolphthalein as the indicator, and the volume consumed was recorded as V1. Afterwards, saponification of pectin was initiated by adding 20 mL of 0.5 M NaOH. When the reaction was completed, 20 mL of 0.5 M HCl was added, and the excess HCl was neutralized with 0.1 M NaOH; the volume consumed was recorded as V2. The volume of V1 was consistent with the content of GalA without esterification, and V2

was consistent with the content of esterified GalA in the pectin sample. The esterification degree of each pectin sample was determined by three groups of independent experiments.

The calculation of DM content is:

$$\mathbf{DM} = \frac{\mathbf{V2}}{\mathbf{V1} + \mathbf{V2}} \times \mathbf{100}\% \quad (1)$$

2.5. Determination of DM by FT-IR

The DM of pectin was determined by FT-IR as previously described [29]. In the FT-IR spectrum, specific bands at 1740 and 1630 cm^{-1} corresponded to the absorption of esterified and free carboxyl groups, respectively. The DM content was proportional to the ratio of the area from the band at 1740 cm^{-1} over the sum of the areas from the bands at 1740 and 1630 cm^{-1}. To quantify the DM of different pectin samples, a calibration curve was constructed based on pectin standards of known DM content (0, 22, 44, 66 and 88%) that were prepared by mixing the appropriate quantities of CP-HG and CP-HM-HG. Each pectin sample (2 mg) was mixed with KBr (100 mg) and then pressed into a 1 mm pellet for FT-IR measurement. The FT-IR spectrum was obtained using a Spectrum Two FT-IR spectrometer average of 4 scans with a spectral resolution over the range of 4000–400 cm^{-1} (Perkin Elmer, USA). From each standard and CP pectin, three independent samples were taken, their FT-IR spectra were recorded, and the area of interest measured.

Results were analyzed and calculated by using OMNIC software (version 9.2, Thermo Fisher Scientific, USA). The FT-IR spectra were smoothed ('automatic smooth' function) and baseline-corrected ('automatic baseline correct' function) in OMNIC software. In absorbance mode, the peaks of 1740 and 1630 cm^{-1} were deconvoluted using the curve-fitting method with the Levenberg–Marquardt algorithm and the Gaussian function for the peak shapes.

2.6. Determination of DM and DAc by HPLC

To determine the DM and DAc levels in esterified pectin samples, the samples were saponified with NaOH to produce methanol and acetic acid, which were then detected and quantified by HPLC using a C18 column (Grace Prevail, 5 μm, 4.6 × 250 mm), essentially as previously reported [17] with some modifications. The pectin sample (5 mg) was suspended in 0.2 M NaOH (0.5 mL) and stirred for 2 h at 4 °C. The reaction solution was then neutralized with 0.2 M H_2SO_4 (0.5 mL) and filtered through 0.45 μm Milliporemembranes and injected (20 μL solution) onto the HPLC.

Methanol and acetic acid were used as external standards to generate the calibration curve for quantifying samples. Standard solutions of methanol and acetic acid with concentrations of 5 mM, 10 mM, 15 mM, 20 mM, and 25 mM were prepared and injected onto the HPLC. Using a refractive index detector at 25 °C, the elution was performed with 4 mM H_2SO_4 at a rate of 0.7 mL min^{-1}. For each standard and CP pectin, three independent samples were taken and their HPLC elution curves were recorded.

The DM and DAc content of pectin samples were calculated as follows:

$$\mathbf{DM} = \frac{\mathbf{C(MeOH)} \times \mathbf{176} \times \mathbf{10^{-3}}}{\mathbf{m} \times \mathbf{GalA}\%} \mathbf{DAc} = \frac{\mathbf{C(HOAc)} \times \mathbf{176} \times \mathbf{10^{-3}}}{\mathbf{m} \times \mathbf{GalA}\%} \quad (2)$$

Here, C(MeOH) and C(HOAc) are the concentrations of MeOH and HOAc; "176" is the molecular weight of GalA in pectin; "m" represents the weight of HG pectin to be determined, and the GalA content was determined by monosaccharide composition analysis.

2.7. Statistical Analysis

Differences between methods of determining the DM or DAc were analyzed using one-way ANOVA (Statistical Software Origin 8, OriginLab, USA). The differences were considered as significant at $p < 0.05$.

3. Results and Discussion

3.1. Preparation of Standard Pectin Samples

In this study, we used citrus pectin (CP) as the raw material to prepare standard pectin samples. CP contains GalA (76.7%) as the major sugar, with small amounts of Gal (13.7%), Rha (3.3%), Glc (3.1%) and Ara (1.7%) (Figure 1). To prepare unesterified HG-type pectin as a standard, CP was de-esterified using alkali saponification and hydrolyzed by acid to remove neutral sugars. The obtained species, termed CP-HG, mainly contained GalA (≥97%). CP-HG was then methyl-esterified with sulfuric acid–methanol to yield high methyl-esterified pectin, termed CP-HM-HG. The monosaccharide composition of CP-HG was similar to CP-HM-HG.

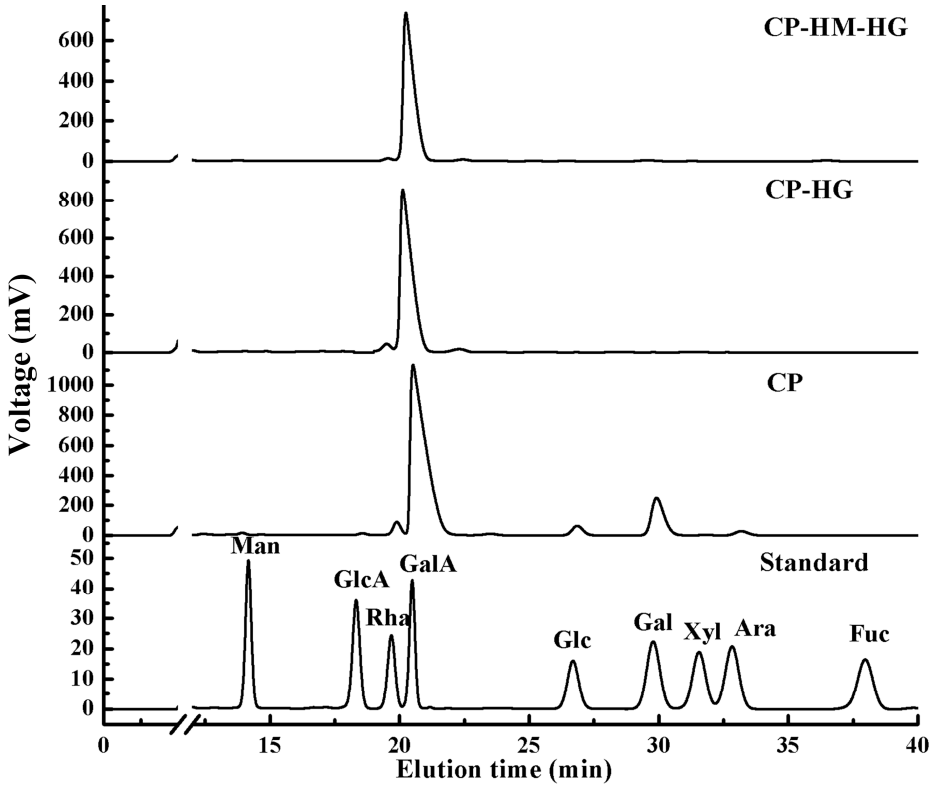

Figure 1. Monosaccharide composition of CP, CP-HG and CP-HM-HG.

3.2. Determination of DM by Titration

The DM values of CP, CP-HG and CP-HM-HG were determined by using the titration method. The volumes of NaOH consumed during the titration of different samples are listed in Table 1. According to Equation (1) in Section 2.4., the DM of CP-HG was 0.0%, consistent with results from alkali de-esterification. The DM content of CP-HM-HG was calculated to be 88.0%, suggesting that it is a high methyl-esterified HG. Acetyl groups were not present in CP-HG and CP-HM-HG due to alkali saponification. The DM content of CP was determined to be 47.0%, and this was further analyzed by using other methods.

Table 1. The titration of V1, V2 and the determination of DM for three pectins.

Pectin	V1 (mL)	V2 (mL)	DM (%)
CP	12.7 ± 0.12	11.3 ± 0.20	47.0 ± 0.63
CP-HG	26.3 ± 0.32	0.0 ± 0.01	0.0 ± 0.01
CP-HM-HG	3.3 ± 0.15	24.3 ± 0.24	88.0 ± 0.59

The results are shown with the associated standard error of means (± SEM) of triplicate values from the average. V1 represents the content of GalA without esterification, V2 represents for the content of GalA esterification.

3.3. Determination of DM by FT-IR

In the FT-IR spectrum, the DM content of pectin was found to be proportional to the ratio of the area of the band at 1740 cm^{-1} over the sum of the areas from the bands at 1740 and 1630 cm^{-1}. To calculate the DM content of different pectin samples, standard curves were established based on pectin with known DM content. In this study, HG standards with different DMs (DM = 0.0%, 22.0%, 44.0%, 66.0%, 88.0%) were prepared by mixing appropriate ratios of CP-HG (DM = 0.0%) and CP-HM-HG (DM = 88.0%), which were then detected by FT-IR (Figure 2a). Then, the peak areas at 1740 cm^{-1} and 1630 cm^{-1} were fitted and integrated (Figure 2b). According to the peak area ratio of A1740/(A1740 + A1630) and corresponding DM content of pectin, the calibration curve was made as follows: DM = 0.0095A1740/(A1740 + A1630), R^2 = 0.9941 (Figure 2c). We then used the FT-IR method to determine the DM content of CP (Figure 2d), and the result was 47.6 ± 0.67%, which was close to the DM content determined by titration.

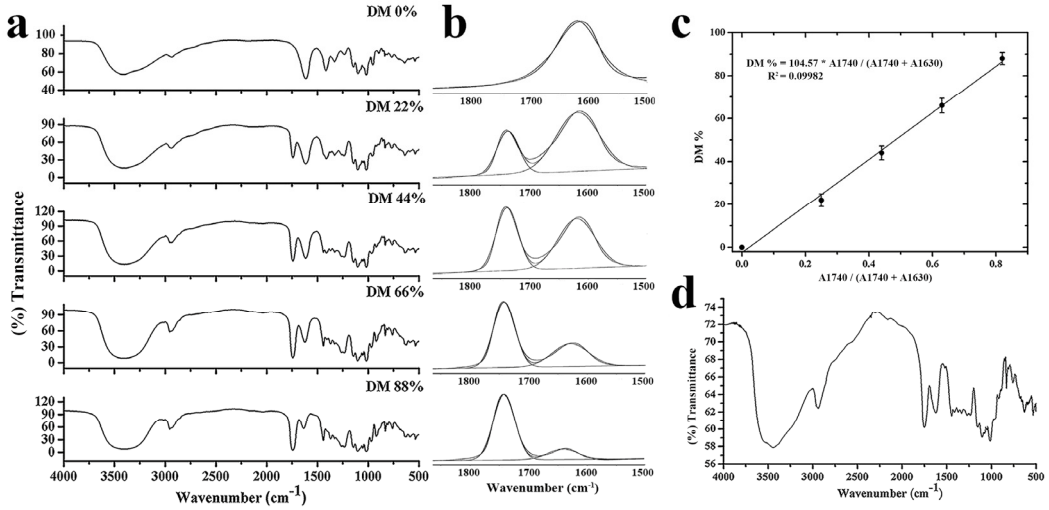

Figure 2. (**a**) FT-IR spectra of HG pectin standards with different DM values. (**b**) The two peak areas of A1740 and A1630 with the Levenberg–Marquardt algorithm and the Gaussian function by OMNIC. (**c**) Linear correlation between DM and A1740/(A1740 + A1630) of standard pectins. The equations and squared correlation coefficients (R^2) of the linear fit are displayed on the graphs. (**d**) FT-IR spectrum of CP pectin. Values are the mean of three independent experiments.

3.4. Determination of DM and DAc by HPLC

HG-type pectin is usually methyl-esterified by methanol at *O-6* carboxyl of GalA, but it can sometimes be acetylated at *O-2* or *O-3* of GalA by acetic acid. The titrimetric and FT-IR methods can determine the DM content of HG pectins, whereas these methods cannot be used to determine DAc content. For the characterization of HG-type pectin, it is better to find a way to simultaneously determine levels of DM and DAc. According to the literature, HPLC analysis following alkali saponification is a method used for determining pectin DM and DAc content simultaneously. In the present study, we adopted this approach with some modifications.

In this regard, methyl-ester and acetyl groups were hydrolyzed by alkali to produce methanol and acetic acid, and these compounds were then detected by HPLC with an RI detector. NaOH neutralization was performed by using 0.2 M H_2SO_4 rather than cation exchange resin. H_2SO_4 was also the eluent for HPLC analysis; therefore, neutralization was simplified by using our procedure. We referred to G.A. Luzio and R.G. Cameron's method for improvement, shortening the sample pretreatment and injection time on this basis [24]. Then, de-esterified pectin was precipitated by adding $CuSO_4$ or isopropanol, as in the literature [17]. However, pectin cannot be completely precipitated by using this approach. At the same time, CuSO4 or isopropanol can also produce higher solvent peak, which may cause error to the DM measured. Column and liquid phase systems may also

be damaged. Here, we did not remove pectin before HPLC analysis, because pectin will not be preserved on the C18 column or guard column. Pectin was usually eluted in under 5 min. Therefore, the modified procedure is simpler and more convenient.

In order to quantify the content of released methanol and acetic acid, external standards were used, and the calibration curve was based on the peak area of standard methanol or acetic acid solutions from HPLC. The results of the determination of methanol and acetic acid at different concentrations by HPLC are shown in Figure 3a. As can be seen, the elution times for methanol and acetic acid were 6.1 min and 9.2 min on HPLC, respectively, which was less than 10 min for each analysis. The peak area was proportional to the concentration of methanol or acetic acid (Figure 3b,c). Therefore, the standard curve was drawn between methanol or acetic acid to their peak areas. The regression equation used for the calibration curve was C(MeOH) = A(MeOH)/906.47 (R^2 = 0.9993) and C(HOAc) = A(HOAc)/5032.3 (R^2 = 0.9997), where C represents the concentration of methanol or acetic acid, and A represents the peak area for methanol or acetic acid.

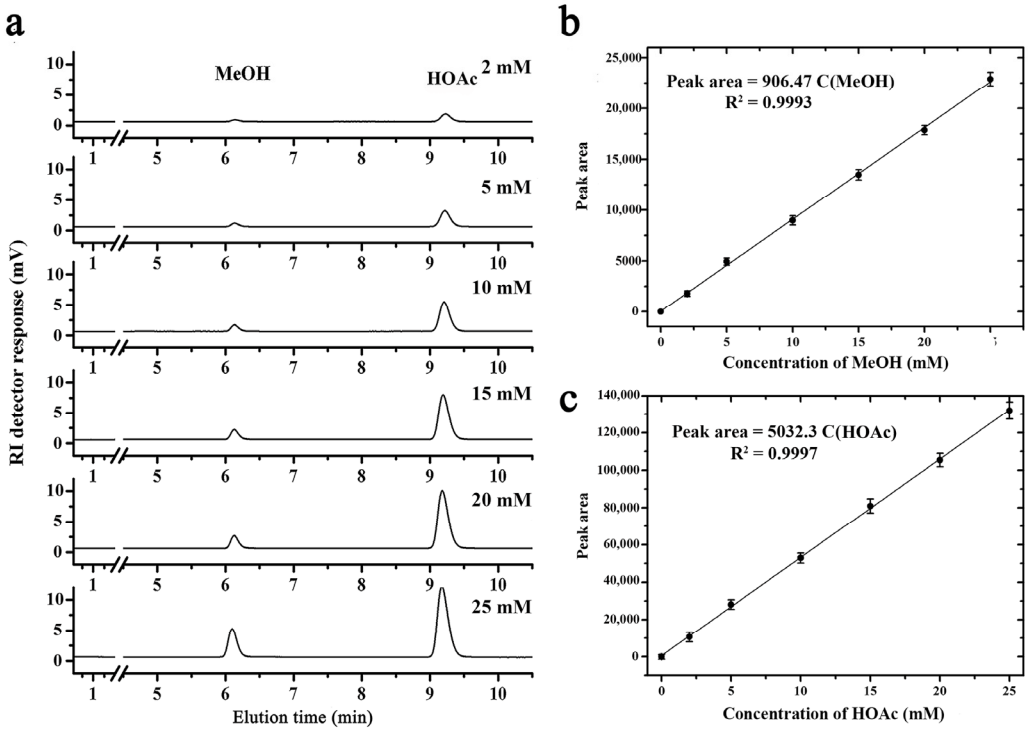

Figure 3. (**a**) Elution curves of methanol and acetic acid with known concentrations on HPLC. (**b**) Standard curve determination between the concentration and peak area of MeOH. (**c**) Standard curve determination between the concentration and peak area of HOAc. The equations and squared correlation coefficients (R^2) of the linear fit are displayed on the graphs. Values are a mean of three independent experiments.

HG pectin standards with different DMs measured by titration (DM = 0.0%, 22.0%, 44.0%, 66.0%, 88.0%) were saponified with alkali and then determined by HPLC. The elution peaks of methanol from different HGs are shown in Figure 4a. No elution peaks were detected at 9.2 min for acetic acid in HPLC, suggesting that there was no acetylation in these HG pectins. By calculating the peak areas of methanol, their concentrations could be determined according to the above calibration curve. Based on Equation (2) in Section 2.6, the DMs of HG standards were calculated as 0%, 23.7 ± 0.24%, 44.5 ± 0.56%, 67.3 ± 0.38% and 84.2 ± 0.67%, respectively. The results between HPLC and titration were not significantly different ($p > 0.05$). These values are consistent with results from the titration method. We also measured the DM of CP as 48.0 ± 0.43%, which had no significant differences compared with the DM of 47.0% and 47.6%, determined by the titration and FT-IR methods, respectively ($p > 0.05$). Comparison of pectin with different DM determined by HPLC is shown in Figure 4b. Meanwhile, the DAc content of CP was determined to

be 1.6 ± 0.14%, indicating that there are only small amounts of acetyl groups in CP. Thus, both DM and DAc could be determined simultaneously by using HPLC.

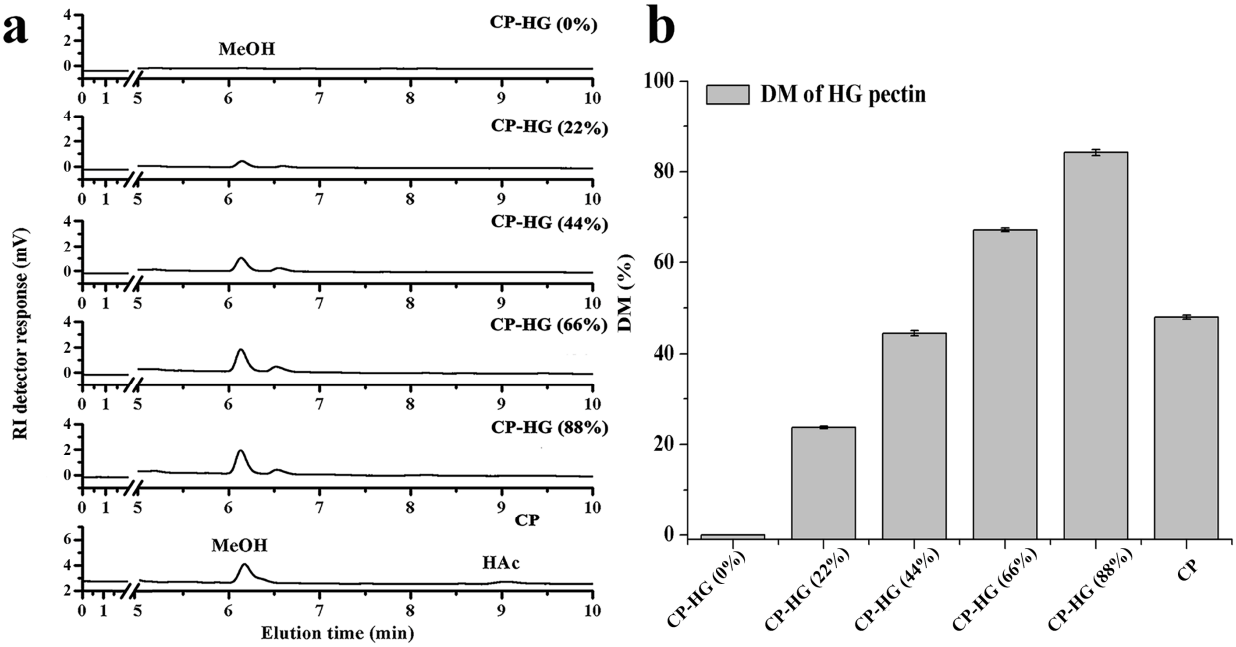

Figure 4. (**a**) Elution profiles of methanol and acetic acid released from different pectins on HPLC. (**b**) The DM results of different pectin by HPLC detection.

According to the above analysis, the DM content of pectin can be determined by the titration, FT-IR, and HPLC methods, and the DAc content can be determined by using the HPLC approach. Comparisons of these three methods are summarized in Table 2. Titration is easy to perform and can be used to determine the DM content in pectin when other instruments are not available in the laboratory; however, it is not suitable with small quantities of samples. The FT-IR method is simple and accurate, and it only needs a few milligrams of a sample. In addition, this method does not destroy the sample; it can be recovered. However, neither the titration nor FT-IR methods can be used to determine DAc content. When the degree of acetylation in the sample is high, the result of DM determination by the titration or FT-IR method approach will be affected. Therefore, these two methods are not suitable for pectin with high acetylation content. Compared with the titration and FT-IR approaches, the HPLC method can be used to determine DM and DAc content in pectin simultaneously, and only a few milligrams of sample will be required. Therefore, if the pectin sample has a high degree of acetylation and HPLC is available, this method is better to use for determining DM and DAc content.

Table 2. Comparison of different methods for the determination of DM and DAc.

	Titration	FT-IR	HPLC
Determination of DM	Yes	Yes	Yes
Determination of DAc	No	No	Yes
Sample amount	500 mg	2 mg	5 mg
Instrument	Burette	Infrared spectrometer	HPLC
Operation	Simple	Simple	Complicated
Sample recyclability	No	Yes	No
Standard sample required	No	Pectin with known DM	MeOH and HOAc with known concentration

4. Conclusions

In this paper, titration, FT-IR and HPLC methods for the determination of DM and DAc content in pectin were compared and optimized. Commercial citrus pectin (CP) was used for the preparation of a series of pectin standards with different DM contents. Our results indicate that the DM contents of CP determined by titration, FT-IR and HPLC methods were mostly consistent, whereas the titration method required more sample than the other two approaches. The DAc content of CP can be determined by using the HPLC method, which is suitable for simultaneously determining DM and DAc content. In our study, the treatment of samples prior to HPLC analysis has been simplified and optimized compared with previous reports in the literature. Based on these comparisons, one can select the appropriate method for determining DM and DAc content in pectins.

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