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FT-IR spectroscopic determination of the degree of esterification of cell wall pectins from stored peaches and correlation to textural changes

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Abstract

The FT-IR spectroscopic study of peach cell walls revealed the existence of two peaks absorbing at 1749 and 1630 cm^{-1} assigned, respectively, to the absorption of the esterified and non-esterified carboxyl groups of the pectin molecules. A linear relationship between the degree of esterification [(number of esterified carboxylic groups/number of total carboxylic groups) × 100] and the ratio of the area underneath the peak at 1749 cm^{-1} over the sum of the areas underneath the two peaks, at 1749 and 1630 cm^{-1}, was established using the FT-IR spectra of standard compounds. The use of the 2nd derivative and curve-fitting techniques allowed the elimination of spectral interferences from other cell wall components. The degree of esterification (D.E.) of pectins from Redhaven peaches immediately after harvest and during storage was evaluated from FT-IR data. During storage at 0°C, the D.E. remained practically constant up to 35 days. During storage at higher temperatures it decreased approaching a final value — the same for all temperatures. This value was reached after 22 days of storage at 5°C, 15 days at 15°C and after only 6 days at 20°C. The changes in the degree of esterification during storage correlated well to fruit firmness.

Keywords: Cell wall; FT-IR spectroscopy; Pectin; Degree of esterification; Determination

1. Introduction

Among the plant cell components, the cell walls probably have the most important influence on the textural properties of plant tissue (Ilker and Szczesniak, 1990). The macromolecular constituents present in the plant cell wall fall into three groups classified on the basis of their solubility in water rather than their chemical composition: celluloses, hemicelluloses and pectins. During storage of fruits, important physiological and textural changes such as softening occur (Robertson et al., 1990a;1990b;1992) . These changes are related to changes in the cell wall composition and structure mostly due to enzymatic modifications of the pectic molecules: demethoxylation because of pectinesterase activity and hydrolysis of glycosidic bonds because of polygalactouronase activity. In order to elucidate the mechanism of fruit softening at the molecular level, the monitoring of these chemical changes as a function of the storage time is essential.

Since the degree of esterification — defined as (number of esterified carboxylic groups/number of total carboxylic groups) × 100 — is among the most important properties for the characterization of the pectic molecules, several methods have been proposed for its determination. These methods can be classified into three different groups. Methods of the first group generally use alkaline hydrolysis to split the ester linkage and liberate the methoxy group as methanol (Voragen et al., 1986). The degree of esterification is then calculated from the ratio of methanol divided by total uronic acids (Wood and Siddiqui, 1971; Voragen et al., 1983; Mcfeeters and Armstrong, 1984) or from the change in the amount of carboxyl groups before and after the base-catalyzed methyl-ester hydrolysis (Mizote et al., 1975). The methods of the second group are based on the selective reduction of the esterified galacturonic group to galactose (Maness et al., 1990) or 6,6-dideuteriogalactose (Kim and Carpita, 1992). The degree of esterification is then calculated either from the colorimetrically determined decrease of the galacturonic acid content of the sample after the reduction or from the ratio of galactose produced by the reduction divided by total galacturonic acid — both contents determined with GC or GC–MS. Both the alkaline hydrolysis and the selective reduction methods present certain disadvantages, mainly due to the complexity of the

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procedures involved. A more trouble-free approach is the instrumental analysis, which directly determines both the amount of ionized carboxylate and of total carboxylic groups without spliting the ester linkage. Presently the determination of these two quantities has been achieved by HPLC (Ploger, 1992) and either ^{1}H-NMR spestroscopy or ^{13}C-NMR spectroscopy (Grasdalen et al., 1988). These instrumental methods are simple and fast, but require isolation of the pectic molecules — using multiple extractions — prior to the determination of the degree of esterification. This is a time-consuming procedure which also runs into the risk of an incomplete extraction of pectins. Further, the isolation procedures of the pectic substances have been shown (McCann et al., 1992) to cause de-esterification of these molecules so that the validity of the degree of esterification determined after such a treatment is questionable.

Fourier transform infrared spectroscopy is a method suitable for monitoring chemical changes in cell walls and more specifically changes in the degree of esterification. The absorption of the different types of functional groups involved (i.e. the methyl ester carboxylic group and the carboxylate group) are located at different wavenumbers. This paper describes the development of a method for determining the degree of esterification of pectin molecules using mid-FT-IR spectroscopy. The main advantages of this method over the existing ones are its simplicity, speed and being non-destructive to the sample. The method was then applied to monitoring the changes in the degree of esterification of pectins in Redhaven peaches during storage. The changes in the degree of esterification as determined with this spectroscopic method correlate well to the tissue firmness of the fruit.

2. Experimental

2.1. Preparation of standard samples

Pectin standards with a known degree of esterification, 31%, 68% and 93%, were obtained from SIGMA (Germany). Subsequent pectin standards with a known degree of esterification 44%, 50%, 57%, 62%, 75%, 80% and 85% were prepared by mixing appropriate amounts of the three commercial standards. The pectin samples were then dissolved in phosphate buffer and the pH was adjusted to 5.5. The solution was freeze-dried and the FT-IR spectrum of the residue was recorded.

2.2. Peaches

'Redhaven' peaches harvested on July 6, 1994 in Macedonia, Greece were stored at 0°C, 5°C, 15°C and 20°C.

2.3. Preparation of cell wall materials

Cell wall materials were prepared according to Selvendran (Selvendran, 1975) modified as follows: 10 g fresh peach tissue was homogenized with 100 ml of a 1.5% SDS solution for 2 min in a blender. A few drops of octanol were added to eliminate foam and the homogenization continued for an additional 3 min in an Ultraturrax apparatus. The homogenized material was filtered through miracloth and the residue was washed out with two volumes of a 0.5% SDS solution containing 5 mM $Na_2S_2O_5$. The residue, in 100 ml of a 0.5% SDS solution containing 5 mM $Na_2S_2O_5$, was treated in a ball-mill for a 18 h period, at a temperature of 2°C. The product was centrifuged for 15 min at 18 000 rpm at 2°C. The residue was washed twice with 200 ml of water (followed by centrifugation), and was resuspended in 150 ml of a phenol–acetic acid–water (2:1:1) (W:V:V) solution. The suspension was homogenized for 2 min in an Ultraturrax apparatus, its pH adjusted to 5.5 and then centrifuged for 15 min at 18 000 rpm at 20°C. The residue which was washed twice with 100 ml of water (followed by centrifugation each time) was considered as the cell wall material.

2.4. FT-IR spectroscopy

The cell wall materials obtained with the above procedure were first freeze-dried and then subjected to an additional dehydration before recording the spectra. This dehydration was carried out by exposing the samples to a vacuum and trapping the subliming water vapors in a trap immersed in a solution of acetone with solid CO_2.

All FT-IR spectra were collected with a Nicolet Magna 750 FT-IR spectrophotometer equipped with a DTGS detector, at 4 cm^{-1} resolution and 100 interferograms were co-added to obtain a high signal to noise ratio. The solid state spectra of both pectins and cell wall materials were recorded using a Spectra Tech Diffuse Reflectance accessory against a KBr background. The samples of the standard compounds were treated exactly in the same way (dissolution, pH adjustment, freeze-drying) as the unknown samples.

From each standard three independent samples were taken and their FT-IR spectra were recorded and the area of interest measured. The average of three such areas was plotted against the degree of esterification of the sample [Fig. 1(a) and (b)].

2.5. Analysis of the FT-IR spectra

The FT-IR spectra were smoothed using the Savitsky–Golay algorithm without specifying the number of points with the 'automatic smooth' function and then the baselines were corrected automatically with the 'automatic baseline correct' function. Thus the root mean squared (RMS) noise of every spectrum was calculated. Then using the 2nd derivative analysis in the region 1780–1540 cm^{-1}, five peaks were determined at 1755, 1657, 1634, 1619 and 1551 cm^{-1} [Fig. 2(b) and (d)]. The aforementioned functions were included in the software which accompanied the equipment of the spectrophotometer (OMNIC 3.1). Using the

wavenumbers of the previous peaks, except of that at 1551 cm^{-1} which does not relate to the carboxyl groups but only to proteins, as a guide, the region from 1780 to 1580 cm^{-1} was deconvoluted using the curve-fitting method with the Levenberg–Marquardt algorithm and the Gaussian function for the peak shapes. During deconvolution the maxima of the deconvoluted peaks were at 1755, 1657, 1634 and 1611 cm^{-1}. By using the RMS noise calculated earlier, the areas of these peaks were calculated [Fig. 3(a) and (b)]. The PEAKSOLVE software (version 1.05) was used for the implementation of the above calculation (Galactic industries corporation).

2.6. Flesh firmness measurement

Flesh firmness was measured (in Newtons) using an Instron Universal Machine fitted with an 8 mm plunger which travelled as far as 15 mm deep into the fruit at cross-head speed of 50 mm min^{-1}. A 1 mm thick disc of skin was removed from the equator of the fruit at the point of puncture before the beginning of the measurement.

3. Results and discussion

The FT-IR spectrum of the cell walls from Redhaven peaches immediately after harvest is shown in Fig. 4. The region between 3500 cm^{-1} and 1800 cm^{-1} presents two major peaks centered at about 3455 cm^{-1} (corresponding to the absorption due to stretching of the hydroxyl groups) and at 2920 cm^{-1} (corresponding to the C–H stretching of the CH_2 groups). A second region below 1500 cm^{-1} is the 'fingerprint region' and the absorptions cannot unambiguously be assigned to any particular vibration because they correspond to complex interacting vibrating systems.

The third region between 1800 and 1500 cm^{-1} is of special interest with regards to the evaluation of the degree of esterification, since it allows the observation of infrared absorption by the carboxylic acid and the carboxylic ester groups of the pectin molecules (Stewart and Morisson, 1992). The examination of this spectral region reveals the existence of two bands centered at 1749 and 1630 cm^{-1}. In order to utilize FT-IR spectroscopic results to monitor the structural changes in pectins with respect to carboxylates, it

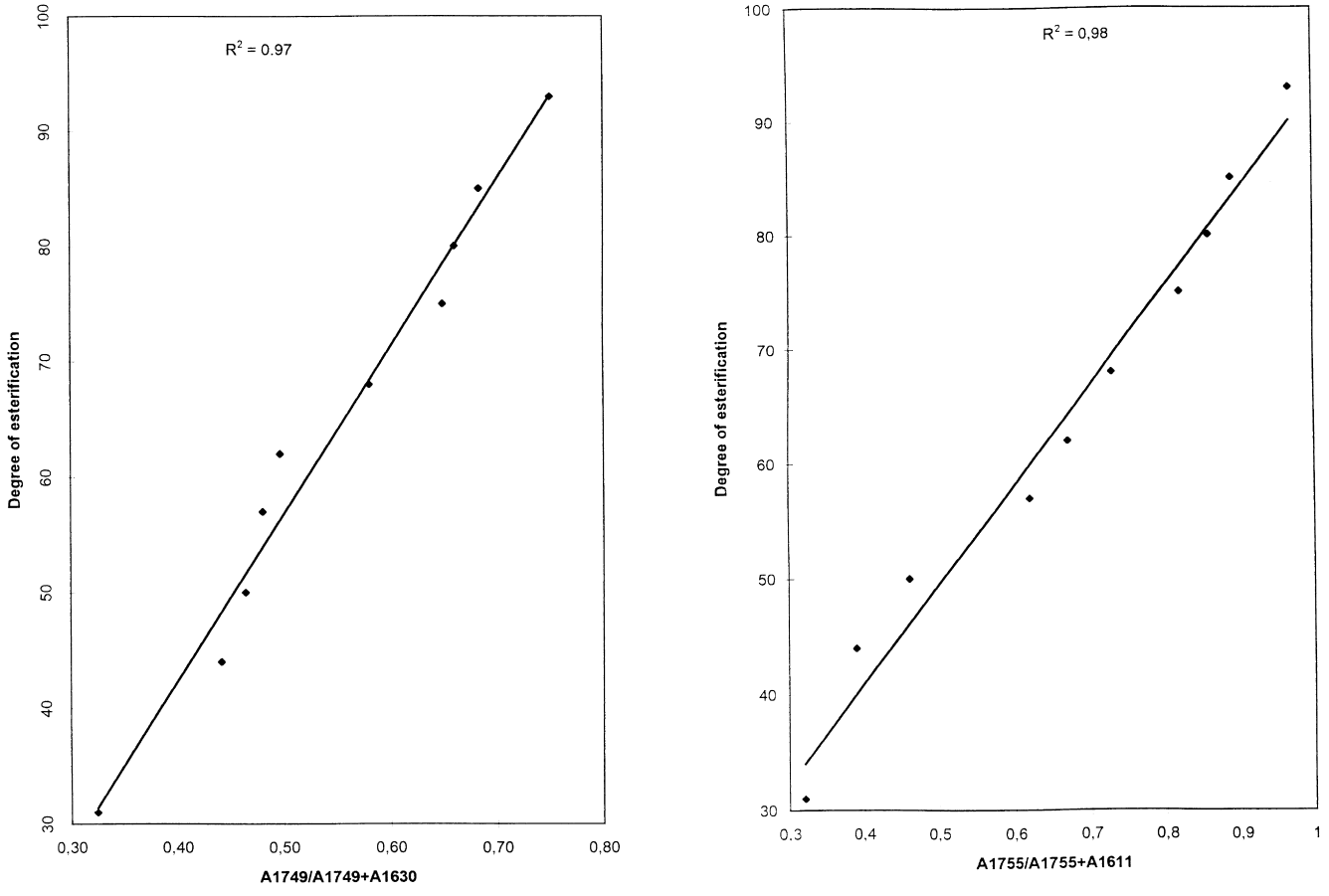


Fig. 1. (a) Calibration curve without deconvolution analysis of the FT-IR spectra: ratio of the peak area at 1749 cm^{-1} over the sum of the peak areas 1749 and 1630 cm^{-1} versus degree of esterification of pectins (%). (b) Calibration curve with deconvolution analysis of the FT-IR spectra: ratio of the peak area at 1755 cm^{-1} over the sum of the peak areas at 1755 and 1611 cm^{-1} versus degree of esterification of pectins (%).

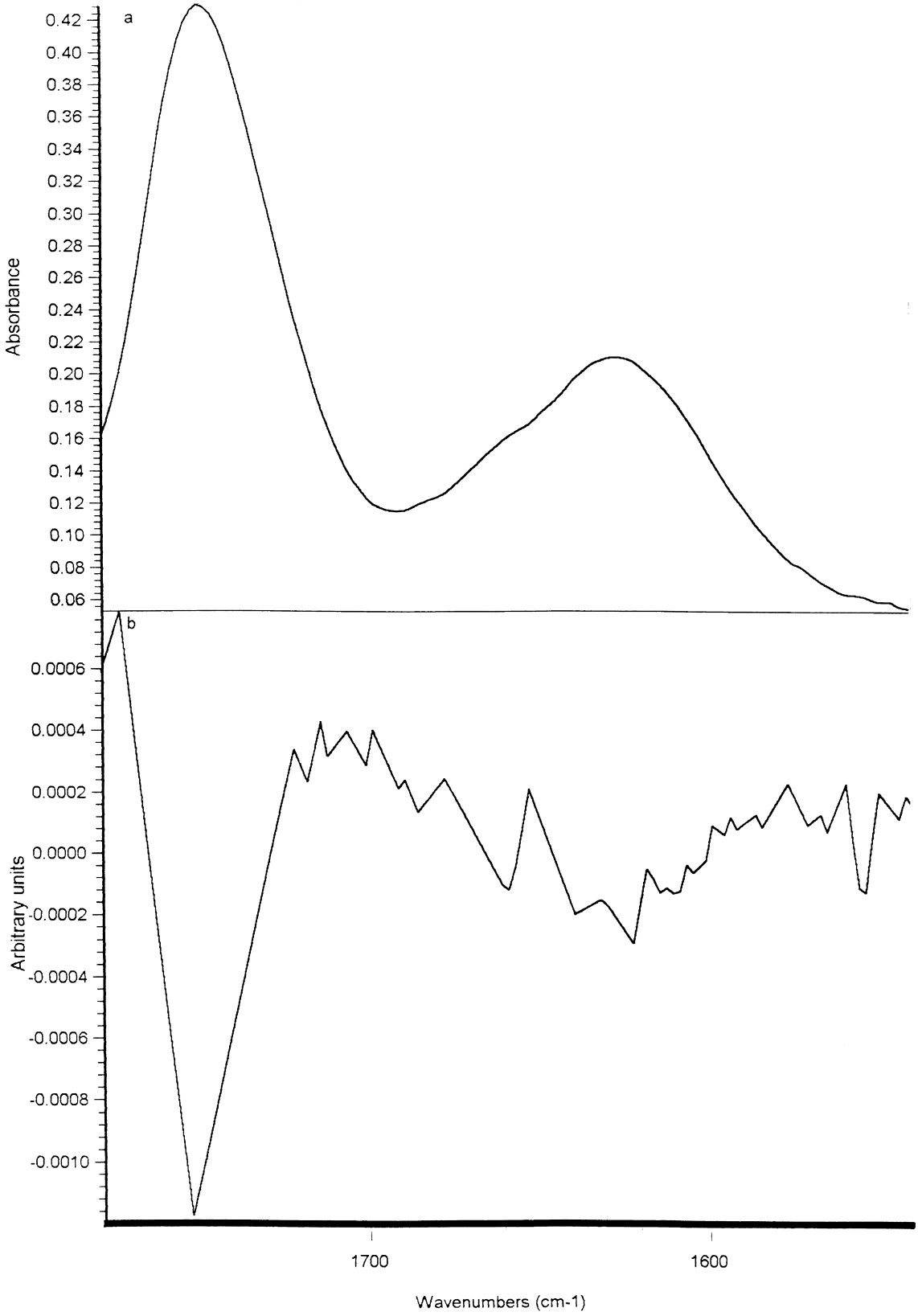
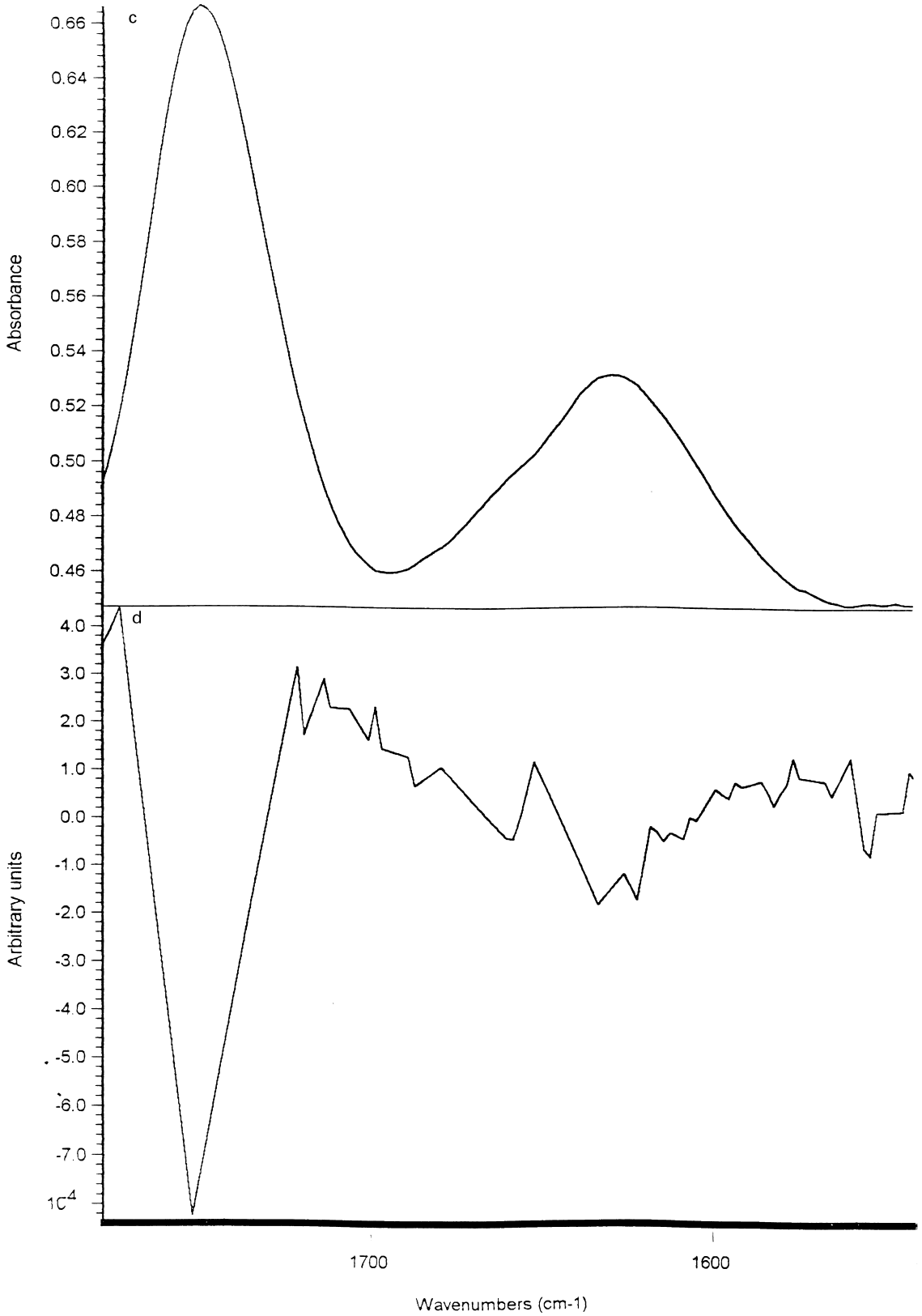


Fig. 2. (a) Zoom of the spectrum from 1780 to 1540 cm^{-1} of the cell wall material (CWM) obtained from Redhaven peaches immediately after harvest; (b) the 2nd derivative of the above spectrum; (c) zoom of the spectrum from 1780 to 1540 cm^{-1} of the standard with an 85% degree of esterification; and (d) the 2nd derivative of the above spectrum.

is important to clearly assign these two absorption bands. The solid state FT-IR spectra of standard compounds such as polygalacturonic acid, its sodium salt and the K^+ salt of a pectin with a degree of esterification equal to 93% were recorded and it was found that both the COOH and $COOCH_3$ groups of pectins absorb at the same frequency 1749 cm^{-1}, while the ionized carboxylate group (COO^-) absorbs at 1630 cm^{-1} (spectra not shown). According to



the literature (Bociek and Welti, 1975; Venyaminov and Kalnin, 1990), the carboxyl ester groups and protonated carboxyl acid groups in solution absorb at about 1740 cm^{-1} whereas the corresponding carboxylate groups absorb at about 1600 cm^{-1}. Thus these absorptions are shifted at higher wavenumbers when the compounds are in the solid state. The same shift has been observed (Costantino et al., 1997) between the solid state and the aqueous solution FT-IR spectra of glycine. Likewise, it is concluded that concerning the cell wall material FT-IR spectra, the band at 1749 cm^{-1} corresponds to the absorption of the esterified carboxylic groups of the pectin molecules while the band at 1630 cm^{-1} is attributed to the absorption of the carboxylate anions. Since the pH of the suspension of the cell wall material was 5.5 and the pK_a of polygalactouronic acids is 3.38 (Ravanat and Rinaudo, 1980), it is clear that all the non-esterified carboxylic groups are in the form of carboxylate ions.

Since the degree of esterification is defined as the (number of esterified carboxylic groups/number of total carboxylic groups) × 100, it is inferred that the ratio of the area of the band at 1749 cm^{-1} over the sum of the areas of the bands at 1749 and 1630 cm^{-1} should be proportional to the degree of esterification. If the appropriate calibration curve relating the ratio of areas $A_{1749}/(A_{1749} + A_{1630})$ to the degree of esterification is established, the degree of esterification of the pectic molecules of the cell walls can be

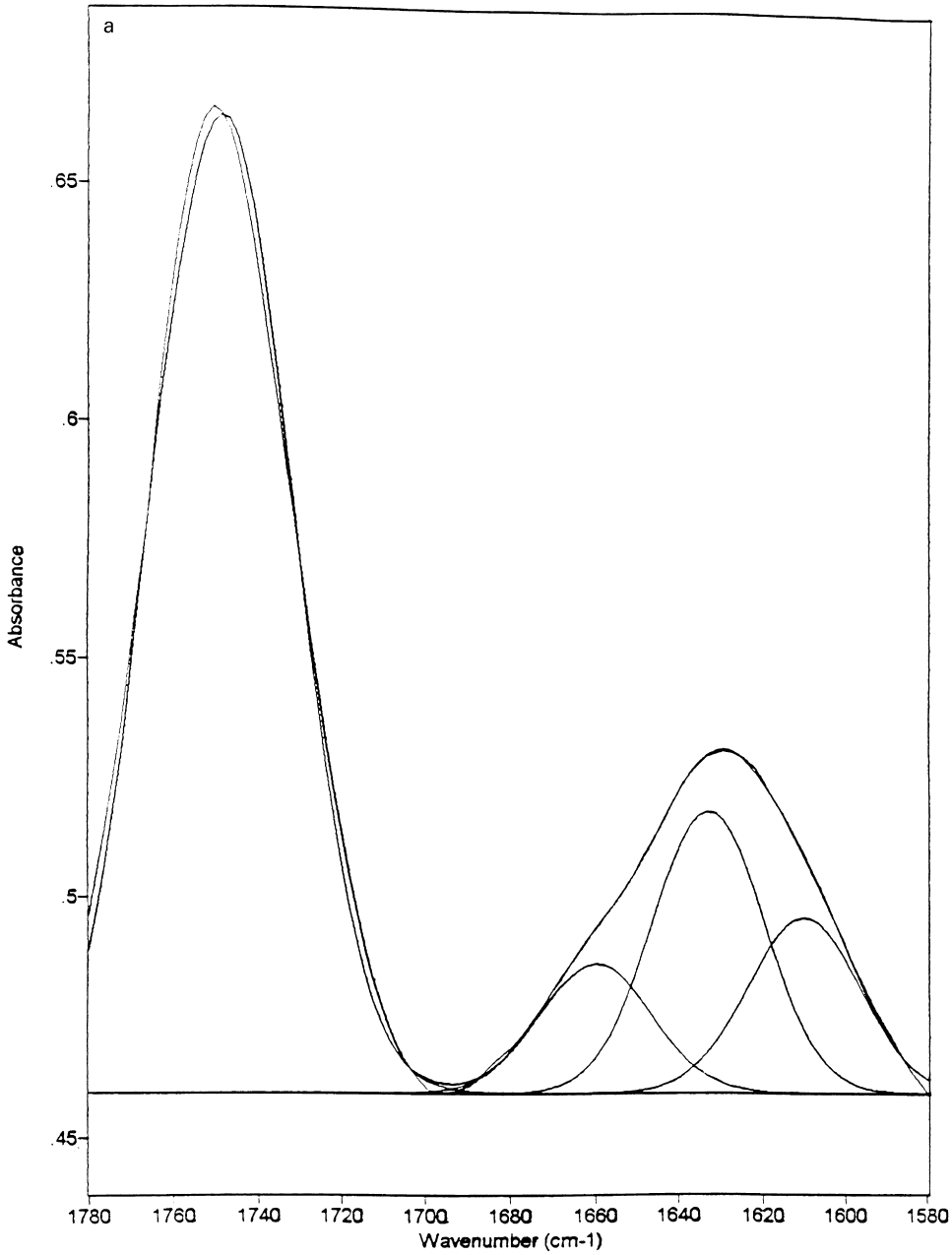
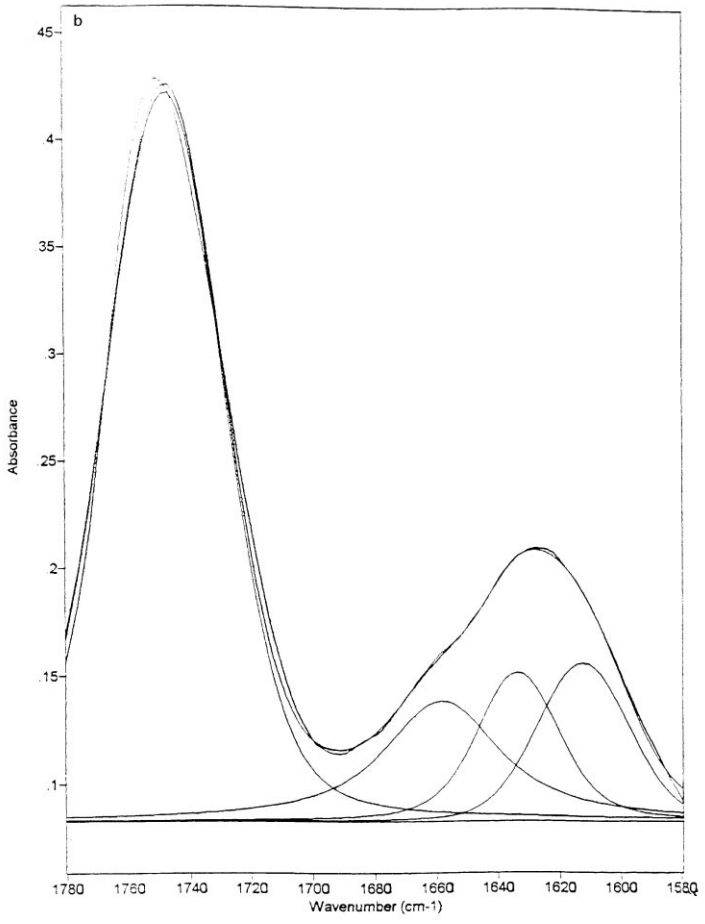


Fig. 3. (a) Deconvoluted spectral region from 1780 to 1580 cm^{-1} of the standard with an 85% degree of esterification; and (b) deconvoluted spectral region from 1780 to 1580 cm^{-1} with of the cell wall material (CWM) obtained from Redhaven peaches immediately after harvest.

determined from FT-IR spectroscopic data. In order to construct this calibration curve the FT-IR spectra of a series of standard pectins of known degree of esterification were recorded and the areas of the bands at 1749 and 1630 cm^{-1} were determined using the built-in (OMNIC 1.1) software of the FT-IR instrument. The limits of the band at 1749 cm^{-1} were set from 1830 to 1695 cm^{-1} and those of the band at 1630 cm^{-1} from 1695 to 1570 cm^{-1}, while a line connecting the points of minimal absorbance left and right of the two bands was used as a common baseline. For every degree of esterification, three different samples were prepared indepedently, their FT-IR spectra were recorded and the ratio $A_{1749}/(A_{1749} + A_{1630})$ calculated. For every triplet of samples the coefficient of variation of the ratios was around 3%, indicating good reproducibility. Using these data the calibration curve presented in Fig. 1(a) was constructed. The correlation coefficient was 0.97. The standard error in the determination of the degree of esterification value of an unknown sample using this calibration curve equaled 4.1. Thus with this calibration curve it was possible to rapidly determine the degree of esterification of pectins from cell walls based on the ratio of the areas of the bands at 1749 and 1630 cm^{-1} of their FT-IR spectra with a reasonable accuracy.

The FT-IR method was then applied to evaluate the degree of esterification of pectins of cell walls from Redhaven peaches immediately after their harvest and to study the changes in the degree of esterification during storage. Of course a number of questions had to be addressed before the calibration curve obtained from commercial pectin samples could be used for the evaluation of peach cell wall samples. First the peak at 1749 cm^{-1} which was used in constructing the calibration curve was characteristic of the absorption of the alkyl esters but aromatic esters which have been reported to occur in the cell walls of rice and sweet corn absorb at 1720 cm^{-1} and interference may be expected (Sene et al., 1994). However, peach cell walls do not contain aromatic esters since the 2nd derivative analysis of the 1749 cm^{-1} centered band revealed the absence of a peak at 1720 cm^{-1} [Fig. 2(b)]. It was also important to check for the presence of interfering components — mainly phenolics and proteins — on the peach cell walls, which absorb in the spectral area used for the evaluation of the degree of esterification: The absence of phenolic compounds — which would absorb in this region — was confirmed by the absence of the caracteristic for these combounds (Sene et al., 1994) Raman bands at 1605 and 1635 cm^{-1}. On the other hand a 2nd derivative analysis revealed [Fig. 2(b)] the existence of small absorptions at 1657 and 1551 cm^{-1} indicating the presence of minor quantity of proteins. However,



as it is shown in the last part of this article their interference is very small.

The following storage temperatures were chosen for the present study: (a) 0°C which is the optimum storage temperature for peaches according to the ISO International Standard for peaches (Robertson et al., 1990b); (b) 5°C in order to simulate maintenance of a cold-chain during wholesale distribution (Shewfelt et al., 1987); (c) 15°C; and (d) 20°C.

The FT-IR spectra of cell walls obtained from peaches stored at the specified temperatures for various days were recorded. A comparison of these spectra showed that, during storage, significant changes occurred in the 'carboxylic' spectral region i.e. from 1850 to 1550 cm^{-1}. The intensity of absorption of the band at 1749 cm^{-1} was diminished while the intensity of the band at 1630 cm^{-1} was enhanced (Fig. 5). These changes reflected a net decrease of the degree of esterification of peach cell wall pectins during storage. This observation was in accordance to findings of Sterling and Kalb (1959) who observed a decrease in the ester content of the pectic substances during ripening of Elberta peaches. At the same time the shape of the band at 1630 cm^{-1} also changed as the absorption maximum was shifted to higher frequencies, probably due to complexing of the pectic carboxylate groups with calcium (Tajmir-Riahi, 1983; Nara et al., 1995).

The ratio of the areas of the bands $A_{1749}/(A_{1749} + A_{1630})$ was calculated from the FT-IR spectrum of every sample and using the calibration curve of Fig. 1(a) the degree of esterification of peach pectins was determined: immediately after the harvest it was 83%. The changes in the degree of esterification as a function of storage time are reported in Fig. 6(a). At 0°C, the degree of esterification changed very slowly and after 35 days of storage it was still 76%. For storage at higher temperatures the degree of esterification declined faster and finally reached a limiting value of aproximately 63%. This plateau value was the same for all storage temperatures studied. However, the rate of the decrease of the degree of esterification did depend on the storage temperature. At 5°C it took 22 days for the degree of esterification to reach this value, while it took only 15 days at 15°C and 6 days at 20°C. The emerging picture from the FT-IR spectroscopic data was in agreement with the results of Dawson et al. (1992) that the degree of esterification of pectins of cell walls of peach fruits stored at 20°C decreased from 79% to 67% in 6 days at storage. Fishman et al. (1993) studied changes in some chemical parameters of peaches (*Prunus persica L.*) during storage at room temperature

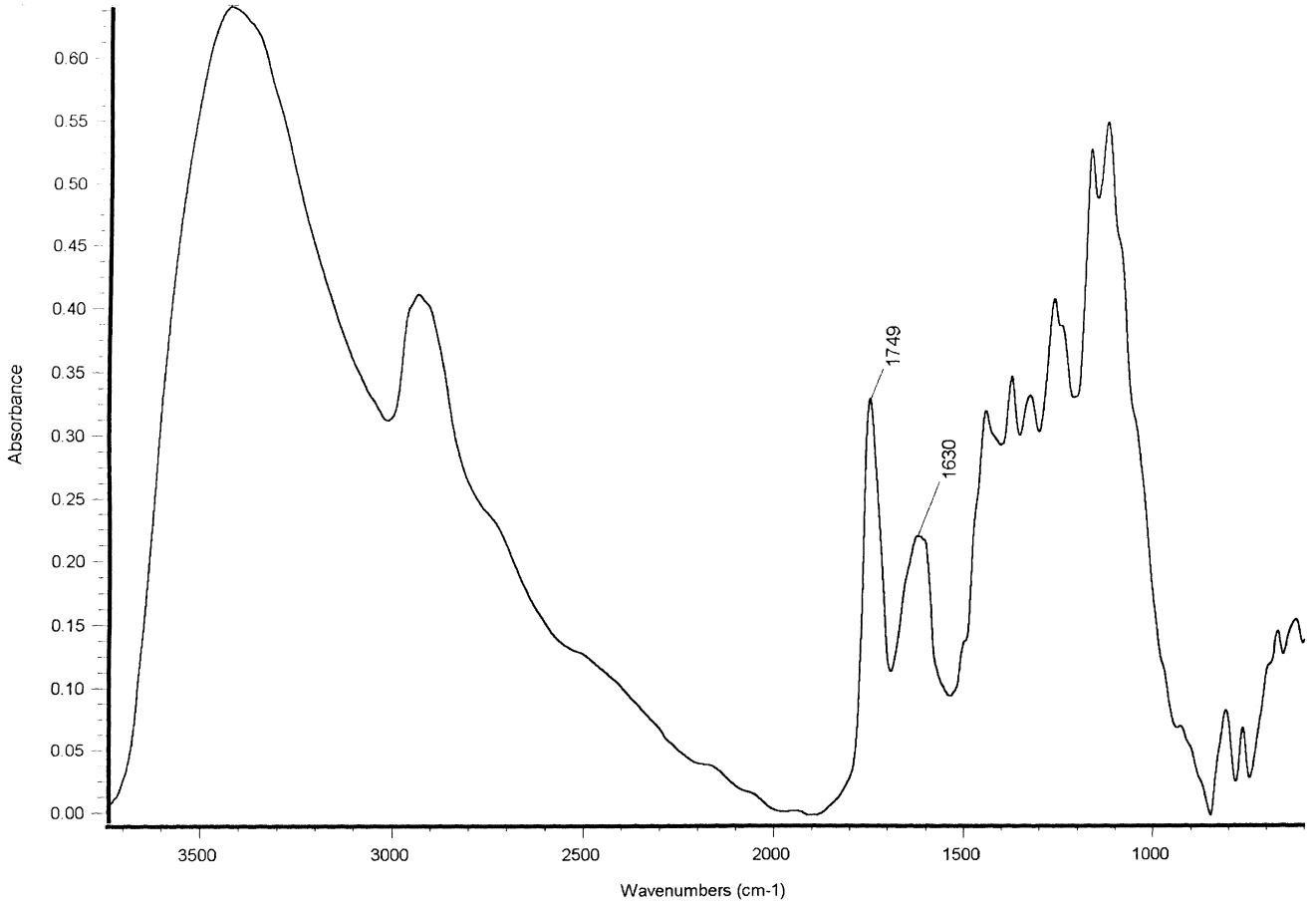


Fig. 4. FT-IR spectrum of the cell wall material (CWM) obtained from Redhaven peaches immediately after harvest.

(25°C ± 2°C). According to their results a number of physicochemical parameters changed dramatically during the first 6 days of storage at this temperature. These parameters included: (a) weight of the cell wall as a percentage of the total fruit weight; (b) weight of pectins as a percentage of the total cell wall weight; and (c) most important the molecular weight distribution of the pectic molecules. Our results indicated that the degree of esterification was another important chemical parameter that changed dramatically after 6 days of storage of peach fruits at ambient temperature.

The changes in firmness of peaches during storage were also studied in order to correlate changes in the degree of esterification of pectins to texture of the fruits. The trend of changes in firmness of peaches during storage is shown in Fig. 7. Practically no change of firmness was observed in peaches stored at 0°C up to 35 days whereas at temperatures above 0°C a softening of peaches ocurred. In all cases firmness of peaches reached a minimum plateau value. This plateau value was the same regardless of the storage temperature, but the rate of softening was faster at higher temperatures. The overall picture [Fig. 6(a) and (b)] was clearly of the same type as the one of the evolution of the degree of esterification indicating a correlation between these two phenomena. The texture of peaches as well as storage life could then be predicted by determining the degree of esterification of the peach pectins.

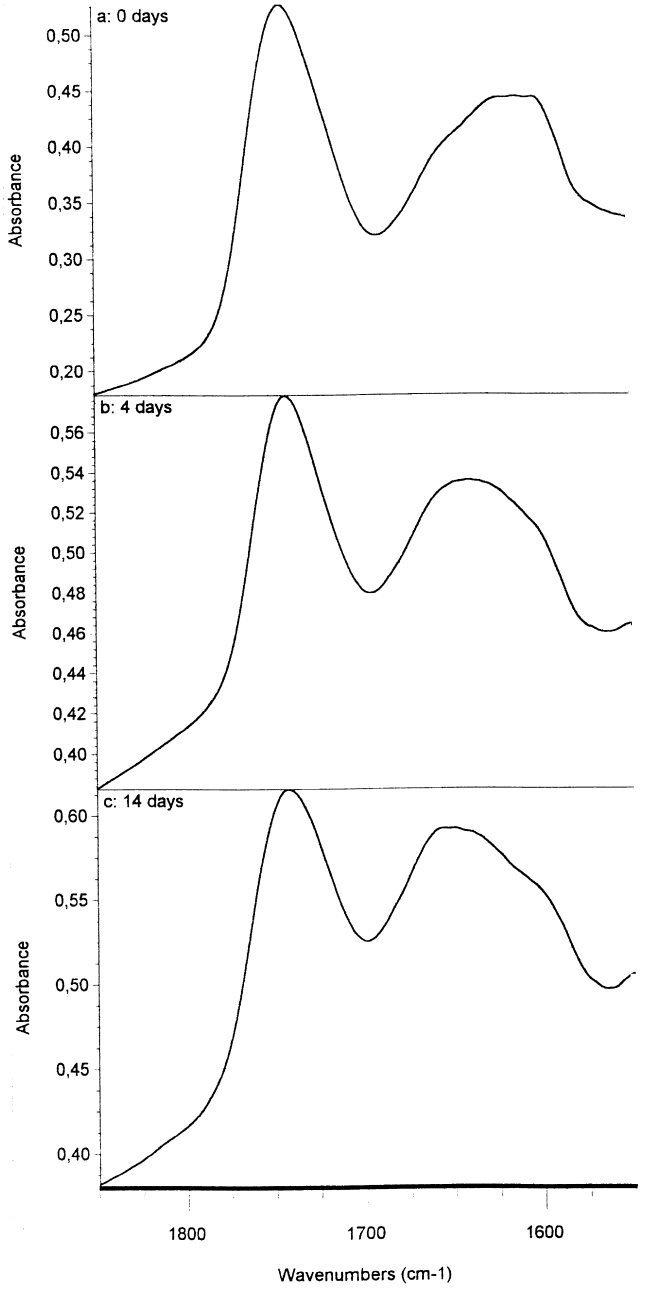


Fig. 5. FT-IR spectra in the 1850–1550 cm^{-1} region of cell wall material from peaches stored at 15°C for: (a) 0 days; (b) 4 days; and (c) 14 days.

From a technological point of view, it seems that storage at 5°C — which is typical during commercial distribution of peaches — is not satisfactory for preserving the texture quality of peaches during postharvest handling althought color and flavor development have been shown to be inhibited at this temperature (Anderson, 1979). The fact that at 0°C the degree of esterification remains practically constant further supports the ISO's statement that the optimum storage temperature for peaches is − 1°C–2°C (ISO, 1980).

More information about the cell wall components can be extracted from the FT-IR peach cell wall spectra with the use of mathematical techniques of spectral data treatment. In particular two points in the FT-IR method developed merit further examination: these are the extent to which the accuracy of determination of the degree of esterification of cell wall pectins is affected by the presence of: (a) the protein compounds — amide I band centered at 1650 cm^{-1} (Sene et al., 1994); and (b) water — the H–O–H bending absorption centered at 1640 cm^{-1} (Marton and Sparks, 1967; Abbott et al., 1987). Apparently both these compounds, if present, would contribute to the intensity of the 1630 cm^{-1} band, which was used for the determination of the degree of esterification and could introduce an error. In order to exclude the interference from these compounds and to maximize the accuracy of the FT-IR method the FT-IR spectroscopic data were further analyzed using the 2nd derivative analysis and the curve-fitting techniques. The 2nd derivative analysis of the 1630 cm^{-1} band — in both peach cell wall FT-IR spectra and the FT-IR spectra of commercial pectins used as standards — revealed [Fig. 2(b)] that this band was in fact composed of three subpeaks. The first one with max at 1657 cm^{-1} and corresponded to the amide I band indicating the presence of proteins. This was confirmed from the fact that the 2nd derivative analysis of the corresponding region revealed the existence of a 1551 cm^{-1} peak (amide II band), too. The second sub-peak centered at 1634 cm^{-1} corresponded to water absorption. The third peak located at 1611 cm^{-1} (at 1619 cm^{-1} using the 2nd derivative) was assigned to the actual carboxylate stretching absorption of the pectin ester group. Evidently only the intensity of this third peak was

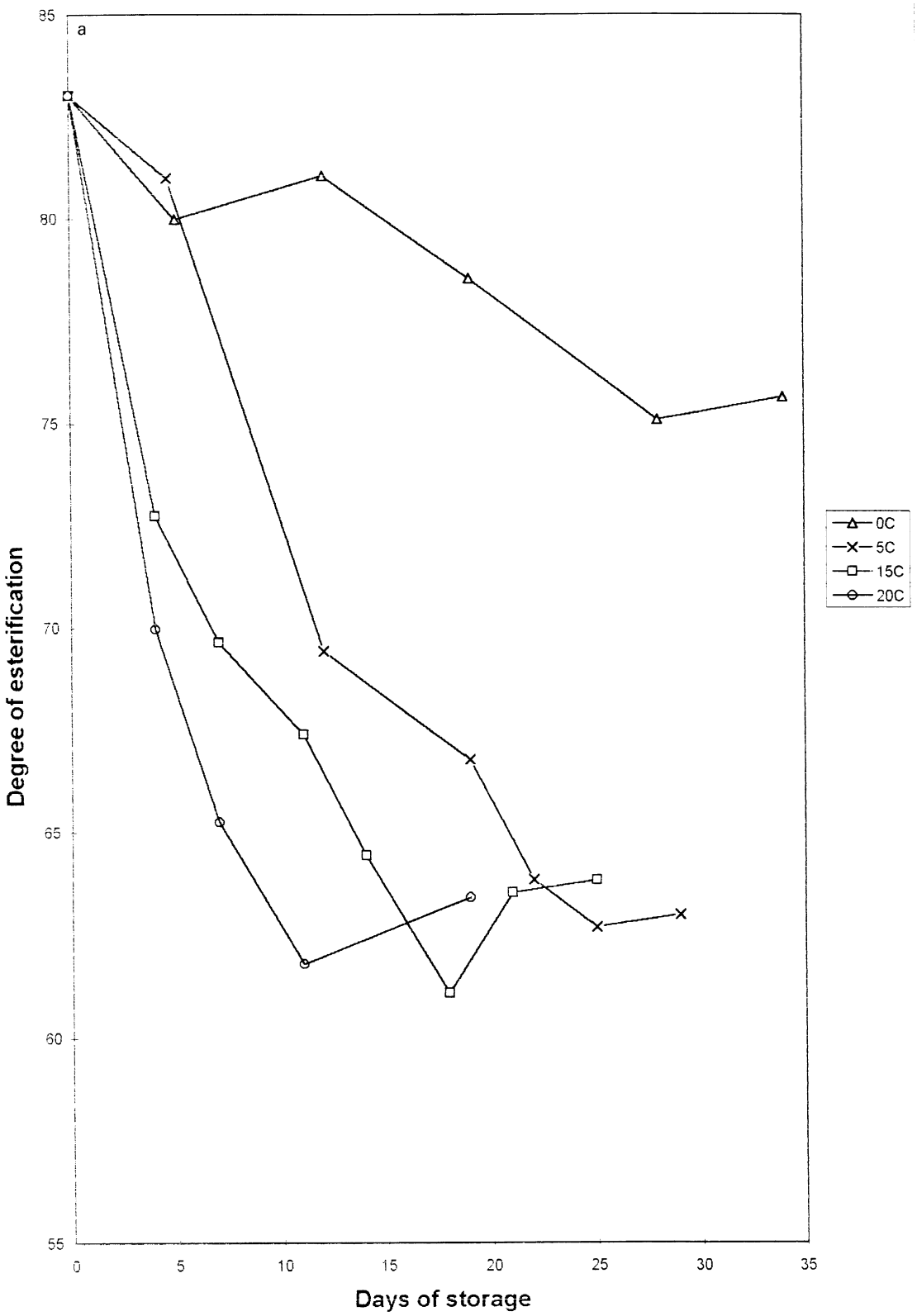
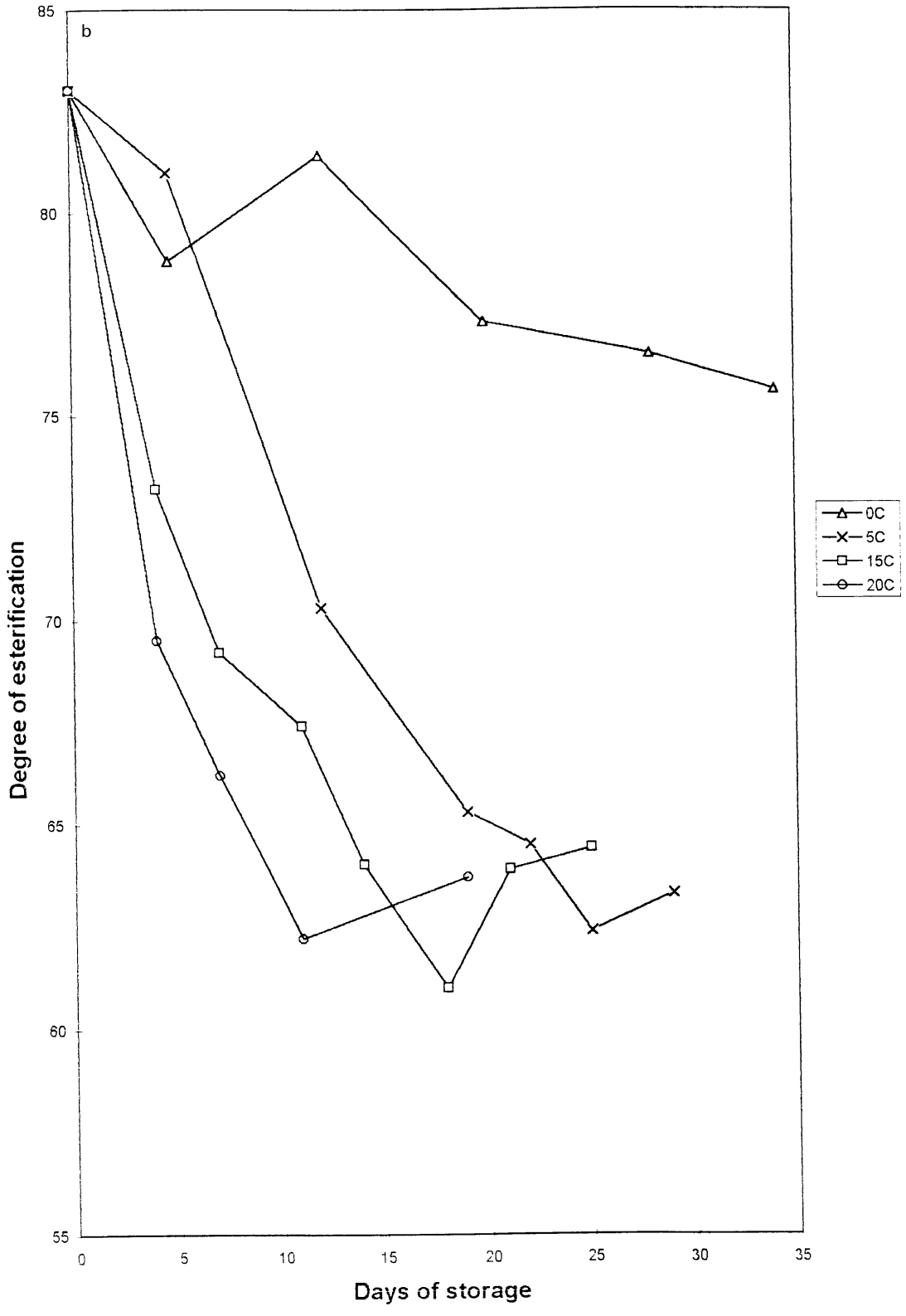


Fig. 6. (a) Changes in the degree of esterification (%) of pectins of peaches stored at different temperatures (0°C, 5°C, 15°C and 20°C) as a function of storage time without deconvolution analysis of spectra. (b) Changes in the degree of esterification (%) of pectins for the peaches stored at different temperatures (0°C, 5°C, 15°C and 20°C) as a function of storage time with deconvolution analysis of spectra.

related to the degree of esterification of pectins. Then the curve-fitting algorithm was used to subtract the interference of water and proteins and allowed the mathematical deconvolution of the 1630 cm^{-1} composite band — of the FT-IR spectrum of each standard — into these three component bands. A calibration curve was constructed relating the ratio of the band areas of each standard to its degree of esterification. This calibration curve was similar to the previous one, but this time the area of the deconvoluted component peak of the carboxylate A_{1611} was substituted for A_{1630} — the



composite band that also involved spectral contributions from water and protein. This curve is shown in Fig. 1(b) and the correlation coefficient (0, 98) was only slightly improved over the one obtained with the nondeconvoluted peak. The same curve-fitting algorithm was used to deconvolute the 1630 cm^{-1} band of the peach cell wall FT-IR spectra [Fig. 3(b)] and construct the calibration curve for the evaluation of the degree of esterification of the cell wall pectins of peaches. The plot of the degree of esterification calculated from deconvoluted spectra as a function of storage time is presented in Fig. 7. The differences are minor relative to the results obtained from nondeconvoluted bands, and the overall trend did not change. It seems that the absorbed water and protein content of peach cell walls did not differ so much from

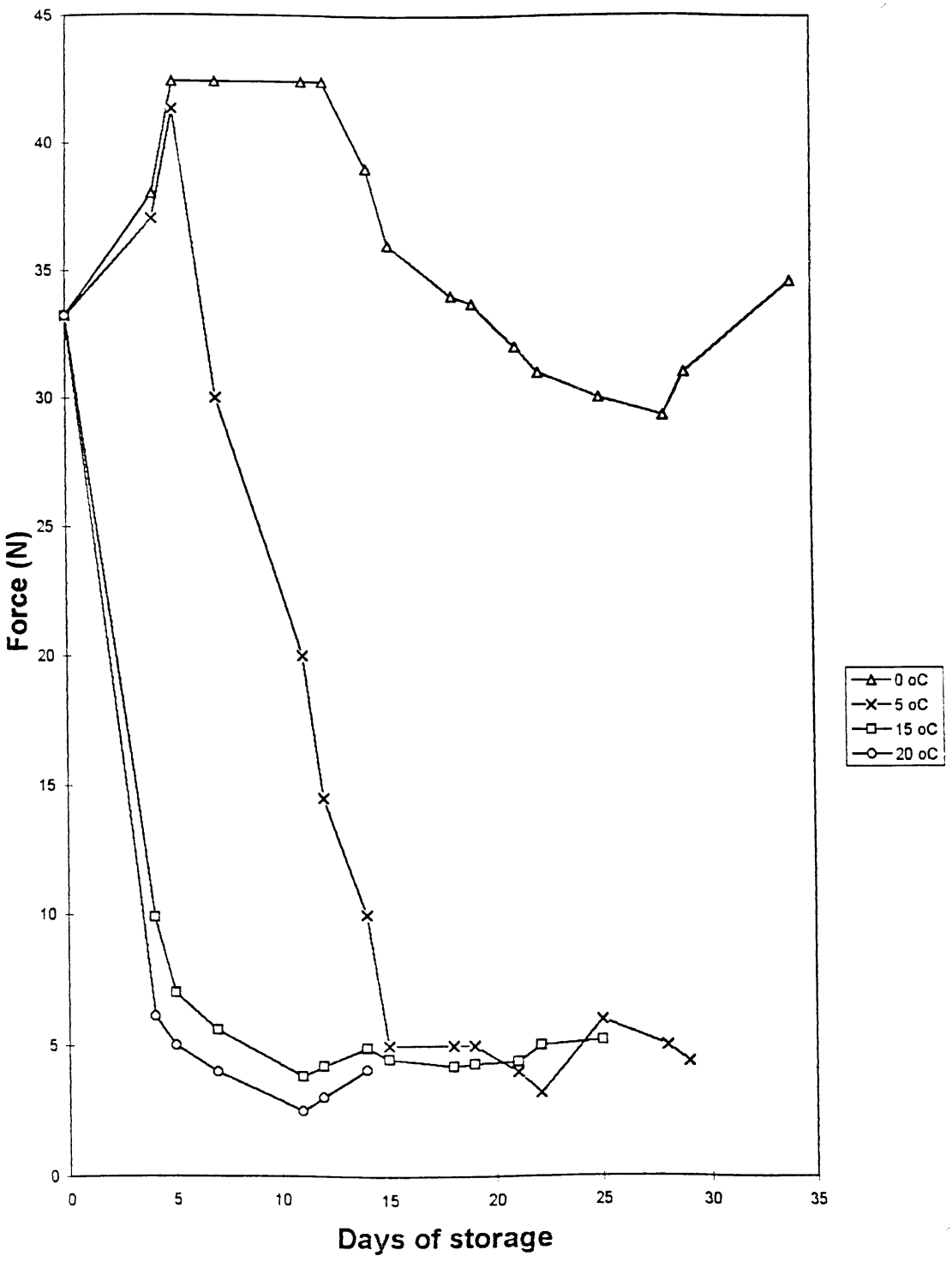


Fig. 7. Changes in the firmness of tissue of peaches stored at different temperatures as a function of storage time.

the absorbed water and protein content of the pectin standards used for establishing the calibration curve. The effect thus of the presence of these compounds in the accuracy of the determination of the degree of esterification of cell wall pectins is minimal if any. The use of the non-deconvoluted FT-IR spectra seems thus the preferred choice for the evaluation of the degree of esterification, since it is simpler and faster compared with the curve-fitting procedure.

4. Conclusions

The FT-IR spectroscopic method described in the present paper belongs to the group of instrumental methods for the determination of the degree of esterification of pectins with minimal processing and the main advantages of speed and simplicity. It is advantageous over the other existing instrumental methods in that it is not necessary to isolate the pectins before measuring the degree of esterification, since the FT-IR spectra of crude cell wall preparations are used for the determination. It was possible to employ spectral data treatment techniques — such as curve-fitting — in order to completely eliminate the interferences from other cell wall components — such as water and proteins. However, it was found that at least in the case of peach cell wall material the results obtained without this treatment are very satisfactory. The FT-IR spectroscopic investigation is proven as a method well-suited for the fast monitoring of chemical changes — such as demethoxylation of pectins — occuring in fruits during storage.

Acknowledgements

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