Available online at www.sciencedirect.com

SCIENCE DIRECT®

Food Chemistry 91 (2005) 551–555

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Determination of the degree of esterification of alkaline de-esterified pectins by capillary zone electrophoresis

Chii-Ming Jiang [a], Shih-Chuan Liu [b], Min-Chang Wu [c], Wei-Hsien Chang [d], Hung-Min Chang [d,*]

[a] *Department of Sea Food Science, National Kaohsiung Institute of Marine Technology, Kaohsiung 811, Taiwan*
[b] *Department of Diet and Restaurant Management, Chung-Shan Medical University, Taichung 402, Taiwan*
[c] *Department of Food Science, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan*
[d] *Graduate Institute of Food Science and Technology, National Taiwan University, 59, Lane 144, Keelung Road, Sec 4 PO Box 23-14, Taipei 10617, Taiwan*

Received 18 February 2004; received in revised form 17 September 2004; accepted 10 October 2004

Abstract

Capillary zone electrophoresis (CZE) was used to determine the correlation between the migration times and alkaline-de-esterified pectins (ADPs) with various degrees (0%, 21%, 47%, 56%, 77% and 93%) of esterification (DEs) using 50 mM phosphate buffer (pH 6.5) as carrier electrolyte. It was observed that pectins with higher DE exhibited shorter migration time, regardless of the capillary length used. Good linearity was obtained with a correlation coefficient (r) as high as 0.994 and 0.998 when a capillary tubing with an effective length of 30 and 60 cm was used, respectively. In addition, separation of pectins was completed in 8 min using a tubing with an effective length of 30 cm. Correlation efficient was also found to be 0.995 when pectin mixture was separated by CZE, suggesting the efficiency and possible utilization of CZE for determination of pectin DE.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: Degree of esterification; Random-type de-esterified pectin; Capillary zone electrophoresis

1. Introduction

Pectic substances are polymers composed mainly of (1 → 4)-α-D-galacturonopyranosyl units, and are found in the middle lamella of plant cells. One of the differences between pectic substances is their content of methyl esters, or degree of esterification (DE), which decreases as fruit or vegetable ripening take place. The DE is defined as the percentage of esterified D-galacturonic acid residues per total number of D-galacturonic acid residues. The DE of pectic substances is usually between 60% and 90%, which depends upon the species, tissues and maturity of the plant tissues (van Buren, 1991).

Alkaline treatment of pectin is usually accompanied with the occurrence of β-elimination in pectin molecules (Ishii & Yokotshuka, 1971); however, well control of reaction temperature and alkaline level during treatment would result in totally random (homogeneous)-type de-esterified pectin (Black & Smit, 1972). On the other hand, pectinesterases (PEs) from higher plants, such as tomato and citrus, release methanol from pectic substances and form blockwise (heterogeneous)-type de-esterified pectin (Heri, Neukom, & Deuel, 1961; Kohn, Furda, & Kopec, 1968; Kohn, Heinrichova, & Malovikova, 1983a, Kohn, Markovic, & Machova, 1983b). Similar to the actions of acid, alkaline, and ethanolic ammonia, some microorganisms such as *Aspergillus*

* Corresponding author. Tel.: +886 2236302312776; fax: +886 223620849.
E-mail address: changhm@ccms.ntu.edu.tw (H.-M. Chang).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.10.003

japonicus produce PEs to form pectin products with random distribution of deacrboxylic acid (Ishii, Kiho, Sugiyama, & Sugimoto, 1979).

The difference in carboxylic acid distribution in pectin molecules resulted in variations in pectin properties such as jellying. Commercial pectic substances usually have DE values between 30% and 76%, and the determination of DE has been a subject of intensive research. Distinction between random- and blockwise-type de-esterified pectin was performed by conventional ion-exchange chromatography (IEC) (Heri et al., 1961; Hong, 1995), high-performance liquid chromatography (de Vries, Rombouts, Voragen, & Pilnik, 1983), determination of stability constant of calcium pectate (Kohn et al., 1968), activity coefficients of calcium ion in calcium pectate solution (Kohn et al., 1983a, 1983b) as well as enzymatic methods in combination with chemicals (Tuerena, Taylor, & Mitchell, 1982; de Vries et al., 1983; de Vires, Hansen, Soderberg, Glahn, & Pederson, 1986). However, those methods appeared to be lengthy and complex.

The alcohol oxidase method developed by Klavons and Bennett (1986) was based on the oxidation of methanol, released from D-galacturonic acid by alkali hydrolysis, to formaldehyde, in the presence of oxidase, which was then reacted with coloring reagent for spectrophotometric quantification. In addition, titration (Mizote, Odagir, Toei, & Tanaka, 1975), borohydride (Maness, Ryan, & Mort, 1990) and Fourier transform infrared (FTIR) (Komae, Sone, Kakuta, & Misaki, 1990) methods have also been proved to be useful. However, these methods all suffer from a major drawback, the lengthy time required. Thus, a rapid method for determining the DE of pectin efficiently is urgently needed.

Capillary electrophoresis (CE) is a newly developed separation technique. It provides advantages of rapid separation with high resolution and efficiency and requires nanoliter levels of sample and media (Kutnink, Skala, Sauberlich, & Omaye, 1985; Taso & Salimi, 1982; Tsai, Li, & Chang, 1998). Among CE techniques, capillary zone electrophoresis (CZE) is the most popular and is based on the difference of electrophoretic mobility that results from the varied charge numbers and particle sizes between electrolytes under applied voltage (Tsai et al., 1998). Under applied voltage, electrolytes are separated by the double effects of their own electrophoretic mobilities and the electroosmotic flow, which results from the electrical double layer in an uncoated fused silica capillary (Kuhn, 1990; Kuhn & Monning, 1992). This method has also proved to be effective in the separation of charged particles (Chiari & Nesi, 1993; Tsai et al., 1998). Neutral solutes are usually separated by micellar electrokinetic chromatography in the presence of anionic surfactants such as Na-dodecyl sulfate (Emmer, Jansson, & Roeraade, 1991). Therefore, most polysaccharides and sugars that lack charges are difficult to be separated by CZE. Pectins with C_6 carboxyl groups in D-galacturonic acids are negatively charged in neutral pH, and the quantity of charge varies with the pectin DE (Christensen, 1986).

In the present research, alkaline-de-esterified pectins (ADPs) with various DEs were prepared and separated by CZE to investigate the relationship between the migration time and pectin DE to establish a rapid method for the determination of DE of pectins in a mixture. In addition, the effect of capillary length on the migration time of ADPs with various DEs was also studied.

2. Materials and methods

2.1. Materials

Citrus pectin (DE = 68%) was the product of Sigma (St. Louis, MO, USA). Methanol, isopropanol, acetone and sulfuric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA), while NaOH was from Merck (Darmstadt, Germany). The other chemicals were of analytical grade.

2.2. Preparation of pectins with various DEs

The DE of citrus pectin from Sigma was raised to 93% with the method described by Sajjaanatakul, van Buren, and Downing (1989), and thus the obtained pectin was treated with various levels (0.005–0.05 N) of NaOH solutions (Heri et al., 1961; Black & Smit, 1972) for 30 min to prepare alkaline-de-esterified pectins (ADPs) with DE of 77%, 68%, 56%, 47%, 33%, 21% and 0%, as determined by the method described below. Temperature (4 °C) was closely controlled to avoid the possible β-elimination and de-polymerization of pectin samples during alkali treatment. The pectins thus obtained were dialyzed against de-ionized water (Mili-Q system, Milipore, Osaka, Japan) at room temperature (26 ± 2 °C)for 12 h and then freeze-dried.

2.3. Determination of pectin

Pectin content of the freeze-dried powders was determined with the method described by Blumenkrantz and Asboe-Hansen (1973). Briefly, pectin solutions (0.5 mL) were mixed thoroughly with 3.0 mL of 0.0125 M Na-tetraborate solution (in c-sulfuric acid) in an ice bath, followed by heating in a boiling water bath for 5 min. The reaction mixture was cooled in an ice bath and was mixed well with 0.05 mL of 0.15% *m*-phenylphenol/0.5% NaOH solution. After resting for 5 min, the absorbance of the reaction mixture at 520 nm was recorded. Different levels (0–100 μg/mL) of D-galacturonic acid (Sigma) solutions were used to construct the standard curve (r^2 = 0.98) for the calculation of pectin content

in each sample. Triplicate samples were each analyzed twice.

2.4. Determination of pectin DE

Pectin DE was determined by the method described by Mizote et al. (1975). A pectin sample (2 g) was mixed well with 90 mL of distilled water, 10 mL of c-sulfuric acid, and 100 mL of isopropanol with slight stirring by a magnetic stirrer for 15 min. Subsequently, the pectin residues obtained by filtration with a Whatman No. 2 filter paper with the aid of an aspirator was rinsed first with 300 mL of 65% isopropanol, followed by 200 mL of isopropanol and 50 mL of acetone to make pectin powders.

The powder (0.5 g) thus obtained was moistened first with 65% isopropanol and then dissolved in 100 mL of distilled water with slight stirring by a magnetic stirrer. Volume (*a* mL) of 0.1 N NaOH solution was recorded during the titration of pectin solution to pH 7.5, and the pectin solution was then mixed well with 30 mL of 0.1 N NaOH for 30 min, followed by the addition of 30 mL of 0.1 N sulfuric acid. Volume (*b* mL) of 0.1 N NaOH was recorded during the titration of pectin solution to pH 7.5. DE (%) = $(b/a + b) \times 100\%$. Triplicate samples were each analyzed twice.

2.5. Apparatus and electrophoretic conditions

Capillary electrophoretic separations were conducted on an electrophoresis instrument (P/ACE system 5500, Beckman Instruments Co., Palo Alto, CA, USA), which was equipped with a diode-array detector with a detective wavelength of 192 nm. Uncoated fused silica capillary tubing (effective length, 30 and 60 cm; inner diameter, 75 μm, Beckman) was rinsed successively with 0.1 N HCl and 0.1 N NaOH for 10 min each, followed by rinsing with de-ionized water and carrier electrolyte solution (50 mM phosphate buffer, pH 6.5) before use. The capillary tubing was rinsed first with 0. 1 N NaOH, and then with de-ionized water and carrier electrolyte solution for 3 min each between analyses. During separation, capillary tubing was kept at a constant temperature of 25.0 ± 0.1 °C by means of a fluorocarbon liquid continuously circulated through the cartridge. The applied voltage was 15 kV. Sample introduction was performed using the pressure option for 5 s. Data collection was carried out with the Gold Chromatography data system version 8.1.

Pectin samples (5.0 mg/mL) with various DEs were dissolved in de-ionized water, followed by centrifugation (10,000*g*, 4 °C, 20 min) to remove the possible insoluble portions prior to use. Carrier electrolyte buffer (50 mM phosphate, pH 6.5) was filtered through a 0.45-nm membrane filter prior to use. De-ionized water used in the present study was prepared with a Mili-Q system (Millipore, Osaka, Japan). In an effort to investigate the effect of capillary length on the relationship between migration time and pectin DE, capillaries with effective lengths of 30 and 60 cm were used.

3. Results and discussion

Fig. 1(a) represents the migration times of ADPs with different DEs using a capillary tubing with an effective length of 30 cm. Pectin with 93% DE showed a migration time of 3.2 min, while those for 77%, 56%, 47%, 21% and 0% were 3.8, 4.7, 5.2, 6.5 and 7.7 min, respectively. The effect of viscosity, varied with pectic substances with different DEs, on the electrophoretic migration time was negligible due to the minimal (nanoliter) level of electrolytes in the carrier electrolyte.

ADPs with higher DE possesses lower extent of carboxylic acid groups and thus, exhibits lower negative charges in a carrier electrolyte buffer with a pH of 6.5 in the present study. The stronger the negative charge of the electrolyte is, the slower is the observed mobility of an electrolyte toward a cathode due to the presence of strong electroosmotic flow (EOF). This can be expressed as $V_{obs} = V_{EP} + V_{EOF}$; where V_{obs}, V_{EP} and V_{EOF} are the observed mobility, electrophoretic mobility and electroosmotic flow, respectively (Kuhn, 1990; Kuhn & Monning, 1992).

The electrophoregram of each ADP was relatively broad compared to those of pure chemicals such amino acids and ascorbic acid (Tsai et al., 1998; Liao, Wu, Wu, & Chang, 2000). Citrus pectins used in the present study were alkaline-treated and randomly de-esterified (Sajjaanatakul et al., 1989); their DE values, as determined with the method described by Mizote et al. (1975), were averaged values. Variations in DEs and molecular weights in the pectin samples could have led to significant broadening of the migration time of pectin during electrophoretic operation.

High linear correlation ($r = 0.994$) between migration times and pectin DEs was observed (Fig. 1(b)) when a capillary tubing with 30 cm effective length was used, and similar results ($r = 0.998$) were observed when the tubing was 60 cm long (Fig. 1(c)). It suggests that the capillary tubing length is not influential on the correlation between migration time and pectin DE. High correlation coefficients imply the possible utilization of CZE for the analysis of pectin DE. As compared to the migration times of pectins in the electrophoretic profiles conducted with a shorter capillary length (Fig. 1(b)), those of the corresponding pectins were longer (Fig. 1(c)). Increase in the capillary length enhanced the resolution of electrolytes by reducing dissipation of the Joule heat, and elevated the theoretical plate numbers; however it prolonged the migration times of electrolytes, as reported previously (Chang, Tsai, & Li, 1998; Peterson,

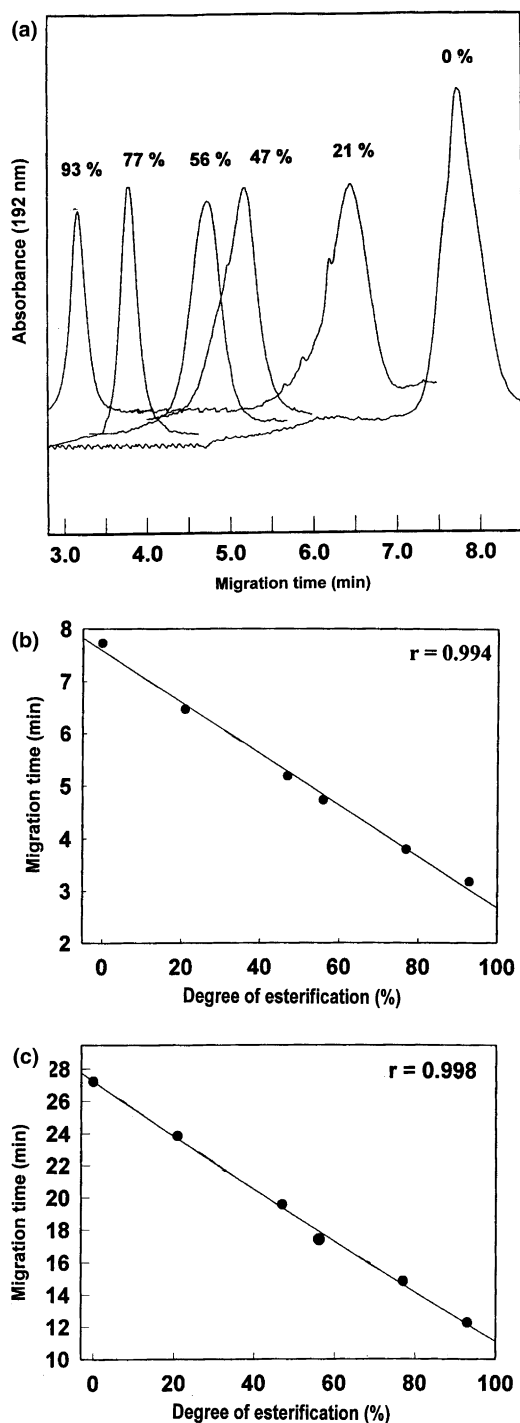

Fig. 1. Capillary zone electrophoregrams (a) of alkaline-de-esterified pectins with various DEs and the correlation coefficients (b) and (c) between migration time and pectin DE. Experimental conditions: pectin concentration, 5 mg/mL; column, uncoated fused silica column with an effective tubing length of 30 cm for (a) and (b) and of 60 cm for (c) (inner diameter, 75 μm); carrier electrolyte solution, 50 mM phosphate (pH 6.5); applied voltage, 15 kV; wavelength, 192 nm.

1993). However, long capillary tubing provided some advantages such as more efficient separation of a mixture of pectin samples with different DEs over the short tubing method due to the increase in resolution of analyte (Chang et al., 1998). Of note, quantification of individual pectin could also be achieved from the corresponding peak area by previous construction of a calibration curve.

ADPs with various DEs (93%, 68%, 47%, 33% and 0%) were mixed and then separated by a CZE with an effective capillary length of 30 cm. As shown in Fig. 2(a), pectins were eluted in the order of 93%, 68%, 47%, 33% and 0%, a decreasing order of DE, in 8 min, similar to that in Fig. 1(a). The correlation between DE value and migration time was good with a coefficient (r) as high as 0.995 (Fig. 2(b)). Thus, it is clear that CZE is suitable for the determination of pectin DE, providing the advantages of economy, speed and, more important, high resolution of the pectin mixture with various DEs.

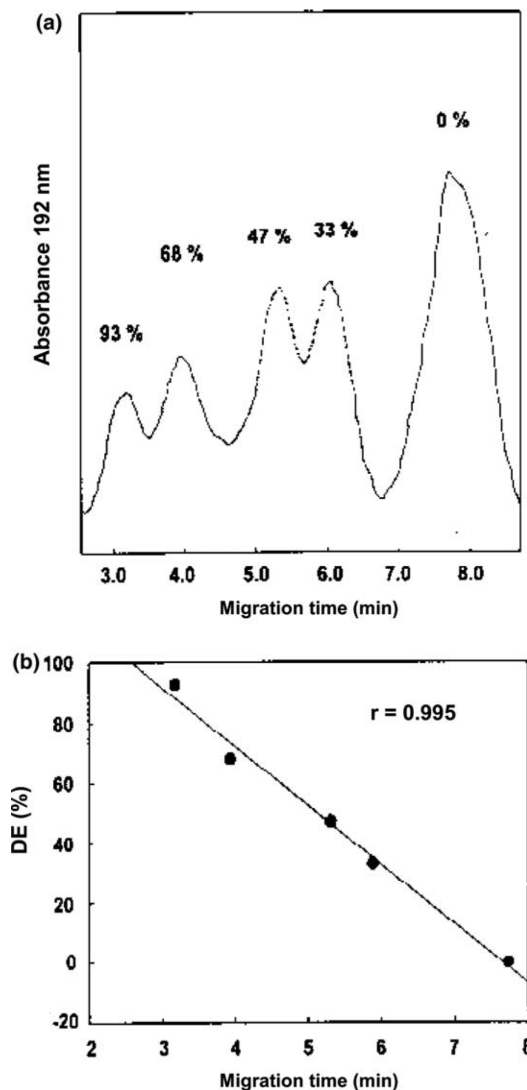

Fig. 2. Capillary zone electrophoregrams (a) of mixed alkaline de-esterified pectins with various DEs and the correlation coefficient (b) between migration time and pectin DE. Experimental conditions were the same as in Fig. 1, except the effective tubing length was 30 cm.

Linear correlations were observed when randomly de-esterified pectins with various DEs were applied to the CZE, regardless of the effective capillary lengths used. Thus, CZE is effective to separate and determine the DE value of randomly de-esterified pectins, prepared with acid or alkaline, in mixture. In other words, in addition to high speed and efficiency, CZE separation was also available for the DE determination of pectin mixtures with various DEs, comparing to the averaged DE determination by the conventional methods. The random type de-esterification results in homogeneously high negative charged areas in pectin molecules in neutral pH. However, it is noteworthy that, besides DE, the molecular weight of pectin samples may influence the migration time during CZE operation. Moreover, method developed in the present study is not available for the determination of DE of pectin mixtures from PE and alkaline de-esterified sources. Due to the great variations in molecular weights and charges of commercial pectin and other carbohydrate samples, such as starch, gum and cellulose, application of CZE method developed appears to be difficult.

Acknowledgement

Financial support for this study from the National Science Council of the Republic of China under Grant NSC-85-2321-B-002-046 is greatly appreciated.

References

Black, S. A., & Smit, C. J. B. (1972). The effect of demethylation procedures on the quality of low-ester pectins used in dessert gels. *Journal of Food Science, 37*, 730–735.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acid. *Analytical Biochemistry, 54*, 484–489.

Chang, H. M., Tsai, C. F., & Li, C. F. (1998). Enantiomeric separation of dans-DL-amino acids by γ-cyclodextrin-modified micellar capillary electrophoresis. *Journal of Agricultural and Food Chemistry, 46*, 4598–4603.

Chiari, M., & Nesi, M. (1993). Determination of total vitamin C in fruits by capillary zone electrophoresis. *Journal of Chromatography, 645*, 197–200.

Christensen, S. H. (1986). Pectins. In M. Glicksman (Ed.). *Food hydrocolloids* (Vol. III, pp. 205–210). FL: CRC Press Inc.

de Vires, J. A., Hansen, M., Soderberg, J., Glahn, P. E., & Pederson, J. K. (1986). Distribution of methoxyl groups in pectins. *Carbohydrate Polymers, 6*, 165–169.

de Vries, J. A., Rombouts, F. M., Voragen, A. G. J., & Pilnik, W. (1983). Distribution of methoxyl groups in apple pectic substances. *Carbohydrate Polymers, 3*, 245–250.

Emmer, A., Jansson, M., & Roeraade, J. (1991). Improved capillary zone electrophoretic separation of basic proteins, using a fluorosurfactant buffer additive. *Journal of Chromatography, 547*, 544–550.

Heri, W., Neukom, H., & Deuel, H. (1961). Chromatographie von pektinen mit verschiedener verteilung der methylestergruppen auf den fadenmolekeln. *Helvetica Chimica Acta, 239*, 1939–1943.

Hong, H.L. (1995). Preparations of blockwise and random de-esterified pectins of different degrees of esterification, and comparison of their physico-chemical properties. Ph.D. dissertation. Graduate Institute of Food Science and Technology, National Taiwan University.

Ishii, S., Kiho, K., Sugiyama, S., & Sugimoto, H. (1979). Low-methoxyl pectin prepared by pectinesterase from *Aspergillus japonicus*. *Journal of Food Science, 44*, 611–615.

Ishii, S., & Yokotshuka, T. (1971). Maceration of plant tissues by pectin transliminase. *Agricultural Biological Chemistry, 35*, 1157–1162.

Klavons, J. A., & Bennett, R. D. (1986). Determination of methanol using alcohol oxidase and its application to methylester content of pectin. *Journal of Agricultural and Food Chemistry, 34*, 597–599.

Kohn, R., Furda, I., & Kopec, Z. (1968). Distribution of free carboxyl groups in the pectin molecule after treatment with pectin esterases. *Collection of Czechoslovak Chemistry Communications, 33*, 264–268.

Kohn, R., Heinrichova, K., & Malovikova, A. (1983a). Binding of calcium and copper (II) ions to oligogalacturonic acids. *Collection of Czechoslovak Chemistry Communications, 48*, 1922–1927.

Kohn, R., Markovic, O., & Machova, E. (1983b). Deesterification mode of pectin by pectinesterase of *Apsergillus foetidus*, tomatoes, and alfalfa. *Collection of Czechoslovak Chemistry Communications, 48*, 790–794.

Komae, K., Sone, J., Kakuta, M., & Misaki, A. (1990). Purification and characterization of pectinesterase from *Ficus awkeotsang*. *Agricultural Biological Chemistry, 54*, 1469–1473.

Kuhn, W. G. (1990). Capillary electrophoresis. *Analytical Chemistry, 62*, 403R–414R.

Kuhn, W. G., & Monning, C. A. (1992). Capillary electrophoresis. *Analytical Chemistry, 64*, 389–407.

Kutnink, M. A., Skala, J. H., Sauberlich, H. E., & Omaye, S. T. (1985). Simultaneous determination of ascorbic acid, isoascorbic acid (erythorbic acid) and uric acid in human blood by high-performance liquid chromatography. *Journal of Liquid Chromatography, 8*, 31–46.

Liao, T., Wu, J. S. B., Wu, M. C., & Chang, H. M. (2000). Epimeric separation of L-ascorbic acid and D-isoascorbic acid by capillary zone electrophoresis. *Journal of Agricultural and Food Chemistry, 48*, 37–41.

Maness, N. O., Ryan, J. D., & Mort, A. J. (1990). Determination of the degree of methyl esterification of pectins in small samples by selective reduction of esterified galacturonic acid to galactose. *Analytical Biochemistry, 185*, 346–351.

Mizote, A., Odagir, H., Toei, K., & Tanaka, K. (1975). Determination of residues of carboxylic acids (mainly galacturonic acid) and their degree of esterification in industrial pectins by colloid titration with cat-floc. *Analyst, 100*(November), 822–827.

Peterson, T. E. (1993). Separation of drug stereoisomers by capillary zone electrophoresis with cyclodextrin. *Journal of Chromatography, 630*, 353–361.

Sajjaanatakul, T., van Buren, J. P., & Downing, D. J. (1989). Effect of methyl content on heat degradation of chelator-soluble carrot pectin. *Journal of Food Science, 54*, 1272–1276.

Tsai, C. F., Li, C. F., & Chang, H. M. (1998). Enantiomeric separation of dansyl derivatized-DL-amino acids by β-cyclodextrin-modified micellar electrokinetic chromatography. *Journal of Agricultural and Food Chemistry, 46*, 979–985.

Taso, C. S., & Salimi, S. D. (1982). Differential determination of L-ascorbic acid and D-isoascorbic acid by reversed-phase high performance liquid chromatography with electrochemical detection. *Journal of Chromatography, 245*, 355–358.

Tuerena, C. E., Taylor, A. J., & Mitchell, J. R. (1982). Evaluation of a method for determining the free carboxyl group distribution in pectins. *Carbohydrate Polymers, 2*, 193–197.

van Buren, J. P. (1991). Function of pectin in plant tissue structure and firmness. In R. H. Walter (Ed.), *The chemistry and technology of pectins* (pp. 4–10). New York: Academic press, Inc..