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Analytical Methods

Combined enzymatic and colorimetric method for determining the uronic acid and methylester content of pectin: Application to tomato products

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Abstract

A simple procedure for determining the galacturonic acid and methanol contents of soluble and insoluble pectins, relying on enzymatic pectin hydrolysis and colorimetric quantification, is described. Pectin samples are incubated with a commercial pectinase preparation, Viscozyme, then the galacturonic acid content of the hydrolyzed pectin is quantified colorimetrically using a modification of the Cu reduction procedure originally described by Avigad and Milner. This modification, substituting the commonly used Folin–Ciocalteau reagent for the arsenic containing Nelson reagent, gives a response that is linear, sensitive, and selective for uronic acids over neutral sugars. This method also avoids the use of concentrated acids needed for the commonly used *m*-phenylphenol method. Methanol, released by the action of the pectin methylesterase found in the Viscozyme, is quantified using alcohol oxidase and Purpald. This combined enzymatic and colorimetric procedure correctly determined the galacturonic acid and methanol content of purified, soluble citrus pectin. Application of the procedure to water insoluble pectins was evaluated with water insoluble material from apples and oranges. In both cases good agreement was obtained between this method and commonly used methods based on chemical pectin hydrolysis. Good agreement between these procedures was also found in the analysis of both soluble and insoluble pectins from several tomato products.

Keywords: Pectin; Tomato; Uronic acid; Methanol; Pectin methylesterase; Degree of esterification

1. Introduction

Pectins are a major constituent of plant cell walls and greatly affect the quality of many plant-derived food products. While tremendous variability in overall pectin composition exists across the plant kingdom, in many common fruits and vegetables galacturonic acid comprises >80% of the total pectin carbohydrate, with neutral sugars, primarily galactose, arabinose, rhamnose, and xylose, making up the remainder (Ridley, O'Neill, & Mohnen, 2001). Pectin galacturonic acid is present in three polymeric forms: homogalcturonan (HG), a linear polymer of α 1–4 linked galactuonic acids containing variable levels of methylesterification of the carboxyl group and acetylation on C-2; rhamnogalacturonan I (RG-1), a repeating disaccharide of galacturonic acid and rhamnose; and rhamnogalacturnon II (RG-II), a homogalacturonic backbone with numerous complex sidechains containing rhamnose and other neutral sugars. How these various polymers are attached to each other is still an open question (Vincken et al., 2003). HG the most abundant of the three polymers and the degree to which it is methylesterified has a large impact on the functional properties of the pectin.

During thermal processing of plant material both chemical and enzyme catalyzed reactions can occur that change pectin solubility, polymer size, and degree of methylesterification.. These changes can have important consequences for product quality. For example, reducing the degree of pectin methylesterification by activating endogenous pectin

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methylesterase (PME) activity, results in processed material with a firmer texture. This is because the lower degree of pectin methylesterification both increases Ca^{2+} cross-linking of the pectin chains and makes the pectin less susceptible to thermal cleavage by β-elimination (Stolle-Smits, Beekhuizen, Recourt, Voragen, & Van Dijk, 2000). Changes in pectins during processing can also affect quality in fluid products like juices and sauces. In tomatoes, hydrolysis of pectin by the combined action of PME and polygalacturonase (PG) will result in decreased pectin polymer size and a loss of fluid viscosity if these enzymes are not thermally inactivated at the time of homogenization in the so-called hot-break process. The heat input during the hot-break and the additional heating required to concentrate the juice to paste can cause other changes in the pectins, including solubilization and breakdown (Hurtado, Greve, & Labavitch, 2002; Xu, Shoemaker, & Luh, 1986). To follow the changes in pectin that occur during processing a simple method for quantifying the amounts of soluble and insoluble pectin, and assessing the degree of methyl esterification is thus desirable.

Several methods are available for quantifying and characterizing pectin. Most commonly a preparation containing the cell wall material is isolated, usually by ethanol precipitation, to separate the polymeric material from any monomeric galacturonic acid or other soluble small molecules that would interfere with subsequent the galacturonic acid analysis. This alcohol insoluble residue is then sequentially extracted with water, chelators, and finally high pH to produce fractions each containing a portion of the total pectic material. These fractions can then be hydrolyzed and analyzed for their methanol, uronic acid, and neutral sugar contents. Results are typically reported as the amounts of these constituent per amount of alcohol insoluble residue. Assuming that the pectin precipitation by the alcohol is quantitative, and that the yield of alcohol insoluble residue per amount of plant material is reported, the amount of pectin in the plant material can be calculated.

Quantifying the galacturonic acid content of the various fraction is most commonly done by first hydrolyzing the pectic material in hot concentrated acid, then quantifying the resulting anhydrogalacturonic acid residues colorimetrically with *m*-hydroxydiphenyl (Blumenkrantz & Asboe-Hansen, 1973). Since both the hydrolysis of the pectin and the reaction with *m*-hydroxydiphenyl occur in concentrated sulfuric acid, a rapid and simple procedure for quantifying the uronic acid content of pectin is possible (Ahmed & Labavitch, 1977). Improvements to reduce the interference by neutral sugars have been developed (Filisetti-Cozzi & Carpita, 1991). A different approach is to enzymatically hydrolyze the pectin to galacturonic acid using mixtures of pectin degrading enzymes. The galacturonic acid content can then be determined colorimetrically (McComb & McCready, 1952) or by anion exchange HPLC (Matsuhashi, Shin-ichi, & Hatanaka, 1992; Rumpunen, Thomas, Badilas, & Thibault, 2002; Garna, Mabon, Nott, Wathelet, & Paquot, 2006). Several different preparations of highly active pectin degrading enzymes are commercially available and have been shown to give complete pectin hydrolysis. In addition to eliminating the safety and disposal problems associated with the use of hot concentrated sulfuric acid, these enzymatic procedures have the advantage that they are specific for pectin and require mild conditions. Unlike acid hydrolysis, where the galacturonic acid released from the pectin can be further degraded, the galacturonic acid formed from enzymatic hydrolysis is stable.

Several methods are available for determining the degree of pectin methyl esterification. With pure pectins a simple titration procedure can be used (Schultz, 1965). With less pure preparations, such as alcohol insoluble residues, the methyl esters can be hydrolyzed under mild alkaline conditions and the amount of methanol produced determined. Chemical quantification of methanol can be done by first oxidizing it to formaldehyde, either with permanganate (Wood & Siddiqui, 1971) or enzymatically with alcohol oxidase (Klavons & Bennett, 1986), then be determining the formaldehyde with one of several colorimetric reagents (Anthon & Barrett, 2004). The methanol can also be quantified by GC (Bartolome & Hoff, 1972; Savary & Nunez, 2003). An alternative approach is to treat the partially methylesterified pectin with $NaBH_4$ which reduces the methylesterified galacturonic acids to galactose. The total galacturonic acid content with and without $NaBH_4$ reduction can then be determined and the methylester content calculated (Maness, Ryan, & Mort, 1990).

Our goal was to develop a simple method for determining the both amount and degree of methyl esterification of soluble and insoluble pectin, suitable for use with products such as tomato paste and juices. To eliminate the need for concentrated acids, we have used enzymatic pectin hydrolysis. Galacturonic acids can then be quantified with a modified version of the colorimetric procedure of Milner and Avigad (1967) in which uronic acids are quantified reductometrically at pH 4.8. In this assay neutral reducing sugars do not interfere, because at pH 4.8, only uronic acids and certain uncommon ketose sugars, but not most aldoses, will reduce copper. In the original procedure the reduced copper was quantified with the arsenomolybdate reagent of Nelson (1944). Here, we show here that the readily available Folin–Ciocalteau reagent works equally well, eliminating the need for arsenic in the assay. Since the mixtures of pectin degrading enzymes used to depolymerize pectin also contain pectin methylesterase activity, the amount of methanol in the hydrolyzed sample can be determined along with the galacturonic acid content to give the degree of methylesterification. In addition, we have examined the use of centrifugation through gel filtration media, a procedure commonly used to desalt protein solutions, as a method for separating pectin polymers from small molecules (e.g. methanol, galacturonic acid, and reducing sugars) that would interfere with the quantification of the pectin uronic acid and methylester content.

2. Materials and methods

2.1. Materials

All reagents were obtained from Sigma (St. Louis, MO). The mixture of pectin degrading enzymes used here is referred to by its Novozymes trade names of "Viscozyme". This product is sold by Sigma (catalog #V2010) as "Cell wall degrading enzyme complex". Micro bio-spin columns, containing Biogel P6DG, were obtained from Bio-Rad (Hercules, CA). Microwave hot-break juice was prepared from field grown processing tomatoes (variety not known) according to the procedure in (Garcia & Barrett, 2006). A 28 °Brix hot-break paste was obtained from a local manufacturer. Canned tomato juice (prepared from cold break paste), apples (cv. Fuji), and oranges (navel type) were purchased at a local market. *Arabidopsis thaliana* seeds (Col-0 wild-type) were obtained from Lehle Seeds (Round Rock, TX).

2.2. Galacturonic acid assay

A buffered copper solution was prepared by adding 23.2 g NaCl, 3.2 g sodium acetate, and 1.0 mL glacial acetic acid to 80 mL water. Once dissolved, 0.5 g $CuSO_4$ is added, the pH adjusted to 4.8 with NaOH, and the final volume brought to 100 mL. This solution is stable for weeks at room temperature. For the assay, equal volumes of this solution and the sample are mixed, giving final reagent concentrations in the assay of 2 M NaCl, 0.2 M acetate, and 10 mM $CuSO_4$. In our standard assay, sample and assay solution volumes of 0.1 mL each are mixed in 12×75 test tubes, then the tubes are covered with glass marbles and placed in an aluminum heating block at 100 °C. A diluted Folin–Ciocalteau reagent is then prepared by mixing 1 mL of 2 N Folin–Ciocalteau with 39 mL of water. After 40 min, the samples are removed from the heat and 8 volumes (0.8 mL in our standard assay) of the 40-fold diluted Folin–Ciocalteau reagent is added. We routinely made this final addition immediately after removing the samples from the heat but the timing of this addition does not appear to be critical. A colored product forms immediately; absorbance was measured at 750 nm. Where the BCA reagent was used the procedure was the same, except instead of adding the diluted Folin–Ciocalteau reagent, 0.8 mL of solution "A" from the procedure described in Waffenschmidt and Jaenicke (1987), containing 5.0 mM BCA in pH 10.1 carbonate buffer, was added. Absorbance was measured at 560 nm.

2.3. Methanol assay

Methanol was determined using alcohol oxidase and Purpald as described previously (Anthon & Barrett, 2004). Samples, in a volume of 100 μL, were mixed with 90 μL of 0.2 M phosphate buffer (pH 7) in a 12×75 test tube. The assay was started by the addition of 10 μL of alcohol oxidase (0.01 U/mL in 0.2 M phosphate buffer, pH 7) and incubated at 30 °C in a water bath. After 10 min, 0.2 mL of freshly prepared 5 mg/mL Purpald in 0.5 M NaOH was added and the mixture incubated for an additional 40 min at 30 °C. The samples were then removed from the water bath and brought to a final volume of 1.0 mL by the addition of 0.6 mL of water. Absorbance at 550 nm was then determined.

2.4. Enzymatic pectin hydrolysis

Solutions of Viscozyme as supplied contain large amounts of non-protein material that interferes with the methanol and galacturonic acid assays. This material must be removed from the enzyme preparation by dialysis or gel filtration prior to use. For this we used Bio-Rad micro bio-spin columns equilibrated with 25 mM acetate buffer (pH 4.8) and centrifuged according to the manufacturer's recommendations. To ensure complete removal of the contaminating material the desalting was done twice. The protein concentration of the undiluted desalted enzyme preparations was 12.9 mg/mL for Viscozyme, as determined with the Bradford reagent using BSA as a standard. This solution was then diluted to a protein concentration of 0.5 mg/ml with acetate buffer (pH 4.8) and stored at −20 °C. This enzyme solution and could be thawed and refrozen repeatedly without apparent loss of activity. On the day of use an aliquot of this enzyme solution was diluted 50-fold with acetate buffer (pH 4.8) to give the final 10 μg/mL solution used in the assay.

In our standard procedure for pectin digestion, a 50 μL aliquot of sample, containing up to 100 μg of pectin, was mixed with 300 μL of 25 mM acetate buffer (pH 4.8) in a 1.5 mL centrifuge tube. A 50 μL aliquot of a 10 μg/mL solution of desalted Viscozyme, in 25 mM acetate buffer was then added (final enzyme concentration of 1.25 μg/mL) and the samples incubated for 24 h at 30 °C. Appropriate sized aliquots (usually 50 μL) of the enzyme digested pectin were then removed, diluted to 100 μL with water and then analyzed for methanol and galacturonic acid as described above. Since the sensitivities of the methanol and galacturonic acid assays are similar, the same sized aliquot was generally used for both. For pectin with a low degree of esterification a larger aliquot may be needed for the methanol determination.

2.5. Desalting of soluble pectin

The procedure for desalting solutions of soluble pectin with Bio-Rad micro bio-spin columns, containing Bio Gel P6DG, was based on the manufacturer's recommendations. Columns were equilibrated with water by applying 0.5 mL of water, centrifuging for 1 min at 1000*g*, and discarding the eluted buffer. This procedure was repeated 5 times prior to use. As supplied, or after prolonged storage at 4 °C, we found that these columns were contaminated with material that reacted positively in the Purpald assay.

Washing 5 times with water prior to use completely removed this interfering material.

For desalting of pectin, 0.5 mL of water was applied to a washed column, and the column was centrifuged for 2 min at 1000*g*. The column was then transferred to a clean 1.5 mL centrifuge tube and 50 μL of sample was applied. To elute the sample, the column was then centrifuged for 4 min at 1000*g*. The eluted sample (50 μL) was then analyzed for pectin content by the addition of 300 μL of 25 mM acetate buffer (pH 4.8) and 50 μL of a 10 μg/mL solution of desalted Viscozyme, and then incubated as described above. After use, the columns were again washed 5 times with water before a final 0.5 mL of water was applied and the columns stored at 4 °C.

2.6. Pectin analysis by chemical hydrolysis

Pectin containing samples were hydrolyzed with concentrated sulfuric acid and analyzed for uronic acids with *m*-phenylphenol following the procedure in Filisetti-Cozzi and Carpita (1991). Alkaline hydrolysis for methanol was done by mixing 100 μL of sample with 200 μL of 0.5 M NaOH and incubating at room temperature for 1 h. The sample was then neutralized with 100 μL of 1 M HCl and analyzed for methanol as described above.

2.7. Preparation of Arabidopsis seed mucilage

Seed mucilage was prepared by water extraction as described (Macquet, Ralet, Kronenberger, Marion-Poll, & North, 2007). Fifty micrograms of seed was soaked in 0.5 mL of water and incubated for 4 h at 50 °C. The sample was vigorously vortexed, centrifuged (3 min, 8000*g*) and the supernatant collected. Aliquots of the supernatant were desalted on Bio Rad micro bio-spin columns and analyzed for pectin content by hydrolysis by Viscozyme.

2.8. Preparation of insoluble pectins from apples and oranges

Water insoluble residues from apple and orange were prepared based on the procedure given in Ahmed and Labavitch (1977). Apple parenchyma tissue (skin and core removed) and orange tissue (skins removed) were homogenized for 30 s in a blender with an equal weight of water, then ground for an additional 30 s with a polytron. The homogenate was centrifuged 10 min at 10,000*g*, the supernatant discarded, and the pellet washed once with water. This washed pellet was then resuspended in water and collected by vacuum filtration on filter paper. The residue was then washed three times with acetone and then water before being allowed to air dry. Portions of this air-dried residue (20–50 mg) were then resuspended in 1.0 mL of water then aliquots of the suspension analyzed for pectins by both the enzymatic and chemical hydrolysis methods.

2.9. Analysis of tomato juice

The tomato paste was reconstituted back to juice by diluting with water to 5° Brix before analysis. The microwave hot-break juice and canned tomato juice were analyzed without any dilution. For each of the juices, 200 μL aliquots were pipetted with a positive displacement pipetter into 1.5 mL centrifuge tubes, then centrifuged for 5 min at 16,300*g*. The supernatants were removed and saved, then the pellets were resuspended in 1.0 mL of water and re-centrifuged. The pellet was then washed one additional time with water before the pellet was finally resuspended in 1.0 mL of water. A 100 μL aliquot of the resuspended pellet was removed and mixed, in a 1.5 mL centrifuge tube, with 250 μL of 25 mM acetate buffer (pH 4.8) and 50 μL of a 10 μg/mL solution of desalted Viscozyme, as described above. After incubating 24 h at 30 °C, the sample was centrifuged to remove insoluble material, then 50 μL aliquots of the supernatant were analyzed for methanol and galacturonic acid. Aliquots (50 μL) of the juice supernatants were desalted, enzymatically hydrolyzed, and analyzed for pectin content as described above for soluble pectin. Each juice was analyzed at least in triplicate.

3. Results and discussion

3.1. Determination of galacturonic acid

In the procedure developed by Milner and Avigad (1967), galacturonic acid, but not most other reducing sugars, will reduce copper at pH 4.8. The reduced copper could then be quantified with the arseno-molybdate reagent of Nelson (1944). We examined whether two other reagent commonly used to quantify reduced copper, the Folin–Ciocalteau reagent or BCA, could be used as alternatives. Both the Folin–Ciocalteau and BCA reagents gave a colored product when added to galacturonic acid, heated with copper at pH 4.8; glucose did not form a color. The response linear up to at least 50 nmol of galacturonic acid with the Folin–Ciocalteau reagent. BCA gave a significantly higher absorbance, but the response was not linear. It is possible that by changing reagent concentrations or other reaction conditions this non-linearity could be eliminated to give an extremely sensitive assay for galacturonic acid. This extra sensitivity was not needed for pectin analysis so the BCA reagent was not pursued further.

Detecting reduced copper with the Folin–Ciocalteau reagent offers several advantages over the Nelson reagent. It is available commercially and does not contain arsenic. Unlike the original protocol with the Nelson reagent, in the procedure described here, no precipitate forms when the Folin–Ciocalteau reagent is added, eliminating the need to centrifuge the samples prior to reading absorbance. Reaction conditions were found to be similar to those given by Milner and Avigad. The reaction requires a high salt concentration during the heating step, but not the 28% sodium sulfate as called for in the original procedure. We

obtained the full color response with as little as 1.0 M NaCl in the buffered copper solution. The original procedure called for heating the samples for 10 min at 100 °C. However, we have found that full color development requires longer heating times and we routinely use 40 min.

To be useful for quantifying the galacturonic acid derived from pectin, the response of the assay towards the neutral sugars found in pectin needs to be minimal. Relative to the response of galacturonic acid in the copper reduction assay with the Folin–Ciocalteau reagent, xylose gave 6% as much color while arabinose, rhamnose, galactose, and mannose were all gave 2% or less (Table 1). The low response by these sugars agrees with the results of Milner and Avigad (1967) and indicates that they will cause negligible interference in the quantification of galacturonic acid from hydrolyzed pectin. Of the other sugars examined only glucuronic acid and glucosamine gave a response comparable to galacturonic acid. Fructose gave a small response, while glucose did not. Potential interfering substances other than sugars were also examined. It is well known that the Folin–Ciocalteau reagent can be reduced by proteins, ascorbic acid, and phenolic compounds. Indeed, the principal use of the Folin–Ciocalteau reagent is in assaying for these substances. Both ascorbic acid and the phenolic compound, gallic acid, produced amount of color similar to that formed by an equal amount of galacturonic acid (Table 1).

Protein also produced a color. On an equal weight basis, however, the response of BSA is only about 5% that of galacturonic acid. This interference would thus only become a problem in analyzing samples where the amount of protein equals or exceeds the amount of pectin. This is not the case in most fruits and vegetables. If samples did contain high protein levels the protein could be removed by methods such as TCA precipitation, after the pectin had been hydrolyzed. High levels of protein also interfere in the *m*-hydroxydiphenyl assay for galacturonic acid (Van den Hoogen et al., 1998).

3.2. Enzymatic hydrolysis of soluble pectin

Several different commercial pectinase preparations have been successfully used for the complete hydrolysis of pectin. We examined Viscozyme, a product of Novozymes that is reported to contain multiple pectin degrading enzymes including pectin methylesterase and polygalacturonase and to be capable of complete pectin hydrolysis. The galacturonic acid content of a pectin sample was determined by incubating the sample with the pectin degrading enzyme, then quantifying the amount of galacturonic acid produced with the copper Folin procedure. Samples were incubated at 30 °C with various amounts of added enzyme, then assayed after 1, 4, or 24 h for galacturonic acid content. When the final concentration of added enzyme was equal to at least 10 μg/mL of protein, the hydrolysis of the pectin was complete in one hour with no additional accumulation of galacturonic acid at 4 or 24 h (Fig. 1). At lesser amounts of enzyme, hydrolysis was not complete until 4 or 24 h. After 24 h of incubation the average galacturonic acid content was 9.7 ± 0.4 μg, which is very close to the expected value of 9.85 μg, calculated from the galacturonic acid content of this pectin as reported by the supplier (78.8%) and the amount of digested pectin used per galacturonic acid assay (12.5 μg). This indicates that complete hydrolysis of the pectin occurs with this enzymes, in agreement with previous reports (Garna et al., 2006).

One complication with the copper reduction galacturonic acid assay is that, as noted above, protein interferes, giving a small positive response. If used in combination with enzymatic pectin hydrolysis a background correction is necessary to subtract off the absorbance in the samples due to the protein content of the added enzymes. This can easily be done by including a blank in the assay that includes the enzyme only. Fortunately, in the range of protein concentrations needed for complete pectin hydrolysis, the size of this correction is small. At 10 μg/mL of added protein this background absorbance was less than 0.05 (Fig. 1). If a 10-fold lower protein concentration is used, complete pectin hydrolysis is obtained in 24 h and the

Table 1
Absorbance at 750 nm of various substances relative to galacturonic acid

Substance	Relative absorbance	Substance	Relative absorbance
Galacturonic acid	100	Glucuronic acid	98
Glucose	1	Glucosamine	55
Arabinose	0	Fructose	8
Galactose	2	Pyruvate	43
Mannose	1	Ascorbic acid	92
Rhamnose	1	Gallic acid	95
Xylose	6	BSA (25 μg)	12

In all cases except BSA, the amount of material used was 50 nmol.

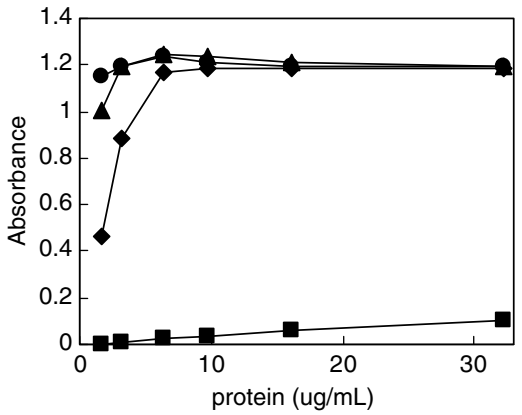

Fig. 1. Uronic acids formed from pectin versus the amount of added Viscozyme. Samples were incubated for 1 h (♦), 4 h (▲), or 24 h (●) then uronic acid content determined using the Folin–Ciocalteau reagent. Also indicated is the background absorbance formed in this assay by the added enzyme alone (■). Other data points are the net absorbances after subtracting off this background.

absorbance due to protein is negligible, so no background correction is necessary. This is our preferred procedure as described in Section 2.

3.3. Methanol content

The enzyme preparation used for pectin hydrolysis contains PME activity. Aliquots of the enzyme-digested pectin can thus be analyzed for methanol to determine the methyl ester content of the pectin. Pectin samples treated with Viscozyme, at the same levels as used for pectin hydrolysis, produced quantities of methanol consistent with complete de-esterification (Table 2). After 1 h of incubation at 12.5 μg/mL of added enzyme, or 24 h incubation at 1.25 μg/mL, the amount of methanol produced was in agreement with the methoxy content of this pectin as determined by alkaline hydrolysis. Unlike the galacturonic acid assay, the protein content of the pectin enzymes did not affect the methanol assay.

3.4. Rapid desalting of soluble pectin

Determining the galacturonic acid content of a soluble pectin sample requires that small interfering molecules, such as ascorbic acid or galacturonic acid monomers, be removed from the solution prior to assay. This is typically done by either precipitating the pectin with ethanol or by dialysis. As an alternative, we examined whether centrifugation of the pectin through gel filtration media would also work as a means for separating soluble pectin from interfering small molecules. A citrus pectin solution containing 100 μg of pectin (50 μL of 2 mg/mL) was desalted on a Bio-Rad micro bio-spin column equilibrated with water. Both the material passing through the column and an aliquot of the starting material were then enzymatically digested and analyzed for galacturonic acid and methanol. Based on either galacturonic acid or methanol content, the recovery of the pectin through this procedure was near 100% (Table 3). In separate experiments, 50 μL of 5 mM solutions of either galacturonic acid or methanol were applied to the columns. In these cases the recovery was about 1%, indicating almost complete retention of these small molecules under these conditions, in agreement with the manufacturer specifications. Although these columns are sold as a single-use disposable product, we have found that if washed several times and re-equilibrated with water immediately after use they may be reused many times without loss of performance.

This rapid desalting procedure is preferable to either dialysis or ethanol precipitation for the separation of soluble pectin from small interfering molecules. Dialysis is much slower and leads to dilution of the sample. Ethanol precipitation, in addition to being time consuming, does not easily lend itself to quantitative recovery of the pectin. The precipitate formed by soluble high-methoxy pectin in 80% ethanol highly disperse and gelatinous. Collecting this precipitate either by centrifugation or filtration without loss of material is difficult. The main limitation with this method is that the amount of sample that may be applied to the Bio-Rad micro bio-spin columns is very small (75 μL). This amount of material is, nevertheless, more than adequate (by at least a factor of 10) for the analysis of pectin from pectin rich sources like tomato serum. If larger samples are required, it is possible to purchase or construct larger columns, packed with either Bio-Gel P6DG or Sephadex G-25, and centrifuge them in a swinging bucket rotor as described (Helmerhorst & Stokes, 1980). We have used such larger columns, with bed volumes up to 6.0 mL, for desalting up to 1.0 mL of pectin solutions. This is a useful method not only for pectin analysis but also for preparing pectin solutions free from any contaminating methanol. Such pectin solutions are desirable, for example, if the pectin is to be used as a substrate in a PME assay based on methanol production (Anthon & Barrett, 2004).

Table 2
Pectin de-esterification by enzyme and alkaline treatment

Treatment		Methanol (g/100 g pectin)	
		1 h	24 h
No enzyme		0.1 ± 0.01[a]	0.1 ± 0.01
Viscozyme	1.25 μg/mL	5.1 ± 0.1	11.1 ± 0.4
	12.5 μg/mL	11.3 ± 0.1	11.6 ± 0.1
0.5 M NaOH		11.6 ± 0.1	

[a] Mean ± s.e., $n = 3$.

Table 3
Recovery of pectin, galacturonic acid and methanol through desalting on Bio-Gel

Sample applied to column		Before desalting	After desalting	Recovery (%)
Citrus pectin	GalA	78.5 ± 1.4[a,b]	76.2 ± 0.4	97
	MeOH	11.8 ± 0.2	11.2 ± 0.2	95
Galacturonic acid		48.5	0.63 ± 0.33	1
Methanol		8.0	0.08 ± 0.04	1

Fifty μL aliquots of 2 g/L citrus pectin were desalted by centrifuging through Bio-Rad micro bio-spin columns. These samples, along with samples of the starting material were then enzymatically digested and analyzed for methanol and galacturonic acid content. In separate experiments 50 μL aliquots of 5 mM galacturonic acid or methanol were applied to the columns and the amount of galacturonic acid or methanol in the eluted material determined.

[a] μg.

[b] Mean ± s.e., $n = 3$.

3.5. Enzymatic hydrolysis of rhamnogalacturonans

The majority of the galacturonic acid (>90%) in most fruit and vegetable pectins is present as HG, which is readily hydrolyzed by the enzymes in Viscozyme. To quantify the total galacturonic acid, however, it is necessary that RGI and RGII polymers also be hydrolyzed. A previous

analysis of the neutral sugar content of apple pectin following digestion with Viscozyme showed substantial amounts of rhamnose, indicating that Viscozyme does indeed contain activities necessary for rhamnogalacturonan hydrolysis (Garna, Mabon, Wathelet, & Paquot, 2004). To test whether complete RGI hydrolysis occurs with Viscozyme under our standard assay conditions, we applied our assay to the water soluble mucilage from Arabidopsis seeds, a polymer that has previously been shown to consist entirely of RGI (Macquet et al., 2007). A sample of this mucilage was obtained by soaking a known weight of seed in water, then collecting a portion of the soaking solution and analyzing the polymeric material in it for galacturonic acid content. When analyzed by our enzymatic hydrolysis method, a value of 9.1 ± 0.3 mg galacturonic acid g^{-1} seed was obtained. This value is in excellent agreement with the previously reported values of 9.1 (Macquet et al., 2007) and 9.9 (Usadel, Kuschinsky, Rosso, Eckermann, & Pauly, 2004), obtained for this same accession of Arabidopsis seed using chemical hydrolysis of the seed mucilage and quantification with *m*-phenylphenol. The similarity of the results obtained by enzymatic and chemical hydrolysis confirms that Viscozyme can indeed hydrolyze RGI.

We did not determine whether Viscozyme can also hydrolyze RGII. It has previously been shown that the amount of rhamnose recovered from apple pectin following Viscozyme hydrolysis is similar to that obtained by chemical hydrolysis, implying that both RGI and RGII are enzymatically hydrolyzed (Garna et al., 2004). On the other hand, treatment of fruit and vegetable juices with several other commercial enzyme preparations, similar to Viscozyme, left pectin residues consisting almost exclusively RGII, indicating that while both HG and RGI are hydrolyzed, RGII is uniquely resistant to enzymatic hydrolysis (Doco, Williams, Vidal, & Pellerrin, 1997). Since these authors did not specifically test Viscozyme, it is possible that Viscozyme contains RGII hydrolases not found in other commercial preparations and that RGII is hydrolyzed in our assay. However, even if RGII is not hydrolyzed by Viscozyme, the error this causes in the determination of total galacturonic acid is minimal for most fruits and vegetables because the total amount of RGII is small. For example, in apple juice the total amount of galacturonic acid present as enzyme resistant RGII was determined to be 132 μg g^{-1} fresh weight (Doco et al., 1997). This compares to a total pectin galacturonic acid content of 4500 μg g^{-1} fresh weight for apples of the same variety (Renard, 2005). The underestimation of total galacturonic acid content by not hydrolyzing RGII will be less than 3%. The RGII content of tomato juice is lower than that of apple juice (Doco et al., 1997), indicating that the potential error when applying this method to tomato products will be even smaller. For pectins that contain substantially greater amounts of RGII the error would be larger and our assay may not be suitable.

3.6. Analysis of water insoluble pectins

The above results show that enzymatic hydrolysis can be used for the determination of the galacturonic acid content of water soluble pectins, in agreement with other previous reports. However, a substantial portion of the total pectin in most fruits and vegetables is water insoluble and this insoluble pectin may not be as good of a substrate for the pectin degrading enzymes as the soluble pectin. To see if enzymatic digestion would be a suitable method for analyzing these insoluble pectins, water insoluble residues were prepared from apples and oranges then analyzed for pectin galacturonic acid and methanol content using both traditional methods based on chemical hydrolysis and the enzymatic procedure described here. For both the apple and orange residues the two methods gave similar results for the determination of galacturonic acid and methanol (Table 4). Good agreement between enzymatic and chemical hydrolysis was also reported in the determination of the uronic acid content of alcohol insoluble residues from quince (Rumpunen et al., 2002). The high degree of methylesterification with the apple residue is consistent with previous reports, where degrees of methylesterification of 80% and higher have been found (Fischer & Amando, 1994; Knee, 1978; Massiot & Renard, 1997). This level likely depends on the variety and condition of the apple, as well as the extraction method, and slightly lower values (70–80%) have also been reported (Renard, 2005; Renard, Voragen, Thibault, & Pilnik, 1990). The degree of methylesterification of the orange residue was lower than for the apple, consistent with the generally lower degree of methylesterification of citrus versus apple pectins (May, 1990).

3.7. Application to tomato products

The combined procedure for pectin analysis, involving the desalting of the soluble pectins on BioGel, the enzymatic digestion with viscozyme, and quantification of galacturonic acid with copper and the Folin–Ciocalteau reagent, was applied to tomato juice. Three different juices with similar Brix levels were analyzed: a microwave hot-break

Table 4
Comparison of enzymatic versus chemical hydrolysis in the determination of the methanol and galacturonic acid composition of the water insoluble residues from apple and orange

	Hydrolysis	Galacturonic acid (nmol/g residue)	Methanol (nmol/g residue)	DM (%)
Apple	Chemical	220 ± 55[a]	192 ± 18	88
	Enzymatic	215 ± 21	183 ± 15	85
Orange	Chemical	113 ± 18	80 ± 12	71
	Enzymatic	107 ± 16	79 ± 12	73

Degree of methylesterification (DM) was calculated from the ratio of the methanol and galacturonic acid content.
[a] Mean ± s.e., $n = 3$.

Table 5
Pectin composition of tomato juices

Tomato juice	Brix	Hydrolysis	Galacturonic acid (μg/g)		Methanol (μg/g)		DM (%)	
			Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble
Microwave hot-break juice	5.0	Chemical	1549 ± 103[a]	2081 ± 90	178 ± 9	229 ± 11	71	68
		Enzymatic	1622 ± 69	1851 ± 67	172 ± 15	219 ± 14	65	73
Diluted hot-break paste	5.0	Chemical	1938 ± 223	2108 ± 286	209 ± 13	222 ± 8	66	65
		Enzymatic	2051 ± 239	2098 ± 64	189 ± 14	230 ± 8	57	67
Juice made from cold break paste	5.6	Chemical	678 ± 9	554 ± 75	14 ± 0	30 ± 2	12	33
		Enzymatic	534 ± 19	457 ± 51	11 ± 1	27 ± 2	12	37

The soluble and insoluble fractions from tomato juice were analyzed for methanol and galacturonic acid content after either chemical or enzymatic treatment to hydrolyze the pectins. Results are expressed as μg/g juice.
[a] Mean ± s.e., $n = 3$.

juice; a juice prepared by reconstituting commercial hot-break paste with water; and a commercial tomato juice, derived from cold break paste. The juices were first separated into soluble serum and insoluble pellet fractions by centrifugation. These two fractions were then analyzed for methanol and galacturonic acid content by both enzymatic and chemical hydrolysis.

As with the apple and orange pectins, similar amounts of galacturonic acid were obtained when the tomato samples were analyzed by either chemical or enzymatic pectin hydrolysis (Table 5). The greatest discrepancy between the two methods was in the amount of galacturonic acid found with the insoluble fraction from cold break paste. This was the only difference in means between the two methods that was significant ($p < 0.05$). The lower value after enzymatic hydrolysis could indicate that in the cold break paste a portion of the galacturonic acid was present in a polymeric form that is resistant to enzymatic hydrolysis. Since most of the readily hydrolyzed HG originally present in the tomato was broken down by endogenous tomato enzymes during the cold break process, this enzyme resistant polymeric galacturonic acid would comprise a larger portion of the total galacturonic acid in the cold break paste and thus cause a greater error. A slightly lower amount of methanol was also found after the enzymatic hydrolysis of the cold break paste, indicating that a small proportion of the methylesters may also be resistant to enzymatic hydrolysis. These apparent underestimates of methanol and galacturonic acid were similar such that the calculated degrees of methylesterification were the same for the two hydrolysis methods.

The pectin compositions of the three juices were consistent with the known changes in pectins that occur during tomato processing. The microwave hot-break juice and the hot-break paste had the higher total level of galacturonic acid and the higher degrees of pectin methylesterification than the juice derived from cold break paste. This is expected because the pectin degrading enzymes, PME and PG, which are inactivated in the hot-break process, are active during the cold break, causing de-esterification and de-polymerization of the pectins. The degrees of methylesterification for the soluble pectins from the cold and hot-break pastes, 37% and 67%, respectively, are in general agreement with the 28.5% and 62% previously reported (Chou & Kokini, 1987) for juices prepared from these two types of paste. Surprisingly, a recent report (Lin et al., 2005) has claimed that the degrees of methylesterification of the soluble pectins from both hot and cold break pastes are less than 15%. While it is possible that different manufacturing processes produce pastes with very different levels of pectin esterification, such low values seem unlikely, especially for a hot-break paste where pectin methylesterase is rapidly inactivated.

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