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Gas chromatography–mass spectrometry method for determining the methanol and acetic acid contents of pectin using headspace solid-phase microextraction and stable isotope dilution

Brett J. Savary*, Alberto Nuñez

Eastern Regional Research Center, Agricultural Research Service, US Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA

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Abstract

A simple, fast, and direct procedure was developed for the simultaneous determination of the methanol and acetic acid present as esters in the plant cell wall polysaccharide pectin. After base-hydrolysis of esters and acidification of pectin samples, headspace solid-phase microextraction (SPME) was performed using a Carboxen-PDMS fiber assembly. Methanol and acetic acid were separated by gas chromatography with a Chrompak PoraPlot Q capillary column and detected using electron impact mass spectrometry with selected ion monitoring. Stable deuterated isotopomers (d_3-methanol and d_3-acetic acid) were used as internal standards and for constructing calibration curves, providing accurate and absolute quantification of analytes. The methanol and acetic acid contents in 1 mg quantities of fruit and vegetable pectins were readily quantified by this procedure.

Keywords: Headspace analysis; Stable isotope dilution; Solid-phase microextraction; Pectin; Methanol; Acetic acid; Polysaccharides

1. Introduction

Pectin is a complex of acidic polysaccharides that form an interpenetrating network in the plant cell wall [1]. They are an important food hydrocolloid and traditionally used in gelling and thickening applications [2]. Pectin is composed primarily of linear homogalacturonan (α-1,4-galacturonic acids) chains interspersed with branched rhamnogalacturonan (α-1,4-galacturonic acid to α-1,2-rhamnose) chains (the neutral sugar branches are attached through rhamnose residues) [3,4]. In homogalacturonans, galacturonic acids are extensively esterified with methanol at C6-carboxyl groups and variably esterified with acetic acetic at C2/C3 hydroxyl groups. Galacturonic acids in rhamnogalacturonan may be similarly esterified with acetic acid. The methanol and acetic acid contents of pectin extracted from citrus peel is about 12 and 0.2%, respectively, and about 9 and 5%, respectively, from sugar beet root [3]. The contents can vary considerably by species, tissue type, age, and by extraction and processing conditions [5,6]. The methanol and acetic acid contents are often indicated by degree of esterification (DE), the percent mole ratio to anhydrogalacturonic acid determined for a pectin. Specifically, they are

* Corresponding author. Tel.: +1-215-233-6458; fax: +1-215-233-6406.
E-mail address: bsavary@arserrc.gov (B.J. Savary).

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represented as degree of methoxylation (DM) and degree of acetylation (DA), respectively. The DM is a primary factor influencing the conditions and mechanism for gelling by commercial pectins [2]. Most commercial pectins are produced from citrus peel, and pectins with lower DM are prepared by chemical treatments [2]. Hydrolysis of methylesters using the enzyme pectin methylesterase has been investigated as an alternative means to chemical deesterification and may provide pectins with unique functional properties [3,7,8]. Acetyl esters generally act to inhibit pectin gelling [9]; but treatment with enzyme preparations containing pectin acetylesterase can be used to improve gelling properties of pectin extracted from sugar beet [10,11].

A standard titration method is used for determining the degree of methyl esterification and anhydrogalacturonic acid content of commercial pectins [12], but this is subject to error by contribution of acetic acid esters and putative non-methyl galacturonosyl carboxyl esters [13]. Chromatographic and spectroscopic methods have been reported for determination of pectin DE [14–18]. Direct determination of methanol content of pectin is frequently cited by colorimetric assay based on oxidation of methanol released from pectin and derivatization of the resulting formaldehyde with pentane-2,4-dione [19]. Improvements have included enzymatic oxidation of the methanol [20] and adaptation to HPLC with derivatization of formaldehyde by condensation with 2,4-dinitrophenylhydrazine [21]. A GLC–FID method was developed to measure methyl esters in plant cell walls and pectin [22] using a Carbowax 20 M packed column. A HPLC method based on an ion-exchange resin column provided separation of both methanol and acetic acid [23], but suffered in sensitivity and specificity due to refractive index detection. An improved HPLC method was reported recently [24]. Although any of these methods can be used to measure enzymatic hydrolysis by pectin methylesterase, titration assay is most conveniently used to determine standard units of enzyme activity [25]. Probably, because of the low acetyl content in commercial pectins, methods for acetic acid determination are less advanced. Colorimetric assays commonly used are the Hestrin method [26] or hydroxamic acid reaction [27]. A commercial enzyme assay kit (Boehringer–Mannheim) has been used to measure acetate released from pectin by chemical or enzymatic hydrolysis [11]. The GLC–FID method reported for methyl ester content was also proposed for use in determining acetyl ester content [22]. More recently, GC with a Porapak QS column was used for enzymatic or chemical treatment of pectin samples worked up from ion-exchange and solvent extraction [10], but no details were reported for the methodology.

Quantification of a particular analyte by GC analysis requires the use of internal standards. Deuterated isotopomers of analytes provide an ideal internal standard when they are used in conjunction with mass spectrometer detectors, providing direct and accurate determination of concentration by stable isotope dilution assay [28]. This compensates for losses during sample workup, thereby reducing error in determinations, and coupled with the selectivity of MS in selected ion mode, can provide unequivocal identity by fragment pattern in the presence of unresolved contaminants [29]. The recent development of solid-phase microextraction (SPME) fiber systems facilitates the selective extraction of individual analytes from a solution based on their affinity with a specific fiber adsorbant. SPME was introduced originally for application in environmental analysis [30], and it has found wide use in biomedical, forensic, and food analysis applications [31,32]. The SPME technique uses a polymer-coated fused silica fiber that provides sample extraction, concentration, and transfer to the chromatograph in a single step. Headspace sampling is preferred over direct immersion as it avoids contact with organic polymers that can degrade fiber performance and lifetime, and it eliminates introduction of non-volatile contaminants. Differences in factors such as concentration, volatility, and partition equilibria in headspace sampling for methanol and acetic acid could possibly introduce bias in quantitative determinations, but such limitations are overcome with stable isotope-labeled internal standards [29]. We report here an integrated gas chromatography–mass spectrometry (GC–MS) method for the simple, fast, direct, and simultaneous determination of methanol and acetic acid released from pectin. This method exploits the availability of: (1) fully deuterated forms of methanol (d_3-MeOH) and acetic acid (d_3-HOAc) for use as internal isotopomer standards and (2) suitably selective SPME fibers for headspace capture of methanol and acetic acid.

2. Experimental

2.1. Materials and reagents

All chemicals and solvents were of analytical grade and purchased from Sigma (St. Louis, MO, USA) and Burdick and Jackson (Muskegon, MI), respectively, unless otherwise indicated. Deuterated standards were purchased from Aldrich: acetic-d_3 acid-d (99.9 at.% D) and methyl-d_3 alcohol-d (99.8 at.% D). Solid-phase microextraction fibers were purchased from Supelco (Belfont, PA): 75 μm Carboxen-PDMS (5–7318) and 65 μm Carbowax-DVB (5–7312). Fibers were conditioned as recommended by manufacturer prior to use. HPLC-grade water used as diluent was sparged with helium. Apple (73.5% anhydrogalacturonic acid equivalents, AGA; 9.7% methoxyl) and citrus pectins (72.1% AGA; 8.1% methoxyl) was purchased from Sigma; methylated lime pectin (Grindsted URS: 89.0% AGA; 81.5% methoxyl) was a gift from Danisco USA (New Century, KS), sugar beet pectin (Classic RU 301: 65% AGA; 6.2% methoxyl; 3.5% acetyl) a gift from Herbstreith and Fox KG (Neuenbuerg, Germany). Samples of *Aloe vera* and onion pectin were gifts from Rose Chau and Marshall Fishman, USDA-ARS, Wyndmoor).

2.2. Sample preparation and SPME procedure

Pectins were treated by dissolution in water, freezing, and then lyophilization to remove residual solvents from commercial pectin processing. Fresh pectin solutions were prepared at 5 mg/ml by dissolving in water with brief heating (60 °C) and sonication. For calibration samples, vials (4 ml volume) received sugar beet pectin (1 mg), d_3-methanol (2.0 μmol) and d_3-acetic acid (0.500 μmol) internal standards, and varying quantities of unlabelled analytes (0.10–8.0 μmol methanol and 0.05–2.0 μmol acetic acid). The final volume was 1 ml and 0.100 M monobasic sodium phosphate (pH 2.0 with sulfuric acid). Vials were capped and warmed to 40 °C in an aluminum block heater, then the needle of the SPME device was inserted through the septum and the fiber was exposed to the headspace vapor for 15 min. Thereafter, the fiber was retracted and immediately transferred to the GC injection port for sample desorption. The fiber remained exposed in the injection port at least 10 min between samples. In a control experiment with pectin solutions having no standards added, no methanol or acetic acid was detected, demonstrating negligible ester hydrolysis occurred under these sampling conditions. Two samples were prepared for each sample point and each run in duplicate ($n = 4$).

For pectin analyses, vials received 0.200 ml pectin (1 mg), 0.200 ml of 1.0 M NaOH, and 0.100 ml d_3-standards (2.00 μmol of MeOH and 0.500 or 0.050 μmol of HOAc). The vials were immediately capped and heated at 40 °C for 1 h, and then placed on ice. Vials then received 0.5 ml of 0.4 M sulfuric acid (final pH <2.0). Headspace-SPME sampling was performed as described for calibration samples. Duplicate samples for each pectin were prepared and analyzed in triplicate (total analyses, $n = 6$).

2.3. Gas chromatography–mass spectroscopy detection

The GC–MS system consisted of a 5890 Series II Plus gas chromatograph with a Mass Selective Detector (Hewlett-Packard, San Fernando, CA) fitted with a PoraPLOT Q capillary column, 25 ml × 0.25 mm i.d., film thickness 8 μm (Chrompack, Raritan, NJ) and a narrow bore (0.75 mm) SPME injection liner (Supelco). All injections were splitless with the injector set at 300 °C and detector at 250 °C, using helium as carrier gas at 1 ml/min. The oven temperature gradient profile was 40 °C (1.6 min) to 250 °C (5 min) at 50 °C/min and held at temperature for 5 min. The detector was set at electron impact ionization mode (70 eV) with data collected using the selected ion recording for selected ions at 1.2 scans/s. Methanol concentrations were calculated by plotting the peak area ratios (normal to deuterated forms) for base ion pairs (m/z $29/30_d$) over the indicated range of concentration ratios. Acetic acid concentrations were similarly calculated using peak area ratios of base ion pairs (m/z $43/46_d$) over the indicated range of concentration ratios.

3. Results and discussion

3.1. Headspace sampling by SPME

Carbowax-DVM SPME fiber is recommended by the manufacturer for applications involving alcohols

and polar compounds (MW 40–275) and was determined to be most effective for headspace sampling of ethanol and other volatile compounds in blood [34]. Carboxen-PDMS fiber was reported as most sensitive for adsorbing small molecules and organic acids from coffee sample headspace [35], and it was recommended for analysis of methanol or formic acid in bodily fluids [36]. Both fiber types were compared in this study for their ability to bind methanol and acetic acid in sample headspace at ambient and elevated temperature (Fig. 1). Carboxen-PDMS proved a much better adsorbent than Carbowax-DVB for both analytes. The sensitivity for acetic acid increased further by a factor of three with elevated temperature during sampling. Methanol binding was not increased at higher temperature. Carboxen-PDMS fiber sampling was selected for calibrating standard curves and analysis of methanol and acetic acid released from pectin samples. Optimizations such as added salts, increased temperature, and exposure time have provided increased sensitivity for extracting volatile compounds from sample headspace by SPME [31,32,34]. However, no further optimization of headspace extraction was attempted in this study since the system was sufficiently sensitive for the range of methanol and acetic acid contents possible in 1 mg of pectin.

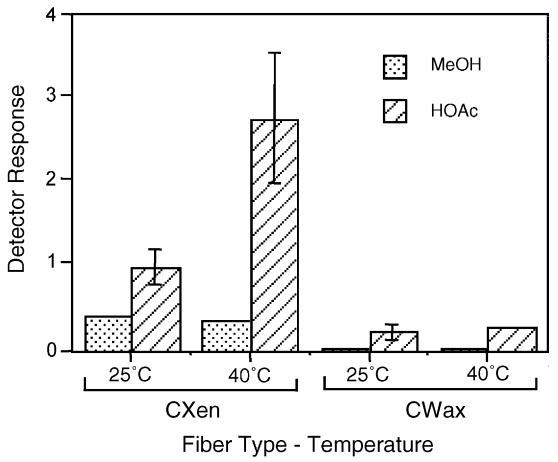

Fig. 1. Headspace sampling for methanol and acetic acid with Carboxen-PDMS (CXen) and Carbowax-DVB (CWax) SPME fibers at two temperatures. Samples in 4 ml vials contained 1 mg pectin in 1 ml volume at pH 2.0 and spiked with 60 μg deuterated standards.

3.2. GC separation

Although a range of GC columns have been used for analyses that have included methanol and acetic acid, generally more polar columns are preferred for resolving such volatile compounds. Derivatization of organic acids, such as methylation of the acid group, is generally recommended for improved elution and separation, but such manipulations introduce additional steps in the analytical procedure. A PoraPLOT Q column provided separation of polar or non-polar volatile compounds in the range of C_1–C_7 with little influence by the polarity or boiling point of the molecule and, in general, separated compounds primarily by molecule size without the need for derivatization [37]. We evaluated this PoroPLOT Q column to separate a mixture of methanol and acetic acid in the natural and deuterated forms. Suitable retention times and resolution of methanol and acetic acid were obtained in a 10 min program. Methanol eluted at about 4.75 min (approximately 180 °C) while acetic acid eluded at about 6.33 min (250 °C), as shown in the total ion current chromatogram in Fig. 2. A PoroBond Q column was also evaluated, but it was found unsuitable due to excessive tailing of the acetic acid peak.

3.3. Ion selective mass detection and calibration curves

Deuterated forms (d_3) of methanol and acetic acid are available commercially, inexpensive, and highly-labeled (99.8 at.% D). Coupled with a mass selective detector, these isotopomers can be used as near ideal internal standards to provide direct and accurate quantification by stable isotope dilution assay [28,29]. The electron impact spectra for the natural and deuterated form of methanol and acetic acid are shown in Fig. 3. The base ions (100% relative intensity) are observed at *m/z* 29 $[HCO]^+$ and 30 $[DCO]^+$ for methanol and d_3-methanol, respectively, and *m/z* 43 $[CH_3CO]^+$ and 46 $[CD_3CO]^+$ for acetic acid and d_3-acetic acid, respectively. Molecular ions are observed at *m/z* 32 (25%) and 35 (32%) for methanol and d_3-methanol, respectively. Correspondingly, the molecular ions for both forms of acetic acid are at *m/z* 60 (51%) and 63 (46%). These sets of ion pairs (molecular and base) have the relative abundance necessary for use in the stable isotope dilution method

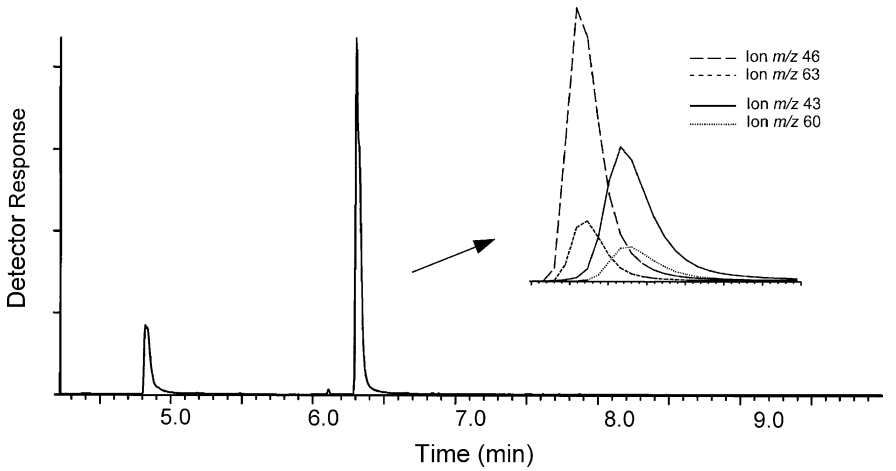

Fig. 2. Total ion chromatogram from GC–MS for separation of methanol and acetic acid mixture. Insert: selected ion chromatogram overlays for acetic acid (*m*/*z* 43, 60) and d_3-acetic acid (*m*/*z* 46, 63).

[28] and provide two quantitative alternatives for each analyte, and comparison of analyte determinations using each can be used to provide a qualitative means to assess for cross-ion contamination.

The reconstructed ion chromatograms obtained for the selected ions for the deuterated and natural forms for both compounds indicated a partial resolution of the isotopomeric mixture by the PoraPLOT Q column. This is shown for acetic acid in the insert in Fig. 2. Deuterated forms eluted slightly before unlabelled forms. Because of this partial separation of isotopomer pairs, quantification of analytes was based on peak area ratios determined from reconstructed ion peak area chromatograms rather than ion intensity height ratios as described in the stable isotope dilution method [28,29].

The calibration curves generated for methanol and acetic acid quantification are plotted for the selected

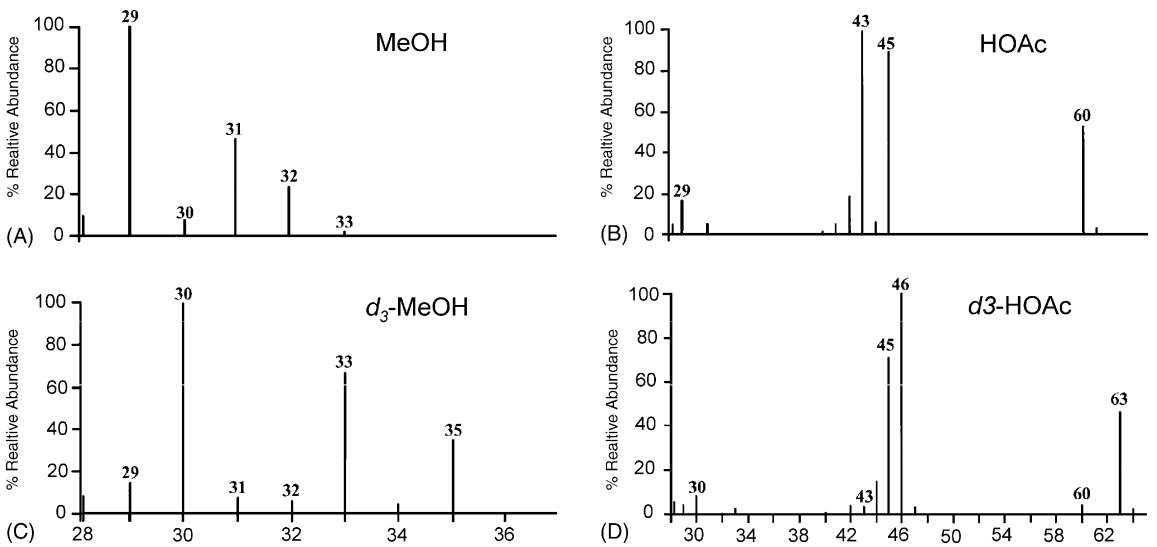

Fig. 3. Electron impact (70 eV) mass spectra for: (A) methanol; (B) acetic acid; (C) d_3-methanol; (D) d_3-acetic acid.

base ion peak area ratios versus the concentration ratios (Fig. 4). For methanol determination, the concentrations used cover the possible content range found per mg in commercial pectins. The methanol calibration curve represented in Fig. 4A was described by a second-order polynomial fit ($r^2 = 0.989$) over the concentration range indicted for the base ion pairs, providing the calculation of concentration (μmol/ml) in 1 mg pectin samples: $x = (0.165y^2 + 0.386y - 0.037)2.00$ μmol, where y is the ratio of peak areas (m/z 29/32). For pectins with low methanol content (<1.00 μmol/mg), the amount of d_3-methanol internal standard was reduced to 1.00 μmol and the methanol content calculation adjusted accordingly.

The calibration curve similarly generated for acetic acid is shown in Fig. 4B ($r^2 = 0.991$), providing the calculation of acetic acid concentration (μmol/ml) from 1 mg pectin samples: $x = (0.056y^2 + 0.761y - 0.221)0.500$ μmol/ml. The mid-range of acetic acid concentrations (0.500 μmol) used was selected for pectins having moderately high acetyl ester content (e.g. from sugar beet pulp). A 10-fold lower quantity of d_3-acetic acid was used as internal standard to pectin samples having very low acetyl ester content (e.g. from citrus peel), and the calculations were adjusted accordingly. The calibration curves were essentially unchanged at this lower range (data not shown), but in practice we found a lower limit of determination of about 0.0125 μmol acetic acid/mg pectin.

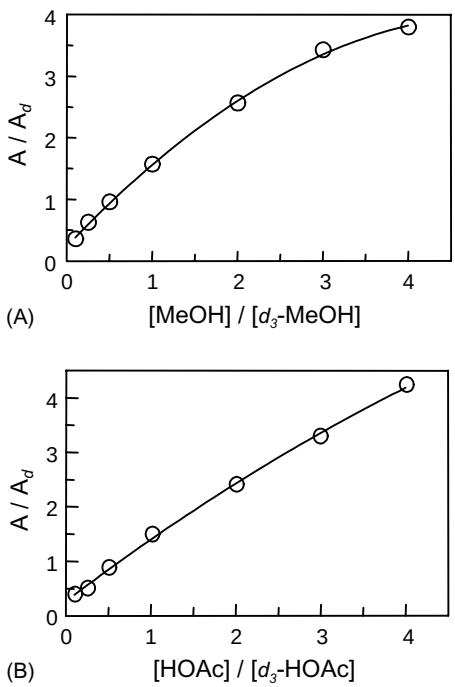

Fig. 4. Calibration curves for methanol (2.00 μmol/ml) and acetic acid (0.500 μmol/ml): (A) methanol and d_3-methanol (base ions m/z 29 and 30_d, respectively); (B) acetic acid and d_3-acetic acid (base ions m/z 43 and 46_d, respectively). Graphed as selected ion peak area ratios (natural to deuterated) against concentration ratios (natural to deuterated), and data fitted by second-order polynomial. Each concentration prepared in duplicate and subsequently determined in triplicate.

3.4. Determination of methanol and acetic acid contents of pectin

The GC–MS method and calibration curves were applied to quantify the methanol and acetic acid contents from a range of fruit and vegetable pectin samples—apple, citrus, and methylated lime pectin, and sugar beet, *Aloe*, and onion, respectively. Samples were saponified with alkali to hydrolyze pectin esters and subsequently acidified to convert acetate to acetic acid. Determinations for methanol and acetic acid contents are indicated in Table 1. Contents ranged from a high of 3.70 μmol methanol/mg methylated lime pectin and 0.440 μmol acetic acid/mg sugar beet pectin to low of 0.427 μmol methanol/mg *Aloe* pectin and 0.018 μmol acetic acid/mg methylated lime pectin.

Separation of methanol and acetic acid peaks from a vegetable-type pectin, represented by sugar beet pectin, is shown in Fig. 5A. The methanol content determined by this GC–MS method matched that provided by the manufacturer using customary methods. Sugar beet pectin is distinctive for being rich in acetyl esters [3,27,38], and the residual acetic acid content in the commercially-prepared pectin was found to be 2.5% (Table 1). The acetic acid content determined by GC–MS is indicated at about 27% less than the content reported by the manufacturer using the couple–enzyme assay method [11]. Because this GC–MS method provides a direct and specific determination by internal isotopomer standards, the higher content determined here is believed to be more accurate and the indirect enzyme-based method.

Table 1
Methanol and acetic acid contents determined for fruit and vegetable pectin samples[a]

Pectin	Determined content[b]	Determined composition (%)[c]	Labeled composition (%)[d]
Apple			
Methanol	2.85 ± 0.27	9.11 ± 0.88	9.7
Acetic acid	0.101 ± 0.013	0.61 ± 0.08	ND[e]
Degree methylation[f]		68.2 ± 6.5	72.6
Degree acetylation[f]		2.43 ± 0.32	ND
Citrus			
Methanol	2.59 ± 0.10	8.31 ± 0.33	8.1
Acetic acid	0.036 ± 0.005	0.21 ± 0.03	ND
Degree methylation		63.3 ± 2.5	61.7
Degree acetylation		0.87 ± 0.12	ND
Methylated lime			
Methanol	3.70 ± 0.31	11.8 ± 1.0	13.2
Acetic acid	0.018 ± 0.003	0.11 ± 0.02	ND
Degree methylation		73.2 ± 6.0	81.5
Degree acetylation		0.35 ± 0.06	ND
Sugar beet			
Methanol	1.86 ± 0.12	5.98 ± 0.40	6.2
Acetic acid	0.440 ± 0.025	2.54 ± 0.15	3.5
Degree methylation		53.1 ± 3.6	55.4
Degree acetylation		12.6 ± 0.7	16.6
Aloe vera[g]			
Methanol	0.427 ± 0.017	1.37 ± 0.05	ND
Acetic acid	0.352 ± 0.067	2.11 ± 0.40	ND
Onion[g]			
Methanol	0.932 ± 0.008	2.99 ± 0.03	ND
Acetic acid	0.087 ± 0.003	0.52 ± 0.02	ND

[a] Each pectin sample prepared in duplicate and subsequently analyzed in triplicate.
[b] Methanol and acetic acid contents determined as μmol per 1 mg pectin (±standard deviation).
[c] Methanol and acetic acid compositions determined as mg per 1 mg pectin (±standard deviation).
[d] Manufacturer's determinations (mg/mg pectin) using official titration method for methylester content and using enzyme assay for acetic acid content.
[e] ND: not determined.
[f] Degree of esterification is percent molar ratio with galacturonic acid equivalents. Galacturonic acid contents were determined by manufacturer.
[g] Microwave "flash" extracted pectin [40] isolated from commercial processing residues.

Commercial fruit-type pectin such as that from citrus peel are generally extracted in a highly methylated state, while having a very low acetyl ester content [27,38]. Both the commercial citrus (Fig. 5B) and a methylated lime (not shown) pectins gave strong methanol signals. The methanol content calculated for the two citrus pectins was consistent with determinations provided by the manufacturers using titration-based determination methods. No acetic acid content was provided by the manufacturers. Very low amounts of acetic acid were detected in both samples, such as that represented by citrus pectin (Fig. 5B), and its identity was confirmed unequivocally by the EI–mass spectrum of the acetic acid peak (Fig. 3C). The acetic acid content in citrus peel is thus determined directly at about 72-fold lower mole content compared to methanol. These results, thereby demonstrate a rapid, simple, and direct determination for both methanol and acetic acid contents of pectin, providing a considerable improvement over the time-consuming and labor intensive titration, enzyme and chemical assays [12,19,20,27,32].

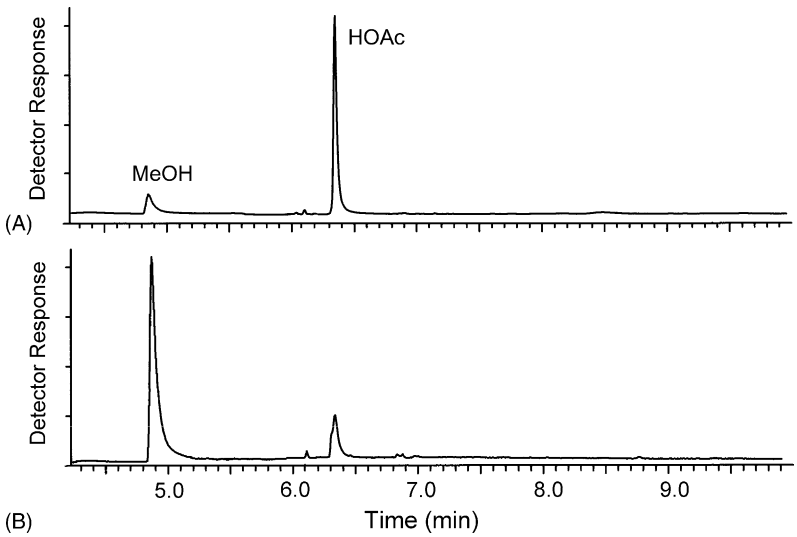

Fig. 5. Selected ion chromatograms (non-deuterated base ions *m/z* 29 and 43) of representative vegetable and fruit pectins: (A) sugar beet pectin; (B) citrus pectin.

During preliminary analyses, total ion current chromatograms showed contaminant peaks after headspace sampling of pectin samples. Such peaks represented solvents (particularly *iso*-propanol) used in pectin manufacture. Although these contaminants did not interfere in GC–MS analysis of pectin, it was preferred to eliminate them with a pretreatment of pectin samples using lyophilization. Solvent contaminants were otherwise persistent through heating samples at 100 °C for 2 h under vacuum (data not shown, and noted in [38]). The susceptibility of this highly sensitive analytical system to organic solvent contamination from the laboratory requires utmost care in preparation of solutions and handling of transfer instruments and containers. In particular, trace levels of acetone (which can be introduced from cleaning of syringes and injection sleeves) can contribute to a baseline shift for acetic acid determinations. This can result in significant error in quantifying the low content of acetic acid present in certain pectins such as that from citrus peel, if not recognized and properly controlled for.

Pectin methylesterases and acetylesterase are enzymes highly specific for their corresponding ester substituents in homogalacturonan [39]. We treated a sugar beet pectin with an orange peel enzyme extract that contained both a salt-independent pectin methylesterase isoenzyme in addition to acetylesterase activity [11,25,33]. In this preliminary experiment, we demonstrated recovery of both methanol and acetic acid from the headspace of the enzyme–pectin reaction mixture (data not shown). These results therefore indicate this GC–MS method can be used for directly determining kinetic properties of esterases specific to pectin. Similarly, uncharacterized enzyme extracts from plant and microbial sources may be screened for novel pectin esterase activities. Finally, this method should be directly applicable for determining the acetic acid ester content and corresponding enzyme activities for other types of polysaccharides such as *O*-acetylated xylans.

4. Conclusion

Headspace-SPME coupled to GC–MS with selected ion monitoring is an effective method for determining the methanol and acetic acid contents in pectin. Use of deuterated internal standards provided an accurate and absolute determination of both analytes. The method described may be adapted for measuring corresponding esterase activities and ester contents in other classes of *O*-acetylated polysaccharides.

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