JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Simple and Validated Quantitative ^{1}H NMR Method for the Determination of Methylation, Acetylation, and Feruloylation Degree of Pectin

Judith Müller-Maatsch,[†] Augusta Caligiani,*[,†] Tullia Tedeschi,[†] Kathy Elst,[§] and Stefano Sforza[†]

[†]Department of Food Science, University of Parma, Parma, Italy

[§]Flemish Institute for Technological Research, Mol, Belgium

ABSTRACT: The knowledge of pectin esterification degree is of primary importance to predict gelling and other properties of pectin from different sources. This paper reports the development of a simple and rapid ^{1}H NMR-based method for the simultaneous quantitative determination of methylation, acetylation, and feruloylation degree of pectin isolated from various food sources. Pectin esters are hydrolyzed in NaOH/D_2O, and the obtained methanol, acetic acid, and ferulic acid are directly measured by ^{1}H NMR. High accuracy, repeatability, and reproducibility of the method were obtained, and the analysis time is reduced as compared to conventional chromatography- or titration-based methods.

KEYWORDS: *pectin, quantitative ^{1}H NMR, methylation degree, acetylation degree, feruloylation degree*

INTRODUCTION

Pectins are a group of polysaccharides ubiquitously found in plants. They are present in the cell walls located in the middle lamella and primary and secondary cell walls. The chemical structure of pectin is heterogenic, based on the origin, location in the plant, and extraction method.[1] The main residue is galacturonic acid (GalA), α-1,4-glycosidically linked. Beyond this standard structural feature, different pectic structures have been described in the literature: homogalacturonan and rhamnogalacturonan I are the most cited ones. Homogalacturonan is a simple linear α-1,4-linked galacturonic acid polysaccharide, whereas in the backbone of rhamnogalacturonan I the galacturonic acid is partly substituted with α-1,2-linked rhamnose. In addition, several side chains containing sugars such as xylose, arabinose, glucose, fucose, mannose, or galactose have been found to be linked to the backbone structure. Pectin (Figure 1) can be furthermore methyl-esterified on the galacturonic acid carboxylic acid moiety, acetyl-esterified at the O2/O3-position, or, less commonly, ferulyl-esterified on side chains.[2] Ferulylated pectin has been reported in sugar beet,[3] spinach,[4] and glasswort.[5]

The degree of esterification has an impact on the physical properties of pectin: emulsion formation,[6] surface tension, stabilization of tissue,[7,8] and gel characteristics.[9] Pectin with a degree of methylation of >50% is known to form gels under low pH and high sugar concentrations, whereas pectin with a lower degree of methylation gells with a bipolar cation such as calcium.[10] Acetylation, like methylation, decreases the affinity of pectin for cations, which has an impact on the gelling ability of pectin.[11,12] Furthermore, it has an effect on the surface activity,[13] emulsion stability, and viscosity.[14] Ferulic acid esters play a significant role in the plant cell wall because they form bonds between polysaccharides and proteins, and pectin esterified with ferulic acid as in sugar beet has been reported as an emulsifying agent.[15] More recent evidence also points to the role of ferulic acid and other phenolics associated with pectin in fiber fermentability and gut health.[16]

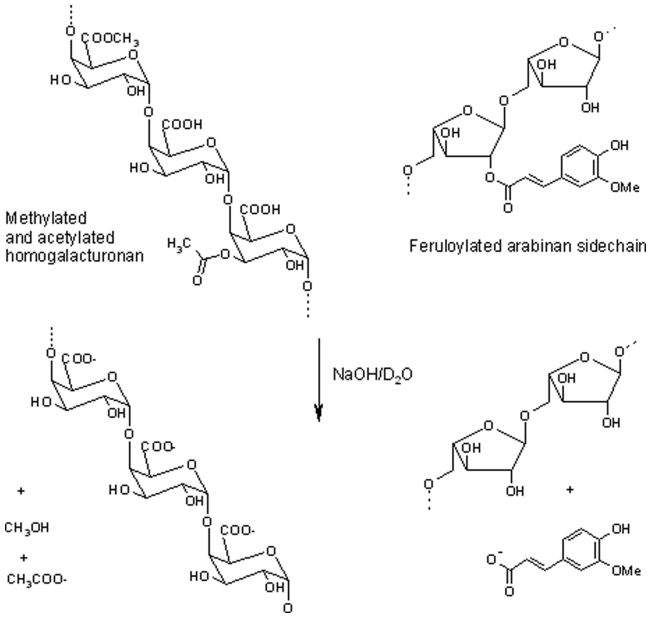

Figure 1. Structures of the main esterified oligosaccharides of pectin and saponification reaction.

Different approaches to measure the degree of methylation of pectin are published in the literature.[17] Most of the methods are based on using alkaline hydrolysis of the ester bonds; methanol is released from the galacturonic acid by incubation with sodium hydroxide solution. The content of methanol in the

Received: June 6, 2014
Revised: August 1, 2014
Accepted: August 19, 2014
Published: August 19, 2014

samples has been detected using HPLC,[18,19] GLC,[20] GC headspace,[21] GC-MS,[22] and spectrophotometric methods[23,24] or FTIR.[25]

Another approach, mostly used in industry, is basic titration, again based on the methanol release from ester induced by the basic environment.[26,27]

Methods combining both acetic acid and methanol determination are limited. There have been attempts measuring both using HPLC[18,19] or GC-MS[22] for the detection after saponification. No methods were found allowing the simultaneous detection of methylation, acetylation, and feruloylation degree of pectin. Ferulic acid in pectin was generally determined by spectroscopic methods[28] or HPLC-UV methods.[29]

NMR was extensively used to study pectin and, more in general, food carbohydrates at a structural level,[30] whereas the application of this technique as a quantitative analytical tool in carbohydrate determination is still limited, despite its huge possibilities.[31]

With regard to the determination of the degree of esterification in pectin by NMR, few studies can be found in the literature,[32−34] and only two papers report the simultaneous measurement of the methylation and acetylation degrees of pectin by NMR.[35,36] In most of these studies, the NMR analysis is performed on intact pectin and the degree of methylation is based on the integration of the signals of the protons adjacent to the carboxylate in free or esterified form. However, the determination of the methylation degree of intact pectin is affected by difficulties related to the low spectral resolution due to the gelling properties of pectin. In fact, in an attempt to reduce the poor resolution, all of the NMR experiments reported in the literature are registered at high temperatures (60−90 °C). The low spectral resolution leads to an enlargement and a deviation from the Lorentzian form of the NMR signals, which become more difficult to integrate, thus affecting the error of the measurement. Bedouet et al.[36] explored the possibility to determine acetylation and methylation degrees of pectin by ^{1}H NMR measurement after saponification of pectic material directly in the NMR tube. However, also in this case experiments were performed at 80 °C, due to the known jellying properties of pectin also after saponification. Neumuller et al.[37] proposed a quantitative NMR-based method to determine phenoyl and acetyl esters of different polysaccharides as corn fiber and potato starch after saponification. The method was in good agreement with a conventional HPLC analyses, with the advantage of significantly reduced analysis time.

Here, we suggest a simpler method for the simultaneous detection of methylation, acetylation, and feruloylation degrees of pectin, which consists of an easy preparation of the sample for ^{1}H NMR analysis by performing an alkaline saponification (Figure 1) on the pectin isolated from the food sources. This step allows very resolved NMR spectra to be obtained at room temperature, where the peaks of methanol, acetic acid, and ferulic acid can easily be integrated and quantified with respect to the amount of pectin.

MATERIALS AND METHODS

Materials and Chemicals. Standard of pectin from citrus peel, deuterium oxide, 3-(trimethylsilyl)propionate-d_4 (TSP), methanol, acetic acid, ferulic acid, galacturonic acid, sulfamic acid, potassium hydroxide, sodium tetraborate, *m*-hydroxydiphenyl, sodium hydroxide, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), phenol, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), dimethyl sulfoxide, porcine pancreatic α-amylase, and sulfuric acid, of analytical grade were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Three samples of pectin with different methylation degrees determined by titration were provided by Silvaextracts (Cuneo, Italy) as freeze-dried powders. Sugar beet flakes, pea hulls, olive pomace, grape pomace, and apple cake were provided by IGV (Nuthetal, Germany) as part of the NOSHAN EU project.

Methods of Analysis. *Extraction of Soluble Pectic Polysaccharides by a Chelating Agent Solution.* The isolation of cell wall was performed following the instructions of Melton and Smith[38] for samples containing starch. To inactivate enzymes, the samples were homogenized (Ultraturrax T-50 basic, IKA-Werke, Staufen im Breisgau, Germany; 2−3 min, 4000 rpm) with 80% phenol−0.5 M HEPES buffer (w/v). Soluble cell matrix contents were solubilized by 50 mM HEPES buffer (pH 6.7) and separated by centrifuge (Eppendorf AG, Hamburg, Germany; 20 min, 3220*g*, RT, HDPE centrifuge bottles). The pellet was treated (incubation for 6 and 24 h) with DMSO 90% (v/v) and further incubated for 1 h at 40 °C with 200 U of porcine pancreatic α-amylase stabilized by 20 mM HEPES buffer with 20 mM $CaCl_2$ (pH 6.9), to remove starch. Cell walls were treated twice (6 and 12 h) with 50 mM CDTA in 50 mM potassium acetate buffer (pH 6.5). The supernatants were separated by centrifuge, combined, and dialyzed with 0.1 M ammonium acetate buffer (pH 6.5, 1 day, three changes, 4 °C) followed by H_2O (3 days, three changes at 4 °C). All samples were freeze-dried and stored at −18 °C.

Galacturonic Acid Determination. The galacturonic acid content was determined with *m*-hydroxydiphenyl as described by Melton and Smith[39] using galacturonic acid as standard. Pectin samples were hydrolyzed using 1 mL × 2 concentrated sulfuric acid, mixed, and cooled in an ice bath. Bidistilled water, 0.5 and 5 mL, was added, respectively, centrifuged (Eppendorf 5810R, Eppendorf, Augsburg, Germany), and the supernatant was used for spectrophotometric measurements (UV−vis Lambda BIO 20 spectrophotometer, PerkinElmer, Waltham, MA, USA). To 400 μL of supernatant or standard were added 40 μL of 4 M sulfamic acid/potassium sulfamate solution (pH 1.6) and 2.4 mL of 75 mM sodium tetraborate/sulfuric acid solution. The mixture was heated (100 °C) for 20 min and then cooled. To the sample control was added 80 μL of 0.5% NaOH to determine the sugar coloring. To the sample and standard was added 80 μL of 8.8 mM *m*-hydroxydiphenyl in 0.5% NaOH. The absorbance was measured after 10 min of incubation time at 525 nm against a water blank.

Methylation, Acetylation, and Feruloylation Degree. Pectic polysaccharides were incubated (2 h, room temperature) with 1 mL of 0.4 M NaOH in D_2O and 0.1 mL of internal standard (TSP, 0.2 mg/mL in D_2O). The supernatant was centrifuged (Centrifuge Eppendorf 5810R, Eppendorf, Augsburg, Germany), clarified (nylon syringe filter system, 0.4 μm), and transferred in NMR tubes. ^{1}H NMR spectra were acquired on a Varian-Inova 600 MHz spectrometer, equipped with a triple-resonance inverse probe (HCN), operating at 599.736 MHz for proton. Spectra were collected at 298 K, with 32K complex points, using a 90° pulse length. Sixty-four scans were acquired with a spectral width of 7196.8 Hz, an acquisition time of 2.53, and a relaxation delay (d1) of 5 s. The experiments were carried out with water suppression by low-power selective water signal presaturation during 5 s of the relaxation delay. The accuracy of the quantitative data was assured by the relaxation delay, determined by the longitudinal relaxation time (T1) measurements, which is set in order to allow the complete relaxation of the nuclei. The T1 time was determined by the T1 inversion recovery pulse sequence consisting of a 180° pulse followed by a variable delay (τ), a 90° pulse, and an acquisition.

The NMR spectra were processed by MestreC software. The spectra were Fourier transformed with FT size of 64K and 0.2 Hz line-broadening factor, phased and baseline corrected, and referenced to the TSP peak (0 ppm). The quantitative determination of acetic acid, methanol, and ferulic acid was obtained by manual integration of the

corresponding signals (1.920 ppm for acetic acid, 3.358 ppm for methanol, and 6.233 ppm for ferulic acid) and comparison with the TSP area. Integrals were converted in mass value (mg) according to the following formula, as previously reported.[40]

$$A\ x \times \frac{\text{EW}\ x}{\text{mg}\ x} = A\ \text{TSP} \times \frac{\text{EW TSP}}{\text{mg TSP}}$$

$A\ x$ is the spectral area of the analyte, A TSP is the spectral area of the internal standard, EW x is the equivalent weight of the analyte, EW TSP is the equivalent weight of the internal standard, and EW = (molecular weight/number of hydrogens in the signal).

Through the content of methanol and the content of galacturonic acid, the degree of methylation (or acetylation/feruloylation) was calculated, using the following equation:

$$\text{degree of methylation} = \frac{\text{mol of methanol}}{\text{mol of galacturonic acid}} \times 100\%$$

Linearity, Limit of Detection, and Limit of Quantification. The linearity of the method was checked at a range between 0.1 mM (detection limit) and 100 mM for each analyte. Three replicates were performed for each concentration. Each solution was subjected to the entire experimental protocol. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated utilizing the S/N ratio methods, based on the determination of the peak to peak noise.[41] LOD and LOQ were therefore calculated as the minimum concentrations producing a recognizable peak with a signal-to-noise ratio of, respectively, 3.3 or 10.

Accuracy, Precision, Recovery, and Robustness of the Method. The accuracy of the analyte's recovery was determined by assaying samples with known concentrations of methanol, acetic acid, and ferulic acid, both as pure compounds and as spiked matrix. Accuracy was determined by the following equation: accuracy = $(p^2 + b^2)^{1/2}$, where p is precision and b is the bias (difference between NMR value and the true or "accepted" value). Precision was evaluated both intraday and interday (intermediate precision), and the data were compared after three consecutive analyses (intraday) and from data obtained over a 15 day period (interday). The precision was expressed as coefficient of variation (CV%). Recovery of analytes was determined by spiking a sample of sugar beet pectin with pure compounds. The evaluation of robustness was performed by varying two parameters of the analytical procedure as hydrolyzing volume and time of hydrolysis.

RESULTS AND DISCUSSION

Development and Optimization of the Sample Preparation. The proposed method involves pectin saponification (Figure 1) followed by separation of the polysaccharidic component by filtration and direct ^{1}H NMR analysis at room temperature. The filtration step is necessary to eliminate from the solution the de-esterified polysaccharidic polymers. In fact, preliminary tests aiming to evaluate the possibility of analyzing directly the solution after saponification showed that pectin gave very poorly resolved spectra, also when de-esterified. For this reason, in the method we proposed, the polysaccharidic components were separated by filtration prior to NMR analysis. In a previous paper,[36] the authors determined the acetylation and methylation degree of pectin by ^{1}H NMR measurement of the product of in-tube saponification of pectic material, and in an attempt to reduce the poor resolution of pectin solutions, NMR experiments were registered at high temperatures (80 °C). However, from a technical point of view, 80 °C is the maximum permitted temperature for many common NMR probes, thus limiting the applicability of the methods. Moreover, this approach is not optimal when the goal is the accurate determination of methanol, because at high temperatures a repartition of methanol in the headspace of the NMR tube may occur, and this might represent a delicate point that affects the robustness of the procedure.

The method proposed was initially tested on a standard of pectin sample, namely, citrus peel pectin with a declared methylation degree of 63−66%. To obtain satisfactory efficiency, the effect of the main variables involved in the hydrolysis of pectin, time of hydrolysis and volume of NaOH in D_2O, were varied to determine the optimal conditions allowing the complete reaction. Variable volumes of NaOH in D_2O (1, 2, and 3 mL) and different incubation times (30, 60, 120, and 180 min) were tested. The results were evaluated by applying two-way ANOVA at a p level of 0.05, considering also the interactions of the two factors. ANOVA results showed that the incubation time had a significant effect in enhancing the hydrolysis degree (p = 0.007), whereas the NaOH in D_2O volume and the interactions among the parameters were not found to be significant (p = 0.079 and p = 0.629, respectively). The incubation times of 30 and 60 min gave results significantly different from those obtained with incubation times of 120 and 180 min (as determined by post hoc Tukey-HSD tests). Thus, the use of 1 mL of NaOH in D_2O, to avoid deuterated solvent consumption, and 120 min of incubation, to minimize analysis time, was chosen in the final method.

Method Validation. The optimized method was subjected to validation in terms of precision, accuracy, linearity, detection and quantitation limits, repeatability, reproducibility, recovery, and robustness following recommendations of the International Conference on Harmonization (ICH (2005)): validation of analytical procedures, text and methodology; harmonized tripartite guideline, Q2(R1)). The validation tests were performed on a pure solution of methanol, acetic acid, and ferulic acid.

To determine the accuracy and precision of the methanol, acetic acid, and ferulic acid analysis by ^{1}H NMR, a solution containing all of the analytes was obtained by weighing the compounds and dissolving them in the hydrolyzing solution (1 mL of 0.4 M NaOH in D_2O and 0.1 mL of 0.2 mg/mL TSP in D_2O as internal standard) and analyzed by ^{1}H NMR in the experimental conditions previously reported. The same solution was used to spike a real matrix of sugar beet pectin. Measured results of the standard solution were in agreement with the amounts weighed in the range of concentrations of 0.1−100 mM, a range that corresponds with the final in-tube concentration of the analytes in real samples of pectin. Linearity was demonstrated in the same range. The instrumental quantification limit (LOQ, signal-to-noise ratio >10) in the experimental conditions reported was about 0.1 mM for methanol and acetic acid and 0.2 mM for ferulic acid, whereas the LOD was obtained at 0.03 mM for methanol and acetic acid and at 0.05 mM for ferulic acid (S/N ratio 3.3).

Standard solutions were also subjected to the entire experimental protocol to verify eventual loss of methanol and acetic acid during the various steps of sample preparation. Results are reported in Table 1 as precision, bias, and accuracy. A spiked matrix (sugar beet) was also analyzed, and the corresponding recoveries are reported in Table 1.

Reproducibility and Robustness of the Method. To determine the interday reproducibility, a pectin standard, after the first measurement, was kept refrigerated and reanalyzed by ^{1}H NMR spectrometer after 3, 7, and 14 days. On each day three repetitions of the experiment were performed. The data were treated with one-way ANOVA for each analyte, and the results showed that no significant differences had arisen in the

Table 1. Precision (Percent; Standard Deviation), Accuracy, Bias, and Recovery of the NMR Measurement of Methanol, Acetic Acid, and Ferulic Acid

	methanol	acetic acid	ferulic acid
precision (CV%)[a]	2.22	1.94	2.01
bias[a]	0.024	0.093	0.002
accuracy[a]	2.22	1.94	2.00
recovery %[b]	93 ± 2	96 ± 2	90 ± 2

[a]Calculated on a standard solution subjected to the entire experimental protocol. [b]Calculated on sugar beet pectin spiked with standard solution and subjected to the entire experimental protocol.

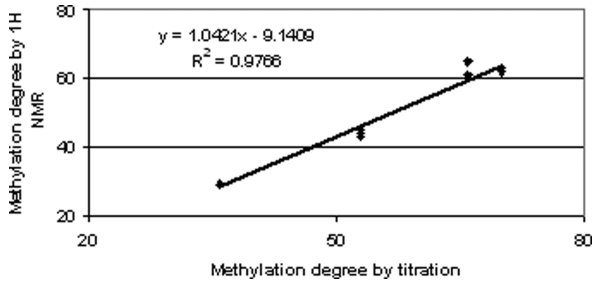

Figure 2. Correlation observed between the results obtained from the titration method and the ^{1}H NMR-based method for citrus peel pectin methylation degree.

time interval under study. The coefficients of variation (CV) calculated on all 12 data (3 repetitions for 4 days) were 2.4% for acetic acid and 2.5% for methanol.

The data obtained from method optimization trials were analyzed to test the robustness of the method: the non-significant effect of the hydrolyzing agent volume and of hydrolysis time in the range of 120−180 min, as reported above, demonstrated that the method is robust to the variation of these factors. In this case the CV% calculated for methanol was 3.9%.

Comparison of ^{1}H NMR-Based Method and Titration Method. The method was applied to three commercial pectin samples from citrus peel with different values of methylation, certified by the producer (Silvaextracts) and determined by titration. The titration method[26] is based on the determination of the ratio between the volume of NaOH used for the titration of the carboxylic groups "covered" by methyl groups (V_2) on the sum of V_2 + the volume of NaOH used for the titration of free carboxylic groups (V_1).

$$\text{degree of methylation} = \frac{V_2}{V_1 + V_2} \times 100\%$$

Results obtained by the proposed ^{1}H NMR method (determined in triplicate) compared to the values provided are shown in Table 2.

The values determined are in line with the data provided by Silvaextracts. The correlations between couples of data were obtained by applying the regression analysis and are shown in Figure 2 for methylation degree. A good correlation (R^2 = 0.9766) was obtained. It can be observed that in general data from NMR were lower than those obtained by titration. The differences between the two methods can be ascribed to interfering substances present in pectin able to react with the sodium hydroxide, thus "inflating" the methylation degree. Also, similar differences between the method of titration and saponification combined with HPLC were reported previously.[18,19] The method here presented, based on the actual visualization of methanol and acetic acid, is expected to be more accurate.

As compared to the HPLC methods reported in the literature, the ^{1}H NMR-based method here proposed presents the advantages of reduced time of analysis and the possibility to obtain in a single analysis data on methylation, acetylation, and feruloylation, which generally require separate chromatographic analyses. Another important advantage is that NMR allows absolute quantitative data to be obtained, avoiding the need of reference compounds and calibration curves.

Analysis of Real Samples. The validated method was applied to samples of soluble pectin extracted by chelating agent solutions from different food waste sources: sugar beet flakes, apple cake, pea hulls, olive pomace, and grape pomace. Figures 3 and 4 show the ^{1}H NMR spectra for sugar beet flakes and olive pomace. ^{1}H NMR spectra show the optimal resolution of methanol, acetic acid, and ferulic acid, which can be easily detected and quantified in samples of various origins. It is worth noting that besides signals of methanol and acetic acid, it is possible to detect in the spectra some other minor signals (evidenced in the spectra enlargements) that correspond to other chemical molecules linked to the pectin backbone. In the case of pectin originating from sugar beet, the main minor signals are in the aromatic zone and can be attributed to ferulic acid analogues.

The degree of methylation, acetylation, and feruloylation determined in the samples of industrial byproducts having variable levels of esterification degrees are reported in Table 3. Feruloylation degree is expressed with respect to galacturonic acid to make the data compatible with methylation and acetylation, even if ferulic acid in pectin can be linked also to arabinose side chains.

Results showed that, as expected, methanol is the main compound linked via ester bond to the pectin backbone. Sugar beet flakes and pea hulls contain also a significant amount of acetic esters, whereas ferulic acid esters were detected only in sugar beet.

Table 2. Methylation and Acetylation Degree (Mole Percent of Galacturonic Acid) of Standard Citrus Peel Pectin Determined by Titration or ^{1}H NMR

	standard[a]		low methylation degree[b]		medium methylation degree[b]		high methylation degree[b]	
	titration	^{1}H NMR	titration	^{1}H NMR	titration	^{1}H NMR	titration	^{1}H NMR
galacturonic acid	75	77 ± 1[c]	80.8	85 ± 2[c]	86.2	81.8 ± 0.3[c]	84.4	78 ± 3[c]
methylation degree	63−66	61.1 ± 0.4	36.1	29.1 ± 0.2	53.5	44 ± 1	69.5	62.4 ± 0.4
acetylation degree	nd	1.0 ± 0.1	nd	0.10 ± 0.01	nd	0.40 ± 0.01	nd	2.4 ± 0.1

[a]Citrus peel pectin purchased from Sigma-Aldrich. [b]Citrus peel pectin provided by Silvaextract. [c]Galacturonic acid content (%) determined by colorimetric method.

dx.doi.org/10.1021/jf502679s | J. Agric. Food Chem. 2014, 62, 9081−9087

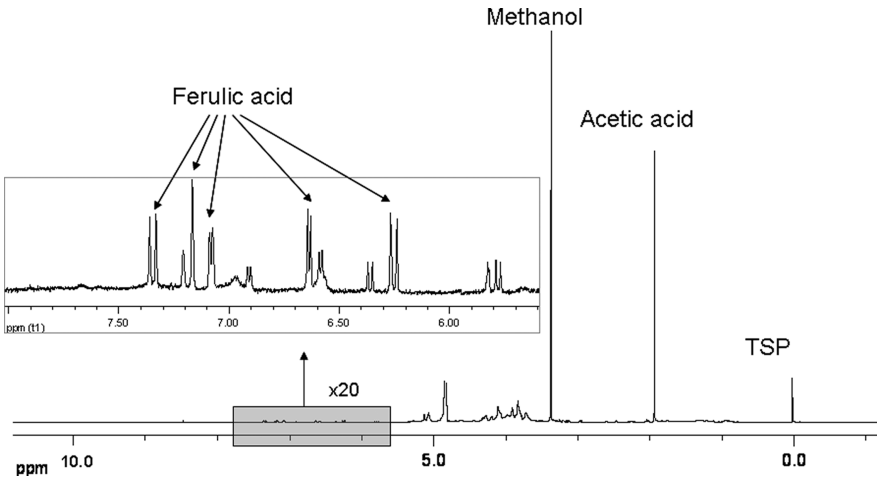

Figure 3. [1]H NMR spectrum of pectin sample (source: sugar beet flakes). Experimental conditions are reported under Materials and Methods.

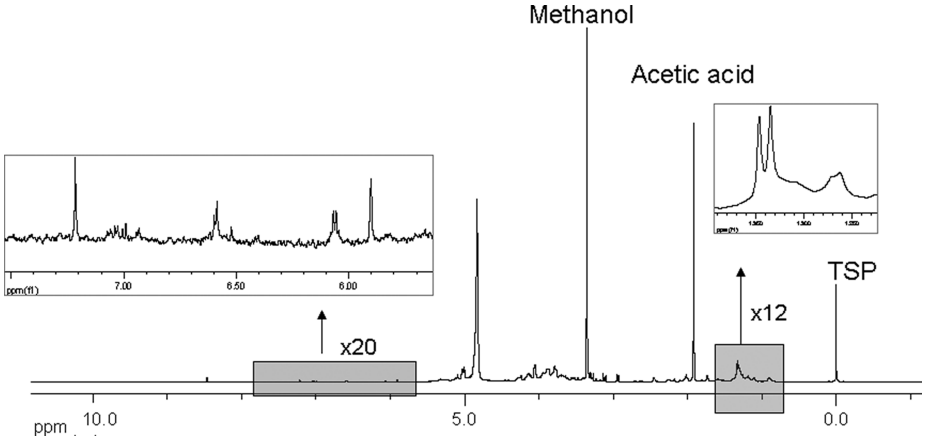

Figure 4. [1]H NMR spectrum of pectin sample (source: olive pomace). Experimental conditions are reported under Materials and Methods.

Table 3. Galacturonic Acid Content (Percent) and Methylation, Acetylation, and Feruloylation Degrees (Mole Percent of Galacturonic Acid) of Some Industrial Byproducts Determined by NMR[a]

	galacturonic acid content	methylation degree	acetylation degree	feruloylation degree
sugar beet flakes	67.1 ± 0.9	29.1 ± 0.8	18.0 ± 0.5	0.268 ± 0.001
pea hulls	44.6 ± 0.8	29.83 ± 0.03	10.5 ± 0.9	nd
apple cake	40.8 ± 0.5	40 ± 1	5.6 ± 0.1	nd
olive pomace	72 ± 1	10.5 ± 0.5	7.5 ± 0.1	nd
grape pomace	89 ± 2	5.0 ± 0.2	1.0 ± 0.1	nd

[a]Galacturonic acid content was determined spectrophotometrically.

The values determined for pea hull sample using our method are in line with the values obtained by similarly extracted pectic polysaccharides of this byproduct reported previously[42] (methylation degree of 24%).

For sugar beet samples, lower values for methylation degree were observed with respect to the data published by Renard and Thibault[43] (29.1% for our sample vs 52%). This can be attributed to the different sample origins: the current sample was dried sugar beet flakes, whereas the sample analyzed by Renard and Thibault[43] was fresh sugar beet pulp. This indicates that the process of drying sugar beet pulp to obtain flakes can actually lower the degree of methylation. The same behavior is also observed by comparing the feruloylation degree obtained (0.2%) that is lower with respect to previously published data.[28]

The method is currently being applied to a wide number of different food and byproduct samples, showing that it is suitable for all food matrices.

The overall data demonstrate that the quantitative [1]H NMR methodology proposed can be considered a high-throughput analysis that combines the determination of methylation, acetylation, and also feruloylation degree of pectin using a small sample amount and short preparation time. Furthermore, the method can be applied to pectic polysaccharides originating from a wide range of sources and having very different physical properties. It can detect small amounts of methanol, acetic acid, and ferulic acid.

The method is expected to be more accurate with respect to titration in determining the methylation degree without confusing it with other esterified forms present in pectin and also offers the advantage to obtain structural information on other compounds possibly linked via ester bond to pectin.

AUTHOR INFORMATION

Corresponding Author

*(A.C.) Mail: Department of Food Science, University of Parma, Parco Area delle Scienze 17A, 43124 Parma, Italy. Phone: +39 0521 905407. Fax: +39 0521 905472. E-mail: augusta.caligiani@unipr.it.

Funding

The research leading to these results has received funding from the European Union, Seventh Framework Program (FP7/2007-2013), under Grant Agreement 312140 (Functional and Safe Feed from Food Waste − NOSHAN).

Notes

The authors declare no competing financial interest.

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