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Short communication

High-performance liquid chromatography of methanol released from pectins after its oxidation to formaldehyde and condensation with 2,4-dinitrophenylhydrazine

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Abstract

A procedure was developed to measure the content of methanol in pectins after the base-catalysed hydrolysis of galacturonic acid methyl esters and oxidation of released methanol with potassium permanganate followed by condensation of the resulting formaldehyde (HCHO) with 2,4-dinitrophenylhydrazine (DNPH) dissolved in acetonitrile. The constant yields of resultant formaldehyde 2,4-dinitrophenylhydrazone (HCHO-DNPH derivative) were obtained at molar ratios of DNPH/HCHO higher than 5. The separation of the HCHO-DNPH derivative from DNPH reagent was achieved by isocratic reversed-phase HPLC equipped with the spectrophotometric detector set at a wavelength of 351 nm. The calibration curve was linear in the methanol concentration range between 0.04 and 15 μmol/ml (R=0.9995). The total recovery from pectin solutions spiked with methanol was equal to 100.6±5.1%.

Keywords: Derivatization, LC; Pectins; Methanol; Formaldehyde; Dinitrophenylhydrazine

1. Introduction

The functional properties of pectic substances, especially their gelling strength, depend among other factors on the degree of methyl esterification (DM) [1]. The DM is defined as a molar ratio of methanol to galacturonic acid contained in pectins. In native pectins about 70% of carboxyl groups of galactopyranosyl uronic acid residues are methyl-esterified. Alkaline or enzymatic deesterification of pectins leads to the liberation of methanol. The determination of the content of methyl ester groups in pectins is of considerable significance. In analytical respects it allows for better understanding of the structure and properties of this polysaccharide. It also enables foresighting the amount of methanol that potentially may be released from pectins in the technological process.

Most commonly used methods of the DM determination are based on an alkaline titration of carboxyl groups liberated during pectin demethoxylation [2,3]. Prior to the analysis, pectin preparations have to be converted into the free acidic form. Moreover, for pectins containing acetyl groups the appropriate correction of titration results is required. The content of methyl ester groups can be also estimated by the copper binding procedure based on the amount of copper ions (Cu^{2+}) reacting with

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carboxyl groups of pectins before and after saponification [4].

The amount of methanol released from pectins as a result of deesterification can be determined colorimetrically after its oxidation to formaldehyde followed by the derivatization with pentane-2,4-dione as a chromophore forming reagent [5]. The accuracy of selected titrimetric and colorimetric methods of the DM determination in pectins has already been discussed [6].

Both gas–liquid chromatography [7] and high-performance liquid chromatography (HPLC) [8] were applied to estimate methanol in relation to pectin methyl ester hydrolysis. In the latter case the separation of methanol and acetic acid liberated after saponification of pectins was achieved on an ion-exchange resin column. The refractive index detector used for the quantitative determination of methanol was not very sensitive therefore relatively high concentration of pectin was needed for analysis.

Another procedure for the DM determination is based on the reduction of free carboxyl groups of galacturonic acid residues to aldehyde groups with sodium borohydride. The concentration of methyl ester groups in this case can be calculated either from the increase in galactose content assayed by gas–liquid chromatography or from the change in galacturonic acid content measured colorimetrically [9,10].

In recent years several high-performance liquid chromatographic methods for the determination of low-molecular-mass aldehydes and ketones after their condensation with 2,4,-dinitrophenylhydrazine (DNPH) have been developed [11–14]. Considerable attention has been paid to measurement of formaldehyde concentration after this compound was discovered to be an important intermediate in the oxidation process of hydrocarbons present in the atmosphere [15,16]. In the polluted atmosphere the derivatization of formaldehyde to HCHO-DNPH derivative allows the determination of this compound in the ppb range [15].

The procedure described in this paper involves the oxidation of methanol released from pectin with permanganate and condensation of resulting formaldehyde with DNPH. Since both reactions require strong acidic conditions it was possible to couple the oxidation step directly to the derivatization of HCHO to HCHO-DNPH. To prevent the precipitation of derivatives the DNPH reagent was prepared in acetonitrile. The aqueous–acetonitrile reacting mixture may be directly injected onto the column [17]. The HCHO-DNPH derivative was separated from DNPH reagent by reversed-phase HPLC and quantitatively measured with a spectrophotometric detector.

2. Experimental

2.1. Materials and reagents

Acetonitrile, HPLC reagent (J.T. Baker, Deventer, The Netherlands); methanol, analytical-reagent grade (POCH, Gliwice, Poland); 2,4-dinitrophenylhydrazine, analytical-reagent grade (Aldrich, Steinheim, Germany); potassium permanganate, analytical-reagent grade (POCH); sodium arsenite, analytical-reagent grade (International Enzymes, Berkshire, UK); sodium hydroxide, pure (POCH); sulfuric acid, analytical-reagent grade (PCh Cheman, Warsaw, Poland) were used as received. Water from a Millipore ion-exchange unit (Milli-Q) was used.

Two industrial pectins, namely Pectin classic CU 401 USP from citrus fruit (galacturonic acid content – 85%; methoxyl content – 8.3%) and pectin classic AU 202 from apple (galacturonic acid content – 78%; methoxyl content – 9.4%) were kindly supplied by Herbstreith & Fox, Neuenbürg, Germany. Three commercial pectins, i.e., apple pectin (galacturonic acid content – 76%; methoxyl content – 7.3%) from Sigma (St. Louis, MO, USA), pectin ex citrus fruit rind, pure (Koch-Light Labs., Colnbrook, UK) and low-methoxyl Rapid Pectin (Pacific Pectins, Oukhurst, CA, USA) were also investigated. The water content in pectins was determined gravimetrically after drying 2 g of substance at 105°C for 2 h.

2.2. Sample preparation

Pectin solutions (0.1%, w/v) were prepared by gentle overnight stirring of 0.25 g of dry substance in 250 ml of deionized water. For each of investigated pectin preparations galacturonic acid content was estimated by the *m*-phenylphenol method according

to Blumenkrantz and Asboe-Hanssen [18]. Deesterification of pectin samples was performed at the room temperature in alkaline conditions by adding 0.25 ml of 1.5 *M* NaOH solution to 0.5 ml of 0.1% pectin solution. The reaction time was set to 30 min. The traces of free methanol in pectins were estimated without their demethoxylation and in this case 0.25 ml of water instead of NaOH solution was added to each sample.

The oxidation of methanol released from pectins was carried out according to the procedure described by Wood and Siddiqui [5]. The pectin samples after alkaline saponification were precooled in an ice-water bath. Then 0.25 ml of 2.75 *M* solution of sulfuric acid was added to each sample and thoroughly mixed. Next 0.2 ml of 2% aqueous solution of $KMnO_4$ was added and the samples were left for 15 min. Finally 0.2 ml of 0.5 *M* solution of sodium arsenite in 0.06 *M* H_2SO_4 was added with the aim of destroying an excessive amount of permanganate. The thoroughly mixed solution was left for 1 h at the room temperature. The derivatization of resulting formaldehyde was carried out by adding to each sample 1.4 ml of DNPH solution in acetonitrile. The DNPH solution was prepared by dissolving 0.15 g of 2,4-dinitrophenylhydrazine in 100 ml of acetonitrile and stored in brown glass flask. The reacting mixture was kept at the room temperature for 30 min. Then a 20-μl volume of the solution was injected directly onto the chromatographic column.

2.3. Calibration

The calibration curve was plotted taking peak areas of the HCHO-DNPH derivative obtained for methanol standard solutions at different concentrations after its oxidation to formaldehyde. A stock standard solution containing 10^{-2} mol/l of methanol was prepared in deionized water. The standard solutions were obtained by proper dilution of the stock sample. During investigations of standard methanol solutions the saponification step was omitted and instead of sodium hydroxide solution the equivalent volume of water (0.25 ml) was added. The solutions containing methanol in the concentration range from 0.04 to 15 μmol/ml were analyzed. After oxidation of methanol with permanganate the condensation of resulting formaldehyde with DNPH was carried out. The concentration of DNPH added was at least five-times higher than the concentration of methanol in each sample.

2.4. Determination of recoveries

For the determination of recoveries a 0.5-ml aliquot of 0.1% aqueous pectin solution was added to 0.25 ml of standard solutions containing methanol in the concentration range from 0.1 to 5 μmol/ml. The oxidized samples after condensation with DNPH were analyzed by HPLC.

2.5. High-performance liquid chromatography

The Knauer liquid chromatograph consisting of a Model 64 pump, a 250 mm×4.6 mm I.D. chromatographic column, Spherisorb S ODS1, packed with 5 μm particles, a variable-wavelength spectrophotometric detector built into the Knauer Compact system and a 20 μl injection valve was used. Peak areas were determined by means of a Hewlett-Packard Model 3395 integrator. Chromatographic separation was carried out isocratically at room temperature with acetonitrile–water (45:55, v/v) as eluent at a flow-rate of 1 ml/min. The mobile phase, prepared by mixing measured volumes of the solvents, was ultrasonically degassed before use. The spectrophotometric detector was set at 351 nm, i.e., at the maximum of absorbance of the HCHO-DNPH derivative having a molar extinction coefficient of 18 200 [11,19].

3. Results and discussion

3.1. HPLC analysis

The determination of methanol released in pectins submitted to alkaline deesterification was carried out after its oxidation to formaldehyde. For this purpose the procedure developed by Wood and Siddiqui [5] was applied. The resulting formaldehyde was condensed with DNPH giving the HCHO-DNPH derivative. Satisfactory separation of the HCHO-DNPH derivative from the DNPH substrate was achieved using a 250 mm long chromatographic column packed with ODS1 Spherisorb S, 5 μm material.

With the mobile phase of acetonitrile–water (45:55, v/v) and a flow-rate of 1 ml/min the retention times were about 5.1 and 9.0 min for the DNPH and HCHO-DNPH derivatives, respectively. Fig. 1 shows the chromatograms obtained for methanol standard solution (A) and for apple pectin after its alkaline deesterification, oxidation and derivatization (B).

3.2. Calibration curve

The calibration curve was generated for standard solutions containing up to 15 μmol/ml of methanol (the highest concentration examined). Peak areas evaluated by the integrator from the appropriate chromatograms were plotted as the function of methanol concentration. Linear fitting calculations for $n=30$ measurements gave the formula: $y=0.17\cdot 10^6+14.59\cdot 10^6 x$, where y is peak area (arbitrary units) and x denotes the methanol concentration in μmol/ml. The following regression data were obtained: standard deviation of the slope $0.09\cdot 10^6$, standard deviation of the intercept $0.07\cdot 10^6$, standard

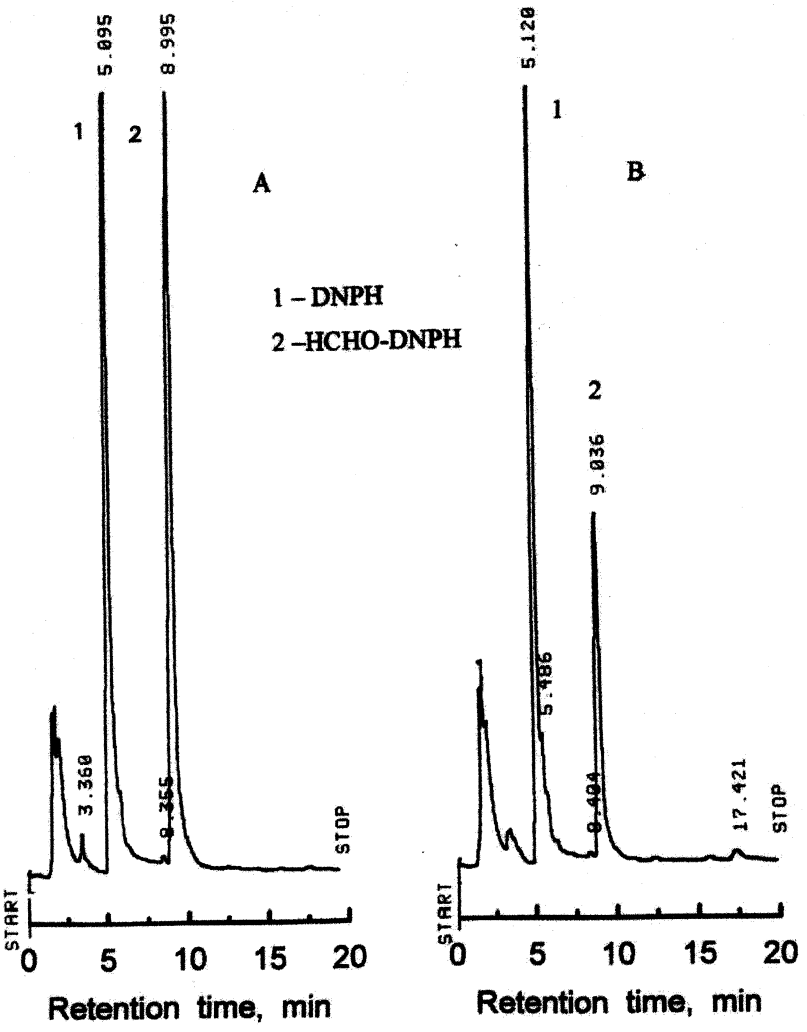

Fig. 1. HPLC separation of the HCHO-DNPH derivative and DNPH reagent on a Spherisorb S column. Chromatographic conditions: column, ODS1 (5 μm), 250 mm×4 mm I.D; mobile phase, acetonitrile–water (45:55, v/v); flow-rate 1.0 ml/min; detection wavelength, 351 nm; temperature, ambient; injection volume, 20 μl. (A) Obtained for methanol standard solution (0.2 μmol/ml) after its oxidation with permanganate and derivatization to HCHO-DNPH by adding of DNPH in acetonitrile (1.5 μmol/ml); (B) obtained for pectin AU 202 in 0.1% aqueous solution after alkaline deesterification, oxidation and derivatization with DNPH in acetonitrile.

error $0.28 \cdot 10^6$, correlation coefficient $R=0.9995$. The lowest concentration measured was equal to 0.04 μmol/ml and corresponds to the smallest clearly detectable peak area of methanol which is significantly different (3 SD) from the peak area of the blank standard solution prepared with use of water instead of methanol.

3.3. Optimization of the derivatization procedure

The frequently used procedures of formaldehyde derivatization with DNPH are based on using the DNPH reagent dissolved in aqueous solutions containing hydrochloric or sulfuric acid [11,14,15]. Recently the derivation technique has been improved by dissolving DNPH in acetonitrile. Thus the long and time consuming steps of filtration and washing the precipitate of the HCHO-DNPH derivative formed in aqueous conditions is eliminated. The addition of DNPH solution in acetonitrile to the aqueous sample solution offers the advantage of obtaining derivatives that may be injected directly onto the chromatographic column [13,17].

The reaction between formaldehyde and DNPH carried out in acidic aqueous–acetonitrile solution at room temperature was optimized with respect to the DNPH-to-HCHO molar ratio. The yield of HCHO-DNPH derivative was determined at various times after the reaction was initiated. Two different initial concentrations of methanol, namely, 0.15 and 0.30 μmol/ml and at the constant concentration of DNPH equal to 1.5 μmol/ml were prepared. After certain time intervals the volume of 20 μl of each sample was injected onto the column. The results obtained confirm that the derivatization of formaldehyde proceeds nearly quantitatively within 15 min. Nevertheless, most analyses were carried out after the reaction time of 30 min. The HCHO-DNPH derivative obtained is stable at room temperature for at least 48 h.

The efficiency of the reaction between HCHO and DNPH depends also on the molar ratio of both reagents. Therefore this dependence has been studied more precisely. For this purpose a number of samples containing constant amount of methanol (0.4 μmol/ml) were oxidized with permanganate. The derivatization of HCHO was performed by adding 1.4 ml of DNPH solution in acetonitrile containing different concentrations (1–30 μmol/ml) of this reagent. After the reaction time of 30 min a 20-μl volume of the reaction mixture was injected onto the column and the corresponding peak areas were recorded. The results showed that the constant reaction yields were obtained at molar ratios of DNPH/HCHO higher than 5. Below this value the efficiency of formaldehyde derivatization decreased. A similar dependence was observed by Tuss et al. [15], although under different reaction conditions. The constant reaction yield of 98% was obtained by these authors at molar ratios of DNPH/HCHO higher than 40. The explanation of decreased efficiency of derivatization at low molar ratios of reagents given by Tuss et al. is based on the possibility of partial dissociation of HCHO-DNPH derivative in acidic aqueous solutions. The authors confirmed this conclusion by the observation of decreasing HCHO-DNPH concentration and increasing DNPH content after dissolving pure derivative in 1 *M* HCl solution.

In the proposed procedure, derivatization of formaldehyde is carried out in water–acetonitrile solution (50:50, v/v), where the dissociation of HCHO-DNPH should be limited and a higher derivatization efficiency is reached at lower molar ratio of reagents.

3.4. Recovery and reproducibility

Recovery was evaluated for 0.1% pectin solutions spiked with different levels of methanol (0.1–5 μmol/ml). Prior to derivatization, each sample was oxidized using the described procedure. The results obtained show that the recoveries ranged from 95.5 to 105.0% (mean value for three determinations of each sample was 100.6 ± 5.1).

3.5. Application

The described procedure was applied to the determination of methanol released after alkaline deesterification of five different pectin preparations. Typical chromatogram obtained for apple pectin AU 202 is presented in Fig. 1B, while quantitative results for all pectins studied are reported in Table 1. There are detectable traces of methanol measured in pectins analyzed without their alkaline demethoxylation. The presence of trace amounts of free methanol is

Table 1
Assay results of different pectin preparations

Pectin sample	Water content (%)	Galacturonic acid, g/100 g dry substance	Methanol concentrations, g/100 g dry substance		Methoxyl content, % of dry substance
			1[a]	2[b]	
Pectin classic CU 401	6.0	85[c]	0.28	7.80±0.10	8.3[c]
Pectin classic AU 202	7.1	78[c]	0.44	8.86±0.08	9.4[c]
Apple pectin	9.9	76[c]	0.39	7.71±0.09	7.3[c]
Citrus pectin	11.8	83	0.36	6.94±0.10	Data not available
Rapid pectin	14.0	88	0.08	2.06±0.07	Data not available

[a] 1 – Methanol content determined in 0.1% pectin solution without alkaline deesterification.
[b] 2 – Methanol content determined in 0.1% pectin solution after alkaline deesterification in 0.5 *M* NaOH (mean value±standard deviation for five determinations).
[c] Content declared by suppliers.

probably due to the slow course of pectins deesterification in acidic conditions required for methanol oxidation with permanganate. As can be seen from Table 1 the content of methanol released after alkaline deesterification of pectins can be measured quite precisely. The differences between measured values of methanol content and the values of methoxyl content declared by the suppliers of pectins are lower than 10% and may be explained by the use of different analytical procedures.

4. Conclusions

Taking advantage of, firstly, the specific reaction conditions of methanol oxidation and condensation of formaldehyde with DNPH and, secondly, the suitability of resulting derivatives for analysis by HPLC, the procedure for determination of methanol released from pectins was developed. It was shown that the derivatization of formaldehyde resulting from methanol oxidation in pectin solutions to HCHO-DNPH derivative is almost quantitative after the reaction time of 30 min at the room temperature. Applying DNPH in acetonitrile solutions allowed for providing the derivatization reaction with the DNPH/HCHO molar ratio as low as 5 and for direct injection of reacting mixture onto the chromatographic column. Moreover, the separation of the HCHO-DNPH derivative from the DNPH reagent was achieved with the eluent composed of acetonitrile and water at the proportion quite similar to the ratio of both solvents in the reacting mixture. The proposed procedure may also be applied for methanol measurements after deesterification of pectins with pectin methylesterases, as well as for determination of activity of these enzymes.

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