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Method for Rapid Extraction of Pectic Substances from Plant Materials

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ABSTRACT

A method is described for the rapid extraction of pectic substances from alcohol insoluble solids (AIS) from material of plant origin, especially fruit. Samples of AIS can be prepared for galacturonic acid assay within 60 min using extraction with 0·5M HCl in a Fibertec-1 system (Tecator) for 30 min. The extraction conditions are carefully standardised and operator error is reduced by the elimination of transfer steps, particularly during filtration. The results obtained for plant-derived alcohol insoluble solids containing from 10% to 33% pectic substances were in close agreement with those obtained by enzymic hydrolysis using a commercially available enzyme preparation (Ultrazyme). The method will have application in the rapid, routine estimation of pectic substances in plant materials.

INTRODUCTION

Pectic substances are found as an integral part of the primary cell wall and middle lamella of higher plants and hence have significance in a number of areas of research including plant physiology, especially with respect to growth, food science in connection with textural changes occurring during both maturation and processing of fruits and vegetables and nutrition with regard to dietary fibre analysis. The more commonly used term 'pectin'

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designates those pectic substances soluble in water and capable of forming gels under suitable conditions (Pilnik & Voragen, 1970). Pectic substances are complex polymers composed of a backbone of partially methylated 1–4 linked α-D-galactopyranosyluronic acid residues with some 1–2 linked α-L-rhamnopyranose residues. Some of the hydroxyl groups on C_2 and C_3 of the galactopyranosyluronic acid residues may be acetylated and neutral sugars are present as covalently linked side chains (Pilnik & Voragen, 1970).

The problems associated with the assay of such heterogeneous polymers are attested to by the wide range of methods reported in the literature. Two principal areas of uncertainty exist; first, the extraction of pectic substances from plant materials and, secondly, the reliability of subsequent assays.

Extraction can be effected by chemical or enzymic methods, either separately or combined. Ammonium oxalate/oxalic acid extraction followed by hydrolysis of the extracted pectic substances using polygalacturonase (Dekker & Richards, 1972) has found wide application but is time consuming and extraction may be incomplete. Sulphuric acid has also been used to solubilise pectic substances (Ahmed & Labovitch, 1977; Selvendran *et al.*, 1979) but requires careful control of the conditions used to prevent clumping and/or charring of the sample. Voragen *et al.* (1983) compared the sulphuric acid method of Ahmed & Labovitch (1977), with both an enzymic hydrolysis technique using two commercial enzymes ('Ultrazyme' and 'Maxazyme') and an alkali procedure employing sodium hydroxide and ethylenediamine tetraacetic acid. For a range of plant materials the latter method consistently gave lower values for pectin content which were attributed to incomplete extraction in the alkaline medium. Enzyme hydrolysis of plant material with a mixture of pectic (i.e. polygalacturonase and pectinesterase) and non-pectic (i.e. cellulase and hemicellulase) enzymes is preferable to pectic enzymes alone as this ensures a more complete disruption of cell wall material and hence extraction of pectic substances.

Following extraction, pectic substances are commonly assayed as galacturonic acid by the colorimetric method of Dische (1947) using carbazole. However, this method is known to be susceptible to interference from the relatively low levels of neutral sugars often found in extracts of pectic substances. Recently, a more specific colorimetric assay using 3,5-dimethylphenol reported by Scott (1979) has found increasing application and is now generally regarded as the preferred analytical procedure.

Alternative assay methods utilise directly the carboxyl group of the galacturonate monomer eliminating the need for extraction of the pectic substances from the alcohol-insoluble solids prior to its assay. The copper binding method of Keijbets & Pilnik (1974), which measures Cu^{2+} binding to the carboxyl group, enables both pectin content and its degree of

esterification to be determined. This method is, however, time consuming and liable to interference, especially from starch.

Direct titration of the carboxyl groups was used by Warren & Woodman (1973) for analysis of pectic substances in potatoes, but the lack of specificity renders this assay liable to interference from non-pectic substances.

A comparison by Katan & van de Bovenkamp (1981) of the copper-binding assay and an enzyme hydrolysis extraction technique followed by the carbazole assay gave inconclusive results. These authors recommend the use of at least two different methods to determine pectic substances in foodstuffs.

A decarboxylation method using hydriodic acid and absorption of the released carbon dioxide into barium hydroxide solution has been described (Bylund & Donetzhuber, 1968). The reaction is continually monitored by recording the change in conductivity from which the uronic acid content can be calculated. Although assays can be carried out within 45 min, careful calibration is required.

The requirement for an effective but relatively rapid method of extracting pectic substances is clear. Industrially, extraction is effected by dilute mineral acids (i.e. nitric or hydrochloric), eliminating problems of degradation and decarboxylation associated with stronger acid concentrations. In the present study a method was devised for the extraction of total pectic substances from plant material with dilute HCl and carefully standardised heating conditions using a Fibertec-1 system (Tecator).

MATERIALS AND METHODS

Plant materials

Samples of apple peel (var. Bramley) were obtained on two separate occasions from a local commercial source (peels A and B). These were frozen and kept at −20°C prior to extraction.

Orange peel albedo was prepared from oranges purchased locally. The flavedo was removed with a carborundum peeler and the albedo separated by hand. Samples were frozen and kept at −20°C prior to extraction.

Pears (var. Beure Hardy) were purchased from a local retail outlet. The fruit were cored and the peel and flesh used directly for the preparation of alcohol-insoluble solids.

Preparation of alcohol-insoluble solids (AIS)

Fruit material (50 g) was homogenised in sufficient warm (65–70°C) 98% ethanol to give a final concentration of 80% ethanol, refluxed for 60 min and

allowed to stand overnight (16 h). The sample was filtered using a Buchner funnel (Whatman No. 1) and washed with 80% ethanol. The residue was air-dried overnight at room temperature, vacuum-dried at 45°C for 24 h and ground to pass through a 1 mm sieve.

Enzyme digestion

Alcohol-insoluble solids (0·2 g) were suspended in distilled water (20 ml) or 0·05M succinate buffer, pH 5·0, and dispersed by ultrasonication for 5 min. Ultrazyme 100 G (1 ml of 1% solution: Novo Enzyme Products Ltd.) containing pectinesterase, polygalacturonase, pectin lyase, cellulase, cellobiase and hemicellulase was added, the suspension mixed and then incubated overnight (16 h) at 30°C. The resulting digest was made up to a volume of 50 ml with distilled water.

Pectin extraction using HCl

Alcohol-insoluble solids (0·2 g) were suspended in HCl (30 ml, 0·1–1·0M) and extracted under reflux for 2 h. The sample was filtered through Whatman No. 1 filter paper, washed with warm (60–70°C) HCl of the same molarity and the filtrate made up to a volume of 50 ml with distilled water.

Pectin extraction using the Fibertec-1

Extraction of pectic substances was carried out in a Fibertec-1 (Tecator Ltd, Thornbury, Bristol) as used by Asp *et al.* (1983) for the analysis of dietary fibre. Alcohol-insoluble solids (0·2 g) were weighed into a sintered glass filter (porosity 3) and fitted into the Fibertec-1. The samples were refluxed for 30 min in HCl (100 ml, 0·1–1·0M), filtered and washed with warm (60–70°C) HCl of the same molarity (3 × 30 ml). The filtrate was made up to volume (usually 500 ml) with distilled water.

Fibertec-1 system

The Fibertec-1 (Tecator Ltd, Thornbury, Bristol) is a semi-automated system for crude fibre (Weende) and detergent fibre (e.g. Van Soest) methods. There are two separate units, the cold extraction unit for defatting, etc., and the hot extraction unit for refluxing. Only the latter was used in this work and will be described. The hot extraction unit consists of electrically heated porous crucibles clamped below water-cooled reflux columns. Reagents (up to two) can be heated to 94°C by continuous circulation through heat exchangers. Control valves located below the crucibles are used to select for

the addition of each reagent, to remove the filtrate by evacuation or apply positive pressure to prevent 'matting' in the crucible. Once the filtrate has been removed the residual material can be hot water washed from an inlet above the reflux column. The temperature and time of extraction/reflux are controlled by the instrument panel on the side, enabling accurate standardisation of heating regimes.

Pectin assay

Pectic substances were determined in the filtrates obtained above as galacturonic acid by the modified method of Scott (1979) in which the tubes were heated at 98°C for 10 min in a dri-block heater (Griffin) instead of a water bath as this was found to improve reproducibility. In this method, the colorimetric reagent 3,5-dimethylphenol selectively reacts with 5-formyl-2-furancarboxylic acid formed from the uronic acid on heating in concentrated H_2SO_4. D-Galacturonic acid monohydrate (Sigma) was employed as a standard and results calculated as galacturonic acid monohydrate equivalents.

RESULTS AND DISCUSSION

Enzymic digestion

Concentrations of galacturonic acid found in the AIS from the two samples of apple peel were similar (Table 1). Although hydrolysis was complete after an incubation period of 4 h, samples were routinely incubated overnight

TABLE 1
Time Course for Enzyme Digestion of Apple Peel AIS

Time of digestion (h)	*Galacturonic acid concentration (% of AIS)*	
	Sample A	*Sample B*
1	16·2 ± 0·48[a]	16·2 ± 0·58
2	18·6 ± 0·11	19·4 ± 0·81
4	20·4 ± 0·40	21·7 ± 0·04
6	20·8 ± 0·38	20·0 ± 0·04
8	20·4 ± 0·63	19·6 ± 0·61
24	19·8 ± 0·17	20·3 ± 0·34

[b] Results: mean of three replicates ± SEM.

(16 h). Enzyme digestion carried out in the presence of 0·05M succinate buffer, pH 5·0 (optimum pH for polygalacturonase), gave similar levels of galacturonic acid (mean of three replicates ±SEM; 20·0 ± 0·25 and 21·2 ± 0·88 for samples A and B, respectively) to the unbuffered system. An unbuffered system therefore appears adequate for enzymic hydrolysis.

Production of unsaturated products by the lyase was examined over the incubation period by measuring change in OD at 235 nm. The level of unsaturated product was consistently calculated at 24–25% of the total galacturonic acid determined for each of the materials examined. Dilution of the sample solutions with standard galacturonic acid gave results as expected from calculation based on the same colour development with the 3,5-dimethylphenol reagent for both the saturated and unsaturated products. This suggests that lyase activity will have little effect on the total level of galacturonic acid determined.

Pectin extraction using HCl

Extraction with HCl at concentrations between 0·1M and 1·0M yielded similar concentrations of galacturonic acid for both samples of apple peel (Table 2). However, the results obtained were less reproducible as indicated by the larger SEM, and generally slightly lower than those obtained by enzyme digestion (Table 1). The variability is probably attributable to the manual transfers required during filtration whereas the lower values

TABLE 2
Galacturonic Acid Content of Apple Peel AIS Determined using Acid Extraction

HCl concentration (M)	*Galacturonic acid concentration* (% *of AIS*)	
	Sample A	*Sample B*
0·1	16·5 ± 0·23[a]	15·2 ± 1·94
0·2	18·7 ± 1·01	18·2 ± 1·46
0·3	18·2 ± 0·53	19·7 ± 0·98
0·4	17·0 ± 1·76	17·5 ± 2·32
0·5	18·3 ± 0·90	18·8 ± 0·04
0·6	17·5 ± 1·91	18·3 ± 0·68
0·7	19·4 ± 0·37	20·4 ± 0·85
0·8	20·1 ± 0·23	20·1 ± 1·12
0·9	18·7 ± 1·86	17·0 ± 1·16
1·0	19·2 ± 0·83	20·0 ± 0·00

[a] Results: mean of three replicates ± SEM.

TABLE 3
Effect of Reflux Time on Extraction of Pectic Substances from Apple Peel A AIS using the Fibertec-1

Reflux time[a] (*min*)	*Galacturonic acid concentration* (*% of AIS*)
15	15·6 ± 0·50[b]
20	17·9 ± 0·33
30	18·9 ± 0·15
40	18·8 ± 0·07
60	18·6 ± 0·00

[a] Using 0·5M HCl.
[b] Results: mean of three replicates ± SEM.

obtained probably reflect the reduced specificity of acid extraction compared to the enzymic digestion, i.e. incomplete extraction and/or degradation of the galacturonate monomer.

Pectin extraction using the Fibertec-1 system

The optimum reflux period for extraction of pectic material from apple peel with the Fibertec-1 and 0·5M HCl is 30 min (Table 3). Lengthening the reflux period did not result in any increase in the galacturonic acid extracted but did reduce variability as shown by the decreasing SEM obtained. Extraction with a range of HCl concentrations from 0·1 to 1·0M for 30 min gave similar concentrations of galacturonic acid for both samples of peel (Table 4), but with a greater degree of precision than was obtained with the manual technique (Table 2). Optimum extraction of pectic substances was found for the same HCl concentrations (0·2 to 1·0M) with both the manual and Fibertec extraction techniques.

The residues from pectin extraction of apple peel AIS with the Fibertec-1 and 0·5 and 1·0M HCl were re-extracted by enzyme digestion to assess efficiency of extraction (Table 5). The total pectic substances recovered with both HCl concentrations were comparable to the total determined by enzyme digestion alone (Table 1). Recovery of pectic substances by the Fibertec technique was between 93% and 96% of that determined by enzyme hydrolysis. The recovery of pectin (citrus Grade I sigma) with the Fibertec-1 was 100 ± 0·92% and 102 ± 0·33% (mean of three replicates ± SEM) on extraction with 0·5 and 1·0M HCl, respectively.

Two other fruit materials, orange albedo and pear, containing different quantities of pectic substances, were examined by HCl extraction, both manual and Fibertec-1 techniques, and enzyme hydrolysis (Table 6). For

TABLE 4
Effect of HCl Concentration on the Amount of Galacturonic Acid Extracted from Apple Peel AIS using the Fibertec-1

HCl concentration (M)	*Galacturonic acid concentration* (*% of AIS*)	
	Sample A	*Sample B*
0·1	16·6 ± 0·21[a]	17·6 ± 0·49
0·2	18·4 ± 0·69	19·9 ± 0·52
0·3	16·8 ± 0·34	18·1 ± 1·14
0·4	18·0 ± 0·77	17·8 ± 0·97
0·5	19·4 ± 0·40	18·3 ± 0·11
0·6	17·7 ± 0·78	17·3 ± 0·42
0·7	19·0 ± 0·36	18·5 ± 0·63
0·8	18·3 ± 0·61	19·4 ± 0·42
0·9	19·4 ± 0·37	19·9 ± 0·22
1·0	21·0 ± 0·34	22·1 ± 0·15

[a] Results: mean of three replicates ± SEM.

both these materials extraction with 0·5M HCl and the Fibertec-1 gave results comparable to those obtained using enzyme digestion.

From the above data it is concluded that the extraction of pectic substances from AIS with 0·5M HCl and a reflux time of 30 min in the Fibertec-1 system has several advantages over previously described

TABLE 5
Total Pectic Substances Determined in Apple Peel AIS

Extraction method	*Galacturonic acid concentration* (*% of AIS*)	
	Sample A	*Sample B*
Fibertec: 0·5M HCl[a]	19·3 ± 0·08[b]	21·3 ± 0·17
Residue: Enzyme[c]	1·33 ± 0·02	1·26 ± 0·02
Total	20·7 ± 0·09	22·6 ± 0·16
Fibertec: 1·0M HCl	19·8 ± 0·55	20·2 ± 0·17
Residue: Enzyme	0·93 ± 0·05	1·10 ± 0·02
Total	20·8 ± 0·60	21·3 ± 0·17

[a] Method as described in experimental section using molarity of HCl indicated.
[a] Results: mean of three replicates ± SEM.
[c] Enzyme digestion of residue from Fibertec hydrolysis as described in experimental section.

TABLE 6
Comparison of Methods for Extraction of Pectic Substances from Orange Albedo and Pear

Method of extracting pectin	*Galacturonic acid concentration (% of AIS)*	
	Orange albedo	*Pear*
Fibertec		
0·1M HCl	31·8 ± 0·00[a]	10·0 ± 0·00
0·5M HCl	32·3 ± 0·25	11·0 ± 0·38
1·0M HCl	34·9 ± 0·13	11·8 ± 0·27
Enzyme digestion	32·8 ± 0·70	10·6 ± 0·14
Acid extraction 0·5M HCl	33·0 ± 2·50	9·82 ± 0·15

[a] Results: mean of three replicates ± SEM.

methods. Operator errors due to transfer of material during filtration are eliminated, conditions of hydrolysis can be carefully controlled and standardised and the use of dilute HCl minimises degradation and decarboxylation of the extracted pectic substances. However, the main advantage of this method is its rapidity, since samples of AIS can be prepared for galacturonic acid assay within 1 h. This method should have particular application in the routine analysis of pectic substances present in plant materials and possibly foodstuffs.

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