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Assessment of methanolysis for the determination of sugars in pectins

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ABSTRACT

A procedure for the determination of galacturonic acid and the main neutral sugars in pectins involves enzymic hydrolysis followed by methanolysis and h.p.l.c. The usefulness of this method was demonstrated by comparison of the results obtained by (*1*) methanolysis in methanolic M HCl without enzymic prehydrolysis, (*2*) methanolysis in methanolic 72% H_2SO_4 with pretreatment for 3 h with aqueous 72% H_2SO_4, (*3*) colorimetric determination of galacturonic acid, and (*4*) g.l.c. of the alditol acetates of the neutral sugars released by acid hydrolysis under various conditions.

INTRODUCTION

Determination of the sugar composition of pectins generally involves acid hydrolysis, and the various stabilities of the monosaccharides liberated require different optimum conditions of hydrolysis[1,2]. Neutral sugars released are generally converted into the alditol acetates[3,4] and analysed by g.l.c. Because acid hydrolysis of glycuronosyl linkages is difficult[5] and the production of volatile derivatives of uronic acid is laborious, their content is often obtained by colorimetry[6,7], decarboxylation[8], or titrimetry[9].

Methanolysis is as efficient as hydrolysis for the cleavage of glycosidic linkages and causes less destruction[10], but the release of uronic acids from pectins[11], xanthan[11], or alginates[12] is incomplete. In order to improve the methanolysis of pectins, an enzymic prehydrolysis step has been proposed[13] with analysis of the resulting methyl glycosides by h.p.l.c.[14,15], which is less tedious than g.l.c. of the corresponding trimethylsilyl ethers[16–18].

We now present an assessment of methanolysis and of the results obtained for apple, citrus, and sugar-beet pectins in comparison with those obtained by colorimetric determination of galacturonic acid and g.l.c. of the alditol acetates of neutral sugars under various conditions.

EXPERIMENTAL

General. — The h.p.l.c. system involved a Waters 590 programmable pump, an Erma ERC-3310 degasser, a Touzart/Matignon pulsation dumper, a Valco C 6 W

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injection valve equipped with 10-μL loop, an Erma ERC-7510 differential refractometer, and a Shimadzu CR 4 A integrator.

Instant methanolic HCl kit (acetyl chloride and anhydrous methanol) was purchased from Alltech/Applied Science. The Reacti-Therm Heating/Stirring Module, Reacti-Vap Evaporator, and Reacti-Vials (10 mL) sealed with Teflon-lined septa were from Pierce and Warriner. In order to avoid leaks, top screw caps (73803-24400) from Kimble (U.S.A.) were used in place of open top screw caps from Pierce.

Sugar-beet pectin and high-methoxyl citrus pectin (degree of methylation, 72%) were obtained from Kobenhavns Pektinfabrik (Copenhagen, Denmark), and apple pectin (degree of methylation, 28%) from Unipectine (Redon, France). Sugar-beet and citrus pectins were purified by precipitation with ethanol (4 vol.) from their aqueous 2% solutions, whereas apple pectin was purified by precipitation with cupric ions[19]. All the samples were dried at 40° over P_2O_5, under vacuum for at least 24 h.

The enzyme used was a commercial liquid preparation (SP 249), obtained from Novo (Denmark), and purified by precipitation with $(NH_4)_2SO_4$ (90% saturation) in order to eliminate contaminants, especially low-molecular-weight carbohydrates. After centrifugation, the pellet was washed with $(NH_4)_2SO_4$ at 90% saturation (40 mL/mL of initial preparation) and the proteins were resolubilised in 50mM acetate buffer (pH 4.5; 1 mL/mL of initial preparation). This solution was dialysed at 4° against water to a conductivity < 10 μSiemens/cm, then for 4 h against two changes of 50mM acetate buffer, and diluted in ultrapure water to a protein concentration[20] of ~ 1 mg/mL.

Rohament P (Rohm) was dialysed at 4° for 48 h against several changes of water, freeze-dried, and then stored at 4° before use.

Proposed method. — Reactions were conducted in the Reacti-Therm Heating/Stirring Module. Dried pectin (10 mg) was incubated in the Reacti-Vial for 1 h at 45° with purified SP 249 (400 μL, 1 mg of protein/mL). The hydrolysate was concentrated to dryness with methanol (2 $\times$ 500 μL) at 45° in a stream of air using the Reacti-Vap Evaporator. Methanolic M HCl (2 mL) that contained dimethyl L-tartrate[15] (2 mg/mL) as internal standard was added, the Reacti-Vial was sealed with a Teflon-lined septum in the screw cap, and methanolysis was carried out for 16 h at 85° with stirring (as enzymic hydrolysis). After neutralisation with Ag_2CO_3 (120 mg) and centrifugation (5000*g* for 5 min), the supernatant solution was concentrated (40°, under vacuum), and the dry residue was dissolved in water (1 mL). The resulting methyl glycosides were analyzed by h.p.l.c. on a Merck Superspher end-capped C_{18} cartridge (25 $\times$ 0.4 cm) by elution with water at 0.9 mL/min and monitoring by differential refractometry.

Others methods. — Pectins were depolymerized by treatment with 72% H_2SO_4 in methanol after pretreatment with aqueous 72% H_2SO_4 for 3 h according to the procedure of Roberts *et al.*[21].

Enzymic degradation with Rohament P and subsequent methanolysis (methanolic 0.8M HCl, 60°, 4 h) were carried out as described by Ford[22].

Sugars liberated enzymically from the pectins were analyzed on a column of Aminex HPX 87 (H^+) resin (Bio Rad) by elution with 5 $\times$ 10^{-4}M H_2SO_4 (0.6 mL/min,

50°), whereas the contaminating sugars in the enzymic preparation were analyzed on a column of Aminex HPX 87 P by elution with water (0.6 mL/min) at 85°.

Galacturonic acid was determined colourmetrically by the *m*-hydroxybiphenyl method[7].

De-esterification was carried out in 0.05M NaOH at 4°. After various times, the solution was brought to pH 5, dialysed against distilled water, and freeze-dried.

Reduction and acetylation of the neutral sugars and the subsequent g.l.c. were carried out according to Blakeney *et al.*[4]. Pectins were hydrolysed variously with 2–6M CF_3COOH and HCl, and 1–3M H_2SO_4 for 1–6 h at 100–120°.

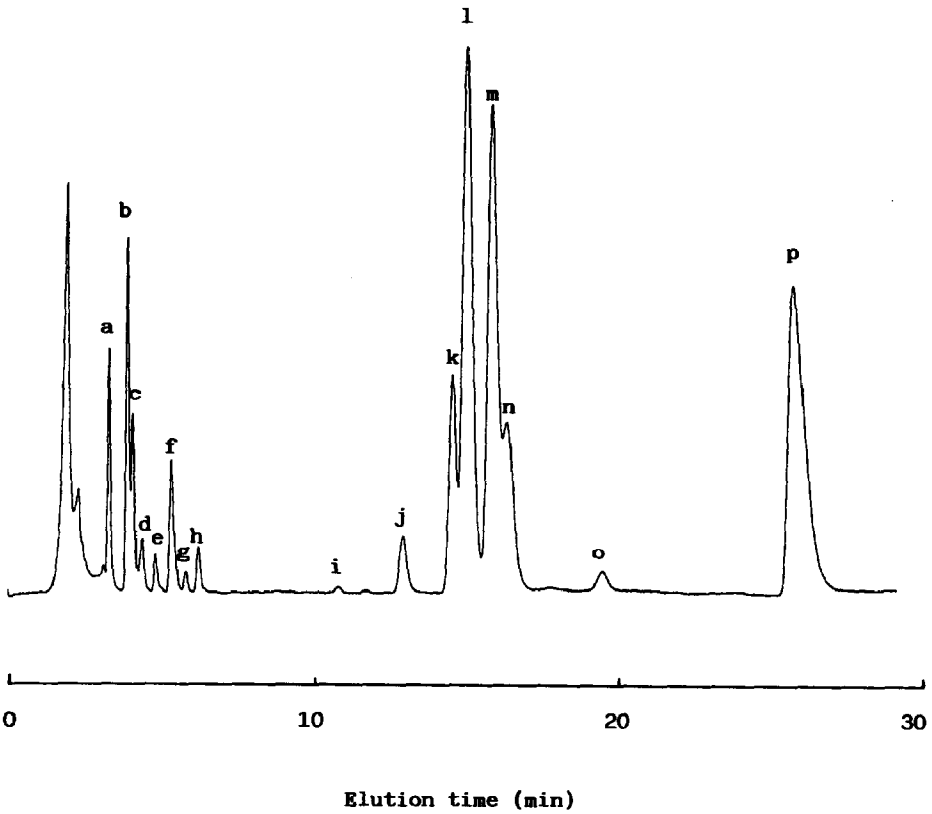

Fig. 1. H.p.l.c. of methanolysis products from apple pectin: a, methyl α- and β-D-galactopyranosides; b, methyl α-D-galactofuranoside and methyl α-D-glucopyranoside; c, methyl β-D-glucopyranoside; d, methyl β-D-galactofuranoside and methyl α-L-arabinofuranoside; e, methyl β-L-arabinofuranoside and methyl α-D-mannopyranoside; f, methyl α- and β-L-arabinopyranosides and methyl α-D-xylopyranoside; g, methyl β-D-mannopyranoside and methyl α- and β-D-mannofuranosides; h, methyl β-D-xylopyranoside; i and j, methyl L-rhamnosides; k and l, methyl (methyl α- and β-D-galactofuranosid)uronates; m and n, methyl (methyl α- and β-D-galactopyranosid)uronates; o, unknown; p, dimethyl L-tartrate (internal standard).

RESULTS AND DISCUSSION

Methanolysis. — The dimethyl L-tartrate, used as internal standard, was stable under the experimental conditions and the area of the corresponding peak was related linearly (correlation coefficient 0.994) to concentration up to 4 mg/mL.

Since methanolysis of sugars yields a mixture of glycosides[14], the response factors of standard sugars relative to that of the internal standard were calculated from the areas under the main and well-separated peaks (a, f, g, h, and j, for galactose, arabinose, mannose, xylose, and rhamnose, respectively; Fig. 1). The sums of the areas under peaks b + c and k + l + m + n were used for the quantification of glucose and galacturonic acid, respectively, in order to take into account an eventual decrease in the resolution of the column. In the determination of glucose, problems can arise due to the presence of methyl α-D-galactofuranoside and methyl α-D-glucopyranoside in peak b. As the proportions of the areas of the peaks corresponding to the derivatives from the standard sugar (galactose) are constant for concentrations of <5 mg/mL, it is possible to determine, from the area of the peak a, the area corresponding to methyl α-D-galactofuranoside under peak b and thence to determine the amount of glucose. A similar problem arises when xylose is not a minor component (as in apple pectin) for the determination of arabinose from the peak f. The relative response factors for standard sugars multiplied by the factor for the conversion monomer/polymer are indicated in Table I. The values obtained for each sugar were similar in the range 5–25 mg, indicating a good linearity of the responses and stability of the methyl glycosides. Although the relative response factors of such neutral sugars as galactose reached a plateau during methanolysis for 3 h, the reaction was carried out for 16 h in order to reach a constant relative response factor for D-galacturonic acid. Furthermore, the proportions of the different peaks corresponding to the methyl glycosides from each sugar were constant only when these values were reached.

The yields of the sugars released from the pectins treated for 16 h with methanolic M HCl at 85°[10] are shown in Table II. The amount of fucose was too low to be detectable by h.p.l.c. under the conditions used. The content of rhamnose was higher than that obtained previously by g.l.c. on similar beet pectin[23] or citrus pectin[24], whereas the amounts of the other neutral sugars were similar.

Methanolysis according to the conditions of Roberts *et al.*[21] (especially the use of Drierite as drying agent) led to difficulties in the determination by h.p.l.c. of galactose, glucose, arabinose, and xylose because of interfering salts. Only peaks corresponding to rhamnose and galacturonic acid could be measured unambiguously and results similar to those of the other methanolysis method were obtained. The amounts of galacturonic acid measured by these methods were significantly lower than those estimated colorimetrically (*cf.* Table II). It was concluded that methanolysis cannot completely depolymerise the polygalacturonic backbone of the pectins. Enzymic hydrolysis was therefore studied as a preliminary step.

TABLE I

Response factors of standard sugars relative to the internal standard (dimethyl L-tartrate) after methanolysis with methanolic M HCl for 24 h at 85° and h.p.l.c. analysis on C_{18}

Sugar	*Weight (mg)*	*Peak*	*Relative response factor*
Galactose	10.70	a	0.83
	15.07		0.89
	15.18		0.84
	15.78		0.89
	25.58		0.85
Glucose	10.43	b + c	0.62
	15.10		0.61
	20.97		0.65
	25.10		0.65
Arabinose	10.21	f	0.93
	15.20		0.96
	20.29		0.96
	25.37		0.95
Mannose	10.10	g	0.64
	15.61		0.68
	19.87		0.68
	24.65		0.69
Xylose	10.69	h	1.05
	14.86		1.05
	20.95		1.01
	26.50		1.00
Rhamnose	9.29	j	0.74
	12.35		0.74
	18.15		0.72
	22.81		0.72
Galacturonic acid	4.95	k + l + m + n	0.70
	9.44		0.70
	10.32		0.67
	13.99		0.67
	18.07		0.69
	18.33		0.69

Studies of enzymic prehydrolysis. — Rohament P has been used by Ford[22] in order to depolymerise pectins. Under the conditions described, a release of only 2–8% of galacturonic acid was obtained for our pectins. These poor yields were due to the fact[25] that the preparation was rich in polygalacturonase but poor in pectinesterase which is necessary for an appreciable degradation of pectins.

Another commercial preparation, SP 249, was preferred because it contains several other activities which are[26] mainly polygalacturonase (EC 3.2.1.15, specific

TABLE II

Yields (%) of sugars released by methanolysis (without enzymic prehydrolysis) and of galacturonic acid estimated colorimetrically in sugar beet, apple, and citrus pectins

Pectin	*Method*[a]	*GalA*	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
Sugar beet	A	38.5	5.1	<0.1	3.1	0.1	<0.1	7.2	trace
	B	42.6	4.0						
	C	57.8							
Citrus	A	47.4	1.4	<0.1	1.0	0.1	<0.1	2.2	trace
	B	47.2	1.5						
	C	79.2							
Apple	A	51.5	2.1	<0.1	1.0	1.2	<0.1	2.5	3.5
	C	76.8							

[a] A, Methanolysis for 16 h at 85° in methanolic M HCl and subsequent h.p.l.c.; B, methanolysis according to Roberts *et al.*[21] and subsequent h.p.l.c.; C, colorimetric method according to ref. 7.

activity: 270 nkat/mg of proteins), pectin esterase (EC 3.1.1.11, 87 nkat/mg), arabinanase (EC 3.2.1.99, 173 nkat/mg), α-L-arabinofuranosidase (EC 3.2.1.55, 14 nkat/mg), β-D-galactosidase (EC 3.2.1.23, 10 nkat/mg), and galactanase (EC 3.2.1.89), and because it is poor in pectin lyase (EC 4.2.2.10, 9.3 nkat/mg) and devoid of pectate lyase (EC 4.2.2.2, 0.2 nkat/mg). However, the SP 249 liquid preparation required purification, since chromatography of the supernatant solution, after precipitation of proteins by heating, revealed appreciable amounts of glucose (1.5 mg/mL), galactose (1.8 mg/mL), and mannose (2.8 mg/mL). The precipitation of proteins by ammonium sulfate removed the main low-molecular-weight sugars and only traces of contaminating sugars remained (< 0.04 mg/mL). However, methanolysis of the purified preparation revealed a contaminant (*cf.* peak g in Fig. 1) that may have been mannose derived from the enzyme (glycoprotein). Since this peak may hinder the integration of peaks f and h if too much enzyme is present in the mixture and when the column has lost some efficiency, the optimum concentration of enzyme required to depolymerise the pectins was determined.

Because the yields of neutral sugars and galacturonic acid released by SP 249 in 50mM acetate buffer (pH 4.5) and in ultrapure water (pH 5) were identical at 45°, the assays were performed in aqueous solutions. The yields of galacturonic acid and neutral sugars are shown in Table III. The presence of free rhamnose in the hydrolysates demonstrated the presence of an enzyme that cleaved linkages between rhamnose and galacturonic acid. However, the amounts of rhamnose did not increase after hydrolysis for 3 h and the values were lower than those obtained by methanolysis alone (*cf.* Table II). The amounts of arabinose in citrus and apple pectins were low, and therefore rhamnose and arabinose peaks were not well resolved and were quantified together. The release of galacturonic acid, whatever the pectin, reached a plateau after 1 h for concentrations of protein of ⩾0.65 mg/mL and was 90–95% of the values obtained colorimetrically.

TABLE III

Yields (%) of monosaccharides released by hydrolysis of pectins at 45° with different amounts of SP 249

Pectin	*Protein (mg/mL)*	*Time (h)*	*GalA*	*Gal*	*Rha*	*Ara*	*Glc*
Sugar beet	13.10	0.5	47.1	6.0	2.2	2.1	<0.1
		1	50.0	6.9	2.7	2.4	<0.1
		3	51.6	7.5	3.4	2.6	<0.1
		6	52.4	7.9	3.4	2.8	<0.1
	0.65	0.5	43.7	4.9	1.8	1.8	<0.1
		1	49.0	6.1	2.4	2.3	<0.1
		3	51.2	6.9	3.2	2.5	<0.1
		6	52.3	7.3	3.6	2.7	<0.1
Citrus	13.10	0.5	68.9	3.0	1.3	0.6	0.1
		1	69.9	3.1	2.0[a]		0.1
		3	71.4	3.2	2.2		0.1
		6	71.6	3.3	2.2		0.1
	0.65	0.5	66.7	1.8	0.5	0.3	<0.1
		1	67.9	1.9	0.5	0.3	<0.1
		3	69.1	2.0	0.6	0.6	<0.1
		6	67.6	1.9	0.7	0.3	<0.1
	0.26	0.5	47.2	1.7	0.3	0.2	<0.1
		1	68.3	1.9	0.4	0.3	<0.1
		3	69.7	2.0	0.4	0.4	<0.1
		6	73.1	2.1	0.5	0.5	<0.1
	0.13	0.5	27.8	1.5	0.3	0.1	<0.1
		1	45.8	1.7	0.3	0.2	<0.1
		3	71.4	2.0	0.4	0.3	<0.1
		6	73.1	2.0	0.4	0.4	<0.1
Apple	13.10	0.5	70.7	3.9	2.5[a]		3.1
		1	70.6	3.9	2.7		3.1
		3	71.2	4.2	3.2		3.2
		6	71.9	4.4	3.2		3.2
	0.65	0.5	67.5	2.2	1.1		2.4
		1	68.2	2.3	1.2		2.6
		3	69.1	2.5	1.4		2.8
		6	68.6	2.5	1.4		2.8
	0.26	0.5	50.7	2.0	0.8		2.1
		1	64.4	2.1	0.9		2.4
		3	67.1	2.2	1.4		2.8
		6	68.8	2.3	1.1		2.7
	0.13	0.5	30.5	1.6	0.6		2.0
		1	43.1	1.8	0.7		2.2
		3	60.1	2.0	0.8		2.6
		6	63.3	2.0	0.8		2.7

[a] Rhamnose + arabinose, see text.

Enzymic prehydrolysis and methanolysis assays. — The yields of sugars after enzymic prehydrolysis followed by methanolysis for 24 h or 16 h at 85° in methanolic 0.5M, M, 2M, and 4M HCl are shown in Table IV. Methanolic 0.5M HCl did not depolymerise the pectin completely, and 2 and 4M HCl destroyed some of the galacturonic acid and the neutral sugars. Maximum amounts of galacturonic acid and rhamnose were obtained by the use of methanolic M HCl. All these results were in agreement with those of Chambers and Clamp[10]. Under these conditions, the amounts of galacturonic acid released from beet, citrus, or apple pectins were higher than that obtained by methanolysis or enzymic hydrolysis alone (*cf.* Tables II and III, respectively) and were close to the content measured by colorimetry (Table II). There were no differences in the amounts released after 16 and 24 h with methanolic M HCl and so the method proposed used methanolic M HCl for 16 h. As the enzyme mixture contained a low, but significant, amount of pectin-lyase activity, which could lead to undectable unsaturated compounds, the highest esterified pectin (citrus pectin) was also analysed by the proposed method after de-esterifications at a temperature which minimised chain cleavages by β-elimination[27]. The yield of galacturonic acid was in the range 73.0–78.9% for degrees of methylation varying from 0% to 53%. These results showed that pectin-lyase activity had no significant effect in the determination of saturated galacturonic acid.

G.l.c. assays. — Previous results[13] showed that methanolysis yielded more rhamnose than was determined by g.l.c. under standard conditions for acid hydrolysis of pectins[2,4] (*e.g.*, 2 M CF_3COOH, 1–2 h, 120°), whereas the results for the other sugars were similar. In order to confirm the values obtained by methanolysis for the rhamnose content, the effects of the nature and molarity of the acid used (CF_3COOH, HCl, and H_2SO_4) and of the period of hydrolysis on the yields of sugars were studied. The results obtained on sugar-beet pectins are indicated in Table V. The yields of fucose, arabinose, xylose, mannose, galactose, and glucose released did not depend markedly on the origin of the pectins or on the conditions of hydrolysis; arabinose was degraded during hydrolysis by 2M H_2SO_4 at 100°. The maximum of release for all these sugars was obtained within 1 h of hydrolysis and the amounts of sugars determined in this way were close to those obtained after enzymic prehydrolysis and methanolysis (*cf.* Table IV).

TABLE IV

Yields (%) of the sugars released from pectins by enzymic prehydrolysis with purified SP 249 and subsequent methanolysis at 85° under different conditions

Pectin	*HCl* (M)	*Time* (*h*)	*GalA*	*Rha*	*Ara*	*Xyl*	*Gal*	*Glc*
Sugar beet	0.5	24	50.9	4.6	3.1	0.1	7.0	tr
	1	24	57.5	5.1	3.1	0.2	7.5	tr
	1	16	57.4	5.1	3.2	0.2	8.1	tr
	2	24	50.0	4.5	2.7	0.1	4.6	tr
	4	24	42.5	4.4	2.7	0.1	5.1	tr
Apple	1	24	75.9	2.2	1.0	1.4	2.6	3.6
Citrus	1	24	77.1	1.4	1.1	0.2	2.4	tr

TABLE V

Neutral sugar composition (%) of sugar beet pectin obtained by g.l.c. after acid hydrolysis under various conditions

Hydrolysis	*Time (h)*	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*
M H_2SO_4 at 100°	1	1.2	0.1	3.4	0.2	0.0	7.4	0.1
	2	1.9	0.1	3.3	0.2	0.0	7.8	0.1
	3	3.3	0.1	3.6	0.2	0.1	8.2	0.1
	4	4.2	0.1	3.4	0.2	0.0	8.1	0.1
	5	4.3	0.1	3.3	0.3	0.0	8.1	0.1
	6	5.2	0.1	3.4	0.3	0.0	7.9	0.1
2M H_2SO_4 at 100°	1	1.7	0.1	2.1	0.1	0.0	6.2	0.1
	2	3.9	0.2	2.6	0.2	0.0	7.0	0.1
	3	4.5	0.1	2.8	0.3	0.0	7.5	0.1
2M HCl at 100°	1	2.9	0.1	3.1	0.2	0.1	7.8	0.2
	2	5.0	0.2	3.8	0.2	0.0	9.3	0.1
	4	5.3	0.1	3.4	0.2	0.0	8.8	0.2
2M CF_3COOH at 120°	1	1.6	0.1	3.3	0.1	0.1	7.6	0.1
	2	4.8	0.1	3.1	0.2	0.0	7.8	0.1
	3	5.5	0.2	3.6	0.2	0.1	7.8	0.1
	4	5.0	0.2	3.4	0.3	0.1	8.0	0.1
	5	5.4	0.1	3.1	0.2	0.1	7.5	0.1
3M CF_3COOH at 120°	1	3.9	0.1	3.1	0.2	0.3	7.1	0.1
	2	4.2	0.1	3.5	0.3	0.0	8.5	0.2
	3	4.8	0.1	3.3	0.3	0.0	7.6	0.1
	4	5.5	0.2	3.7	0.3	0.0	8.1	0.1

In contrast, the rhamnose content varied with the experimental conditions used, in agreement with the results of Selvendran *et al.*[29,30]. Rhamnose was released slowly and in a sigmoidal manner that may be characteristic of its linkages with galacturonic acid residues. The maximum value was obtained with 2M CF_3COOH after at least 3 h of hydrolysis at 120°. This is a longer period than that generally used for the determination of the neutral sugars in pectins. The rhamnose contents were close to those obtained by enzymic prehydrolysis and methanolysis. The use of HCl and H_2SO_4 gave lower values for the same periods of hydrolysis; this was not due to degradation but to slow release of rhamnose because the contents increased with increase in the time of hydrolysis. Because the methyl esterification of the carboxyl groups of the galacturonic acid may strengthen the glycosidic linkages[31], de-esterification was carried out prior to acid hydrolysis. The rhamnose content, measured after de-esterification and the hydrolysis with CF_3COOH, was not changed. Therefore, hydrolysis for at least 3 h at 120° with 2M CF_3COOH is recommended for the determination of neutral sugars in pectins. Similar results were obtained with citrus and apple pectins (results not shown). The sum of the sugar contents in these pectins was not 100% because of the presence of such substituents as methyl and acetyl groups, protein, and inorganic material (ash). These components may represent up to 20% of the weight of pectins[32].

The above results show that methanolysis may be used for the analysis of neutral and acidic sugars in pectins only if a preliminary enzymic hydrolysis is carried out. The resulting methyl glycosides could be analysed by h.p.l.c., but fucose could not be quantified under our experimental conditions. In the determination of mannose, it is necessary to take account of the mannose liberated from the enzyme. The results obtained for different pectins are close to those obtained by colorimetry for the galacturonic acid and by g.l.c. of the alditol acetates of the neutral sugars after hydrolysis by 2M CF_3COOH for 3–4 h at 120°. This period of time is longer than that used generally and therefore the amount of rhamnose in the pectins obtained by other conditions of hydrolysis may be underestimated. It is important to determine accurately the rhamnose content in pectins since it has a potential role in forming "kinks" and thus affects the physical properties of these polysaccharides.

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