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# Comparison of the Structural Features of Apple and Citrus Pectic Substances

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## *ABSTRACT*

*Pectic substances were extracted from Alcohol Insoluble Solids from lemon peel (albedo) and fractionated by ion exchange chromatography and gelfiltration. The pectin molecules contained rhamnose, arabinose, galactose, glucose and galacturonic acid residues; xylose residues were almost absent. Degradation with purified pectolytic enzymes and subsequent gelfiltration of the resulting pectin fragments showed that the neutral sugar side chains were present in 'hairy regions' (blocks of neutral sugar side chains). The distribution of the methoxyl groups was studied by HPLC analysis of enzyme-degraded pectins. Some influence of native pectinesterase on the distribution of the methoxyl groups was found. The results are compared with those of similarly extracted and purified apple pectic substances.*

## INTRODUCTION

In previous papers, investigations of the structure of apple pectic substances have been reported (de Vries *et al.*, 1981, 1982, 1983*a*, *b*, 1984). The extraction and purification of pectins from apple Alcohol Insoluble Solids (AIS) was described (de Vries *et al.*, 1981) and a model of apple pectin molecules was presented (de Vries *et al.*, 1982). In this model, the molecules consist of 'hairy regions' (rhamnogalacturonan segments carrying the neutral sugar side chains) and 'smooth regions' (homogalacturonan segments). The distribution of the methoxyl groups was studied (de Vries *et al.*, 1983*b*) and it was found by high-pressure liquid chromatography (HPLC) of enzyme-degraded pectins that the distribution of the methoxyl groups could be a random one. The extractability and the molecular weight (MW) of the apple pectic substances were found to depend on storage time (de Vries *et al.*, 1983*b*).

In this paper, structural studies on lemon peel pectic substances are reported. The methods developed in the above mentioned studies were applied to lemon peel pectic substances; in this way, apple and citrus pectic substances extracted and purified according to the same methods could be compared.

## METHODS

### *Analytical methods*

The anhydro-uronic acid (AUA, MW 176) content of pectin fractions was determined by an automated *m*-hydroxydiphenyl/sulphuric acid assay (Thibault, 1979). The amount of AUA in the AIS-preparations was determined according to Ahmed & Labavitch (1977). The neutral sugars were analysed gas-chromatographically as their alditol acetates (Albersheim *et al.*, 1967; Darvill *et al.*, 1975) after hydrolysation in 2 N TFA for 1 h. The methoxyl content was determined by gas chromatographic analysis of the methanol released on alkaline de-esterification (1 h at room temperature; 0·1 M KOH). Methanol was converted to methyl nitrite and determined according to the method of Versteeg (1979).

### *Extractions*

AIS (10 g) was extracted three times for 30 min (while being continuously stirred) with 300 ml of 0·05 M Na-acetate buffer (pH = 5·2) at room temperature and the whole procedure was repeated at 70°C; extraction with 0·05 M EDTA and 0·05 M $NH_4$-oxalate in 0·05 M Na-acetate buffer followed (again three times, 70°C, 30 min). The extracts were filtered and the pectins were precipitated with ethanol at a concentration of 70%. These three extracts are referred to as the cold buffer, hot buffer and oxalate extracts.

### *Enzymic degradation*

Highly purified pectin lyase ('type 2', van Houdenhoven, 1975) and pectate lyase (Rombouts *et al.*, 1978) were used. Enzyme reaction conditions were as follows.

Pectin lyase (EC 4.2.2.10; poly(methoxygalacturonide)lyase); 0·2 mg ml$^{-1}$ substrate and 0·002 units ml$^{-1}$ (limited degradation) or 3 units ml$^{-1}$ (extensive degradation) in 10 mM sodium citrate buffer, pH = 5·2, at 30°C for 4–10 h. The extent of degradation was determined spectrophotometrically at 235 nm, assuming $\epsilon_{235} = 5500\ \text{M}^{-1}\ \text{cm}^{-1}$ for the esterified unsaturated product.

Pectate lyase (EC 4.2.2.2; poly(1,4-α-D-galacturonide)lyase); 1 mg ml$^{-1}$ substrate and 15 units ml$^{-1}$ enzyme in 25 mM sodium carbonate buffer, pH = 6·9, at 30°C for 4 h.

The extent of degradation (percentage of bonds broken) was determined spectrophotometrically at 235 nm, while $\epsilon_{235} = 4800\ \text{M}^{-1}\ \text{cm}^{-1}$ (McMillan & Vaughn, 1964) was assumed for the de-esterified unsaturated product and $\epsilon_{235} = 5500\ \text{M}^{-1}\ \text{cm}^{-1}$ (Edstrom & Phaff, 1964) for the esterified unsaturated product.

### *Gel filtration*

An amount of 10–50 mg of (degraded) pectin was dissolved in about 2 ml water and applied to a 80 × 2·5 cm column of Sephacryl S-300 (Pharmacia). The void volume (Blue Dextran) of the column was 120 ml; the included molecules (glucose) appeared at an elution volume of 300 ml. The eluent was 0·1 M Na-phosphate, pH = 5·1, or water. The flow rate was 0·3 ml min$^{-1}$, controlled by an LKB peristaltic pump. Experiments were performed at room temperature.

### *High-pressure liquid chromatography (HPLC)*

A Spectra Physics Liquid Chromatograph (SP 8000) equipped with a Schoeffel 770 variable wavelength detector was used. Oligomeric pectin fragments were separated on a 250 × 4·6 mm I.D. LiChrosorb 10NH$_2$ (Merck) column with a Vydac 501 SC quard column (100 × 2·1 mm I.D., 37–44$\mu$, Chrompack). Na-acetate buffers of varying pH and concentration were used as eluents. The exact conditions depended on the age and the condition of the column (Voragen *et al.*, 1982).

### *Ion-exchange chromatography*

About 100 mg of pectin sample was dissolved in 5 mM Na-phosphate buffer, pH = 5·1, and applied to a 10 × 0·4 cm column of DEAE-cellulose (Whatman DE 52). After washing thoroughly with the starting buffer, the pectins were eluted from the column with a linear gradient of 5–300 mM Na-phosphate buffer of pH = 5·1 (200 ml). Experiments were performed at room temperature.

**TABLE 1**
Characteristics of Alcohol Insoluble Solids (AIS) from Lemon Albedo and Ripe Apple Cortex

| | *AIS (% of fresh wt)* | *AUA (mg/g AIS)* | *AUA (mg/g fresh wt)* | *DE (%)* |
|---|---|---|---|---|
| Lemon albedo | 7·3 | 207 | 15·1 | 71 |
| Ripe apple cortex | 2·1 | 284 | 6·0 | 70 |

*Note.* AUA, anhydro-uronic acid; DE, degree of esterification. The preparation of the ripe apple AIS was described by de Vries *et al.* (1981).

### *Preparation of AIS*

Lemons (*Citrus limon*) from Italy were purchased from a local greengrocer. The fruits were peeled (removal of the flavedo), after which the albedos were removed. The albedos were then mashed in a mincer and portions of 1 kg were extracted three times with 2·5 litres of 96% alcohol at 70°C. The AIS-preparation was air-dried overnight after solvent drying with acetone, ground in a hammer mill with a 10 $\mu$m sieve and stored at $-40$°C. Table 1 gives some characteristics of the AIS obtained by this procedure.

## RESULTS AND DISCUSSION

Table 1 shows that there are differences between AIS from lemon peels and from apples. Lemon peels contain more pectin than apples, but the content in the AIS is lower. These differences can be explained by the difference in cell size: the cells of apple cortical tissue are large and the cell walls are relatively thin. The AUA content of lemon peel AIS (207 mg/g) is about the same as reported by Sinclair (1961) for orange albedo AIS (196 mg/g). The results of the pectin extractions are given in Table 2. Also in this table, a comparison can be made between apple AIS and lemon peel AIS.

The most important difference is that between the oxalate extracts in both cases: from lemon peel AIS more pectin can be extracted by oxalate. But also in this case, the degree of esterification (DE) is high

TABLE 2

Pectin Fractions Obtained from AIS from Ripe Apple Cortical Tissue and from Lemon Albedo by Fractional Extraction

| *Extract* | *Amount extracted* | | | | *Degree of esterification, %* | |
|---|---|---|---|---|---|---|
| | *AUA (mg)/AIS (g)* | | *%* | | | |
| | *apple cortex* | *lemon albedo* | *apple cortex* | *lemon albedo* | *apple cortex* | *lemon albedo* |
| Cold buffer | 30 | 17 | 11 | 8 | 80 | 74 |
| Hot buffer | 30 | 37 | 11 | 18 | 76 | 70 |
| Oxalate | 31 | 61 | 11 | 30 | 78 | 70 |
| Total | 91 | 115 | 33 | 56 | | |

*Note.* AUA, anhydro-uronic acid. Conditions during the extractions are described in the text.

(70%). It must be said, however, that we did not investigate this 'oxalate effect' any further. Therefore the question whether this effect is specific for oxalate or not, remains open. Hence, it cannot be concluded that, in lemon peel cell walls, more pectin has been rendered insoluble by $Ca^{2+}$-binding than in apple cell walls. The purification of the pectin from the oxalate extract of lemon albedo AIS on DEAE-cellulose is shown in Fig. 1 and Table 3.

The neutral sugar composition of the pectin pools is expressed as moles sugar residues/mole arabinose residues to make comparison with apple pectin fractions easier (Table 4).

The pectin molecules contained rhamnose, arabinose, galactose and glucose residues. Only trace amounts of xylose residues were present. This is the most striking difference between the pectins from the oxalate extracts of apple and citrus AIS (Table 4). In a crude pectin extract from grapefruits, Kawabata (1977) also reported the absence of xylose residues.

Aspinall *et al.* (1968) found only traces of xylose residues in DEAE-cellulose purified lemon peel pectin. In the crude cold water extract of lemon peels, however, they found (after partial hydrolysis) the disaccharide xylose-(1,3)-galacturonic acid. This suggests that in citrus

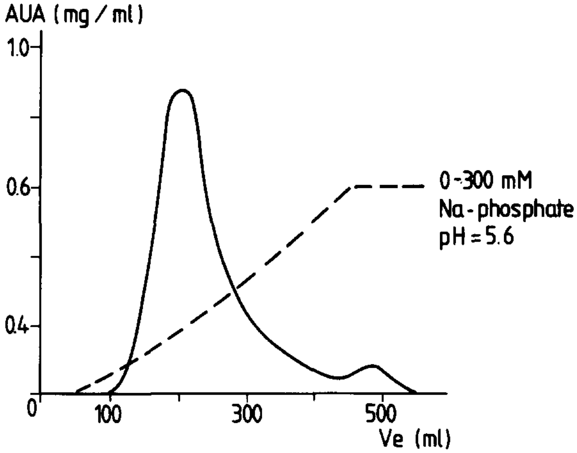

**Fig. 1.** Fractionation of the pectin from the oxalate extract of AIS from lemon peels on DEAE-cellulose. AUA, Anhydro-uronic acid; Ve, elution volume. The DEAE-cellulose column was eluted by a linear gradient of 5-300 mM Na-phosphate buffer, pH = 5·1 (400 ml).

TABLE 3
Neutral Sugar Composition of Citrus Pectin Pools Shown in Fig. 1

| *Sugar composition* | *Elution volume (ml)* | | | |
|---|---|---|---|---|
| | 100-190 | 190-250 | 250-400 | 400-500 |
| Neutral sugar content (moles/mole of galacturonate residues) | 0·09 | 0·12 | 0·15 | 0·0 |
| Moles rhamnose/mole arabinose | 0·09 | 0·05 | 0·09 | 0·1 |
| Moles galactose/mole arabinose | 0·55 | 0·59 | 0·26 | 0·3 |
| Moles glucose/mole arabinose | 0·10 | 0·05 | 0·25 | 0·2 |
| Percentage of total anhydro-uronic acid | 28 | 30 | 37 | |

pectin some xylogalacturonan regions are present, as has been shown to be the case for apple pectic substances (de Vries *et al.*, 1982). It may be that in citrus xylogalacturonans occur only in some parts of the cell wall. It is also possible that these pectin fragments containing xylose play a role as 'elicitor' in host–parasite relations of the plant (Albersheim *et al.*, 1981).

**TABLE 4**

Neutral Sugar Composition of the DEAE-Cellulose Purified Pectin from the Oxalate Extracts of AIS from Ripe Apple Cortex and Lemon Albedo

| *Sugar composition*[a] | *Ripe apple cortex* | *Lemon albedo* |
|---|---|---|
| Rhamnose/arabinose | 0·09 | 0·10 |
| Xylose/arabinose | 0·09 | 0·00 |
| Glucose/arabinose | 0·10 | 0·20 |
| Galactose/arabinose | 0·35 | 0·43 |

[a] Moles of neutral sugar residues/moles of arabinose residues.

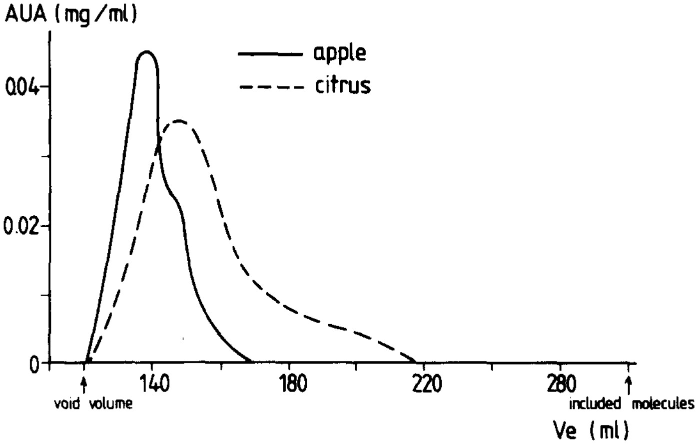

**Fig. 2.** Gel filtration of DEAE-cellulose purified pectin fractions from the oxalate extracts of AIS from lemon peels and from ripe apples. AUA, Anhydro-uronic acid; Ve, elution volume. The eluent was 0·1 M Na-phosphate buffer, pH = 5·1.

Both in citrus and apple pectic substances the ratio of galactose/arabinose in the neutral sugar side chains is not constant (Table 3 and de Vries *et al.*, 1981). In both cases this inconsistency is caused by the presence of two types of arabinogalactans: 1,3/1,6-linked and 1,4-linked galactans (Aspinall & Cottrell, 1970; de Vries *et al.*, 1982).

Figure 2 suggests that the molecular weight (the hydrodynamic volumes) of citrus pectin is lower than that of apple pectin. The molecular weight of apple pectic substances, however, decreases during

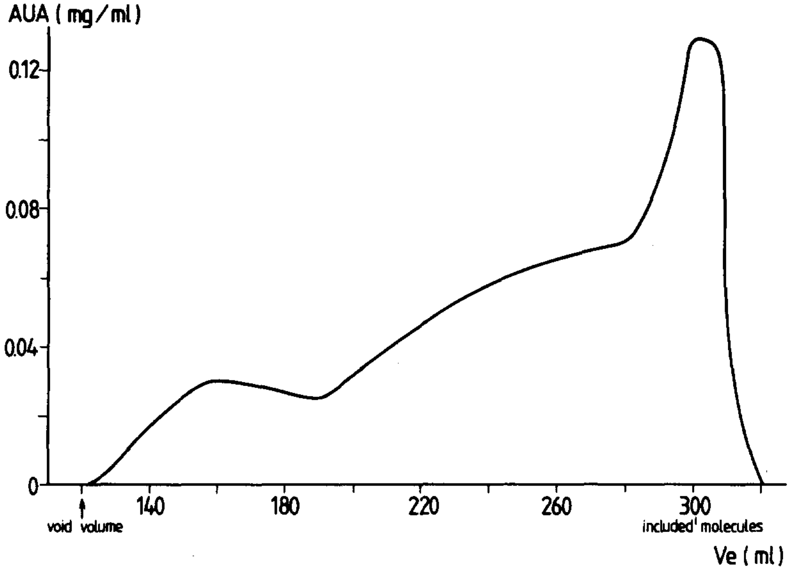

Fig. 3. Gel filtration of a pectin lyase degraded citrus pectin. AUA, anhydrouronic acid; Ve, elution volume. The substrate was a DEAE-cellulose purified pectin from the oxalate extract of AIS from lemon peels. Limited degradation with pectin lyase as described in the Results and Discussion section (extent of degradation was 7%); the eluent was water.

storage of the apples (de Vries *et al.*, 1983*b*). The interpretation of the data in Fig. 2 is also hampered by the fact that the oxalate fractions probably represent different sub-fractions of the pectic substances present (see Table 2).

Figure 3 shows the result of limited enzymic degradation and subsequent gelfiltration of a citrus pectin fraction from the oxalate extract. Nearly all the neutral sugar residues appear in elution volumes, where high-molecular-weight fragments can be expected (Table 5). It can be concluded that in citrus pectin the neutral sugar side chains are concentrated in regions that cannot be degraded by pectin lyase, the so-called 'hairy regions', exactly as was shown for apple pectic substances (de Vries *et al.*, 1982). Aspinall & Cottrell (1970) found small amounts of molecules rich in neutral sugars in their lemon peel extracts. Zitko & Bishop (1965) reported the presence of two types of molecules in commercial citrus pectin: one type was rich in neutral sugars and the other was poor in neutral sugars. Our results show that these two types

**TABLE 5**
Neutral Sugar Content of Citrus Pectin Pools Shown in Fig. 3

| *Sugar* | *Elution volume (ml)* | | |
|---|---|---|---|
| | 120-180 | 180-280 | 280-320 |
| Percentage of total AUA | 7 | 53 | 40 |
| Neutral sugar content (moles neutral sugar residues/mole of galacturonate residues) | 1·31 | 0·01 | 0·00 |

are fragments of the same molecules and that low degrees of degradation result in the appearance of the two types of molecule reported by Zitko & Bishop (1965). Aspinall *et al.* (1970) and Aspinall & Cottrell (1970) studied the distribution of the methoxyl groups of lemon peel pectic substances. It can be concluded from their work that an intermolecular distribution exists which may be similar to the one for apple pectic substances (de Vries *et al.*, 1983*a*).

We studied the intramolecular distribution of the methoxyl groups by extensive degradation of pectin with pectin lyase and subsequent fractionation of the partially esterified oligogalacturonides by HPLC as shown in Fig. 4. Using the HPLC system, the partially esterified oligogalacturonides are separated according to their number of non-esterified galacturonic acid residues (de Vries *et al.*, 1983*a*). In Fig. 4, *a*, *b* and *c* indicate pectin fragments with 0, 1 and 2 free (non-esterified) galacturonate residues.

In Table 6 a comparison is made between the HPLC patterns of pectin fractions from apple and lemon peel. The differences appear to be small, which may indicate that no substantial differences between the intramolecular distributions of the methoxyl groups of apple and lemon peel pectin exist.

It was shown in a previous paper (de Vries *et al.*, 1983*b*) that the HPLC patterns of apple pectins do not differ much from those of 'trans-esterified' pectins (pectins esterified to 95% and subsequently de-esterified to 70% in cold alkali). This suggests that apple and citrus pectins have a random distribution of methoxyl groups.

When the whole AIS from both apple and lemon peel is degraded by pectate lyase and the resultant pectin fragments are analysed by HPLC

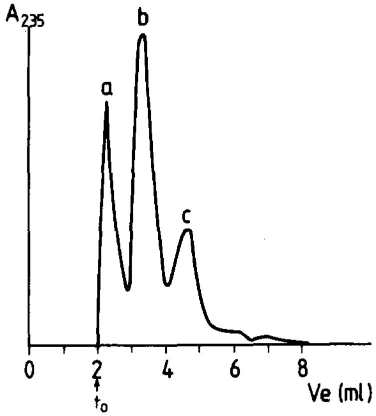

**Fig. 4.** High-pressure liquid chromatograms (HPLC) of citrus pectin degraded with pectin lyase. $A_{235}$, Absorbance at 235 nm; Ve, elution volume. HPLC conditions are as described in the text. The substrate was a pectin fraction from the oxalate extract of AIS from lemon peels. The extensive enzymic degradation is described in the Results and Discussion section.

**TABLE 6**

Percentage of Total AUA Present in the Peaks of the High-Pressure Liquid Chromatograms of Pectin lyase Degraded Substrates

| *Pectin fragment* | *AUC, in extract, %* | |
|---|---|---|
| | *Apple* | *Lemon* |
| *a* | 13 | 17 |
| *b* | 48 | 40 |
| *c* | 30 | 28 |
| '*d*' | 9 | 15 |

*Note.* The substrates were DEAE-cellulose purified pectin fractions from the oxalate extracts of AIS from ripe apples (de Vries *et al.*, 1981) and from lemon albedo. Figure 4 gives the high-pressure liquid chromatogram for the pectin from lemon albedo; *a*, *b* and *c* are pectin fragments with 0, 1 or 2 non-esterified galacturonate residues (see Fig. 4 and de Vries *et al.*, 1983*b*) '*d*' = 100 − (*a* + *b* + *c*).

**TABLE 7**

Amounts of Di- and Trigalacturonic Acid Present in De-esterified Pectate Lyase Digests of AIS from Ripe Apples and from Lemon Albedo, Expressed as Percentage of the Total Amount of Uronic Acid Residues Present in the AIS

| | *Ripe apple AIS* | *Lemon albedo AIS* |
|---|---|---|
| Digalacturonate | 0·02 | 0·79 |
| Trigalacturonate | 0·00 | 0·26 |

*Note.* The pectate lyase digests (for conditions see text) were alkali-saponified and the degree of polymerisation of oligomers present was determined by HPLC (de Vries *et al.*, 1983*b*).

(after de-esterification), differences between apple and lemon peel can be detected (Table 7). In the case of lemon peel AIS, small amounts of di-galacturonic acid and tri-galacturonic acid were found. In a previous paper (de Vries *et al.*, 1983*a*), it was shown that this occurrence of di- and tri-galacturonic acid is typical of pectins de-esterified by (citrus) pectinesterase. It can be concluded from Table 7 that citrus pectinesterase influences the distribution of the methoxyl groups in the citrus fruits. This influence, however, is very small: the resultant decrease of the overall DE is only 1 or 2% (Table 7).

## CONCLUSIONS

In conclusion our results show that apple and citrus pectin molecules are very similar. In both cases the neutral sugar side chains are located in 'hairy regions'. The distribution of the methoxyl groups is probably the same in both cases, namely a random one. A difference is that citrus pectin contains few xylose residues.

It must be realised, however, that pectin structural parameters are influenced by factors like fruit storage conditions (O'Beirne *et al.*, 1981), and conditions during extraction. Differences among batches of commercial pectins may result from these factors.

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