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Characterisation and selectivity of divalent metal ions binding by citrus and sugar-beet pectins

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A scale of selectivity for the binding of calcium and some heavy metal ions by citrus and sugar-beet pectins was set up by pH-measurements. The same order of selectivity was found for the two pectins, decreasing as follows: $Cu^{2+} \sim Pb^{2+} \gg Zn^{2+} > Cd^{2+} \sim Ni^{2+} \geq Ca^{2+}$. Binding isotherms for Ca^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} ions have shown a greater binding level when the ionic strength decreased and when the pectin concentration increased in the presence of 0.1 M $NaNO_3$. By comparing binding isotherms, the same order of selectivity was found as by pH-studies. Scatchard plots and Hill index evaluation showed for all ions and all pectins anticooperative interactions in water. In the presence of 0.1 M $NaNO_3$, citrus pectins displayed cooperative interactions for all metal ions. In contrast, for sugar-beet pectins, cooperative interactions only occured with Cu^{2+} and Pb^{2+}. With Ca^{2+}, Ni^{2+} and Zn^{2+} sugar-beet pectins displayed Scatchard plots which could not be distinguished from an anticooperative binding. This difference of behaviour could be related to the presence of acetyl groups decreasing the affinity of Me^{2+} for sugar-beet pectins. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

The extensive use of heavy metals in a large number of industrial applications and the more and rigorous drastic standards imposed for industrial waste streams have led to renewed interest in the removal/recovery of these toxic metals. Indeed, conventional methods have reached their limits and numerous methods are being tested. Among these methods, biosorption by dead microorganisms, algae or fungi (Volesky, 1990) or by natural polyelectrolytes, such as alginates and pectins, seems to be promising due to their high cation exchange capacities. Binding properties of charge-bearing biopolymers are therefore being studied for this purpose for a few years (Zhang *et al.*, 1994; Bosco *et al.*, 1994; Axelos *et al.*, 1994; Mitani *et al.*, 1995).

Pectins are anionic polysaccharides present in the primary cell walls and intercellular regions of higher plants (Voragen *et al.*, 1995). The main feature of pectins is a linear chain of α-(1→4)-linked D-galacturonic acid units with varying amount of methoxyl esters. Their methoxyl content, expressed as their degree of methylation (DM), implies specific industrial applications (Nelson *et al.*, 1977). The galacturonosyl backbone is interrupted by the insertion of (1→2)-linked α-L-rhamnose units (typical amounts of 1–4%), on which side-chains composed by neutral sugars are attached. Sugar-beet pectins are characterised by higher rhamnose, arabinose and galactose contents than citrus or apple pectins, and by acetylation of the secondary hydroxyl functions of the galacturonosyl residues up to 35%. Despite a higher proportion of 'hairy' regions (groups of rhamnosyl units carrying side chains), causing higher amounts of neutral sugars in the case of sugar-beet pectins, pectins have similar 'smooth' regions (homogalacturonic regions) composed of ~100 galacturonosyl units (Thibault *et al.*, 1993).

The affinity of the binding of pectins or derivatives with metal ions has been extensively investigated. Heavy metal binding by oligomeric fragments of citrus pectins (DP 1 to 9), in the presence of KNO_3 as the supporting salt, was reviewed (Kohn, 1987 and references therein): intramolecular electrostatic bonds for Ca^{2+}, Sr^{2+} and Zn^{2+} ions and complexes formation for Cd^{2+}, Cu^{2+} and Pb^{2+} explained the much higher affinity of such fragments towards the latter ions. Moreover, only one type of copper complex with oligomers is formed without a positively charged intermediate (Kohn *et al.*, 1983).

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Binding of Ca^{2+}, Sr^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} to citrus pectins would involve, whatever the DM, a stoechiometric bond wherein one metal cation is bound to two free carboxyl functions (Malovikova & Kohn, 1983). The high selectivity of pectinates towards Pb^{2+} at the ionic strength $I = 0.30$ M was also noticed even with DM as high as 85%; binding constants were found to be independent of the DM at least with DM $\leq$ 50% (Malovikova & Kohn, 1979). In contrast to strongly bound cations like Pb^{2+}, Sr-, Ca- and Cd-pectinates complex formation (Kohn & Luknar, 1975; Malovikova & Kohn, 1982) depends strongly on the DM of pectins indicating different interactions: pure electrostatic interactions would be progressively replaced by chelate formation with decreasing DM.

More attention has been paid to the study of the binding mechanisms to explain the specific affinity of pectins according to the divalent cations. Thus, binding process may be characterised by its cooperative or anti-cooperative modes i.e. an increase or a decrease in the affinity of the ligand for the polymer as the binding reaction proceeds, respectively. A different and less specific binding mechanism was shown by circular dichroism for Cu^{2+} compared to Cd^{2+}, Ni^{2+}, Pb^{2+} or Ca^{2+} (Thom *et al.*, 1982). Gelation of pectins induced by adding Cu^{2+}, Cd^{2+}, Co^{2+}, Ba^{2+}, Sr^{2+} and Ca^{2+}, as followed by light-scattering measurements, indicated a less specific association mechanism with Cu^{2+}, Cd^{2+} and Co^{2+} (Thibault & Rinaudo, 1986). This different behaviour was also noticed by Axelos *et al.* (1994). Moreover, Schlemmer & Decker (1993) have shown cooperativity in the binding of copper ions by pectins whatever the DM but no indication of the ionic strength of the solvent was given. Both anti-cooperativity (Lips *et al.*, 1991) or cooperativity (Braudo *et al.*, 1992) of the binding of calcium ions by pectate and pectinate were claimed. However, it was demonstrated that both types of interactions could actually occur between citrus pectins and calcium ions according to the solvent conditions (Garnier *et al.*, 1994). An anti-cooperative character in water occurred where there was a cooperative character in presence of 0.1 M NaCl.

The aim of the present work was to set up a scale of selectivity for the binding of divalent metal ions by pectins ($Me^{2+} = Ca^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}$) by a rapid method involving pH-measurements. Pectins with similar DM but from citrus and sugar-beet, were utilized in order to check the influence of the degree of acetylation. The binding was quantified and characterised by determining free Me^{2+} ion concentrations by potentiometry or by means of a dye spectrophotometric method; various concentrations of pectins and metal ions were studied both in salt-free solutions or in the presence of a supporting salt.

EXPERIMENTAL

Materials

Sugar-beet pectins (B58) were provided by Copenhagen Pectin Factory Ltd (Denmark). High-methoxyl citrus pectins (DM = 74) were obtained from SBI (Beaupte, France) and further deesterified to DM = 54 by saponification according to Thibault & Rinaudo (1985). The acidic form of pectins was obtained by treatment with 0.1 M HCl as described previously (Michel *et al.*, 1984). Metal cations were used as their nitrate salts; $Cu(NO_3)_2{\cdot}2.5H_2O$, $Cd(NO_3)_2{\cdot}4H_2O$, $Ni(NO_3)_2{\cdot}6H_2O$, were purchased from Aldrich, $Pb(NO_3)_2$ and $Ca(NO_3)_2{\cdot}4H_2O$ from Merck and $Zn(NO_3)_2{\cdot}7H_2O$ from Fluka. Tetramethylmurexide (TMMX) was purchased from Sigma and used without further purification.

Characterisation of pectins

The galacturonic acid (GalA) content of the samples was measured colorimetrically by the *m*-hydroxydiphenyl method (Thibault, 1979). The degrees of methylation DM and acetylation DAc were calculated as the molar ratio of methanol and acetic acid respectively, as determined by HPLC (Voragen *et al.*, 1986), to GalA.

Neutral sugars were analysed by GLC as alditol acetate derivatives (Englyst & Cummings, 1984).

The theoretical cation exchange capacity (CEC_{theo}) was calculated from the GalA content and the DM. The experimental CEC (CEC_{exp}) of pectins (acidic form, 100 mg in 50 ml of 0.1 M $NaNO_3$) was determined by titration with 0.05 M NaOH. CECs were expressed as $mequiv{\cdot}g^{-1}$ of dry matter.

The weight-average molecular weights $\bar{M}_W$ of the pectins were determined on very dilute solutions in 0.1 M NaCl by static light scattering experiments. The solutions were filtered through 0.45 μm pore size Millipore-durapore filters and then slowly through 0.20 μm pore size Anotop-25 filters (Whatman Lab. Division). Static measurements were carried out on a Malvern K7025 instrument in combination with an ion argon Spectra-Physics laser operating with vertically polarized light (wavelength λ = 514.5 nm), over the angular range 30–150° at 25°C, using a refractive index increment extrapolated to 0.146 $g\,ml^{-1}$ from values usually determined for polysaccharides in aqueous solutions (Ousalem *et al.*, 1993).

The structural charge density parameter ξ was calculated from the following equation (Lifson & Katchalsky, 1954):

$$\xi = \frac{e^2/bDkT}{(1 - DM/100)} = 1.61(1 - DM/100)\,\text{at}\,25°\text{C},$$

where e is the electron charge, k the Boltzmann constant, T the absolute temperature, b the length of the monomeric GalA unit (0.435 nm) (Rees & Wight, 1971) and *D* the dielectric constant of the solvent.

pH-measurements

All pH measurements were performed at 25.0±0.1°C with a pH-meter LPH430T (Radiometer Analytical S.A.) fitted out with a combined pH-electrode Ingold (type U402-S7/120) and a temperature probe (XT 130, Radiometer). The pH-meter response was calibrated before each experiment. Pectin samples in the acidic form were dissolved in 0.1 M $NaNO_3$ with gentle stirring overnight at 4°C. Experiments were carried out with pectin solutions at a stable initial pH, corresponding either to native pH or adjusted to ~5.1 by adding 0.05 M NaOH. pH measurements were performed sequentially as follows: addition of the Me^{2+} ion (initial solution at 2.10^{-2} equiv.l^{-1}), 15 min of stirring, 15 min of rest, pH measurement, new addition of metal, stirring, rest, pH measurement and so on.

Binding quantification

Experimental

Pectins in acidic form were dissolved either in water or in 0.1 M $NaNO_3$ with gentle stirring overnight at 4°C and then the pH of the pectins solutions was adjusted to pH ~7.2 by adding 0.05 M NaOH. Varying amounts of the Me^{2+} ion (initial solution at $2 \cdot 10^{-2}$ equiv.l^{-1}) were added to 20 ml aliquots of the pectins solutions and the mixture was stirred for 2 h.

Potentiometric method

Cu^{2+}, Pb^{2+} and Ca^{2+} activity was determined by potentiometry on the reactant mixtures after 15 min of stirring and stabilization of the response. Ion selective electrodes for Cu^{2+}, Pb^{2+} and Ca^{2+} (XS290, XS310 and F2112, respectively, Radiometer) were used with a saturated calomel electrode (XR 100, Radiometer) fitted out with a protection extension (XL 100, Tacussel) filled with 1 M KNO_3, connected to a potentiometer (LPH430T, Radiometer Analytical S.A.).

Calibration was carried out before each experiment with standard copper, lead and calcium nitrate solutions. Activities were calculated from the concentration of the calibrating solutions, and free ion concentrations were obtained from the measured activities in the experiments using the Debye-Hückel theory formula:

$$-\log \gamma_{Me^{2+}} = 0.5085 z^2_{Me^{2+}} \sqrt{I}/(1 + 0.3281 a_{Me^{2+}} \sqrt{I})$$

where $\gamma_{Me^{2+}}$ = activity coefficient of the species, Me^{2+}, $z_{Me^{2+}}$ = valence of the species Me^{2+}, I = ionic strength of the solution, $a_{Me^{2+}}$ = the effective diameter of the hydrated ion corresponding to 0.45 nm for Pb^{2+} and to 0.60 nm for the others (Skoog & West, 1976), then $[Me^{2+}]_f = (Me^{2+})_f/\gamma_{Me^{2+}}$ where $[Me^{2+}]_f$ is the concentration of free species Me^{2+} and $(Me^{2+})_f$ is the activity of the free species Me^{2+}.

Spectrophotometric method

Zn^{2+}, Ca^{2+} and Ni^{2+} activities were determined spectrophotometrically after equilibration and centrifugation (20 000 g for 20 min) in order to eliminate precipitates. The free Zn^{2+}, Ca^{2+} and Ni^{2+} activities were determined by means of a dual-wavelength spectrophotometric method (DWSM) (Kohn & Furda, 1967; Kwak & Joshi, 1981), using TMMX as an activity probe for the metal ion. A TMMX concentration of $2 \cdot 10^{-5}$ mol l^{-1} was used for Me^{2+} activity determinations.

Absorbance measurements were made in 1 cm path length cells, by using a S-1000 spectrophotometer (Secomam, France). Absorbances A of the sample and standard solutions were read at the wavelength corresponding to the maximum of absorption of the complexes (λ = 462, 467 and 493 nm for Zn, Ni and Ca-TMMX complexes, respectively) and at the wavelength λ' = 530 nm corresponding to the maximum of absorption of the TMMX itself. Concentration of free Me^{2+} ions $[Me^{2+}]_f$ was calculated using calibration curves ϕ vs (Me^{2+}) where $\phi = A_\lambda/A_{\lambda'}$ and from activity coefficients calculated as described above.

RESULTS AND DISCUSSION

Characterisation of the pectins

The main features of the pectins are shown in Table 1.

Citrus pectins were deesterified in order to have a similar DM (54) to sugar-beet pectins (58). However, their structural charge density parameter ξ and their CEC differed due to a slightly different DM and a

Table 1. Main characteristics of the pectins

	B58	C54
Galacturonic acid	700	911
Rhamnose	45	11
Fucose	1	tr
Arabinose	46	15
Xylose	2	1
Mannose	tr	tr
Galactose	57	38
Glucose	3	3
DM	58	54
DAc	14	0
$CEC_{theo.}$	1.67	2.38
$CEC_{exp.}$	1.65	2.38
$M_w (+10^3)$	290	171
ξ	0.676	0.741

GalA and neutral sugars contents in mg g^{-1}, DM and DAc in percentage, CEC in mequiv g^{-1} of dry matter, $\bar{M}_W$ in g mol^{-1}; ξ is a dimensionless number; tr: traces.

different GalA content, respectively. B58 pectins had a degree of acetylation of 14%. The presence of acetyl groups is an important structural feature of sugar-beet pectins (Voragen *et al.*, 1986).

Experimental CEC values were found to be in very good agreement with values calculated from GalA contents and DM, showing that only the galacturonate groups are responsible for ionic binding.

Rhamnose, arabinose and galactose contents were higher for B58 than for C54 as already found (Axelos & Thibault, 1991; Thibault *et al.*, 1993). Weight-average molecular weight $\bar{M}_W$ was found to be higher for sugar-beet pectins than citrus pectins in agreement with previous result (Thibault *et al.*, 1993). However, these authors showed that both pectins present regions of similar length (~100 galacturonosyl units) capable of ion binding.

Selectivity scale

The method we used to compare the binding of various Me^{2+} ions to pectins was based on the fact that the exchange between the protons of pectins in acidic form and the Me^{2+} ions leads to an increase in the protons concentration in solution which is more pronounced for more strongly bound ions. It can therefore be assumed that the more the pH decreases, the higher the affinity of pectins for a given Me^{2+} ion.

The experiments were carried out with pectins in 0.1 M $NaNO_3$ in order to screen ionic effects. The experimental data are presented by plotting the variation of the pH corrected for dilution, ΔpH, against the ratio $[Me^{2+}]_t$ / $[COO^-]$ (ranging from 0 to 2) where $[Me^{2+}]_t$ is the total metal concentration (equiv l^{-1}) and $[COO^-]$ the pectin concentration in equiv l^{-1}.

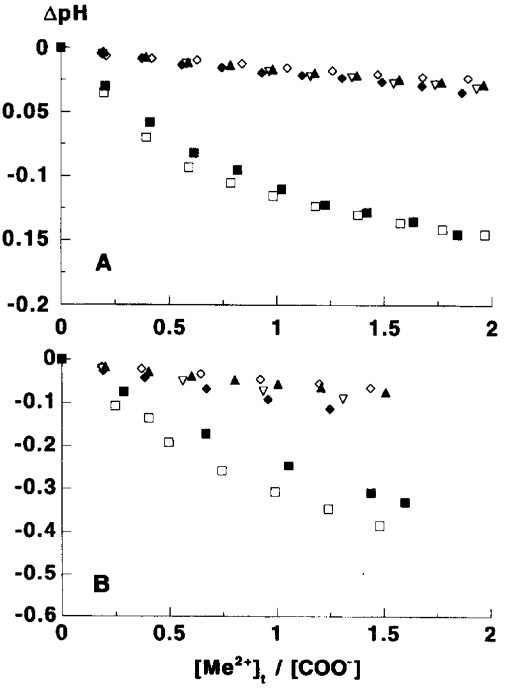

Fig. 1. Effect of metal ions binding on the pH for sugar-beet pectins B58 at 2 mequiv l^{-1} in 0.1 M $NaNO_3$ at 25°C: pH 3.1 (A) and pH 5.1 (B), (□) Cu^{2+}, (■) Pb^{2+}, (◆) Zn^{2+}, (▽) Cd^{2+}, (▲) Ni^{2+}, (◇) Ca^{2+}.

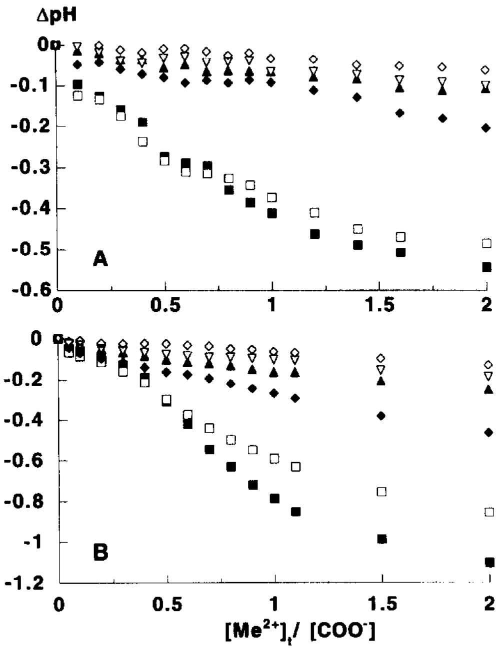

Fig. 2. Effect of metal ions binding on the pH for citrus pectins C54 at pH 5.1 in 0.1 M $NaNO_3$ at 25°C: 2 mequiv l^{-1} (A) and 4 mequiv l^{-1} (B), symbols as in Fig. 1

Experiments were first performed on B58 at 2 mequiv l^{-1} (Fig. 1a) at pH ~3.1. Two groups of metal ions could be distinguished. A first one, composed of Ca^{2+}, Ni^{2+}, Cd^{2+} and Zn^{2+} was characterised by a low and quasi-linear variation. The second group, composed of Pb^{2+} and Cu^{2+}, showed a much higher pH variation which tended to a plateau in the final stages with no significant difference between the two ions. In order to differentiate the metals in each group, the same experiments were performed at pH ~5.1 (Fig. 1b). At this pH, the amount of protons to be replaced by Me^{2+} ions is lower, but the ΔpH values were more marked due to the logarithmic character of the pH-scale.

In the same way, C54 solutions (Fig. 2A) at 2 mequiv l^{-1} were also adjusted to pH ~5.1 and the same two groups of metal ions were identified. Using higher pectin concentration i.e. 4 mequiv l^{-1} (Fig. 2b), it became easier to differentiate between the weakly bound metals.

Starting at pH 5.1, a steeper drop in the pH, accentuated at 4 mequiv l^{-1}, appeared as the beginning of pectin precipitation was observed. For both pectins precipitation occurs at $[Me^{2+}]_t/[COO^-]$ ~ 0.4 for Cu^{2+} and Pb^{2+}. For the other four metal ions, it occurs only for C54 at $[Me^{2+}]_t/[COO^-]$ = 0.8–1. B58 did not precipitate in the presence of Zn^{2+}, Cd^{2+}, Ni^{2+} or Ca^{2+} confirming that Cu^{2+} and Pb^{2+} were considerably more strongly bound.

Both pectins exhibit the same behaviour towards the two groups of metals with higher magnitude of pH-variation for C54 due to its higher ξ. No significant difference in the selectivity appeared between B58 and C54.

The following decreasing scale of selectivity may therefore be drawn independently of the origin of the pectin:

$$Pb^{2+} \sim Cu^{2+} \gg Zn^{2+} > Cd^{2+} \sim Ni^{2+} \geq Ca^{2+}$$

This affinity order is in agreement with the rule for the sequences of complex stability from the Irving–Williams concept based on ligand field or simple-crystal field theory (Cotton & Wilkinson, 1976). According to this rule, the stability of complexes metal cations-oxygen donor groups decreases in the series: $Pb^{2+} > Cu^{2+} > Zn^{2+} \sim Ni^{2+} > Cd^{2+} > Ca^{2+}$.

The scale of selectivity set up by pH-measurements in this work does not necessitate any calculation of thermodynamic constants derived from theories on the binding of cations by polymers. This scale is also in agreement with results found for polygalacturonic acid (Jellinek & Chen, 1972; Deiana *et al.*, 1983) which showed that the stability of the complex decreases as follows: $Cu^{2+} \gg Cd^{2+} \geq Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$. The binding constants have been determined by potentiometric titration from the Bjerrum's theory which assumes that complex formation follows a two-step process. This was confirmed for copper ions by Debongnie *et al.* (1987). However, in case of oligomeric fragments of citrus pectins, Kohn (1987) showed that Cd^{2+} was more strongly bound than Zn^{2+} by using activity coefficients, degrees of association and the multiple-equilibria theory.

The presence of acetylated hydroxyl functions on C_2/C_3 of the galacturonosyl units of pectins did not modify the selectivity scale towards metal cations, though it did modify their behaviour since the sugar-beet pectins did not precipitate with the more weakly bound cations.

Quantification of binding

The experimental data obtained by activity measurements are presented as binding isotherms $[Me^{2+}]_b/[COO^-]$ *vs* $[Me^{2+}]_t/[COO^-]$ where $[Me^{2+}]_b$ is the bound cation concentration (equiv l^{-1}) calculated from the measured activity, and $[Me^{2+}]_t$ is the total cation concentration (equiv l^{-1}). A total binding of the added ion would occur according to an ideal isotherm ('stoechiometric isotherm') symbolized by a straight line on the graph.

Binding isotherms were found to have the same features for B58 (Fig. 3) and C54 (results compilated in Fig. 5), according to the ionic strength, the pectin concentration and the metal ion. Titrating calcium either by potentiometry or the DWSM was found to give the same results. The interpretation is only detailed for B58 and was therefore valid for C54.

Influence of the ionic strength

In water, where polyelectrolyte effects take place, binding isotherms were independent of the pectin concentration (Fig. 3) and followed a master curve as already noticed for low-methoxyl pectins (Garnier *et al.*, 1994). Me^{2+} ions were totally bound at the lower ratios $[Me^{2+}]_t/[COO^-]$ up to ~0.3 in the case of Zn^{2+}, Ni^{2+} and Ca^{2+}, and ~0.6 for Cu^{2+} and Pb^{2+}. Binding isotherms almost follow the "stoechiometric isotherm' for the latter two ions. For a given polymer concentration, binding is less in the presence of supporting salts than in water.

This confirms previous results (Lips *et al.*, 1991; Garnier *et al.*, 1994) that showed a progressive decrease in the binding of Ca^{2+} ions by pectic acid with increasing concentration of NaCl. Similar results were also obtained for La- (Mattai & Kwak, 1984) and Zn-dextransulfate (Mattai & Kwak, 1981). The decreasing binding is due to the presence of Na^+ ions which compete with Me^{2+} for binding to pectins.

Influence of pectin concentration

In the presence of a supporting salt, the binding of the Me^{2+} ions increased with the polymer concentration (Fig. 3). Indeed, binding isotherms tended towards the 'stoechiometric isotherm' as pectins concentration increased, and this was more or less marked according to the metal ion. The competitive interactions Me-pectins *vs* Na-pectins increased with the ratio $[Me^{2+}]/[Na^+]$ thus explaining the increased binding level. Jellinek & Sangal (1972) also showed that the higher the ratio polygalacturonate/Cu^{2+} the higher the percentage of complexed Cu^{2+} ions. Binding isotherms of the system $ZnCl_2/NaCl$/dextransulfate presented the same features (Mattai & Kwak, 1981).

Influence of the nature of the metal ion

Binding isotherms of the five metal ions in the presence of supporting salt or in water for B58 and C54 at 2 mequiv l^{-1} are compiled in Figs 4 and 5, respectively. For a given polymer concentration, the levels of the binding isotherms, depending of the metal ions, were more easily differentiated in 0.1 M $NaNO_3$ than in water. Thus, the same order of selectivity as obtained by pH studies was found for both pectins by simple comparison of the level of the five binding isotherms: $Cu^{2+} \sim Pb^{2+} \gg Zn^{2+} \sim Ni^{2+} \geq Ca^{2+}$. This scale confirmed that Cu^{2+} and Pb^{2+} are more strongly bound than the other three cations for both pectins. Nevertheless, C54 was shown to bind slightly more metal cations than B58 due to its higher ξ value.

Characterisation of the type of interaction Me-pectins

In order to characterise the type of binding, experimental data from binding isotherms were plotted in

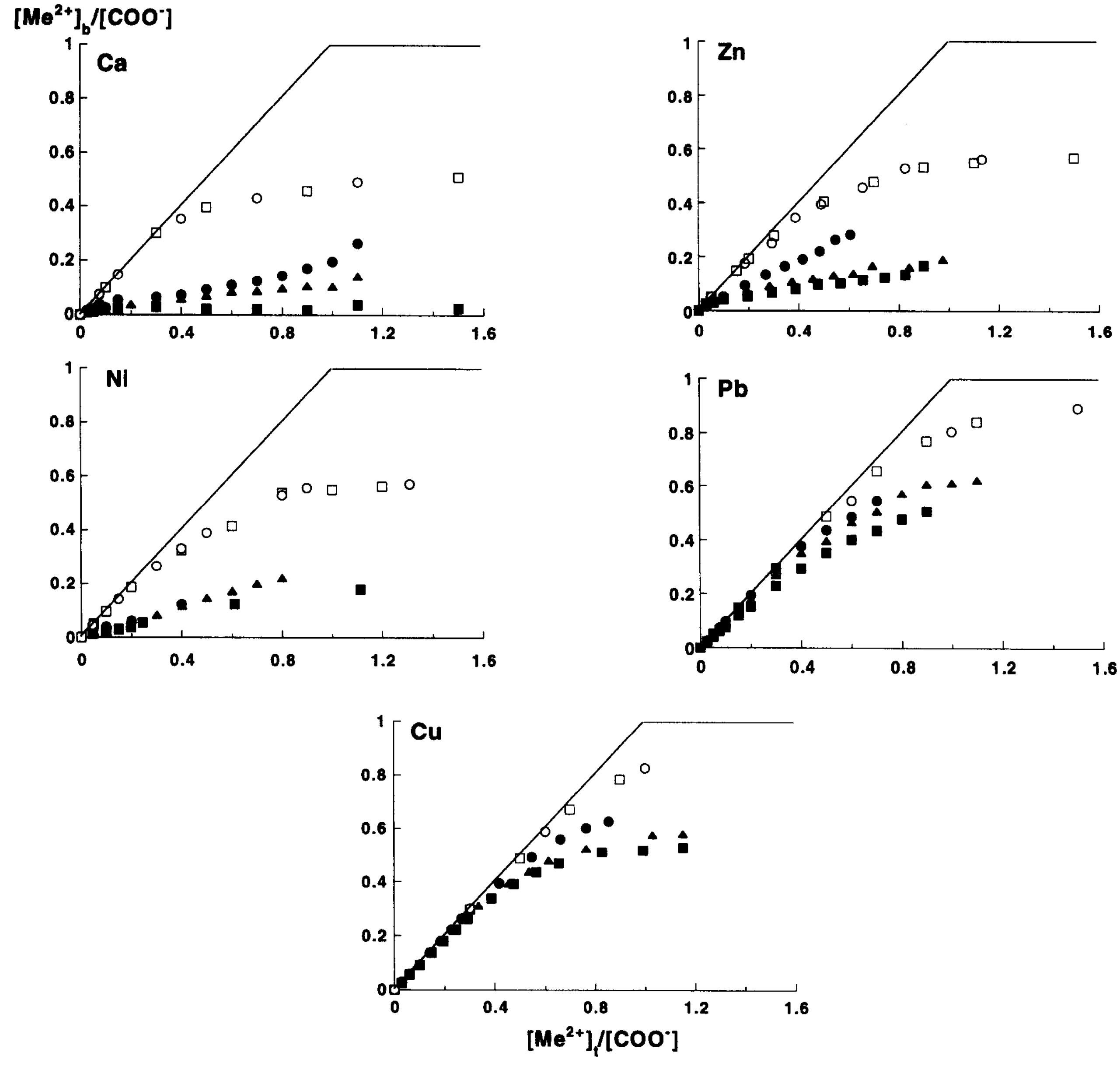

Fig. 3. Influence of the ionic strength and the polymer concentration on the binding isotherm of B58 in water and in 0.1 M $NaNO_3$ at 25°C: (■) 2 mequiv l^{-1}, (▲) 4 mequiv l^{-1}, (●) 8 mequiv l^{-1}. Full symbols: 0.1 M $NaNO_3$, empty symbols: water; (—): stoechiometric isotherm.

terms of Scatchard plots (Scatchard, 1949), ν/L *vs* ν where ν is the ratio $[Me^{2+}]_b/C_p$, L corresponds to $[Me^{2+}]_f$ and C_p to the pectin concentration. In this case, $[Me^{2+}]$ was expressed in mol l^{-1} and C_p in mole of chains l^{-1} in order to express the results with the same variables as used with the Hill approach.

Results are shown for Zn^{2+} and Cu^{2+} ions with B58 (Fig. 6) and C54 (Fig. 7) at 2 mequiv l^{-1}. The same results were obtained for the five cations whatever the pectin concentrations. Results for Ca^{2+} or Ni^{2+} and Pb^{2+} ions are represented by the plots obtained for Zn^{2+} and Cu^{2+} respectively, since they follow the same characteristics.

Scatchard plots obtained for all five metal ions in water showed a clear concave curvature whatever the pectins.

In the presence of supporting salts, Cu^{2+} and Pb^{2+} ions, which are the two more strongly bound ions, gave convex-shaped curves with B58 and C54 whereas a concave curvature was observed with Ca^{2+}, Zn^{2+} and Ni^{2+} with B58 despite the presence of supporting salt. C54 exhibited always convex-shaped curves with the three latter cations.

Either anti-cooperative interactions or the presence of more than one class of binding sites can explain such a concave curvature (Cantor & Schimmel, 1980). However in this case, the hypothesis of more than one class of binding sites is unlikely. Indeed, pectins have

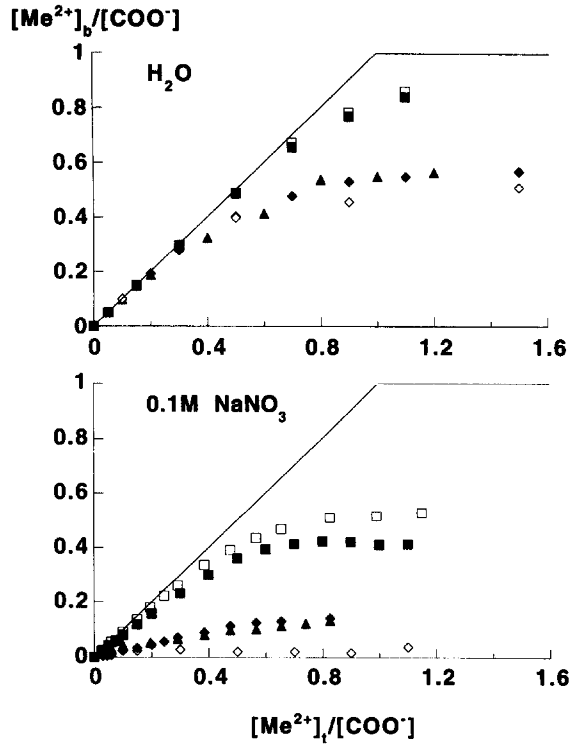

Fig. 4. Influence of the metal ion on the binding isotherms of B58 at 2 mequivl^{-1} in water or in 0.1 M $NaNO_3$ at 25°C: symbols as in Fig. 1; (—): stoechiometric isotherm.

the same class of binding sites for all metal ions in 0.1 M $NaNO_3$. An anti-cooperative mode of interactions was therefore assumed in the case of concave-shaped curves. On the other hand, a cooperative mode of binding is always distinguished by a convex curvature of the plot.

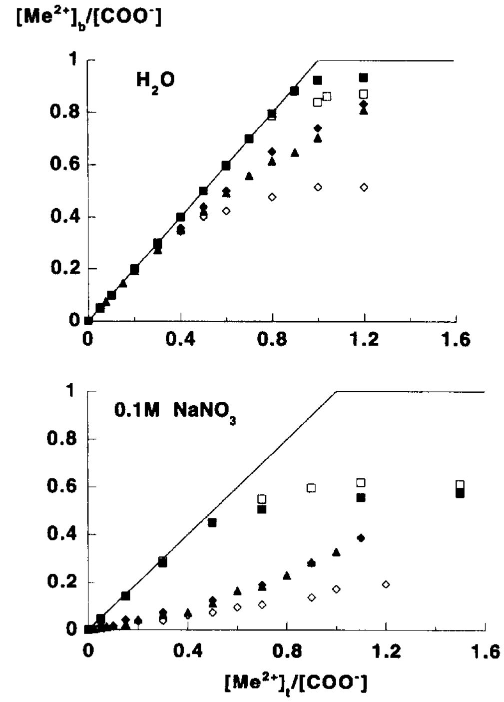

Fig. 5. Influence of the metal ion on the binding isotherms of C54 at 2 mequivl^{-1} in water or in 0.1 M $NaNO_3$ at 25°C: symbols as in Fig. 1; (—): stoechiometric isotherm.

The results found for calcium ions with C54 confirm previous results obtained on apple and citrus pectins (Garnier *et al.*, 1994; Braudo *et al.*, 1992). Anticooperativity in water was also proposed by other authors (Mattai & Kwak, 1986; Lips *et al.*, 1991; Garnier *et al.*, 1994).

As in the case of B58, anticooperativity in the presence of ionic strength was also found for Zn-dextransulfate interactions (Mattai & Kwak, 1981) and its character increased with decreasing ionic strength. Using the equilibrium dialysis technique for studying the binding of copper ions by pectins (DM =0, 52, 72%, structure and origin not specified), Schlemmer & Decker (1993) have pointed out the cooperativity of the binding process. By plotting experimental data in the form of Adair curves (Cantor & Schimmel, 1980), they showed that three different successive modes existed through the binding range (cooperative and non-cooperative binding following by a non-specific association), and this independently of the DM.

Discrepancies still remain with previous works on pectins and other polysaccharides. The binding of calcium ions by pectate, monitored by the DWSM (Mattai & Kwak, 1986), was shown to be anticooperative in the presence of supporting electrolyte in agreement with the results obtained by Lips *et al.* (1991) with apple pectins in presence of excess NaCl, using ion-specific electrode. Some evidence was lately given, by comparing interaction of Mg^{2+} and Ca^{2+} counterions on polygalacturonate, for a cooperative process in the case of Ca-polygalacturonate interactions causing chain-chain association even in salt-free solutions (Malovikova *et al.*, 1994).

In order to further interpret our results obtained from Scatchard plots, we used the semi-empirical approach developed by Hill (Cantor & Schimmel, 1980) and defined by the equation:

$$\ln L = -(1/\alpha_H)\ln[(n_H/\nu) - 1] + \ln K_H$$

where n_H is the number of binding sites per molecule, K_H their apparent dissociation constant (expressed in mole) and α_H the Hill index (dimensionless number) which depends on the type of interactions during the binding process.

Values of the Hill equation parameters, obtained from reliable fitting of the experimental data with $r > 0.99$, are gathered in Tables 2 and 3 for B58 and C54 respectively, at different concentrations either in water or in 0.1 M $NaNO_3$. The value of n_H in the equation was estimated from a theoretical value calculated from the following relationship (Garnier *et al.*, 1994):

$$n_H = \overline{DP}_w \times (GalA/1000) \times (1 - DM/100)$$

with $\overline{DP}_w = \bar{M}_w/176$ (where 176 corresponds to the molar weight of the dehydrated galacturonosyl unit).

Hill plots fitted from experimental data obtained by

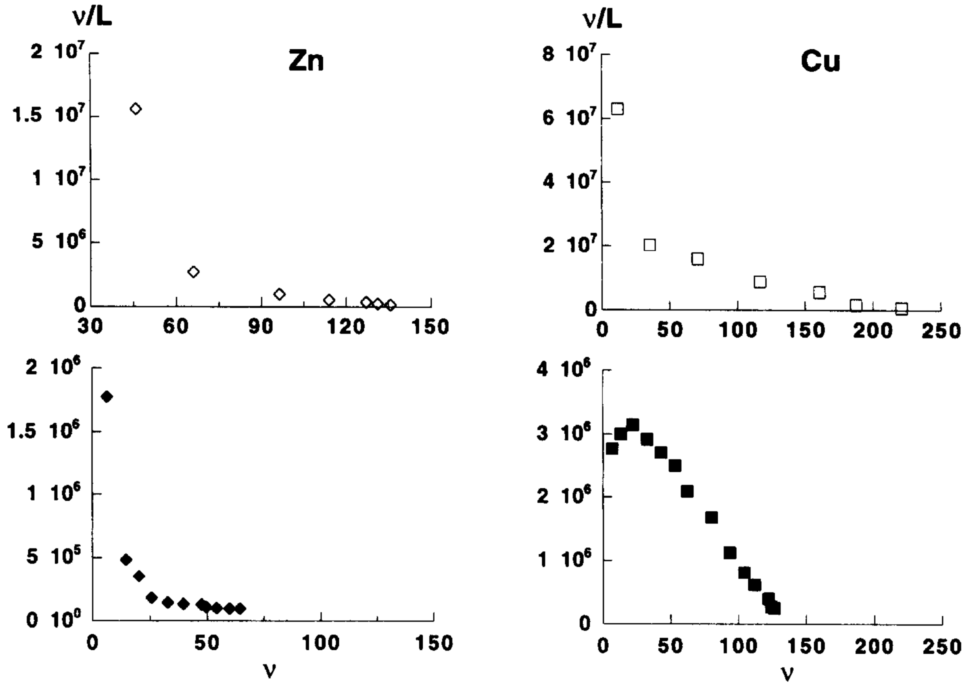

Fig. 6. Influence of the type of pectins and the metal ion on Scatchard representation for B58 at 2 mequiv l^{-1} and 25°C: (◇) Zn^{2+} (□) Cu^{2+}. Full symbols: 0.1 M NaNO$_3$, empty symbols: water.

both types of titration of calcium ions gave similar α_H values (pectin concentration of 4 mequiv l^{-1} in water: $\alpha_H = 0.59$ by DWSM and 0.58 by potentiometry, average value of two experiments) showing again that the results do not depend on the mode of titration.

In water, values of $\alpha_H < 1$, indicating an anti-cooperative process, were found for Me-pectins interactions whatever the pectins and the metal ion as expected from Scatchard plots. The anti-cooperative character tended therefore to decrease (increasing α_H values) together with the apparent association constant values (decreasing K_H^{-1} values) when the polymer concentration increased.

In the presence of supporting salt, values of $\alpha_H > 1$,

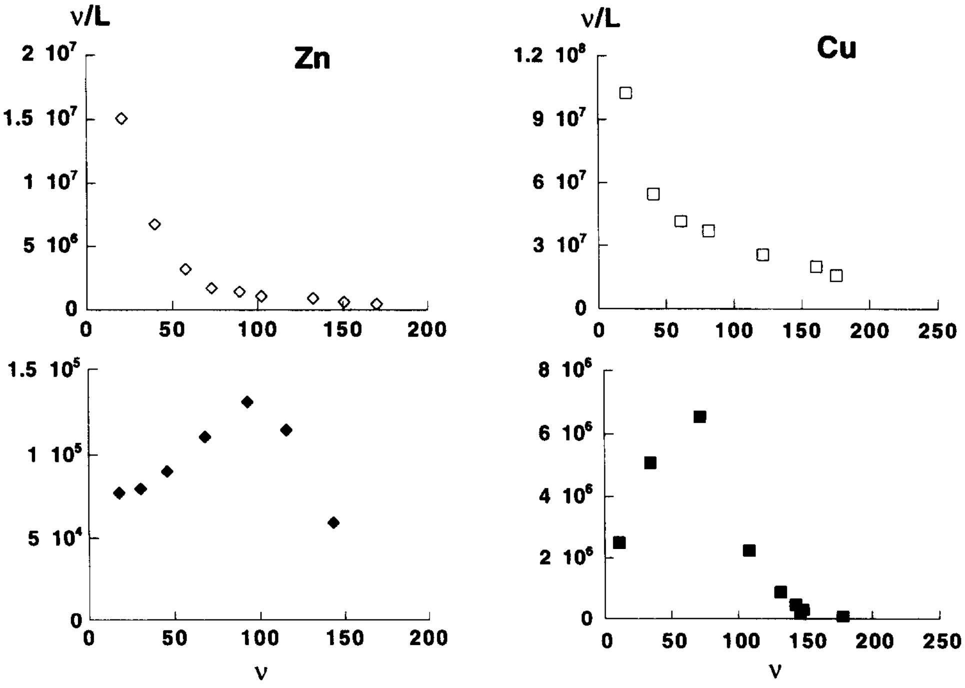

Fig. 7. Influence of the type of pectins and the metal ion on Scatchard representation for C54 at 2 mequiv l^{-1} and 25°C: (◇) Zn^{2+}, (□) Cu^{2+}. Full symbols: 0.1 M NaNO$_3$, empty symbols: water.

Table 2. Values of the Hill equation parameters for sugar-beet pectins (B58)*

	1 mequiv l^{-1}		2 mequiv l^{-1}		4 mequiv l^{-1}		8 mequiv l^{-1}		12 mequiv l^{-1}	
	α_H	K_H^{-1}	α_H	K_H^{-1}	α_H	K_H^{-1}	α_H	K_H^{-1}	α_H	K_H^{-1}
Zn water			0.35	306			0.35	5		
0.1 M $NaNO_3$	0.37	3	0.47	38	0.54	61	0.87	96		
Ni water			0.37	171			0.42	16		
0.1 M $NaNO_3$	0.79	54	0.85	71	0.89	86				
Ca water			0.25	33			0.28	1		
0.1 M $NaNO_3$			0.48	2	0.59	5	0.65	10	0.78	19
Cu water			0.62	13 869			0.70	1125		
0.1 M $NaNO_3$	1.10	4614	0.96	4957	1.13	5751	0.93	5930		
Pb water			0.61	12 860			0.79	1103		
0.1 M $NaNO_3$	0.83	798	0.88	1390	1.06	2720	0.91	3950	1.11	6122

*K_H^{-1} in mole^{-1}.

Table 3. Values of the Hill equation parameters for citrus pectins (C54) at 2 mequiv l^{-1}*

	α_H	K_H^{-1}
Zn water	0.41	641
0.1 M $NaNO_3$	1.16	359
Ni water	0.49	885
0.1 M $NaNO_3$	1.31	293
Ca water	0.21	61
0.1 M $NaNO_3$	1.11	7
Cu water	0.67	49 708
0.1 M $NaNO_3$	2.24	45 796
Pb water	0.48	37 520
0.1 M $NaNO_3$	1.23	10 620

*K_H^{-1} in mole^{-1}.

attesting a cooperative mode of the binding process, were found for C54 whatever the metal ion. However, with B58, values of $\alpha_H \geq 1$ were found for Cu^{2+} and Pb^{2+} whereas values of $\alpha_H \ll 1$ but increasing with C_p were found for Ca^{2+}, Ni^{2+} and Zn^{2+}, confirming results obtained from Scatchard plots.

The discrepancies between the clear convex-shaped Scatchard plots and values of α_H close to 1 may be ascribed to the fitting of experimental data by the Hill equation. Indeed, Hill indexes, found by this method, may underestimate the degree of cooperativity in the case of very asymmetric Scatchard curves.

Evaluation of the formation constants was also dependant on the precision of the fitting of experimental data. The apparent association constants K_H^{-1} had higher values for each cation in water than in 0.1 M $NaNO_3$ as expected from their respective level of binding isotherms. The following order of affinity may be therefore established by comparing K_H^{-1} values between cations and was similar to that evidenced by pH-studies and by comparing binding isotherms: $Cu^{2+} \sim Pb^{2+} \gg Zn^{2+} \sim Ni^{2+} > Ca^{2+}$.

As previously reported (Garnier *et al.*, 1994), cooperative interactions would only take place when two polymeric chains are close enough to form junction zones i.e. when polyelectrolyte effects are screened at high ionic strengths. Hence, the cations that are more weakly bound (Zn^{2+}, Ni^{2+} and Ca^{2+}) by simple electrostatic interactions (outer-sphere complexes), and the more strongly bound cations, are both capable of cooperativity. Nevertheless, the anticooperative interactions found for B58 in the presence of supporting salts can not be explained by such an assumption. The difference of behaviour between B58 and C54 in the presence of supporting salts may be ascribed to the presence of acetylated hydroxyl functions on C_2/C_3 of the galacturonosyl units of sugar-beet pectins. The higher proportion of 'hairy' regions in sugar-beet pectins should not play a significant role since both pectins have similar 'smooth' regions. The presence of acetyl functions probably creates some steric hindrance decreasing the affinity of Me^{2+} for sugar-beet pectins. Indeed, values of K_H^{-1} were lower with B58 than with C54 for all cations. Acetyl groups on sugar-beet pectins (DAc = 31.3%) did not appear to play an important role in the dissociation of the carboxyl functions (Thibault, 1986) but Kohn & Furda (1968) noticed that acetyl derivatives of pectins had a lower binding constant with calcium ions than the original pectins. This decreasing affinity can be related to the poor gelling power of the acetylated pectins (Thibault, 1986). Only the cations (Cu^{2+} and Pb^{2+}) that are the more strongly bound, by formation of largely covalent bonds (inner-sphere complexes), are able to favour the fixation on a neighbouring binding site which leads to the formation of junction zones, allowing cooperativity.

CONCLUSION

Binding of some divalent cations by pectins from citrus and sugar-beet of close DM has shown the same clear scale of selectivity: $Pb^{2+} \sim Cu^{2+} \gg Zn^{2+} > Cd^{2+} \sim$

$Ni^{2+} \geq Ca^{2+}$ as can be found with other polycarboxylates (Axelos *et al.*, 1994). This is the first report of a difference between the type of interactions during the binding process of divalent cations by pectins of different origin: citrus and sugar-beet pectins. This difference was interpreted in terms of difference of chemical structure, namely the presence of acetyl groups close to ionic sites on sugar-beet pectins. Further studies of binding of divalent cations could be carried out with pectins which possess varying degrees of substitution, as acetylation, to evaluate the impact on the binding process and to link this parameter to the gelation phenomenon.

Lastly, our results showed that pectins have high capacities for retention of heavy metals with a particular scale of selectivity making pectins or pectins-containing cell-wall matrix suitable for their use in purification of industrial streams containing toxic metals.

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