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Multivariate analysis of uronic acid and neutral sugars in whole pectic samples by FT-IR spectroscopy

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Abstract

The sequential extraction of the cell wall material (CWM) of olive and orange pulps and its subsequent fractionation by ethanol precipitation and anion-exchange chromatography gave a wide range of cell wall polysaccharide fractions. The fractions rich in pectic polysaccharides, characterised by the presence of uronic acid, rhamnose, arabinose and galactose monosaccharides released by acid hydrolysis, were selected, 13 from olive and 11 from orange. The FT-IR spectrum of a pectic polysaccharide sample shows characteristic absorbances in the region between 1200 and 850 cm^{-1}; samples rich in uronic acid show two intense peaks at 1110 and 1018 cm^{-1}. Although the peaks are readily observable, simple quantification is difficult because of the underlying spectral structure. To verify if this region is useful for the characterisation of the pectic polysaccharides, a multivariate analysis was performed. This region allows prediction of the uronic acid and neutral sugars' pectic side chains of the two distinct fruits. In order to highlight the selective wavenumbers for the determination of the sugar components of the pectic polysaccharides from olive and orange, it was performed on a selection of variables based on a mathematical method that uses the signal to noise ratio. This study showed that the FT-IR spectra have the potential to be an important source of information for a quick evaluation of the polysaccharide composition of samples of pectic origin.

Keywords: FT-IR spectroscopy; Pectic polysaccharides; Multivariate analysis; Variable selection; Uronic acid; Neutral sugars

Abbreviations: Ara, arabinose; CDTA, *trans*-1,2-cyclohexane-diamine-*N*,*N*,*N*′,*N*′-tetraacetate; CR, cellulose-rich residue; CWM, cell wall material; FT-IR, Fourier transform infrared; Gal, galactose; NIPALS, non-linear iterative partial least squares; PC, principal component; PCA, principal component analysis; PCR, principal component regression; PLS, partial least squares regression; Rha, rhamnose; RMSEP, root mean square error of prediction; Ur.Ac, uronic acid

1. Introduction

'Pectic substances' is the general term used to refer to a range of polysaccharides consisting mainly of esterified residues of galacturonic acid (pectin), its de-esterified residues (pectic acid), and its salts (pectate). Also included are a range of neutral and highly branched arabinose- and/or galactose-rich polymers that are closely associated as side chains of the rhamnogalacturonan backbone (Selvendran, 1985; Bacic et al., 1988). Plant cell walls of non-lignified tissues of fruits and vegetables are sources of pectic substances (McDougall et al., 1996).

The uronic acid content of the pectic substances can be quantified by various chemical methods. Usually, the monosaccharides are released by acid hydrolysis not withstanding the difficulty of achieving quantitative results due to the stability of the glycosyluronic acid linkage (Selvendran and O'Neill, 1987; Fry, 1988; Quigley and Englyst, 1994). Other methods of hydrolysis, such as the combination of enzymic hydrolysis and methanolysis have also been proposed (Quemener et al., 1993). After hydrolysis, the methods most used for quantification of uronic acid are colorimetric methods that involve the reaction with *m*-hydroxydiphenyl in concentrated sulphuric acid media (Blumenkrantz and Asboe-Hansen, 1973; Filizetti-Cozzi and Carpita, 1991). The use of HPLC for quantification of the uronic acid is also possible (Quemener et al., 1993; Quigley and Englyst, 1994). Neutral sugars such as arabinose,

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galactose, rhamnose and xylose, usually present in pectic substances can be quantified after hydrolysis by gas chromatography as their alditol acetates (Selvendran and O'Neill, 1987; Coimbra et al., 1996a). Analysis of the monosaccharide composition of the pectin substances by traditional chemical methods is time consuming and expensive.

The use of infrared spectroscopy on pectic substances was previously applied to distinguish between high and low methoxyl contents (Reintjes et al., 1962), and was proven a useful tool to distinguish and evaluate the methoxyl content of different commercial pectins with high and low levels of esterification (Haas and Jager, 1986). The quantification of pectins by infrared spectroscopy was proposed by Bociek and Welti (1975) using the ester band at 1740 cm^{-1}, the amide I band at 1650 cm^{-1}, and the carboxylate band at 1607 cm^{-1}. The FT-IR was also proposed to be used for monitoring cell wall extraction providing both information about the solubilized polymers and about the structure of the remaining insoluble material (McCann et al., 1992). The carbohydrates show high absorbances in the region 1200–950 cm^{-1} which is within the so-called 'fingerprint' region, where the position and intensity of the bands are specific for every polysaccharide, allowing its possible identification (Filippov, 1992). In this region it is not possible to assign an absorbance at a specific wavenumber to a specific bond or functional group due to overlaps. The traditional univariate calibration does not allow reliable predictions. The multivariate calibration can be a useful method to overcome this problem. Recently, FT-IR spectra and multivariate analysis, namely Partial Component Analysis (PCA), was used to evaluate the authenticity and discrimination of products of vegetable origin (Kemsley et al., 1994; Defernez et al., 1995; Briandet et al., 1996).

Partial Least Squares Regression (PLS) is a procedure used to model the relationship between a set of predictor variables X (N objects $\times$ K variables) and a set of response variables Y (N objects $\times$ M response). In this study there is only one response in Y (concentration), thus Y has N (objects) $\times$ 1 (response) dimensions. This procedure was applied to construct calibration models for quantification of the uronic acid/neutral sugars ratio in pectins from olive and orange pulps using FT-IR spectroscopy. The PLS regression procedure has the advantage of accepting more variables than objects in the data and of avoiding the problem of collinearity among variables. In contrast with other multivariate regression methods such as Multilinear Regression and Principal Component Regression (PCR), the PLS procedure also uses the information present in Y to build the prediction model. The classical PLS regression method is a special case of Non-linear Iterative Partial Least Squares (NIPALS) method, in which the information explained by each dimension is subtracted from the X matrix in an iterative process, until all the important variance is extracted (Geladi and Kowalski, 1986).

The PLS regression procedure may be written as:

$$Y = XB + F$$

where Y are the dependent variables, X the independent variables (spectra), B is the B coefficients matrix and F the error matrix. The regression model is generated by calculating the B coefficients matrix which minimises the F error matrix.

The main difference between this and other regression procedures is in the way the B matrix is calculated. In the PLS procedure the information present in the Y matrix is used in the calculation of B coefficients.

Usually the infrared spectra obtained in many studies involve the use of many wavelengths (or wavenumbers), as opposed to the number of observations. The presence of non-linearities and the fact that normally the spectra obtained have more than one component involved in the analysis, leads to the need for regression procedures such as PLS and PCR. However, it is desirable to find a method that can highlight those wavelengths that are highly selective for the component that one wishes to characterise. Using only those highly selective wavelengths has the advantage of using simple least squares analysis and thus focuses only on the main characteristics of a component.

2. Materials and methods

2.1. Sample origin

Pectic material from olive and orange pulps was obtained from the respective cell wall materials by sequential extraction with aqueous solutions following the methods described by Coimbra et al. (1996a,b) (Fig. 1). The cell wall material (CWM) prepared from olive and orange pulps (1 g) were sequentially extracted with (i) water, 100 ml at 20°C for 2 h (sample 14); (ii) 50 mM *trans*-1,2-cyclohexane-diamine-*N*,*N*,*N*′,*N*′-tetraacetate Na salt (CDTA), 100 ml, pH 6.5 at 20°C for 6 h (CDTA-1, sample

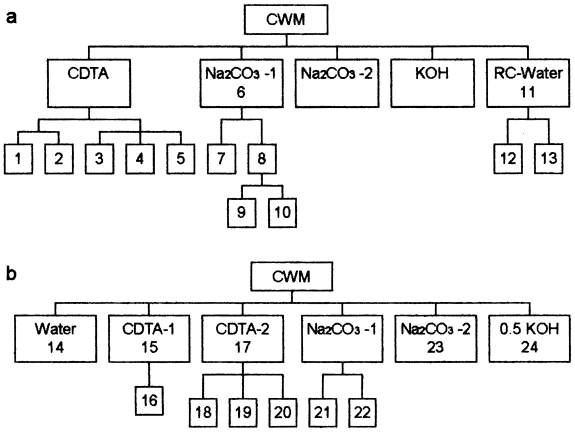

Fig. 1. Experimental design of the samples obtained from (a) olive; and (b) orange cell wall material.

15); (iii) 50 mM CDTA (100 ml), pH 6.5 at 20°C for 2 h (sample 17); (iv) 50 mM Na_2CO_3 + 20 mM $NaBH_4$ (100 mL) at 1°C for 16 h (sample 6); (v) 50 mM Na_2CO_3 + 20 mM $NaBH_4$ (100 ml) at 20°C for 3 h (sample 23); (vi) 0.5 M KOH + 20 mM $NaBH_4$ (75 ml) at 1°C for 2 h (sample 24); (vii) 1 M KOH + 20 mM $NaBH_4$ (75 ml) at 1°C for 2 h; (viii) 1 M KOH + 20 mM $NaBH_4$ (75 ml) at 20°C for 2 h; (ix) 4 M KOH + 20 mM $NaBH_4$ (75 ml) at 20°C for 2 h; and (x) 4 M KOH + 3.5% H_3BO_3 + 20 mM $NaBH_4$ (75 ml) at 20°C for 2 h. The alkali extractions were carried out with O_2-free solutions under argon. After each extraction, solubilized polymers were separated from the insoluble residue by centrifugation (CDTA and Na_2CO_3 extracts) or by filtration through G1 glass sinter (KOH extracts). All extracts were filtered through GF/C and dialysed exhaustively; Na_2CO_3 and KOH extracts were acidified to pH 5 with acetic acid prior to dialysis. Precipitates formed during dialysis of alkali extracts were collected separately. The cellulose-rich residues (CR) remaining after the final alkali extracation (4 M KOH + borate) were suspended in water (25 ml) and the solutions were acidified to pH 5 and dialysed. The supernatants of the CR dialysis were collected separately by centrifugation from the CR1 residue (sample 11). All extracts collected after dialysis were concentrated and freeze-dried. In order to have a wide range of separated polymers, several selected fractions were submitted to graded precipitation with ethanol. The polymers were dissolved in water and precipitated by the addition of ethanol, as described by Coimbra et al. (1996b). The samples obtained by graded ethanol precipitation were 3, 4 and 5 (from CDTA-1); samples 7 and 8 (from sample 6); samples 12 and 13 (from sample 11); sample 16 (from sample 15); samples 18, 19 and 20 (from sample 17); and samples 21 and 22 (from Na_2CO_3-1). Some fractions (CDTA-1 and sample 8) were submitted to anion-exchange chromatography, DEAE-Trisacryl M columns, as described by Redgwell and Selvendran (1986). The samples obtained by anion-exchange chromatography were samples 1 and 2 (from CDTA-1) and samples 9 and 10 (from sample 8).

2.2. Carbohydrate analysis

Neutral sugars were released by Saeman hydrolysis (Selvendran et al., 1979) and analysed as their alditol acetates by gas–liquid chromatography (Blakeney et al., 1983; Harris et al., 1988) using a Hewlett-Packard 5890 with a split injector (split ratio 1:60) and a FID detector. A 25 m column CP-Sil-43 CB, (Chrompack, Holland) with I.D. 0.15 μm and 0.20 μm film thickness was used. With the injector and detector operating at 220°C, the following temperature program was used: 180°C for 5 min and 200°C for 20 min, with a rate of 0.5°C/min. Linear velocity of the carrier gas (H_2) was set at 50 cm/s at 200°C. Uronic acids were determined colorimetrically by a modification (Coimbra et al., 1996a) of the Blumenkrantz and Asboe-Hansen (1973) method.

2.3. FT-IR spectra

FT-IR spectra of the pectic materials were obtained at a resolution of 8 cm^{-1}. The samples were incorporated into KBr (spectroscopic grade) and pressed into a 1-mm pellet. Spectra were recorded at the absorbance mode from 4000 to 400 cm^{-1} in a Nicolet Magna-IR 550 (for the olive samples) and Brucker IFS-55 (for the orange samples). Three replicate spectra were collected for each sample.

2.4. Data pretreatment

Each spectrum was baseline corrected, normalised, and centred. The spectra were transferred via a JCAMP.DX format (Rutledge and McIntyre, 1992) into the data analysis software package developed in our laboratories. The data analysis treatment consisted initially of a PCA followed by a PLS1.

The method adopted to perform the wavenumber selection was proposed by Brown (1992). The kernel of this method uses a subset of q wavenumbers ($j \in \{1..q\}$) that minimises the length of a confidence interval for a known composition k. It selects q' wavenumbers that correspond to the q' largest values of signal-to-noise ratio $\frac{\beta_j^2}{\sigma_j^2}$. The procedure, discussed by Brown et al. (1991) gives the following formulation:

$$\frac{c(\eta)}{\sqrt{\left(\sum_{1}^{q'} \frac{\beta_j^2}{\sigma_j^2}\right)}} \quad (1)$$

where $1 - \eta$ is the confidence level and $c(\eta)$ is the square root of the tabulated chi-squared values on q' degrees of freedom. It can be seen that the number of wavenumbers is a monotonically decreasing function of the significance level η (Brown, 1992).

The methodology to choose the q' wavenumbers involves the sorting in decreasing order of the ratio $\frac{\beta_j^2}{\sigma_j^2}$ until Eq. (1) is minimised. If a wavenumber is selective for a component, then this ratio will be large if the other components or the matrix effect are not important for this wavenumber. This is related to the fact that the presence of other effects at this wavenumber will increase the estimates of σ.

3. Results and discussion

3.1. Selection of samples

The sequential extraction of the cell wall material (CWM) of olive and orange pulps and its subsequent fractionation by ethanol precipitation and anion-exchange chromatography gave a wide range of fractions rich in cell wall polysaccharides. The determination of its carbohydrate composition allowed the assessment of the type of polymers present in the different fractions. The fractions rich in pectic

polysaccharides, characterised by the presence of uronic acid (Ur.Ac), rhamnose (Rha), arabinose (Ara) and galactose (Gal) monosaccharides released by acid hydrolysis, were selected, 13 from olive and 11 from orange. Table 1 shows the sugar composition of the selected pulp cell wall pectic fractions. The olive samples show that the major sugar component of its pectic polysaccharides are Ur.Ac and Ara. These two residues vary considerably according to the sample; Ur.Ac ranges from 19 to 77 mol% and Ara from 19 to 75 mol%. Gal and rhamnose are minor constituents in olive pulp pectic polysaccharides. The orange samples show that the major sugar components of its pectic polysaccharides are Ur.Ac, Ara and Gal. Ara and/or Gal occur in pectic polysaccharides as the sugar components of neutral side chains and are the major neutral sugars in these polymers (de Vries et al., 1984). Ur.Ac content ranges from 21 to 86 mol%, Ara, from 6 to 50 mol%, and Gal, from 2 to 22 mol%. The amount of neutral sugars ranges from 11 to 63 mol%. The last column of Table 1 gives the percentage of the total sugars present in each sample, on a weight percentage basis. The results obtained showed that the recovery of the glycosidic residues after acid hydrolysis, for the majority of the samples, was not 100%. Olive pectic fractions showed a recovery that ranged from 50 to 91% and orange pectic fractions showed a recovery from 55 to 100%. Experiments by other authors showed that the acid hydrolysis of the glycosidic linkage of the component sugar residues may not be complete (Selvendran and O'Neill, 1987; Fry, 1988; Quigley and Englyst, 1994) but, if submitted to a large extension, several labile sugars such as the Ur.Ac and the Ara residues may degrade. Olive pectic samples present more acid liability than orange pectic samples (P. Vasco, M.A. Coimbra, M. Barros and I. Delgadillo, unpublished results). These results allowed us to infer that the extension of acid hydrolysis is dependent on the type and origin of the pectic samples. When the Ur.Ac was expressed as a weight fraction of the total starting material, the different extensions of the acid hydrolyses were reflected in the total amount of the sugar residues and poor correlation with the FT-IR spectra was obtained. However, when it was expressed as mol% of the recovered material, much better correlation was obtained. This supports the assertion that there was variable recovery of total sugars.

3.2. FT-IR spectra

Fig. 2a shows the FT-IR spectrum of a pectic polysaccharide sample rich in Ur.Ac. The spectrum shows absorbances at wavenumbers characteristic of cell wall pectic polysaccharides (McCann et al., 1992); 3440 cm^{-1} OH, 1750 cm^{-1} ester, 1630 cm^{-1} carboxylate, and 1200–850 cm^{-1} carbohydrate. Fig. 2b shows the 1200–850 cm^{-1} region of three pectic samples with different amounts of Ur.Ac: the sample richest in Ur.Ac (Fig. 2a, spectrum III) shows two intense peaks at 1100 and 1018 cm^{-1}. In the sample with the lowest Ur.Ac content (Fig. 2a, spectrum I) the intensity of these peaks diminished; spectrum II, with an intermediate Ur.Ac content, shows peaks with intermediate intensity. Although the peaks are readily

Table 1
Sugar composition of cell wall pectic polysaccharides from olive and orange pulps

Sample number	Origin	Ur.Ac. (mol%)	Ara (mol%)	Gal (mol%)	Neutral sugars (mol%)	Total (mol%)	Total sugar (weight %)
1	Olive	77	19	1	20	98	71
2		75	23	1	24	100	55
3		69	27	1	28	99	51
4		72	23	2	25	100	69
5		63	30	1	31	96	56
6		50	43	3	46	98	72
7		50	41	3	44	97	50
8		52	41	2	43	96	78
9		19	71	4	75	97	58
10		25	68	3	71	99	80
11		30	62	3	65	97	78
12		25	70	3	73	100	84
13		21	75	2	77	100	91
14	Orange	62	24	2	26	89	100
15		77	19	2	21	99	80
16		70	16	6	22	94	99
17		86	6	5	11	97	59
18		75	13	4	17	94	55
19		81	12	3	15	97	62
20		69	12	13	25	97	91
21		61	20	16	36	100	93
22		52	30	12	42	100	100
23		36	41	22	63	100	100
24		21	50	9	59	81	92

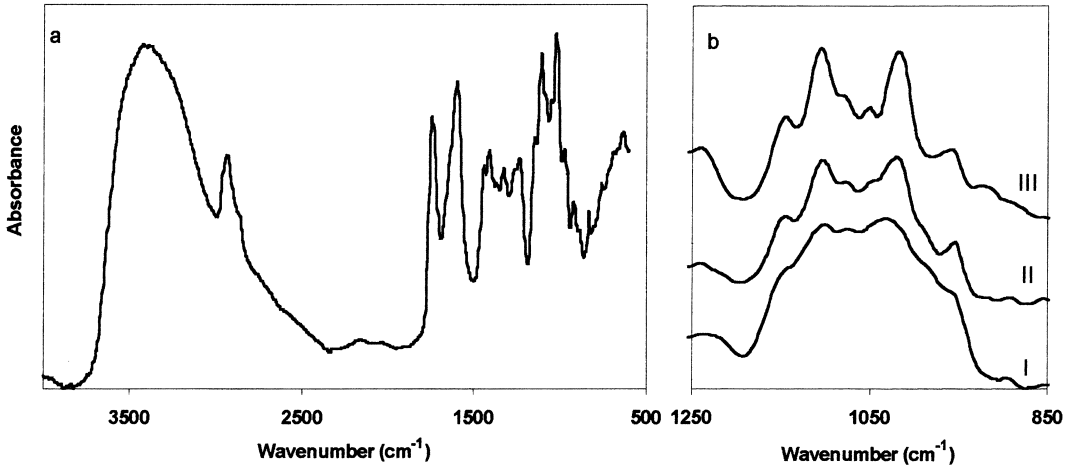

Fig. 2. (a) FT-IR spectrum of the 4000–500 cm^{-1} region of a orange pectic polysaccharide sample. (b) FT-IR spectra of the 1250–850 cm^{-1} region of three olive pectic samples with distinct uronic acid content (I, 21 mol%; II, 50 mol%; III, 77 mol%).

observable, simple quantification is difficult because of the underlying spectral structure. To verify if this region is useful for the characterisation of the pectic polysaccharides, a multivariate analysis was performed. As this region is not directly related to the degree of esterification nor acetylation of carbohydrates, the samples were used independently of the amount of esterified and acetylated residues present.

3.3. Analysis of the olive data set

To study the internal data structure, a PCA was performed on this data set. The scores plot (Fig. 3a) showed three distinct groups and PC1, which explains 61.4% of the data variability, is related to the relative amount of Ur.Ac and neutral sugars present in the samples.

In order to establish a calibration model and the identification of the most important wavenumbers for the evaluation of Ur.Ac and neutral sugars, PLS1 and variable selection were conducted. As the amount of Ur.Ac and neutral sugars on a molar percentage basis are highly anti-correlated, only the Ur.Ac was used in the calibration procedure. In order to remove undesirable scale variations (Martens and Naes, 1989), the molar percentages were recalculated only for Ur.Ac, Ara, and Gal. The PLS1 regression procedure was applied using the amount of Ur.Ac as mol% in the *Y* vector. Four factors, estimated by internal

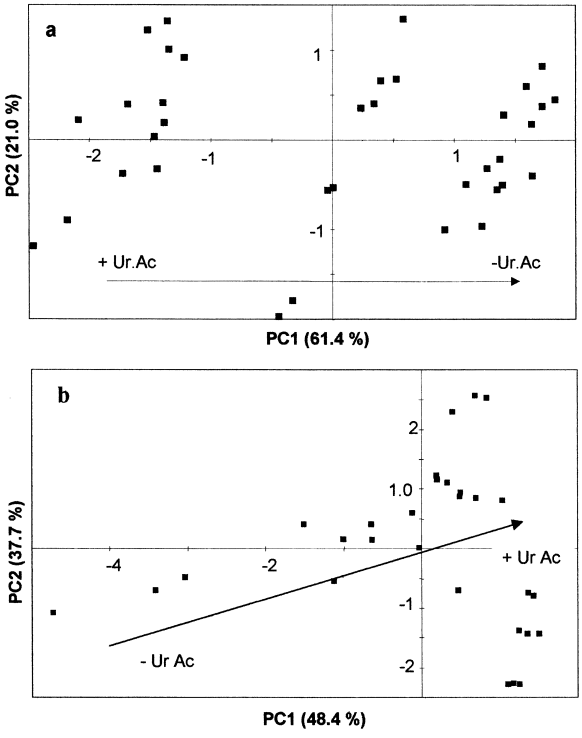

Fig. 3. Scores scatter plot (PC1 vs PC2) for (a) olive data set; (b) orange data set.

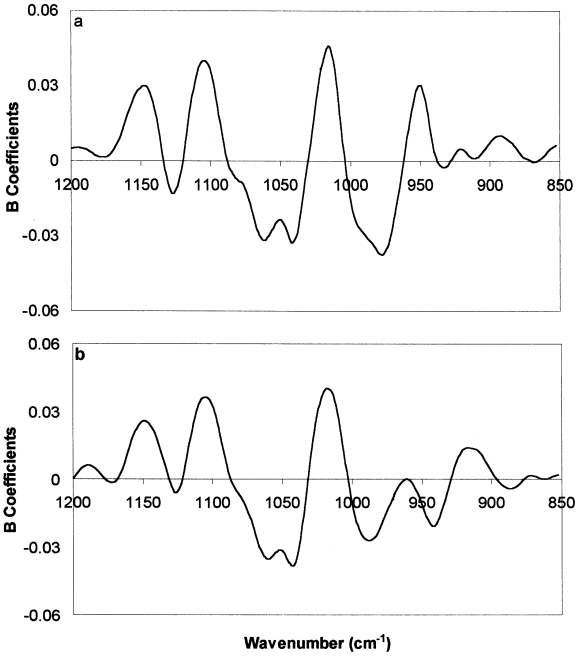

Fig. 4. *B* coefficients plots for determination of Ur.Ac for (a) olive data set; (b) orange data set.

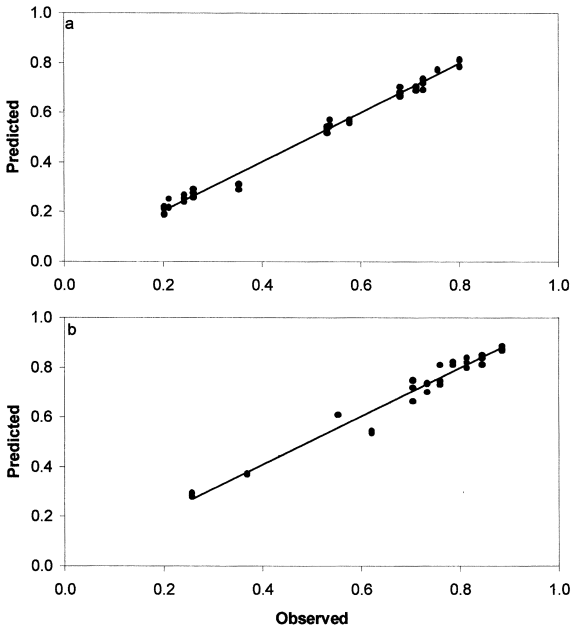

Fig. 5. Regression lines for the relative amount of Ur.Ac in (a) olive data set; (b) orange data set.

cross-validation, were required to obtain a predictive ability. Fig. 4a shows the *B* coefficients vector plot with which it is possible to characterise the most important wavenumbers with regard to the prediction of Ur.Ac and, at the same time, the neutral sugars. Positive peaks are related to samples with a high Ur.Ac content and a low neutral sugars content; conversely, negative peaks are related to samples with high neutral sugars content and low Ur.Ac. content. The regression model obtained (Fig. 5a), had a correlation of 0.989 and a RMSEP (root mean square error of prediction) of 2.01%.

The method for variable selection, as explained in the Materials and Methods section, was applied to select the most important wavenumbers for the determination of Ur.Ac. In decreasing order of importance, the relevant wavenumbers obtained were: 1011, 1038, 1041, 1045, 1061, 1065, 1099, 1103, 1107, 1111, 984 cm^{-1} (Fig. 6a). When a PLS1 regression model was applied in order to evaluate the predictive ability of these selected wavenumbers for the determination of Ur.Ac, it was found that two factors were necessary, and a correlation of 0.967 and a RMSEP of 3.54% were obtained. These results showed that the selected

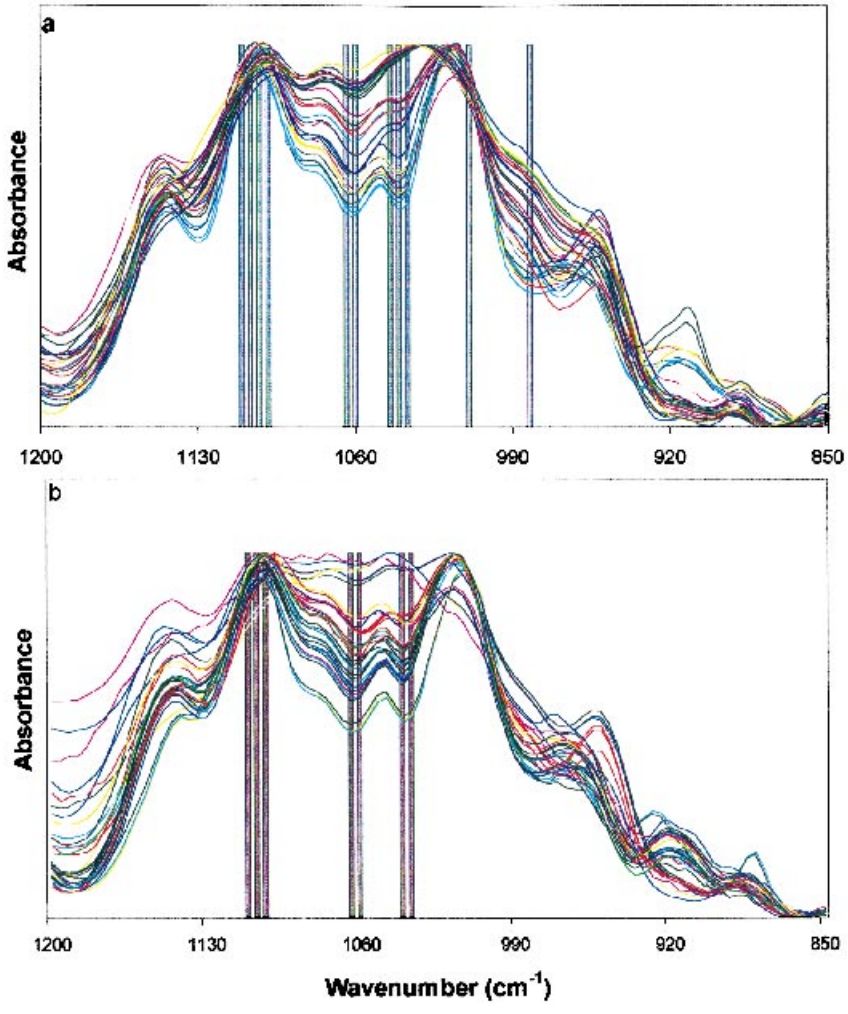

Fig. 6. Selected wavenumbers from (a) olive data set; (b) orange data set.

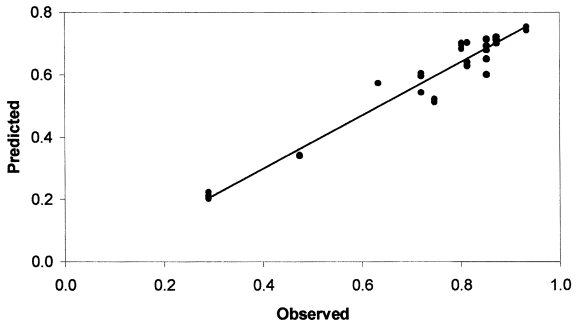

Fig. 7. Regression line of the model transfer from olive to orange.

wavenumbers were highly related to the relative amount of Ur.Ac and neutral sugars, and can be used for a quick evaluation of the relative content of these sugar residues in olive pectic samples.

3.4. Analysis of the orange data set

To study the internal data structure of the orange data set a PCA was performed. The scores plot of the first two PCs (Fig. 3b), as observed in olive, showed that PC1, which explains 48.4% of the data variability, is related to the relative amount of Ur.Ac and neutral sugars.

The PLS1 regression model build for this data set showed that the dependent variables, Ur.Ac and the sum of the Ara and Gal content (neutral sugars) are highly anti-correlated. Therefore, the amount of Ur.Ac and neutral sugars were expressed as relative mol%. The relative amount of Ur.Ac was used in the *Y* vector as a dependent variable. Three factors, estimated by internal cross-validation, were required to obtain a predictive ability. Fig. 4b shows the *B* coefficients vector plot, from which it is possible to characterise the most important wavenumbers with regard to the prediction of Ur.Ac and, therefore, the neutral sugars. Positive peaks are related to samples with a high Ur.Ac content and a low neutral sugars content; conversely, negative peaks are related to samples with high neutral sugars content and low Ur.Ac. content. The regression model obtained (Fig. 5b), had a correlation of 0.972 and a RMSEP of 2.38%.

The most important wavenumbers for the prediction of the relative amounts of Ur.Ac and neutral sugars, in decreasing order of importance, were: 1037, 1041, 1061, 1065, 1103, 1107, 1111 cm^{-1} (Fig. 6b). To evaluate the predictive ability of these wavenumbers, a PLS1 regression procedure was applied with only these selected wavenumbers (three PCs were still required). A correlation of 0.958 and a RMPSEP of 2.94% were obtained. These results, not very different from those obtained with the all the wavenumbers, showed that the selected wavenumbers were highly related to the amount of Ur.Ac and neutral sugars, and can be used for a quick evaluation of the relative content of sugar residues in orange pectic samples.

3.5. Model transfer

The comparisons of the *B* coefficients vector (Fig. 4a,b) obtained from the olive and orange data sets show slightly similar profiles in the region between 1200 and 995 cm^{-1}. The region between 995 and 850 cm^{-1} shows a very distinct profile, which can be due to the presence of higher amounts of Gal. In order to see if it was possible to predict the amount of Ur.Ac and neutral sugars for the orange pectic samples using the regression model of the olive pectic samples, the regression model for the olive data set was applied. The regression model obtained (Fig. 7), had a correlation of 0.948 and a RMSEP of 11.64%.

3.6. Identification of Ur.Ac, Ara and Gal

In order to compare the contribution of each glycosidic residue to the FT-IR spectra, a tentative identification was

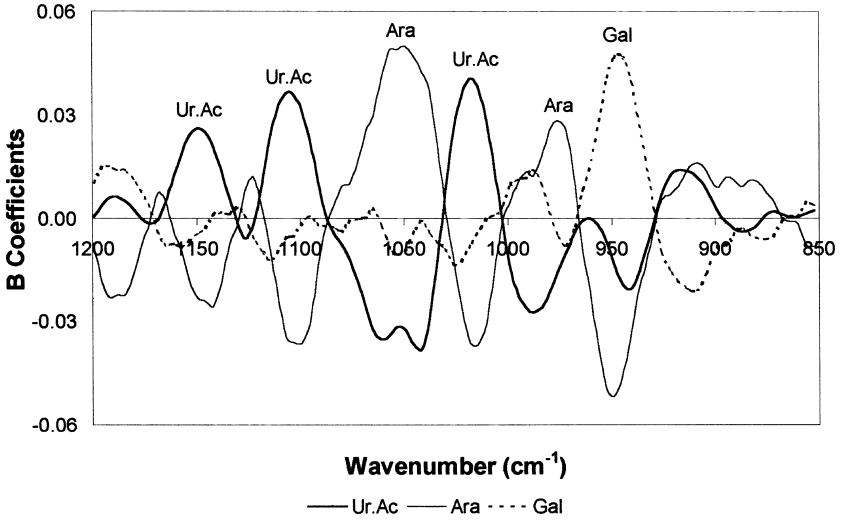

Fig. 8. *B* coefficient plots for the relative amounts of Ur.Ac, Ara, and Gal for orange data set.

performed by multivariate analysis for the relative amount of each sugar, Ur.Ac, Ara, and Gal, using the orange data set. Fig. 8 shows that, for Ur.Ac, the main absorbance regions are at 1150, 1110, and 1020 cm^{-1} (thick line); for Ara, the main absorbance regions are at 1060–1040 and 975 cm^{-1} (thin line); for Gal, the main absorbance region is at 945 cm^{-1} (dotted line).

3.7. Concluding remarks

The FT-IR spectra in the region 1200–850 cm^{-1} allow the prediction of Ur.Ac and neutral sugars in the pectic polysaccharide samples of two distinct fruits. The most important wavenumbers in this region were identified and were characterised based on the normalised Ur.Ac and neutral sugars content.

Characteristic absorbance regions can be identified for Ur.Ac, Ara, and Gal in the FT-IR spectra of orange pectic samples.

This study showed that the FT-IR spectra have the potential to be an important source of information for a quick evaluation of the polysaccharide composition of samples of pectic origin.

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