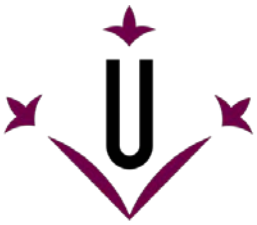

Post-storage cell wall metabolism in two sweet cherry (Prunus avium L.) cultivars displaying different postharvest performance.

Journal:	*Food Science and Technology International*
Manuscript ID:	FSTI-14-0139.R1
Manuscript Type:	Research Article
Subject category:	Physical, Chemical and Sensory Properties
Date Submitted by the Author:	n/a
Complete List of Authors:	Belge, Burcu; University of Lleida, Chemistry Comabella, Eva; University of Lleida, Chemistry Graell, Jordi; University of Lleida, Food Technology Lara, Isabel; University of Lleida, Chemistry
Keywords:	Cell wall polysaccharides, Cold storage, Enzymes, Fruit postharvest, Fruit storage
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POST-STORAGE CELL WALL METABOLISM IN TWO SWEET CHERRY (*Prunus avium* L.) CULTIVARS DISPLAYING DIFFERENT POSTHARVEST PERFORMANCE

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Abstract

The biochemical processes underlying firmness loss of sweet cherry (*Prunus avium* L.) fruit are poorly understood. Studies on cell wall metabolism of sweet cherry have been generally undertaken during on-tree development or at harvest maturity, while published reports on postharvest changes are scarce and fragmentary. In this work, cell wall modifications after storage at 0 ºC were studied in two cherry cultivars ('Celeste' and 'Somerset') displaying different postharvest potential. Firmness was largely determined by the yields of the Na_2CO_3- and KOH-soluble fractions, enriched in covalently-bound pectins and in matrix glycans, respectively, and correlated well with ascorbic acid contents. The yields of these two cell wall fractions were correlated inversely with PME and EGase activities, indicating a relevant role of these two enzymes in postharvest firmness changes in sweet cherry. The amount of solubilised cell wall materials was closely associated to the contents of dehydroascorbic acid, suggesting a possible role for oxidative mechanisms in cell wall disassembly. These data may help understanding the evolution of fruit quality during the marketing period, and give hints for the design of suitable management strategies to preserve key attributes.

Keywords:

Ascorbate; cell wall; cold storage; enzymes; *Prunus avium* L.; postharvest

INTRODUCTION

Keeping and handling potential of sweet cherry (*Prunus avium* L.) fruit is limited, owing to rapid postharvest deterioration and to high susceptibility to infections and mechanical injuries. These fruit must be harvested fully ripe in order to achieve good eating quality, surface colour and soluble solids content (SSC) being the main criteria commonly used to define harvest maturity (Romano et al., 2006). While the eating quality of sweet cherries is dependent upon SSC, titratable acidity (TA), SSC/TA ratio, aroma and flavour, handling options are determined largely by firmness, and indeed rapid softening rates of these fruit are closely related to their high perishability. These characteristics restrict drastically their storage potential and marketing possibilities after harvest.

Ripening-related firmness loss is commonly attributed to modifications in cell wall composition and structure, driven by the cooperative, tightly-regulated action of numerous related proteins. Profound differences exist reportedly in the extent and enzyme regulation of ripening-related modifications of cell wall polysaccharides among fruit species or even cultivars (Goulao and Oliveira, 2008). For sweet cherry, the biochemical processes underlying postharvest firmness loss are poorly understood. Size exclusion chromatography studies revealed negligible pectin depolymerisation during ripening of cherry fruit, suggesting that fruit softening does not depend on this event (Batisse et al., 1994). Moreover, texture differences between firm and soft cherry fruit were also not related to depolymerisation of the pectin backbone, but rather to differences in the structure and size of the pectin side-chains (Batisse et al., 1996; Salato et al., 2013). A survey of six cherry genotypes with differing firmness levels indicated that cell walls of firmer cultivars contained higher amounts of alcohol-insoluble residues, of tightly-bound pectins and, although not consistently for all genotypes examined, of xyloglucans (Choi et al., 2002a).

A few studies have also addressed the changes in some cell wall-modifying enzyme activities in relation to a putative role in firmness loss of cherry fruit. Polygalacturonase (PG) activity increases reportedly late during sweet cherry development, suggesting that early fruit softening taking place at the onset of fruit ripening is unrelated to PG-mediated pectin disassembly, while carboxymethyl (Cx)-cellulase (currently termed endo-β-1,4-glucanase), β-galactosidase and β-glucosidase activities might be involved in the initiation of the softening process (Choi et al., 2002b). Deposition of active β-glucosidase in the cell wall has been demonstrated during ripening of cherry fruit, and the enzyme characterised to possess hydrolytic activity against complex glycans (Gerardi et al., 2001). The highest β-galactosidase activity during on-tree development of 'Bing' fruit was observed approximately two weeks prior to fruit maturity, and suggested to contribute to cell wall hydrolysis during sweet cherry fruit softening (Andrews and Li, 1994). Functional characterisation of the enzyme demonstrated high specificity for *p*-nithophenyl-β-D-galactopyranoside, together with increased activity during ripening (Gerardi et al., 2012).

Studies on cell wall metabolism of sweet cherry have been undertaken during on-tree development or at harvest maturity, while we are not aware of any published reports on postharvest changes in cell walls of these fruit. Additionally, there is experimental evidence that non-enzymatic mechanisms may be involved in the oxidative scission of cell wall polysaccharides in other fruit species such as tomato, strawberry, banana or longan, and particular attention has been focused on the possible role of ascorbic acid and its oxidation derivatives (Fry, 1998; Agius et al., 2003; Cheng et al., 2008; Duan et al., 2011). For these reasons, we undertook this study on cell wall modifications during cold storage of 'Celeste' and 'Somerset' sweet cherry fruit, with special emphasis on the possible role of enzymatic and non-enzymatic factors on postharvest cell wall disassembly.

MATERIALS AND METHODS

Plant material and postharvest handling

Cherry fruit (*Prunus avium* L. 'Celeste' and 'Somerset') were hand-collected in 2011 (May 23rd and June 7th, respectively) from a family-led orchard located in Corbins, in the area of Lleida (NE Spain), at commercial maturity on the basis of size and colour according to the usual practices in the producing area. Samples were selected for uniformity and absence of visible defects and damages, transported directly to the laboratory, and stored at 0 ºC and 92% relative humidity under regular air for up to 14 ('Celeste') or 28 ('Somerset') days. Fruit were analysed immediately after harvest and upon removal from storage, with or without 3 additional days at 20 ºC to simulate commercial shelf life. Tissue samples from the pericarp of 30 fruit (three replicate samples × 10 fruit per sample) were taken at each sampling date, frozen in liquid nitrogen, freeze-dried, powdered, and kept at -80 ºC for subsequent biochemical analyses.

Analysis of standard quality attributes

Firmness was measured with a Durofel DFT 100 durometer (Agro-Technologie, Forges Les Eaux, France) fitted with a 5.64-mm tip, on two opposite faces on the cheek region of 30 fruit, and results were expressed as Durofel units (1, no resistance - 100, maximum resistance). Weight of 30 cherries was determined at harvest and at each analysis date in order to determine weight loss (%) regarding harvest date. Fungal decay was expressed as a percentage of fruit affected. For the assessment of juiciness, three replicate samples (10 fruit each) were stoned and squeezed until no more juice was released. After filtration, the volume of juice recovered was measured, and expressed as mL 100 g^{-1} fresh weight. Soluble solids

content (SSC) and titratable acidity (TA) were assessed in juice obtained as described above. SSC was determined with a hand-held refractometer (Atago, Tokyo, Japan), and results expressed as ºBrix. For TA determination, 10 mL of juice were diluted in 10 mL distilled water, and titrated with 0.1 M NaOH to pH 8.1; results were given as g malic acid L^{-1}. Skin colour was determined at two opposite equatorial points of 30 fruit using a portable tristimulus colorimeter (Chroma Meter CR-200, Minolta Corp., Osaka, Japan), with CIE D_{65} illuminant and 8 mm aperture diameter. Lightness (L^*) values were recorded, and hue angle was calculated from a^* and b^* parameters as arctg b^*/a^*.

Extraction, fractionation and analysis of cell wall materials

The phenol:acetic acid:water (2:1:1, w/v/v) (PAW) method was used for the extraction of cell wall materials (CWM) from lyophilised tissue (3 g) according to Redgwell et al. (1992). The pellet obtained after PAW extraction was resuspended in water and centrifuged again. The PAW and water wash supernatants were combined, dialysed (mol. wt. cut-off 7000) for two days against Milli-Q water at 4 ºC, and centrifuged again to sediment out the precipitate formed during the dialysis. The combined supernatant (henceforth, PAW-soluble fraction; PAW_{sf}) was recovered, lyophilised and weighed. The PAW-insoluble pellet was washed twice in acetone, recovered by vacuum filtration, lyophilised and weighed to determine yield of CWM, expressed as % (w/w) FW. For further fractionation, CWM (100 mg) from each replicate were extracted sequentially with water, 0.05 M cyclohexane-trans-1,2-diamine tetra-acetate (CDTA), 0.05 M Na_2CO_3, and 4 M KOH as described previously (Selvendran and O'Neill, 1987), in order to fractionate water-soluble pectin, non-covalently-bound pectin, covalently-bound pectin and matrix glycans (hemicelluloses), respectively. Each fraction was filtered through Miracloth, extensively dialysed (mol. wt. cut-off 7000) against Milli-Q water at 4 ºC, lyophilised and weighed. Yields were expressed as % (w/w) CWM.

Samples of total CWM, CDTA- and Na_2CO_3-soluble fractions (30-35 mg each) were hydrolysed with 12 M sulphuric acid for further analysis as described previously (Ortiz et al., 2011a). Uronic acid content in the hydrolysate was measured by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) using galacturonic acid as a standard. Total neutral sugars were estimated at 490 nm by the phenol-sulphuric acid assay (Dubois et al., 1956), with galactose as the standard.

The colorimetric ascorbate assay (Gillespie and Ainsworth, 2007) was used to analyse the contents of ascorbic (AA) and dehydroascorbic (DHA) acid in samples (40 mg) of lyophilised tissue. Determinations were done in triplicate, and data given as μmol gDW^{-1}.

Extraction and assay of cell wall-modifying enzyme activities

For the extraction of polygalacturonase (exo-PG; EC 3.2.1.67 and endo-PG; EC 3.2.1.15), pectinmethylesterase (PME; EC 3.1.1.11), pectate lyase (PL; EC 4.2.2.2) and endo-1,4-β-D-glucanase (EGase; EC 3.2.1.4) activities, a 10% (w/v) pulp homogenate was prepared by homogenising 100 mg of freeze-dried pulp tissue in an extraction buffer prepared according to Lohani et al. (2004). PG activity was determined on apple pectin (d.e. 70-75%) as described previously (Pathak and Sanwall, 1998), with galacturonic acid (GalUA) as a standard. One unit (U) of PG activity was defined as the liberation of 1 μmol of GalUA min^{-1}. PME activity was measured according to Hagerman and Austin (1986). For the assay, the reaction mixture contained enzyme extract, apple pectin and bromothymol blue prepared as described previously (Alonso et al., 1997). One unit (U) of PME activity was defined as the decrease of one unit of A_{620} min^{-1}. PL activity was assayed with apple pectin as the substrate according to Moran et al. (1968) as modified by Lohani et al. (2004). One unit (U) of PL activity was defined as the increase of one unit of A_{235} min^{-1}. For the assessment of EGase activity, the DNS method (Miller, 1959), with carboxymethylcellulose as the assay substrate,

was used to determine the amount of reducing sugars released, with glucose as a standard. One unit (U) of EGase activity was defined as the release of 1 μmol of glucose min^{-1}.

For the extraction of β-galactosidase (β-Gal; EC 3.2.1.23), β-xylosidase (β-Xyl; EC 3.2.1.37) and α-L-arabinofuranosidase (AFase; EC 3.2.1.55) activities, a 10% (w/v) pulp homogenate was prepared by homogenising 100 mg of freeze-dried pulp tissue in extraction buffer prepared according to previous work (Vicente et al., 2005). β-Gal, β-Xyl and AFase activity assays were undertaken in the crude extract as described in Vicente et al. (2005) and Wei et al. (2010), respectively. One unit (U) of β-Gal was defined as the liberation of 1 μmol of *p*-nitrophenol min^{-1} from *p*-nitrophenyl-β-D-galactopyranoside. One unit (U) of AFase or β-Xyl activity was defined as the liberation of 1 nmol of p-nitrophenol min^{-1} from *p*-nitrophenyl-α-L-arabinofuranoside or *p*-nitrophenyl-β-D-xylopyranoside, respectively.

Total protein content in the crude extracts was determined with the Bradford (1976) method, using BSA as a standard. All analyses were done in triplicate, and results were expressed as specific activity (U mg^{-1} protein).

Statistical and multivariate analysis

Results were treated for multiple comparisons by analysis of variance (ANOVA), followed by the least significant difference (LSD) Fisher's test at $P \leq 0.05$ with the Minitab 16 software package (Minitab Inc., UK). Partial least square regression (PLSR) was also used as a predictive method to relate a matrix of dependent variables (*Y*) to a set of explanatory variables (*X*) in a single estimation procedure, using the Unscrambler version 9.1.2 software (CAMO ASA, 2004).

RESULTS AND DISCUSSION

The usual standard indicators used to evaluate commercial quality of fruit indicated that samples were picked at a suitable maturity stage according to the standards in the producing area (Table 1). 'Somerset' fruit were in average larger and heavier than 'Celeste' cherries, and also displayed higher firmness, juiciness, SSC and TA values, besides being darker and redder. 'Celeste' cherries were analysed after 0, 7 and 14 days of cold storage. Because 'Somerset' fruit display better keeping potential, the analyses on this cultivar were undertaken after storing the samples at 0 ºC during 0, 14 and 28 days. For clarity, Tables and Figures show results corresponding to 0 and 14 days of storage uniquely. However, all samples (2 cultivars × 3 storage periods × 2 shelf life periods) were used for the development of the regression models.

Fruit quality and cell wall materials after cold storage

Firmness of 'Celeste' fruit declined significantly after 14 days at 0 ºC, whereas that of 'Somerset' cherries increased (Table 2), indicating a tightening effect of cold temperature on fruit tissues as observed elsewhere for 'Pájaro' strawberries (Lara et al., 2004). 'Somerset' samples actually retained high levels of this attribute (> 80 Durofel units) throughout the whole experimental period of 28 days (not shown). In addition, weight loss rates were lower in 'Somerset' than in 'Celeste' fruit, but in contrast 'Somerset' cherries suffered from higher incidence of fungal infections (Table 2).

Insoluble and PAW-soluble cell wall materials were extracted from fruit samples in order to assess possible relationships to some shelf life potential-related attributes. Different trends were observed for each cultivar considered. For 'Celeste', increased yields of insoluble cell wall materials (CWM) and decreased amounts of PAW-soluble materials (PAW_{sf}), indicative

of the degree of *in vivo* solubilisation of cell wall polysaccharides, were observed in fruit after a two-week cold storage period (Table 3). These changes resulted in remarkably augmented CWM:PAW_{sf} ratios as compared with values at harvest (120.8 *vs*. 22.2) in spite of decreased fruit firmness. For 'Somerset', the increase in firmness after cold storage of fruit was accompanied by a two-fold increment in CWM yields recovered from fruit pericarp. However, firmness remained at similar levels after fruit was kept 3 days at 20 ºC thereafter, even though CWM yields remained similarly high and PAW_{sf} yields were lower (Table 3), leading likewise to increased CWM:PAW_{sf} ratios (132.3 *vs*. 76.1). This disagrees with previous observations on fruit displaying non-melting as well as melting softening patterns, such as apple (Ortiz et al., 2011a) and nectarine (Ortiz et al., 2010), for which we found declining CWM:PAW_{sf} ratios with concomitant firmness loss during on-tree ripening, reflecting progressive solubilisation of cell wall polymers during the process. However, the biochemical mechanisms underlying firmness changes may be different during ripening and during the postharvest period of fully ripe fruit. These discrepancies may also be placed in connection with the view that noticeable differences exist among fruit species in the mechanisms involved in ripening-related firmness loss (Goulao and Oliveira, 2008), and suggest that additional processes besides cell wall modifications may play a role in sweet cherry fruit softening. One of such mechanisms may be related to water loss rates, which in turn are dependent upon the properties of the fruit cuticle. Indeed, there is experimental evidence for some fruit species that firmness loss shows good correspondence to the alterations in cell turgor taking place during ripening (Lara et al., 2014). The water-proofing properties of the cuticle depend to a large extent on the composition of cuticular waxes. For fruit species within the *Solanaceae* family, such as tomato or pepper, it has been reported that the ratio of alkanes to triterpenoids (Vogg et al., 2004) or to triterpenoids plus sterols (Parsons et al., 2012) correlated inversely to dehydration rates. Our own results (unpublished data)

indicated ratios of 0.18 and 0.33 for 'Celeste' and 'Somerset' at harvest, respectively, which would be consistent with higher firmness and lower water loss rates in the latter (Table 2).

Cell wall status may be more properly described by the specific composition and structure of the cell wall polymers, rather than by CWM:PAW_{sf} ratios. When the insoluble cell wall materials obtained from the samples were fractionated and analysed, cultivar-related differences were found, which might be related to the different keeping potential in each case. For both cultivars, yields of the chelator-soluble fraction ($CDTA_{sf}$), enriched in pectins bound non-covalently to the cell wall, did not change after keeping the samples at 0 ºC during 14 days. However, different responses were observed for each cultivar considered after the simulated commercialisation period at 20 ºC subsequent to cold storage. While for 'Celeste' fruit lower $CDTA_{sf}$ yields were found as compared with non-refrigerated samples kept 3 days at 20 ºC immediately after harvest, the opposite was found for 'Somerset samples (Table 3). Differences were observed as well in the evolution of yields of the carbonate-soluble fraction (Na_2CO_{3sf}), representative of covalently-linked pectins, which decreased after cold storage of 'Somerset fruit while remaining at similar levels in 'Celeste' samples (Table 3). Moreover, the percentages of both pectin-containing fractions over total insoluble materials were generally higher in 'Somerset' than in 'Celeste' samples, which had considerably lower firmness and more intense weight loss (Table 2). When the content of uronic acids was analysed in total insoluble materials as well as in pectin-containing fractions, it was found that uronic acid content in the Na_2CO_3-soluble fraction increased in both cultivars after storing the fruit at 0 ºC during 14 days. In contrast, uronic acids in the chelator-soluble fraction decreased after cold storage in comparison with levels at harvest, although 'Somerset' cherries were found to display higher contents than 'Celeste' fruit in all the sampling dates considered (Fig. 1). Differences were also observed in the dynamics of the potassium hydroxide-soluble fraction (KOH_{sf}), which contains mainly cell wall xyloglucans, 'Somerset' samples generally

displaying also higher contents of this cell wall fraction (Table 3). Although levels of ascorbic acid, both reduced (AA) and oxidised (DHA), showed similar dynamics in both cultivars in response to storage conditions, very different contents were observed in each case, 'Somerset' samples displaying higher AA and lower DHA contents in comparison with 'Celeste' fruit (Table 4).

In order to assess possible relationships to firmness and other quality attributes, cell wall composition data, together with AA and DHA contents, were used as the potentially explanatory variables (*X*-matrix) to develop a regression model. The corresponding correlations loadings plot (Fig. 2) shows that firmness was associated closely to yields of the Na_2CO_3-soluble fraction, enriched in covalently-linked pectins, to the levels of uronic acids in the chelator-soluble fraction of pectins and, to a lower extent, to KOH_{sf} yields. This agrees with a previous report on six sweet cherry cultivars differing in firmness, which demonstrated that softer fruit had lower levels of these two fractions (Choi et al., 2002a). Interestingly, firmness was also related to AA contents, while more intense cell wall solubilisation, as indicated by higher yields of PAW- and water-soluble materials, was associated to dehydroascorbate (DHA) contents. Since AA and DHA contents were assessed in lyophilised pericarp tissue rather than in isolated cell wall fractions, these data should be considered with caution. However, they are consistent with previous reports that ascorbate-induced hydroxyl radicals can cause non-enzymatic, oxidative scission of cell wall polysaccharides and thus contribute to fruit softening (Fry, 1998; Cheng et al., 2008; Duan et al., 2011), and suggests a role for ascorbate in softening of cherry fruit. Actually, a highly positive correlation between total antioxidant activity and ascorbic acid content has been reported for cherry fruit (Serrano et al., 2005).

Cell wall-modifying enzyme activities after cold storage

Ripening-related modifications in cell wall composition and structure are commonly attributed to the finely-tuned, coordinated action of a number of specific enzymatic and non-enzymatic proteins on different cell wall polysaccharides, which eventually lead to cell wall disassembly. The controlling mechanisms involved in this event, though, appear to vary widely among species (Goulao and Oliveira, 2008). Historically, depolymerizing enzymes acting on the pectin backbone, such as PG and PME, have been considered to play a central role in fruit softening. More recently, pectate lyase (PL), a lyase which removes demethylated uronic acid residues from the pectin backbone through a β-elimination mechanism, has received increasing attention, and has been shown to contribute to firmness loss in some fruit species, including banana (Domínguez-Puigjaner et al., 1997), strawberry (Jiménez-Bermúdez et al., 2002), nectarine (Ortiz et al., 2010), peach (Ortiz et al., 2011b) and apple (Ortiz et al., 2011a, 2011c). Because PME catalyses the demethylation of galacturonosyl residues, which is required for subsequent PG and PL depolymerising action (Bennett and Labavitch, 2008), this enzyme activity is believed to be essential for ripening-related firmness loss. When these activities were analysed, cultivar-specific differences were observed in their evolution after harvest.

Cold storage did not cause significant changes in PME activity in 'Celeste' fruit, but levels increased during the subsequent simulated shelf life period at 20 ºC (Fig. 3A), while in 'Somerset' samples similar PME activity levels were observed both at harvest and in cold-stored fruit (Fig. 3B). PG activity increased in 'Celeste' cherries after cold storage (Fig. 3A), whereas similar levels as compared with those at harvest were observed in 'Somerset' fruit (Fig. 3B), maybe in relation to the retention of higher firmness in these fruit (Table 2). For both cultivars, PL activity levels did not show significant differences after cold storage, but an increase was found when fruit were kept at 20 ºC thereafter for 3 days (Table 5).

Current experimental evidence shows that cell wall disassembly is far more complex than initially thought, and that other cell wall proteins may contribute significantly and decisively to this event. Actually, textural differences between crisp and soft cherry fruit are apparently related to structure and composition of pectin side-chains rather than to depolymerisation of the pectin backbone, which has been reported to be very limited (Batisse et al., 1996). Indeed, the highly branched structure of pectins is believed to control pore size in the cell wall, thus restricting the access of pectin backbone-acting enzymes to their substrates, and protecting cell wall polysaccharides from extensive depolymerisation by pectolytic enzymes (Goulao and Oliveira, 2008), even though high levels of pectolytic activity may be present. Removal of these galactosyl- and arabinosyl-containing pectin side-chains thus increases cell wall permeability and favours the access of PG, PL and PME to their backbone substrates. For cherry fruit, it has been reported that higher firmness was associated with higher degree of pectin branching (Batisse et al., 1996), although a recent study in which the degree of pectin branching in fruit of a soft ('Newstar') and a firm ('Sweetheart') cultivar was compared found the opposite (Salato et al., 2013). β-Gal and AFase act on these neutral sugar-rich pectin side-chains, and may therefore have a major role in pectin solubilisation.

A cell wall-associated sweet cherry β-galactosidase (β-Gal) has been characterised to be active against complex glycans (Gerardi et al., 2012). Because the enzyme was the main glycosidase activity detected in sweet cherry extracts at different ripening stages, and increased notably during ripening, it was suggested to play a significant role in fruit softening. In this work, β-Gal activity levels rose in 'Celeste' samples both after cold storage and after 3 days of simulated commercial life at 20 ºC (Fig. 3A), whereas cold storage induced a sharp increase in activity levels of 'Somerset' fruit (Fig. 3B). AFase activity, which cleaves arabinosyl residues from pectin side-chains, was also assessed, and found to follow dissimilar trends in both cultivars, with significantly increased levels being found in 'Celeste' fruit after

harvest regardless of temperature, whereas in 'Somerset' samples cold storage did not cause significant differences in AFase activity (Table 5).

Since data indicated that firmness was associated to the yield of specific cell wall fractions and to ascorbate content (Fig. 2), an additional regression model was developed in order to aid the visualisation of relationships among the variables assessed. Yield and composition of cell wall materials isolated from fruit were taken as the Y variables, with the assessed pectolytic and non-pectolytic enzyme activities, together with AA and DHA contents, as the potentially explanatory (*X* matrix) variables. The correlations loading plot for this model (Fig. 4) shows that the yields of the Na_2CO_3-soluble fraction, found to relate closely to fruit firmness (Fig. 2), were correlated inversely to PME activity levels, suggesting a role in firmness loss. Actually, PME activity was also well correlated to PAW_{sf} and W_{sf} yields, indicative of the degree of solubilisation of cell wall materials, and associated to higher DHA contents. This is interesting in regard of previous evidence that de-esterified pectin is more susceptible to ascorbate-induced scission in vitro than methylesterified pectin, and that the ability to release ascorbate into the apoplast increases during ripening of tomato fruit (Dumville and Fry, 2003).

PG activity was apparently unrelated to firmness loss (Fig. 4), and PL activity levels were moreover generally higher in firmer fruit (Tables 2 and 5), which de-emphasises the role of these enzymes in postharvest firmness changes of cherry fruit. High levels of PG and PL activity might be not really of consequence for extensive changes in cell wall polysaccharides, as their action is dependent upon the availability and accessibility to their demethylated backbone substrate.

Removal of methyl moieties by PME also confers an anionic charge to polyuronides, and contributes to changes in the electric charge and to the acidification of the apoplast, thus modifying the activity and mobility of other cell wall proteins. Indeed, the pH optimum of 4.0

reported for sweet cherry β-Gal (Gerardi et al., 2012) is consistent with the idea of increased activity upon ripening-associated decrease of apoplastic pH (Almeida and Huber, 1999). Yet results do not support a key role for β-Gal in postharvest deterioration of 'Celeste' and 'Somerset' cherries, firmer fruit actually displaying higher activity levels (Fig. 4). This result is not in accordance with the proposed role on sweet cherry softening (Gerardi et al., 2012), and it also disagrees with previous results on other fruit species (Ortiz et al., 2011a, 2011b). However, Gerardi et al. (2012) reported that β-Gal activity increased at early stages of sweet cherry ripening but decreased in over-ripe fruit. Similar results were obtained for nectarine (Ortiz et al., 2010), where β-Gal levels increased at the initial phase of softening, prior to the onset of the melting-like drop in firmness, to remain steady thereafter. Thus, the association between firmness and β-Gal activity might be indicating this early role in softening-leading events. No apparent association to firmness changes was found either for AFase activity, which is also considered to contribute to these controlling mechanisms of cell wall porosity. However, it was found to relate closely to the yields and to total neutral sugar contents in the $CDTA_{sf}$, which is consistent with previous observations that a part of insoluble cell wall materials are reallocated transiently from the carbonate- to the chelator-soluble fraction of pectins during softening of some fruits (reviewed in Goulao and Oliveira, 2008; Duan at el., 2011).

Similarly to the observations for PME, the matrix glycan-degrading EGase activity was inversely correlated to firmness levels and to the contents of ascorbic acid, again suggesting that antioxidant activity may have a role in preventing extensive cell wall disassembly. This is also consistent with the finding that higher yields of the KOH-soluble fraction of insoluble cell wall materials, which is enriched in hemicellulosic polymers, were associated to higher firmness values (Fig. 2). Contradictory reports on the relevance of this non-pectolytic activity for fruit softening have been published (Goulao and Oliveira, 2008), and it has been suggested

that it might be related rather to cell wall extensibility at early stages of fruit development. Activity levels decreased progressively during fruit development of 'Mondial Gala' apples (Goulao et al., 2007) and 'Snow Queen' nectarines (Ortiz et al., 2010), and were reportedly higher in firmer 'Golden Reinders' apples (Ortiz et al., 2011c). However, results of the present study suggest that EGase activity may have a relevant role in postharvest firmness loss of sweet cherry fruit, in agreement with observations during fruit development (Choi et al., 2002b).

As the concluding remarks, total amount of insoluble cell wall materials was not a good predictor of firmness in 'Celeste' and 'Somerset' cherries. Further fractionation of these materials revealed that firmness was largely determined by Na_2CO_{3sf} yields, representative of the amount of covalently-bound pectins, and to a lesser extent by KOH_{sf} yields, which contain mainly the matrix glycans. Firmness was also associated to higher levels of ascorbic acid, whereas the degree of solubilisation of cell wall materials was closely related to the contents of dehydroascorbic acid, which suggest a possible role of oxidative mechanisms in cell wall disassembly. PME and EGase activities, respectively a pectin- and a hemicellulose-degrading enzyme, were inversely correlated with the yields of the firmness-determining Na_2CO_3- and KOH-soluble fractions, as well as with ascorbic acid contents. These data may help understanding the evolution of fruit quality during the marketing period, and thus identifying key involved factors which may give hints for the design of suitable management strategies to preserve key attributes.

REFERENCES

Agius F, González-Lamothe R, Caballero JL, Muñoz-Blanco J, Botella MA and Valpuesta V. (2003). Engineering increased vitamic C levels in plants by overexpressing of a D-galacturonic acid reductase. *Nature Biotechnology* 21: 177-181.

Almeida DPF and Huber DJ. (1999). Apoplastic pH and inorganic ion levels in tomato fruit: a potential means for regulation of cell wall metabolism during ripening. *Physiologia Plantarum* 105: 506-512.

Alonso J, Howell N and Canet W. (1997). Purification and characterisation of two pectinmethylesterases from persimmon (*Diospyros kaki*). *Journal of the Science of Food and Agriculture* 75: 352-358.

Andrews PK and Li S. (1994). Partial purification and characterization of β-D-galactosidase from sweet cherry, a nonclimacteric fruit. *Journal of Agricultural and Food Chemistry* 42: 2177-2182.

Batisse C, Fils-Lycaon B and Buret M. (1994). Pectin changes in ripening cherry fruit. *Journal of Food Science* 59: 389-393.

Batisse C, Buret M and Coulomb PJ. (1996). Biochemical differences in cell wall of cherry fruit between soft and crisp fruit. *Journal of Agricultural and Food Chemistry* 44: 453-457.

Bennett AB and Labavitch JM. (2008). Ethylene and ripening-regulated expression and function of fruit cell wall modifying proteins. *Plant Science* 175: 130-136.

Blumenkrantz N and Asboe-Hansen G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry* 54: 484-489.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* 72: 248-254.

Cheng G, Duan X, Shi J, Lu W, Luo Y, Jiang W and Jiang Y. (2008). Effects of reactive oxygen species on cellular wall disassembly of banana fruit during ripening. *Food Chemistry* 109: 319-324.

Choi C, Toivonen P, Wiersma PA and Kappel F. (2002a). Differences in levels of pectic substances and firmness in fruit from six sweet cherry genotypes. *Journal of the American Pomological Society* 56: 197-201.

Choi C, Wiersma PA, Toivonen P and Kappel F. (2002b). Fruit growth, firmness and cell wall hydrolytic enzyme activity during development of sweet cherry fruit treated with gibberellic acid (GA_3). *Journal of Horticultural Science and Biotechnology* 77: 615-621.

Domínguez-Puigjaner E, Llop I, Vendrell M and Prat S. (1997). A cDNA clone highly expressed in ripe banana fruit shows homology to pectate lyases. *Plant Physiology* 114: 1071-1076.

Duan X, Zhang H, Zhang D, Sheng J, Lin H and Jiang Y. (2011). Role of hydroxiyl radical in modification of cell wall polysaccharides and aril breakdown during senescence of harvested longan fruit. *Food Chemistry* 128: 203-207.

Dubois M, Gilles KA, Hamilton JK, Rebers PA and Smith F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 28: 350-356.

Dumville JC and Fry SC. (2003). Solubilisation of tomato fruit pectins by ascorbate: a possible non-enzymic mechanism of fruit softening. *Planta* 217: 951-961.

Fry SC. (1998). Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochemical Journal* 332: 507-515.

Gerardi C, Blando F, Santino A and Zacheo G. (2001). Purification and characterisation of a β-glucosidase abundantly expressed in ripe sweet cherry (*Prunus avium* L.) fruit. *Plant Science* 160: 795-805.

Gerardi C, Blando F and Santino A. (2012). Purification and chemical characterisation of a cell wall-associated β-galactosidase from mature sweet cherry (*Prunus avium* L.) fruit. *Plant Physiology and Biochemistry* 61: 123-130.

Gillespie KM and Ainsworth EA. (2007). Measurement of reduced, oxidized and total ascorbate content in plants. *Nature Protocols* 2: 871-874.

Goulao LF and Oliveira CM. (2008). Cell wall modifications during fruit ripening: when a fruit is not the fruit. *Trends in Food Science and Technology* 19: 4-25.

Goulao LF, Santos J, de Sousa I and Oliveira CM. (2007). Patterns of enzymatic activity of cell wall-modifying enzymes during growth and ripening of apples. *Postharvest Biology and Technology* 43: 307-318.

Hagerman AE and Austin PJ. (1986). Continuous spectrophotometric assay for plant pectin methyl-esterase. *Journal of Agricultural and Food Chemistry* 34: 440-444.

Jiménez-Bermúdez S, Redondo-Nevado J, Muñoz-Blanco J, Caballero JL, López-Aranda JM, Valpuesta V, Pliego-Alfaro F, Quesada MA and Mercado JA. (2002). Manipulation of strawberry fruit softening by antisense expression of a pectate lyase gene. *Plant Physiology* 128: 751-759.

Lara I, García P and Vendrell M. (2004). Modifications in cell wall composition after cold storage of calcium-treated strawberry (*Fragaria* × *ananassa* Duch.) fruit. *Postharvest Biology and Technology* 34: 331-339.

Lara I, Belge B and Goulao LF. (2014). The fruit cuticle as a modulator of postharvest quality. *Postharvest Biology and Technology* 87: 103-112.

Lohani S, Trivedi PK and Nath P. (2004). Changes in activities of cell wall hydrolases during ethylene-induced ripening in banana: effect of 1-MCP, ABA and IAA. *Postharvest Biology and Technology* 31: 119-126.

Miller GL. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* 31: 426-428.

Moran F, Nasuno S and Starr MP. (1968). Extracellular and intracellular polygalacturonicacid trans-eliminase of *Erwinia carotovora*. *Archives of Biochemistry and Biophysics* 123: 298-306.

Ortiz A, Graell J and Lara I. (2011a). Preharvest calcium applications inhibit some cell wall-modifying enzyme activities and delay cell wall disassembly at commercial harvest of 'Fuji Kiku-8' apples. *Postharvest Biology and Technology* 62: 161-167.

Ortiz A, Vendrell M and Lara I. (2011b). Softening and cell wall metabolism in late-season peach in response to controlled atmosphere and 1-MCP treatment. *Journal of Horticultural Science and Biotechnology* 86: 175-181.

Ortiz A, Graell J and Lara I. (2011c). Cell wall-modifying enzymes and firmness loss in ripening 'Golden Reinders' apples: A comparison between calcium dips and ULO storage. *Food Chemistry* 128: 1072-1079.

Ortiz A, Seymour GB, Tucker GA and Lara I. (2010). Cell wall disassembly during the melting phase of softening in 'Snow Queen' nectarines. *Postharvest Biology and Technology* 58: 88-92.

Parsons EP, Popopvsky S, Lohrey GT, Lu S, Alkalai-Tuvia S, Perzelan Y, Paran I, Fallik E, Jenks MA. (2012). Fruit cuticle lipid composition and fruit post-harvest water loss in an advanced backcross generation of pepper (*Capsicum* sp.). *Physiologia Plantarum* 146: 15-25.

Pathak N and Sanwall GG. (1998). Multiple forms of polygalacturonase from banana fruits. *Phytochemistry* 48: 249-255.

Redgwell RJ, Melton LD and Brasch DJ. (1992). Cell wall dissolution in ripening kiwifruit (*Actinidia deliciosa*). *Plant Physiology* 98: 71-81.

Romano GS, Cittadini ED, Pugh B and Schouten R. (2006). Sweet cherry quality in the horticultural production chain. *Stewart Postharvest Review* 2: 6 (paper no. 9)

Salato GS, Ponce NMA, Raffo MD, Vicente AR and Stortz CA. (2013). Developmental changes in cell wall polysaccharides from sweet cherry (*Prunus avium* L.) cultivars with contrasting firmness. *Postharvest Biology and Technology* 84: 66-73.

Selvendran RR and O'Neill MA. (1987). Isolation and analysis of cell walls from plant material. In: Glick D (ed) *Methods of Biochemical Analysis*, *vol. 32*. New York: John Wiley Interscience, pp. 25-153.

Serrano M, Guillén F, Martínez-Romero D, Castillo S and Valero D. (2005). Chemical constituents and antioxidant activity of sweet cherry at different ripening stages. *Journal of Agricultural and Food Chemistry* 53: 2741-2745.

Vicente AR, Costa ML, Martínez GA, Chaves AR and Civello PM. (2005). Effect of heat treatments on cell wall degradation and softening in strawberry fruit. *Postharvest Biology and Technology* 38: 213-222.

Vogg G, Fischer S, Leide J, Emmanuel E, Jetter R, Levy AA, Riederer M. (2004). Tomato fruit cuticular waxes and their effect on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid β-ketoacyl-CoA synthase. *Journal of Experimental Botany* 55: 1404-1410.

Wei J, Fengwang M, Shi S, Qi X, Zhu Z and Yuan J. (2010). Changes and postharvest regulation of activity and gene expression of enzymes related to cell wall degradation in ripening apple fruit. *Postharvest Biology and Technology* 56: 147-154.

FUNDING ACKNOWLEDGEMENT

B. B. is the recipient of a FI-DGR grant from AGAUR (Generalitat de Catalunya). This work was supported through the AGL2010-14801/ALI project, funded by the Ministerio de Ciencia e Innovación (MICINN) of Spain. Fruit samples were provided by J. M. Camats (Cireres de Corbins Camats-Carpi).

DECLARATION OF CONFLICTING INTERESTS

The Authors declare that there is no conflict of interest.

Table 1. Maturity and quality indices of 'Celeste' and 'Somerset' sweet cherry fruit at commercial harvest.

Parameter	'Celeste'	'Somerset'
Weight (g)	9.02 ± 1.43	10.49 ± 1.49
Diameter (mm)	27.95 ± 3.23	30.73 ± 2.08
Firmness (Durofel units)	77.7 ± 8.25	80.43 ± 5.89
Juiciness (mL 100g^{-1} FW)	61.07 ± 6.35	65.37 ± 7.62
SSC (º Brix)	16.07 ± 0.57	19.43 ± 0.91
TA (g L^{-1})	6.70 ± 0.35	7.32 ± 0.38
Hue (º)	21.88 ± 2.82	16.97 ± 1.46
Lightness (L*)	33.51 ± 0.17	27.01 ± 0.32

Values represent means ± SD of 30 or three (SSC, TA, juiciness) replicates.

Table 2. Firmness (Durofel units), weight loss (%) and decay incidence (%) in 'Celeste' and 'Somerset' sweet cherries at harvest and after cold storage.

Cultivar	Days[a]	Firmness	Decay	Weight loss
'Celeste'	**0**	77.7 a	0.0	-
	0+3	72.1 ab	0.0	9.6 c
	14+0	68.5 b	0.0	15.8 b
	14+3	69.9 b	6.7	22.4 a
'Somerset'	**0**	80.4 b	0.0	-
	0+3	77.2 b	0.0	2.4 c
	14+0	86.5 a	8.5	7.2 b
	14+3	82.1 ab	21.4	15.4 a

Values are the means of 30 (firmness, weight loss) replicates. Decay incidence was evaluated as a percentage on the total number of fruit. For a given cultivar, means followed by different letters within the same column are significantly different at $P \leq 0.05$ (LSD test).

[a] Days at 0 ºC + days at 20 ºC.

Table 3. Yield of insoluble (CWM) and PAW-soluble (PAW_{sf}) cell wall materials (% FW), and of fractions isolated from insoluble cell wall materials (% CWM) in 'Celeste' and 'Somerset' sweet cherries at harvest and after cold storage.

				CWM fractions			
Cultivar	**Days[a]**	**CWM**	**PAW_{sf}**	**W_{sf}**	**$CDTA_{sf}$**	**Na_2CO_{3sf}**	**KOH_{sf}**
'Celeste'	**0**	1.086 c	0.049 ab	11.197 a	16.510 a	8.613 b	0.938 c
	0+3	1.302 c	0.058 a	6.552 ab	17.710 a	13.674 a	2.406 a
	14+0	2.412 b	0.020 c	2.438 b	15.572 ab	7.860 b	0.948 c
	14+3	3.751 a	0.029 bc	1.588 b	11.964 b	7.258 b	1.833 b
'Somerset'	**0**	1.186 b	0.022 b	3.495 a	18.204 ab	17.136 a	3.738 a
	0+3	2.205 a	0.019 b	2.409 ab	17.232 b	9.660 b	1.267 b
	14+0	2.153 a	0.028 a	1.078 b	17.451 b	10.098 b	1.912 ab
	14+3	2.117 a	0.016 c	2.767 ab	20.098 a	14.750 ab	3.690 a

Values are the means of three replicates. For a given cultivar, means followed by different letters within the same column are significantly different at $P \leq 0.05$ (LSD test).

[a] Days at 0 ºC + days at 20 ºC.

Table 4. Content of ascorbic and dehydroascorbic acid (μmol gDW^{-1}) in 'Celeste' and 'Somerset' sweet cherries at harvest and after cold storage.

Cultivar	Days[a]	AA	DHA
'Celeste'	**0**	9.041 a	5.532 b
	0+3	11.766 a	6.629 a
	14+0	2.567 b	0.240 d
	14+3	8.134 a	1.082 c
'Somerset'	**0**	13.850 a	0.524 b
	0+3	17.573 a	2.056 a
	14+0	6.884 b	0.017 c
	14+3	13.966 a	0.253 b

Values are the means of three replicates. For a given cultivar, means followed by different letters within the same column are significantly different at $P \leq 0.05$ (LSD test).

[a] Days at 0 ºC + days at 20 ºC.

Table 5. Specific activities (U mg protein^{-1}) of some pectolytic and non-pectolytic cell wall-modifying enzymes in 'Celeste' and 'Somerset' sweet cherries at harvest and after cold storage.

Cultivar	Days[a]	PL	AFase	β-Xyl	EGase
'Celeste'	**0**	1.026 b	36.920 c	80.935 b	11.943 ab
	0+3	1.294 b	45.845 a	104.401 ab	18.708 a
	14+0	1.652 ab	40.926 b	144.240 a	15.580 a
	14+3	2.137 a	43.517 ab	114.650 ab	6.712 b
'Somerset'	**0**	2.712 b	35.207 b	129.844 b	13.851 ab
	0+3	3.151 b	38.402 b	156.394 ab	11.574 b
	14+0	2.287 b	39.003 b	60.025 c	15.545 a
	14+3	4.688 a	46.910 a	183.751 a	7.788 c

Values are the means of three replicates. For a given cultivar, means followed by different letters within the same column are significantly different at $P \leq 0.05$ (LSD test).

[a] Days at 0 ºC + days at 20 ºC.

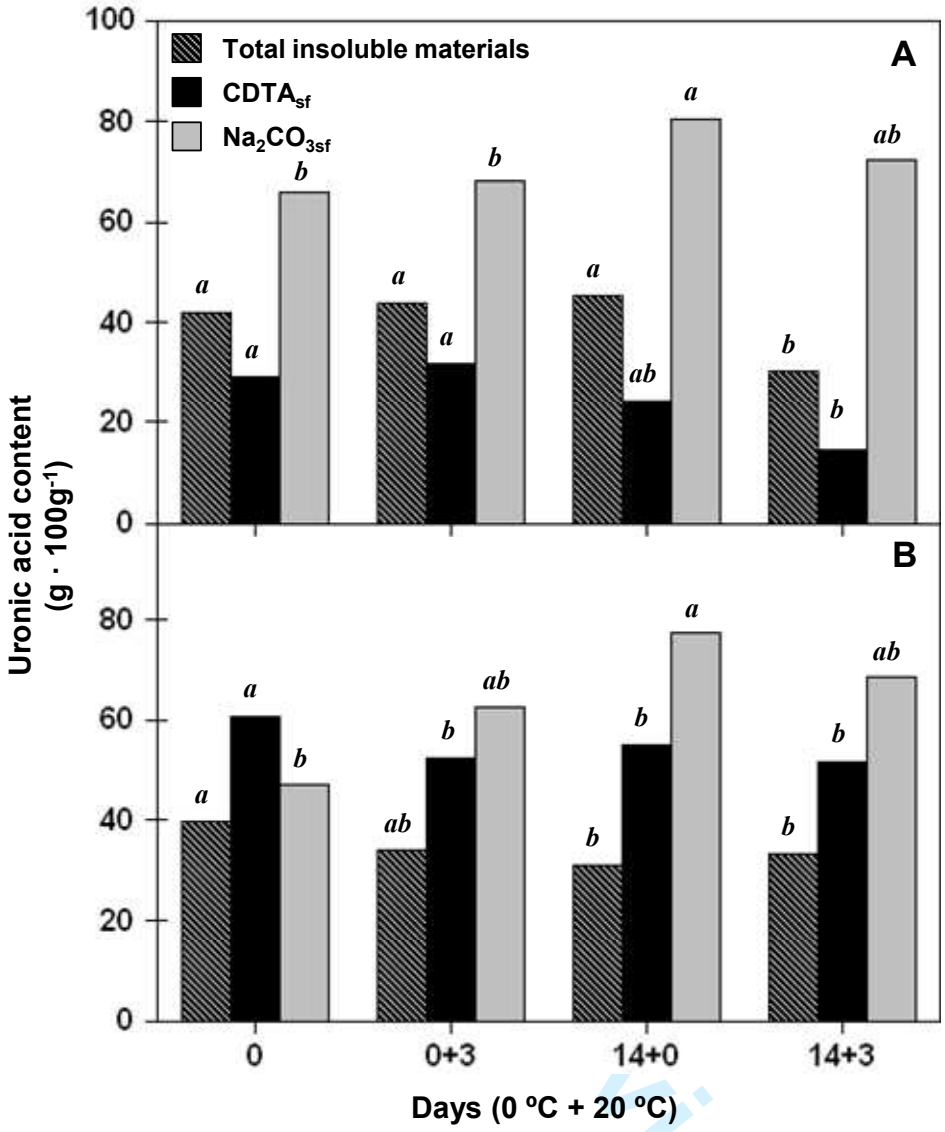

Figure 1. Uronic acid content (% w/w) in total insoluble cell wall materials and in pectin-containing fractions isolated from 'Celeste' (**A**) and 'Somerset' (**B**) sweet cherries at harvest and after cold storage. Bars represent means of three replicates. For a given fraction, values bearing different letters are significantly different at $P \leq 0.05$ (LSD test).

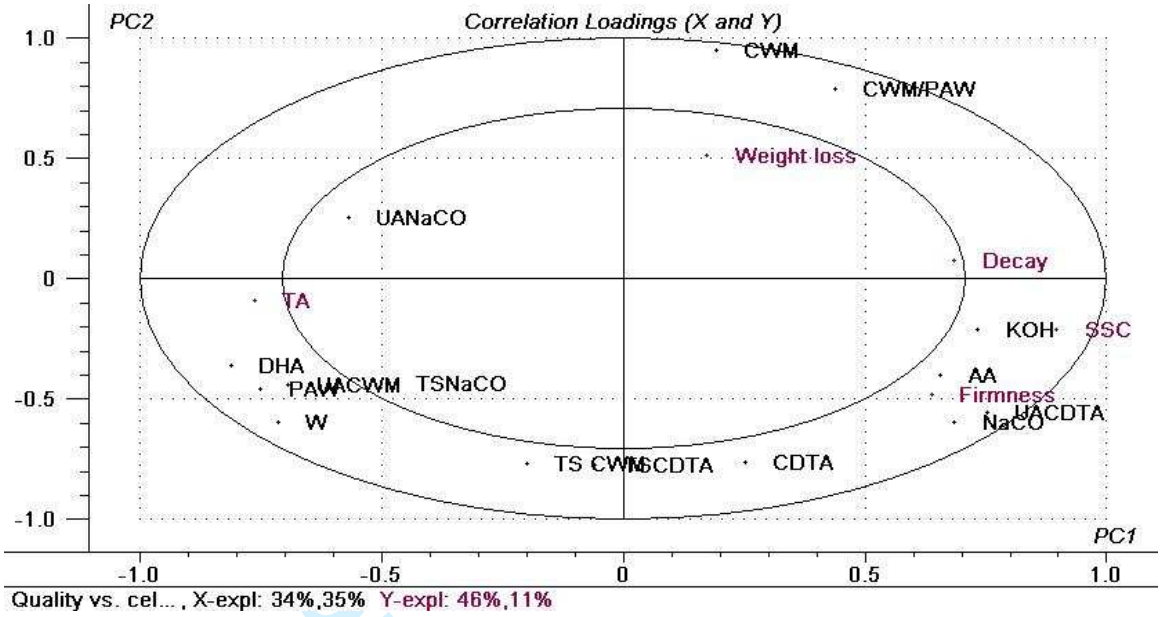

Figure 2. Correlation loadings plot of PC1 *vs*. PC2 corresponding to a PLSR model for quality attributes (*Y* variables) vs. yield and composition of cell wall fractions (*X* variables) isolated from 'Celeste' and 'Somerset' cherries after cold storage at 0 ºC (AA, ascorbic acid; DHA, dehydroascorbic acid; UA and TS, uronic acid and total sugar content in a given cell wall fraction, respectively).

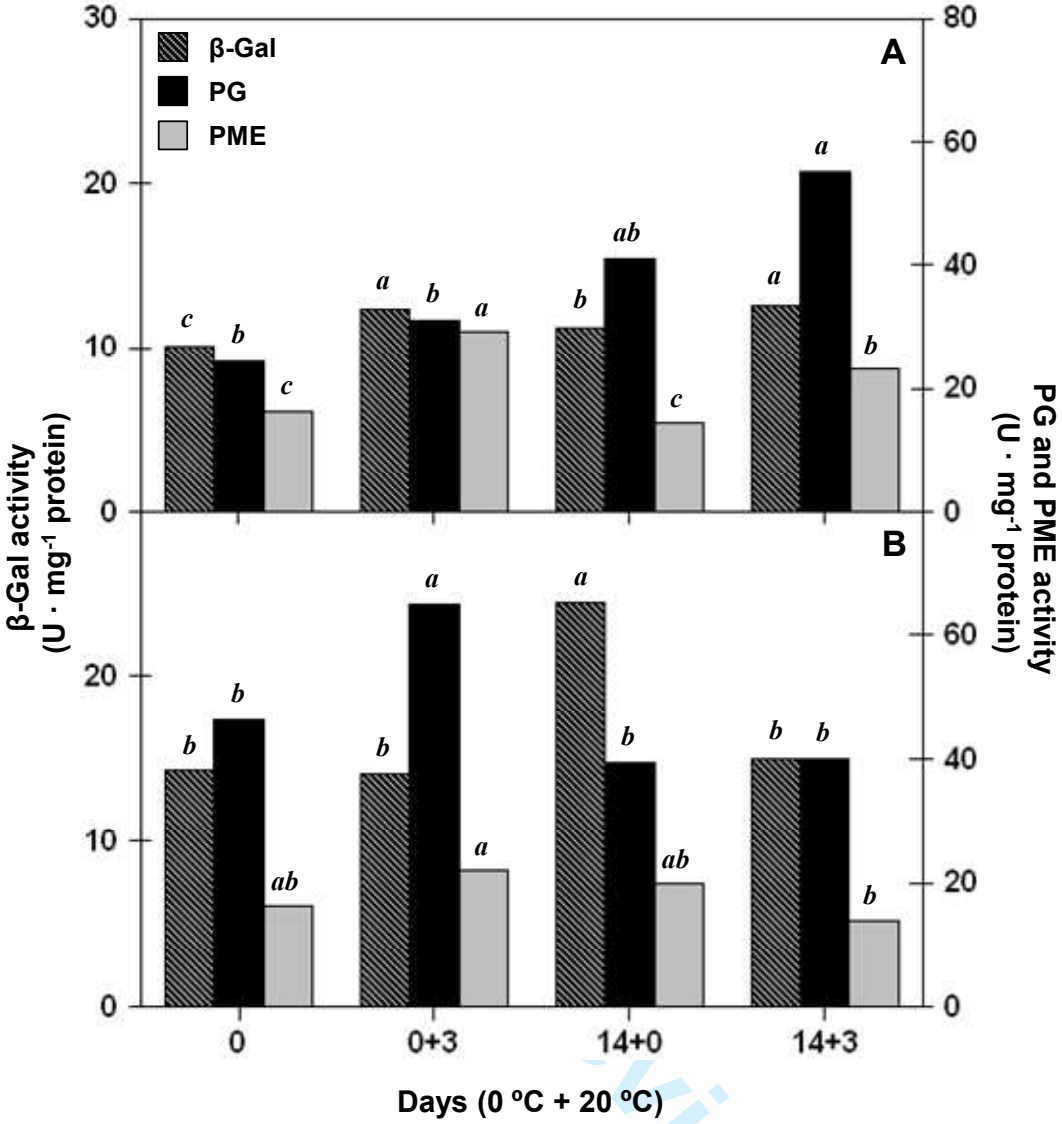

Figure 3. β-Galactosidase, polygalacturonase and pectinmethylesterase activities in 'Celeste' (**A**) and 'Somerset' (**B**) sweet cherries at harvest and after cold storage. Bars represent means of three replicates. For a given enzyme activity, values bearing different letters are significantly different at $P \leq 0.05$ (LSD test).

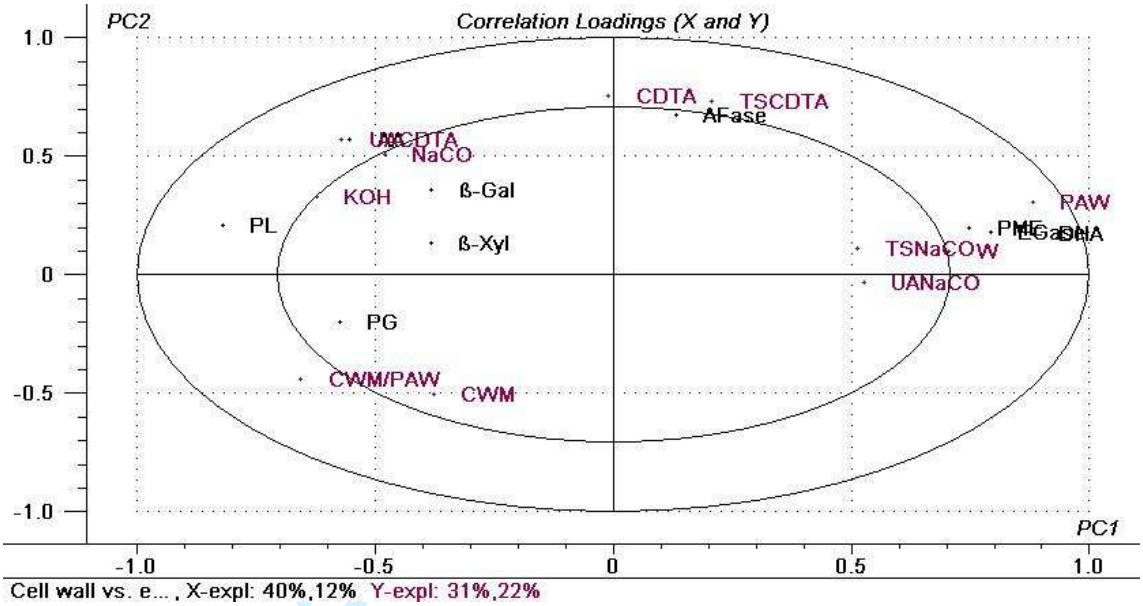

Figure 4. Correlation loadings plot of PC1 *vs*. PC2 corresponding to a PLSR model for yield and composition of cell wall fractions (*Y* variables) vs. cell wall-modifying enzyme activities and ascorbic acid content (*X* variables) in 'Celeste' and 'Somerset' cherries after cold storage at 0 ºC (AA, ascorbic acid; DHA, dehydroascorbic acid; UA and TS, uronic acid and total sugar content in a given cell wall fraction, respectively).