
RELATIONSHIP BETWEEN QUALITY PARAMETERS AND INTERNAL DISORDERS IN PEAR BY MEANS OF MULTIVARIATE ANALYSIS

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ABSTRACT: Multivariate analysis was used to establish the relationship between quality parameters and two post harvest disorders (brown heart and core browning) that occurred during pear storage in two different years. The damage percentage for each disorder was related to length of storage, storage CO₂ concentration and values of maturity indexes: colour, firmness, sugar concentration (SSC) and acidity. Direct counting clearly indicated harvest date and CO₂ concentration as determining factors for the incidence of disorders. The PCA model distinguished two groups: healthy and damaged fruits. Within the damaged group, the model also clearly discriminated between fruit with brown heart and fruit with core browning. In this scheme, core browning appears before 3 months of storage and brown heart appears later. We also established a complementary PLS model that quantified the importance of each variable and predict the percentage of disorder. According to this model, brown heart negatively correlated with acidity over the two years, while core browning correlated with firmness and to a lesser extent with acidity. We conclude that core browning and brown heart are two different disorders related to specific changes in quality parameters.

Key words: brown heart, core browning, quality parameters, principal component analysis, partial least squares.

INTRODUCTION

Controlled-atmosphere (CA) of pears has received considerable attention, not only for its desirable effects in extending storage life, but also due to its harmful effects including the induction of physiological disorders (Richardson et al., 1997). In such atmospheres, pears may develop core browning (CB) and brown heart (BH). The severity of these disorders depends on the particular CA conditions and length of storage. The higher the level of CO₂ and longer the duration of storage, the greater the incidence of disorders (Pintó et al., 2001).

Core Browning in pears is characterised by softening and browning: first of the core and later of the flesh (Lammertyn et al, 2001). In advanced stages, lesions usually extend and may

affect the entire pulp (Hall and Scott, 1977). In brown heart, lesions may dry out to form large cavities in the flesh. The occurrence of these disorders is influenced by: the weather, orchard-related factors, harvest date, post harvest treatment and the conditions and duration of storage (Roelofs and de Jager, 1997). Grant et al., 1996, observed that internal browning (IB) is also linked to advanced fruit maturity and that in the late-harvested fruits the pulp's resistance to gas diffusion may account for the increase in the incidence of such disorders.

A great inconvenience of CB and BH is that their symptoms are internal and not externally visible. The economic losses may be high because consumers are disappointed when buying pears with good appearance but with severe internal disorders. Furthermore, no detection system is available to characterize the damaged fruit. Further research is needed to determine a suitable detection system, if possible, should be non-destructive, that could preferably be used to reliably predict the susceptibility fruit to CB and BH.

Several techniques have so far been proposed for this purpose. Ascorbic acid (AA), for example, seems to be a good marker of CB in Conference pears (Veltman et al., 1999). In this cultivar, browning begins when the AA level is below a certain threshold (Eccher Zerbini et al., 2002). Volatile compounds such as ethanol, acetaldehyde and ethylene have also been described as good indicators for freezing injury, heat stress and water stress (Forney and Jordan, 1988; Kimmever and Kozlowski, 1982). Elevated concentrations of ethanol, for example, indicate physiological damage in heat-treated fresh broccoli (Forney and Jordan, 1998). Their potential use as indicators of CB and BH has, however, yet to be confirmed. Chlorophyll fluorescence has also been used as an indicator of chilling stress in green pepper (Lurie et al., 1994), cucumber (Van Kooten et al., 1992) banana and mango (Smillie et al., 1987). On a limited scale, this technique may detect physiological disorders related to low O₂ or high CO₂ damage in stored apples (DeEll et al., 1995; Mir et al., 1998). Similarly, fluorescent imaging seems a very promising tool for the detection of early stress or disease in apple fruit. Using fluorescent imaging it was possible to visualise internal browning in Jonagold fruit before the apparition of visually evident symptoms (Ciscato et al., 2000). Time-domain reflectance spectroscopy (TDSR) has proven a useful technique for non-destructive identification of mealiness in apples (Valero et al., 2001).

These last techniques are good diagnostic tools for detecting physiological disorders during fruit storage. They are, however, very sophisticated and too expensive and difficult to use in the packinghouse. We therefore need more practical methods of detecting BH and CB disorders. These might involve the use of quality parameters. According to Crisosto et al. (1994), skin colour can be an indicator of internal browning in Chinese pears. For Song et al. (2000), surface colour and soluble solids did not highly correlate with flesh browning resulting from heat and freeze stress and only titratable acidity was affected by the heat treatment (Song et al., 2000).

This study sought to determine whether quality parameters might characterise CB and BH-damaged fruits. Correlations between these parameters and the incidence of disorder were established applying multivariate analysis. A further objective was to determine whether BH and CB are two different disorders. We finally established a marker for each disorder that could be easily used by packinghouses.

MATERIALS AND METHODS

Plant material

Blanquilla pears were grown in Lleida, Spain, and harvested in two consecutive years (1998 and 1999). In the first year, pears were harvested on 12th August. In 1999, pears were harvested on two different dates: 4th August (harvest 1) and 11th August (harvest 2). The orchard characteristics were as follows: tree age - 18 years old; spacing - 5 x 2.5; and rootstock - franc. Fruit was picked free of defects from three trees (blocks) and according to a completely randomised design. In 1998, fruit was harvested 3 days after the optimal commercial date, following local recommendations (firmness, SSC and acidity values). In the second year, in order to ensure the maximum number of disorders, fruit was harvested 3 days before the commercial harvest date (harvest1=H1) and 1 week after this date (harvest 2=H2). Fruit from each harvest and block was randomly selected and put in controlled atmosphere storage as described below.

Storage

After harvest, fruit was separated into two lots and stored at -1°C and 92 % RH, at 2 % O₂ + 0.7 % CO₂ (standard CA conditions) or at 2 % O₂ + 5 % CO₂ (inducing storage conditions). In 1998, the fruit samples were evaluated after three months of storage and in the second year after two, four and six months of storage (S1, S2 and S3 respectively).

Incidence of disorders

Core browning (t1) and brown heart (t2) were evaluated by longitudinally and transversally cutting 200 pear samples for each storage condition. For each condition, the incidence of disorder was expressed as both a percentage of damaged fruit and a percentage of damaged tissue. For 1998, pear samples were only examined for brown heart and scored according to the following scale: I0 = no symptoms, I1 = very slight (discrete brown spots on the core), I2 = slight (core completely brown without cavities in the flesh), I3 = moderate (core brown + slight cavities in the flesh), I4 = severe (brown core + large cavities in the flesh) and I5 = very severe symptoms (core + flesh completely damaged). For 1999, both core browning and brown heart were estimated and results were expressed using the following classification: g0 = healthy or slightly damaged fruit (< 20%), g1 = acutely damaged fruit (≥ 20%), t0 = healthy fruit, t1 = fruit with core browning, t2 = fruit with brown heart and %alt = percentage of damaged tissue.

Evaluation of quality parameters

For the first year, the fruit (n = 30) was analysed at harvest and after three months of storage and the following quality parameters were determined: flesh firmness (firm), titratable acidity (acid), skin colour (L*, a*, b*), soluble solid concentration (SSC) and incidence of brown heart (%alt). For the second year, all the same quality parameters were determined except skin colour, and these were related to the incidence of brown heart and core browning. Firmness was measured on two opposite peeled sides using an Effegi tester (FT 3279) fitted with an 8 mm diameter plunger. Results were expressed in kg. Colour was measured on two opposite sides of each fruit, placing the head of a portable tristimulus colorimeter (Chromameter CR-

200, Minolta, Japan) at the midpoint between the stem and the end of the calyx and recording fruit chromaticity in the L* a* and b* space coordinates (McGuire, 1992). Soluble solid concentration (SSC) was measured using the juice of a quarter of the fruit and mixing that of five different fruits (values are the means of six different samples). SSC was determined by measuring the refractive index with a digital refractometer (Atago PR-100), and using juice directly extracted from the pulp: results were expressed in percentage terms. Acidity was assessed as follows: 10 ml of the juice previously extracted for SSC were diluted with 10 ml of H₂O and titrated with 0.1 M NaOH using 1 % phenolphthalein. Acidity was expressed in grams of malic acid per litre of juice.

Chemometrics

Data were analysed using Principal Component Analysis (PCA) in order to establish a preliminary descriptive relationship between internal disorder and quality parameters. Partial Least Squares Regression (PLSR) was also used to quantify this correlation. These analyses were carried out using Unscrambler v. 6. 11b software (Camo AS (ed.) 1996).

For 1998, the data set incorporated two category variables to identify CO₂ concentration during storage (cam7 and cam5), two category variables for healthy and damaged fruit (g0 and g1) and three category variables to identify types of disorder (t0, t1 and t2). Data for 1999 included all the above variables plus five more: three corresponding to length of storage (S1, S2 and S3) and two corresponding to harvest date (H1 and H2). In addition to these category variables, the quantitative variables used were: percentage of core browning or brown heart (%alt), firmness (the mean of two measurements, firm), acidity (acid), soluble solid content (SSC) and chromaticity values (L*, a*, b*). Category variables were codified using a discrete value, with a value of +1 when samples were included in the category and -1 when they were excluded. The data for 1998 included 60 samples and 14 variables; that for 1999 included 72 samples and 16 variables. As the variables were measured in different units, there were large differences between them with respect to the corresponding mean values, variance and standard deviation. To avoid these differences, the data were subsequently centred (by subtracting the mean) and weighted with the inverse of the standard deviation.

RESULTS AND DISCUSSION

Effect of CA storage on disorders

For both the years studied, fruit kept under standard CA conditions (0.7% CO₂) did not develop disorders, whereas fruit kept under high CO₂ was damaged (Figure 1). There were, however, important differences between the two years. For 1998, the recorded incidence of BH was only slight (result not shown). For 1999, the two disorders appeared at different times: CB appeared first, at 2 months and BH appeared later (Figure 1b). The extent of BH damage was proportional to the duration of storage and the stage of maturity at harvest (Figure 1). The more mature the fruit was, the greater was the incidence of brown heart. These results confirmed those of Larrigaudière et al. (1998) and Lenthéric et al. (1999), and showed that the stage of maturity at harvest played an important role in determining the incidence of disorders. As a consequence, the model subsequently developed was established using only fruit from the late harvest and that had been stored at high CO₂ concentrations.

Multivariate analysis for the incidence of brown heart in 1998

For 1998, principal component analysis (PCA) was used to obtain a global overview of the samples after 3 months of storage. The score plot of PC1 vs. PC2 showed two clearly differentiated groups by PC1. One corresponded to the samples labelled I0, I1 and I2 (healthy or slightly damaged fruit) in the left half of the space, and the other included samples labelled I3, I4 or I5 (acutely damaged fruit) located in the right half of the PC space (Figure 2a). The first two PC's explained 43 % of the variance in the data and the third explained a further 10 %. Although these values were not very high, they were higher than the variances obtained when we used the three studied variables separately: this result shows that the model could be useful for our objectives.

The loading plot of PC1 vs. PC2 from the same PCA model showed a very negative correlation with respect to PC1 between the percentage of disorder and the acidity. The rest of the variables were mainly related to PC2 and consequently only slightly correlated with the variable studied (Figure 2b).

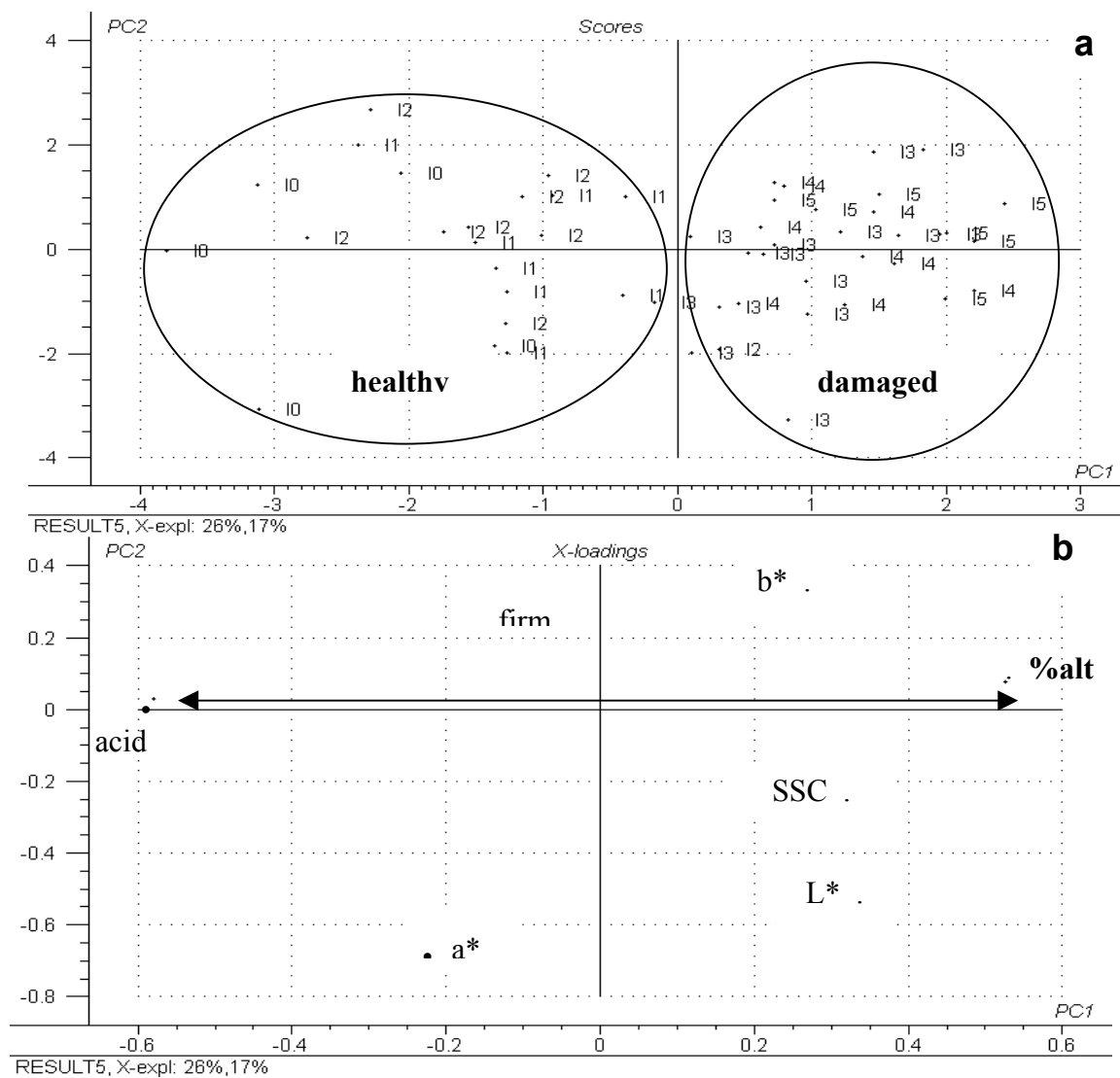


Figure 2.- Score and loading plots of PC1 vs. PC2 for brown heart in 1998. (a) Score plot of samples labelled according to intensity of damage: healthy or slightly damaged fruits (I0, I1 and I2) and acutely damaged fruit (I3, I4 and I5). (b) Loading plot of PC1 vs. PC2. Sixty samples and seven variables are included and labelled using codes defined in the text.

Another model, called Partial least squares (PLS), was used to predict the percentage of disorder in new samples. The loading plot of this PLS model (Figure 3a) showed that PC1 was strongly influenced by the variable percentage of disorder, which correlated very negatively with acidity. PC2 explained only 5 % of the variance and was influenced by some colourimetric parameters (L^* , a^* and b^*). The rest of the variables (SSC and firmness) displayed smaller loading values and seemed to be less important for determining the disorder percentage. The regression coefficients shown in figure 3b were obtained using 3 PC's as suggested by full cross validation. Acidity presented the highest negative correlation ($r=-0.71$) with the incidence of disorder and was the most important variable for predicting BH in 1998. This result confirms data already presented for the PCA model. In PC1 vs. PC2, the PLS model explain up to 75 % of the total variance in the percentage of brown heart (Table 1). The prediction error (RMSEP) for the validation samples was of 10.71 units and the correlation between the predicted and measured values was 0.85 (Table 1). These are both rather good values for qualitative purposes and showed that, even with only 3 PC's, the present model was adequate for predicting the incidence of brown heart in new samples.

From the results obtained for this first year, it can be concluded that colourimetric parameters, firmness and SSC cannot predict BH disorder. The incidence of disorder had a much clearer correlation with acidity values: these seem to be the best marker for predicting damage. As shown in Figure 1, the two kinds of disorder were clearly separated. This result favours the hypothesis of the presence of two different disorders. To test this hypothesis, we carried out two separate analyses in 1999: one for BH and a second for CB. The colourimetric parameters, which had showed very low loading values, were not included in this second study.

Table 1.- Parameters of the partial least squares regression (PLSR) model for the prediction of the percentage of disorder (Y-variable) in function of quality parameters (X-variables). BH: brown-heart; CB: core browning.

	BH- year 1	BH- year 2	CB- year 2
N° of PLS factors	3	2	2
RMSEP^a	10.71	11.10	7.06
Correlation^b	0.85	0.85	0.9
% of Y variance explained^c	75	9	85
% of X variance explained^d	8	43	70

^a Root mean squares error of prediction (in the same units as the values)

^b Correlations between predicted and measured values

^c Amount of Y-variance explained for two first PLS factors

^d % of variation in X data that explain variations in Y

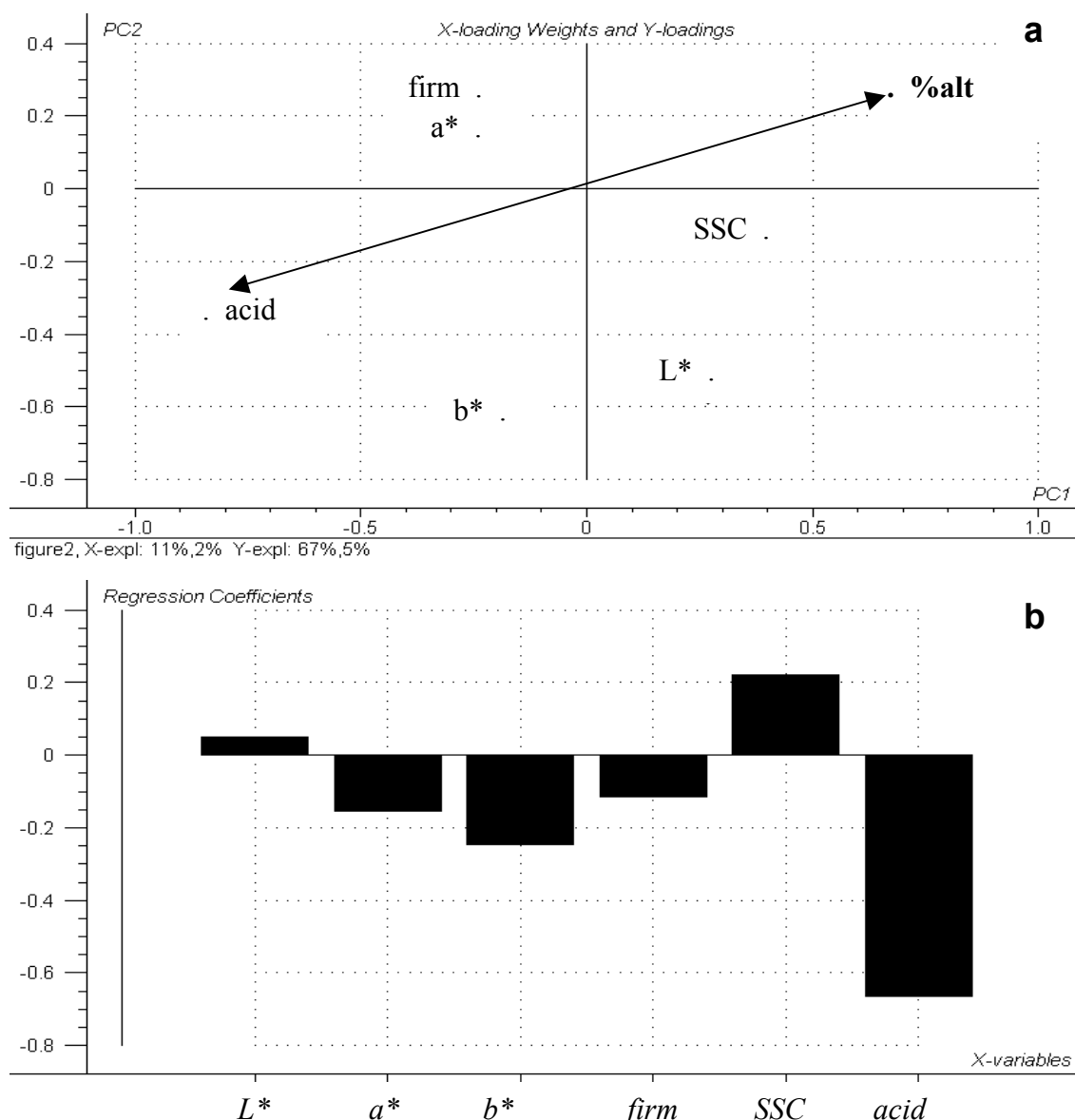


figure2, X-expl: 11%,2% Y-expl: 67%,5%

Figure 3.- PLS model for brown heart in 1998. **(a)** Loading plot of PC1 vs. PC2. **(b)** Regression coefficients between percentage of disorder and all other variables. Sixty samples and seven variables are included and labelled using codes defined in text.

Multivariate analysis of the incidence of brown heart and core browning in 1999

Full-data PCA model for quality parameters

The global PCA model according to the two principal components (PC1 and PC2) is shown in Figure 4a. As for 1998, two groups are separated according to PC1 scores: healthy samples (g0) on the left and damaged samples (g1) on the right. The disorder percentage had a strong influence upon the differentiation of the sample and explained 29 % of the variability. Following PC2, it was also possible to distinguish two groups of samples (Figure 4b) defined by type of disorder (t1= core browning and t2=brown heart). Length of storage also strongly characterised the samples in PC2 (Figure 4c). In this scheme, core browning

correlated with short-term storage (S1), whereas brown heart correlated with longer storage (S2 and S3).

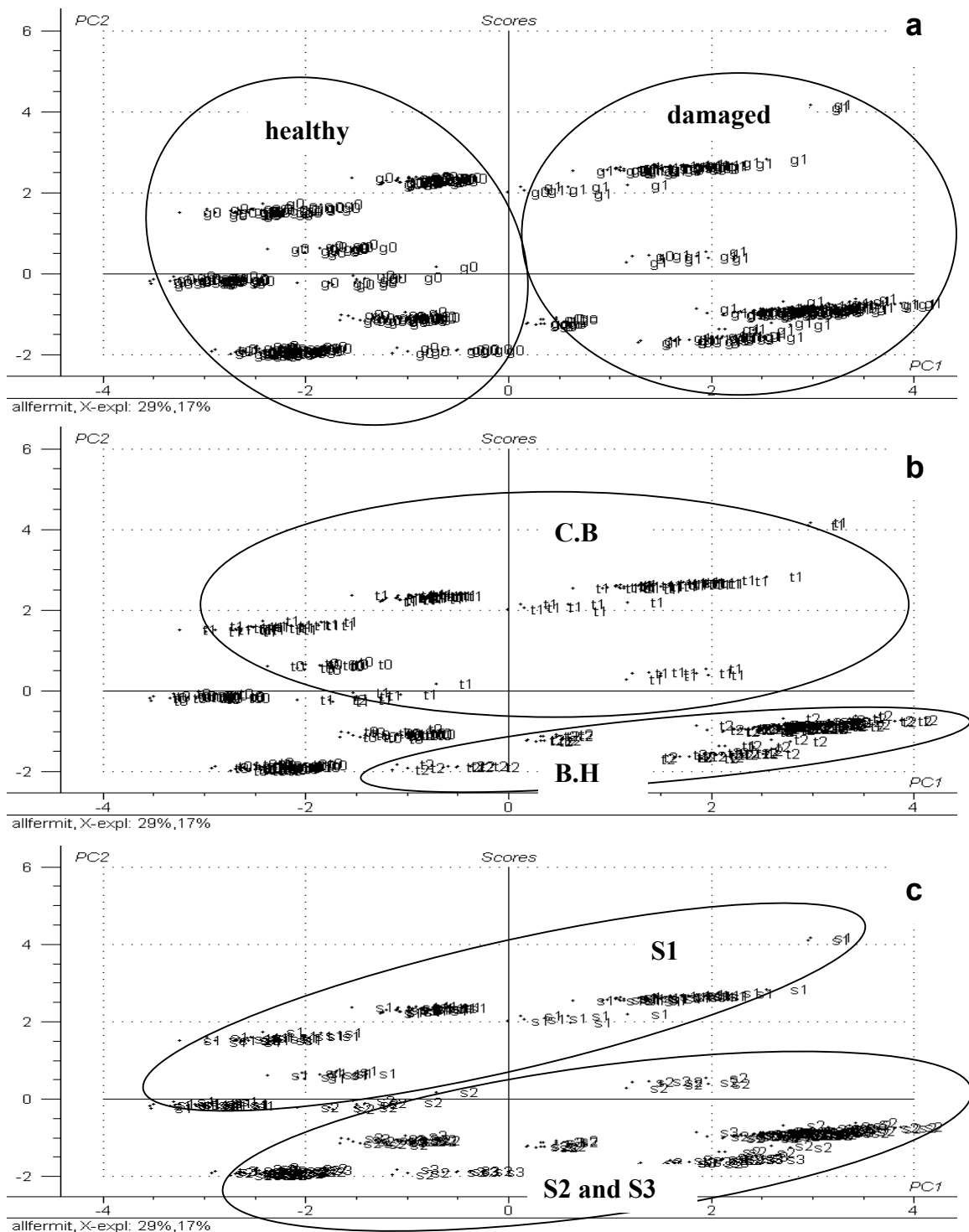


Figure 4.- Score plots of PC1 vs. PC2 from full-data PCA for 1999. **(a)** Score plot for samples labelled according to intensity of damage: healthy (g0) and damaged fruit (g1). **(b)** Score plot for samples labelled according to type of disorder: core browning (t1) and brown heart (t2). **(c)** Score plot for samples labelled according to length of storage (s1, s2 and s3). Seventy-two samples and thirteen variables are included and labelled using codes defined in the text.

The loading plot of PC1 vs. PC2 for this PCA model (Figure 5) confirmed previously described results. Taken together, the two models indicated that core browning and brown heart are two different disorders. This confirmed our observation that some pears are affected by core browning but do not have cavities, whereas others have cavities but show no signs of browning. Our results differed from those of Roelofs and DeJager, 1997, who noted the presence of cavities in fruit affected by internal browning and said that they were also likely within areas previously affected by internal browning. The question therefore remains: Are CB and BH associated with the same disorder? To answer this question, we conducted a specific PLS analysis for each disorder.

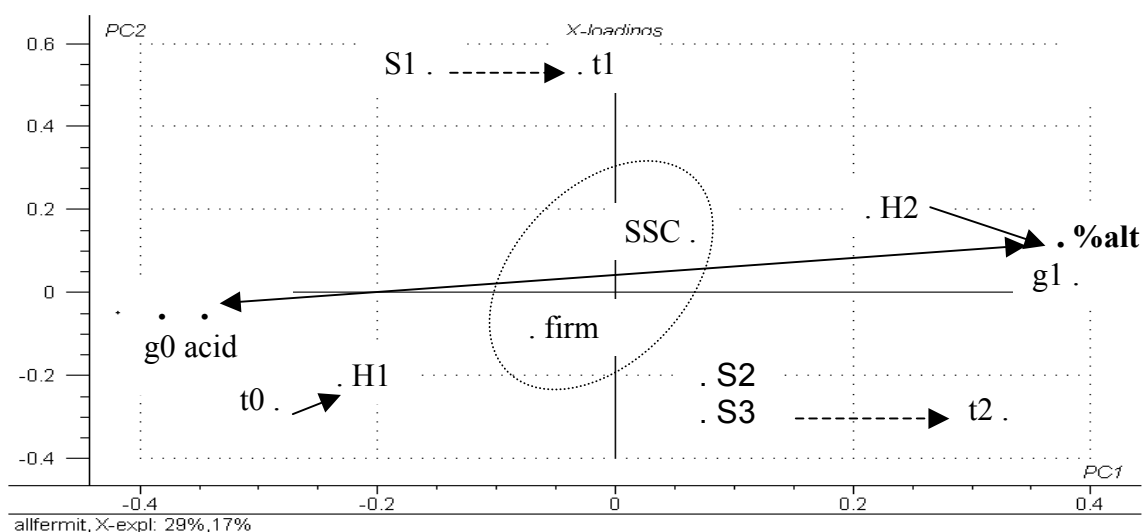


Figure 5.- Loading plot of PC1 vs. PC2 from full-data PCA for 1999. Seventy-two samples and thirteen variables are included and labelled using codes defined in the text.

PLS model for brown heart in 1999

Figure 6a shows the loading plot of the percentage of BH disorder obtained using the two PLS factors that provide the best explanation of variance (see also Table 1). The percentage of disorder and solid soluble content displayed a positive loading value with respect to PC1 (figure 6a), whereas acidity correlated negatively with the disorder in the PC1 direction. Firmness displayed a high positive loading value with respect to PC2 (which only explained 7 % of the variance) and can therefore be considered unimportant for predicting the disorder (the smallest loading value in direction PC1). The prediction error of the validation samples between the quality parameters and the percentage of disorder (RMSEP) was 11.10 (Table 1) and the correlation coefficient for predicted vs. measured values was 0.85 (Table 1). These two values suggest that this is a satisfactory model for predicting new incidences of disorder in the fruit.

As in the case of the 1998 study, regression coefficients relating to quality parameters (Figure 6b) showed that acidity was the parameter which had the most important negative correlation with respect to the disorder. SSC also showed an important positive correlation with incidence of the disorder. The increase in SSC may be regarded as a consequence of ripening and the correlation observed here probably reflects the link between harvest date (when fruit is mature) and the percentage of alteration (Figure 5). In general, the more mature fruit is, the more sensitive it becomes to brown heart (Roelofs and DeJager, 1997). Our results support this theory.

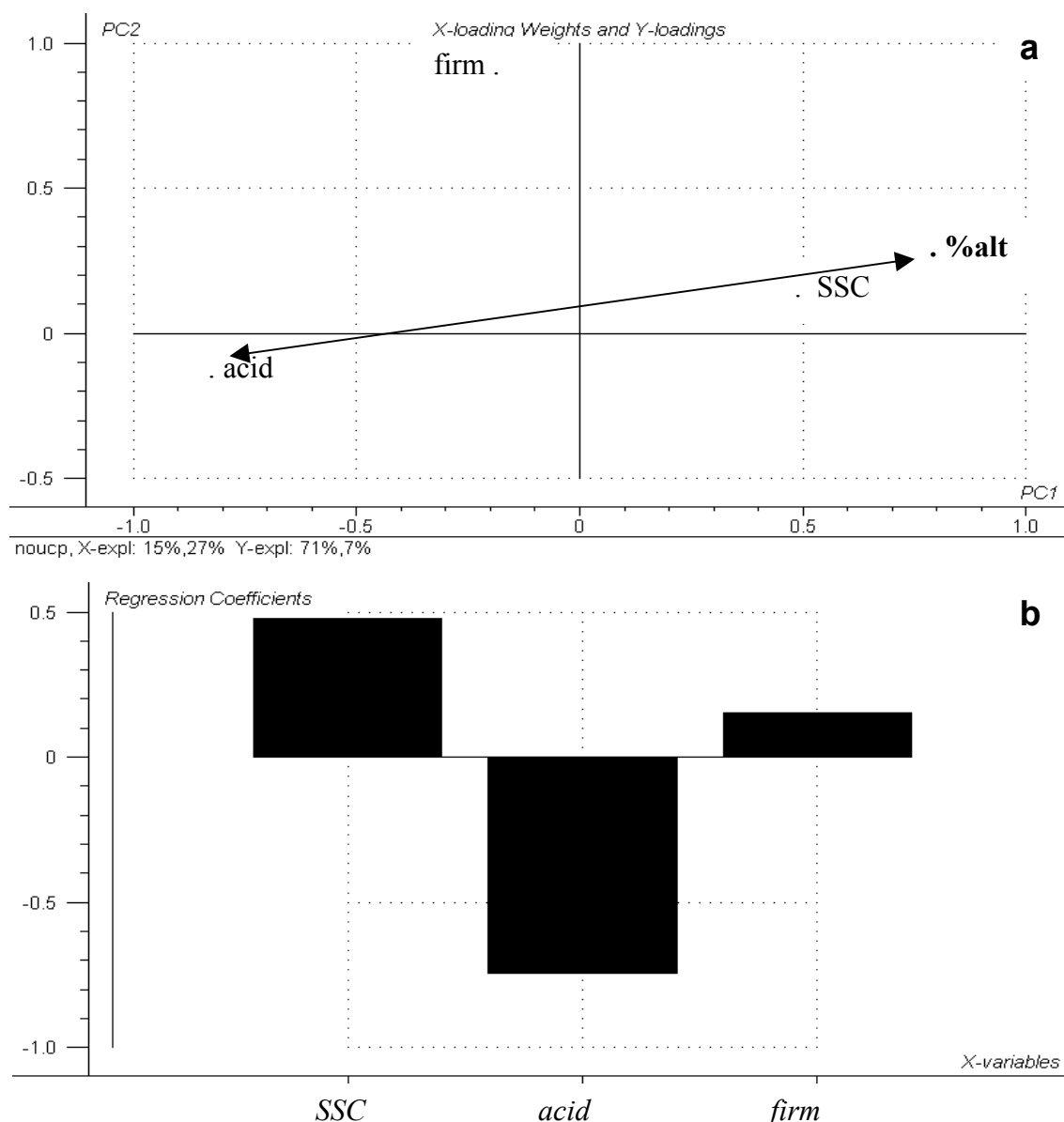


Figure 6.- PLS model for brown heart in 1999. **(a)** Loading plot PC1 vs. PC2 from PLS model of variable percentage of disorder. **(b)** Regression coefficients between percentage of disorder and all other variables. 18 samples and 4 variables are included and labelled using codes defined in the text.

PLS model for core browning in 1999

The loading plot for the first two PLS factors accounted for up to 85 % of total variation in the incidence of core browning (table 1 and figure 7a). This value was higher than for brown heart (in both years), but more information about the X-variables is needed to explain the model (Table 1). The prediction error (RMSEP) of the validation samples was 7.06 and the correlation coefficient between the predicted and measured samples was 0.90 (Table 1). Both values showed the accuracy of the model for estimating the incidence of CB in new fruit.

In the case of CB, the variable that most closely correlated with the percentage of disorder was fruit firmness (Figure 7a). This quality parameter correlated negatively in both directions (PC1 and PC2). Solid soluble content only depended on PC2 and acid content displayed a

smaller loading value and appeared to be less important for determining the incidence of disorder.

Regression coefficients for the variables obtained using 2 significant PCs are presented in figure 7b. These coefficients confirmed the results observed in figure 7a and again showed that the most important variable for explaining the incidence of CB was fruit firmness, while acidity provided indications to a lesser extent. It is widely recognised that maturity at harvest determines the sensitivity of fruit to core browning during storage (Lentheric et al., 1999; Veltman et al., 2000). On the basis of our results, CB appears to differ from BH. CB is mainly the consequence of a senescence process, whereas BH is probably due to oxidative damage in which acids, and particularly ascorbic acid, seem to play an underlying role (Larrigaudière et al., 2001)

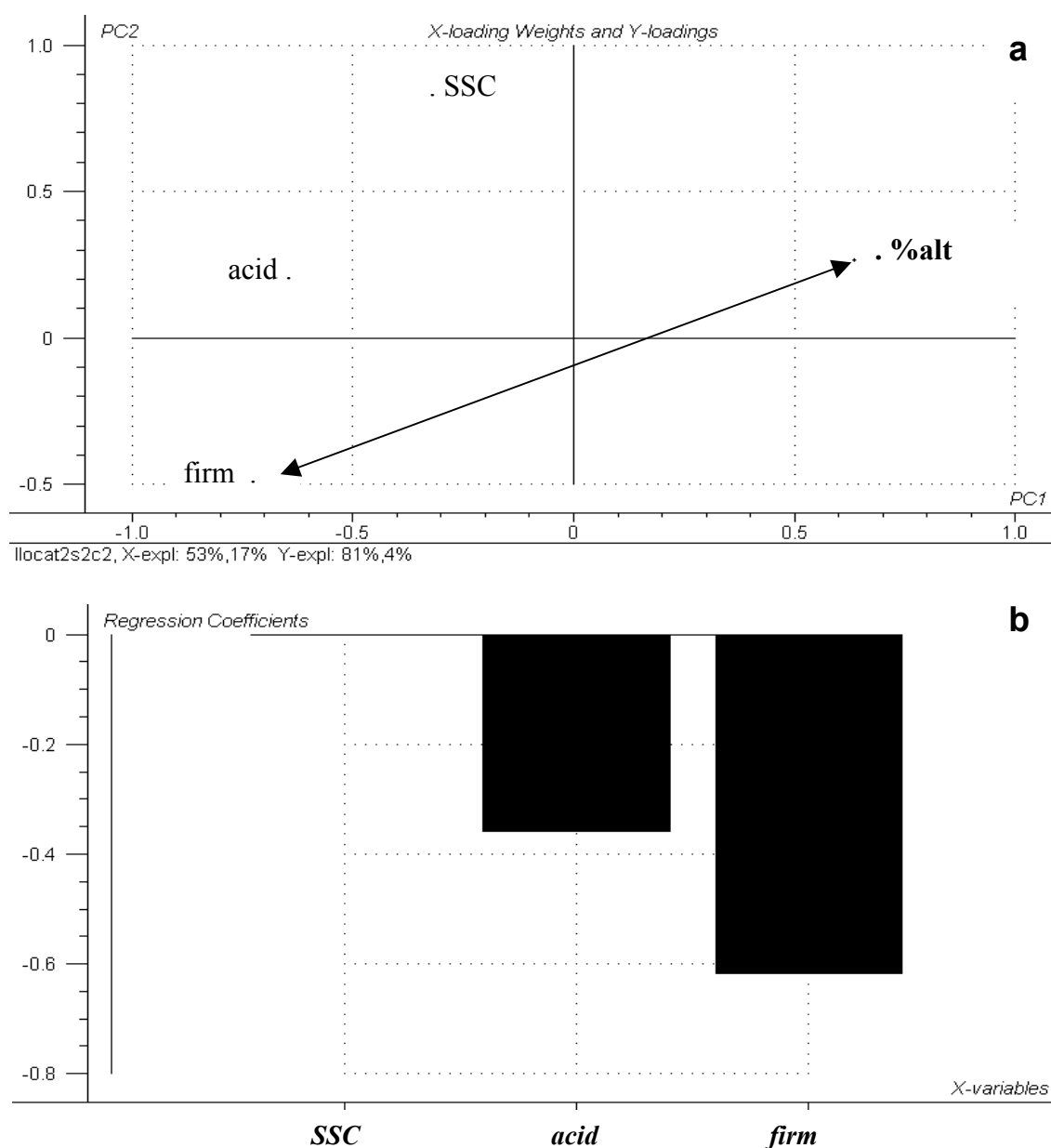


Figure 7.- PLS model for core browning samples in 1999. **(a)** Loading plot PC1 vs. PC2 from PLS model of variable percentage of disorder. **(b)** Regression coefficients between percentage of disorder and all other variables. 24 samples and 4 variables are included and labelled using codes define in the text.

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Capítol 5

Multivariate analysis of the metabolic pathways involved in core browning and brown heart disorders in pears

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MULTIVARIATE ANALYSIS OF THE METABOLIC PATHWAYS INVOLVED IN CORE BROWNING AND BROWN HEART DISORDER IN PEARS

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ABSTRACT: In this work multivariate analysis was used to characterize and differentiate two important post harvest disorders that occurred in pear during storage: brown heart and core browning. Damage percentage for each disorder was correlated with length of storage and storage CO₂ concentration. Disorder incidence was also correlated with ACC and MACC content, acetaldehyde, ethanol, and finally with levels of ascorbate and glutathione. The first characterization of samples (PCA model) clearly distinguished two groups related with CO₂ concentration: healthy and damaged fruits. Within the damaged group, the model also discriminated between fruits with brown heart and those with core browning. Core browning first appeared during storage, while brown heart appeared later. To complement these initial results, we established a PLS model to quantify the importance of each variable and predict the percentage of disorder. According to this model, core browning correlated strongly with ACC, and also positively, although less with fermentative metabolites, but it correlated negatively with ascorbate levels. In contrast, brown heart only showed a clear negative correlation with ascorbate levels. Collectively, these results show that core browning and brown heart are two different disorders and that they involve different metabolic pathways.

Key words: brown heart, core browning, ACC, MACC, ethanol, acetaldehyde, ascorbate, glutathione, principal component analysis, partial least squares.

INTRODUCTION

Some pear varieties, such as Blanquilla are susceptible to internal disorders (1). These appear in controlled atmospheres (CA) and especially at high CO₂ levels. Core browning (CB) and brown heart (BH) are among the most serious post-harvest problems affecting this variety (2, 3).

These two disorders are characterized by browning of first the core and later the flesh (4). Core browning first appears in the form of damp, brown areas in the mid-cortex of the flesh (5). Lesions subsequently tend to extend and may affect the entire pulp. In the case of brown heart, the tissue becomes dry and cavities form in the pulp. In both cases, the symptoms are similar in various pear cultivars (6). One argument in favor of the hypothesis of different

disorders is the fact that the symptoms frequently appear separately. Late harvesting and high CO₂ storage can increase susceptibility to disorders (7, 8). Climatic factors are also important (9) and delayed CA storage reduces the incidence of BH (10). Are BH and CB two different disorders? The question remains open, so that one of the objectives of this study was to clarify this point.

For the present, the mechanisms involved in these physiological disorders remain unclear. The main physical cause appears to be excessive concentrations of CO₂ during storage. Pesis et al. (11) showed that CO₂ resulted in alcohol and acetaldehyde accumulation in damaged tissue. Another hypothesis centers on the role of the oxidative processes: changes in antioxidant metabolism are a general feature of a plant's response to changing environmental conditions (12). These changes lead to an enhancement in active oxygen species (AOS) such as O₂⁻ and H₂O₂ (13), which can indiscriminately cause lipid peroxidation and protein denaturation (14). To prevent such damage, plants are equipped with several enzymatic and non-enzymatic antioxidant defense systems. Among the non-enzymatic antioxidants, ascorbate (AsA) plays a key role in the destruction of AOS, while glutathione (GSH) is essential for the regeneration of ascorbate. In response to stress, plants normally increase components of this antioxidative system (15, 16). A decrease in ascorbate is often associated with a reduced capability to prevent oxidative damage (17) and may also be related to the induction of BH disorder during CA storage (18, 10).

May CB and BH be related to other biochemical processes, such as ethylene biosynthesis? One argument that shows that ethylene probably is involved is the fact that a 1-MCP treatment significantly reduced the incidence of CB and BH in Blanquilla pears (19). This result supports the involvement of ethylene in the induction of BH and CB disorder. But, is ethylene the most relevant pathway that causes these disorders?

To answer to these questions, we studied the relationship between the fermentative, oxidative and ethylene metabolisms and the incidence of BH and CB disorder by means of multivariate analysis. The ultimate objectives were to determinate whether CB and BH are two different disorders and which pathways are involved in each case.

MATERIAL AND METHODS

Plant material

Pears (*Pyrus communis* cv. *Blanquilla*) were harvested in summer 1999, from an orchard planted in 1980 in Lleida (Spain). Fruits were harvested three days after the optimal commercial date and according to local recommendations (firmness, soluble solid concentrations, and acidity values). Fruits were selected on the basis of size and absence of defects, and stored in experimental chambers (22m³) for two (S1) and four (S2) months at -1°C and 92% RH under the following conditions: controlled storage conditions (cam7, 2% O₂ + 0.7% CO₂) and disorder inducing storage conditions (cam5, 2% O₂ + 5 % CO₂).

Estimation of internal disorders

After 2 and 4 month's storage, pears (n=200) were cut longitudinally and transversally in order to examine the incidence of CB and BH. Severity symptoms for each disorder were scored using a classification in which: t0 = healthy fruits, t1 = fruits with core browning, t2 = fruits with brown heart, g0 = slightly damaged fruits (< 20%), g1 = acutely damaged fruit (≥ 20%) and %alt = % of damaged area.

Determination of ascorbate (AsA) and glutathione levels

Total ascorbate was extracted from twelve pears following the protocol of Brubaker et al. (20) mixing 25 g (fresh weight) of pulp with 75 ml of twice distilled water and 10 ml of a solution of 10 % (w/v) metaphosphoric acid and 5 % 2,3-mercaptopropanol. The homogenate was centrifuged at 20,000 g for 25 min and water was added to the supernatant to a final volume of 200 ml. All procedures were carried out at 1 °C and in the dark. Analyses were carried out by high-performance liquid chromatography (HPLC) with a μ Bondapak/carbohydrate column (Waters Associates) and employing absorbance detection (Applied Biosystems UV detector 783A) at 254 nm.

Glutathione was determined using the Degoussé et al. spectrophotometric method (21). The pulp (40 g fresh weight, n=12) was homogenized at 4° C in 80 ml of oxygen-free extraction buffer, consisting of 0.15 % (w/v) sodium ascorbate and 20 mM EDTA in 0.1 M Tris buffer (pH 8.0). Ten ml of 10 % SDS diluted in the extraction medium was immediately added to stabilize the sulphhydryl groups. The homogenate was centrifuged at 30,000 g for 15 min. A 5 ml aliquot of the supernatant was mixed with 1.25 ml of 50 % TCA, and after precipitation (10 min at ambient temperature), samples were centrifuged as above. The deproteinised supernatant (0.5 ml) was mixed with 1 ml of 1 M Tris buffer (pH 8.0) and 0.1 ml of 10 mM DTNB in 0.2 M sodium phosphate buffer. After 5 min the absorbance at 412 nm was measured with a Kontron 922 spectrophotometer. The molar extinction coefficient used was $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ (22), and A_{412} was corrected for the color of a blank sample prepared without DTNB (replaced by 0.2 M sodium phosphate buffer, pH 7.0) and for the color of a blank reagent prepared without extract (replaced by extraction buffer).

Volatile measurement

Ethanol and acetaldehyde contents were determined according to Volz et al. (23). Volatiles were extracted from the flesh juice of 12 separate fruits, immediately after removal from storage. Juice samples (5 ml) were stored at -25 °C until analysis. Samples were subsequently put in a 10 ml test tube with a screw cap and incubated in a water bath at 60° C. After 60 min, a headspace sample was taken with a 1 ml glass syringe in order to determine acetaldehyde and ethanol concentrations. This was done using a gas chromatograph (HP 5890H, Hewlett Packard) equipped with a flame ionization detector (at 200 °C) and a column (2 mm x 2 m) containing 5 % Carbowax on 60/80 Carbopack (Supelco, Bellefonte, PA, USA) at 85 °C. Tissue concentrations were calculated using a standard curve, which was generated by injecting standard solutions of known concentrations.

Determination of ACC and MACC concentrations

Approximately 0.15 g of freeze-dried pulp (n=12) was taken in order to analyze ACC and MACC contents. For this, ACC samples were extracted under reflux for 15 min with 80 % ethanol, which was removed under vacuum at 40 °C. Free ACC content was measured directly on the aqueous extract according to the Lizada and Yang (24) method. Malonyl ACC (MACC) was extracted from the same aqueous extract, which had been previously hydrolyzed with 6 N HCl at 100 °C for 3 hours (25). MACC was taken as the difference between total and free ACC concentrations. Yields were about 85%.

Chemometrics

PCA (Principal Component Analysis) is a multivariate statistical technique used to reduce the number of variables and facilitate interpretation of data based on data visualization techniques. In this work, PCA was used to study the relationships between disorder incidence and fermentative, oxidative and ethylene metabolisms. Partial least squares regression (PLSR) was also used to analyze the correlation between the percentage of disorder and the rest of the variables. These analyses were carried out using Unscrambler v. 6.11b software (26).

The data set included eight categories of variables: two relating to the chamber (cam7 and cam5), three for length of storage (S0, S1 and S2), two for healthy and damaged fruits (g0 and g1) and three identifying the types of disorder (t0, t1 and t2). Category variables were codified using discrete values, with a value of +1 being attributed when samples were included in this particular category and -1 when they were excluded. In addition to these category variables, quantitative variables were: percentage of core browning or brown heart (%alt), ascorbate content (AA), glutathione content (GSH), acetaldehyde content (ACETAL), ethanol content (ETANOL), ACC and MACC levels. The resulting data matrix contained 72 samples and 15 variables. As the variables were measured in different units, they differed greatly with respect to mean values, variance and standard deviation. To remove these differences, the data were subsequently centered (by subtracting the mean) and then weighted with the inverse of the standard deviation.

RESULTS AND DISCUSSION

First exploration of the data obtained from the PCA model

A PCA model is used to explain which variables are really important and to describe variations in the data matrix. Data variance is described by means of linear combinations of the original variables: called principal components or PCs.

According to our PCA analysis, it was possible to explain 60% of the variance with reference to the first two principal components (PC1: 36 %; PC2: 24 %) and a further 13 % by reference to the third. Such high values showed the accuracy of the model. The score plot for PC1 vs. PC2 (figure 1A) clearly separated two groups along the PC1 axis: healthy (t0) fruits

and damaged (t1 and t2, full lines) ones. Within this second group, fruits respectively affected by CB or BH were clearly differentiated along the PC2 axis (dotted lines). In Figure 1B, two groups that represent storage conditions with low CO₂ (cam7) and high CO₂ concentrations (cam5) were also separated by PC1. Following PC2, it was finally possible to distinguish two groups of samples (figure 1C) defined by length of storage (S1 and S2), which corresponded with core browning and brown heart respectively (Figure 1A and 1C). According to these results, storage time seems to have a major influence upon the differentiation of the disorder while high CO₂ only determines the incidence of disorders.

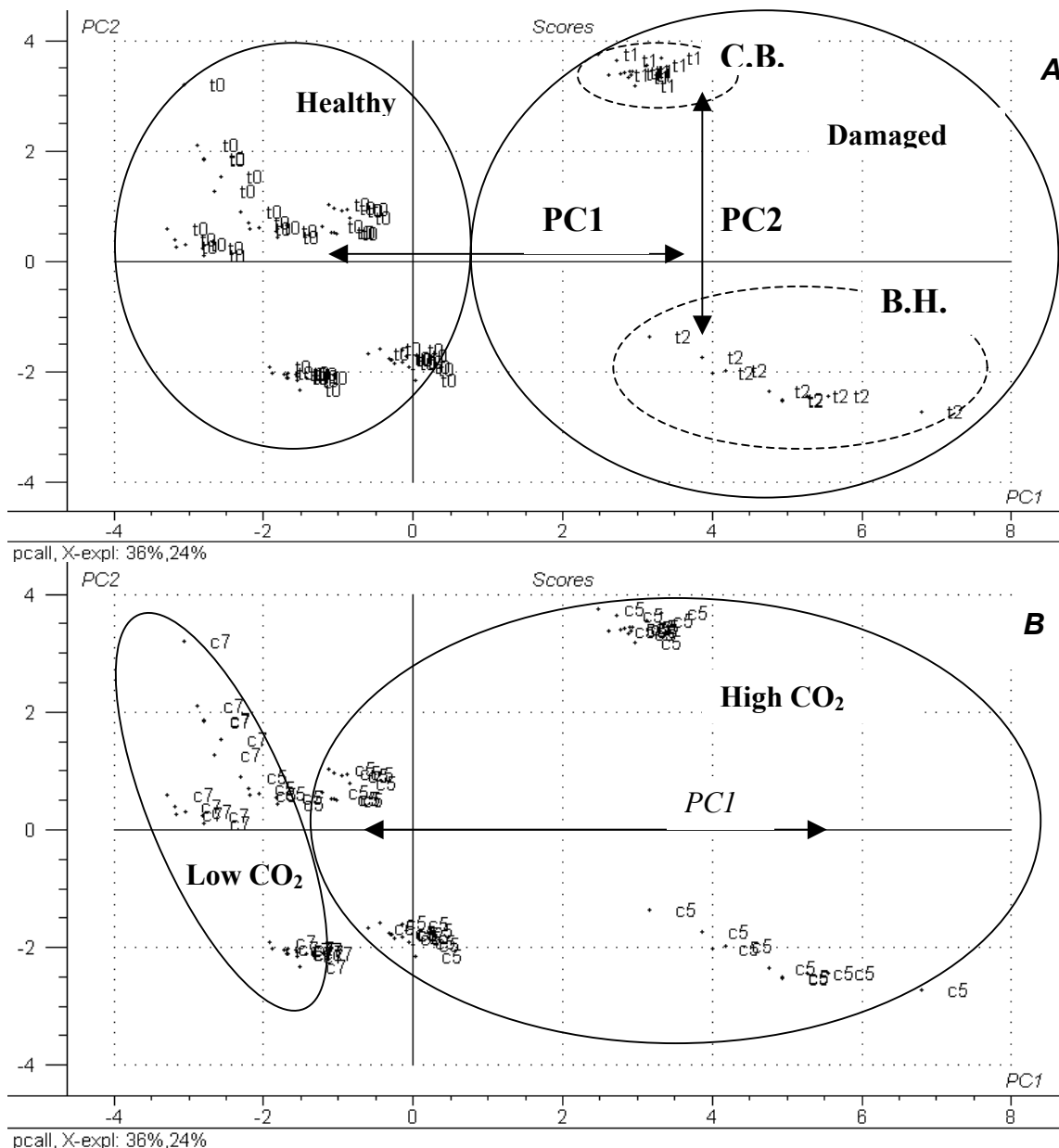


Figure 1.- Score plots for PC1 vs. PC2 from full-data PCA. (**A**) Samples labeled according to intensity of damage: healthy (t0) and damaged fruits (t1 and t2), and according to type of disorder: core browning (t1) and brown heart (t2). (**B**) Samples labelled according to level of CO₂: low CO₂ (c7) and high CO₂ (c5). Seventy-two samples and fifteen variables are included and labeled using codes defined in the text.

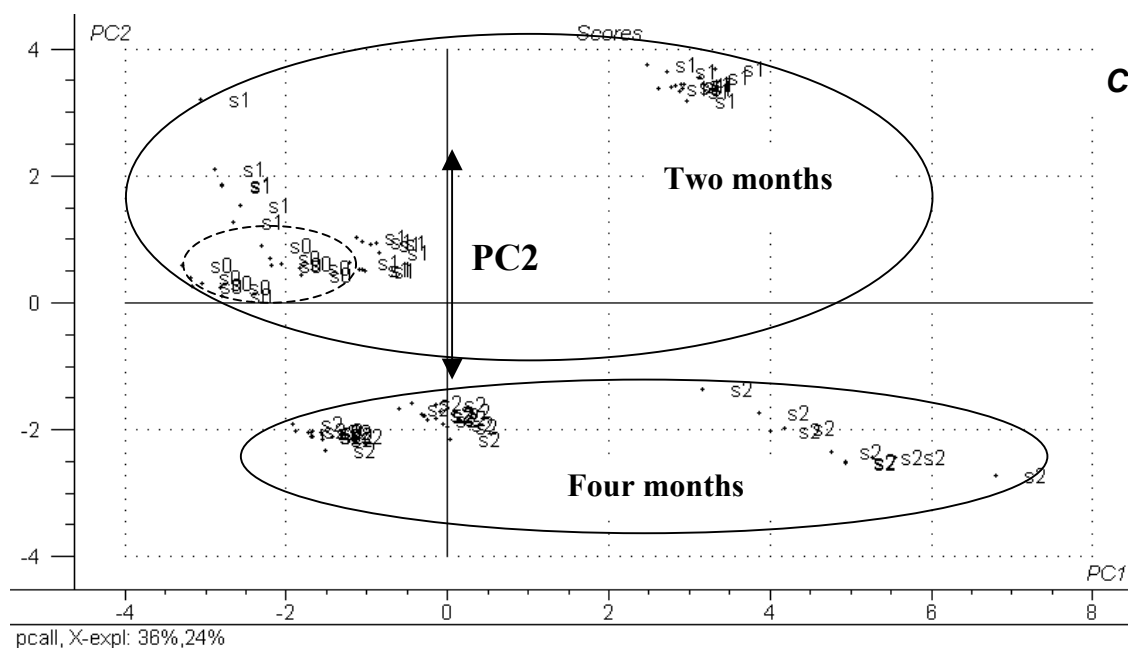


Figure 1 (C).- Score plot for PC1 vs. PC2 from full-data PCA. Samples labeled according to length of storage (s0,s1 and s2). Seventy-two samples and fifteen variables are included and labelled using codes defined in text.

When we examined the loading plot for PC1 vs. PC2 (Figure 2), two groups appeared to have a clear negative correlation according to PC1. The group on the right-hand side was defined by the percentage of alteration, which showed a clear correlation with high levels of CO₂ and damaged fruits (g1). The group on the left-hand side was defined by the level of ascorbate and showed a correlation with low levels of CO₂ and healthy fruits (g0). An acutely negative correlation was found between percentage of disorder and ascorbate content.

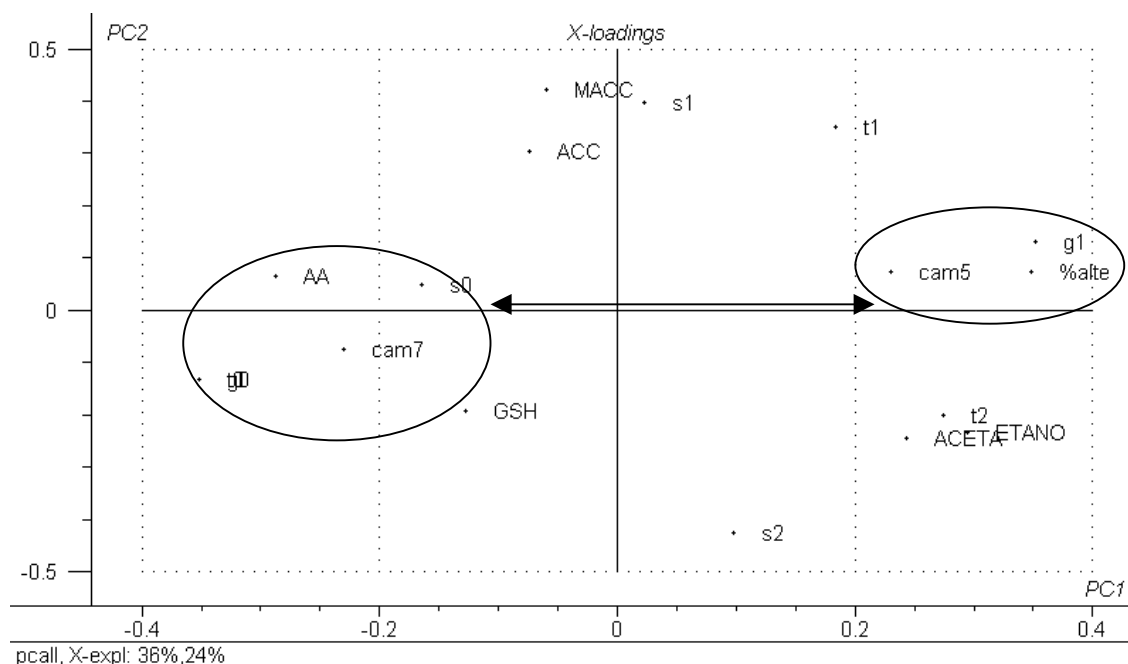


Figure 2.- Loading plot for PC1 vs. PC2 from full-data PCA. Seventy-two samples and fifteen variables are included and labelled using codes defined in the text.

As previously described (Figure 1A), the type of disorder (t1 and t2) was mainly described by PC2. PC2 was also acutely defined by the length of storage (Figure 1C). This result shows the relationship between the type of disorder and storage duration. Core browning mainly appeared at the beginning of storage (S1) whereas brown heart appeared later (S2). This difference provides an argument for distinguishing between the two disorders.

According to this preliminary characterization, ACC and MACC levels exhibited slight correlations with CB (t1, Figure 2 dotted lines). The fermentation products, acetaldehyde and ethanol also exhibited some correlations with BH (t2, Figure 2 dotted lines).

To complement this study, a new PCA model was established removing the variable harvest date and storage condition at low CO₂ that did not influence the disorder variance.

PCA analysis of samples stored with high CO₂ levels

The scores and loading plots for samples stored for two and four months with high rates of CO₂ are shown in Figure 3. The first two PCs explained up to 70% of the variance in the matrix (PC1: 45 % and PC2: 35 %). This result was better than the first one previously obtained when comparing all samples. The model clearly distinguished two groups by PC2 (Figure 3A): one corresponding to healthy samples (t0) and other including all the damaged samples (t1 and t2, full lines). According to PC1, the second group also contained two separate sub-groups of samples that could be defined by type of disorder (dotted lines).

The fruit samples were characterized according to the relative positions of their variables on the loading plot (Figure 3B). This characterization mainly depended on the type of disorder according PC1 (t1 and t2, thin dotted arrow) and percentage of damage according to PC2 (%alt, thick dotted arrow). PC1 also clearly characterized the samples with respect to storage time. Core browning and brown heart correlated positively with different variables: CB (t1) with ACC metabolism and BH (t2) with fermentative metabolism. As previously shown, BH exhibited a clearly negative correlation with ascorbic acid both by PC1 and PC2.

Overall, these results were consistent with the hypothesis that BH and CB are two different disorders, but they were not conclusive. We therefore proceeded to conduct a specific analysis of each disorder using a PLS model.

Influence of different metabolic pathways on the incidence of core browning following the PLS model

PLS was used to model the incidence of core browning in samples subjected to high CO₂ conditions for two months. The model sought to explain the maximum variance in the percentage of damage observed in the primary factors or PCs.

The loading plot using the first two PCs explained up to 94% of the variance for core browning (Figure 4A). The variable percentage of alteration presented a clear correlation with the variable ACC and an acutely negative correlation with ascorbic acid (AA). With the exception of GSH content, the rest of the variables also correlated positively with the incidence of CB.

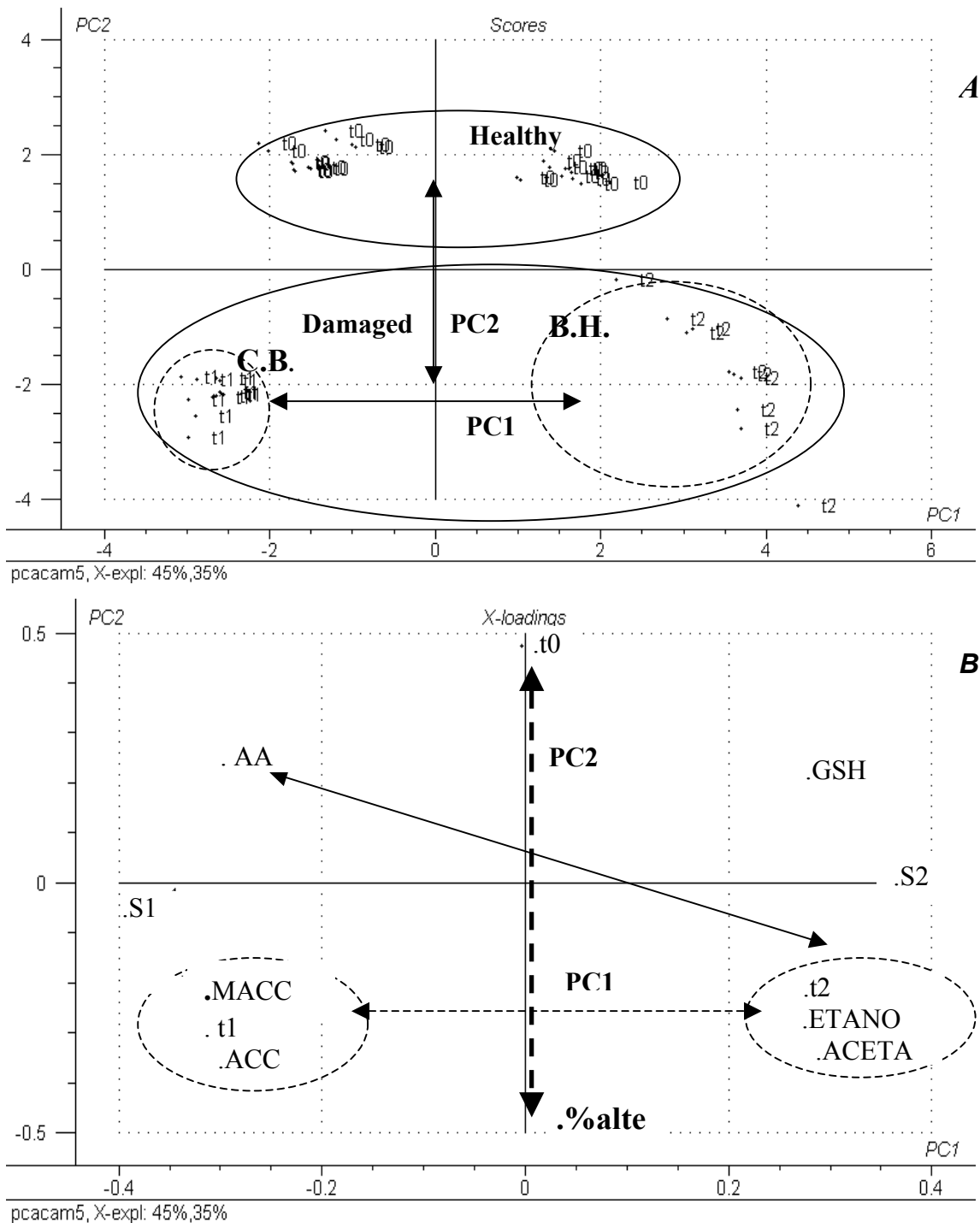


Figure 3.- (A) Score plot for PC1 vs. PC2 using data corresponding to storage with high levels of CO₂. **(B)** Loading plot for PC1 vs. PC2 using data corresponding to storage with high levels of CO₂. Forty-eight samples and twelve variables are included and labelled using codes defined in the text.

Figure 4B shows the predicted percentage of disorder vs. the measured percentage of disorder using full cross-validation. This model clearly identified two separate groups: healthy (t0) and damaged samples (t1). This separation was both clear and accurate, particularly considering the correlation coefficients between predicted and measured values (0.95) and with respect to the RMSEP = 4.77 (root mean square error of prediction).

The regression coefficients obtained for each variable (figure 4C), for the two PCs used in this study, showed their importance in predicting the percentage of damage. These results confirmed those shown on the loading plot for the same PLS model (Figure 4A). The precursors of ethylene and the fermentative products correlated positively with the percentage of damage and therefore seemed have a role in the CB disorder. Ascorbate content correlated negatively with damage, which seemed to indicate the importance of the non-enzymatic antioxidant system in the incidence of this disorder.

According to this scheme, the CB disorder seems to be related to three biochemical processes: ethylene metabolism, fermentative process and oxidative damage.

As CB is known to be a consequence of the ageing process (3, 27), it seems logical to find a correlation between CB and ethylene metabolism. The putative role of ethylene in the incidence of CB was also confirmed by our own results (19): we observed that 1-MCP, a known inhibitor of ethylene receptors, significantly reduced the incidence of CB in this variety. Both results suggest a putative relationship between ethylene and CB, though this hypothesis remains to be confirmed.

Although ethylene metabolism may be a determining factor in the incidence of CB, this parameter alone cannot completely explain the disorder. Another explanation may involve the fermentative and oxidative pathways, especially when respiration switches from the aerobic pathway to fermentation and when acetaldehyde and ethanol accumulated resulting toxic for the fruit (11). Changes in fermentation have often been associated with tissue browning and cell death (28, 29). At the same time, CA storage with high CO₂ levels may be considered as a source of oxidative stress (7). To prevent this type of stress, fruits possess an antioxidative system mainly formed by ascorbate (AsA) and glutathione (GSH). As shown in our results, both AsA and GSH levels correlated very negatively with incidence of the disorder: these correlations reflected the importance of oxidative processes in the incidence of CB.

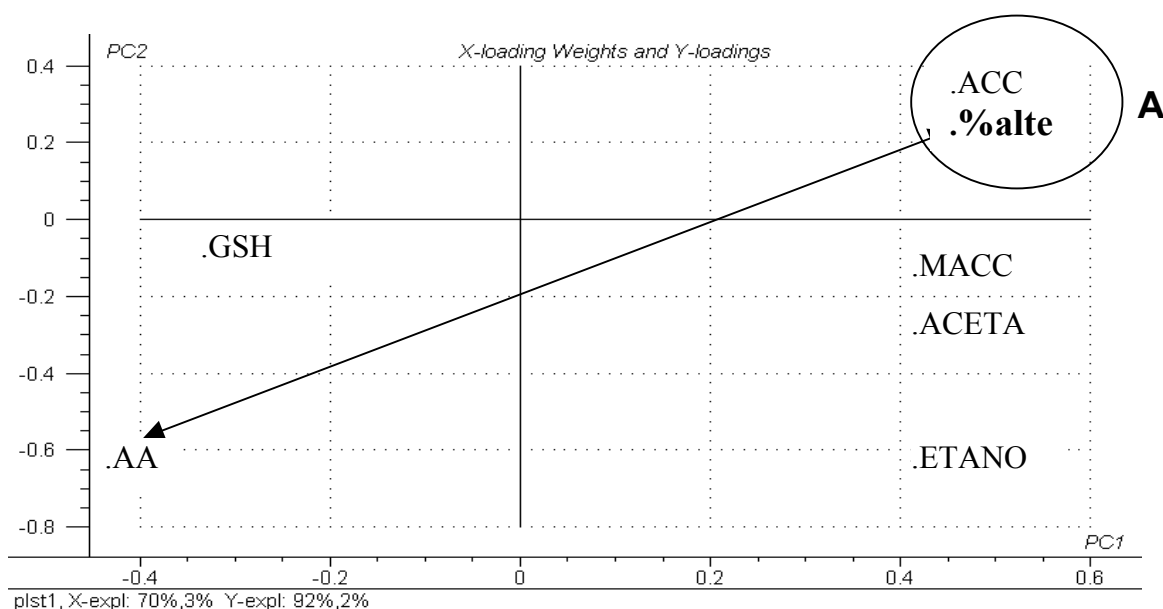


Figure 4.- PLS model using data corresponding to core browning. (**A**) Loading plot for PC1 vs. PC2 from variable percentage of disorder derived from PLS model. Twenty-four samples and six variables are included and labelled using codes defined in the text.

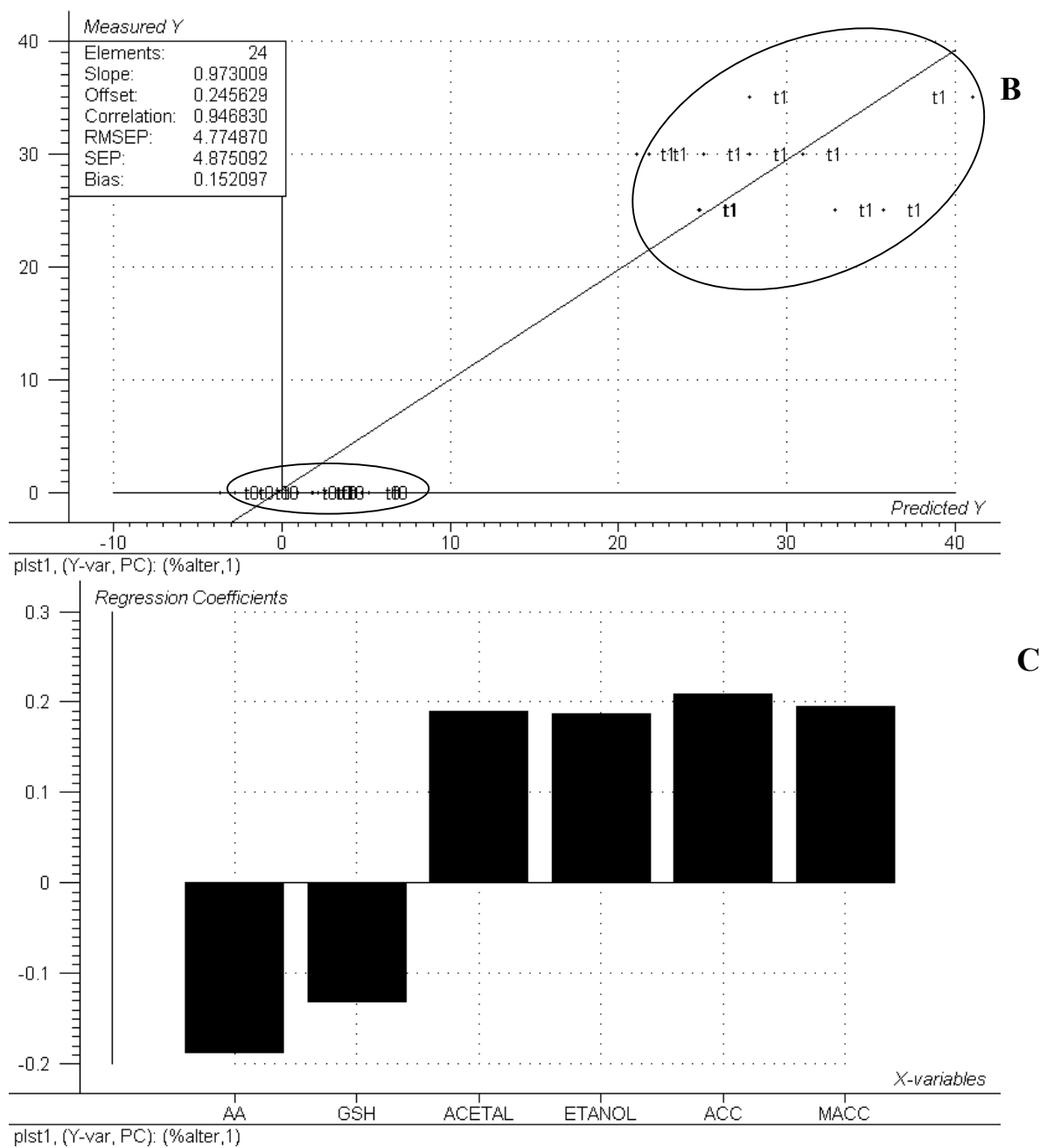


Figure 4.- PLS model using data corresponding to core browning. (**B**) Predicted vs. measured plot for percentage of disorder. (**C**) Regression coefficients for percentage of disorder versus all other variables. Twenty-four samples and six variables are included and labelled using codes defined in the text.

Influence of different metabolic pathways on the incidence of brown heart following the PLS model

When we established the PLS model for BH (figure 5), the loading plot obtained using the first two PLS factors explained up to 87 % of the variance (Figure 5A). The ascorbic acid level correlated very negatively with the incidence of brown heart according to both PC1 and PC2 (figure 5A). Glutathione content also presented a weak negative correlation with respect to damage, whereas ethanol content correlated only slightly.

The correlation between predicted and measured values was high ($r=0.89$) and the RMSEP (9.46) was also very good (Figure 5B). These two values suggest that this is a satisfactory model for predicting new incidences of disorder in the fruit.

The regression coefficient plot (Figure 5C) showed that ascorbate had the highest relative value and was the most important variable involved in the incidence of brown heart. Ascorbate plays an important role in the detoxification of activated oxygen (30). It can remove harmful radicals and may also participate in the removal of H_2O_2 . In Conference pears, ascorbate levels decrease under stress conditions, particularly when the fruits are stored in CA (7, 10). This decrease in ascorbate probably leads to oxidative damage, membrane alteration, and finally to brown heart (31). The lower is ascorbate content, the more susceptible fruit becomes to brown heart. Glutathione content also correlated negatively with the disorder, whereas ethanol content correlated positively. In contrast with CB, ACC did not appear to be a relevant factor for explaining the incidence of the disorder.

The statistical models presented in this work confirmed the experimental results quoted in the literature. They showed that, after three or four months of storage with high CO_2 levels, CA induced changes in AsA levels, which clearly correlated with the incidence of brown heart. Glutathione content did not seem to be a limiting factor but may have played an important role in the regeneration of AsA.

Taken together, our results apparently indicate that CB and BH are two different disorders. CB mainly results from an ageing process which involves the three pathways studied in this work. In contrast, BH appears to be mainly determined by oxidative processes and by a reduction in ascorbate levels. The models described here clearly show these differences. They may therefore be considered as a first step towards the prediction and detection of these two disorders in Blanquilla pears.

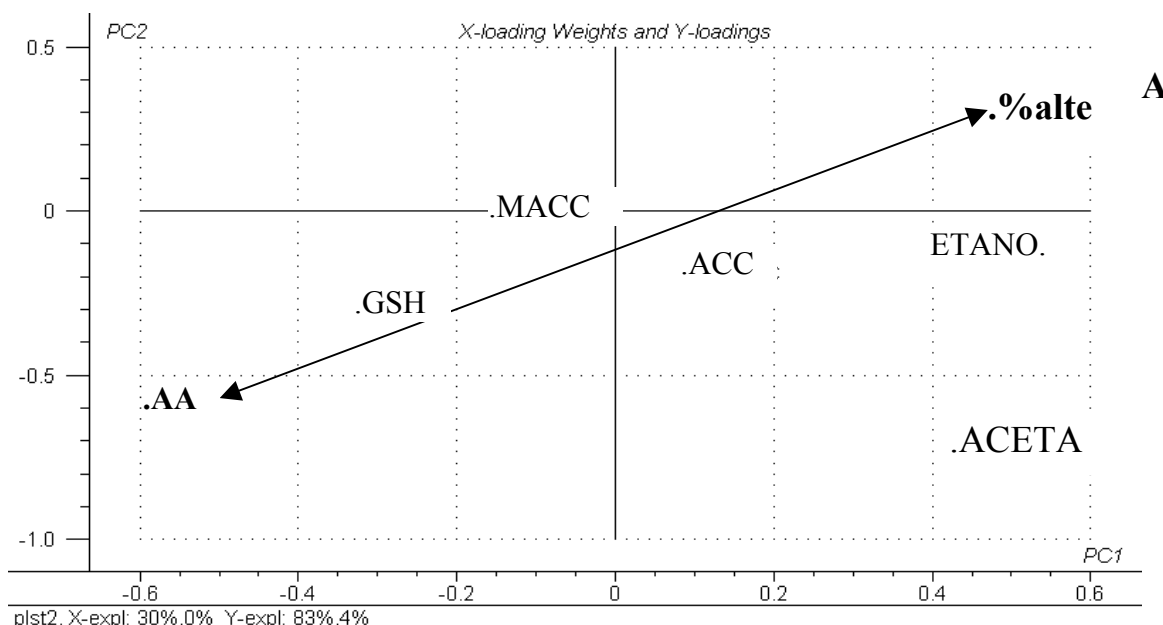


Figure 5.- PLS model using data corresponding to brown heart. (A) Loading plot for PC1 vs. PC2 from variable percentage of disorder derived PLS model. Twenty-four samples and six variables are included and labelled using codes denifed in the text.

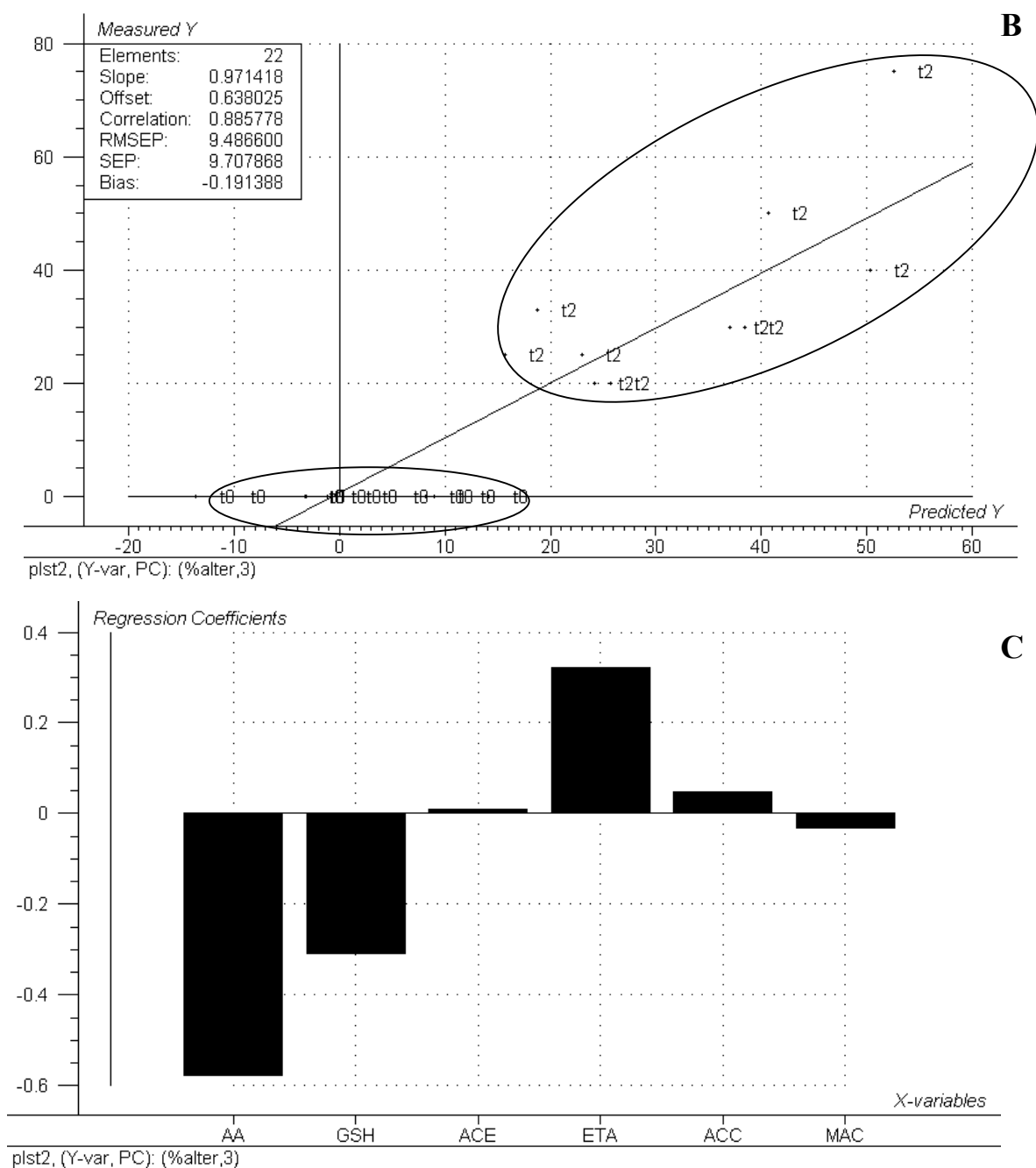


Figure 5.- PLS model using data corresponding to brown heart. **(B)** Predicted vs. measured plot for percentage of disorder. **(C)** Regression coefficients for percentage of disorder versus all other variables. Twenty-four samples and six variables are included and labelled using codes defined in the text.

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