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EXPLORING REFLECTANCE SPECTRA OF APPLE SLICES AND THEIR RELATION TO ACTIVE PHENOLIC COMPOUNDS

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Key words: browning, diffuse reflectance, cultivars, redox, chlorogenic acid, oxidase

Matured apple fruits of 11 cultivars were a source of fresh flesh and of an extremely variable phenolics composition in the biological medium of cut apple tissue. The content of epicatechin, chlorogenic acid, phloretin-xyloglucoside, and procyanidin B2 were quantitatively determined in extracts from tissue using HPLC method. Diffuse reflectance spectra were recorded every two minutes from the moment of flesh cutting till 12 min after, when enzymatic browning occurred. A two-beam reflectance spectrophotometer was used to collect the data within a wavelength range from 220 nm to 400 nm. The differential spectra calculated from diffuse absorbance data in the course of the browning kinetics were correlated with the phenolics concentration in apples. Chemometric graphs and analysis pointed to a specific time period and specific spectra bands linked to phenolics degradation (310 nm, 332 nm, 346 nm) or with the activation of certain new chromophores (258 nm, 280 nm, 310 nm, 380 nm) in apple tissue. The levels of biologically active compounds in fresh tissue will be correctly predicted from three linear regressions of diffuse-absorbance at characteristic bands of the spectra and browning time (from 0 to 12 min).

INTRODUCTION

The content of phenolics in apple fruits has been studied extensively [Oleszek *et al.*, 1988; Varoquaux & Wiley, 1994; Awad *et al.*, 2000; Piyasena *et al.*, 2002; Awad & de Jager, 2003]. Absorption techniques commonly involve spectrophotometric measurement on solutions obtained after crushing the tissues and removal of solids. Traditional methods for its determination consisted in measurement of total phenolics because of their association with browning reactions. Such measurements estimate the soluble pigments and are usually performed near 400 nm corresponding to the absorption maxima of catechol quinones [Sapers & Douglas, 1987]. The method makes no allowance for the diverse spectral characteristics of the absorbing species whose absorption maxima may vary [Kuczyński & Varoquaux, 1992]. Measurement of the insoluble polymerised pigments bound to membranes can be evaluated by reflectance measurements [Kuczyński & Varoquaux, 1992; Oszmiański *et al.*, 1995].

The need for profiling and identifying individual phenolic compounds has seen traditional methods based on colorimetry replaced by reverse phase HPLC, currently representing the most popular and reliable technique for phenolics analysis [Oleszek *et al.*, 1988; Oszmiański *et al.*, 1988; Escarpa & González, 1999]. Elution of compounds is typical, that is, polar compounds elute first, followed by those of decreasing polarity. Detection is based on absorption of ultraviolet or fluorescence [Rodríguez-Delgado *et al.*, 2001] at wavelengths characteristic of the class of phenolic compounds. Hence, an elution order can be developed and the spectrometry of time-course for specific set of apparatus.

At the subcellular level, the occurrence of phenolics in soluble, suspended and colloidal forms, and in combination with cell wall components, may have significant impact on their extraction [Guyota *et al.*, 2002]. Phenolic compounds are highly reactive species and methods of protecting the compounds from oxidation and other deteriorative processes have included the addition of antioxidants during the extraction and the use of inert atmospheres, therefore their quantitative recovery becomes particularly problematic [Robards *et al.*, 1999].

In physiological terms, in tissue systems, phenolics appear to be reactive oxygen species while polyphenol oxidases and free radicals are the main oxidants in tissue when the phenolics act as substrates and antioxidants, respectively. It is now generally accepted that the initial step in enzymatic oxidation consists in the hydroxylation of monophenols into o-diphenols, and o-diphenols into quinones. To be successful as an antioxidant in flesh, the phenoxy radical formed in the above reactions must not initiate the formation of further radicals but must rather be a relatively stable species without areas suitable for attack by molecular oxygen [Hotta *et al.*, 2001].

One of the biggest problems is associated with the difficulty of analytical measurement of the substrate concentration and browning which can be followed either by absorption spectrometry or reflectance methods, and those results were not conclusive due to the limitation of the methodology used. We suggest that the susceptibility of apples to browning illustrates those complex interactions, and that it is not possible to solve problems in the polyphenol model system (*in vitro*) only. Studies of enzymatic browning directly on the cut tissue are still less reported [Kuczyński *et al.*, 1993, 1994; Kuczyński, 2001].

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In the present work, a diffuse reflectance method was presented for the determination of phenolic composition in apple flesh during browning reactions after cutting. The procedure allows baseline resolution of major phenolic groups found in apple tissue. Thus, the objectives of this experiments were: (1) to collect the UV-VIS spectra directly on the cut flesh during browning; (2) to isolate phenolics from the flesh and to detect them by HPLC; (3) to compare the phenolics concentration with their activities near fruit flesh biosystem as their relation to UV spectra; (4) to develop the time-course diffuse absorbance method as a chemometric analysis for providing basic information on the enzymatic browning compounds. The technique should also help to verify the mechanism by which agents inhibit melanosis in suppression technologies.

MATERIAL AND METHODS

Plant materials. The preliminary results [Kuczyński, 1995] provided important information in order to obtain high differentiation in enzymatic browning and, as we supposed, substrate content regarding the best use of the apple varieties investigated for direct analytical method testing [Kuczyński 1999, 2001] as also with respect to high nutritional value for fresh apple consumption [Podsędek *et al.*, 2000; Leja *et al.*, 2003]. The trees were grown at the same trial plantation of the Albigowa Experimental Station Orchard (Podkarpackie voivodship). The apple fruits were cold stored for a long time to reach full consumption maturity and the best quality. Fruits were conditioned for at least 36 h at 18-20°C, after which they were ready to be moved to the minimal processing (fresh-cutting) technology [Varoquaux & Wiley, 1994].

For the purpose of the phenolics study only 12 cultivars were taken; *Bancroft* (BCF), *Gloster* (GST), *Idared* (IDR), *Golden Delicious* (GDL), *Starkrimson* (STN), *Spartan* (SPT), *Gala* (GAL), *Jonagold* (JOG), *Boiken* (BOI), *Melrose* (MEL), *Elstar* (ELS), *Šampion* (SAM), and all the cultivars at 2 separate terms of storage for the same full consumption maturity.

Browning description. The method is based on the diffuse reflectance spectra of the cut surface [Kuczyński *et al.*, 1993]. Three apples of each cultivar were cut in half along the stem axis. The halves were positioned in a Petri dish, cut side down, under a 20 mm cutting tube so that plugs could be bored. A traverse cut was made in the plug, at least 5 mm from the skin, to exclude the effects of bruising. Just at the start of measurements a cutting was made of a 1 mm slice to refresh the sample and three replications for each plug were done using refreshing cut surfaces before recording.

The reflectance spectra were recorded on Shimadzu UV-2101 PC double-beam spectrophotometer, equipped with an ISR-260 integrating sphere (60 mm in diameter) assembly and with Hamamatsu R445U Photomultipler Tube. The instrument was standardized against a $Ba(SO_2)_3$ etalon for the region of 200 nm to 400 nm. Drift was stabilized at less than 0.0004 Abs, photometric repeatability was better than 0.001 Abs for a light beam 3 mm wide and 5.5 mm high, with scan speed 200 nm/min and slit at 5 nm.

The results were expressed in absorbance (Abs) values calculated from the formula: Abs = log(1/R), where R is the diffuse reflectance. Browning was monitored by calculating difference of absorbance spectra from data taken immediately after cutting and every 2 min until 16 min. They were twice smoothed by a simple moving average procedure on 5 points.

Extraction of phenolic compounds. Apple flesh used in the "browning description" procedure was peeled and cut into cubes (20 g) and fast frozen (-20°C) with the same quantity of 99% ethanol (plus sodium metabisulphite to obtain 3 mmol/L final concentration [Kuczyński & Varoquaux, 1992]). Frozen material was incubated for one month, then homogenised and sonificated under cooling, then the pulp was centrifuged. Ethanol extracts were used for the HPLC analysis.

HPLC analysis were curried out using a Waters 600E liquid chromatograph equipped with Waters 486 VV-VIS detector. Before the HPLC analysis, the phenolic extracts were fractionated by solid-phase extraction into acidic and neutral polyphenols [Oszmiański *et al.*, 1988]. The separation of apple phenolics was carried out with RP_{18} SPHERI-5 OD-224, 4.6x220 mm column C_{18} from Brownlee Labs. The elution conditions were as follows: flow rate 1 mL/min; temperature 20°C; detection at 280 nm; solvent A - 2.5 mL acetic acid in 97.5 mL water; solvent B - a mixture of acetonitrile and solvent A (4:1); linear gradient from 0 to 10% B in A for 10 min, from 10 to 20% B up to 30 min and from 20 to 40% B up to 35 min. The column was then washed for 15 min with 100% B and re-used. All analyses of phenolic compounds (mg/100 g fresh flesh): epicatechin (Epi), chlorogenic acid (Chlra), procyanidin B2 (B2) and phloretin-xyloglucoside (Phlxg) were triplicated and performed in 2 separate series.

Intercellular spaces (%) were quantified as effective porosity of apple pieces [Samotus *et al.*, 1986; del Valle *et al.*, 1998]. Apple plugs (20 mm in height and 20 mm in diameter) were bored from equivalent fruits used in the "browning description" procedure. Pure water was vacuum infiltrated into the fruit tissue. Once the desired vacuum pressure of 9.3 kPa (70 mmHg) was reached, the system conditions were maintained for additional 2 min.

After removing trapped intercellular gases from the fruit piece, on-off valves were closed and air was allowed into the system and water entered the porous fruit flesh for an elapsed time of 5 min. The volume of intercellular spaces (%) was calculated on the basis of measurements of the geometrical volume of samples, the weight of sample flesh before infiltration and the weight after imbibition.

Correlation coefficients were calculated for each of the absorbance differences *versus* intercellular spaces and phenolics concentration. The significance level calculated for each correlation coefficient was a primary source of information about the reliability of the linear relation between substrate found in our samples *versus* the spectra differences. Specifically, the significance level represents the probability of error that is involved in accepting our observed result as valid. In order to facilitate identifying significant coefficients on the "map of probability" that are significant at the same desired level (5%, 1% or 0.1%), they were highlighted with shades of gray of different density. These results were the basis for

a comprehensive statistical analysis concerning the relationship between concentration, absorbance differences and the time of the redox process.

Canonical correlation explains the relationship between two sets of variables: flesh substrates and spectra differences by finding a small number of linear combinations for each set of variables. The linear combinations are those that contain the highest loadings (correlations) possible between the sets. The coefficients of these linear combinations are canonical weights. For sets of spectra data, linear combinations were used to calculate scores for canonical variates and, on the basis of the scores, a linear function was found that approximated the substrate concentration in the tissue.

Cluster analysis. Clustering was started from the "tree clustering" procedure. Then, after data analysis and from our experience, "K-means clustering" was used that (1) minimized variability within clusters and (2) maximized variability between clusters.

All statistical analyses were performed and graphs generated using Statgraphics PL v. 5.5 [StatSoft, 2000].

RESULTS

Composition of the apple flesh

Great differences observed in the composition of the apple flesh, in the intercellular spaces and the phenolics content, are presented in Table 1. Intercellular spaces differentiated cultivars and ranged from 14.5 to 58.3%. For the investigated cultivars, at full consumption maturity, epicatechin was the main phenolic compound and its concentrations varied from 7 to 44.8 mg/100g. Another main compound, chlorogenic acid, varied in concentration from 3.8 to 22.7 mg/100 g. The dominant proanthocyanidins B2 ranged from 9.3 to 19.7 mg/100 g. The levels of phloretin-xyloglucoside were up to 7.3 mg/100 g.

Cluster analysis selected 5 groups of cultivars characterised by differentiated concentrations of browning substrates (Table 1):

1) Bancroft - the highest concentration of epicatechin, chlorogenic acid and phloretin-xyloglucoside;

2) Gloster, Idared, Golden Delicious - the lowest concentration of phloretin-xyloglucoside, high level of chlorogenic acid, and an intermediate content of other substrates;

3) Spartan, Gala, Jonagold - intermediate phenolics content for the cultivars studied;

4) Boiken - the highest concentration of phloretin--xyloglucoside, the lowest concentration of epicatechin, and high level of chlorogenic acid;

5) Šampion, Melrose, Elstar - high concentration of epicatechin and procyanidin B2, very low level of chlorogenic acid.

Diffuse absorbance spectra

Changes in the absorbance differences calculated from diffuse reflectance spectra are very complex [Kuczyński *et al.*, 1994]. Only significant correlation between the initial concentration of phenolic compounds and the absorbance differences helps us to find specific bands. The "map of probability" (Figure 1) clearly indicates the formation of chromophores (as the correlation + sign and % of probability) and their disappearance (as correlation - sign and % of probability) in spectra bands.

The main substrate is obviously oxygen from intercellular spaces. Oxygen is the best natural substrate of PPO; its conversion dominated the absorbance differences from the 1st minute of browning capacity. Intercellular spaces are indicated as a significant increase at bands of 272 nm, 258 nm, at the main browning band of 380 nm, and as a decrease at 320 nm and 332 nm. The second main factor on the time axis is phloretin-xyloglucoside at bands (increase) of 258 nm, 280 nm, 272 nm and 365 nm. The next are epicatechin (decrease at 346 nm, increase at 380 nm) and procyanidin B2 (increase at 310 nm). Such a global and fast result is known as the non-enzymatic coupled oxidation mechanism, *i.e.* the o-quinones, enzymatically produced from some phenols, were oxidized to other phenols. Chlorogenic acid was finally degraded (at bands of 310 nm and 332 nm) as the last on a time axis and took part in the broad band (380 nm) of browning. Only higher concentrations of epicatechin significantly increased the absorbance (in the broad band of browning) in the visible region of the spectrum from the 1st minute.

From the "map of probability" characteristic spectrum bands for the compounds: oxygen, chlorogenic acid, epicatechin, procyanidin B2, phloretin-xyloglucoside were found (Figure 1). Initial concentrations of these substrates formed statistically significant changes in diffuse absorbance at UV spectrum, and finally in VIS. Moreover, the characteristic periods of browning were determined (up to 12 min at 20°C), *i.e.* the time during which a substrate takes an active part in the changes of the absorbance spectrum profile.

Clusters for kinetics of pre-selected spectra bands (Figure 2)

The differences of absorbance in selected bands of substrate were collected for all cultivars samples. Cluster analysis performed for this data divided the cultivars into 4 classes. The observations of absorbance differences of flesh indicate that enzymatic browning starts from the first minute. Those changes are still not observable visually, until the absorbance difference at 380 nm is lower than

TABLE 1. Browning substrates of flesh (mg/100 g) and intercellular spaces (%) at full maturity of apple fruits, presented as five clusters of cultivars (values are means and standard deviation, minimum, maximum).

Cultivars	Intercellular spaces (%) (SD)	Epicatechin (SD)	Chlorogenic acid (SD)	Procyanidin B2 (SD)	Phloretin-xyloglucoside (SD)
BCF	**47.6** (0.7)	**35.2** (13.6)	18.4 (5.4)	11.6 (6.5)	1.7 (0.9)
GST, GDL, IDR	31.7 (6.9)	20.3 (8.3)	20.8 (4.5)	11.8 (1.0)	0.9 (0.1)
SPT, GAL, JOG	31.6 (4.4)	13.9 (2.9)	14.8 (1.9)	10.8 (1.2)	1.5 (0.4)
BOI	**49.3** (1.0)	15.7 (9.1)	18.6 (2.5)	10.5 (1.1)	**5.1** (3.3)
SAM, MEL, ELS	27.2 (5.2)	27.3 (4.7)	6.5 (2.9)	16.4 (2.7)	1.0 (0.3)
average (SD)	34.3 (9.4)	21.5 (9.3)	15.0 (6.5)	12.6 (3.2)	1.7 (1.8)

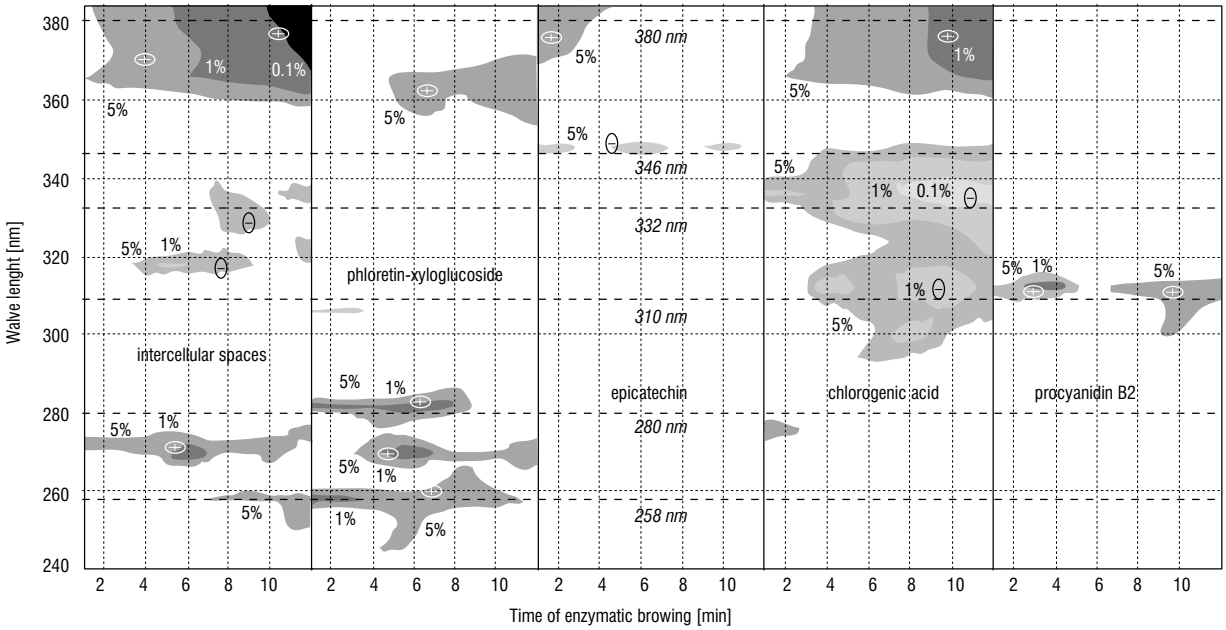

FIGURE 1. The "map of probability" for diffuse absorbance differences against volume of intercellular spaces and concentrations of phenolic compounds in apple flesh.

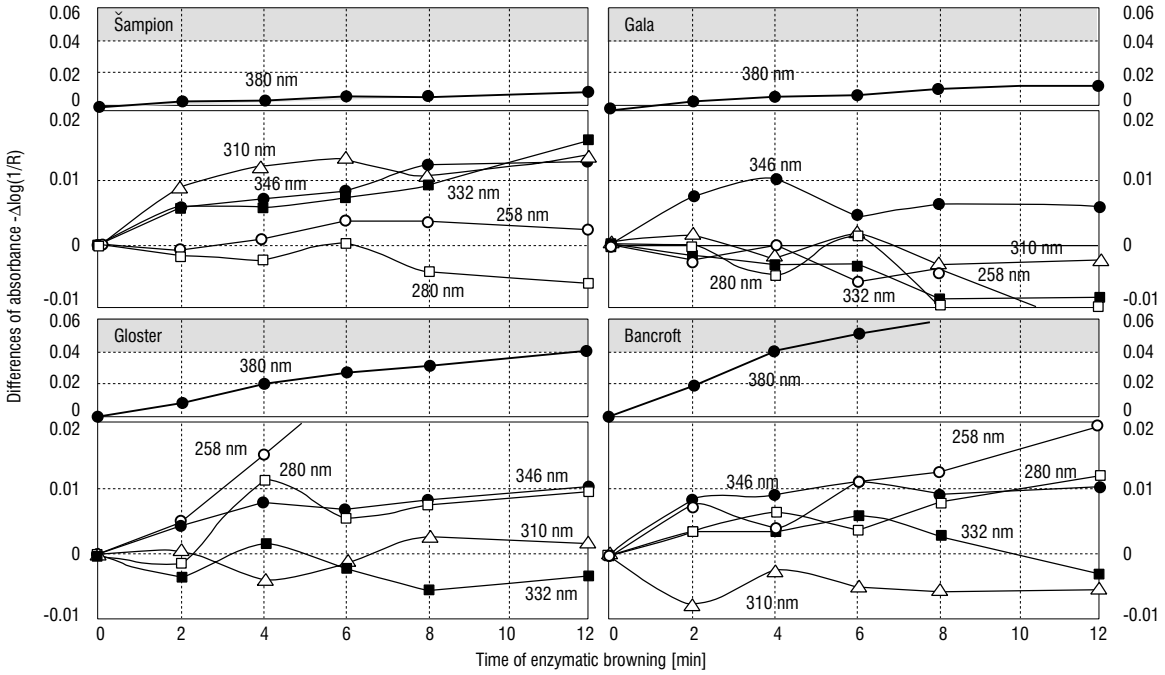

FIGURE 2. Kinetics of chromophores found as compounds related to enzymatic browning of apple flesh, presented as four types of clusters for pre-selected spectra bands.

0.04 [Kuczyński & Varoquaux, 1996] and only become noticeable after 6 min in strongly browning cultivars like *Bancroft* (BCF). Cultivars with a weak browning capacity, like *Šampion* (SAM), are still near 0.01 absorbance differences.

Changes of absorbance observed in the BCF cultivar are a classic example of enzymatic browning known from studies on model catechine/chlorogenic acid solutions [Oszmiański & Lee, 1990] and compounds isolated from the apple [Oszmiański & Lee, 1991; Amiot *et al.*, 1992]. A characteristic feature here is a strong absorbance increase just after 2 min. at the 380 nm band, which is accompanied by a drop in absorbance in the ultraviolet band at 310 nm. This browning process can be attributed to fast oxygenation of high level of chlorogenic acid (diffuse absorbance decrease at 310 nm) and to follow epicatechin (diffuse absorbance increase at 346 nm), as well as phloretin-xyloglucoside (diffuse absorbance increase at 258, 280 nm). The *Šampion* (SAM) variety is characterized by the slowest rate of diffuse absorbance increase within the visible range (at 380 nm) but a rapid increase within the invisible bands of the spectrum, at 310 nm and 332 nm.

Changes in the absorbance of GST apples at 380 nm are somewhat similar to fast changes in the BCF cultivar. However, for GST the increase in absorbance within the 258 nm band is much faster, and there is no decrease of absorbance at 310 nm. This was observed at a considerably higher concentration of epicatechin at approximately the same level of chlorogenic acid and other phenolic compounds.

TABLE 2. Factor structure in substrate variable set and factor structure in time-course spectra band set.

Time_band		Root 1	Root 2	Root 3
	Average (SD)	Factor loadings (Canonical weights)		
0_Abs332	1.10 (0.10)	0.17 (0.19)	**0.77** (0.51)	-0.06 (0.08)
2_dAbs258	0.003 (0.006)	-0.27 (0.82)	0.26 (-0.16)	**-0.60** (-0.82)
2_dAbs280	0.001 (0.004)	-0.06 (-0.17)	0.39 (0.08)	**-0.55** (0.22)
2_dAbs310	0.001 (0.005)	**-0.45** (0.14)	0.06 (-0.04)	-0.01 (0.00)
2_dAbs346	0.005 (0.003)	0.33 (0.05)	-0.12 (0.54)	-0.46 (-0.27)
2_dAbs380	0.010 (0.014)	**-0.36** (-1.86)	0.61 (2.02)	0.09 (-1.25)
4_dAbs258	0.006 (0.007)	-0.27 (-1.66)	0.43 (-0.16)	**-0.56** (-0.71)
4_dAbs280	0.003 (0.005)	-0.02 (0.65)	0.56 (0.38)	-0.37 (-0.43)
4_dAbs346	0.008 (0.005)	0.20 (0.74)	-0.27 (0.05)	-0.37 (-0.75)
4_dAbs380	0.020 (0.023)	-0.33 (2.12)	**0.72** (-3.14)	0.04 (4.91)
6_dAbs258	0.009 (0.010)	-0.27 (-0.55)	0.44 (-0.68)	-0.40(-0.02)
6_dAbs280	0.003 (0.006)	0.09 (-0.09)	0.47(-0.08)	-0.50 (-0.29)
8_dAbs258	0.009 (0.012)	-0.21 (0.14)	0.52 (1.25)	-0.33 (0.88)
8_dAbs332	0.002 (0.008)	-0.08 (-1.18)	-0.54 (0.48)	-0.10 (-0.42)
8_dAbs346	0.009 (0.007)	0.08 (0.27)	-0.26 (-0.94)	-0.40 (0.62)
12_dAbs280	0.005 (0.007)	0.12 (0.97)	0.54 (-0.24)	-0.12 (0.80)
12_dAbs380	0.041 (0.034)	-0.27 (-1.22)	**0.83** (1.67)	-0.02 (-3.85)
Factor redundancy		5.7%	23.4%	11.6%
Total redundancy		43.1%		

Substrate	Root 1	Root 2	Root 3
	Factor loadings		
Chlra	0.19	**0.87**	0.11
B2	**-0.81**	-0.43	0.41
Phlxg	-0.33	0.34	**-0.88**
Epi	-0.58	0.19	0.65
Total redundancy	92.0%		

Spectra for GAL have a slow increase in absorbance within the visible range at 380 nm, somewhat like *Šampion* (SAM); however that is accompanied by a fast increase of absorbance only in the band of 346 nm and little decrease others.

Canonical correlations analysis

Total redundancy values can be interpreted so that, based on all canonical roots, given the spectra set as variables, we can account for, on the average, 92% of the variance in the variables of the phenolics substrates (Table 2). Likewise, we can account for 43.1% of the variance in the spectra related items, given the phenolic substrates related items. These results suggest a fairly strong overall relationship between the items in the two sets, *i.e.* initial concentrations of phenolics and time-course spectra bands. From the sequential significance test we conclude that only 3 canonical roots are statistically significant, and this should be examined.

Factor structure in substrate

From canonical factor loadings (Table 2) we know how the roots can be interpreted, and whether they are correlated with the variables in the two sets. As we can see in Table 2, the three substrate items - B2 at Root 1, Chlra at Root 2 and Phlxg at Root 3 show substantial loadings, that is they correlate highly with those factors. From factor redundancy, the first canonical root (B2) extracts an average of about 5.7% of the variance from the spectra items. The Root 2 (Chlra) extracts an average of about 23.4% of the variance from the spectra items and Root 3 (Phlxg) extracts an average of about 11.6% of the variance from the spectra items.

Factor structure in time-course spectra band set

In the factor structure spectra-time course (Table 2), the first canonical root (B2) is marked by high loadings on the bands-time items 2_dAbs310, 2_dAbs380, 4_dAbs380. The second root (Chlra) is marked by high loadings on the bands-time items 0_Abs332, 4_dAbs380, 12_dAbs380, and the third root (Phlxg) is marked by high loadings on the bands-time items 2_dAbs258, 2_dAbs280, 4_dAbs258.

All the loadings are much lower for bands at long time items, apart from the loadings of the second root (Chlra). Therefore, we can conclude that the significant canonical correlation between the variables in the two sets (based on the first root) is probably the result of a relationship between B2, and short time 310 and general 380 nm. If we consider the third root (Phlxg) as the explanatory variable, we could say that Phlxg affects 258, 280 nm spectra at short time and general 380 nm, but much less at long time with all the bands.

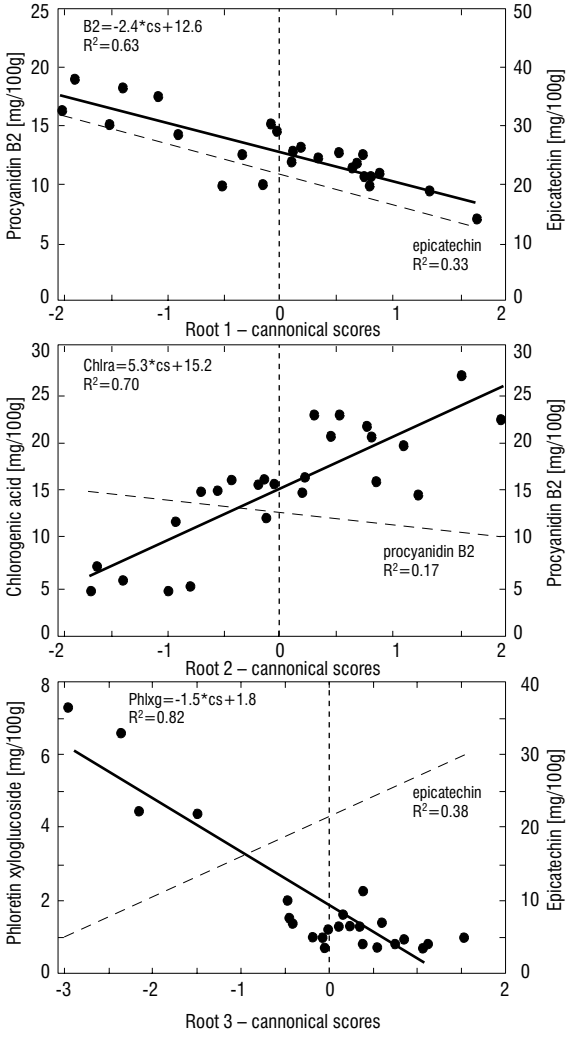

FIGURE 3. Three chemometric calibration curves for phenolic compounds as regression against canonical scores of roots constructed on the basis of time-course diffuse-absorbance spectra bands.

Predictive phenolic compounds

We used canonical weights to compute scores for the canonical variates. The canonical scores for Root 1, 2 and 3 were plotted against the suitable (factor loading determined) phenolic compounds set (Figure 3). Three scatter plots display the statistically significant linear relation of canonical scores against concentrations of phenolics: procyanidin B2, chlorogenic acid, and phloretin--xyloglucoside. The doted lines and the left axis marked data for interacted compounds but presented only the lower coefficient of determination - R^2. Finally, a relationship between initial concentration of phenolic substrates in the flesh against the spectrum of UV-VIS light diffuse reflected from the tissue during 12 min of the kinetics was shown.

The interesting aspect of the scatter plot for phloretin--xyloglucoside in Figure 3 is the clustering of cases. Such a clustering happened because samples from the BOI cultivar, notably, have generally a higher content of phloretin-xyloglucoside.

DISCUSSION

The presented chemometric experiments provide, for the first time to our knowledge, a basis for the spectrophotometrical characterization of phenolic compounds concentration directly in apple flesh as their browning behaviour. The estimation of phenolics concentration on flesh surface provides and insight the dual role of phenolic compounds as antioxidants and as substrates for oxidative browning reactions. In both roles, the key for exact separation of data from absorbance differences spectra and for the evaluation of phenolic compounds content was the consideration of the particular rate of redox reactions. Multiway methods (chemometrics) have been used earlier in the analysis of polyphenolic extracts from vegetables [Bro & Heimdal, 1996].

The phenolic composition of apple flesh is determined by genetic and environmental factors but may be modified by stressed reactions during processing or storage [Burda *et al.*, 1990]. Information on such a fast biosynthesis or degradation of metabolites is essential to understand the interaction between plants and their environment [Leja *et al.*, 2003]. It was concluded from our results that intercellular spaces and the phenolics compounds appear to be one of the most important parameters for the classification of apple cultivars into groups. Apple flesh at full consumption maturity has high levels of biologically active flavan 3-ols, *i.e.* epicatechine [Oleszek, *et al.*, 1988], procyanidin B2, [Guyota, *et al.*, 2002] and cinnamic acids, *i.e.* chlorogenic acid [Oleszek *et al.*, 1988, 1989; Amiot *et al.*, 1992]. The related concentration of flavan 3-ols to cinnamic acids, as was now observed, can be reversed at the handling stage to full maturity stage [Awad *et al.*, 2000; Podsędek *et al.*, 2000; van der Slui, *et al.*, 2001; Guyota *et al.*, 2002]. However, it can be noticed that high volume of intercellular spaces is related to high concentrations of epicatechine or phloretin-xyloglucoside in flesh. Notably, the great differences in the phenolics composition of apple fruits is certainly accepted as its biodiversity and the most important factor for the characterisation of flesh with respect to their nutritional value [Amiot *et al.*, 1992; Tomás-Barberán & Espin, 2001; Leja *et al.*, 2003].

The cultivars used in the study correspond to a wide range of UV-VIS diffuse absorbance spectra with variation between cultivars and within samples [Kuczyński, 1995, Kuczyński & Varoquaux, 1996]. Our analysis suggests that achromatic scattering from flesh surface was the most important factor in this variation [Kuczyński *et al.*, 1993; Sims & Gamon, 2002]. Only calculation of spectrum of absorbance differences permits to eliminate this effect and to follow small changes in the spectra during the redox process [Kuczyński & Varoquaux, 1992].

The spectrum of differences has often been used in analysis of the fruit extract, but it is only applied occasionally today. Indeed, in spite of the many improvements made to this method, the information it provides is often affected by considerable inaccuracy. This is caused, first, by the possibility of interference from non-phenolic substances with similar properties (although this can be remedied to some extent by prior purification of extracts), and second, by the very variable reactivity of the various molecules present, even within a single class of phenolic compounds. As a result, the same overall value obtained may represent extracts whose phenolic compositions are pretty different. These overall methods were soon joined by measurement after the separation (HPLC) at time-course of various compounds of a phenolic extract. Separation of various phenolics in the course of apple flesh browning and the application of the diffuse reflectance method at time-course does not have all such defects.

Situations that give rise to browning are physiological and technological processes [Varoquaux & Wiley, 1994]. Phenolic compounds act as substrates for a number of oxidoreductases, namely, polyphenoloxidases (PPO) and peroxidases (POD). The main oxidative phenomenon involves an initial enzymatic oxidation of phenolic compounds located predominantly in the vacuole by polyphenoloxidases located in cytoplasm. At tissue levels the oxidised bases are determined by the balance between oxidation and repair, but the rate at which the antioxidant behaviour presents is protective function against oxidative spoilage of the cells in living tissue. The common thread is the redox chemistry of the phenolics, which may be beneficially oxidised in preference to living tissue, or detrimentally oxidised during flesh cells spoilage [Kuczyński, 1999]. However, there appears to be no full discussion of how phenolics recovery may be influenced by temperature, the presence of oxygen, solvent, *etc*.

In literature, data on the substrate specificity of PPO show inconsistencies in the apparent K_m values (Michaelis--Menten kinetics) between species, cultivars and experimental data [Robards *et al.*, 1999]. Also direct experiments indicated that the intensity of enzymatic browning was similar in the pulp of fruits PPO activity of 3 000 U/g and with PPO activity of 1 380 U/g [Podsędek, *et al.*, 2000]. These results and our experiments pointed to the full redox nature of the factors which determine the kinetics of browning reactions [Kuczyński, 1999].

As oxidants, the o-quinones will oxidise any other substances with lower reduction potentials. Oxidation will include other phenols, ascorbic acid and sulphur dioxide, and in this process the quinones are themselves reduced to the original phenol [Amiot *et al.*, 1992; Guyota *et al.*, 2002]. The reaction products become quite complex with as few as

two phenols (o-quinones) in admixture [Oszmiański & Lee, 1990, 1991]. So, much of our attention at the present methodical work has been devoted to determining the exact pathway by which enzymatic browning may occur in the flesh. Also, this work has taken a novel approach to the experiments on phenolic compounds by linking what many may see as disparate areas, namely the potential antioxidant activity of these compounds in the flesh and their protective role in oxidative processes of flesh.

In the absence of other substrates, condensation and polymerisation will occur *via* reaction with the corresponding hydroquinone. The latter, polymerised compounds remain bound to the cell membranes and to the walls, and they play their protective role against oxidative environment [Guyota *et al.*, 2002]. In such a place flavonoid phenoxy radicals exhibit reduction potentials and the corresponding parent flavonoids are expected to efficiently inactivate various reactive oxygen species with higher potentials [Kuczyński, 1999]. In summary, the o-quinones will enter along different pathways, according to their oxidative and electrophilic properties [Robards *et al.*, 1999; Hotta *et al.*, 2001].

Many authors have attempted to correlate browning intensity (in VIS region) with factors like the appropriate enzyme activities and substrate content; often with contradictory results [Janovitz-Klapp *et al.*, 1990; Amiot *et al.*, 1992; Podsędek *et al.*, 2000]. Reported examinations showed either an increase or a decrease of diffuse absorbance during browning in the non-visible, ultra-violet region of the spectrum [Kuczyński *et al.*, 1994], as was reported earlier also on extracts [Oleszek *et al.*, 1989]. We presented, on the map of probability, that volume of intercellular spaces and the contents of all investigated phenolics correlated well with changes of absorbance differences in particular UV bands and at particular time as measured by diffuse reflectance method.

The chemometric procedure optimised spectrophotometric time-course data to resolve the mixture of main phenolics in apple flesh [Bro & Heimdal, 1996].For the first time, it can be concluded from analysis of large dataset from apple flesh that time longer than 10 min at a temperature of 18-20°C has to be seen as an artificial factor, because it is not specific either for the rate of browning or for the redox of phenolic compounds.

The light diffuse reflectance techniques [Kuczyński & Varoquaux, 1992; Kuczyński *et al.*, 1993] allow for kinetic determination of the compounds and expression of the results in terms of the most practical units, *i.e.* in the terms of concentration of favourable phytochemicals. That is a new analytical model used in research on biological activity of phytochemicals in cut plant tissue.

The phenolics in fresh cut flesh were quantified by HPLC because the calibration, as for the other methods - NIR reflectance or HPLC, is dependent on the set of apparatus. In practice, before generalizing the precise rates and spectra bands, as adequate for other apparatus set, we should replicate the calibration study. Care and attention were devoted to the stability of spectrophotometer readings (drift), which allowed us to measure at very low levels of absorbance differences. Under the described conditions, this approach allowed good resolution of mixture of three major phenolics commonly encountered in apple fruits - those that are the most abundant.

There is no single method that would be applicable to fulfil all analytical requirements of phenolics in all tissues. The method was developed only for specific experimental objectives - it serves for the study of fresh cut apple flesh. The advantages of the procedure have been contrasted with HPLC currently used for similar applications. However, it is noteworthy that within the context of such methodical work was also introduced a novel conceptual approach that allows practically unlimited manipulation with respect to details of enzymatic reactions near living plant tissue and their recognized kinetics. These two factors permit considerable flexibility of methodological approaches that can be further developed for more specific applications of analysis.

CONCLUSION

This work presents knowledge transfer between biophysics and food analysis. Chemometrics and the now applied spectrophotometer did not allow the resolution of all phenolic compounds of interest in flesh samples. It is noteworthy that the essential element here was the application of an even more sensitive and faster scanning spectrophotometer, which would allow quantitative measurement of very small amounts of phenolics in cut sample. This also shows that the procedure ensures enough specificity as evidenced by high correlation coefficients and the calibration curves. Quantitative approximation was shown on three phenolic compounds in apple flesh. The reflectance method offers a wide range of application in phytochemical research on active biological systems particularly in laboratories that process large number of plant samples.

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