Identification of Red-Fruited Pears by Fourth Derivative Spectroscopy of Intact Lamina[1]

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Abstract

Intact lamina (leaf blade) of young leaves from red-fruited pears were analyzed by fourth derivative attenuance (~absorption) spectroscopy. This method was used to extend electrophoresis based identification procedures. When the two methods were used in combination before fruit development, red-fruited pears were distinguished from the non-red-fruited parent cultivars, and these from other red-fruited and non-red-fruited pear cultivars.

Index words: *Pyrus*, Apple, *Malus*, red-pears, spectroscopy, electrophoresis, pigments, anthocyanins, chemotaxonomy, plant fingerprinting

Introduction

Pear (*Pyrus*) cultivars have red variants, e.g. 'Comice' and 'Regal Red Comice'; 'Anjou' and 'Red Anjou'; 'Bartlett', 'Max Red Bartlett', and 'Sensation Red Bartlett' pears. All cultivars of western pears tested, to date, can be identified by electrophoresis of stem isozymes, (19 and unpublished results). However, the color 'sports' of these cultivars, which have considerable economic importance, are indistinguishable with present electrophoretic techniques. In addition, electrophoretic analysis of apple cultivar stems show that red variants have isozyme patterns indistinguishable from the non-red or less red clone from which these red 'sports' were derived (18). In apple cultivars, red 'sports' not only have red fruit, but also have the same anthocyanin pigments in other tissues which can be used for selection of redder mutants (20). In pears, leaf phenolics generally provide better identification data than those of the bark (6). It was noted that red 'sports' of pears frequently have red stripes on the young leaves in early spring, this matter was investigated for its usefulness in identification.

Fruit color is important in marketing; thus, the pigments that give color have horticultural importance. Fourth derivative spectroscopy is a method to analyze pigments with similar colors. Recent advances in electronics permit the use of standard equipment to measure pigments in intact plant tissues (9, 10, 11). In intact plant tissues variations in amounts of pigment yield linear responses only when the pigments are present in high concentrations (24). When, in intact tissues, pigment concentrations are low or when there is a wide range of concentration levels, linearity of response can only be obtained through the use of ranking procedures (8, 9). Since, what we measure is rank, the measurement obtained is called rank of attenuance (9). Non-parametric statistics uses rank of data rather than the numeric values. When the data is transformed to ranks the same methods of analysis of variation as performed in the more common parametric statistical methods can be used (8). Anthocyanin attenuation rank transformed data has an additional advantage as it permits, using appropriate standard clones, comparison of relative leaf color from one part of the season to another (9), and from one year to the next (unpublished information).

Anthocyanins, mainly cyanidin glycosides, are responsible for the red-color of apple and pear tissues (12, 13, 20, 23, 25). Cyanidin glucosides have *in vivo* spectral maxima which closely approximate that of the extracted pigments in buffer (21), but are on average about 8 nm longer than the maxima of the extracted cyanidin glycosides in acidified methanol (17). Though *in vivo* spectroscopy closely resembles that of the purified components in aqueous solvents (1, 21), most spectra of anthocyanin extracts are determined in acidified methanol (0.1% HCl), because they are unstable in aqueous solutions at vacuolar pH, e.g. pH 5.3 (1). Since anthocyanin color is pH dependent (7, 22, 26) *in vivo* spectroscopy is more appropriate for measurements related to field observations or consumer preferences (9).

Visible spectra bands are quite broad (17, 21, 24) even with advanced equipment (16). However, the intense, but overlapping bands of leaf visible spectra can be resolved by curve analysis. This type of analysis was usually done using leaf fraction preparations or algae containing chlorophyll pigmented moeties, at liquid nitrogen temperatures (4, 5) and with the use of specific computerized treatment of the spectroscopic data (2, 3, 14). However, newer UV-visible microprocessor-equipped spectrophotometers are able to analyze room temperature spectra of intact leaf lamina using fourth derivative spectroscopy without need for further computer assisted analysis of the data (10, 11). Anthocyanin pigments were partially resolved in the intact leaf (9) and this method was improved for this paper.

Materials and Methods

Leaves and stems were collected in April 1986 from the National Clonal Germplasm Repository, and the adjacent orchards of the Department of Horticulture, Oregon State University, Corvallis. Attenuance spectroscopy was done on a Shimadzu 260, (Shimadzu Scientific Instruments, Inc., Columbia MD) equipped with an integrating sphere using

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methods previously described (9). The programs modified to use the fourth rather than the second derivative (10, 11) and to permit accumulation of more spectra, are available on request. The leaves were masked, except for two holes 0.78 cm (0.307 inches) in diameter, with a blackened copper template (11). For direct attenuance measurements the leaves were placed in the sample compartment. To obtain difference spectra and remove contributions of pigments common to both kinds of leaves, the red leaf was inserted so that the red stripes on the leaf were in the sample compartment. The green leaf of the corresponding non-red or less red clone was similarly placed in the reference compartment. Electrophoresis was performed, using stem extracts as previously described (19).

Results and Discussion

Figure 1 shows the difference attenuance spectra of red and non-red leaves. Difference spectra was used since it is a powerful tool in distinguishing spectra between leaves from similar sources (11). The spectra of red and non-red

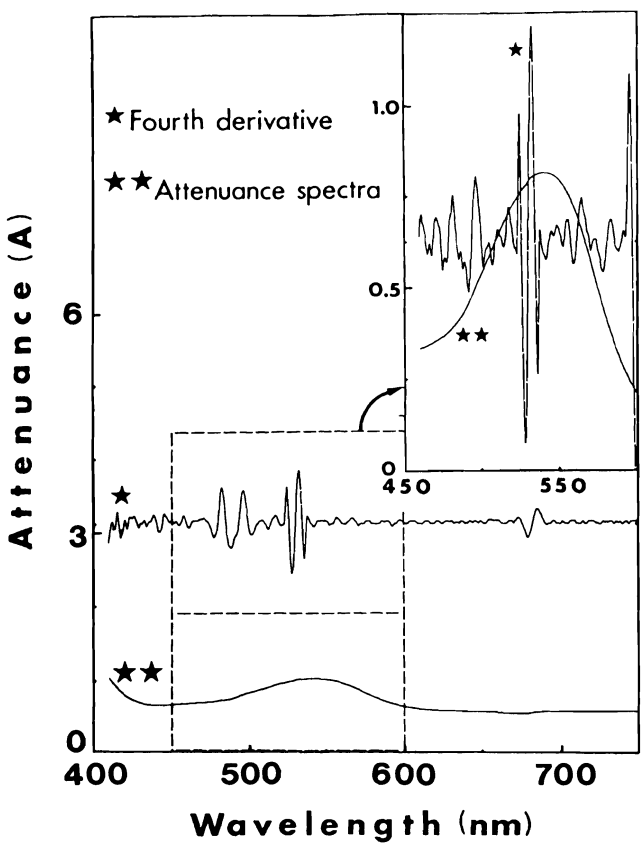

Fig. 1. **Attenuance difference spectrum between young leaves of red fruited pear 'sport' 'Red Anjou' and the parent cultivar 'Anjou.' A young leaf from 'Red Anjou' is compared with an 'Anjou' leaf of the same developmental stage. The 'Red Anjou' leaf is placed in the sample compartment with the red stripe in the path of the beam. The 'Anjou' leaf is placed analogously in the sample compartment. The result is a difference spectra in which the common green pigments cancel each other out, but the difference in red pigments is retained. Attenuance is expressed as absorbance units (A). The less abruptly changing curve which starts at the lower right is the attenuance (~absorption) spectrum, the fourth derivative analysis of the spectrum starts at the middle right. The insert shows the region of the spectrum where anthocyanin absorption occurs.**

leaves are similar except for the anthocyanin region. Pigment spectra are best described as spectral band envelopes (11) rather than a fixed absorption spectra. Spectral band envelopes are the result of broadening of the spectral lines by various factors. In leaves broadening of anthocyanin attenuance spectra envelopes is attributable to the variation in pH of the cell compartments (22) and the pH-absorption interaction with other cell components (1). These envelopes are so broad that in a standard spectrum they appear continuous; however, fourth derivative analysis demonstrates that they are composed of discrete components (Fig. 1).

Determining envelope characteristics is the first step in the development of this method. Figure 2 shows that the pooled fourth derivative spectra maxima yield a histogram composed of 13 discrete envelopes. These envelopes correspond to known anthocyanins measured *in vivo* (17); except for the ~496 nm peak which is probably a carotenoid (15) or carotenoid derivative (Hrazdina, pers. communic.).

Table 1 lists the identification numbers of the isozymic patterns found and shows that the red-fruited 'sports' frequently share stem isozyme patterns with the parent cultivar. Figure 3a shows an example of one of the gels, and Figure 3b shows the schematic representation of each isozymic pattern. Table 1 and Figure 3 illustrate the difficulties encountered when attempting to identify these 'sports' when mature fruit is not present. However, the young leaves of these red-fruited 'sports' frequently have red pigments. Table 2 shows the most intense pigment envelopes found in the difference spectra between the red 'sport' and the corresponding parent strain. Using the data in Tables 1 and 2 in combination we were able to distinguish the parent and red 'sport' from each other and characterize the pigment envelopes of the different red 'sports.' No pigments were found in the leaves of Red Clapp Favorite collected in April 1986.

Apple cultivars were also tested and although the leaf pigmentation was not readily observed by eye, we were able to detect differences spectroscopically. Table 2 shows that apple and pear leaves share the same pigment envelopes, but the most intense pigment envelope varied between cul-

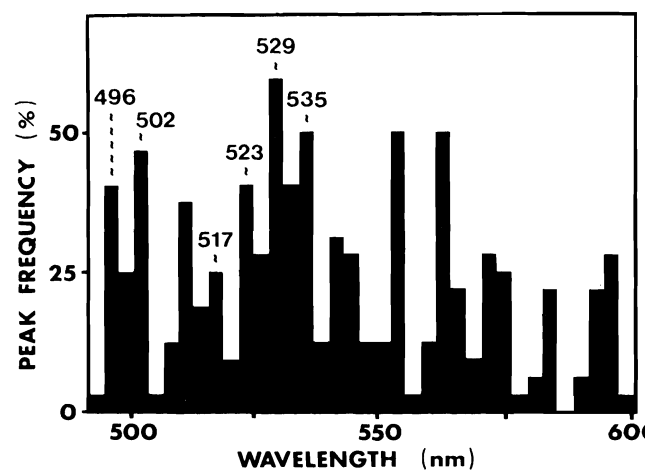

Fig. 2. **Histogram of pooled fourth derivative maxima of all data collected. All detected bands in all apple and pear leaf spectra were used in this compilation. Histogram resolution is 3 nm, since this gave optimal peak definition. Since all bands were weighted equally this histogram represents frequency not intensity.**

Table 1. Isozyme patterns associated with the cultivars investigated. The isozyme patterns are given in Figure 3. Differences in the same group are indicated in bold print.

	Pattern numbers		
	Peroxidase	Esterase	Acid Phosphatase
Pear Cultivars			
Anjou	8	8	6
Red Anjou	8	8	6
Bartlett	6	6	6
Max Red Bartlett	6	6	6
Sensation Red Bartlett	6	6	6
Comice	7	7	6
Regal Red Comice	7	7	6
Clapp Favorite	31	40	8
Red Clapp Favorite	31	40	8
Apple Cultivars			
Granny Smith	30	38	15
Spur Granny Smith	30	38	15
Jersey Mac[z]	29	38	13
Macspur	29	38	14
Starking Delicious	29	39	14
Starkrimson	29	39	14

[z]Macintosh the parent clone was not available at the time of the investigation. Jersey Mac is related to Macintosh, but is not the cultivar from which Macspur was derived.

tivars at the time of our measurements. The relative intensity of the pigment envelopes was much greater in most pear samples than in apples, to the degree that visual determination of red pigments is useful for identification in most pears, but not in apples. It is possible that in apples the association of spectroscopically detectable red pigmentation and spur habit may be useful for future work.

The precise nature of the leaf pigments is unknown. This is not important in production; however, the identity of the pigments would provide information for breeding decisions. Fourth derivative analysis of our data (Table 2) and comparisons to published information on apple and pear fruit anthocyanins (13, 20, 23, 25) suggest that the ~529 nm peak is probably cyanidin 3-galactoside, or a complex of this compound with 7-arabinoside and 3-arabinoside cyanidins, since these compounds all have the same maxima in acidified methanol (25). The presence of the bands at ~517, ~523, and ~535 nm suggests that other anthocyanins are also commonly present. Candidate compounds include the glycosides of delphinidin, pelargonidin and others from the table of *in vivo* absorbances of Ishikura (17). Additional frequent bands shown in Figure 2, indicate the complexity of anthocyanin pigments in these species.

Significance to the Nursery Industry

The identification of pear species and cultivars constitutes an integral part of the program of conservation and characterization at the National Clonal Germplasm Repository (NCGR). The nursery industry, institutional breeding programs, and independent breeders have the NCGR germplasm collections at their disposal. This collection is a source of clonal plant material which contains many desirable characteristics including disease resistance and cold hardiness. In addition, the collection contains accessions with colored

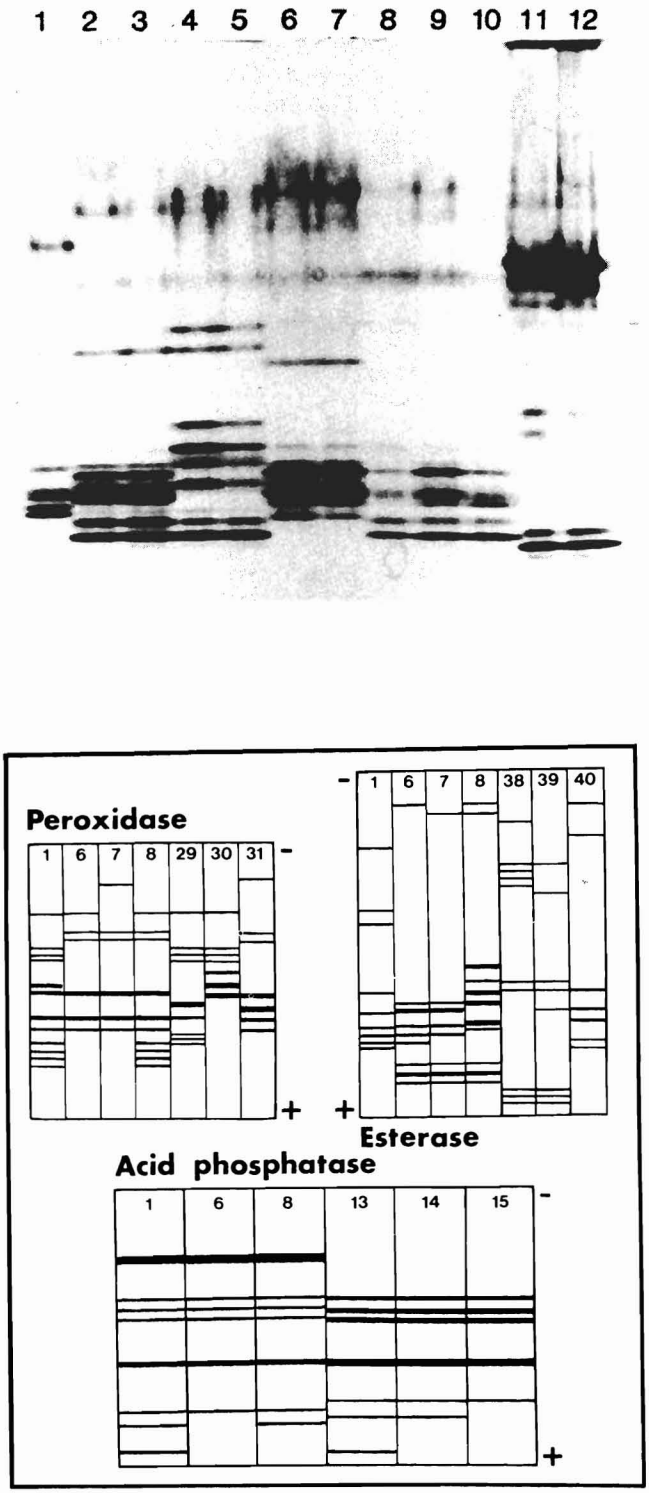

Fig. 3. Electrophoretic patterns of apple and pear cultivars and 'sports': A.) Photograph of gradient polyacrylamide electrophoresis gels showing patterns esterase staining patterns. The sources (from left to right) are: 1, *P. calleryana* (NCGR clone 661); 2, 'Comice' pear; 3, 'Red Comice' pear; 4, 'Anjou' pear; 5, 'Red Anjou' pear; 6, 'Clapp Favorite' pear; 7, 'Red Clapp Favorite' pear; 8, 'Sensation Red Bartlett' pear; 9, 'Max Red Bartlett' pear; 10, 'Bartlett' pear; 11, 'Starking Delicious' apple; 12, 'Starkrimson' apple. B.) Diagramatic representation of patterns pertinent to cultivars and 'sports' discussed in this paper. To find correspondence of numbered patterns to cultivars and clones please see Table 1.

Table 2. The three most intense pigment bands and total *in vivo* anthocyanin content. Data was obtained by fourth-derivative differences spectroscopy between leaves of red-fruited clones and their respective non-red- (or less red-) parent cultivars. This data was analysed by non-parametric statistics and is given as mean attenuation rank and standard deviation of rank (parenthesis), 1 indicates strongest band, 2 is strongest, etc. Total anthocyanin is reported as mean differential attenuation (~absorption) per leaf where Anthocyanin = $[A_{529\ nm}-A_{700\ nm}] \times 1000$ (modified from 9).

	Mean Rank of the Three Most Intense Pigment Bands [reported as band envelope maxima (nm)]						Total Anthocyanin
	~496	~502	~517	~523	~529	~535	(± s.d.)
Pear cultivars							
Red Anjou/Anjou				3.7(0.9)	1.3(0.5)	1.0(0.0)	25(2)
Regal Red Comice/Comice		1.0(0.0)			2.0(0.0)	6.3(1.9)	1.2(1)
Max Red Bartlett/Bartlett	2.0(0.0)				1.5(0.5)	2.3(0.5)	13(7)
Sensation Red Bartlett/Bartlett	2.7(1.2)				1.2(0.4)	2.2(0.7)	6(4)
Apple Cultivars							
Granny Spur/Granny Smith	2.5(0.4)				1.3(0.4)	2.0(0.0)	11(1)
Macspur/Jersey Mac[z]	1.0(0.0)		2.5(0.5)		2.7(1.2)		6(3)
Starkrimson/Starking Delicious	1	3			2		0.3

[z]Macintosh the parent clone was not available at the time of the investigation. Jersey Mac is related to Macintosh, but is not the cultivar from which Macspur was derived.

fruit, leaves, and petals which can be of interest to the landscape/allied nursery industry. In spring, the young leaves of red-fruited pears have bands of red color. A spectroscopic method was developed to be used in combination with electrophoresis of stem tissue to identify these red-fruited cultivars. This paper describes a technique which amplifies the utility of electrophoretic methods by identifying, spectroscopically, the differences between red-fruited 'sports' and their parent cultivars. This method permits the identification of red-fruited 'sports' by leaf spectrophotometric analysis before fruit production. In addition, *in vivo* spectroscopy relates better to consumer preferences and growers' observations than extract analysis. For these reasons this method is expected to be of use in selection of plant materials and in extending electrophoretic methods directed at preventing the costly consequences of mis-labeling clones.

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