A Simple and Rapid Screening Procedure for Identification of Zygotic *Citrus* Seedlings Among Crosses of Certain Taxa[1]

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Abstract. In progenies from crossing browning and nonbrowning *Citrus* taxa, browning was dominant to nonbrowning with certain exceptions. Examining the color of spots formed by pouring homogenates of ground young shoots on blotting paper was the simplest and most rapid procedure for scoring progenies. Presence of enzyme activity and substrate was determined by adding partially purified enzyme and substrate to the spots. Because of taxon-specificity of spot colors, it was concluded that nucellar and zygotic progenies from crosses among polyembryonic taxa could be identified at an early seedling stage, if parents with contrasting spot color were used in crosses.

Facultative nucellar embryony is a mechanism of asexual reproduction common to many *Citrus* taxa. Progenies from selfing or crossing of polyembryonic taxa are mixtures of nucellar (maternal) and zygotic seedlings whose proportions vary with different taxa. Only a few taxa produce seeds with a single zygotic embryo (monoembryonic).

Nucellar and zygotic individuals among the progeny of polyembryonic pistillate parents cannot be identified with certainty until after fruiting unless parents have markedly different vegetative characteristics. Thus, the need for dependable markers to permit early identification of the nucellar and zygotic progeny of polyembryonic *Citrus* taxa has long been recognized. The trifoliate leaf of *Poncirus trifoliata* as a dominant marker has been successfully used for this purpose following intergeneric crosses between *Citrus* and *Poncirus* for many years. However, no such reliable marker is available in the case of inter- and intra-specific crosses of *Citrus*. Furr and Reece (3) reported results applying the "Almen Reagent" color test to water extracts of dried leaf powder to identify nucellar and zygotic seedlings from various taxa. Spot chromatography of 2,2-dimethoxy propane extracts of bark tissue from roots was used by Seele (6) for rootstock identification. Pieringer and Edwards (5) employed infra-red spectroscopy for the same purpose. Isoenzyme profiles of peroxidase and esterase were also shown to be a possible method to identify nucellar and zygotic seedlings when they were as young as 20 days old (4).

Esen and Soost (1) reported the presence of 2 distinct young shoot extract color phenotypes—browning and nonbrowning—in *Citrus*. The browning taxa had one, possibly more, unknown phenolic substrates oxidized by polyphenol oxidase upon tissue homogenation, while the nonbrowning taxa contained neither the substrate nor any detectable enzyme activity. Genetic studies (2) showed that browning was dominant to nonbrowning and was under single gene control. This simple mode of inheritance was for the substrate. Polyphenol oxidase (abbreviated hereafter as PPO) activity showed a continuous variation. These findings, based on a survey of 34 taxa, various F_1 hybrids and an F_2 population of 76 individuals, indicated the possibility of distinguishing nucellar and zygotic progeny from nonbrowning × browning crosses at the young seedling stage. The work reported herein was undertaken 1) to obtain additional documentation for the mode of inheritance of browning, and 2) to develop a simple, rapid screening technique based on the color of young shoot homogenates that can be routinely used for identification of nucellar and zygotic seedlings.

Materials and Methods

The parents and 608 hybrids used in this study were growing in the experimental orchards of the Citrus Research Center of the University of California, Riverside. Progenies resulted from crossing browning and nonbrowning taxa in 3 combinations: browning × browning; browning × nonbrowning (reciprocal); and nonbrowning × nonbrowning. The browning parents were *C. reticulata*, *C. sinensis*, *C. aurantium*, and hybrids from crosses of the first 2 of these species with each other and with nonbrowning species. The nonbrowning parents were *C. grandis*, *C. limon*, *C. aurantifolia*, *Poncirus trifoliata*, *Fortunella margarita*, and *Eremocitrus glauca*. The cultivars of *C. paradisi* were "nonbrowning" because they were deficient in PPO activity even though they contained the substrate. They were "browning", however, if PPO from a known source was added to their homogenates. Although *C. paradisi* is listed as "nonbrowning" in Table 1, the reader should bear in mind its exceptional status. Parents whose progeny were screened with respect to enzymatic browning or its absence are listed in Table 1. Cultivar names of the parents are also provided whenever such information is verified through records. In addition, those hybrids that have attained horticultural use as scion and rootstock cultivars are indicated in the footnote.

The terminal 1 to 3-cm portion (Fig. 1-A) of growing shoots from the spring or summer growth cycle of 1974 were collected in polyethylene bags and kept covered with ice in an insulated container. Tissue homogenates were made on the day of collection by grinding in 0.05 M phosphate buffer (pH 7.2) at room temperature. The ratio of buffer volume to fresh wt was 3:1. The homogenates were poured on white blotting paper (30 x 50 cm) 7–8 cm apart. The spots formed were scored for browning or its absence within 1 hr. Browning of spots was considered evidence for the presence of the phenolic substrate and sufficient enzyme activity (1, 2), however, all nonbrowning hybrids were tested further to determine whether nonbrowning was due to lack of enzyme activity or substrate, or both.

Tests for enzyme and substrate were made by adding one drop of the following solutions to separate areas of the spot formed on paper from nonbrowning parents and hybrids:

1. Purified mushroom PPO (obtained from Worthington Biochemical Corp., Freehold, NJ) and crude PPO (free of phenols) from 'Willow Leaf' mandarin to test for substrate. The use of purified mushroom PPO was discontinued later because of its non-specificity.
2. 15 mM DL 3,4-dihydroxyphenylalanine (DOPA) dissolved in 0.05 M phosphate buffer (pH 6.0) and partially purified substrate (free of enzyme) from 'Willow Leaf' mandarin to test for enzyme.

'Willow Leaf' mandarin was used as a substrate and enzyme source because it had the highest PPO activity among the *Citrus* taxa assayed spectrophotometrically and appeared to be rich in substrate based on qualitative tests (1). The partial purification to obtain the phenol-free enzyme and enzyme-free substrate from 'Willow Leaf' was carried out at 0° to 4°C. Tissue was ground with sterile sand (Standard Ottawa; obtained from Matheson Coleman & Bell) in 0.05 M phosphate buffer (pH 7.2) containing 10 mM L-cystein-HCl, centrifuged for 20 min at 27,000 *g*. The resultant supernatant fluid (10 ml) was immediately placed on a column (2.6 × 25 cm) of Sephadex

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Table 1. The phenotypic distribution of hybrids from crosses of browning and nonbrowning *Citrus* and related taxa.

Cross	No. of progeny	
	browning	non-browning
Browning × Browning		
C. reticulata × *C. reticulata*		
Clementine selfed	29	5[z]
C. reticulata × [*C. sinensis* × *C. reticulata*]		
Clementine × Dweet	170	15[z]
Willow Leaf × King	2[x]	0
C. reticulata × [*C. grandis* × *C. reticulata*]		
Clementine × [Acidless × Frua]	75	3[z]
[*C. grandis* × *C. reticulata*] × *C. reticulata*		
[Acidless × Kinnow] × Kinnow	1	0
[Acidless × Kinnow] × Wilking	16	0
C. reticulata × [*C. paradisi* × *C. reticulata*]		
Clementine × Minneola	84	0
Browning × Nonbrowning		
C. paradisi × *C. reticulata*	21[w]	1
4× Seedy Marsh × Dancy	12	0
4× Seedy Marsh × Kinnow	12	0
C. sinensis × *C. paradisi*	1	0
C. aurantium × *C. grandis*	1	0
C. grandis × *C. sinensis*		
Acidless × Paperrind	1	2
C. grandis × *C. reticulata*		
Deep Red × Clementine	6	1
Acidless × Kinnow	1	0
Acidless × Frua	1	0
Acidless × Wilking	1	0
C. grandis × [*C. sinensis* × *C. reticulata*]		
Acidless × Dweet	10	1
Acidless × [Ruby × Dancy]	4	5[z]
Siamese × Temple	9	0
[*C. grandis* × *C. paradisi*] × *C. reticulata*		
[Acidless × 4× Seedly Marsh] × Dancy	14	0
C. limon × *C. sinensis*	1	0
4× Lisbon × trovita	12	15[z]
C. limon × *C. reticulata*		
4× Lisbon × Kinnow	17	0
C. aurantium × *P. trifoliata*	1	0
C. sinensis × *P. trifoliata*	1	11[v]
C. reticulata × *P. trifoliata*		
Honey × Trifoliate	0	2
Clementine × Trifoliate	8	2
Wilking × Trifoliate	3	0
Eremocitrus glauca × [*C. limon* × *C. sinensis*?]?		
Eremocitrus × Meyer	1	0
Nonbrowning × Nonbrowning		
C. limon × *P. trifoliata*	0	1
C. grandis × *P. trifoliata*		
Kao Panne × trifoliate	0	12
C. grandis × *C. paradisi*		
Acidless × Seedy Marsh	0	10[y]
C. paradisi × *P. trifoliata*	0	3
C. limon × *P. trifoliata*	0	1
C. aurantifolia × *F. margarita*		
West Indian × Oval	0	1[u]
C. aurantifolia × *F. japonica*		
West Indian × Round	0	2[t]

[z] Nonbrowning progeny showing enzyme activity but lacking substrate

[y] Five of these progenies contained substrate; 5 lacked both enzyme activity and substrate.

[x] Cvs. Kinnow and Wilking.

[w] Cvs. Altoona, Clement, Early, Minneola, Orlando, Pearl, Sacaton, Sampson, San Jacinto, Seminola, Sexton, Sunrise, Sunshine, Thorton, Ugli, Wekiwa, Williams, Wilsh, Yalaha, and 3 unnamed tangelos of which 1 (Owari Satsuma × Imperial) was nonbrowning.

[v] Cvs. Carrizo, Coleman, Cunningham, Etonia, Morton, Sanford, Savage, Toyer, Uvalde, Yuma, Rusk, and 1 unnamed citrange (Spanish Sweet Org. × *P. trifolata*) which was the only one browning

Table 1—*Continued*

[u] Cv. Tavares limequat

[t] Cvs. Eustis and Lakeland limequats

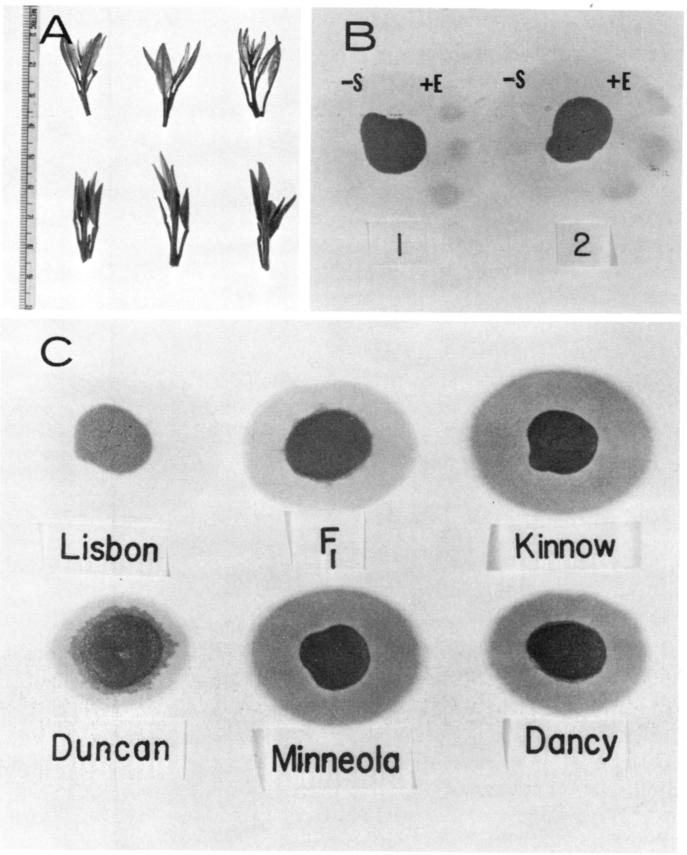

Fig. 1. (A) Young shoot terminals from 'Dancy' mandarin (upper row) and 'Acidless' shaddock (lower row) illustrating material used for making homogenates. (B) Spots of nonbrowning individuals lacking substrate but containing enzyme as evident from three brown spots on right where substrate from 'Willow Leaf' was added in drops. #1 from 'Clementine' × 'Dweet' and #2 from 'Clementine' × ['Acidless' × 'Frua']. (C) Dominance of browning to nonbrowning in F_1 hybrids from 2 nonbrowning × browning crosses, 'Lisbon' × 'Kinnow' and 'Duncan' × 'Dancy', respectively. Note that darker outer spot of 'Duncan' grapefruit is not due to browning but rather from its greenish-olive color.

G-25 (fine, particle size 20–80 μ) equilibrated with the same buffer, and 10 fractions were collected. Fractions containing PPO activity, determined by adding 0.05 ml of the protein fractions to 0.5 ml of DL 3,4-DOPA, were combined and lypholized. The bright yellow (phenol) fractions, which required about 2 hr to elute completely, were tested individually for the substrate by adding the pooled enzyme fraction in a volume ratio of 10:1. Those fractions that browned intensely upon addition of enzyme were pooled and lypholized. The lypholized enzyme and substrate fractions were kept in tightly closed vials at 0° to 4°C. The working solutions to test for enzyme and substrate were prepared by dissolving 20 mg of the protein powder (5 mg/ml used if protein fraction was dialyzed to remove salts prior to freeze-drying) and 10 mg of the substrate powder each in 1 ml of deionized distilled water.

Results and Discussion

Tissue homogenates poured on paper produced 2 circular spots within each other (Fig. 1-C). The inner spot was formed by the solid phase (tissue debris) of the homogenate and the outer one from the diffusion of the liquid phase. In the browning parents and hybrids, browning became visible first in the outer spot within 1 to 10 min depending on PPO activity and probably also on substrate concentra-

tion. Color intensified with time until the spots dried and attained different shades of browning depending on taxa. In the nonbrowning taxa and hybrids, the outer spot colors varied from grayish white to olive green, again depending on taxa.

Of 366 hybrids (and 34 selfs) from browning × browning crosses, 377 were browning and 23 nonbrowning (Table 1). These 23 individuals all had PPO activity as evident from browning when the substrate from 'Willow Leaf' PPO was added (Fig. 1-B). Interestingly enough, all these individuals were from crosses in which 'Clementine' was the pistillate parent. Since browning is dominant to nonbrowning (2), the appearance of nonbrowning individuals among the progeny of 2 browning parents was unexpected. In such individuals either the biosynthetic pathway leading to the substrate is blocked in a prior step or modified, or the substrate is converted to another product not amenable to oxidation by PPO. These possibilities are not unlikely since most *Citrus* taxa are highly heterozygous as a result of accumulation and preservation of genetic variability arising from mutation and hybridization through adventive nucellar embryony and asexual propagation. The involvement of an inhibitor or another enzyme which may reduce the oxidized product of PPO in homogenates is ruled out because browning occurred when equal amounts of tissue from nonbrowning and browning individuals were homogenized together.

The dominance of browning to nonbrowning was evident in progenies from browning × nonbrowning crosses. This dominance was independent of the dosage of the chromosome compliment contributed by the nonbrowning parent. In other words, the intensity of browning was much the same in the progeny of 4x × 2x, 3x × 2x, and 2x × 2x crosses where the browning parent was always diploid. All nonbrowning progeny yielded negative tests for the substrate. Likewise, they lacked PPO activity except 5 hybrids from the cross 'Acidless' × ['Ruby' × 'Dancy'] and 15 from 4x 'Lisbon' × 'Trovita' (Table 1). In most cases there were too few progenies to permit any definite statement about the genotype of parents. The data suggest that the browning cvs. Clementine, Dancy, Kinnow, Wilking, Temple and Dweet are dominant homozygotes (+/+) while Paperrind, Trovita, Honey, the hybrid Ruby × Dancy and, consequently, Ruby are heterozygotes (+/br). Similarly, the cvs. Owari and Imperial which produced the only nonbrowning progeny in crosses between *C. paradisi* and *C. reticulata* may be heterozygotes.

The most puzzling situation occurred in progenies from crossing browning *Citrus* taxa such as *C. sinensis* and *C. reticulata* with *Poncirus trifoliata*. Eleven citranges out of 12 tested were nonbrowning following *C. sinensis* × *P. trifoliata* crosses (Table 1). The nonbrowning hybrids lacked not only substrate but also enzyme activity. The only browning one was a hybrid between 'Spanish Sweet' orange and trifoliate. The 2 citranges, 'Troyer' and 'Carrizo', are known to be hybrids from the cross 'Washington Navel' × trifoliate and the 'Rusk' from 'Ruby' × trifoliate. The orange parent for the remaining citranges is not known. Likewise, 2 hybrids from the cross 'Honey' × trifoliate and 2 out of 8 from 'Clementine' × trifoliate were nonbrowning. Although heterozygosity of the browning parents may account for these results, it does not account for the high proportion of the nonbrowning individuals (11 out of 12) found among progeny from the cross *C. sinensis* × *P. trifoliata*, especially since these individuals were also devoid of enzyme activity. Whether the presence of the Poncirus *genome* with that of *Citrus* disrupts or modifies the enzyme and substrate synthesis in certain genotypes is not known. A similar situation was found in progenies from crossing of monoembryonic *Citrus* taxa with *P. trifoliata* (polyembryonic) where proportions of monoembryonic hybrids were much in excess of the expected proportion based on data from crosses within the genus *Citrus* (J. W. Cameron, personal communication). These results, too, could not be satisfactorily explained by the heterozygosity of one parent (*P. trifoliata*) for polyembryony.

The crosses between 2 nonbrowning taxa produced only nonbrowning progeny, as expected. The hybrids showed no detectable PPO activity. The substrate was present in 5 of 10 hybrids from the cross 'Acidless' pummelo × 'Seedy Marsh' grapefruit but not in other hybrids (Table 1). Thus, the genotype of all nonbrowning taxa except cultivars of *C. paradisi* can be designated as br/br. 'Seedy Marsh' and, most likely, other grapefruit too, must be heterozygous (+/br) for the substrate as evident from the 1:1 segregation observed when it was crossed with 'Acidless' pummelo. In fact, work by Esen and Scora (unpublished) showed that 20 grapefruit cultivars tested lacked PPO activity but contained the substrate.

Our data strongly indicate the potential use of spot colors to identify nucellar and zygotic seedlings from nonbrowning × browning crosses at the early seedling stage. If the staminate parent is heterozygous and shows segregation for the substrate and/or enzyme activity resulting in some non-browning progeny, their hybrid origin may be verified by adding phenol-free enzyme-free substrate to their spots. Only those segregants which lack both the substrate and enzyme activity cannot be distinguished from nucellar progeny. Tests for the substrate must be made by using citrus PPO while the test for the enzyme can be made using 3,4-DOPA or citrus substrate. Purified mushroom 'PPO is non-specific in its activity as it oxidized phenolic fractions not oxidized by PPO from 'Willow Leaf' and also produced browning in homogenates of certain nonbrowning taxa. Substrate and enzyme must not be added to spots sooner than 15–20 min after homogenates are poured; this period is required for the completion of diffusion of homogenate's liquid phase. If added too soon, the substrate and enzyme solutions are diluted during diffusion and the reaction product (browning) may be obscured.

The screening method based on enzymatic browning or its absence as a marker for the identification of nucellar and zygotic seedlings is, we think, superior to those reported in the literature. In addition to its dependability, it is 1) simple because it requires no special skills, 2) inexpensive since it requires no sophisticated equipment, and 3) rapid, for 2 operators may screen as many as 400 seedlings a day if the operation is done in the glasshouse or lathhouse. A fourth advantage is that screening can be done at an early stage, as soon as 0.2 to 0.5 g surplus young growth is available and nucellar seedlings can be eliminated, resulting in considerable savings of space and care.

Hybrid populations used in this study were mostly from controlled pollinations involving monoembryonic pistillate parents. Consequently, the described screening procedure could not be tested on large progenies from polyembryonic parents. The only available population was a mixture of 4x nucellar and 3x zygotic individuals from crossing of 4x 'Ruby' and an unnamed 4x sweet orange with a 2x hybrid. Nucellar and zygotic individuals were not expected to be identified because parents were browning and had similar spot colors. Nevertheless, we positively identified 6 out of 14 individuals from which material was available as hybrids based on variation from that of parents in intensity of browning and color of their inner spots. This identification was also confirmed by chromosome number of hybrids which was not known to us prior to testing. Two other hybrids had spot colors identical with those of 6 nucellar individuals and parents. We screened 50 8-month-old rough lemon and 60 'West Indian' lime seedlings from open-pollination which were judged to be nucellar by their morphological characters; seedlings suspected to be of zygotic origin among these had already been rogued out. All 60 'West Indian' lime seedlings had essentially identical browning spot colors, supporting the morphological conclusion of their nucellar origin. Forty-nine of 50 rough lemon seedlings were browning and had identical spot colors; one seedling was nonbrowning and judged to be zygotic because it was deficient in substrate. A close examination of this seedling with respect to vigor and leaf morphology also indicated its zygotic origin. In addition, the intensity of browning in spots of rough lemon seedlings was much less than that of their pistillate parents grown in the field, suggesting that either the substrate and/or enzyme concentration was reduced under greenhouse conditions. Browning was not influenced by the age of the plant.

Enzymatic browning is taxon-specific (1). The browning taxa are: *C. aurantium*, *C. sinensis*, *C. reticulata*, (and *paradisi* if the condition of its browning is specified). *Citrus limon* and *C. aurantifolia* include both phenotypes; the remaining 9 species are nonbrowning. Thus, a wide array of nonbrowning × browning croses can be made and

nucellar and zygotic progeny from these crosses may then be identified as described. Furthermore, browning and nonbrowning *Citrus* taxa have characteristic inner and outer spot colors. For example, browning species *C. aurantium*, *C. sinensis*, and *C. reticulata* can be distinguished easily from one another by the shade and intensity of browning of their inner and outer spots. Within *C. reticulata*, 2 cultivar-specific shades of browning were observed while no such differences were detectable among cultivars of *C. aurantium* and *C. sinensis*. Inter- and intra-specific differences in the color of inner and outer spots were also observed in the nonbrowning taxa. Spot colors of hybrids from crossing of 2 different nonbrowning taxa were either different from both parents or similar to one of the parents. Thus, it appears that identification of nucellar and zygotic seedlings may be accomplished even following browning × browning and nonbrowning × nonbrowning crosses if parents with contrasting spot colors are used. The dependability of shade and intensity differences of browning between and within the browning taxa as a marker and its biochemical and genetic basis still remains to be fully explored. This is also true with differences between and within nonbrowning taxa.

Taxon-specificity of enzymatic browning was also found to be useful in distinguishing hybrids and variants among both browning and nonbrowning taxa. For example, there were 8 browning accessions of "acid lemons" among 50, and 3 of shaddocks among 39 tested. Since both taxa are nonbrowning, these browning accessions were most likely hybrids from crossing with browning taxa, which was also suspected from their morphological characters. Likewise the parentage of certain presumed hybrids was checked by comparing their spot colors with those of the presumed parents. Results confirmed the presumed origin of several hybrid cultivars. In fact, we propose that the description of *Citrus* cultivars should include information with respect to enzymatic browning or any other chemical information available. This along with information based on morphological characteristics would be useful in checking the identity of certified and patented material as well as that imported from elsewhere.

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Relation of Quality Indices of Individual Blueberries to Photoelectric Measurement of Anthocyanin Content[1]

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Abstract. Individual fruit pH, total acidity (AC as %), soluble solids (SS as %), and SS/AC ratios correlated significantly with anthocyanin contents of 'Wolcott', 'Morrow', 'Jersey', and 'Tifblue' blueberries as measured by light transmission (ΔOD740-800 nm). Fruit orientation with respect to the light path influenced readings. The relationships of the light transmission values to the quality indices differed among cultivars.

Commercial harvesting of blueberries is shifting from the customary hand-harvesting operation to a mechanized one with large over-the-row units. With hand harvesting there is no opportunity, and usually no need, for sorting or grading the fruits because the blue, ripe fruits are selectively removed at several harvests from the bushes and placed directly into the pint container that is shipped to market. In contrast, the mechanical harvester removes any fruits that shake loose when they are subjected to its action. This produces a mass of fruits representing all stages of maturity from the smallest green fruits to the most mature blue fruits on the bushes. Mechanical harvesters also bruise some fruits excessively (12, 13). Consequently, some sorting is required before mechanically-harvested fruits can be packaged for shipment.

In other work, data were developed to document the factors that affect firmness of blueberry fruits (3) and show how fruits can be separated on the basis of firmness with a vibration technique (8). Firmness was not found to be a good index of ripeness because the berries softened noticeably upon turning from green to blue, but thereafter, softened slowly with additional ripening (3). However, only a small amount of bruising decreased firmness considerably. Consequently separate methods for sorting fruits for ripeness and softness (or bruising) are needed.

If harvesting is delayed or if ripe fruits are left on the bushes at one harvest, overripe fruits are included in the next harvest. From a practical point of view, some non-destructive mechanical sorting system capable of screening large numbers of individual fruits is needed. Present sorting-line personnel can identify and manually eliminate green fruits without excessive costs (12, 13) but do not differentiate overripe fruits from other blue fruits. Yet, overripe fruits constitute a greater quality problem than unripe green fruits because of their short shelf-life.

Table 1. Correlation of anthocyanin content of individual blueberries with light transmission difference meter reading.

Anthocyanin content	Correlation coefficient	
	710–800 nm[z]	740–800 nm[y]
μg/berry	+.929	+.916
μg/cm² of berry surface	+.943	+.943
μg/g of berry	+.844	+.972

[z] 50 'Berkeley' fruits.
[y] 24 'Wolcott' fruits.

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