Table VI. Olive Oil Triglyceride Composition of Examined Sample (mol %)

molecular species	present procedure	Vander Wal-Coleman procedure
I, StOP	0.9	1.0
II, PPO	0.2	0.3
III, POP	2.8	2.7
IV, P'OP	0.2	0.2
V, StOO	3.6	4.0
VI, OPO	0.6	0.6
VII, POO	20.1	22.3
VIII, PLSt	0.3	0.1
IX, PLP	0.3	0.3
X, OP'O	0.2	0.2
XI, P'OO	0.7	0.8
XII, OOO	45.6	45.7
XIII, OPL	0.2	0.4
XIV, StOL	0.2	0.4
XV, StLO	0.4	0.5
XVI, PLO	1.9	2.7
XVII, POL	1.5	1.9
XVIII, OOL	7.2	8.0
XIX, P'LO	0.2	0.1
XX, OLO	5.1	5.6
XXI, PLL	0.3	0.2
total:	92.5	97.7

1967) using different procedures and, of course, different samples.

As reported in Table IV, the species OLL and LOL, which belong to the fraction containing glycerides having five double bonds/mol (class A_3), were not determined in this work. According to the procedure of Vander Wal and Coleman, they should have been present in our sample at the rate of 1.0 and 0.3 mol %, respectively.

This omission seemed irrelevant to us, as we were able to prove experimentally that the fraction containing molecules with five double bonds was present in our sample in very low molar percentage.

In order to obtain sufficient quantities of this fraction for the operations described in the experimental part, dozens of chromatographic separations would have been necessary and, although this would been quite possible with slight experimental modifications where necessary, it was completely unwarranted here.

In any case our aim was to reach, by completely experimental means and without any preconceived assumptions, the results that actually were obtained by using the above-described combination of multiple operations (Ag^+ TLC, followed by single fraction lipolysis, oxidation of these fractions, TLC of oxidized products, and then GLC of the methyl esters of the acids present in each of the classes A_3, A_2S, and AS_2 separated by TLC).

LITERATURE CITED

Brockerhoff, H.; Yurkowski, M. *J. Lipid Res.* **1966**, *7*, 62.
Coleman, M. H. *J. Am. Oil Chem. Soc.* **1961**, *38*, 685.
Damiani, P.; Burini, G.; Avellini, P. *Ind. Aliment.* **1977**, *No. 145*, 102.
Dutton, H. J.; Cannon, J. A. *J. Am. Oil Chem. Soc.* **1956**, *33*, 46.
Fedeli, E.; Jacini, G. *Riv. Ital. Sostanze Grasse* **1967**, *44*, 393.
Freyer, F. H.; Ormand, W. L.; Crump, G. B. *J. Am. Oil Chem. Soc.* **1960**, *37*, 589.
Gunstone, F. D.; Padley, F. B. *J. Am. Oil Chem. Soc.* **1965**, *42*, 957.
Hilditch, T. P.; Lea, C. H. *J. Chem. Soc.* **1927**, 3106.
Huebner, U. R. *J. Am. Oil Chem. Soc.* **1961**, *38*, 628.
Kartha, A. R. S. *J. Am. Oil Chem. Soc.* **1961**, *30*, 62.
Kaufmann, H. P.; Wessels, H. *Fete, Seifen, Anstrichm.* **1966**, *68*, 249.
Kuksis, A.; McCarthy, M. J. *Can. J. Biochem. Physiol.* **1962**, *40*, 679.
Litchfield, C.; Farquhar, M.; Reiser, R. *J. Am. Oil Chem. Soc.* **1964**, *41*, 588.
McCarthy, M. J.; Kuksis, A.; Beveridge, J. M. R. *Can. J. Biochem. Physiol.* **1962**, *40*, 1693.
Pelick, N. W.; Supina, R.; Rose, A. *J. Am. Oil Chem. Soc.* **1961**, *38*, 506.
Privett, O. S.; Blank, M. L. *J. Lipid Res.* **1961**, *2*, 37.
Privett, O. S.; Blank, M. L. *J. Am. Oil Chem. Soc.* **1963**, *40*, 70.
Quimby, O. Y.; Willie, R.; Lutton, E. S. *J. Am. Oil Chem. Soc.* **1953**, *30*, 186.
Riemenschneider, R. W. *J. Am. Oil Chem. Soc.* **1954**, *31*, 266.
Scholfield, C. R.; Dutton, H. J. *J. Am. Oil Chem. Soc.* **1958**, *35*, 493.
Scholfield, C. R.; Hicks, M. A. *J. Am. Oil Chem. Soc.* **1957**, *34*, 77.
Subbaram, M. R.; Chakrabarty, M. M.; Youngs, C. G.; Craig, B. M. *J. Am. Oil Chem. Soc.* **1964**, *41*, 691.
Subbaram, M. R.; Youngs, C. G. *J. Am. Oil Chem. Soc.* **1964**, *41*, 445.
Vander Wal, R. J. *J. Am. Oil Chem. Soc.* **1960**, *37*, 18.
Vanhandel, E. *Clin. Chem. (Winston-Salem, N.C.)* **1961**, *7*, 249.
von Rudloff, E. *Can. J. Chem.* **1956**, *34*, 1413.
Youngs, C. G. *J. Am. Oil Chem. Soc.* **1961**, *38*, 62.
Youngs, C. G.; Subbaram, M. R. *J. Am. Oil Chem. Soc.* **1964**, *41*, 218.

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Analysis of Total Phenols Using the Prussian Blue Method

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The method for determining vegetal phenolics based on the formation of the Prussian Blue complex has been reassessed. The color development has not interfered with the common naturally occurring substances except for ascorbic acid. The method is ~20 times as sensitive as the acidified vanillin method and 3 times that of the titanium method. The Prussian Blue method, plus a simple extraction procedure, has been used in order to provide a rapid estimation of the total phenol content in strawberries. Since a remarkable decrease in the levels of phenols was noted at progressive stages of ripeness, they can be considered as a chemical index of ripening.

Our investigation was aimed at finding one or more "warning" substances of the ripening process of fruits and vegetables which may be easily employed by agricultural producers. The search for ripeness indexes comes from the recurrent need to decide when fruits should be harvested. For this purpose total phenols were examined. Phenols which are widely distributed in fruits and vege-

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Table I. Changes in the Mean Content (mg/100 g of Fruit) of Some Ripeness Indexes during Strawberry Ripening

maturity visual skin color	total phenols as epicatechin (our method)	SD[a]	total phenols as epicatechin (vanillin method)	SD	AA	SD	anthocyanins as cyanidin 3-galactoside	SD	total phenols[b] other than anthocyanins	SD
M_1	405.3	5.1	84.5	3.4	26.0	2.1	8.2	0.2	400.0	5.2
M_2	211.6	4.8	41.3	1.9	53.1	1.9	76.9	2.0	162.4	5.0
M_3	147.3	4.1	27.5	0.8	75.9	0.8	140.4	4.1	57.5	4.2

[a] Standard deviation from mean. [b] These values were obtained by deducting the corresponding values of cyanidin 3-galactoside, expressed as epicatechin, from the amount of total phenols (column 2).

tables are important in several respects (Kosuge, 1969; Herrmann, 1976; Joslin and Goldstein, 1964; Golan et al., 1977). Phenolic compounds are important constituents of strawberries (Co and Markakis, 1968; Ryan, 1971; Abers and Wrolstad, 1979), and their qualitative and quantitative evaluation has been investigated by Stöhr and Herrmann (1975). A number of methods are available for measuring phenols (Swain and Hillis, 1959; Price and Butler, 1977; Broadhurst and Jones, 1978; Eskin et al., 1978); however, the Prussian Blue method is the most easily applicable to agricultural products. We modified the procedure of Price and Butler (1977) to improve sensitivity and applied it to strawberries together with a simple, rapid extraction of phenols (Bate-Smith, 1962). Compositional analyses also included the determination of ascorbic acid and anthocyanins. The former was considered because of its interference with the Prussian Blue method; the latter was considered to give better proof of the variations in the concentration of phenols other than anthocyanins during strawberry ripening.

EXPERIMENTAL SECTION

Strawberries. Strawberries (*Fragaria vesca* cv. Pocahontas) were supplied by the CRIOF, University of Bologna, and investigated during two consecutive seasons. They were harvested at three stages of maturity on the basis of subjective evaluation: mature green (M_1), pink (M_2), and ripe red (M_3). Uniform fruits (~200) of each stage of ripeness were handpicked to minimize variability and were immediately frozen in liquid nitrogen. The frozen fruits were crushed homogeneously by means of a hand press, and the powder was stored in liquid nitrogen. A suitable aliquot was then weighed prior to phenolic extraction.

Reagents. All chemicals were reagent grade and were used without any further purification. Phenolic compounds included L-epicatechin (Sigma Chemical Co.), orcinol, hydroquinone, catechol, gallic acid, and resorcinol (Aldrich Europe), and 3,4-dihydroxycinnamic acid, quercetin, pyrogallol, and cyanidin chloride (K & K Rare & Fine Chemicals). All solutions were prepared by using doubly distilled water. All small volumes were measured with Oxford pipets.

Procedure. *Extraction of Total Phenols from Strawberries.* A 5-g sample of crushed fruits was immediately immersed in 2 M HCl in a 1:10 (w/v) ratio to minimize contact with air and was heated for 30 min in a 95 °C water bath. The cooled mixture containing the aglycons was then filtered through a Schleicher & Schüll No. 589[2] filter paper into a 500- or 1000-mL flask. The slush was rinsed with the amount of water necessary to bring the extract to volume.

In order to ensure no losses occurred during the extraction process, a known sample of epicatechin was treated under the same conditions.

Colorimetric Measurements. Three milliliters of the diluted extract was transferred to a 1-cm cuvette and then 200 μL of 0.008 M $K_3Fe(CN)_6$ was added, followed immediately by the addition of 200 μL of 0.1 M $FeCl_3$ in 0.1 M HCl. The optical density was read after 5 min at 700 nm, which is the most suitable wavelength for readings, with a Perkin-Elmer Model 124 spectrophotometer against a blank of identical composition in which the strawberry extract was omitted. The measurements were carried out at a constant temperature of 23 ± 0.05 °C. Five-minutes time for color development was chosen both to allow the color to become more stable and because the rate of reaction was found to be considerably slower after that time. The phenolics content was also determined by using the vanillin method described by Broadhurst and Jones (1978). Results were expressed as milligrams of epicatechin per 100 g of strawberries by constructing a calibration plot for different amounts of epicatechin using the same conditions as in the total phenol analysis. Six separate experiments were performed, and the mean values are summarized in Table I.

Quantitative Determination of Total Anthocyanin. The procedure of Torre and Barritt (1977) was used to determine the total anthocyanin content with a slight modification which consisted in carrying out an ultrasonic disintegration (MSE, 150 W) for 10 min to obtain a more rapid extraction. Absorbance readings were taken at 517 nm, which is known to be the absorbance maximum of pelargonidin 3-glycoside, as it is the major anthocyanin pigment present in the strawberry as reported by Swain (1962). The complete procedure was repeated 3 times, and the means are presented in Table I.

Ascorbic Acid Determination. Ascorbic acid (AA) values were determined by using an aqueous solution of 6% (p/v) metaphosphoric acid as the extracting solvent and the 2,6-dichlorophenol–indophenol procedure as described by Gielfrich and Bernard-Griffiths (1975). The data obtained from four separate experiments are reported in Table I.

RESULTS AND DISCUSSION

Total Anthocyanin and AA Determination. Table I shows anthocyanin and AA levels obtained from strawberries. There was an increase in their concentration during fruit ripening in agreement with the findings of Woodward (1972) and Shorikova and Gorban (1966). Therefore, both substances can be considered suitable indexes of strawberry ripening.

Total Phenol Determination. A 100% recovery of epicatechin was obtained following extraction, which indicated no losses occurred during the extraction procedure.

From Table I it can be seen that there is an appreciable decrease both in the total phenols and in the phenols other than anthocyanins during ripening in accord with the results of Stöhr and Herrmann (1975) and Shorikova and Gorban (1966). Thus, the quantitative evaluation of these compounds can be indicative of the stage of strawberry ripeness. Since, in our method, mixtures of phenols oxidized in different degrees by Fe^{3+} are analyzed, it is nec-

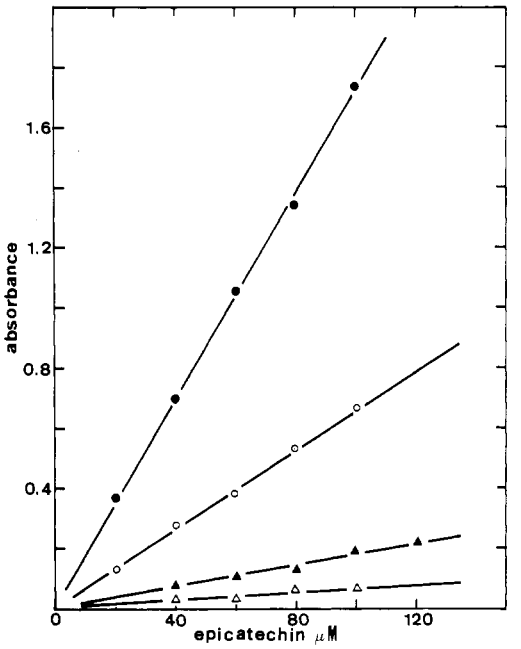

Figure 1. Sensitivity comparison of different procedures for total phenol determination. (●) Our method (readings at 700 nm); (⊙) Eskin et al. method (readings at 430 nm); (▲) Price and Butler method (readings at 720 nm); (△) Broadhurst and Jones method (readings at 500 nm).

essary to be cautious in interpreting the results as emphasized by Price and Butler (1977). However, it must be considered that we are interested in detecting relative variations in "warning" substance content during ripening rather than their absolute values.

We found that the formation of the Prussian Blue complex is affected by AA, so the optical density caused by it must be subtracted from the total absorbance obtained with the colorimetric estimation described above; dosing the exact amount of AA is therefore essential. The interference caused by AA was estimated with a standard curve constructed in the same way as for epicatechin. Table I shows the total phenol content obtained by subtracting the AA contribution. The absorbance at 700 nm for our Prussian Blue method is linear for concentrations of epicatechin up to 10^{-4} M, as can be seen in Figure 1. The sensitivity of the test for epicatechin is sufficient to determine concentrations down to 10^{-5} M.

The vanillin hydrochloride method was employed to estimate tannin phenols in strawberries. This assay is attractive as it is not affected by ascorbate. The values obtained by this procedure showed almost the same trend as those found with the Prussian Blue method, but they were much lower. This was probably due to the presence of considerable phenolic materials which did not respond to vanillin, as well as to the necessity of subtracting the blank, because of its absorption at the same wavelength. In fact, the vanillin test is specific for flavanols (Sarkar and Howarth, 1976), but the possibility of interference by dihydrochalcons and anthocyanins must be kept in mind. On the other hand, the Prussian Blue method revealed more phenols as demonstrated by the positive reaction for the compounds listed in Table II.

A comparison of previously reported methods with the method reported in this paper is shown in Figure 1. Our method is 9 times as sensitive as the other Prussian Blue method (Price and Butler, 1977), 20 times as sensitive as the vanillin method (Broadhurst and Jones, 1978), and 3 times as sensitive as the titanium method (Eskin et al., 1978).

Table II. Molar Absorptivities for the Prussian Blue Reaction

phenolic compd	molar absorptivity	Beer's law plot, r^2
L-epicatechin	1.71×10^4	1.00
orcinol	0	
hydroquinone	1.80×10^4	1.00
catechol	1.82×10^4	0.99
gallic acid	3.62×10^4	1.00
resorcinol	0	
3,4-dihydroxycinnamic acid	1.66×10^4	0.99
pyrogallol	2.82×10^4	1.00
quercetin	8.00×10^4	1.00
cyanidin chloride	2.64×10^4	0.99

CONCLUSION

The formation of the Prussian blue complex offers a sensitive and rapid method for the colorimetric determination of total phenols which can be applied to other agricultural products. The major advantage is that the test is so sensitive that no interfering color is present at the dilutions used. The most relevant disadvantage of this or any other redox method is the lack of specificity, i.e. the existence of interfering substances even though, the simple extraction procedure and the analytical procedure we used, allowed us to eliminate the main interferences other than AA.

LITERATURE CITED

Abers, J. E.; Wrolstad, R. E. *J. Food Sci.* **1979**, *44*, 75.
Bate-Smith, E. C. *J. Linn. Soc. London, Bot.* **1962**, *58*, 39.
Broadhurst, R. B.; Jones, W. T. *J. Sci. Food Agric.* **1978**, *29*, 788.
Co, H.; Markakis, P. *J. Food Sci.* **1968**, *33*, 281.
Eskin, N. A. M.; Hoehn, E.; Frenkel, C. *J. Agric. Food Chem.* **1978**, *26*, 973.
Gielfrich, M. L.; Bernard-Griffiths, M. A. *Sci. Agron. Rennes* **1975**, *53*.
Golan, A.; Varda, K.; Sadowski, A. Y. *J. Agric. Food Chem.* **1977**, *25*, 1253.
Herrmann, K. *J. Food Technol.* **1976**, *11*, 433.
Joslin, M. A.; Goldstein, J. L. *Adv. Food Res.* **1964**, *13*, 179.
Kosuge, T. *Annu. Rev. Phytopathol.* **1969**, *7*, 195.
Price, M. L.; Butler, L. G. *J. Agric. Food Chem.* **1977**, *25*, 1268.
Ryan, J. J. *J. Food Sci.* **1971**, *36*, 867.
Sarkar, S. K.; Howarth, R. E. *J. Agric. Food Chem.* **1976**, *24*, 317.
Shorikova, Yu G.; Gorban, D. F. *Izv. Vyssh. Uchebn. Zaved., Pishch. Tekhnol.* **1966**, *4*, 27.
Stöhr, H.; Herrmann, K. *Z. Lebensm.-Unters. -Forsch.* **1975**, 159, 341.
Swain, T. "The Chemistry of Flavonoid Compounds"; Geissman, T. A., Ed.; Pergamon Press: New York, 1962; pp 513–52.
Swain, T.; Hillis, W. E. *J. Sci. Food. Agric.* **1959**, *10*, 63.
Torre, L. C.; Barritt, B. H. *J. Food Sci.* **1977**, *42*, 488.
Woodward, J. R. *J. Sci. Food Agric.* **1972**, *23*, 465.

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