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Isolation, Characterization, and Antioxidant Activity of *E*- and *Z*-*p*-Coumaryl Fatty Acid Esters from cv. Annurca Apple Fruits

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A total of 12 fatty acid esters of *Z*- and *E*-*p*-coumaryl alcohol were isolated from cv. Annurca apple fruit and characterized. This apple variety is widely cultivated in the south of Italy, and the fruits typically undergoe a reddening treatment after harvest. Structures of the *p*-coumaryl esters were elucidated by GC−MS and ^{1}H and ^{13}C NMR after purification of individual compounds by HPLC. It was found that the esters are localized in the fruit peel. During reddening of the fruit, there was a substantial increase in the amount of esters and particularly in molecular species with unsaturated fatty acids. The individual compounds were tested for antioxidant activity, and over half were shown to be at least as effective as α-tocopherol.

KEYWORDS: ***Malus domestica*** **cv. Annurca; apple fruits; phenolic fatty acid esters; wax; NMR analysis; antioxidant activity**

INTRODUCTION

Annurca is an apple variety grown in the south of Italy and demanded in the local markets for its high quality (*1*). From the agronomical point of view, it has several shortcomings such as bare wood, great vigor, late bearing habit, biennial crop, very short peduncle, and lack of red skin color (*2*). The fruit is medium to small in size with a flat shape. The skin is thick, first yellowish-green and becoming striped brilliant red blush when it ripens. The flesh is quite firm and strong, has an average juiciness, is sweet and slightly acidic, and has an average amount of aroma and good flavor characteristics (*3*, *4*). This apple undergoes a typical reddening treatment. Generally, the unreddened fruits are harvested in October and placed for 25−30 days on a layer of straw or sawdust on the soil and sprayed daily with water. When the sun-exposed surface of the fruit becomes red, they are turned to redden the opposite side (*5*).

In the framework of the Regional Research Center for the Agro-alimentary productions of the Campania Region (Italy), we studied the chemical characteristics of the cv. Annurca apple fruit. In this project, we studied the lipidic component of the fruit. The present study was undertaken to isolate and characterize phenolic fatty acids from the reddened fruit, their localization in the peel of the apple, and their variations during the reddening treatment.

Phenolic fatty acid esters from apples play an important role in natural resistance to scald, and it is hypothesized that they have antioxidant activity. Their abundance seems to be correlated with the apple cultivar and the maturity of the fruits (*6*).

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MATERIAL AND METHODS

Plant Material. Annurca apple fruits were obtained in Sant'Agata de' Goti, near Caserta (Italy), in October 2003, when the fruit had just been harvested (green peel). Tissue samples from a group of these freshly harvested apples were submitted to extraction for analytical analyses. The remaining green fruits were reddened on the straw until November and then stored in a refrigerated chamber at 0 °C and 98% relative humidity.

General Experimental Procedures. NMR spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian 300 Fourier transform NMR spectrometer in $CDCl_3$ at 25 °C. Proton-detected heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC), optimized for $^1J_{HC}$ = 140 Hz, and a gradient heteronuclear multiple-bond coherence (HMBC), optimized for $^nJ_{HC}$ = 8 Hz. UV spectra were obtained on a Perkin−Elmer Lambda 7 spectrophotometer in $CHCl_3$ or EtOH solutions. Electronic impact mass spectra (EI−MS) were obtained with a HP 6890 instrument equipped with a MS 5973 N detector, fitted with 15 m × 0.25 mm i.d., RTX5 fused silica capillary column (Restek, Bellefonte, PA). The column oven was held at 150 °C for 5 min and then increased to 280 °C at 13 °C/min. Injector and detector temperatures were both 290 °C. The carrier gas was N_2, and the flow rate was 1.0 mL/min.

The preparative HPLC apparatus (Shimadzu) consisted of a LC-10AD pump, a RID-10A refractive index detector, and a C-R6A Chromatopac recorder. Preparative HPLC was performed using a 250 × 10 mm i.d., 10 μm, RP-18 Luna column (Phenomenex, Torrance, CA). The analytical HPLC apparatus consisted of a Beckman 127 System Gold pump, a Beckman 166 UV−vis detector, and a Shimadzu Chromatopac C-R6A recorder. HPLC was performed using a 250 × 4.6 mm i.d., 5 μm, RP-18 Luna column (Phenomenex, Torrance, CA). Gas chromatography was performed using a HP 6890 PLUS instrument in split mode with a flame ionization detector. Flash column chromatography (FCC) was performed on Merck Kieselgel 60 (230−400 mesh)

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at 150 kPa. Column chromatography (CC) was performed on Merck Kieselgel 60 (70−240 mesh).

Extraction and Isolation. The reddened "Annurca" apple fruit (5.21 kg) was sliced, frozen in liquid nitrogen, powered in a mortar, and infused in ethanol (6.0 L) for 7 days in a refrigerated chamber at 4 °C in the dark. After the filtration of the hydroalcoholic solution, the fruit were infused in ethyl ether (6.0 L) for 7 days in the same conditions. The Et_2O extract, after filtration, was evaporated using an automatic distillation apparatus. After removal of the ether, we obtained a crude organic extract (4.80 g).

Organic Extract Fractionation. Crude Et_2O extract was chromatographed on silica gel eluting with chloroform and ethyl acetate solutions to obtain fractions I and II.

Fraction I was rechromatographed on SiO_2 by flash chromatography, and the fraction eluted with hexane/acetone (9:1) was purified by RP-18 HPLC eluting with MeOH/MeCN (4:1) to give pure compounds **1c** (2.0 mg), **2d** (5.2 mg), **1d** (3.5 mg), **2e** (2.2 mg), **1e** (1.5 mg), **2f** (2.5 mg), and **1g** (1.1 mg).

Fraction II was chromatographed on an SiO_2 column by flash chromatography eluting with acetone in hexane solutions: fraction eluted with Me_2CO/hexane (19:1) gave a fraction which, after purification on RP-18 HPLC [MeOH/MeCN (4:1)], gave pure compounds **1a** (1.3 mg), **1b** (1.6 mg), **2b** (1.3 mg), **2c** (0.8 mg), and **1f** (1.3 mg).

Compounds Characterization. *E-p-Coumaryl Linolenate (**1a**).* ^{1}H NMR ($CDCl_3$) δ: 7.12 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 6.81 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 11.7 Hz, H-7′), 5.71 (1H, dt, J = 11.7 and 6.6 Hz, H-8′), 5.35 (6H, m, H-9, H-10, H-12, H-13, H-15, and H-16), 4.82 (2H, dd, J = 6.6 and 1.5 Hz, H-9′), 2.80 (4H, m, H-11 and H-14), 2.35 (2H, t, J = 7.8 Hz, H-2), 2.04 (4H, m, H-8 and H-17), 1.31 (10H, m, H-3−H-7), 0.88 (3H, t, J = 6.9 Hz, H-18). ^{13}C NMR ($CDCl_3$) δ: 173.8 (C-1), 155.1 (C-4′), 132.0 (C-7′), 130.2 (C-2′ and C-6′), 130.1−127.9 (C-9, C-10, C-12, C-13, C-15, and C-16), 129.6 (C-1′), 123.9 (C-8′), 114.8 (C-3′ and C-5′), 61.2 (C-9′), 34.0 (C-2), 29.8−29.4 (C-4−C-7), 27.9, (C-8), 26.1 (C-11 and C-14), 25.1 (C-3), 21.2 (C-17), 14.3 (C-18). EI/MS: m/z 410 [M]$^+$. Elemental analysis, found: C, 79.08; H, 9.25. $C_{27}H_{38}O_3$ requires: C, 78.98; H, 9.33.

*Z-p-Coumaryl Linoleate (**1b**).* ^{1}H NMR ($CDCl_3$) δ: 7.11 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.82 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 11.0 Hz, H-7′), 5.70 (1H, dt, J = 11.0 and 6.4 Hz, H-8′), 5.36 (4H, m, H-9, H-10, H-12, and H-13), 4.83 (2H, dd, J = 6.4 and 1.5 Hz, H-9′), 2.77 (2H, m, H-11), 2.33 (2H, t, J = 7.9 Hz, H-2), 2.05 (4H, m, H-8 and H-14), 1.30 (16H, m, H-3−H-7 and H-15−H-17), 0.88 (3H, t, J = 6.9 Hz, H-18). ^{13}C NMR ($CDCl_3$) δ: 173.8 (C-1), 155.1 (C-4′), 132.0 (C-7′), 130.2 (C-2′ and C-6′), 130.1−127.9 (C-9, C-10, C-12, and C-13), 129.6 (C-1′), 123.9 (C-8′), 114.8 (C-3′ and C-5′), 61.2 (C-9′), 33.8 (C-2), 30.4−29.5 (C-4−C-7, C-15, and C-16), 27.7 (C-8 and C-14), 25.8 (C-11), 24.9 (C-3), 22.8 (C-17), 14.2 (C-18). EI/MS: m/z 412 [M]$^+$. Elemental analysis, found: C, 78.01; H, 9.55. $C_{27}H_{40}O_3$ requires: C, 78.60; H, 9.77.

*E-p-Coumaryl Linoleate (**2b**).* ^{1}H NMR ($CDCl_3$) δ: 7.24 (2H, d, J = 9.0 Hz, H-2′ and H-6′), 6.80 (2H, d, J = 9.0 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 15.3 Hz, H-7′), 6.14 (1H, dt, J = 15.3 and 6.6 Hz, H-8′), 5.35 (4H, m, H-9, H-10, H-12, and H-13), 4.70 (2H, d, J = 6.6 Hz, H-9′), 2.77 (2H, m, H-11), 2.33 (2H, t, J = 7.9 Hz, H-2), 2.00 (4H, m, H-8 and H-14), 1.30 (16H, m, H-3−H-7 and H-15−H-17), 0.88 (3H, t, J = 7.0 Hz, H-18). ^{13}C NMR ($CDCl_3$) δ: 173.3 (C-1), 156.0 (C-4′), 135.0 (C-7′), 129.2 (C-2′ and C-6′), 130.1 (C-8′), 130.0−127.9 (C-9, C-10, C-12, and C-13), 129.1 (C-1′), 115.6 (C-3′ and C-5′), 64.2 (C-9′), 33.9 (C-2), 30.5−29.5 (C-4−C-7, C-15, and C-16), 27.9 (C-8 and C-14), 25.7 (C-11), 25.1 (C-3), 22.8 (C-17), 14.2 (C-18). EI/MS: m/z 412 [M]$^+$. Elemental analysis, found: C, 77.92; H, 9.98. $C_{27}H_{40}O_3$ requires: C, 78.60; H, 9.77.

*Z-p-Coumaryl Oleate (**1c**).* ^{1}H NMR ($CDCl_3$) δ: 7.12 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.82 (2, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 12.3 Hz, H-7′), 5.71 (1H, dt, J = 12.3 and 6.6 Hz, H-8′), 5.34 (2H, m, H-9 and H-10), 4.83 (2H, dd, J = 6.6 and 0.9 Hz, H-9′), 2.35 (2H, t, J = 7.2 Hz, H-2), 2.00 (4H, m, H-8 and H-11), 1.29 (22H, m, H-3−H-7 and H-12−H-17), 0.88 (3H, t, J = 6.7 Hz, H-18). ^{13}C NMR ($CDCl_3$) δ: 173.8 (C-1), 155.3 (C-4′), 132.4 (C-7′), 130.3 (C-2′ and C-6′), 130.2 (C-9), 130.0 (C-10), 129.7 (C-1′), 124.2 (C-8′), 115.2 (C-3′ and C-5′), 61.4 (C-9′), 34.3 (C-2), 31.9 (C-16), 29.7−29.1 (C-4−C-7 and C-12−C-15), 25.0 (C-3), 27.2 (C-8 and C-11), 22.7 (C-17), 14.1 (C-18). EI/MS: m/z 414 [M]$^+$. Elemental analysis, found: C, 65.88; H, 10.12. $C_{27}H_{42}O_3$ requires: C, 78.21; H, 10.21.

*E-p-Coumaryl Oleate (**2c**).* ^{1}H NMR ($CDCl_3$) δ: 7.25 (2H, d, J = 8.9 Hz, H-2′ and H-6′), 6.82 (2H, d, J = 8.9 Hz, H-3′ and H-5′), 6.60 (1H, d, J = 15.5 Hz, H-7′), 6.12 (1H, dt, J = 15.5 and 6.7 Hz, H-8′), 5.34 (2H, m, H-9 and H-10), 4.73 (2H, d, J = 6.7 Hz, H-9′), 2.34 (2H, t, J = 7.2 Hz, H-2), 2.01 (4H, m, H-8 and H-11), 1.30 (22H, m, H-3−H-7 and H-12−H-17), 0.87 (3H, t, J = 6.7 Hz, H-18). ^{13}C NMR ($CDCl_3$) δ: 173.4 (C-1), 156.3 (C-4′), 134.4 (C-7′), 130.3 (C-2′ and C-6′), 130.3 (C-9), 130.1 (C-8′ and C-10), 129.9 (C-1′), 115.7 (C-3′ and C-5′), 63.9 (C-9′), 34.1 (C-2), 32.0 (C-16), 29.8−29.2 (C-4−C-7 and C-12−C-15), 24.9 (C-3), 27.3 (C-8 and C-11), 23.0 (C-17), 14.2 (C-18). EI/MS: m/z 414 [M]$^+$. Elemental analysis, found: C, 65.90; H, 10.32. $C_{27}H_{42}O_3$ requires: C, 78.21; H, 10.21.

*Z-p-Coumaryl Stearate (**1d**).* ^{1}H NMR ($CDCl_3$) δ: 7.28 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.82 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 12.0 Hz, H-7′), 5.70 (1H, dt, J = 12.0 and 6.2 Hz, H-8′), 4.84 (2H, d, J = 6.2 Hz, H-9′), 2.36 (2H, t, J = 7.8 Hz, H-2), 1.30 (30H, m, H-3−H-17), 0.88 (3H, t, J = 6.9 Hz, H-18). ^{13}C NMR ($CDCl_3$) δ: 173.2 (C-1), 155.4 (C-4′), 132.4 (C-7′), 130.2 (C-2′ and C-6′), 129.4 (C-1′), 124.2 (C-8′), 115.0 (C-3′ and C-5′), 61.6 (C-9′), 34.2 (C-2), 31.6 (C-16), 29.9−29.1 (C-4−C-15), 24.8 (C-3), 22.9 (C-17), 14.5 (C-18). EI/MS: m/z 416 [M]$^+$. Elemental analysis, found: C, 77.48; H, 10.87. $C_{27}H_{44}O_3$ requires: C, 77.83; H, 10.64.

*E-p-Coumaryl Stearate (**2d**).* ^{1}H NMR ($CDCl_3$) δ: 7.28 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.78 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 15.9 Hz, H-7′), 6.15 (1H, dt, J = 15.9 and 6.9 Hz, H-8′), 4.70 (2H, dd, J = 6.9 and 2.2 Hz, H-9′), 2.37 (2H, t, J = 7.8 Hz, H-2), 1.27 (30H, m, H-3−H-17), 0.89 (3H, t, J = 6.7 Hz, H-18). ^{13}C NMR ($CDCl_3$) δ: 173.2 (C-1), 156.4 (C-4′), 133.9 (C-7′), 130.2 (C-2′ and C-6′), 130.0 (C-8′), 129.4 (C-1′), 115.9 (C-3′ and C-5′), 62.6 (C-9′), 34.1 (C-2), 30.9 (C-16), 29.9−29.1 (C-4−C-15), 24.9 (C-3), 22.7 (C-17), 14.5 (C-18). EI/MS: m/z 416 [M]$^+$. Elemental analysis, found: C, 77.95; H, 10.54. $C_{27}H_{44}O_3$ requires: C, 77.83; H, 10.64.

*Z-p-Coumaryl Arachidate (**1e**).* ^{1}H NMR ($CDCl_3$) δ: 7.12 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 6.81 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 11.7 Hz, H-7′), 5.71 (1H, dt, J = 11.7 and 6.6 Hz, H-8′), 4.82 (2H, d, J = 6.6 Hz, H-9′), 2.35 (2H, t, J = 7.8 Hz, H-2), 1.29 (34H, m, H-3−H-19), 0.88 (3H, t, J = 6.9 Hz, H-20). ^{13}C NMR ($CDCl_3$) δ: 173.5 (C-1), 155.0 (C-4′), 132.4 (C-7′), 130.0 (C-2′ and C-6′), 129.6 (C-1′), 124.4 (C-8′), 115.2 (C-3′ and C-5′), 61.3 (C-9′), 34.0 (C-2), 31.6 (C-18), 29.8−29.1 (C-4−C-17), 24.7 (C-3), 22.6 (C-19), 14.2 (C-20). EI/MS: m/z 444 [M]$^+$. Elemental analysis, found: C, 77.99; H, 11.27. $C_{29}H_{48}O_3$ requires: C, 78.33; H, 10.88.

*E-p-Coumaryl Arachidate (**2e**).* ^{1}H NMR ($CDCl_3$) δ: 7.20 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.80 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 15.9 Hz, H-7′), 6.14 (1H, dt, J = 15.9 and 6.3 Hz, H-8′), 4.70 (2H, dd, J = 6.3 and 2.2 Hz, H-9′), 2.37 (2H, t, J = 7.8 Hz, H-2), 1.26 (34H, m, H-3−H-19), 0.89 (3H, t, J = 6.7 Hz, H-20). ^{13}C NMR ($CDCl_3$) δ: 173.3 (C-1), 156.4 (C-4′), 133.8 (C-7′), 130.1 (C-2′ and C-6′), 130.0 (C-8′), 129.3 (C-1′), 115.6 (C-3′ and C-5′), 62.4 (C-9′), 34.4 (C-2), 31.2 (C-18), 29.9−29.1 (C-4−C-17) 24.7 (C-3), 23.1 (C-19), 14.5 (C-20). EI/MS: m/z 444 [M]$^+$. Elemental analysis, found: C, 77.99; H, 11.27. $C_{29}H_{48}O_3$ requires: C, 77.86; H, 11.45.

*Z-p-Coumaryl Behenate (**1f**).* ^{1}H NMR ($CDCl_3$) δ: 7.12 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.82 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 11.7 Hz, H-7′), 5.71 (1H, dt, J = 11.7 and 7.2 Hz, H-8′), 4.83 (2H, dd, J = 7.2 and 1.8 Hz, H-9′), 2.35 (2H, t, J = 7.8 Hz, H-2), 1.29 (36H, m, H-3−H-21), 0.88 (3H, t, J = 6.9 Hz, H-22). ^{13}C NMR ($CDCl_3$) δ: 173.6 (C-1), 155.3 (C-4′), 131.8 (C-7′), 129.9 (C-2′ and C-6′), 129.6 (C-1′), 124.4 (C-8′), 115.2 (C-3′ and C-5′), 61.8 (C-9′), 34.1 (C-2), 31.5 (C-20), 29.8−29.1 (C-4−C-19), 24.5 (C-3), 22.3 (C-21), 14.1 (C-22). EI/MS: m/z 472 [M]$^+$. Elemental analysis, found: C, 78.11; H, 11.25. $C_{31}H_{52}O_3$ requires: C, 78.76; H, 11.09.

*E-p-Coumaryl Behenate (**2f**).* ^{1}H NMR ($CDCl_3$) δ: 7.28 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.78 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 15.9 Hz, H-7′), 6.15 (1H, dt, J = 15.9 and 6.9 Hz, H-8′), 4.70 (2H, dd, J = 6.9 and 2.2 Hz, H-9′), 2.35 (2H, t, J = 7.8 Hz, H-2), 1.27 (36H, m, H-3−H-21), 0.89 (3H, t, J = 6.9 Hz, H-22).

^{13}C NMR ($CDCl_3$) δ: 173.8 (C-1), 156.0 (C-4′), 133.5 (C-7′), 130.2 (C-2′ and C-6′), 130.0 (C-8′), 129.2 (C-1′), 115.4 (C-3′ and C-5′), 62.2 (C-9′), 34.1 (C-2), 31.5 (C-20), 29.9−29.1 (C-4−C-19), 24.7 (C-3), 22.5 (C-21), 14.3 (C-22). EI/MS: *m/z* 472 $[M]^+$. Elemental analysis, found: C, 78.11; H, 11.25. $C_{31}H_{52}O_3$ requires: C, 78.76; H, 11.09.

*Z-p-Coumaryl Lignocerate (**1g**).* ^{1}H NMR ($CDCl_3$) δ: 7.14 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 6.82 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 6.56 (1H, d, J = 11.7 Hz, H-7′), 5.71 (1H, dt, J = 11.7 and 7.1 Hz, H-8′), 4.83 (2H, dd, J = 7.1 and 1.8 Hz, H-9′), 2.35 (2H, t, J = 7.8 Hz, H-2), 1.26 (40H, m, H-3−H-23), 0.88 (3H, t, J = 6.9 Hz, H-24). ^{13}C NMR ($CDCl_3$) δ: 173.8 (C-1), 155.2 (C-4′), 132.0 (C-7′), 130.1 (C-2′ and C-6′), 129.8 (C-1′), 124.6 (C-8′), 115.6 (C-3′ and C-5′), 64.8 (C-9′), 34.3 (C-2), 31.5 (C-22), 29.8−29.1 (C-4−C-21), 24.2 (C-3), 22.1 (C-23), 14.5 (C-24). EI/MS: *m/z* 500 $[M]^+$. Elemental analysis, found: C, 79.31; H, 11.29. $C_{33}H_{56}O_3$ requires: C, 79.14; H, 11.27.

Gas Chromatographic Analyses. A total of 0.1 mg of each pure ester was dissolved in 0.2 mL of 2 N KOH in methanol in a 1 mL vial. After the solution was stirred for 30 min, 0.8 mL of heptane was added. The solution was mixed by a vortex mixer and centrifuged, using a Beckman GS-15R centrifuge, for 10 min at 4000 rpm. A total of 1 μL of the organic upper phase was analyzed by GC fitted with 100 m × 0.25 mm i.d., 0.2 μm SP2380, fused silica capillary column (Supleco, Bellefonte, PA). The column oven was held at 140 °C for 5 min and then increased to 240 °C at 4 °C/min. Injector and detector temperatures were both 260 °C. The carrier gas was N_2, and the flow rate was 1.0 mL/min.

The fatty acid methyl esters were identified by comparing their retention times with those of the standard fatty acid methyl esters (Supelco 37 Component FAME Mix).

Extraction of "Annurca" Samples for Analytical HPLC Analyses. Samples of about 3 g each of the whole core free Annurca apples, peel and pulp, obtained from both reddened and unreddened fruits, were cut into small pieces, frozen in liquid nitrogen, powdered in a mortar, and lyophilized using a FTS-System Flex-Dry instrument. The powders obtained were extracted by Soxhlet apparatus for 4 h with $CHCl_3$. The crude extracts were dried to have residues, which were solubilized in MeOH, with a final concentration of 0.5 mg/mL. A total of 50 μL of each solution was analyzed by C_{18} HPLC with detection at 258 nm and using a flow rate of 0.5 mL/min. The eluent was MeOH−MeCN (4:1) in isocratic mode.

Antioxidative Activity. The antioxidative activity of each isolated compound was measured by its inhibition of methyl linoleate autoxidation in the bulk phase (*7*). Methyl linoleate (294 mg, 1.0 mmol) containing the isolated compound (each 0.25 μmol; 0.025 mol %, on the basis of methyl linoleate) or α-tocopherol (0.025 mol %, on the basis of methyl linoleate) was placed in a 1.5 cm diameter test tube and incubated at 60 °C in the dark. Methyl linoleate monohydroperoxide was used as the standard peroxide. After 24 h of incubation, each sample (25 μL) was withdrawn and dissolved in 1.0 mL of ethanol. The peroxide value in each sample solution was determined by the iodometric method (*8*).

RESULTS AND DISCUSSION

The metabolites isolated from the Annurca apple fruits have been characterized as the *Z* and *E* isomers of the *p*-coumaryl esters of C18−C24 saturated and unsaturated fatty acids (**Figure 1**). The first isolated compound was identified as *Z-p*-coumaryl oleate (**1c**). The elemental analysis and the EI−MS spectrum, which showed the molecular ion at *m/z* 414, indicated a molecular formula of $C_{27}H_{42}O_3$.

The ^{1}H NMR showed, in the olefinic region, four protons of an AA′BB′ system at δ 7.12 and 6.82, a doublet at δ 6.58 (J = 12.3 Hz), a double triplet at δ 5.71 (J = 12.3 and 6.6 Hz), and two protons as a multiplet centered at δ 5.34. Also present were two protons as a double doublet at δ 4.83 (J = 6.6 and 0.9 Hz), two protons as a triplet at δ 2.35 (J = 7.2 Hz), four protons as a multiplet at δ 2.00, 22 protons at δ 1.29, and a methyl as a triplet at δ 0.88.

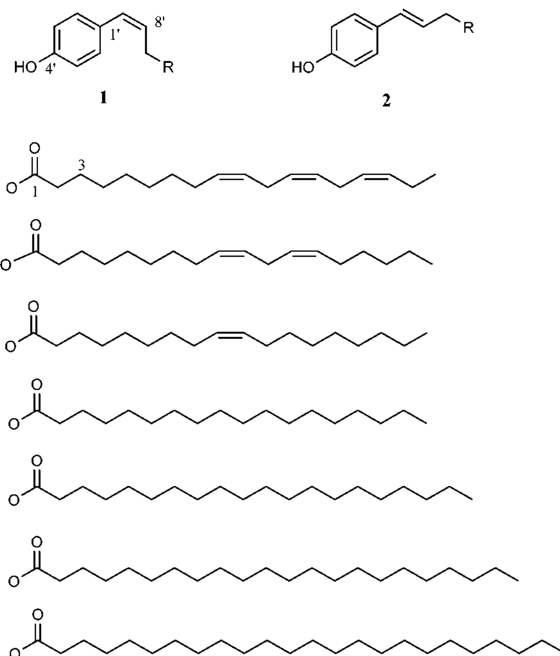

Figure 1. Structures of the *E*- and *Z-p*-coumaryl fatty acid esters isolated from cv. Annurca apple fruits.

The COSY experiment showed correlations between the doublet at δ 7.12 and 6.82 and between the signal at δ 6.58 and the triplet at δ 5.71, which was also correlated with the methylene at δ 4.83. These data suggested the presence of a *p*-coumaryl alcohol moiety, and the coupling constant value of the propenylic chain indicated a *Z* geometry for the double bond. The ^{13}C NMR confirmed the hypothesis, showing two protonated aromatic carbons at δ 115.2 and 130.3 and two tetrasubstituted carbons at δ 129.7 and 155.3, besides the olefinic methynes at δ 132.4 and 124.2 and a methylene carbon at δ 61.4. The HMBC experiment showed correlations between the aromatic protons at δ 7.12 and the carbons at δ 155.3, 129.7, and 132.4 and between the protons at δ 6.82 and the carbons at δ 155.3 and 129.7. Finally, the carbinol protons were correlated with both the olefinic carbons and the carbonyl carbon at δ 173.8, which showed cross-peak with the protons at δ 2.35.

These data suggested the structure of a *Z-p*-coumaryl ester of an unsaturated fatty acid. To identify the acidic moiety, 0.1 mg of the ester was hydrolyzed and methylated by a methanolic KOH and confirmed by GC with authentic samples. The GC analysis revealed the presence of methyl oleate, confirming the hypothesized structure.

Compounds **1a**−**1g** showed similar NMR data for the alcohol moiety, indicating the presence of the *Z* isomer of *p*-coumaryl alcohol. The differences were due to the acidic moieties, which were identified by GC analyses by comparison of the retention time (RT) of the samples with those of the pure standard. The chromatograms of the methyl derivatives of compounds **1a**, **1b**, **1d**, **1e**, **1f**, and **1g** showed peaks at RT 30.81 min (methyl linolenate), 29.14 min (methyl linoleate), 26.63 min (methyl stearate), 29.66 min (methyl arachidate), 32.32 min (methyl behenate), and 35.81 min (methyl lignocerate), respectively.

Compounds **2b**−**2f** were the isomers of compounds **1b**−**1f**. The most abundant, **2d**, was identified as *E-p*-coumaryl stearate. The EI−MS spectrum and the elemental analysis indicated a molecular formula $C_{27}H_{44}O_3$. The ^{1}H NMR showed differences because of the *E* geometry of the propenylic chain. The AA′BB′ system of the 1,4-disubstituted aromatic ring was shifted at δ 7.28 and 6.78 (J = 8.7 Hz), and the propenylic chain signals

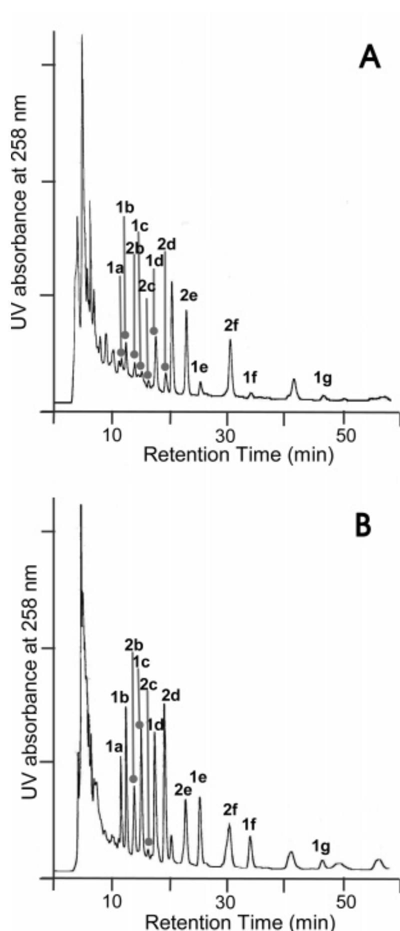

Figure 2. RP_{18}-HPLC analysis of the extracts of the unreddened (A) and reddened (B) whole cv. Annurca apples. Flow rate, 0.50 mL/min; concentration, 0.5 mg/mL; injection volume, 50 μL.

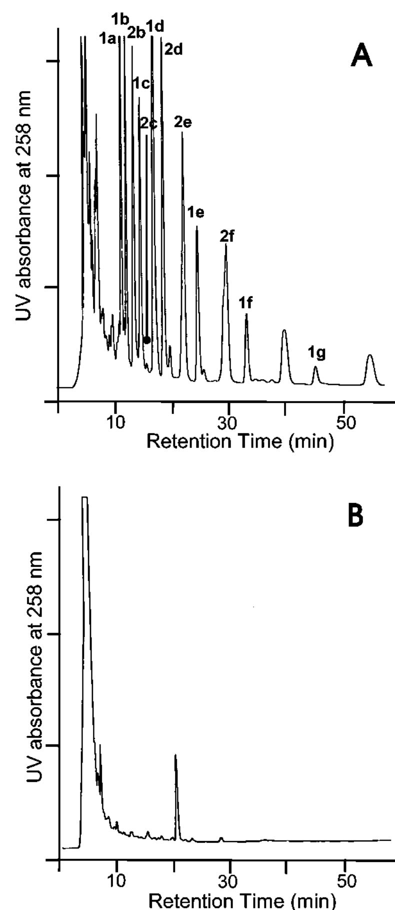

Figure 3. RP_{18}-HPLC analysis of the peel (A) and pulp (B) extract of the reddened fruits of cv. Annurca apples. Flow rate, 0.50 mL/min; concentration, 0.5 mg/mL; injection volume, 50 μL.

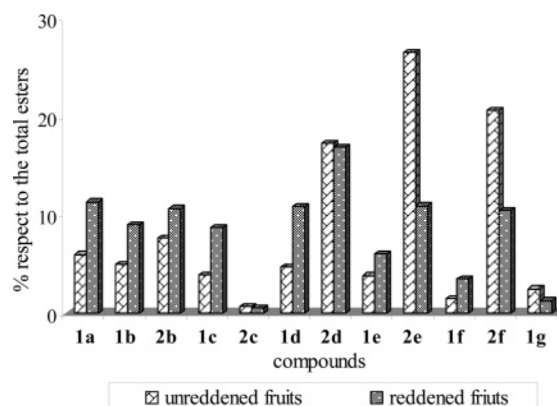

Figure 4. Composition (%) of *p*-coumaryl fatty acid esters of unreddened and reddened cv. Annurca apple fruits.

were present as a doublet at δ 6.58 (J = 15.9 Hz), a double triplet at δ 6.15 (J = 15.9 and 6.9 Hz) and a double doublet at δ 4.70 (J = 6.9 and 1.2 Hz). Two-dimensional experiments confirmed the presence of *E*-*p*-coumaryl alcohol in the molecule. These data were present in the spectra of compound **2b**−**2f**, indicating the same alcoholic moiety in the molecules. The differences were due to the fatty acids. In fact, GC analyses of compounds **2b**, **2c**, **2f**, and **2g** showed peaks with RT at 30.81 min (methyl linolenate), 27.65 min (methyl oleate), 26.63 min (methyl stearate), 29.66 min (methyl arachidate), and 32.31 min (methyl beheate), respectively. Compound **2f** was previously isolated from *Buddleja globosa* stembark (*9*).

Whitaker et al. (*10*) reported the presence of *E*- and *Z*-*p*-coumaryl fatty acid esters in the epicuticular wax from cv. Gala apple. The fatty acids present in the esters isolated from this cultivar were all saturated and had 16−26 carbons. These compounds are present at high concentration in few cultivars as cv. Gala and cv. Annurca apples but are absent in other cultivars.

The esters were isolated from cv. Annurca apple containing unsaturated C-18 acyl groups and saturated fatty acids composed of 18−24 carbons. To localize the esters in the cv. Annurca apple and to understand their variations during the maturation period, 3.0 g each of lyophilized unreddened and reddened whole apples, unreddened and reddened peel, and unreddened and reddened pulp, in triplicate, were exhaustively extracted in a Soxhlet apparatus with $CHCl_3$. The crude extracts were analyzed by HPLC with detection at 258 nm. All of the compounds were identified by comparing of the retention times with those of the pure compounds. The results are reported in **Figures 2** and **3**.

Figure 2 shows the comparison between the unreddened and reddened whole fruits. The content of the *p*-coumaryl fatty acid esters in the reddened apples were greater than those in the green ones. The increase in *p*-coumaryl fatty acid esters of over 300% during reddening has been estimated by integrating all of the ester peaks in the HPLC−UV chromatograms. **Figure 3B** showed the absence of these compounds in the pulp of the apples, while localization in the peel of the fruits was confirmed as seen in **Figure 3A**.

Figure 4 reports the percentage of each compound with respect to the whole *p*-coumaryl fatty acid esters in the

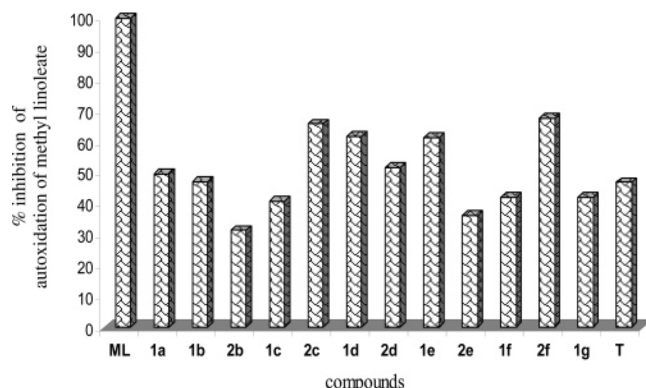

Figure 5. Inhibition of the autoxidation of the methyl linoleate in the presence of the *p*-coumaroyl acids isolated from cv. Annurca apples. ML = methyl linoleate; T = α-tocopherol.

unreddened and reddened fruits. After the reddened treatment, the unsaturated fatty acid esters increased, changing from 23.2% in the unreddened fruits to 40.2% in the mature fruits. Also, the compounds containing the *Z* isomer of the *p*-coumaryl alcohol increased after the maturation procedure, changing from 27.3 to 50.6%. In the literature, *E* isomers of the *p*-coumaryl esters were reported as predominant in the plants. Previous studies reported that isomerization to *E*-*p*-coumaryl alcohol occurs enzymatically as a late step in the pathway (*11*, *12*). The most abundant compounds in the unreddened fruits were *E*-*p*-coumaryl arachidate (27%) and *E*-*p*-coumaryl behenate (21%), while in the reddened fruits, the unsaturated esters and the compounds containing the *Z*-*p*-coumaryl alcohol increased.

The presence of a hydroxyl group in the isolated molecules suggested their possibility to act as antioxidant agents. Therefore, all of the compounds have been tested for their antioxidant activity evaluating their capacity to inhibit the production of peroxides in the methyl linoleate. This test is carried out by oxidizing the methyl linoleate in the presence of the phenolic fatty acid esters and α-tocopherol, generally used as a positive standard in the antioxidant tests. The results are reported in **Figure 5**. All of the compounds showed an inhibition of the peroxides similar to that of α-tocopherol. In particular, compounds **2b** and **2e** showed an antioxidant activity lower that the standard, inhibiting the autoxidation of methyl linoleate between 30 and 40%.

In this study, novel unsaturated phenolic fatty acid esters have been isolated and characterized from cv. Annurca apple fruits. HPLC analysis showed that these compounds were localized in the peel of the fruits, as wax components. During the reddening treatment, we observed an increase of the unsaturated compounds indicating enzymatic involvement, as desaturases, in the maturation phases. The enhancement of the percentage of compound containing the more unstable *E* isomer of the *p*-coumaryl alcohol is not explained as the result of a photochemical process but suggests an enzymatic involvement. C16−C26 saturated fatty acid esters of *p*-coumaryl alchohol have already been identified as a mixture from the epicuticular coating of cv. Gala apple fruits (*10*). These compounds have been previously reported as trace components in plant tissues and utilized in lignin biosynthesis (*11*). The presence of phenolic esters in the peel of apple fruits seems to act as phytoalexins or feeding repellents (*13*). The compounds showed a good antioxidant activity, probably because of the phenolic moiety in the molecules. The antioxidant activities of *E*-*p*-coumaryl palmitate and the *E*-*p*-coumaryl oleate were recently found to be as high as those of commercial antioxidative compounds (*14*).

LITERATURE CITED

(1) Floris, R. *Melo e Pero nella Frutticoltura Italiana ed Europea*. Federchimica Agrofarma: Milano, Italy, 1997; p 17.

(2) Ferrari, M.; Medici D. *Alberi e Arbusti in Italia.* Manuale di Riconoscimento; agricole, 1996; pp 500−502.

(3) Lo Scalzo, R.; Testoni, A.; Genna, A. "Annurca" apple fruit, a southern Italy apple cultivar: Textural properties and aroma composition. *Food Chem.* **2001**, *49*, 3787−3792.

(4) Lo Scalzo, R.; Testoni, A. Caratteristiche qualitative e tipicità del profilo aromatico di mela Annurca. VI Convegno Nazionale sulla Biodiversità "Opportunità di Sviluppo Sostenibile"; Settore Arboreo e Forestale [SA-PO-02]; 2004.

(5) Lintas, C.; Paoletti, F.; Cappelloni, M.; Gabelli, L.; Monastra, F.; Ponziani, G. Agromonic, nutritional, and texture evaluation of "Annurca" apple clones. *Adv. Hortic. Sci.* **1993**, *7*, 165−168.

(6) Whitaker, B. D. Phenolic fatty-acid esters from the peel of "Gala" apples and their possibile role in resistance to superficial scald. *Postharvest Biol. Technol.* **1998**, *13*, 1−10.

(7) Yamauchi, R.; Kato, K.; Oida, S.; Kanaeda, J.; Ueno, Y. Benzyl caffeate, an antioxidative compound isolated from propolis. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 1321−1322.

(8) Yamauchi, R.; Goto, Y.; Kato, K.; Ueno, Y. Prooxidant effect of dihydroxyacetone and reducing sugars on the autoxidation of methyl linoleate in emulsions. *Agric. Biol. Chem.* **1984**, *48*, 843−848.

(9) Houghton, P. J. Phenolic fatty acid esters from *Buddleja globosa* stembark. *Phytochemistry* **1989**, *28*, 2693−2695.

(10) Whitaker, B. D.; Schmidt, W. F.; Kirk, M. C.; Barnes. S. Novel fatty acid esters of *p*-coumaryl alcohol in epiculticular wax of apple fruit. *J. Agric. Food Chem.* **2001**, *49*, 3787−3792.

(11) Yamamoto, E.; Bokelman, G. H.; Lewis, N. G. Phenylpropanoid metabolism in cell walls: an overview. In *Plant Cell Wall Polymers: Biogenesis and Biodegradation*; Lewis, N. G., Paice, M. G., Eds.; American Chemical Society: Washington, DC, 1989; pp 68−88.

(12) Davin, L. B.; Lewis, N. G. Phenylpropanoid metabolism: Biosynthesis of monolignols, lignans and neolignans, lignins and suberins. In *Phenolic Metabolism in Plants*; Stafford, H. A., Ibrahim, R. K., Eds.; Plenum Press: New York, 1992; pp 325−375.

(13) Jakubas, W. J.; Shah, P. S.; Mason, J. R.; Norman, D. A. Avian repellency of conyferyl and cynnamyl derivatives. *Ecol. Appl.* **1992**, *2*, 147−156.

(14) Lee, J. Y.; Yoon, J. W.; Kim, C. T.; Lim, S. T. Antioxidant activity of phenylpropanoid esyters isolated and identified from *Platycodon grandiflorum* A. DC. *Phytochemistry* **2004**, *65*, 3033−3039.

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