trifluoroacetic acid (A) and acetonitrile (B) applied with the following gradient program: isocratic 10% B for 5 min, from 10% to 15% for 15 min, isocratic 15% B for 5 min, from 15% to 18% B for 5 min and from 18% to 35% B for 20 min at a flow rate of 0.5 ml/min. The measurement was carried out using the photodiode detection at 520 nm as the preferred wavelength. The purities of all the anthocaynins and pyranoanthocyanins used in this work, were in every case higher than 94%.

The identity of the anthocyanins and pyranoanthocyanins was confirmed by HPLC using a dual on-line detection by diode array detector and mass spectrometry (HPLC-DAD-MS). MS spectrometry was performed using a Finnigan LCQ equipped with API source, using an electrospray ionization (ESI) interface. Both the auxiliary and the sheath gas were a mixture of nitrogen and helium at flow rates of 1.2, and 6 L/min respectively. The capillary temperature was 195 °C and the capillary voltage was 4 V. The MS detector was programmed to perform a series of three consecutive scans: a full scan from 120 to 1500 amu, an MS^2 scan of the most abundant ion in the full mass and MS^3 of the most abundant ion in the MS^2. The normalized energy of collision was 45%. Spectra were recorded in the positive mode.

3.5 Inhibition of tyrosine nitration

Peroxynitrite solution was prepared in accordance with the published method [28]. A volume of 8 µl of 2.5 mM peroxynitrite solution in 0.05 M NaOH was drawn and mixed rapidly in the injector of the HPLC autosampler with 42 µl 1.0 mM tyrosine solution in 0.11 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.0) containing 40 µM of tested compound. The reaction mixture was injected directly into the HPLC column (Aqua® C18, 5 µm, 150 × 4.6 mm, Phenomenex), the mobile phase, in isocratic conditions, consisted of 90% 40 mM HCOOH and 10% CH_3CN (v/v), at a flow rate of 1 ml/min. The chromatograms were recorded at 276 nm. The activity of the tested compounds was calculated as the percentage of tyrosine nitration relative to the measured peak area of 3-nitrotyrosine of the control.

3.6 Ferric reducing/antioxidant power (FRAP)

The FRAP assay was performed as described previously [16]. FRAP reagent was freshly prepared each day by mixing 10 mM TPTZ, 20 mM $FeCl_3$ and 0.3 M acetate buffer pH 3.6 in the ratio 1:1:10 (v/v). The absorbance of the test components was read at 593 nm (Perkin Elmer UV/VIS Lambda Bio 20) 6 minutes after mixing at room temperature against a blank (FRAP and distilled water). For anthocyanins and pyranoanthocyanins an extra blank was needed. It was prepared by addition of a tested compound in acetate buffer (1:30, v/v). Data were expressed relative to values obtained for Trolox (200 µM) and expressed as Trolox equivalents.

3.7 Trolox equivalent antioxidant capacity (TEAC)

The assay was based on the relative ability of antioxidants to scavenge the cation radical $ABTS^{\bullet+}$ [14]. The radical was generated by the interaction of ABTS (0.15 mM) with the ferrylmyoglobin radical, generated by the activation of metmyoglobin (2.5 µM) with H_2O_2 (0.1 mM). The extent of quenching of the $ABTS^{\bullet+}$ was measured spectrophotometrically at 734 nm and compared to standard amounts of Trolox C. Results are expressed as TEAC value.

3.8 Lipid phase antioxidant activity (TBARS method)

Phospholipid liposomes (final concentration 1 mg/mL) were suspended in 150 mM KCl containing 0.2 mM $FeCl_3$ and the tested compound at a range of concentrations (0–25 μM). Peroxidation was started as described previously [29] with ascorbate addition (final concentration 0.05 mM) in a final volume adjusted to 0.4 mL. Samples were incubated at 37 °C for 40 min and then the reactions were terminated by the addition of 0.8 ml of 20% (w/v) trichloroacetic acid (TCA)/0.4% (w/v) thiobarbituric acid (TBA)/0.25 N HCl and 0.01 ml of butylated hydroxytoluene dissolved in ethanol. The production of thiobarbituric acid reactive substances (TBARS) was measured spectrophotometrically at 535 nm during the 20 min of incubation at 80 °C and expressed as concentration causing 50% inhibition (IC_{50}).

3.9 Statistical analysis

Data are presented as means ± standard deviations (STDEV) of the three independent experiments. The results were processed by using one-way variance analysis (ANOVA). Differences at $p < 0.05$ were considered significant. In addition, simple regression analysis was performed to seek relationships between the different tests.

Acknowledgements

This work was supported by grant AGL-2005-07245-C03-01 from MEC (Ministerio de Educación y Ciencia, Spain), FEDER (EU), CICYT(AGL2002-00167), Ph.D. studentship to MGA (MEC) and by IGA MZ 1A/8666.

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