

Time-Resolved Spectral Studies of Blue–Green Fluorescence of Artichoke (*Cynara cardunculus* L. Var. *Scolymus*) Leaves: Identification of Chlorogenic Acid as One of the Major Fluorophores and Age-Mediated Changes

FERMÍN MORALES,^{*,†} AURÉLIE CARTELAT,[‡] ANA ÁLVAREZ-FERNÁNDEZ,[†]
ISMAEL MOYA,[‡] AND ZORAN G. CEROVIC[‡]

Department of Plant Nutrition, Aula Dei Experimental Station, Spanish Council for Scientific Research (CSIC), Apdo. 202, E-50080 Zaragoza, Spain, and Groupe Photosynthèse et Télédétection, LURE/CNRS, Bât 203, Centre Universitaire Paris-Sud, B.P. 34, 91898 Orsay Cedex, France

Synchrotron radiation and the time-correlated single-photon counting technique were used to investigate the spectral and time-resolved characteristics of blue–green fluorescence (BGF) of artichoke leaves. Leaves emitted BGF under ultraviolet (UV) excitation; the abaxial side was much more fluorescent than the adaxial side, and in both cases, the youngest leaves were much more fluorescent than the oldest ones. The BGF of artichoke leaves was dominated by the presence of hydroxycinnamic acids. A decrease in the percentage of BGF attributable to the very short kinetic component (from 42 to 20%), in the shape of the BGF excitation spectra, and chlorogenic acid concentrations indicate that there is a loss of hydroxycinnamic acid with leaf age. Studies on excitation, emission, and synchronized fluorescence spectra of leaves and trichomes and chlorogenic acid contents indicate that chlorogenic acid is one of the main blue–green fluorophores in artichoke leaves. Results of the present study indicate that 20–42% (i.e., the very short kinetic component) of the overall BGF is emitted by chlorogenic acid. Time-resolved BGF measurements could be a means to extract information on chlorogenic acid fluorescence from the overall leaf BGF.

KEYWORDS: Artichoke; blue–green fluorescence; chlorogenic acid; *Cynara cardunculus* var. *Scolymus*; decay-associated spectra; synchronous fluorescence spectrometry; time-resolved fluorescence

INTRODUCTION

Several spectroscopic methods have been considered for the biophysical characterization of leaves. Among them, the use of a fluorescence signal emitted by plants is currently the object of intense research activity. Chlorophyll fluorescence (ChlF) is usually excited in the visible part of the spectrum. When a leaf is illuminated with ultraviolet (UV) light, it emits not only ChlF but also blue–green fluorescence (BGF), sometimes of higher intensity than the ChlF. The leaf BGF was rediscovered in the 80's (1–5) and has been far less studied than ChlF. The BGF emission spectrum depends on plant species (2, 3, 6), stage of development (1, 2, 7, 8), and anatomy (9, 10). For instance, BGF from the adaxial side of bifacial leaves differs markedly from the abaxial side (9, 10). Also, the BGF emission depends on environmental factors experienced by the plant (1, 11–15).

The leaf BGF has a heterogeneous origin; several fluorophores can fluoresce in this part of the spectrum, unlike ChlF. The blue–green fluorophores are distributed in different

compartments of the leaf and the plant cell (16), complicating further the quest for the origins and causes of BGF variability. The substances identified so far responsible for the leaf BGF can be divided into two main classes, namely, phenolic compounds located mainly in the cell walls and vacuoles of the epidermis that are related to secondary plant metabolism and UV protection (5, 9, 17–19) and pyridine [NAD(P)H] and flavine nucleotides (16, 20, 21), the cofactors of metabolism, which are directly related to the redox state of the plant cell. The phenolic composition of leaves, responsible for UV absorption and BGF emission, is species-dependent; these compounds are usually used as markers for plant chemotaxonomy (22). Therefore, case-by-case experiments have to be carried out.

One way of approaching the problem is combining spectrofluorimetric measurements with lifetime measurements in the subnanosecond time domain, to differentiate more precisely the BGF components (16, 17, 23). They used synchrotron radiation and the time-correlated single-photon counting technique to investigate the spectral and time-resolved characteristics of the BGF emitted from spinach and sugar beet leaves and reported four kinetic components. They also studied excitation and

* To whom correspondence should be addressed. Tel: 34-976-716059. Fax: 34-976-716145. E-mail: fmorales@eead.csic.es.

[†] Spanish Council for Scientific Research (CSIC).

[‡] Centre Universitaire Paris-Sud.

emission spectra of these four kinetic components. Goulas et al. (23) stated that two hydroxycinnamic acid derivatives (ferulic acid and *p*-coumaric acid) and one flavonoid (kaempferol) may contribute to the BGF of spinach leaves. In the case of sugar beet leaves, the BGF is dominated by the emission of ferulic acid (17).

An alternative spectral analytical approach is the use of synchronous fluorescence spectrometry (SFS). SFS was used first by Lloyd (24) to identify different polycyclic aromatic hydrocarbons. This method involves simultaneous scanning of the excitation and emission monochromators, which are synchronized in such a fashion that a constant wavelength ($\Delta\lambda$) is maintained between the excitation and the emission. This technique has been used for the analysis of mixtures of crude oils, pharmaceuticals, and polycyclic aromatic hydrocarbons (24, 25 and references therein).

The aim of this work was two-fold: to identify, by means of excitation and emission fluorescence spectra and time-resolved fluorescence measurements, major compounds responsible for the artichoke leaf BGF and to investigate its age dependence. The spectral and lifetime characteristics of the BGF of these plants have not yet been investigated. Still, they should be determined by the presence of plant phenolics. Decay-associated spectra (DAS) of BGF and synchronized fluorescence spectra were recorded and analyzed. Evidence is presented for chlorogenic acid being responsible for a large part of the artichoke leaf BGF. A way to access to chlorogenic acid fluorescence in artichoke leaves is proposed.

MATERIALS AND METHODS

Plant Material. Artichoke [*Cynara cardunculus* L., var. *Scolymus* (L.) Fiori] plants were purchased as seedlings (approximately 10 cm in size) from a local nursery. Plants were transferred to 1 L pots and grown in a mixture of peat and sand (1:1, by volume) in a shaded greenhouse in the Aula Dei Campus (Zaragoza, Spain). Some plants were transported to LURE (Orsay, France) and maintained in a growth chamber that simulated the diurnal changes in light intensity, temperature, and relative humidity (26). Measurements were carried out when plants were 4–5 months old. Leaves within a plant were numbered from 1 to 7 from the youngest (not fully developed) to the oldest, respectively. Leaves 1–7 have not only a different age, but they are also present on different nodes on the stem, which might have an incidence on their anatomy and physiology. However, age is the major variable among these leaves, and we will therefore ascribe changes mainly to age.

Leaf Morphologic Measurements. Dry mass (DM) and leaf mass per area (LMA) (DM per unit leaf area) were recorded. Leaf disks from young and old leaves were cut with a calibrated cork borer (0.1 cm² per disk), placed in an oven at 60 °C until constant weights, and weighted for DM measurements. Leaf thickness was determined in each leaf with a portable analogic thickness meter (Ascaso, Huesca, Spain). The area of the leaves was estimated by weighing photocopies of each leaf, calibrated with photocopies of a known surface.

Methanolic Extracts of Trichomes and Leaf Disks. Trichomes from the adaxial sides of young artichoke leaves (0.242 cm²) were gently removed from the rest of the leaf tissue with a scalpel and were dissolved in 1 mL of methanol at a controlled room temperature (20 °C).

Phenolic compounds were extracted in warm (at 70 °C) from 0.2 cm² leaf disks with methanol for 30 min. The extract solution was brought to a volume of 10 mL with methanol and then frozen at –80 °C until analysis.

Chemicals. Chlorogenic acid (purity \geq 97%) and caffeic acid (purity \geq 99%) were purchased from Fluka Chemie GmbH (Buchs, Switzerland), and cynarin (1,3-dicaffeoylquinic acid) (purity 98%) was obtained from ChromaDex (Santa Ana, CA), and they were used as standards for high-performance liquid chromatography (HPLC) analyses. HPLC

grade methanol and Uvasol for spectroscopy were purchased from Panreac (Barcelona, Spain) and Merck (Darmstadt, Germany), respectively. All other chemicals used were of proanalysis grade and were obtained from Sigma (St. Quentin Fallavier, France).

Absorption Spectroscopy. Absorption spectra were recorded between 220 and 400 nm with a photodiode array UV–vis spectrophotometer (HP8453, Hewlett-Packard, Les Ulis, France) after cooling the methanolic extracts to room temperature (20 °C). Methanolic extracts were either measured immediately after extraction or stored at –80 °C until analyzed. For comparative purposes, chlorogenic acid (10 μ M in methanol) was also analyzed under similar conditions. All measurements were made in a quartz cuvette of 1 cm optical path (QS, Hellma).

Excitation and Emission Fluorescence Spectra. Spectra were recorded in darkness on the SA4 line of the SUPERACO synchrotron in Orsay (France) as described previously (16, 17, 21) with some modifications. For the excitation part of the apparatus, a double-grating monochromator (6 nm band-pass, H10 D UV, Jobin-Yvon, Longjumeau, France) was used, instead of a single-grating monochromator. For measurements of excitation spectra, a KV418 (Schott) anti-UV filter was placed before the emission monochromator. For measurements of emission spectra, a KV389 (Schott) anti-UV filter was placed before the emission monochromator. Excitation spectra from 220 to 400 nm were recorded by scanning the excitation monochromator and integrating the number of counts during 1.1 s at each new wavelength spaced by 2 nm. Emission spectra from 400 to 600 nm were recorded in the same way by scanning the emission monochromator. For most cases, two forward and two backward scans were averaged. For synchronized fluorescence spectra, both the excitation and the emission monochromators were “scrolling” simultaneously from 220 to 485 nm, with a $\Delta\lambda$ of 110 nm. Emission wavelengths at the beginning and the end of the synchronized fluorescence spectra were, therefore, 330 and 595 nm, respectively. Excitation spectra were corrected on-line for instrumental response using rhodamine B as a quantum meter (27); a part of the excitation beam was deviated toward a cell of rhodamine B (3 g L⁻¹), whose fluorescence was continuously recorded. The lower limit of 220 nm for excitation spectra was imposed by the absorptivity of rhodamine B. Emission spectra were corrected for instrumental response using quinine sulfate as a standard (27) or using an optical radiation calibrator (LI-COR 1800-02, Lincoln, NE). All measurements were made at controlled room temperature (20 °C). For comparative purposes, chlorogenic acid (15 μ M methanol) was also analyzed.

Fluorescence was expressed in quinine sulfate equivalent units (QSEU); 1000 QSEU correspond to the fluorescence of 1 μ M quinine sulfate dehydrate in 1 cm layer 0.105 mol L⁻¹ perchloric acid, excited at 347.5 nm and emitted at 450 nm, under identical measuring conditions. Quinine sulfate is a fluorescence standard of known emission spectrum that is used and distributed by the United States National Institute of Standard and Technology (NIST).

Time-Resolved BGF Measurements. Time-correlated single-photon counting measurements were performed in darkness on the same apparatus, as described previously (16, 17, 21) with some modifications. Decay histograms were acquired until 1200000 counts were accumulated. Thirty-nine nanoseconds was covered by 2.048 channels (0.019 ns per channel). Iterative convolution of BGF decays was performed as previously described (16) using a proprietary program based on the Marquardt search algorithm for nonlinear parameters (28). The correctness of the convolution was judged from the examination of the χ^2 value, distribution of the weighted residuals between the calculated and the experimental function, and the autocorrelation function of the residue (29). Decays were analyzed by both an individual and a global computation approach.

Decay-associated emission spectra were obtained (16) by varying the emission wavelength at which the fluorescence decays were recorded with the excitation wavelength fixed at excitation maximum. Decays were analyzed individually, and then, a global fit was performed to all decays by fitting the best common lifetimes for a four-component model, in a spectrum simultaneously. Then, mean lifetimes for each wavelength were calculated. Finally, the absolute contribution to the emission of the individual component was calculated by multiplying the fractional intensity by the emission spectra at the corresponding wavelength recorded separately. Decay-associated excitation spectra

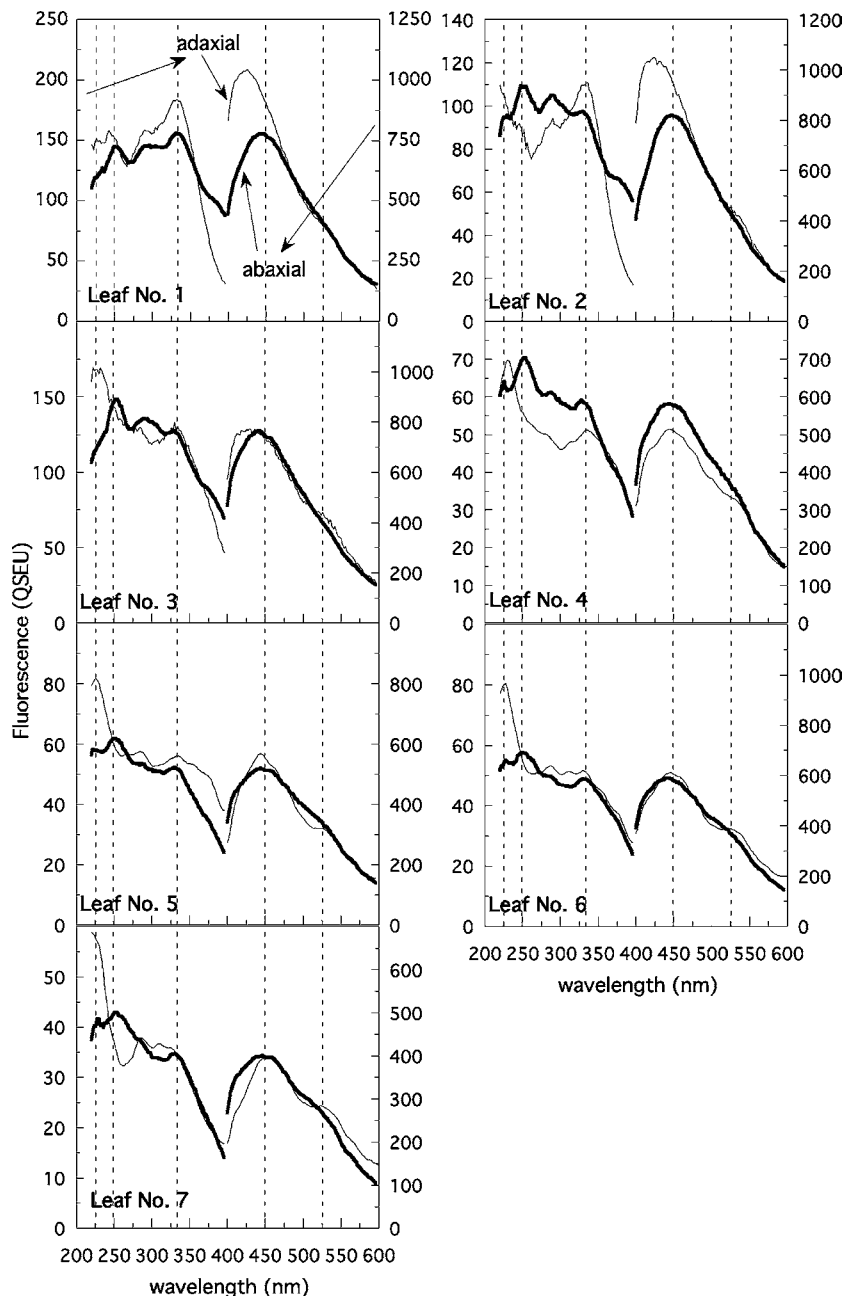


Figure 1. Excitation (from 220 to 400 nm) and emission (from 400 to 600 nm) fluorescence spectra of abaxial (bold lines, right Y-axis) and adaxial (fine lines, left Y-axis) artichoke leaves. Leaves were numbered from 1 to 7, 1 being the youngest leaf and 7 the oldest one.

were recorded with the emission wavelength fixed at the emission maximum and calculated following the same procedure as for the decay-associated emission spectra.

HPLC. A modification of the Adzet and Puigmacia (30) HPLC method was used to determine chlorogenic acid, caffeic acid, and cynarin in artichoke leaf extracts. Prior to analysis, the sample extracts were thawed and filtered through a $0.45\ \mu\text{m}$ PVDF filter. HPLC was performed with a Waters Alliance 2795 HPLC system (Waters Corp., Mildford MA) equipped with on-line degasser, autosampler module ($4\ ^\circ\text{C}$), and column oven ($40\ ^\circ\text{C}$). A Waters 2996 UV-visible diode array detector was used for analyte detection. The column used was an analytical HPLC column Waters Symmetry C18 ($25\ \text{cm} \times 4.6\ \text{mm}$ i.d., $\text{dp} = 5\ \mu\text{m}$) with a 2 cm guard column of the same material. The mobile phase consisted of a gradient system of solution A, MilliQ water-acetic acid (97.5:2.5; v/v), and solution B, HPLC gradient grade methanol-acetic acid (97.5:2.5; v/v), at a flow rate of $1.0\ \text{mL}\ \text{min}^{-1}$. The gradient program was as follows: linear gradient from solution A-B (70:30) to solution A-B (51:49) in 15 min, isocratic at solution A-B (51:49) for 10 min, followed by a linear gradient to solution A-B

(70:30) in 5 min, and finally isocratic at solution A-B (70:30) for 5 min. The injection volume was $15\ \mu\text{L}$. Automated instrumentation was realized with an HPLC data system (HyStar, Bruker Daltonik GmbH, Bremen, Germany). The data were analyzed by a data processing software package (HyStar PostProcessing, Bruker Daltonik GmbH). Peaks were identified by comparison of their retention times and UV absorption spectra with those of phenolic standards in methanol. Quantification was based on peak area measurements at 325 nm. Standard solutions were prepared with chlorogenic acid, caffeic acid, and cynarin. Each phenolic compound was examined by HPLC in order to determine its retention time (4.1, 5.4, and 8.3 min for chlorogenic acid, caffeic acid, and cynarin, respectively). α -Naphthol ($\geq 99\%$ GC; Riedel-de Haën, Seelze, Germany) was used as an internal standard (retention time, t_{R} , 23.0 min). A multipoint calibration was performed in the range of 1.0–80, 0.1–8.0, and 0.8–8.5 μM for chlorogenic acid, caffeic acid, and cynarin, respectively. Linear relationships were as follows: $Y = 8.70X + 15.49$ ($R^2 = 0.9955$) for chlorogenic acid, $Y = 17.37X - 0.49$ ($R^2 = 0.9998$) for caffeic acid, and $Y = 47.41X - 13.00$ ($R^2 = 0.9993$) for cynarin.

We performed HPLC measurements of leaf extracts from different young and old leaves at different times along the year. Data indicate that the composition and concentration of the artichoke leaf phenolic compounds change during the year (not shown). Therefore, we chose those analyses performed at the time of the year in which BGF measurements were made. Thus, HPLC data presented in this work correspond to leaves 1, 4, and 5 ($n = 6, 3,$ and $3,$ respectively), from plants that are all 4–5 months old.

RESULTS

Excitation and Emission Fluorescence Spectra of Artichoke Leaves and Leaf Age. The abaxial sides of artichoke leaves emitted severalfold more BGF than the adaxial sides, and for a given side, the youngest leaves emitted severalfold more BGF than the oldest ones (Figure 1). The leaf excitation spectra showed three peaks, at approximately 230 and 254 nm (UV-C; adaxial and abaxial sides, respectively), 292 (UV-B; in both sides), and 332 nm (UV-A; in both sides) (Figure 1). The relative importance of each peak changed with the leaf age, and thus, in youngest leaves, UV-A excitation was preponderant, as opposed to the oldest leaves that had preponderant UV-C excitation (Figure 1). It should be noted here that the BGF excitation spectra of the adaxial side of leaves 1 and 2 (Figure 1) resembled much to the UV absorption spectra of pure hydroxycinnamic acids in solution (26; see also the UV absorption or the BGF excitation spectra of chlorogenic acid in methanol in Figure 6). In the case of the abaxial sides of the same leaves, a UV-C excitation was added to the hydroxycinnamic acid type UV excitation (Figure 1). The emission maxima changed with the leaf age, and the maxima were at approximately 424 and 460 nm in the adaxial side of youngest and oldest leaves, respectively, whereas in the abaxial side they remained fairly constant (maxima were at approximately 446 ± 4 nm) (Figure 1). Oldest leaves had a shoulder, or secondary maxima, in the green part of the spectrum, at approximately 525 nm; it was more pronounced in the adaxial than in the abaxial side (Figure 1). The appearance of these secondary maxima should not be related to the appearance of a maximum of excitation in the UV-C, since the Stoke's shift is too large. All of these changes decreased the blue (fluorescence at 450 nm) to green (fluorescence at 530 nm) fluorescence ratio with the leaf age from 2.27 to 1.40 in the adaxial side and from 1.94 to 1.53 in the abaxial side.

Time-Resolved BGF of Artichoke Leaves and Leaf Age.

Four kinetic components were resolved on both leaf sides by time-correlated single-photon counting measurements. Fluorescence lifetimes were found to be 0.18, 0.80, 2.49, and 7.64 ns (Figure 2A) and 0.13, 0.80, 2.84, and 8.76 ns (Figure 2B) on the abaxial and adaxial sides, respectively. The relative contribution of each component to the overall BGF changed with the leaf age. On the abaxial side, the contribution of the slowest component increased with the leaf age decreasing slightly the contribution of the two shortest ones (Figure 2A). On adaxial sides of the youngest leaves, the two shortest components contributed to more than 60% of the overall BGF, decreasing their relative contribution with the leaf age and increasing the relative contribution of the two slowest components (Figure 2B). As a consequence of these changes, the mean fluorescence lifetime increased on both leaf sides with the leaf age from 1.6 to 2.3 ns in the abaxial side and from 1.6 to 2.9 ns in the adaxial side (Figure 2).

DAS and Leaf Age. As has already been pointed out, four kinetic components were resolved. At the excitation and emission maxima, the very short kinetic component contributes 25–27 (abaxial, young; Figures 3 and 5), 35–42 (adaxial,

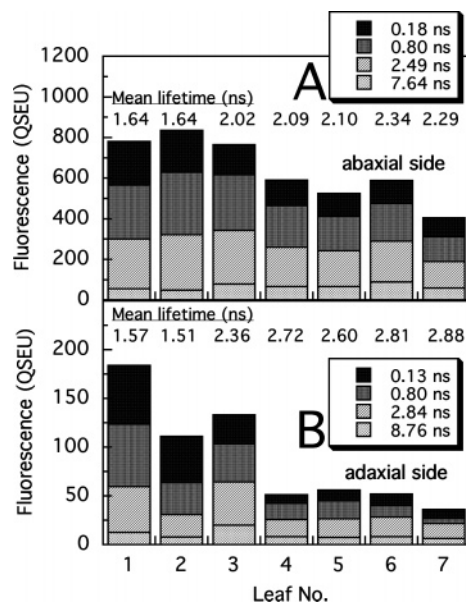


Figure 2. Distribution of the BGF between the four kinetic components resolved by time-correlated single-photon counting measurements. The height of the columns represents the overall BGF of the leaf, and the BGF corresponding to each kinetic component is represented by different shadings. The mean fluorescence lifetimes are also included. Numbers of leaves are as in Figure 1. (A) Abaxial side; (B) adaxial side.

young; Figure 3), 20–22 (abaxial, old; Figure 4), and 23–26% (adaxial, old; Figure 4) to the overall BGF of the leaves. The medium, slow, and very slow kinetic components contribute, at the excitation and emission maxima, with 73–75 (abaxial, young; Figures 3 and 5), 85–65 (adaxial, young; Figure 3), 78–80 (abaxial, old; Figure 4), and 74–77% (adaxial, old; Figure 4) to the overall BGF of the leaves. They have two excitation maxima or shoulders in the UV-C (260 nm) and UV-A (340 nm). Their emission maxima are progressively shifted to longer wavelengths. From Figures 3–5, it can be seen that in artichoke leaves the mean lifetimes depend on the emission or excitation wavelengths. It indicates that the BGF of artichoke leaves is the result of a superposition of several emissions. The very short kinetic component has excitation and emission spectra shapes that resemble those of caffeic acid type hydroxycinnamic acid derivatives (see Figure 6), although excitation and emission maxima were shifted to longer and shorter wavelengths, respectively, when compared to pure acids in methanol. This would mean that the Stoke's shift has been decreased in approximately 30 nm when in vivo measurements are compared to the in vitro ones. This type of change has been previously described for hydroxycinnamic acids and has been ascribed to changes in the microenvironment of the fluorophore [a decrease in the dipole moment of the solvent (18, 31) or an increase in the pH within physiological ranges (31) reduces the hydroxycinnamic acids Stoke's shift]. We can therefore suggest that the very short kinetic component may contain fluorescence of one or several of these hydroxycinnamic acid derivatives. The origin of the other three kinetic components is not clear at present and deserves further investigation.

Analysis of Phenolic Compounds in Leaves and Leaf Age.

In a search for compounds responsible for the leaf BGF, we analyzed phenolic compounds by HPLC. HPLC data from leaf methanolic extracts revealed the presence of cynarin and chlorogenic acid and in both young and old leaves (Table 1). Caffeic acid was not detected (detection limit, $0.45 \mu\text{g cm}^{-2}$). Concentrations of components have been expressed on area,

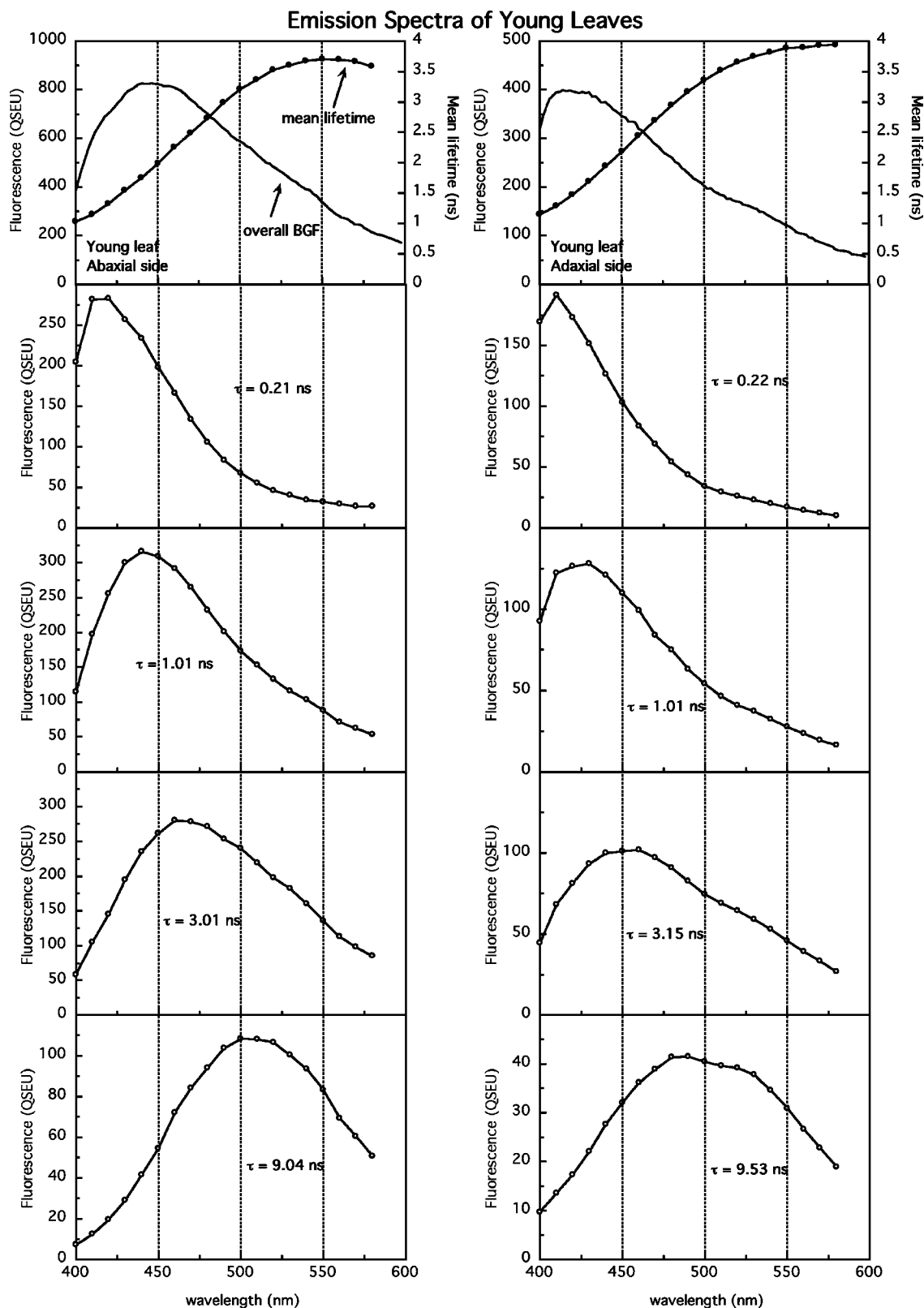


Figure 3. Decay-associated emission spectra from 400 to 600 nm of young artichoke leaves from the abaxial (left panels) and the adaxial (right panels) sides. The top panels report the mean lifetime (solid circles) and the overall BGF spectra (solid line), and the following panels from the top to the bottom show the various components at increasing lifetimes.

DM, volume, and per leaf bases. Chlorogenic acid concentrations were higher in young than in old leaves, irrespective of the basis used to express the data. Cynarin, however, showed slight differences depending on the basis that data were expressed. The concentration of phenolic compounds per leaf

was higher in old leaves than in young leaves and may be due to the fact that old leaves are 2.6-fold larger than young leaves (Table 1).

Absorption and Fluorescence Spectroscopy of Methanolic Extracts of Trichomes and Chlorogenic Acid. The UV

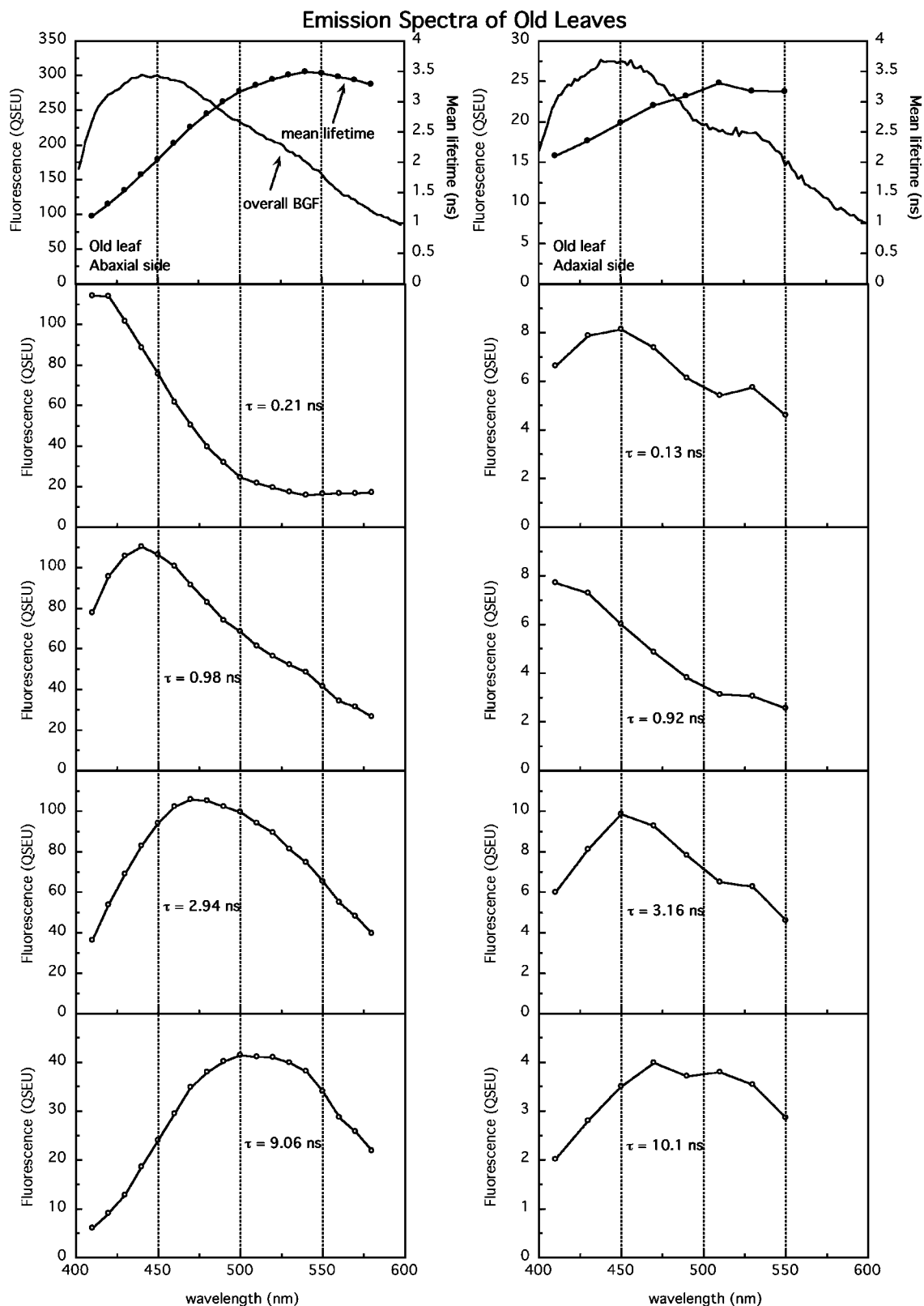


Figure 4. Decay-associated emission spectra from 400 to 600 nm of old artichoke leaves from the abaxial (left panels) and the adaxial (right panels) sides. The top panels report the mean lifetime (solid circles) and the overall BGF spectra (solid line), and the following panels from the top to the bottom show the various components at increasing lifetimes.

absorption spectrum of methanolic extracts of trichomes from the adaxial side of artichoke leaves resembled that of chlorogenic acid in methanol, with maxima at approximately 330 nm and a shoulder, or a secondary maximum, at approximately 300 nm (**Figure 6A**).

Excitation and emission fluorescence spectra of methanolic extracts of trichomes from the adaxial side of artichoke leaves were compared with those of chlorogenic acid in methanol (**Figure 6B**). We found resemblance between them, with identical spectral characteristics (i.e., maxima,

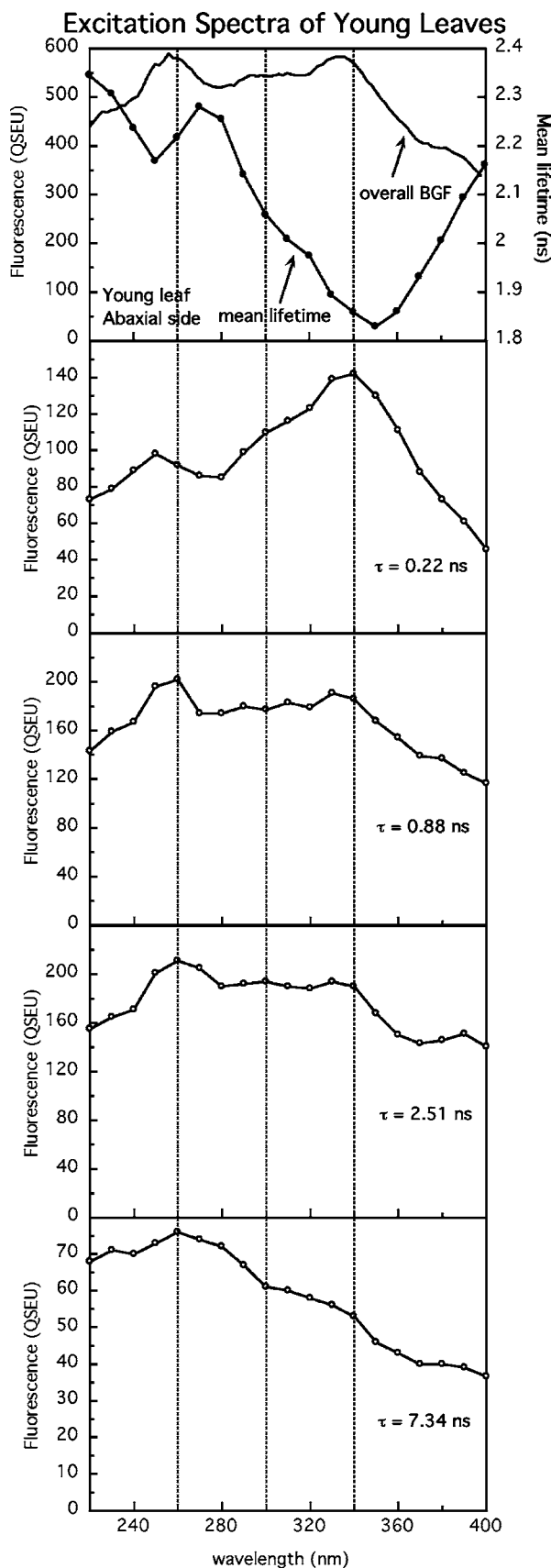


Figure 5. Decay-associated excitation spectra from 220 to 400 nm of young artichoke leaves from the abaxial side. The top panel reports the mean lifetime (solid circles) and the overall BGF spectrum (solid line), and the following panels from the top to the bottom show the various components at increasing lifetimes.

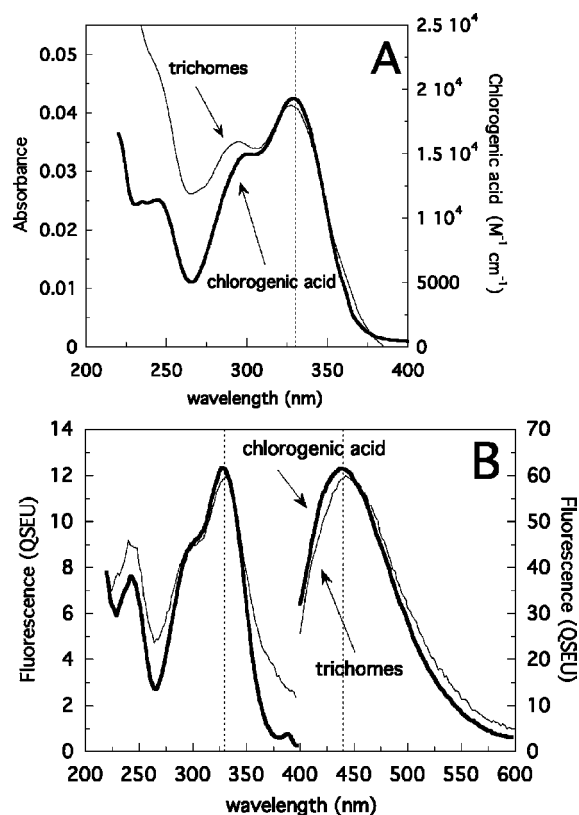


Figure 6. UV absorbance (from 220 to 400 nm) (A) and excitation (from 220 to 400 nm) and emission (from 400 to 600 nm) fluorescence spectra (B) of methanolic extracts of adaxial trichomes from young leaves and pure chlorogenic acid (10 μ M, absorbance; 15 μ M, fluorescence) in methanol. Excitation spectra were measured at emission maxima (440 nm), and emission spectra were measured at excitation maxima (330 nm).

Table 1. Concentrations (and Contents) of Chlorogenic Acid and Cynarin Expressed on Different Bases^a

compound	leaf type		
	young leaf	old leaf	young/old ratio
chlorogenic acid			
mg/cm ²	0.99 ± 0.07	0.63 ± 0.04	1.57
% of DM	4.74 ± 0.31	4.49 ± 0.28	1.06
mg/cm ³	9.68 ± 0.64	7.23 ± 0.45	1.34
mg per leaf	19.21	32.13	0.60
cynarin			
mg/cm ²	0.03 ± 0.00	0.03 ± 0.00	1.00
% of DM	0.16 ± 0.02	0.23 ± 0.01	0.70
mg/cm ³	0.32 ± 0.04	0.36 ± 0.02	0.89
mg per leaf	0.58	1.53	0.38

^a Data are means ± SE ($n = 6$). Conversions between bases are based on LMA of 20.9 and 14.1 mg DM cm⁻², a leaf thickness of 1022 and 876 μ m, and leaf surfaces of 19.4 and 51.0 cm² for young and old leaves, respectively.

shoulder, full width at half-maximum, and form of the spectra).

Synchronized Fluorescence Spectra of Young and Old Artichoke Leaves and of Chlorogenic Acid. An alternative approach to DAS is to use SFS. Synchronized fluorescence spectra were run for young and old leaves (both abaxial and adaxial sides) and for chlorogenic acid (Figure 7). Young leaves had maxima of excitation at approximately 337 and 405 nm (emission wavelengths at 447 and 515 nm, respectively) (Figure 7A). It has to be noted that the relative importance of these maxima changed between the abaxial and the adaxial side. In

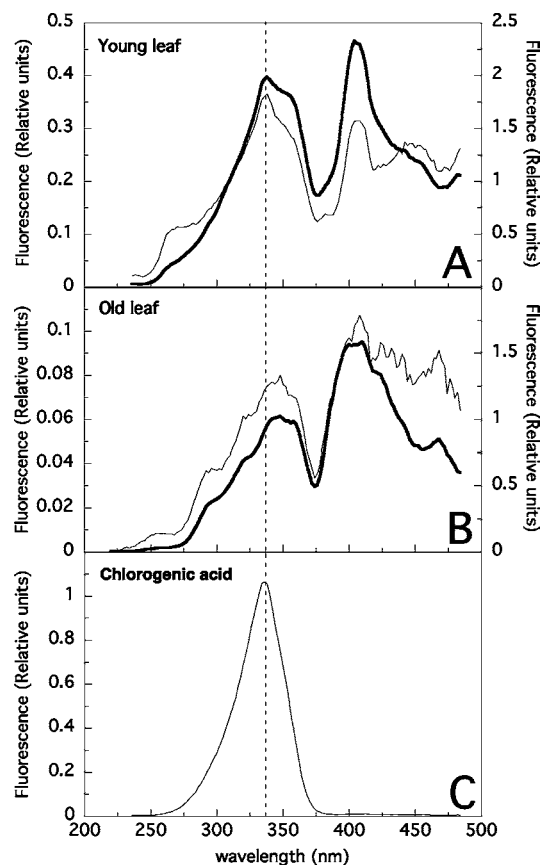


Figure 7. Synchronized fluorescence spectra (from 220 to 485 nm) of abaxial (bold lines) and adaxial (fine lines) young (A) and old (B) artichoke leaves and pure chlorogenic acid (C) (15 μ M) in methanol. $\Delta\lambda$ was 110 nm.

the latter one, a third excitation maxima at approximately 450 nm (emission wavelength at 560 nm) was detected (Figure 7A). In old leaves, the shortest excitation maximum was shifted to longer wavelengths when compared to young leaves (excitation at 348 nm and emission at 458 nm), the second maximum was similar to young leaves, and a third maximum occurred at approximately 468 nm (emission wavelength at 578 nm) (Figure 7B). The relative importance of this third maximum changed between the abaxial and the adaxial side of the leaves. Chlorogenic acid dissolved in methanol had a single excitation maximum at approximately 337 nm (emission wavelength at 447 nm) (Figure 7C).

Relationship between the BGF of the Very Short Kinetic Component and Chlorogenic Acid Concentration. We have compared the BGF of the very short kinetic component of the leaves (abaxial side) (in QSEU, data from Figure 2) to the chlorogenic acid concentration of leaves of different ages (from leaves 1 to 7) on an area basis (Figure 8). We found a close relationship between these two parameters ($R^2 = 0.9999$; Figure 8), and moreover, the relationship between fluorescence and chlorogenic acid concentration does not seem to saturate (Figure 8, insert).

DISCUSSION

Up to very recently, comparisons of BGF measurements made in different laboratories were mostly qualitative, using spectrofluorimeters provided with xenon lamps, or with nitrogen, argon, or frequency tripled Nd:YAG lasers, or with synchrotron radiation as source of excitation. In addition, the emitted BGF was very frequently expressed in relative units (1–3). The use

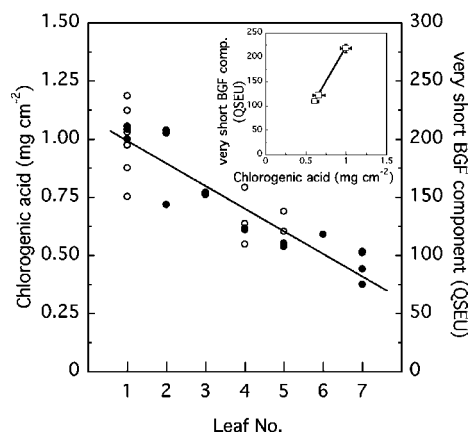


Figure 8. BGF of the very short kinetic component (solid circles) and chlorogenic acid concentrations (open circles) in artichoke leaves of different ages (numbers of leaves as in Figure 1) and the relationship between them (insert).

of relative units may be sufficient when the measuring system geometry is kept constant. Johnson et al. (6) determined a fluorescence yield index, to quantify BGF intensity changes among species, which was calculated by dividing the energy at the peak emission wavelength by the total excitation energy incident on the leaf surface. In this study, a special laboratory spectrofluorimeter was set up on the SA4 line of the Super-ACO storage ring at LURE. It uses synchrotron radiation, which is a pulsed and fully tuneable (white) source, and time-correlated single-photon counting detection (23). It permits measurement of time-resolved excitation and emission fluorescence spectra (16, 17). Very recently, the setup has been improved, allowing quantitative BGF measurements using the BGF of quinine sulfate as a reference standard (5, 26). In this work, we have therefore expressed data on BGF intensity changes as quinine sulfate equivalent units, QSEU.

Artichoke leaves emitted BGF when excited with UV light. In general, the BGF from the adaxial sides of dicotyledonous bifacial leaves is lower than that from the abaxial sides (5). For instance, spinach leaves fluoresce 50 and 150 QSEU in the blue-green when excitation was carried out from the adaxial and abaxial sides, respectively (5). This is, in most cases, the consequence of a larger transmittance for UV-A of the abaxial epidermis, due to the presence of higher concentrations of flavonoids in the adaxial epidermis (32–34). Artichoke leaves also emitted more BGF when excited from the abaxial side (400–800 QSEU) than from the adaxial one (35–200 QSEU). Thus, the BGF abaxial/adaxial ratio was 4 and 11.4 in the youngest and oldest leaves, respectively. In the case of artichoke leaves, we ascribed this larger BGF from the abaxial side to the thick and dense trichome vestment of the abaxial epidermis (as thick as the whole mesophyll in the case of the youngest leaves, revealed by microscopy observations; 35), charged of blue-green fluorophores.

The BGF intensity was shown to depend on plant species (2, 3, 6). For instance, pea leaves, whose cell walls are known to be nonfluorescent (36), have much less BGF (approximately 20 QSEU; 5) than sugar beet leaves (approximately 460 QSEU; 5), whose cell walls are fluorescent because of the presence of esterified ferulic acid (36). Grapevine leaves have a BGF intensity similar to sugar beet leaves (approximately 400 QSEU; 5). Artichoke can be included among the high BGF species (800 QSEU), when the abaxial side of the youngest leaves is measured, or among the low BGF species (30 QSEU), when the adaxial side of the oldest leaves is measured.

The leaf BGF was also shown to depend on stage of development (1, 2, 7, 8). In maize leaves, the concentration of esterified ferulic acid has been reported to increase with age (37). Also, an accumulation of ferulic acid esters can probably explain the changes in the excitation spectrum of BGF in sugar beet leaves with age (5). Moreover, an increased BGF was also recorded in senescent soybean (2) and beech (7) leaves. By contrast, old leaves from artichoke plants fluoresced less than young leaves. This should be at least in part ascribed to a decreased number of epidermal trichomes with leaf age. A natural loss of trichomes with leaf age has been previously reported in *Olea europaea*, *Cydonia oblonga*, and *Eriobotrya japonica* (38). Decreases in the percentage of BGF attributable to the very short kinetic component (from 35–42 to 23–26% in the adaxial side and from 25–27 to 20–22% in the abaxial side; **Figure 2**), the change in shape of the BGF excitation spectra (**Figure 1**), the decrease in leaf chlorogenic acid concentrations (**Table 1**), and the decrease in UV absorbance of leaf methanolic extracts (35) indicate that there is a loss of hydroxycinnamic acids with age relative to other leaf constituents. This does not seem to be a mere trichome dilution effect as leaves grow, since when chlorogenic acid concentrations were expressed on a whole leaf basis, old leaves had more chlorogenic acid than young leaves (**Table 1**).

Wolfbeis (39) reviewed a series of natural compounds that emit BGF upon UV excitation, known to be present in leaves. They are hydroxycinnamic acids (ferulic, caffeic, and sinapic acids), chromones, stilbenes (resveratrol), coumarins (umbelliferone, esculetin, and scopoletin), furocoumarins (psoralen), flavonols, flavones (except 5-hydroxyflavones), isoflavones, flavanones, chalcones, auronones, phenolic acids (salicylic, gentisic, and ellagic acid), nicotinamides [NAD(P)H], flavines (FMN, FAD, and riboflavin), other coenzymes (pyridoxal-5'-phosphate), pterines (folic acid and dihydrofolate), polyenes (phytofluen), quinones (phyllohydroquinone), alkaloids (berberine, quinine, and lysergic acid), and degradation products (kynurenine and polyadenylic acid). Among them, the two main classes of BGF emitters in leaves of different species have been reported to be hydroxycinnamic acids and nicotinamide and flavine nucleotides (5, 9, 16–21).

Some of these compounds have been reported in artichoke leaves, including flavones, flavanones, coumarins, auronones, flavonols, and hydroxycinnamic acids (40–44). Among them, hydroxycinnamic acids are the best candidates to be responsible for part of the artichoke leaf BGF, since the leaf BGF excitation spectra (clearly evident in the youngest ones) and the UV absorption spectra of leaf methanolic extracts (35) match those of these phenolic compounds. Our HPLC data of the leaf extracts revealed the presence of chlorogenic acid and cynarin. On a per area basis, chlorogenic acid is 33- and 21-fold more concentrated than cynarin, in young and old leaves, respectively.

Several lines of evidence indicate that chlorogenic acid is one of the main fluorophores in artichoke leaves. In old leaves, (i) the BGF excitation spectra had a maximum at 330–332 nm (**Figure 1**), (ii) the leaf methanolic extract had a UV absorption maximum at 330 nm (35), and (iii) both match the UV absorption and BGF excitation maximum of chlorogenic acid in methanol (330 nm; **Figure 6**). In young leaves, (i) the excitation spectra of the adaxial side (**Figure 1**) resembled much to the UV absorption spectra of pure hydroxycinnamic acids in solution, (ii) the methanolic extracts of trichomes of the adaxial side had UV absorption and BGF excitation and emission spectra that match perfectly with those of chlorogenic acid in methanol (**Figure 6**), (iii) when excited from the abaxial side, the BGF

seems to present characteristics closer to chlorogenic acid fluorescence, with maxima of excitation at 334 nm and maxima of emission at 444 nm (**Figure 1**), and (iv) in the synchronized fluorescence spectra, both leaves and chlorogenic acid have a similar peak at 337 nm (**Figure 7**). Moreover, among the hydroxycinnamic acids, chlorogenic acid is the only one detected at high concentrations in the methanolic extracts by HPLC (**Table 1**). However, our data indicate that only 20–42% (i.e., the very short kinetic component) of the overall BGF of the artichoke leaves is emitted by chlorogenic acid; 58–80% (i.e., the medium, slow, and very slow kinetic component) remains unexplained. Unfortunately, data on fluorescence lifetimes of most plant phenolics (see above for those reported in artichoke leaves and potentially blue-green fluorophores) are missing from the literature, and the assignment of the medium, slow, and very slow kinetic components should await time-resolved studies of a large variety of candidates.

We have also explored the relationship between the BGF of the very short kinetic component and the chlorogenic acid concentration. Calculations were made multiplying the fractional intensity of this kinetic component by the overall leaf BGF (**Figure 8**). We found a close relationship between these two parameters, which moreover does not seem to saturate. Time-resolved BGF could be a way of extracting information on chlorogenic acid fluorescence from the overall artichoke leaf BGF.

These findings have important implications for artichoke leaf (extracts) producers in natural medicine, since the composition in hydroxycinnamic acid derivatives of the leaves changes with the age of the leaves. Some pharmaceutical companies claim that cynarin is the main constituent of artichoke leaves, whereas other ones remark the high concentration of chlorogenic acid. The former sell artichoke leaf dry matter with cynarin concentrations larger than 2% (www.arkochim.es) or 5% (www.optimalnutrients.com), the latter with cynarin and chlorogenic acid concentrations up to 5 and 15%, respectively (www.lef.org). Differences among companies could arise from their mother leaf material. The age-mediated changes in leaf composition should be, therefore, considered at harvest time.

Chlorogenic acid and cynarin have been shown to have activity against oxidative stress in human leukocytes (45), whereas cynarin has also been shown to have hypocholesterolemic (46), hepatoprotective (47), and, more recently, anti-human immunodeficiency virus-1 (anti-HIV-1; 44) activities. Extracts have been traditionally carried out by placing artichoke leaves in boiling water (50–70 g of fresh leaves per liter) and consuming as infusions. This way favors the conversion of chlorogenic acid into neochlorogenic and cryptochlorogenic acid (30), and the potential benefit for the human health of these two latter acids is not as well-documented in the literature. Therefore, consumers should choose products containing directly dry matter of artichoke leaves or products based on diluted alcoholic extracts from artichoke leaves. Traditionally, drinks based on alcoholic extracts are also used frequently in natural medicine.

ABBREVIATIONS USED

BGF, blue–green fluorescence; DAS, decay-associated spectra; SFS, synchronous fluorescence spectrometry; UV, ultraviolet.

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