(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 387 712 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
04.06.2014 Bulletin 2014/23

(21) Application number: **10732029.3**

(22) Date of filing: **13.01.2010**

(51) Int Cl.:
G01N 33/483 (2006.01) ***G01N 21/64*** (2006.01)
A61B 5/00 (2006.01) ***G01N 21/31*** (2006.01)

(86) International application number:
PCT/US2010/020885

(87) International publication number:
WO 2010/083204 (22.07.2010 Gazette 2010/29)

(54) **NONINVASIVE MEASUREMENT OF FLAVONOID COMPOUNDS IN BIOLOGICAL TISSUE**

NICHTINVASIVE MESSUNG VON FLAVONOID-VERBINDUNGEN IN BIOLOGISCHEM GEWEBE

MESURE NON INVASIVE DES COMPOSES FLAVONOIDES DANS UN TISSU BIOLOGIQUE

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK SM TR

(30) Priority: **13.01.2009 US 352702**

(43) Date of publication of application:
23.11.2011 Bulletin 2011/47

(73) Proprietors:
- **Sharifzadeh, Mohsen**
  Salt Lake City, UT 84108 (US)
- **Ermakov, Igor V.**
  Salt Lake City, UT 84105 (US)
- **Gellerman, Werner**
  Salt Lake City, UT 84105 (US)

(72) Inventors:
- **Sharifzadeh, Mohsen**
  Salt Lake City, UT 84108 (US)
- **Ermakov, Igor V.**
  Salt Lake City, UT 84105 (US)
- **Gellerman, Werner**
  Salt Lake City, UT 84105 (US)

(74) Representative: **Whitfield, Gillian Janette et al**
Astrum ElementOne Limited
Tower House
Fairfax Street
Bristol BS1 3BN (GB)

(56) References cited:
WO-A1-00/78217
WO-A1-03/014714
WO-A2-2006/100685
WO-A2-2009/106524
US-A1- 2004 130 714
US-A1- 2004 233 448
US-B1- 6 289 230
US-B1- 6 421 455
US-B2- 6 965 793

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 2 387 712 B1

Description

FIELD OF THE INVENTION

[0001] The present invention relates generally to optical techniques for measuring compounds found in biological tissue. More specifically, the invention relates to a method and apparatus for the noninvasive detection and measurement of levels of flavonoids and related chemical substances in biological tissue, which can be used as a diagnostic aid in assessing antioxidant status and detecting malignancy risk thereof.

BACKGROUND OF THE INVENTION

[0002] Flavonoids are ubiquitous, naturally occurring polyphenolic compounds that are often responsible for the bright, attractive colors of plants. Concentrated in numerous fruit, vegetables, berries, grains, roots, stems, and also in beverages like tea, coffee, beer and wine, they are taken up with the diet, and are eventually deposited in the living human tissue cells. Flavonoids have generated enormous interest due to their obvious benefits for human health. One motivator is the explanation of the "French Paradox", which is a surprisingly low cardiovascular mortality rate observed in Mediterranean populations, in spite of the relatively high saturated fat intakes. There is compelling evidence now that certain flavonoids present in red wine, which is consumed in relatively high concentrations along with the fat intakes in Mediterranean diets, are indeed responsible for this effect [1]. Probably based on their common antioxidant function, other kinds of flavonoids present in different food sources appear to have a wide range of beneficial effects as well. They have been associated with the scavenging of free radicals, the prevention of DNA damage, protection from UV-light induced tissue damage, the regulation of good and bad cholesterol levels, clearing of arteries, blocking of tumor growth, the promotion of weight control, protection of retinal pigment epithelial cells from oxidative-stress induced death, etc. [2, 3]. Epidemiological studies consistently show that the consumption of flavonoid-rich food lowers the risk of cancers anywhere from 30 to 75 % [2].

[0003] The molecular structure common to all flavonoids includes two aromatic benzene rings on either side of a 3-carbon ring skeleton, C_6 - C_3 - C_6, as illustrated in Figure 1. Depending on the position of carbon double bonds in the C_3 ring, substitution of an OH side group and/or double-bonded oxygen, flavonoids are divided into six main categories. These are *flavonols, flavones, flavanones, catechins, isoflavones,* and *anthocyanidins,* all shown in Figure 2 along with selected representatives and major food sources.

[0004] Flavonoid categories with most compounds are *flavonols and flavones,* both of which have a planar structure due to a double bond in the central C_3 ring. The most prominent and probably the most investigated members are quercetin and kaempferol, found in high concentrations in onions, broccoli, apples, and berries. The third flavonoid category, *flavanones,* is mainly found in citrus fruit. Members of this group are naringenin and hesperetin. A fourth category, *catechins,* is mainly found in green and black tea and in red wine, while the fifth category, *isoflavones,* is relatively narrowly distributed in foods, with soy beans being the primary food source. The last category, *anthocyanidins,* is dominant in cherries, berries, and grapes. Synthesized by plants, flavonoids are often bound to other molecules, such as sugars, in this case forming an inactive glycoside complex. The sugar group is known as the glycone, and the non-sugar group as the aglycone or genin part of the glycoside. As an example, citrus fruit contains hesperidin (a glycoside of the flavanone hesperetin), quercitrin, rutin (two glycosides of the flavonol quercetin), and the flavone tangeritin. In living organisms, like in the human body, enzymes can break up the inactive glycosides if needed, and the sugar and flavonoid components are then made available for use.

[0005] Relatively little is known about the energy levels of flavonoids except that the strong electronic absorption transitions connecting these levels occur at relatively high optical energies in the deep UV to blue spectral region. In flavones and flavonols, two characteristic absorption bands have been described in the literature: a "long-wavelength" band in the 300 - 400 nm region, mostly representing the B-ring absorption, and a "short-wavelength" band in the 240 - 280 nm region, mostly representing the A-ring absorption. Absorption line shapes and strengths of specific flavonoids are thought to depend on the specific number of hydroxyl groups and/or other substitutions as well on their relative positions [4, 5]. For example, comparing the flavonols quercetin and kaempferol with the flavones luteolin and apigenin, it was found that the two flavonols both have a slightly larger (~30 nm) red shift of their long-wavelength, B-ring, absorption bands relative to those of the two flavone members [6]. This was attributed to the fact that the two flavonols have a hydroxyl group attached to their C_3 ring, while the two flavones have no such attachment. For quercetin, the main observed absorption transitions, i.e. those with high oscillator strengths, have been fairly accurately modeled in quantum-chemical configuration interaction calculations, taking into account all excitations from the nine highest occupied molecular orbitals to the nine lowest unoccupied molecular orbitals [7, 8]. The absorption band in the 300 - 400 nm range is shown to be primarily due to a transition between the highest occupied and lowest unoccupied π molecular orbitals, respectively, where the electronic charge density is withdrawn from the B ring to the C=O double bond of the C ring. The transition in the 240 - 280 nm region is assigned to a transition between the second highest and lowest π molecular orbitals, respectively, involving a charge transfer from the region of one aromatic ring through C to the other aromatic ring. No information is given on the existence of energy levels, associated charge distributions, and potential low-energy transitions that could give rise to absorption bands on the long-wavelength side of the B-ring 300-400 nm absorption.

SUMMARY OF THE INVENTION

[0006] The present invention is directed methods and apparatus for the noninvasive detection and measurement of flavonoid compounds and related chemical substances in biological tissue. In particular, the invention makes possible the rapid, noninvasive and quantitative measurement of the concentration of flavonoid compounds, as well as their isomers and metabolites, in biological tissue such as human skin. This is accomplished without the requirement of removing tissue or preparing samples for HPLC and mass spectrometry analysis, as required by prior biochemical "gold standard" techniques.

[0007] The invention can be used in a direct and quantitative optical diagnostic technique, which uses low-intensity, visible-light illumination of intact tissue, provides for high spatial resolution, and allows for precise quantification of the flavonoid levels in the tissue. Such a technique is useful as a biomarker for fruit and vegetable intake, and it can aid in the detection of tissue abnormalities such as malignancy diseases. The optical detection of flavonoids adds to the optical detection of other antioxidant compounds in tissue, such as the Resonance Raman detection of carotenoids in skin [9], and it may be used in combination with the latter to obtain a more general assessment of bioactive compounds present in the measured living tissues. Examples of biological tissues that can be measured non-invasively with the technique of the invention include human skin and mucosal tissue, bodily fluids such as blood serum, urine, and also plant and fruit tissue samples or extracts.

[0008] A noninvasive method of measuring flavonoid levels in biological tissue according to the invention comprises the steps of illuminating a localized region of tissue with light that overlaps the absorption bands of a flavonoid compound; detecting the fluorescence emitted by the flavonoid compound resulting from the illumination; and determining the concentration level of the flavonoid compound based upon the detected fluorescence.

[0009] The tissue is be human skin, preferably on a fingertip or other portion of a hand. The concentration level may be used to assess the antioxidant status of the tissue and/or risk or presence of a malignancy or other disease. The light used for excitation is typically in the 300 to 650 nm spectral region, and the fluorescence emitted by the flavonoid may be characterized using fluorescence spectroscopy or an optical detector, particularly if the light is in the red region of the spectrum and the localized region of tissue is substantially melanin-free.

[0010] The preferred embodiments make use of a previously unknown, low-oscillator strength, optical absorption transition of flavonoids. This transition manifests itself as a long-wavelength absorption feature that extends far into the visible wavelength range, beyond the well-known 300-400 nm B-ring absorption band. This makes it possible to optically excite flavonoids in living human tissue outside the absorption range of other, potentially confounding skin chromophores. Such chromophores, which include carotenoids, blood, elastin, and collagen, commonly generate unwanted, spectrally overlapping, absorption and/or fluorescence responses under optical excitation of the flavonoid A or B ring absorption bands. However, by exciting the tissue flavonoids in their long-wavelength absorption tail outside the absorption range of these other skin chromophores, the invention makes it possible to generate a fluorescence response from the skin that is only due to the flavonoid molecules present in the optically excited tissue volume. As a consequence, fluorescence spectroscopy may be used as a novel non-invasive, optical, quantitative detection method for flavonoids in human tissue such as skin, and to use this information as an aid in the assessment of flavonoid status and potential disease risk.

[0011] A system for measuring flavonoid levels in accordance with the invention includes a source of light for illuminating a localized region of tissue with light that overlaps the absorption bands of a flavonoid compound; a device for detecting the fluorescence emitted by the flavonoid compound resulting from the illumination; and a processor for determining the concentration level of the flavonoid compound based upon the detected fluorescence.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] In order to illustrate the manner in which the above recited and other advantages and objectives of the invention are obtained, a more particular description of the invention briefly described above will be rendered by the reference to specific embodiments thereof, which are illustrated in the appended drawings. Understanding that these drawings depict only typical embodiments of the invention and are not therefore to be considered limiting in scope, the invention will be described and explained with additional specificity and detail through the use of the accompanying drawings in which:

Figure 1 illustrates the basic molecular structure of all flavonoids;

Figure 2 illustrates the six distinct categories of flavonoids, their molecular structure, and their main food sources;

Figure 3 is a general schematic depiction of the apparatus according to the present invention that measures the emission spectra of flavonoids in a variety of samples, including human skin tissue sites;

Figure 4 shows absorption spectra of pure quercetin and kaempferol crystal powder samples;

Figure 5 shows absorption spectra of methanol solutions of quercetin and kaempherol;

Figure 6 shows fluorescence spectra of quercetin crystal powder at blue, green, and red excitation wavelengths;

Figure 7 shows fluorescence spectra of kaempherol crystal powder at blue, green, and red excitation wavelengths;

Figure 8 shows fluorescence spectra of a water solution of quercetin at blue, green, and red excitation wavelengths;

Figure 9 shows fluorescence spectra of a water solution of kaempferol at blue, green, and red excitation wavelengths;

Figure 10 shows absorption spectra of pure apigenin and luteolin crystal powder samples;

Figure 11 shows absorption spectra of methanol solutions of apigenin and luteolin;

Figure 12 shows absorption spectra of diosmin crystal powder and methanol solution;

Figure 13 shows fluorescence spectra of apigenin crystal powder at blue, green, and red excitation wavelengths;

Figure 14 shows fluorescence spectra of luteolin crystal powder at blue, green, and red excitation wavelengths;

Figure 15 shows fluorescence spectra of diosmin at blue, green, and red excitation wavelengths;

Figure 16 shows absorption spectra of hesperidin and naringenin crystal powder samples;

Figure 17 shows absorption spectra of hesperidin and naringenin solutions in methanol;

Figure 18 shows fluorescence spectra of naringenin and hesperidin crystal powder samples;

Figure 19 shows absorption spectra of epicatechin crystal powder;

Figure 20 shows absorption spectra for methanol solutions of catechin, epicatechin, and gallocatechin crystal powder;

Figure 21 shows fluorescence spectra of catechin, epicatechin, and gallocatechin crystal powder samples at blue, green, and red excitation wavelengths;

Figure 22 shows absorption spectra of genistein crystal powder and a methanol solution of genistein;

Figure 23 shows fluorescence spectra of genistein powder at blue, green, and red excitation wavelengths;

Figure 24 shows absorption spectra of pelargonidin chloride crystal powder and two methanol solutions of the compound with low and high concentrations, respectively;

Figure 25 shows fluorescence spectra of pelargonidin powder at blue, green, and red excitation wavelengths;

Figure 26 shows fluorescence spectra of all investigated flavonoids, obtained with blue excitation;

Figure 27 shows fluorescence spectra of all investigated flavonoids, obtained with green excitation;

Figure 28 shows fluorescence spectra of all investigated flavonoids, obtained with red excitation;

Figure 29 shows the bleaching kinetics of quercetin crystal powder under 532 nm and 632 nm excitation;

Figure 30 shows the linearity of the fluorescence intensity of quercetin crystal powder under 632 nm excitation with increasing excitation light power;

Figure 31 shows the fluorescence spectra of living human skin for an inner palm tissue site;

Figure 32 shows the fluorescence spectra of an excised heel skin tissue sample;

Figure 33 shows the fluorescence spectra of collagen;

Figure 34 shows normalized fluorescence spectra of quercetin powder and living skin;

Figure 35 shows the bleaching kinetics for living human skin in comparison with the bleaching kinetics of quercetin;

Figure 36 shows the bleaching kinetics of living human skin;

Figure 37 shows the linearity of the fluorescence intensity of living skin with increasing excitation light power;

Figure 38 shows the repeatability of the skin fluorescence for successive measurements;

Figure 39 shows skin fluorescence spectra for various tissue sites;

Figure 40 shows skin fluorescence spectra for four different volunteer subjects;

Figure 41 shows fluorescence spectra of an onion layer, obtained with blue, green, and red excitation;

Figure 42 shows a comparison of the fluorescence spectrum of an onion layer with fluorescence spectra of quercetin and kaempferol, all obtained under red excitation;

Figure 43 shows fluorescence spectra of a green grape, a red grape, and an onion layer; and

Figure 44 shows a filter-based experimental setup for flavonoid fluorescence measurements.

DETAILED DESCRIPTION OF THE INVENTION

[0013] The present invention uses fluorescence to identify and quantify the presence of flavonoids and similar substances in biological tissue such as skin. In this technique, light is directed onto the tissue, and the fluorescence emitted from the tissue is filtered and detected. The fluorescence intensity can be used as an indicator for the concentration of the flavonoids present in a subject's skin, since the fluorescence intensity can be expected to scale linearly with the concentration of the flavonoids present in the excited tissue volume. A preferred embodiment uses tissue sites such as the palm of the hand that is held against an optical window. The apparatus allows one to continuously measure and display the intensity of the fluorescence. The total time it takes to assess a subject's skin flavonoid level is very brief, amounting to only a few seconds.

[0014] In a method for the noninvasive measurement of flavonoids and related chemical substances in biological tissue according to the current invention, a light source such as a tungsten-halogen lamp, a light emitting diode, or a laser is used, any of which feature light emission with sufficiently high intensity at spectral locations in the wavelength range where absorption bands of the flavonoid compounds occur, i.e. in the 300 to 650 nm spectral region. The fluorescence intensity emitted from the skin flavonoids is proportional to their concentration in the excited tissue volume. Therefore, the fluorescence intensity of the skin flavonoids can be used as an optical measure for the skin flavonoid concentration, and this information can be used to assess the flavonoids antioxidant status of the tissue. The concentration levels can be compared with levels of normal biological tissue to assess the risk or presence of a malignancy disease.

[0015] Figure 1 shows schematically the molecular structure that is common to all flavonoids. It consists of a central ring with three carbon atoms that is connected on either side with an aromatic benzene ring. Figure 2 shows the main categories of flavonoids, their molecular structure, main members of the various categories and their main food sources. Individual flavonoids differ in the number and position of hydroxyl molecules attached at various carbon sites, as well as the existence and positioning of carbon double bonds in the central ring.

[0016] Figure 3 is a general schematic depiction of the apparatus of the present invention for measuring the emission spectra of flavonoids and like substances in biological tissue using fluorescence spectroscopy. The apparatus contains a light source, which in one preferred embodiment of the invention is a light emitting diode, emitting light with ~20 nm bandwidth centered at 627 nm. Alternatively, the light source may comprise other devices for generating light in the spectral range of the flavonoid absorptions. Preferably, in the case of flavonoids, the light source generates light with sufficient intensity at discrete wavelength locations or at certain spectral ranges in the wavelength range 300 - 650 nm, which overlap with the absorption bands of flavonoid compounds. Such light is readily available, e.g., from commercially produced inexpensive slide projector lamps that are suitable filtered, from light emitting diodes, or from lasers.

[0017] The excitation light source is in optical communication with a light beam delivery and collection system that can include various optical components for directing the excitation light to the tissue or sample to be measured and collecting the emitted fluorescence. As shown in Figure 3, the optical components of the apparatus include the light source, a mechanical shutter, a beam splitter that sends part of the excitation light to a monitoring detector, a beam expander, a filter, a lens, a window that is placed against the tissue or sample to be measured, a light collection lens, a filter, a beam contractor, a light collection fiber, a spectrograph, a fluorescence detector, and a computer processor/ monitor. The interaction of these optical components with the light from the light source will be discussed in further detail below.

[0018] The detection part of the apparatus can contain a spectrograph or spectrometer, which serves to spectrally disperse the light components of the flavonoid fluorescence. The spectrally dispersive system can be replaced by various alternative optical components such as diffraction gratings, prisms, holographic filters, dielectric filters, combinations thereof, and the like.

[0019] The spectrally selective system is in optical communication with a detection means such as a light detection system, which is capable of measuring the intensity of the emitted fluorescence as a function of wavelength in the wavelength range of interest, such as the wavelength range characteristic for the flavonoid compounds in human skin. The detection system may comprise, but is not limited to, devices such as a CCD (charge-coupled device) detector array, an intensified CCD detector array, a photomultiplier apparatus, photodiodes, or the like.

[0020] The spectrally selective system and light detection system can be selected from commercial spectrometer systems such as a low-resolution grating spectrometer employing rapid detection with a charge-coupled silicon detector array. For example, a grating spectrometer can be used which employs a dispersion grating with 300 lines/mm, and a silicon detector array with 14 μm individual pixel width. Another suitable spectrometer is a holographic imaging spectrometer, which is interfaced with a CCD detector array, and employs a volume holographic transmission grating. The spectrally selective system and light detection system can also be combined into an imaging system that includes spectrally selective optical elements used in association with a low light level CCD imaging array such as an intensified CCD camera.

[0021] The detected light is preferably converted by a light detection system into a signal that can be visually displayed on an output display such as a computer monitor or the like. It should be understood that the light detection system can also convert the light signal into other digital or numerical formats, if desired. The resulting emitted fluorescence light signals are preferably analyzed via a quantification means such as a quantifying system, which may be calibrated by comparison with chemically measured flavonoid levels from other experiments. The quantifying means may be a computer, preferably one in which data acquisition software is installed that is capable of spectral manipulations, such as the normalization of the spectra to an emission standard, and the determination of concentration values of the flavonoids present in the measured tissue volume. The quantifying system may also comprise a CCD image display or monitor. The quantifying system may be combined with the output display in one computer and can calibrate the results with flavonoid levels obtained with other experiments such as the optical density that is proportional to actual flavonoid levels.

[0022] During operation of the apparatus, a light beam is generated from the light source and is directed through an input optical fiber to the delivery system. Alternatively, the light beam is directed to the light delivery system with the help of mirrors. Part of the excitation light routed toward the system is split off with a beam splitter to monitor its intensity, and the remainder is expanded, filtered, and imaged with a lens through a window onto the sample or tissue volume to be measured. The latter is in contact with the window. The fluorescence emitted from the sample or skin is collected by a lens, and is imaged onto the face of an output fiber that routes the light to a spectrally selective system such as a grating spectrograph. The spectrally dispersed light is directed to a light detection system that measures the light intensity as a function of wavelength in the wavelength range spanning the fluorescence range of all skin flavonoids. Alternatively, the spectrally selective system is skipped, and the fluorescence is routed directly to a light detection system. The light detection system then converts the emitted fluorescence signals into a form suitable for visual display such as on a computer monitor or the like, and the resulting flavonoid emission is analyzed with the quantification system.

[0023] The present invention is particularly useful for the detection of flavonoid content in living human tissue. Humans ingest significant amounts of flavonoids in their diet. After uptake by the human body, they have the ability to modify the body's reaction to allergens, viruses, and carcinogens. They are thought to exhibit anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity. There is strong interest in flavonoids in the food and nutritional supplement industry due to their medicinal properties, especially their potential role in the prevention of cancers and cardiovascular disease. Evidently, the beneficial effects of fruit and vegetables and tea or even red wine can be attributed to a large extent to the inherent flavonoids rather than other compounds. In many cases, specific biochemical and physiological actions have been suggested for the flavonoid compounds. For example, kaempferol has been shown to revert the transformed phenotype of phorbol ester-treated mouse fibroblasts or v-H-ras-transformed NIH 3T3 cells. Another example, apigenin, has been found to inhibit cell proliferation by arresting the cell cycle at the G_2/M phase. Inhibition of growth through cell cycle arrest and induction of apoptosis appear to be related to induction of p53. Inhibitory effects on tumor promotion may also be due to inhibition of kinase activity and the resulting suppression of oncogene expression. It has also been reported to inhibit topoisomerase I catalyzed DNA religation and to enhance gap junctional intercellular communication. A third example, gallocatechin, has been suggested to inhibit the growth and adherence of *P. gingivalis* onto the buccal epithelial cells. A fourth example, genistein, has been shown to be an inhibitor of tyrosine protein kinase, a competitive inhibitor of ATP in other protein kinase reactions, and an antiangiogenic agent, down-regulating the transcription of genes involved in controlling angiogenesis.

[0024] While the microscopic mechanisms of their medicinal benefits are still subject of investigation [10], it is clear that a noninvasive detection method for flavonoids in living human tissue would provide a strong advantage. Current detection methods require mass spectrometry and liquid chromatography methods and as invasive methods are applicable only to biopsied tissue samples and bodily fluids. Noninvasive optical detection, in contrast, allows *in-situ* measurements of undisturbed living human tissue, provide rapid assessment of flavonoid status, serve as biomarker for fruit and vegetable uptake in epidemiological studies, and provide a convenient means for monitoring flavonoid uptake upon dietary modifications and nutritional supplementation

[0025] Various experiments were performed which demonstrate that strong flavonoid fluorescence signals are readily obtainable for various areas of the living human skin using safe light exposure levels. The following examples set forth the apparatus and procedures utilized in these experiments as well as the results derived from them.

EXAMPLE 1

[0026] In order to investigate the potential excitation wavelengths useful for the generation of characteristic flavonoid fluorescence signals, we first measured the absorption characteristics of representative compounds. Crystal powder samples with highest possible purity were obtained from Sigma-Aldrich, Inc. They included quercetin dihydrate and kaempferol for flavonol examples, apigenin, luteolin, and diosmin rutinoside for flavone examples, naringenin and hesperidin (rhamnoglucoside of hesperitin) for flavanones, the catechins gallocatechin and epicatechin, the isoflavone genistein, and the anthocyanidin compound pelargonidin chloride. The manufacturer lists the following synonyms for some of the measured flavonoid compounds.

[0027] For kaempferol: 3,4',5,7-Tetrahydroxyflavone, 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, Robigenin;

for apigenin: 4',5,7-Trihydroxyflavone;
for luteolin: 3',4',5,7-Tetrahydroxyflavone;
for diosmin: 3',5,7-Trihydroxy-4'-methoxyflavone 7-rutinoside;
for naringenin: 4',5,7-Trihydroxyflavanone, (±)-2,3-Dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one;
for hesperedin: Hesperetin 7-rhamnoglucoside, Hesperitin-7-rutinoside;
for catechin: (2S,3R)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol, (-)-*trans*-3,3',4',5,7-Pentahydroxyflavane;
for gallocatechin: (2S,3R)-2-(3,4,5-Trihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol;
for epicatechin: (-)-*cis*-3,3',4',5,7-Pentahydroxyflavane, (2R,3R)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol;
for genistein: 4',5,7-Trihydroxyisoflavone, 5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one;

for pelargordinin chloride: 3,4',5,7-Tetrahydroxyflavylium chloride.

[0028] For each compound we determined the absorption characteristics of samples in crystal powder form, using white-light reflection spectroscopy. Experimental details of the method can be found in pending patent application serial no. 12/134,667. For solutions of the compounds we used a Perkin-Elmer UV/VIS/NIR absorption spectrophotometer.

[0029] In Figures 4 and 5, we show the absorption results for the flavonol compounds quercetin and kaempferol. The absorption bands are very similar and feature prominent broad bands peaking in the 400 to 430 nm range. Importantly, they also feature a weak, but clearly noticeable long wavelength absorption tail in the visible/far-red wavelength region, extending from about 500 to 800 nm. This weak, long-wavelength tail is not only visible for these flavonol compounds in powder form, which could point to a scattering effect, but also for methanol solutions. The absorption spectra obtained for perfectly clear methanol solutions of quercetin and kaempferol, i.e. solutions having no residual suspended flavonol material, are shown in the top panel of Figure 5, where trace (a) belongs to quercetin and trace (b) to kaempferol. The spectra reveal the well-known, three-band absorption pattern of flavonoid solutions in the deep UV/ blue spectral region, characterized by strong optical absorption transitions with maxima near ~ 200 nm, 260 nm, and 380 nm in the case of the two flavonol solutions. Compared to the solid-state form, the long-wavelength absorption bands of the compounds in solution are shifted slightly (several ten nm) to shorter wavelengths. Importantly, when measuring the absorption behavior of the more concentrated solutions in the visible wavelength region, a weak but clearly recognizable absorption tail of the two flavonols is apparent again, stretching up to wavelengths well into the visible/ red wavelength region (up to about 650 nm).

[0030] Next, using the experimental apparatus of Figure 3, we measured the emission behavior of both flavonol compounds. As excitation light wavelengths we tested 488, 532, and 632 nm, all exciting the absorption band on the long wavelength shoulder. The results for a quercetin powder sample are shown in Figure 6. They reveal that for all three excitation wavelengths, a strong fluorescence response is obtained. At 488 nm excitation, which has the strongest overlap with the absorption, a very broad fluorescence band is obtained, with maximum at -600 nm, halfwidth of -150 nm, and measurable fluorescence intensity extending up to about 800 nm in the near infrared region. The sharp peak at 488 nm is an artifact due to leakage of the excitation light into the spectrometer. Under 532 nm excitation, the emission occurs again with a maximum at -600 nm, slightly reduced halfwidth, and strength that is reduced by a factor of five due to the reduced overlap of the excitation with the absorption band. At 632 nm excitation, the fluorescence occurs shifted to a position of the maximum near ~670 nm. This shift appears due to the chopped off short-wavelength emission response. However, due to the fluorescence nature of the emission, the obtainable signal strengths are still relatively large, even under these "extreme" excitation conditions. The Stokes shift between long-wavelength absorption and obtained emission is very small, indicating that the emission originates from the same energy state reached in absorption, and thus suggesting that the oscillator strength of the emission is comparable to that of the absorption transition.

[0031] In Figure 7, the emission behavior is shown for kaempferol under the same three excitation conditions. Compared to quercetin, all effects are very similar except the emission maxima for 488 and 532 nm excitation are shifted slightly (by ~ 40 nm) to shorter wavelengths. Again, the emission range is very large, extending to about 775 nm in the near infrared. Compared to excitation at 488 and 532 nm, the fluorescence intensities obtained with 632 nm excitation are reduced by about one order of magnitude.

[0032] In Figures 8 and 9, the emission behaviors are shown, respectively, for aqueous solutions of quercetin and kaempherol. All signal strengths are weaker compared to the powder samples due to the reduced concentration of the active molecules and there is stronger scattering of the excitation light into the spectrometer, as evidenced by the strong, out-of-scale intensities at the excitation wavelengths. Importantly, however, in both cases the emission behavior is very similar again in terms of spectral shapes and locations of the maxima with respect to the pure powder samples.

[0033] In Figures 10-25 we show absorption and emission results for representative compounds of all other remaining flavonoid categories. In each case, we observe a distinct, long-wavelength absorption tail for the compound in powder form as well as in solution. Also, in each case we obtain again the distinct fluorescence pattern for blue, green, and red excitation, i.e. strong, broadband fluorescence responses in the 600-800 nm region at each excitation wavelength, with slightly reducing bandwidths upon increasing excitation wavelength.

[0034] The absorption and fluorescence properties of the flavones are illustrated in Figures 10-15 for the compounds apigenin, luteolin, and diosmin. In apigenin powder, the absorption tail is more prominent than in luteolin, as is apparent from Figure 10. However, in all cases the absorption tail can be observed with increased-concentration absorption measurements, even in methanol solutions, as is evident from Figures 11 and 12. The fluorescence spectra of the three flavone compounds, shown in Figures 13-15, are very similar under blue, green, and red excitation, differing slightly with regards to their relative intensities, but importantly, high fluorescence intensities in the 660 nm range are obtainable for all three compounds using long-wavelength excitation at 632 nm.

[0035] The absorption properties of the flavanone examples hesperidin and naringenin are shown in Figures

16 and 17, and their fluorescence behavior in Figure 18. In powder form, both compounds exhibit a very strong absorption tail up to -900 nm, as seen in Figure16. For a concentrated methanol solution of naringenin, the tail extends up to about 700 nm. Long-wavelength excitation at 632 nm yields significant fluorescence intensities in the 650 - 700 nm region, as can be seen from Figure 18.

[0036] The absorption behavior for catechins is shown in Figures 19 and 20, and their emission behavior in Figure 21. In powder form, a strong absorption tail exists up to ~650 nm, as illustrated in Figure 19 for epicatechin. Dissolved in methanol, the absorption tail is very weak in all cases, as seen in Figure 20 for catechin, epicatechin and gallocatechin. All compounds, however, exhibit the familiar characteristic strong emission pattern shown in Figure 21.

[0037] The absorption and fluorescence properties of the isoflavone genistein are shown in Figures 22 and 23. In powder form, pronounced band-like absorption tails exists in the 450-900 nm range. In a methanol solution, these tails seem to disappear, as seen in Figure 22, but fluorescence spectra reveal again significant intensities in the 650-700 nm region for this compound.

[0038] The absorption and fluorescence properties of the anthocyanidin example pelargonidin chloride, shown in Figures 24 and 25, differ the most from all other flavonoids. The absorption band tail is very strong, extending up to about 1000 nm in the powder sample. In a methanol solution, pronounced absorption bands appear throughout the visible wavelength region. Optical excitation yields very strong fluorescence responses at all excitation wavelengths, with the maximum of the fluorescence band occurring at ~700 nm.

[0039] The similarity of the fluorescence responses for all investigated flavonoid compounds is summarized in Figures 26-28, where the fluorescence responses are plotted, respectively, for blue (488 nm), green (532 nm), and red (632 nm) excitations, and where the wavelength positions and spectral shapes of the bands can be compared for all investigated compounds. For each excitation wavelength, the spectral shape and position of the compounds is slightly different, with the exception of pelargodinin chloride, which is significantly shifted to longer wavelengths. Importantly, strong fluorescence signals can be obtained in all compounds even with relatively long-wavelength, red excitation.

[0040] Upon excitation the fluorescence intensity is found to decrease slightly over time, an effect illustrated in Figure 29 for quercetin powder with 532 and 632 nm excitation. The fluorescence decay is less severe for increasing wavelengths. This effect is likely due to photoionization of the flavonoid compounds. In Figure 30 the increase of the fluorescence intensity with excitation light power is illustrated for quercetin powder. The increase is seen to stay linear while varying the excitation light power over about two orders of magnitude.

[0041] In Figure 31, we show the results of fluorescence measurements for a palm tissue site of living human skin, obtained under the same excitation conditions and with the same experimental setup. At all three excitation wavelengths, i.e. 488, 532, and 632 nm, broad emission bands are obtained which in positions of the spectral maxima, shapes, halfwidths, and relative strengths are very similar to the behavior of the pure flavonoid samples discussed above. A dip in the emission spectra occurs at around 570 nm, which can be attributed to the absorption of hemoglobin in the living tissue. This dip disappears when measuring a detached heel skin tissue sample, consisting essentially of a bloodless thick and relatively homogenous stratum corneum layer, as can be seen from the corresponding emission spectrum shown in Fig 32. Compared to the pure flavonoid powder samples, the emission intensities in skin are somewhat stronger under blue excitation relative to green and red excitation wavelengths.

[0042] In order to investigate the potential influence of collagen tissue components onto the observed emission behavior, we excited a pure collagen sample (obtained from Sigma Aldrich) under the same excitation conditions. The fluorescence spectra obtained are shown in Figure 33. Under blue excitation, a strong fluorescence band exists with maximum at ~540 nm. Its strength decreases rapidly with increasing excitation wavelength. Under excitation with 532 nm, the intensity drops by a factor of about 200, and under excitation with 632 nm, the obtained response consists essentially only of noise signals. This result is important since it proves that collagen does not generate any confounding fluorescence effects in the wavelength range of interest.

[0043] In Figure 34 we show, for direct comparison, the emission spectrum of living human skin, obtained with 632 nm excitation for an inner palm tissue site, along with the emission spectrum of quercetin. Clearly, the emission characteristics are very similar save for a slightly reduced bandwidth of the fluorescence in skin tissue, and we conclude therefore that it is possible to measure the presence of flavonoids in living human skin without any significant confounding effects. An exception is the influence of melanin, which acts as a passive absorber in skin tissue. This chromophore can be largely avoided, however, by using tissue sites with a thick stratum corneum layer, such the inner palm of the hand, since these sites are virtually free of melanin.

EXAMPLE 2

[0044] To investigate the stability of the skin flavonoids under optical excitation conditions, we investigated potential bleaching effects of the flavonoid emission. The results are shown in Figure 35. They are very similar to the bleaching kinetics of the pure flavonoids. Trace (a) in the top panel of Figure 35 shows the decrease of the skin fluorescence with time under 532 nm excitation, and trace (b), for comparison, the corresponding behavior of pure quercetin powder. Very similar bleaching kinetic is observed in both cases. However, the skin fluorescence decrease is less severe than the decrease in the pure powder sample. This effect could possibly be attributed to the lower concentration of the flavonoid compounds in the skin cells as well as to their increased stability in that environment. The plot in the bottom panel of Figure 35 shows the decrease of the skin fluorescence over time, with the excitation light turned off for 100 seconds after an initial decay period of about 300 seconds. As can be clearly seen, the fluorescence intensity does not recover to its initial level after turning the excitation light back on, thus demonstrating that the decay is irreversible, at least on the time scale of 10 minutes. The irreversible bleaching effect has to be taken into account for the optical detection of skin flavonoid content by choosing excitation in the red wavelength region, by proper reduction of excitation light power, or by decrease of the exposure time. Indeed, this can be best achieved with 632 nm excitation, as illustrated in Figure 36, where the bleaching kinetics is compared for 532 and 632 nm excitation. Clearly, the bleaching is minimized when exciting with the longer wavelength.

[0045] In Figure 37 we plot the variation of skin fluorescence with increasing 632 nm excitation light power. We observe a linear increase of the skin fluorescence intensity with increasing excitation light power from low levels of about 0.3 mW up to 3 mW. Thus, under these conditions, the skin fluorescence response is linear, and it can be used as a measure of flavonoid concentration in the illuminated tissue volume. We established that useful fluorescence measurements of living human skin could be accomplished using a laser power of less that about 2 mW, and an exposure time of 5 seconds. Taking into account an approximately 1.5 mm diameter laser light spot size on the skin, this results in an intensity of about 0.11 W/cm^2 at the skin surface, which is considered safe by ANSI Z136.1-2000 standards [11]. In fact, for the used laser intensity on the skin, the exposure time required for a measurement is about a factor of 1000 below the exposure limit set by this safety standard.

[0046] The repeatability of the skin flavonoid measurements under these conditions is shown in Figure 38, where the skin flavonoid fluorescence intensity in the maximum of the band at ~660 nm is plotted for a palm tissue site of a volunteer subject for 13 repeated measurements. Repeatability is better than about 10%.

EXAMPLE 3

[0047] To test the optimum tissue site for optical detection of skin flavonoids, we investigated several skin tissue sites, using identical excitation and detection conditions. The results are shown in Figure 39 for the tissue sites tip of an index finger, curve (a), tip of a thumb, curve (b), inner volar forearm, curve (c), and inner palm, curve (d). By far the highest flavonoid response is seen to originate from the index finger tissue site. This is likely due to the thin stratum corneum layer in this tissue site, which reduces scattering of excitation and emission wavelengths upon tissue propagation, and therefore allows for increased optical penetration into the tissue volume. However, sufficiently strong fluorescence signals are obtainable even for the palm tissue site, which has the advantage of the thickest stratum corneum among the tested tissue sites. Due to the strong scattering of the stratum corneum this has the advantage of limiting the light penetration to less dermal layers, and thereby reducing the effects of potentially confounding deeper, blood containing skin layers.

EXAMPLE 4

[0048] Using as a skin tissue site the inner palm, and 632 nm excitation, we measured the skin flavonoid emission response in several volunteer subjects. The results are shown in Figure 40. They demonstrate the same spectral shape in all subjects, but a large inter-subject variability in emission strength, and hence flavonoid content. The optical flavonoid detection method is therefore useful to assess skin flavonoid levels non-invasively, to compare flavonoid status between subjects, and to track the status over time.

EXAMPLE 5

[0049] Certain flavonoids are selectively concentrated in certain fruits and vegetables, like quercetin in the outer ring layer of onions. After removal of the outer skin, onion samples are optically clear in the visible wavelength range, and therefore well suited for flavonoid excitation and fluorescence measurements. Using the setup of Figure 3, we measured the emission of a sample under 488, 532, and 632 nm excitation. The result is shown in Figure 41. The emission behavior is very similar again to that of the pure flavonoid samples of Figure 6, featuring almost identical maxima of the emission bands, reduced halfwidths and spectral shifts with increasing excitation wavelengths. In Figure 42, the emission behavior of the onion sample obtained under 632 nm is compared directly with the emission spectra of pure quercetin and kaempferol. Clearly, the shape of the emission spectra is very similar, suggesting that the emission spectra of the onion sample is indeed due to flavonoids, and that the fluorescence spectroscopy method described in this patent application, is suitable also for the noninvasive flavonoids measurement of vegetables having sufficient optical clarity in the wavelength range of interest. Even in samples with potentially confounding concentrations from other pigments, the optical detection method may still be viable. An example is Figure 43, in which the fluorescence spectra obtained with 632 nm excitation are shown for red and green grape samples containing other pigments than flavonoids. The fluorescence of these pigments appears in a narrow band centered at ~680 nm, and is clearly distinguishable from the fluorescence of flavonoids, such as those in the onion sample, which again is shown for comparison, and in which the fluorescence is peaking near 660 nm.

EXAMPLE 6

[0050] For the optical detection of flavonoids in living human tissue it may be possible to facilitate the required instrumentation by eliminating the spectrograph and to use instead a filter based optical setup -- at least in the case of melanin-free skin tissue sites and at red excitation wavelengths avoiding confounding chromophores. Basically, such an instrument which is sketched in Figure 44, and which would be economically very attractive, would consist of a simple excitation beam path that illuminates the tissue site of interest with a filtered light source. Furthermore, it would contain a detection beam path that collects the fluorescence from the skin tissue via collimator, filters the emission with a suitable long pass filter, and quantifies the emission intensity via photodiode/computer combination.

REFERENCES

[0051]

1. Formica JV, Regelson W, Review of the biology of quercetin and related bioflavonoids, Food Chem. Toxicol. 1995; 33:1061-80.

2. Flavonoids: Chemistry, Biochemistry, and Applications, O. M. Andersen and K. R. Markham, Eds., CRC Press, Taylor & Francis Group, LLC, 2006.

3. Hanneken A, Lin F-F, Johnson J, Maher P 2006, Investigative Ophthalmology & Visual Science 47, 3164-77.

4. Rice-Evans CA, Miller NJ, Paganga G, Free Radic. Biol. Med. 1996; 20:933.

5. Bohm BA, Introduction to Flavonoids. Chemistry and Biochemistry of Organic Natural Products. Harwood Academic Publisher: Amsterdam, 1998:200.

6. Jurasekova Z, Garcia-Ramos JV, Domingo C, Sanchez-Cortes S, Surface-enhanced Raman scattering of flavonoids, J. Raman Spectrosc. 2006; 37: 1239-1241.

7. Cornard JP, Merlin JC, Boudet AC, Vrielynck L, http://www3.interscience.wiley.com/journal/56500/issue

8. Cornard JP, Dangleterre, Lapouge C 2005, J. Phys. Chem. A; 109:10044-10051.

9. Gellermann W et al., US patent 6,205,354 B1.

10. Lotito SB, Frei B 2006; Free Radic. Biol. Med. 41 (12): 1727-46.

11. American National Standards Institute, *American National Standard for Safe Use of Lasers,* ANSI Z136.1-2000, Laser Institute of America, Orlando, FL (2000).

Claims

1. A noninvasive method of measuring flavonoid levels in biological tissue, comprising the steps of:

illuminating a localized region of tissue with light that overlaps the absorption bands of a flavonoid compound;
detecting the fluorescence emitted by the flavonoid compound resulting from the illumination; and
determining the concentration level of the flavonoid compound based upon the detected fluorescence,
wherein the tissue is human skin, such as the skin on a fingertip or other portion of a hand.

2. The method of claim 1, including the step of using the concentration level to assess the antioxidant status of the tissue.

3. The method of claim 1, including the step of comparing the concentration level to levels of normal biological tissue to assess the risk or presence of a malignancy or other disease.

4. The method of claim 1, including the step of illuminating the localized region of tissue with light in the 300 to 650 nm spectral region.

5. The method of claim 1, wherein the step of detecting the fluorescence emitted by the flavonoid compound includes the use of fluorescence spectroscopy or the use of an optical detector.

6. The method of claim 5, wherein, when using an optical detector, the light is in the red region of the spectrum.

7. A system for measuring flavonoid levels in biological tissue noninvasively, comprising:

a source of light for illuminating a localized region of tissue with light that overlaps the absorption bands of a flavonoid compound;
a device for detecting the fluorescence emitted by the flavonoid compound resulting from the illumination; and
a processor for determining the concentration level of the flavonoid compound based upon the

detected fluorescence,
wherein the tissue is human skin, such as the skin on a fmgertip or other portion of a hand.

8. The system of claim 7, wherein:

a) the processor is operative to assess the antioxidant status of the tissue based upon the concentration level; and optionally
b) wherein the system further includes a memory for storing flavonoid concentration levels associated with normal biological tissue; and wherein the processor is operative to compare the determined concentration level to the stored levels to assess the risk or presence of a malignancy or other disease.

9. The system of claim 7, wherein the light source is in the 300 to 650 nm spectral region.

10. The system of claim 7, wherein the device for detecting the fluorescence emitted by the flavonoid compound includes a fluorescence spectrograph or an optical detector in electrical communication with the processor.

11. The system of claim 10, wherein, when using an optical detector, the source of light is in the red region of the spectrum.

12. The method of claim 6 or the system of claim 10, wherein the localized region of tissue is substantially melanin-free.

13. The method of claim 1 or the system of claim 7, wherein the source of light is outside the absorption range of potentially confounding skin chromophores and wherein, optionally, the chromophores include carotenoids, blood, elastin and collagen.

14. The method of claim 1 or the system of claim 7, wherein the source of light excites the tissue flavonoids in their long-wavelength absorption tail outside the absorption range of other skin chromophores.

Patentansprüche

1. Nichtinvasives Verfahren zum Messen des Flavonoidgehalts in biologischem Gewebe, das die folgenden Schritte aufweist:

Beleuchten einer örtlich begrenzten Geweberegion mit Licht, das die Absorptionsbanden einer Flavonoidverbindung überlappt;
Ermitteln der sich aus der Beleuchtung ergebenden, durch die Flavonoidverbindung ausgesendeten Fluoreszenz; und
Bestimmen des Konzentrationsniveaus der Flavonoidverbindung basierend auf der ermittelten Fluoreszenz,
wobei das Gewebe menschliche Haut ist, wie zum Beispiel die Haut auf einer Fingerspitze oder einem anderen Teil einer Hand.

2. Verfahren gemäß Anspruch 1, einschließlich des Schrittes der Verwendung des Konzentrationsniveaus, um den Antioxidationszustand des Gewebes festzustellen.

3. Verfahren gemäß Anspruch 1, einschließlich des Schrittes des Vergleichens des Konzentrationsniveaus mit Niveaus von normalem biologischen Gewebe zur Beurteilung des Risikos oder Vorhandenseins einer Malignität oder sonstigen Krankheit.

4. Verfahren gemäß Anspruch 1, einschließlich des Schrittes des Beleuchtens der örtlich begrenzten Region des Gewebes mit Licht im Spektralbereich von 300 bis 650 nm.

5. Verfahren gemäß Anspruch 1, wobei der Schritt des Ermittelns der durch die Flavonoidverbindung ausgesendeten Fluoreszenz die Verwendung von Fluoreszenzspektroskopie bzw. die Verwendung eines optischen Detektors beinhaltet.

6. Verfahren gemäß Anspruch 5, wobei bei Verwendung eines optischen Detektors sich das Licht im Rotbereich des Spektrums befindet.

7. System zum nichtinvasivem Messen des Flavonoidgehalts in biologischem Gewebe, das Folgendes aufweist:

eine Lichtquelle zum Beleuchten einer örtlich begrenzten Geweberegion mit Licht, das die Absorptionsbanden einer Flavonoidverbindung überlappt;
eine Vorrichtung zum Ermitteln der sich aus der Beleuchtung ergebenden, durch die Flavonoidverbindung ausgesendeten Fluoreszenz; und
einen Prozessor zum Bestimmen des Konzentrationsniveaus der Flavonoidverbindung basierend auf der ermittelten Fluoreszenz,
wobei das Gewebe menschliche Haut ist, wie zum Beispiel die Haut auf einer Fingerspitze oder einem anderen Teil einer Hand.

8. System gemäß Anspruch 7, wobei:

a) der Prozessor dahingehend betriebsfähig ist, den Antioxidationsstatus des Gewebes basierend auf dem Konzentrationsniveau zu bewerten; und optional
b) wobei das System darüber hinaus einen Spei-

cher zum Speichern von Flavonoid-Konzentrationsniveaus beinhaltet, die normalem biologischen Gewebe zugeordnet sind; und wobei der Prozessor dahingehend betriebsfähig ist, das ermittelte Konzentrationsniveau mit den gespeicherten Niveaus zu vergleichen, um das Risiko oder das Vorhandensein einer Malignität oder sonstigen Krankheit zu beurteilen.

9. System gemäß Anspruch 7, wobei sich die Lichtquelle im Spektralbereich von 300 bis 650 nm befindet.

10. System gemäß Anspruch 7, wobei die Vorrichtung zum Ermitteln der durch die Flavonoidverbindung ausgesendeten Fluoreszenz einen Fluoreszenzspektrographen oder einen optischen Detektor in elektrischer Verbindung mit dem Prozessor beinhaltet.

11. System gemäß Anspruch 10, wobei bei Verwendung eines optischen Detektors sich die Lichtquelle im Rotbereich des Spektrums befindet.

12. Verfahren gemäß Anspruch 6 oder System gemäß Anspruch 10, wobei der örtlich begrenzte Gewebebereich im Wesentlichen melaninfrei ist.

13. Verfahren gemäß Anspruch 1 oder System gemäß Anspruch 7, wobei sich die Lichtquelle außerhalb des Absorptionsbereichs von sich potentiell störenden Hautchromophoren befindet und wobei die Chromophore optional Carotinoide, Blut, Elastin und Kollagen beinhalten.

14. Verfahren gemäß Anspruch 1 oder System gemäß Anspruch 7, wobei die Lichtquelle die Gewebeflavonoide in ihrem langwelligen Absorptionsende außerhalb des Absorptionsbereichs von anderen Hautchromophoren anregt.

Revendications

1. Procédé non invasif de mesure de niveaux de flavonoïde dans un tissu biologique, comprenant les étapes de :

illumination d'une région localisée de tissu avec de la lumière qui chevauche les bandes d'absorption d'un composé flavonoïde ;
détection de la fluorescence émise par le composé flavonoïde résultant de l'illumination ; et
détermination du niveau de concentration du composé flavonoïde basé sur la fluorescence détectée,
dans lequel le tissu est la peau humaine, telle que la peau d'un bout de doigt ou d'une autre partie d'une main.

2. Procédé selon la revendication 1, comprenant l'étape d'utilisation du niveau de concentration pour évaluer l'état antioxydant du tissu.

3. Procédé selon la revendication 1, comprenant l'étape de comparaison du niveau de concentration aux niveaux de concentration d'un tissu biologique normal pour évaluer le risque ou la présence d'une malignité ou d'une autre maladie.

4. Procédé selon la revendication 1, comprenant l'étape d'illumination de la région localisée de tissu avec de la lumière dans la région spectrale de 300 à 650 nm.

5. Procédé selon la revendication 1, dans lequel l'étape de détection de la fluorescence émise par le composé flavonoïde comprend l'utilisation de la spectroscopie de fluorescence ou l'utilisation d'un détecteur optique.

6. Procédé selon la revendication 5, dans lequel, quand on utilise un détecteur optique, la lumière est dans la région rouge du spectre.

7. Système pour mesurer les niveaux de flavonoïde das un tissu biologique de manière non invasive, comprenant :

une source de lumière pour illuminer une région localisée de tissu avec de la lumière qui chevauche les bandes d'absorption d'un composé flavonoïde ;
un dispositif pour détecter la fluorescence émise par le composé flavonoïde résultant de l'illumination ; et
un processeur pour déterminer le niveau de concentration du composé flavonoïde basé sur la fluorescence détectée,
dans lequel le tissu est la peau humaine, telle que la peau sur un bout de doigt ou une autre partie d'une main.

8. Système selon la revendication 7, dans lequel :

a) le processeur est opérationnel pour évaluer l'état antioxydant du tissu basé sur le niveau de concentration ; et éventuellement
b) dans lequel le système comprend en outre une mémoire pour mémoriser les niveaux de concentration de flavonoïde associés avec un tissu biologique normal ; et dans lequel le processeur est opérationnel pour comparer le niveau de concentration déterminé aux niveaux mémorisés pour évaluer le risque ou la présence d'une malignité ou d'une autre maladie.

9. Système selon la revendication 7, dans lequel la source de lumière est dans la région spectrale de 300 à 650 nm.

10. Système selon la revendication 7, dans lequel le dispositif pour détecter la fluorescence émise par le composé flavonoïde comprend un spectrographe de fluorescence ou un détecteur optique en communication électrique avec le processeur.

11. Système selon la revendication 10, dans lequel, quand on utilise un détecteur optique, la source de lumière est dans la région rouge du spectre.

12. Procédé selon la revendication 6 ou système selon la revendication 10, dans lequel la région localisée de tissu est essentiellement sans mélanine.

13. Procédé selon la revendication 1 ou système selon la revendication 7, dans lequel la source de lumière est à l'extérieur de la plage d'absorption de chromophores de la peau potentiellement confondants et dans lequel, éventuellement, les chromophores comprennent les caroténoïdes, le sang, l'élastine et le collagène.

14. Procédé selon la revendication 1 ou système selon la revendication 7, dans lequel la source de lumière excite les flavonoïdes tissulaires dans leur queue d'absorption de longue longueur d'onde hors de la plage d'absorption d'autres chromophores de la peau.

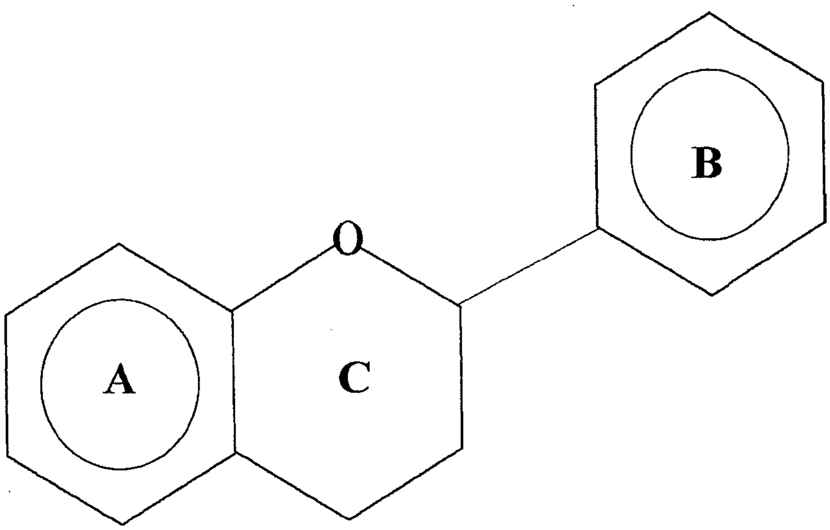


Fig. 1

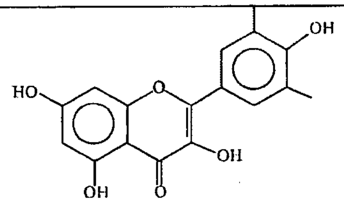
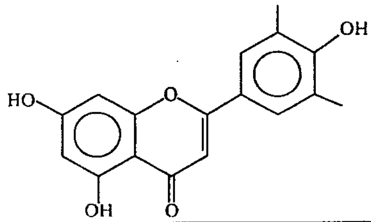
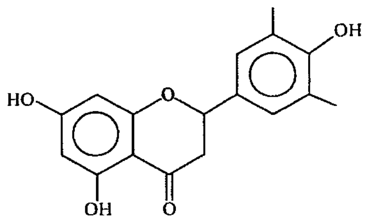
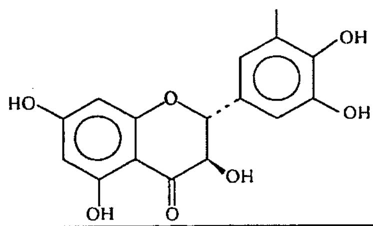
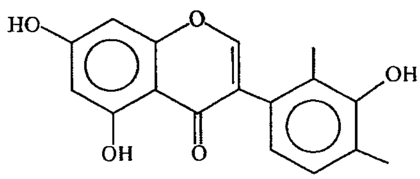
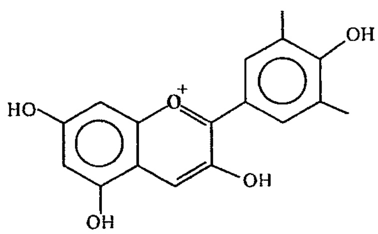
Categories	Molecular Structures	Compounds	Major Food Sources
Flavonols		Quercetin Kaempferol Myricetin	yellow onion, kale, leeks, cherries, broccoli, apples, tea, grapes
Flavones		Apigenin Luteolin Diosmin	parsley, celery, red pepper
Flavanones		Naringenin Hesperitin	citrus fruit
Catechins		Catechin Gallocatechin Epigallocatechin	cocoa, beans, apricots, cherries, grapes, peaches, red wine, cider, tea, blackberries
Isoflavones		Genistein	soy beans
Anthocyanidins		Pelargonidin	blueberries, black currants, grapes, cherries, rhubarb, plums, strawberries, red wine, red cabbage

Fig. 2

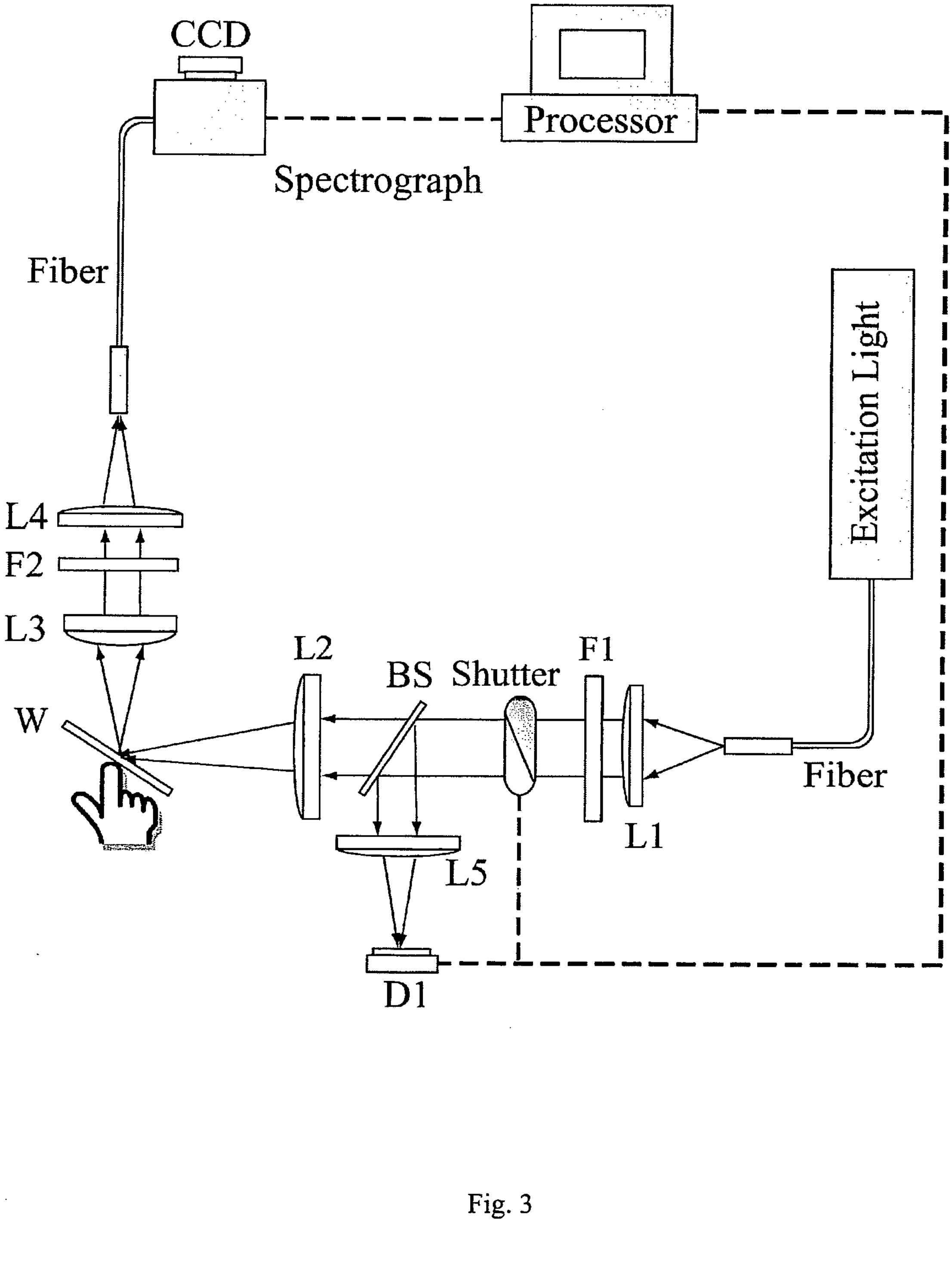

CCD
Processor
Spectrograph
Fiber
Excitation Light
L4
F2
L3
L2
BS
Shutter
F1
W
Fiber
L1
L5
D1

Fig. 3

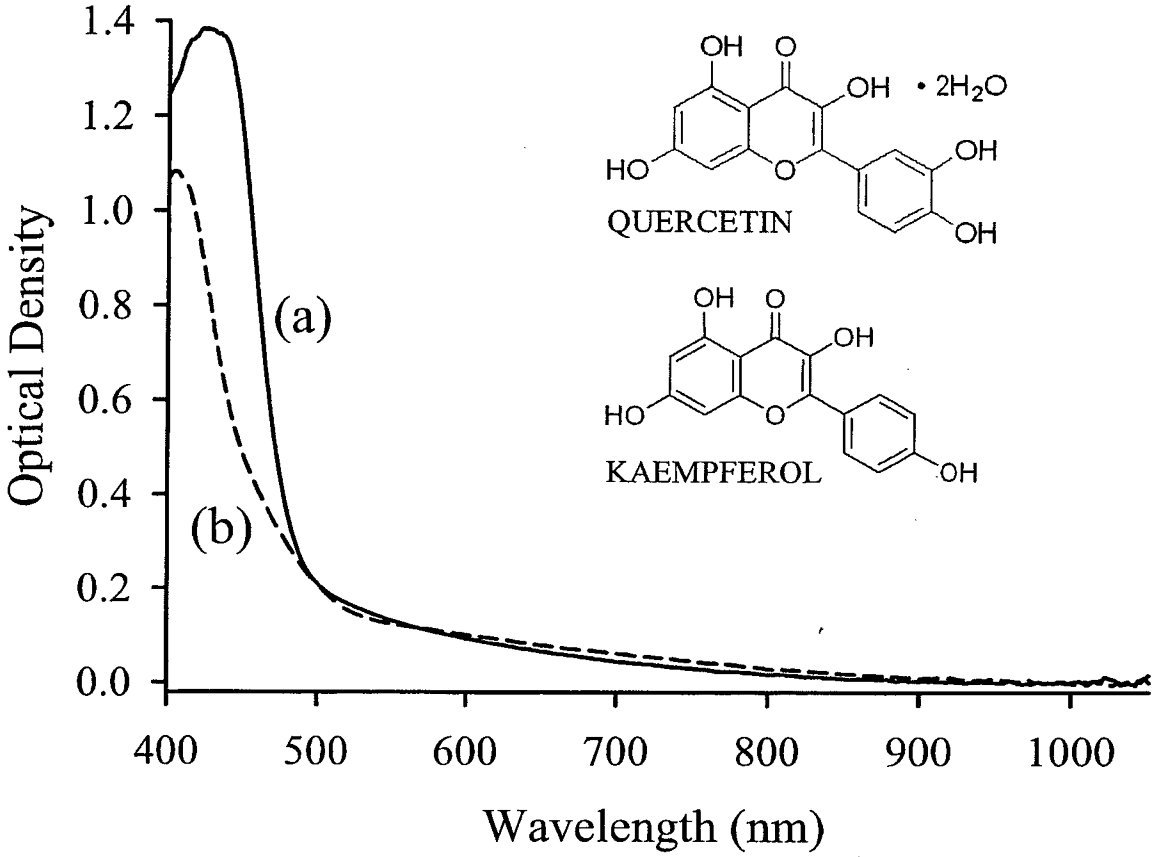

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Optical Density
(a)
(b)
400
500
600
700
800
900
1000
Wavelength (nm)
OH
O
OH
• $2H_2O$
HO
O
OH
QUERCETIN
OH
OH
O
OH
HO
O
KAEMPFEROL
OH

Fig. 4

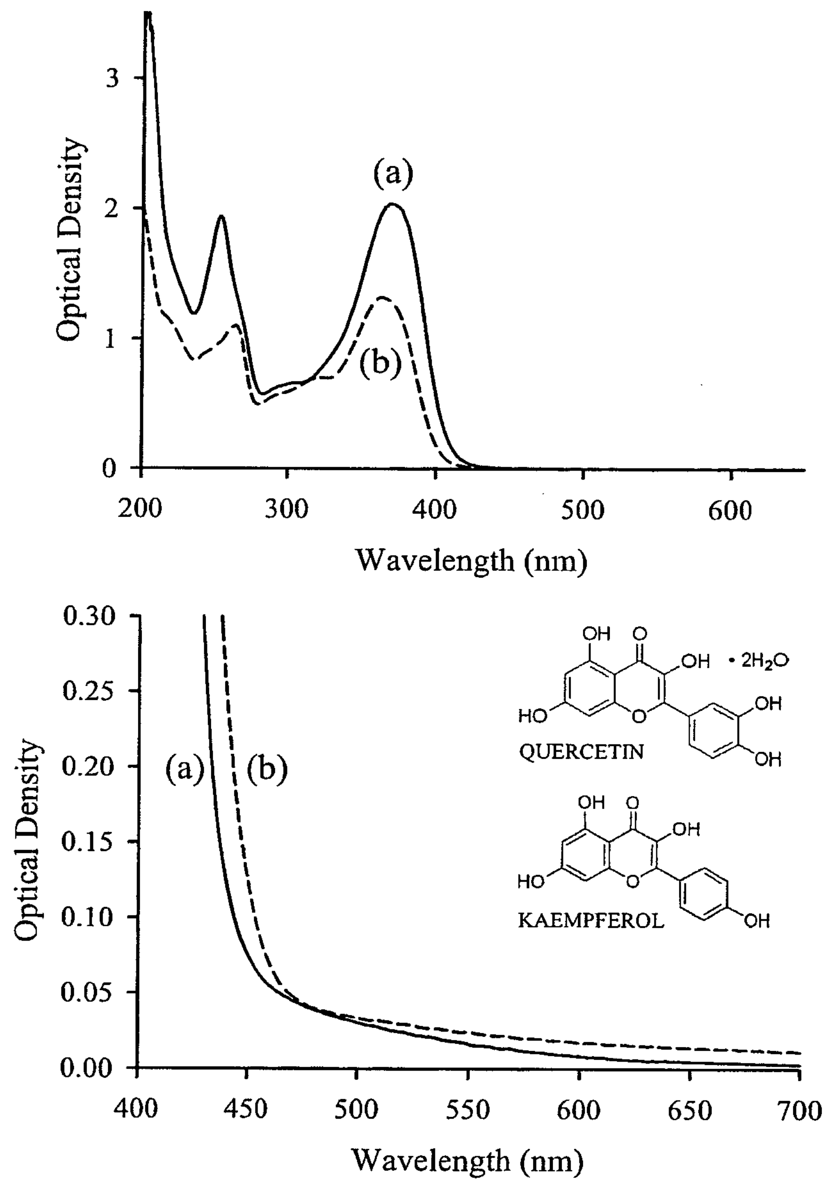

3
2
1
0
Optical Density
(a)
(b)
200
300
400
500
600
Wavelength (nm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Optical Density
(a)
(b)
400
450
500
550
600
650
700
Wavelength (nm)
OH
O
OH
• 2H2O
HO
O
OH
QUERCETIN
OH
OH
O
OH
HO
O
KAEMPFEROL
OH

Fig. 5

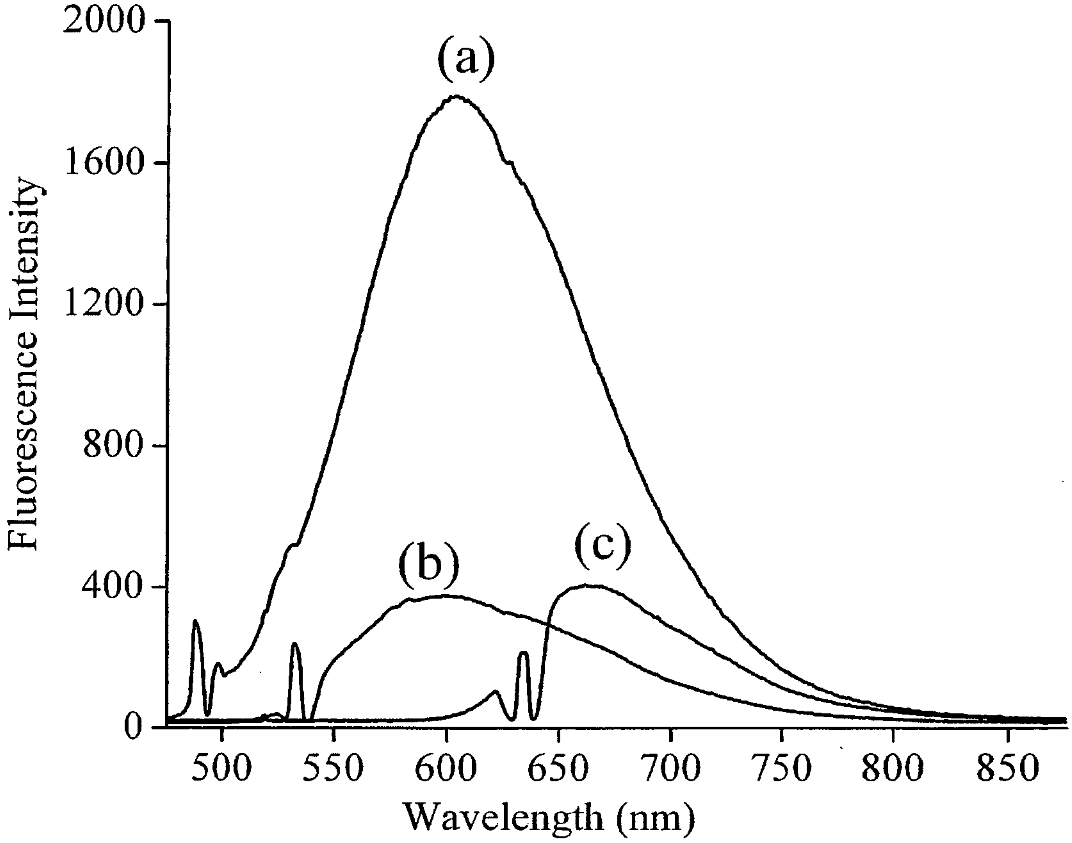

2000
1600
1200
800
400
0
Fluorescence Intensity
(a)
(b)
(c)
500
550
600
650
700
750
800
850
Wavelength (nm)

Fig. 6

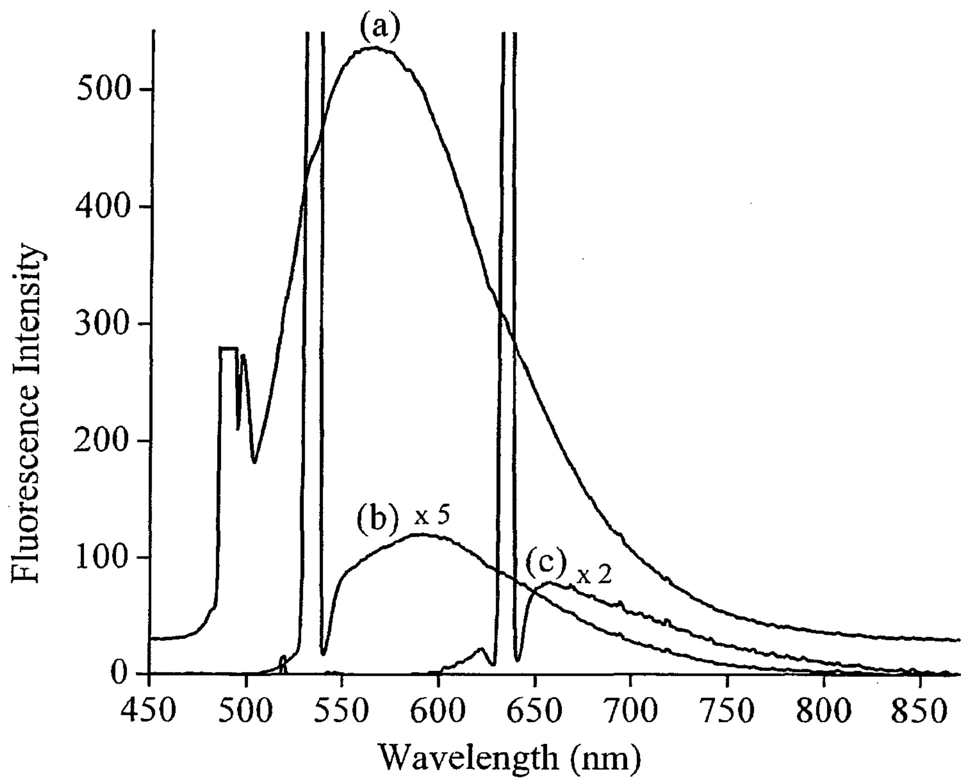

(a)
(b) x 5
(c) x 2
Fluorescence Intensity
Wavelength (nm)
0
100
200
300
400
500
450
500
550
600
650
700
750
800
850

Fig. 7

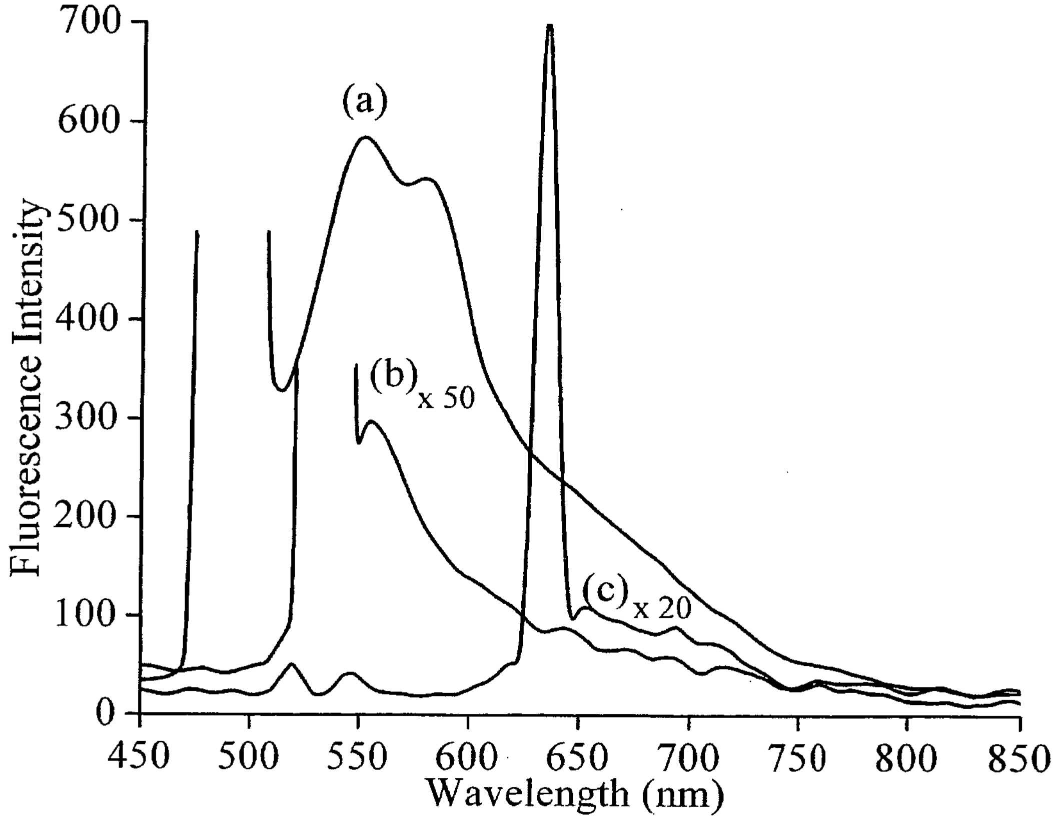

700
600
500
400
300
200
100
0
Fluorescence Intensity
(a)
(b)x 50
(c)x 20
450
500
550
600
650
700
750
800
850
Wavelength (nm)

Fig. 8

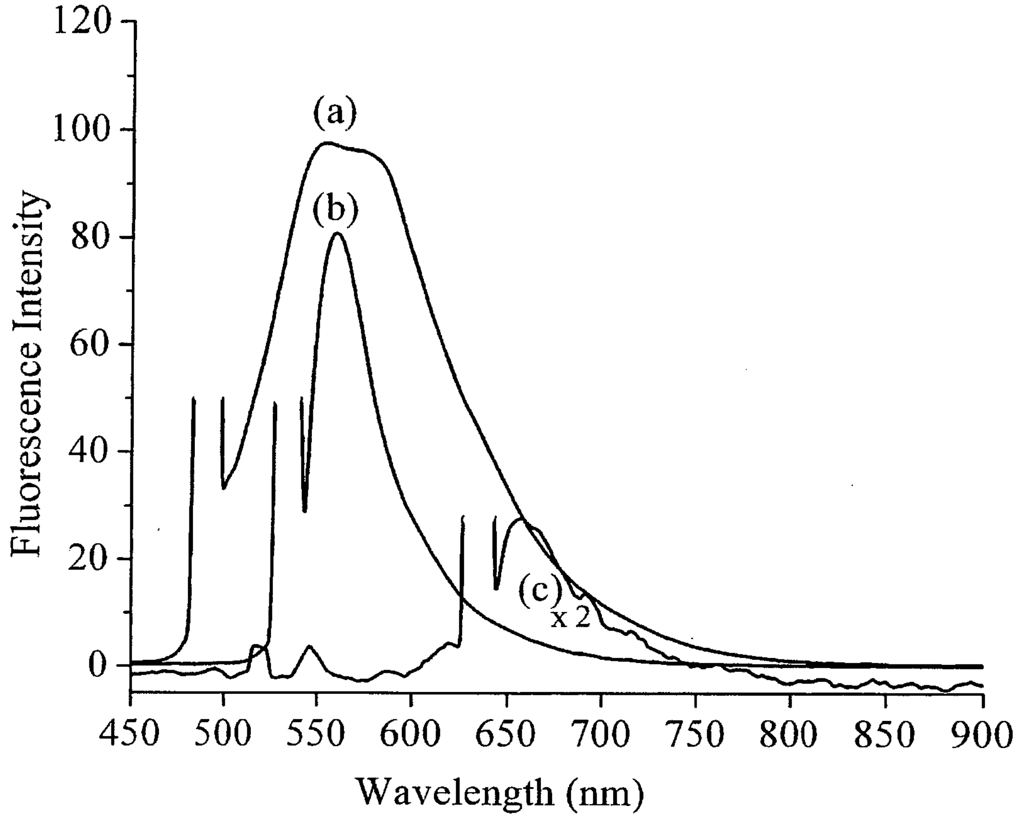

120
100
80
60
40
20
0
Fluorescence Intensity
(a)
(b)
(c)
x 2
450 500 550 600 650 700 750 800 850 900
Wavelength (nm)

Fig. 9

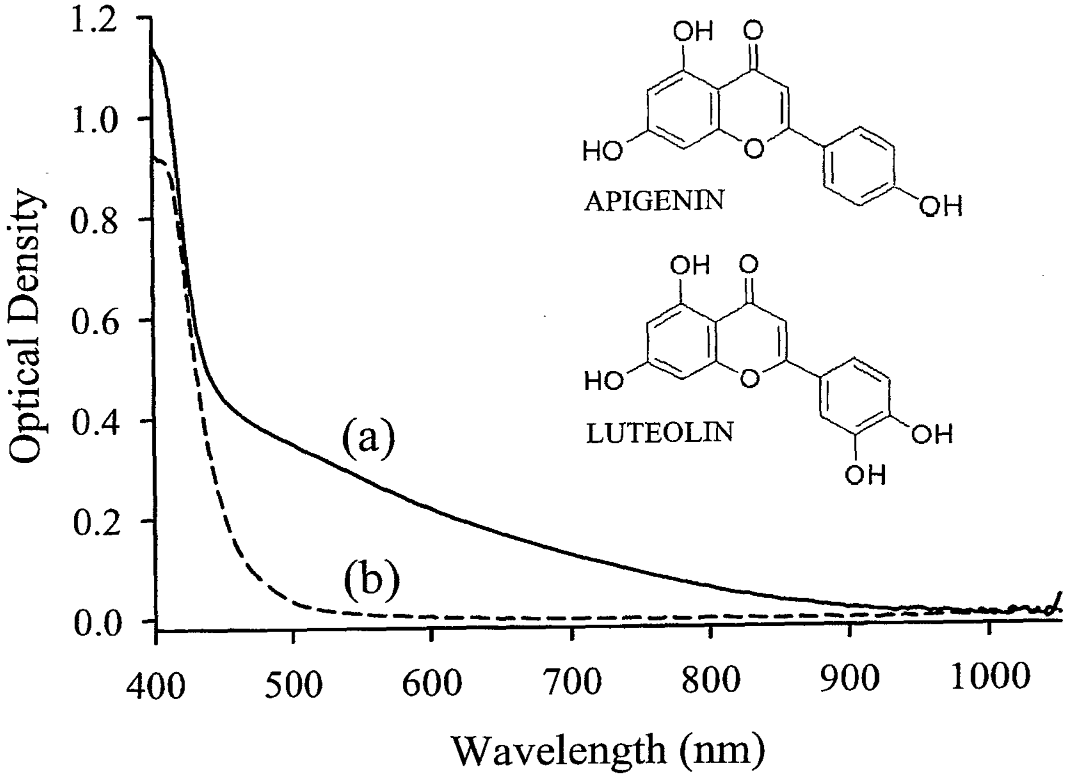

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Optical Density
400
500
600
700
800
900
1000
Wavelength (nm)
(a)
(b)
APIGENIN
LUTEOLIN
OH
O
HO
OH

Fig. 10

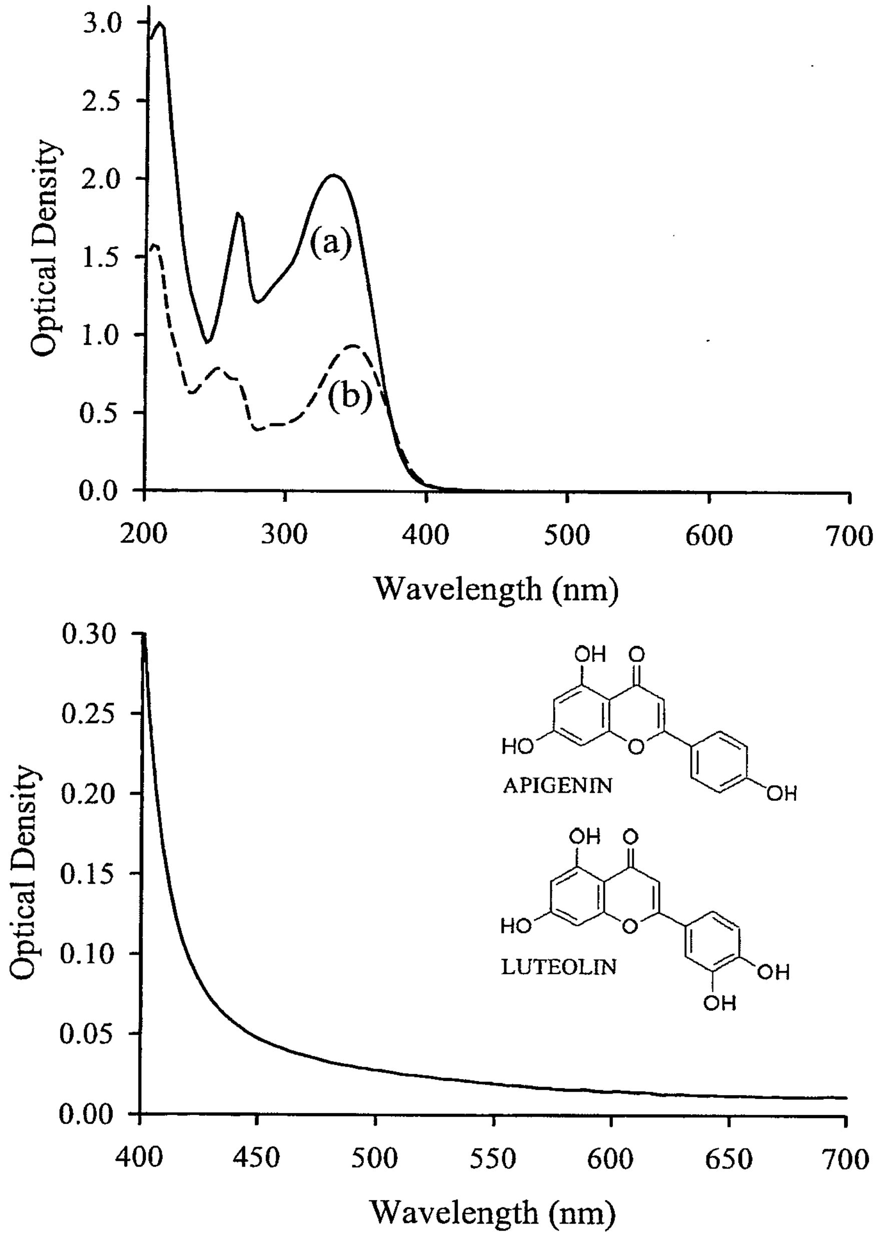
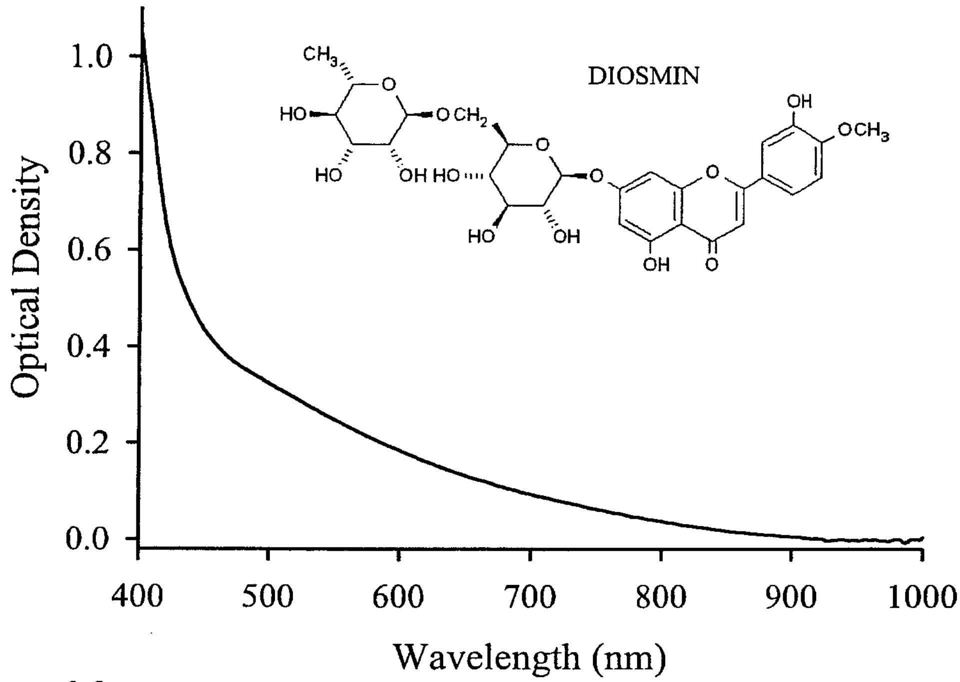

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Optical Density
(a)
(b)
200
300
400
500
600
700
Wavelength (nm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Optical Density
OH
O
HO
O
OH
APIGENIN
OH
O
HO
O
OH
OH
LUTEOLIN
400
450
500
550
600
650
700
Wavelength (nm)

Fig. 11


DIOSMIN
Optical Density
Wavelength (nm)
1.0
0.8
0.6
0.4
0.2
0.0
400
500
600
700
800
900
1000

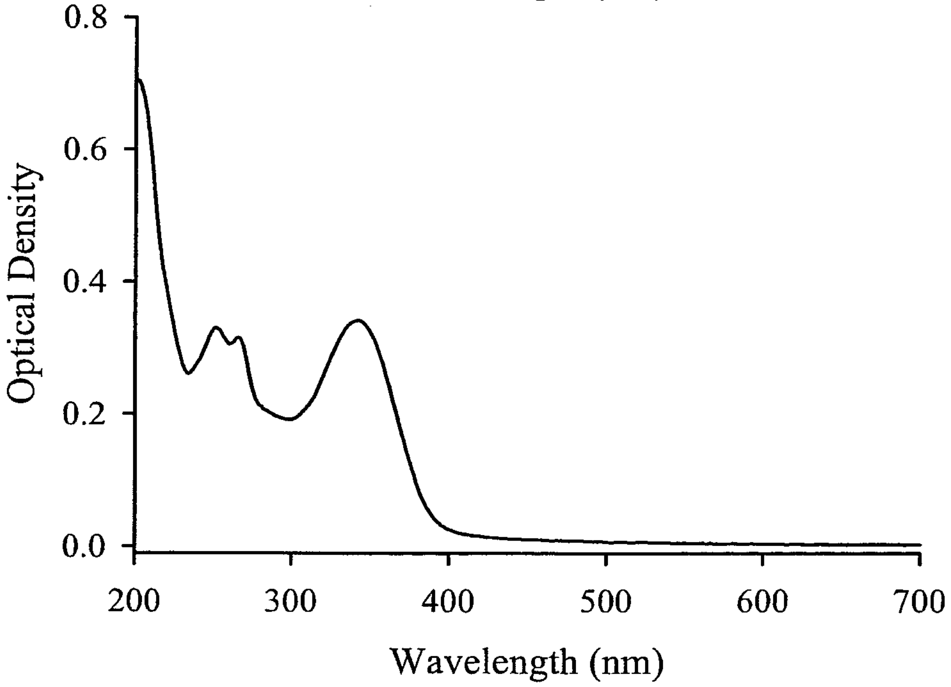

Optical Density
Wavelength (nm)
0.8
0.6
0.4
0.2
0.0
200
300
400
500
600
700

Fig. 12

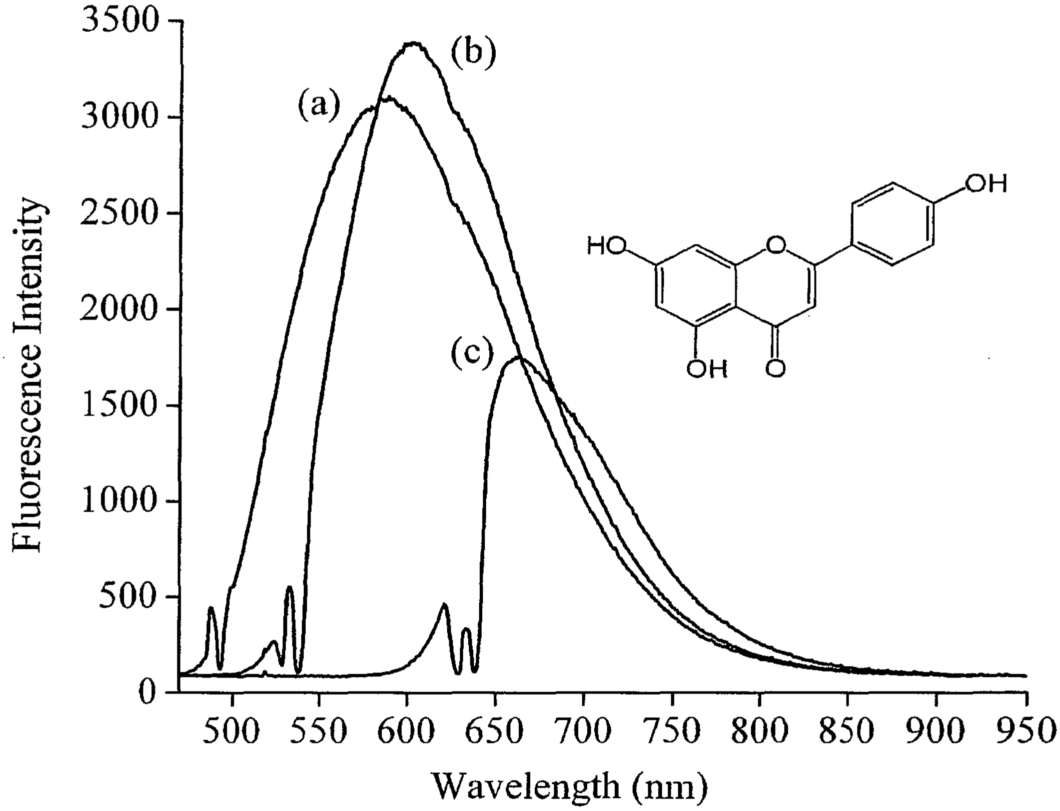

3500
3000
2500
2000
1500
1000
500
0
Fluorescence Intensity
500 550 600 650 700 750 800 850 900 950
Wavelength (nm)
(a)
(b)
(c)
HO
O
OH
OH
O

Fig. 13

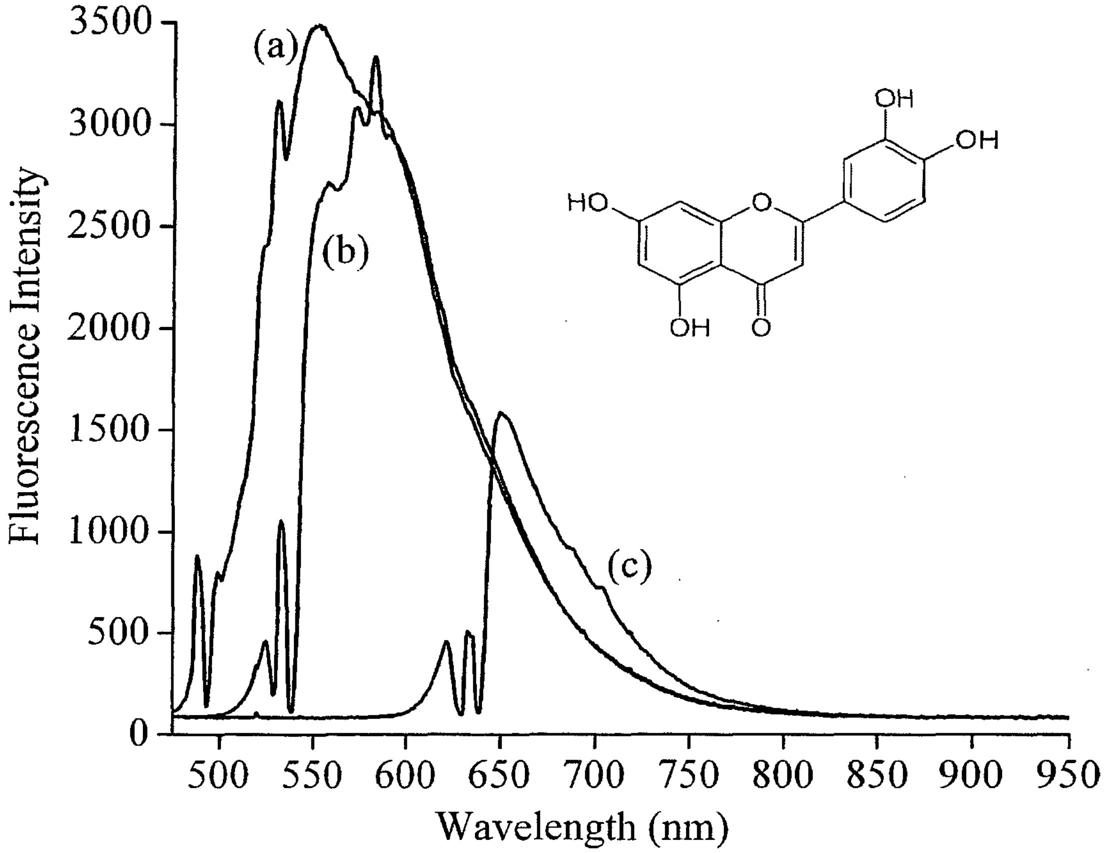

3500
3000
2500
2000
1500
1000
500
0
Fluorescence Intensity
500
550
600
650
700
750
800
850
900
950
Wavelength (nm)
(a)
(b)
(c)
OH
OH
HO
O
OH
O

Fig. 14

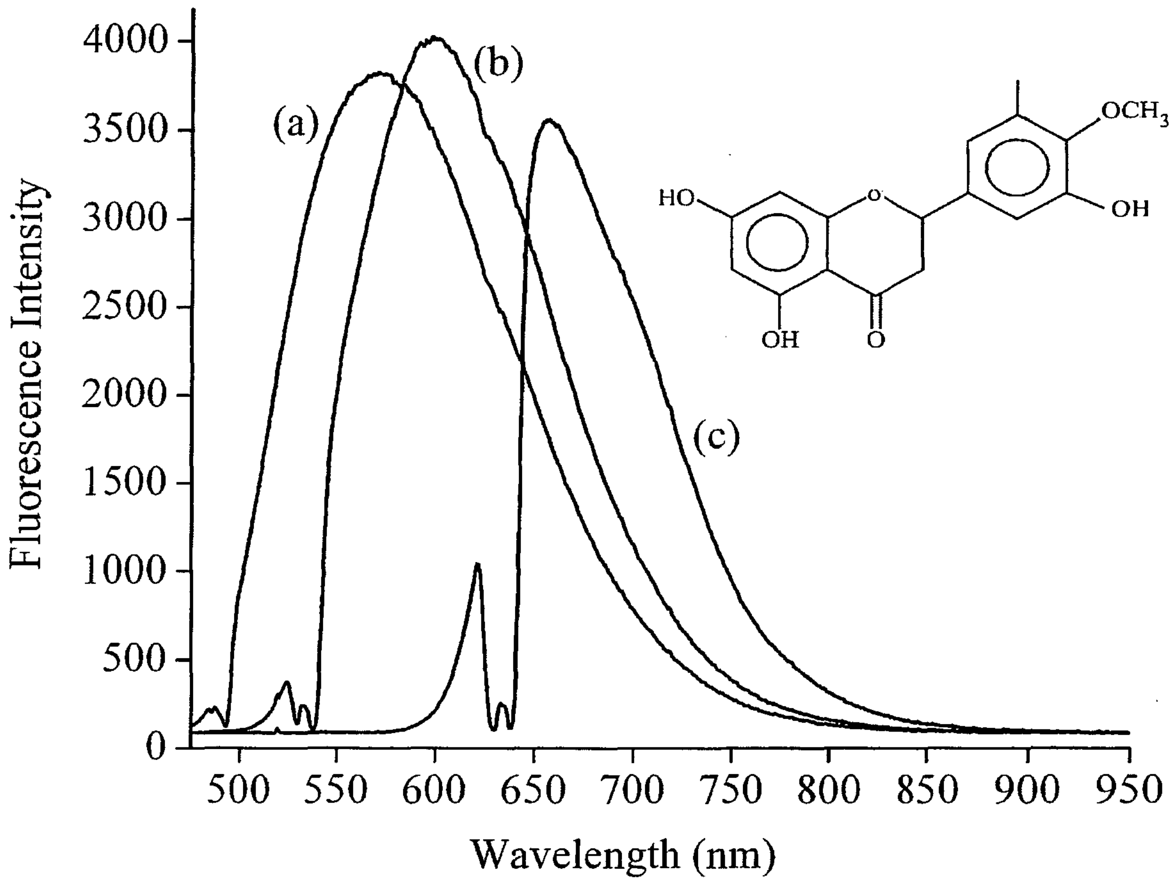

Fluorescence Intensity
Wavelength (nm)
(a)
(b)
(c)
0
500
1000
1500
2000
2500
3000
3500
4000
500
550
600
650
700
750
800
850
900
950
HO
O
OCH3
OH
OH
O

Fig. 15

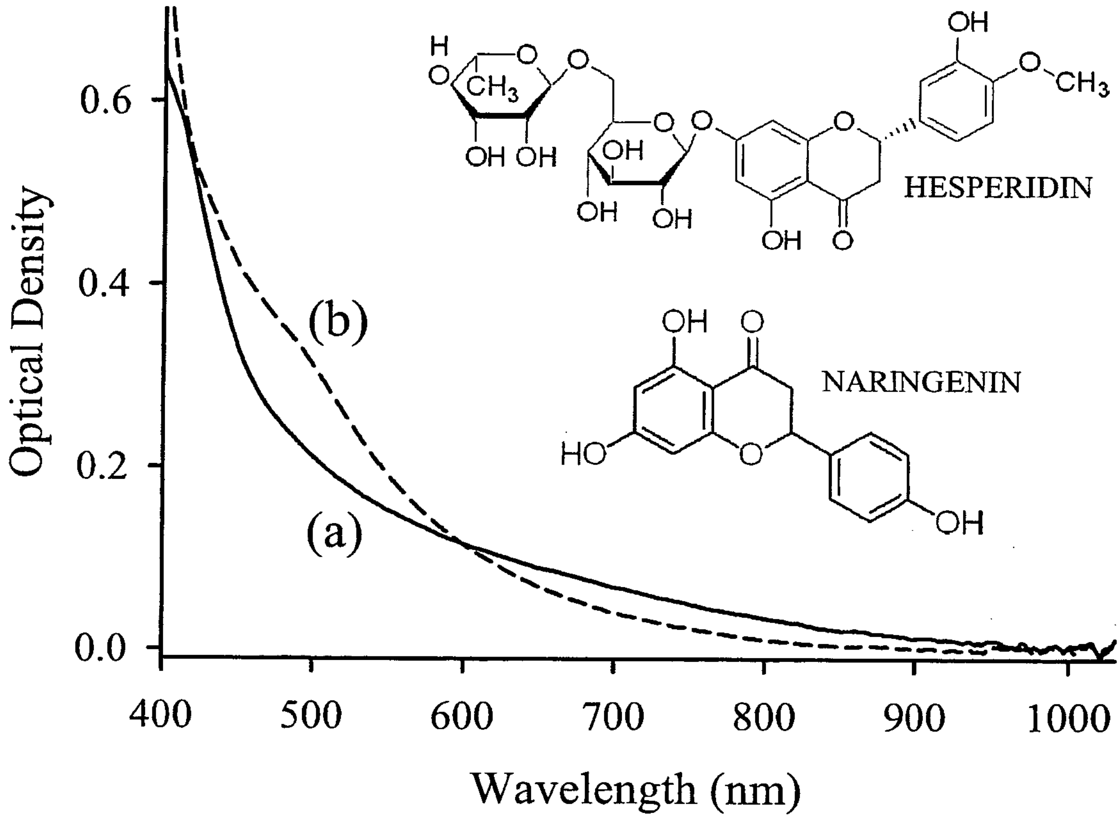

Optical Density
0.6
0.4
0.2
0.0
400
500
600
700
800
900
1000
Wavelength (nm)
(a)
(b)
HESPERIDIN
NARINGENIN

Fig. 16

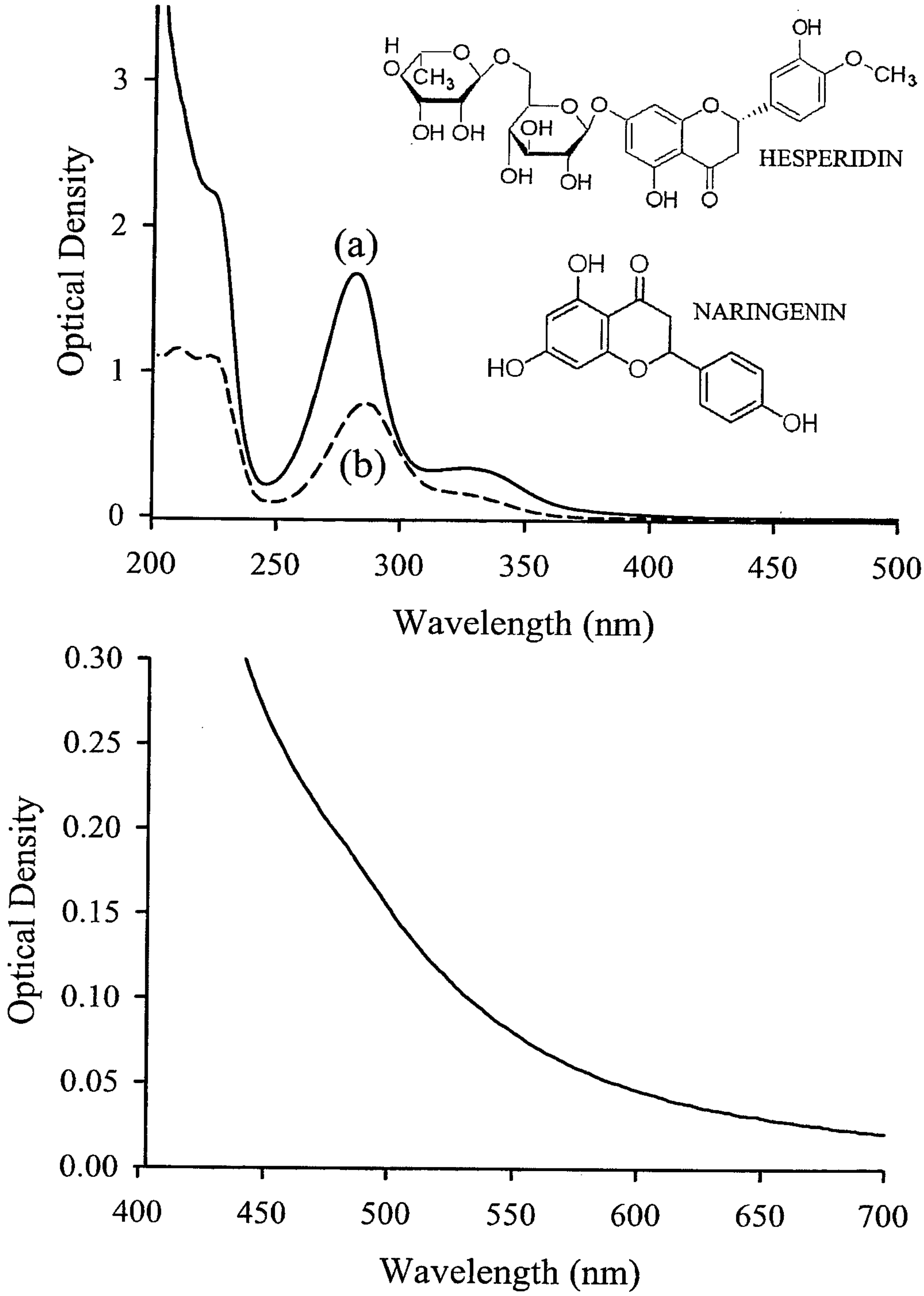
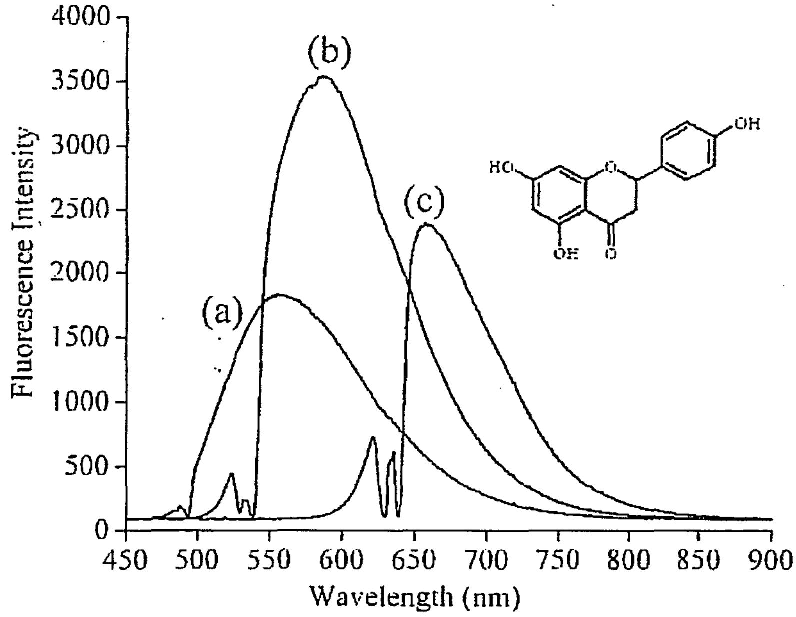

HESPERIDIN
NARINGENIN
(a)
(b)
Optical Density
Wavelength (nm)
Optical Density
Wavelength (nm)

Fig. 17


4000
3500
3000
2500
2000
1500
1000
500
0
450 500 550 600 650 700 750 800 850 900
Wavelength (nm)
Fluorescence Intensity
(a)
(b)
(c)
HO
OH
OH
O

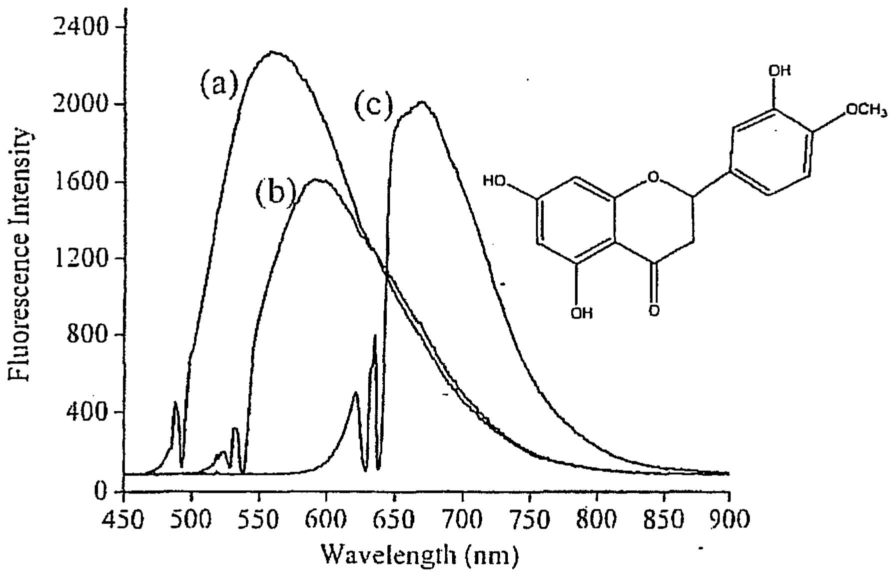

2400
2000
1600
1200
800
400
0
450 500 550 600 650 700 750 800 850 900
Wavelength (nm)
Fluorescence Intensity
(a)
(b)
(c)
OH
OCH3
HO
O
OH
O

Fig. 18

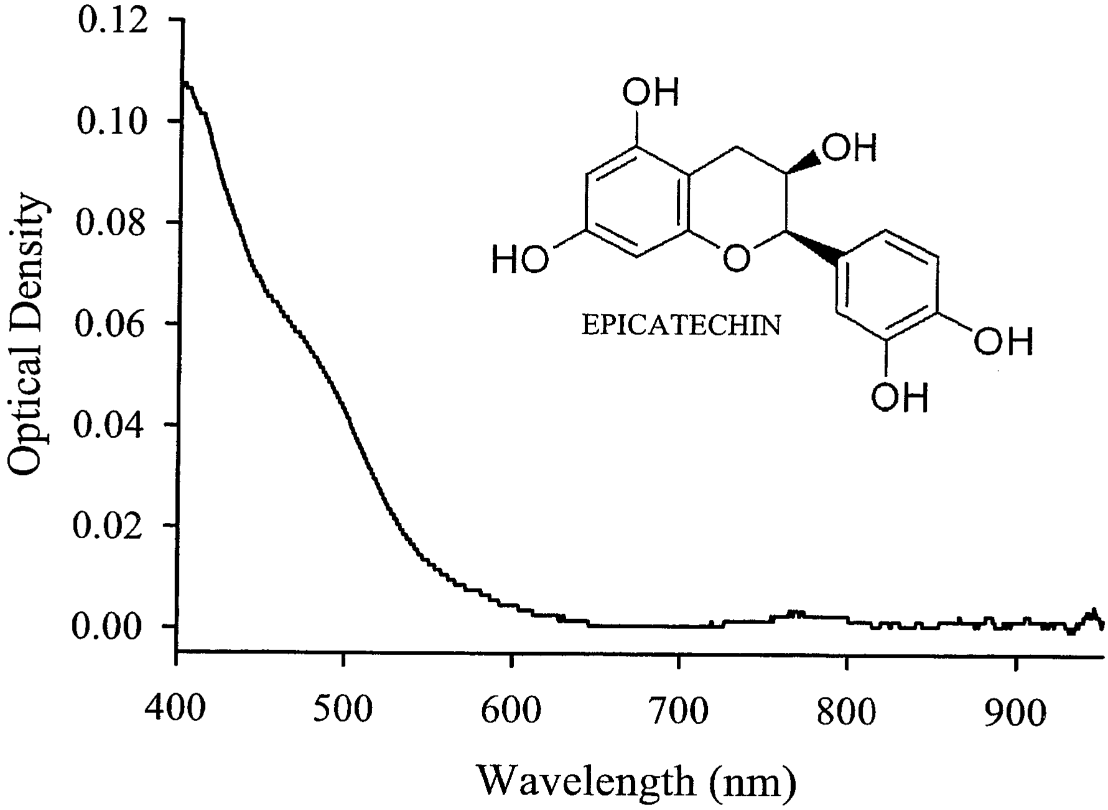
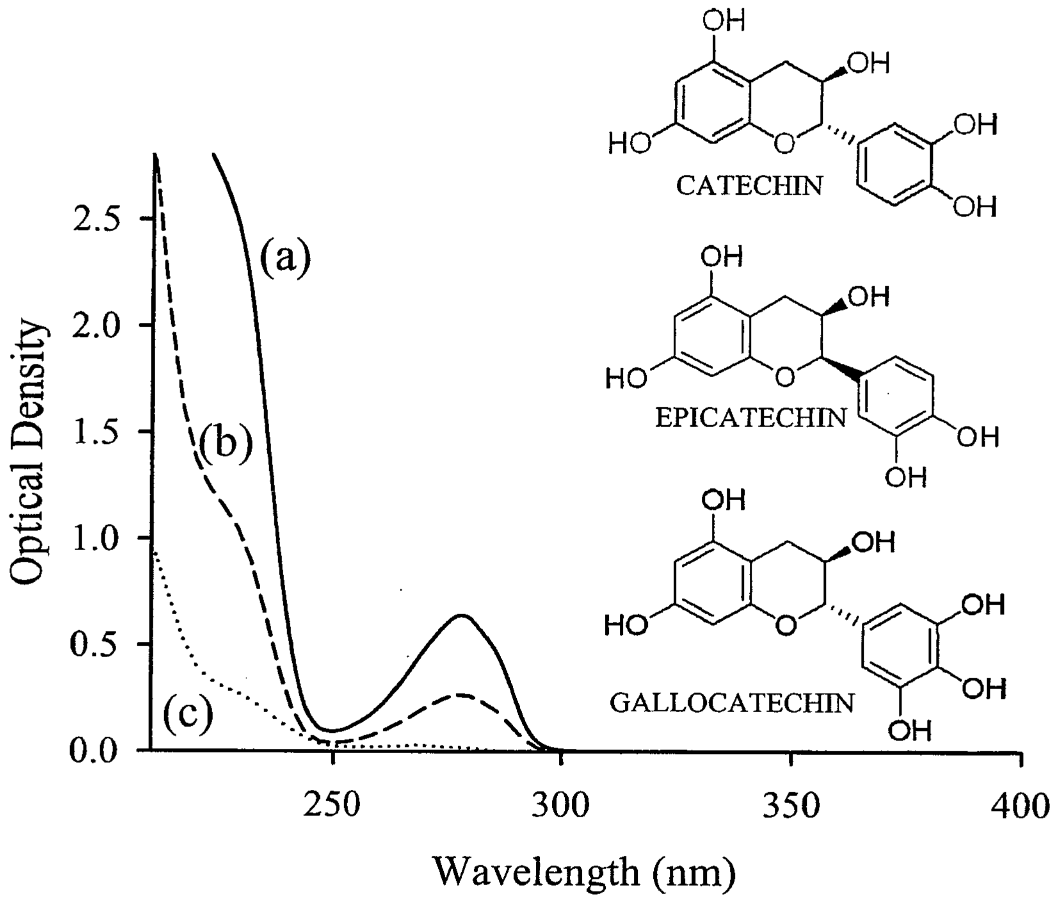

0.12
0.10
0.08
0.06
0.04
0.02
0.00
Optical Density
400
500
600
700
800
900
Wavelength (nm)
OH
OH
HO
O
OH
OH
EPICATECHIN

Fig. 19



OH
OH
HO
O
OH
CATECHIN
OH
OH
OH
HO
O
EPICATECHIN
OH
OH
OH
OH
HO
O
OH
GALLOCATECHIN
OH
OH
(a)
(b)
(c)
2.5
2.0
1.5
1.0
0.5
0.0
Optical Density
250
300
350
400
Wavelength (nm)

Fig. 20

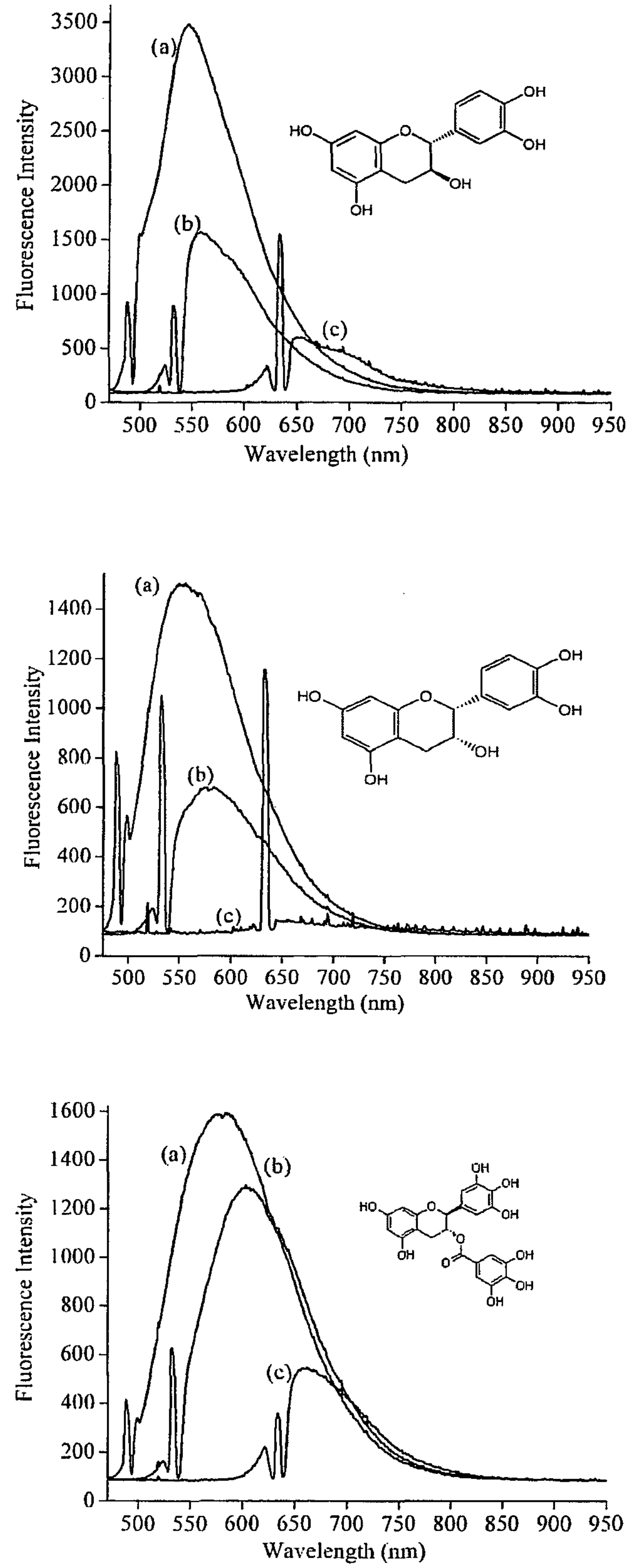


Fig. 21

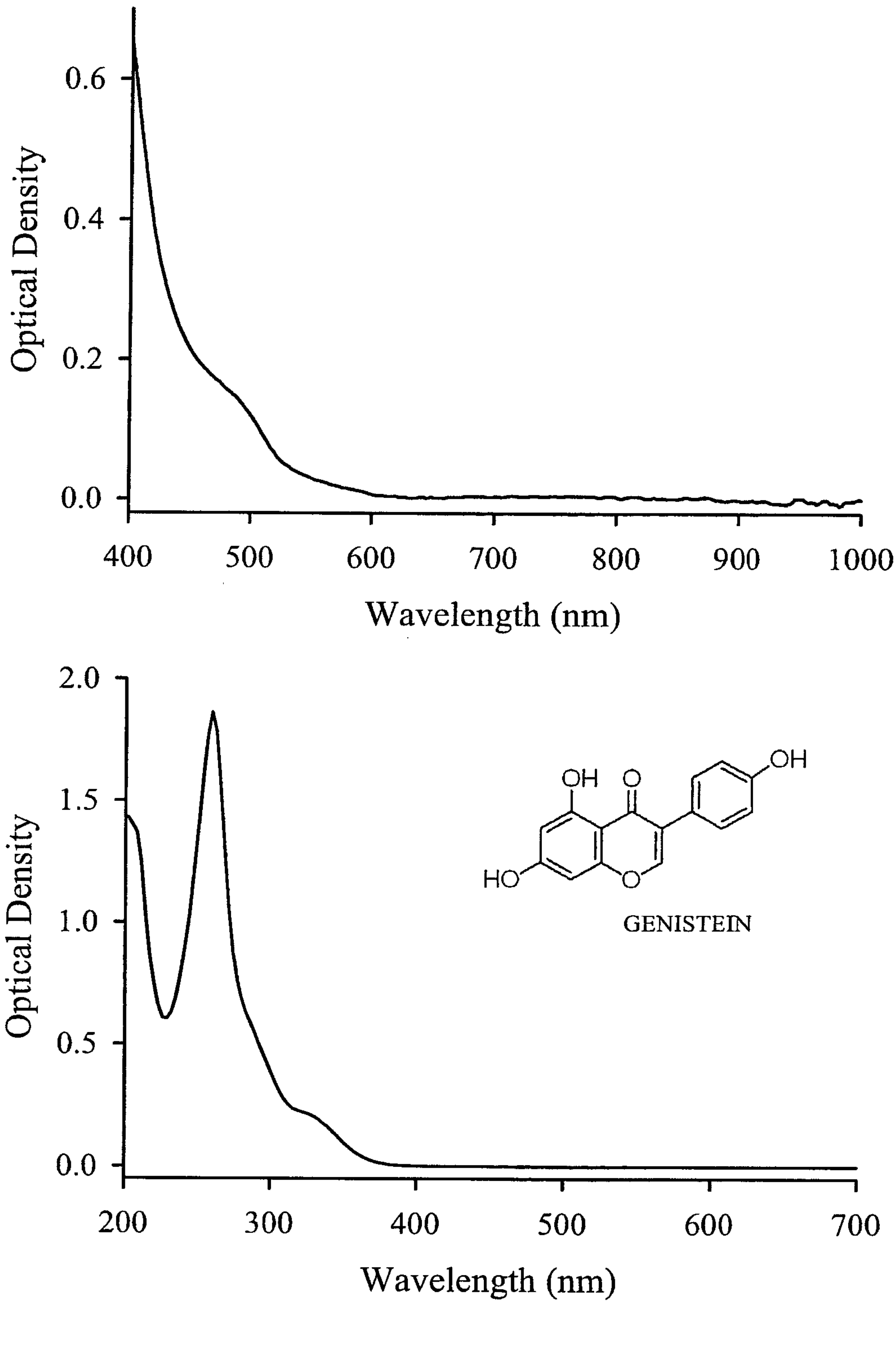

Optical Density
0.6
0.4
0.2
0.0
400
500
600
700
800
900
1000
Wavelength (nm)
2.0
1.5
1.0
0.5
0.0
200
300
400
500
600
700
Wavelength (nm)
OH
O
OH
HO
O
GENISTEIN

Fig. 22

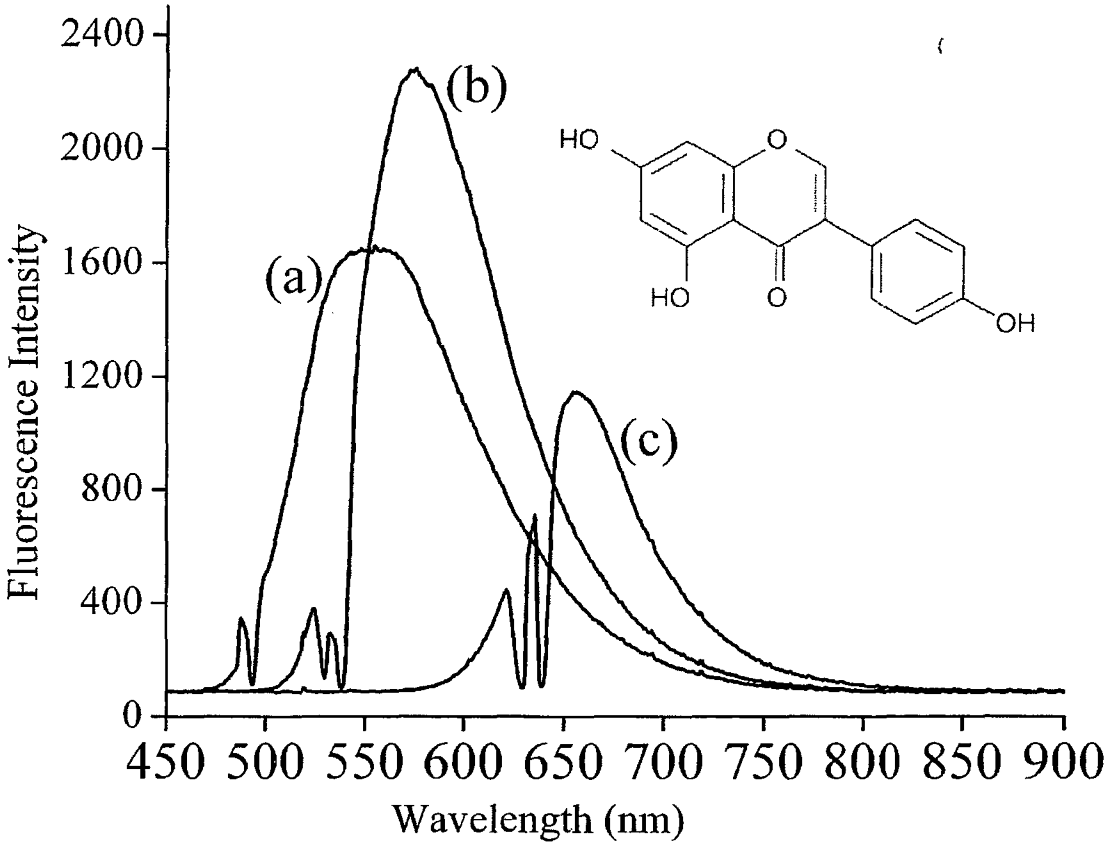

2400
2000
1600
1200
800
400
0
Fluorescence Intensity
(a)
(b)
(c)
HO
O
HO
O
OH
450 500 550 600 650 700 750 800 850 900
Wavelength (nm)

Fig. 23

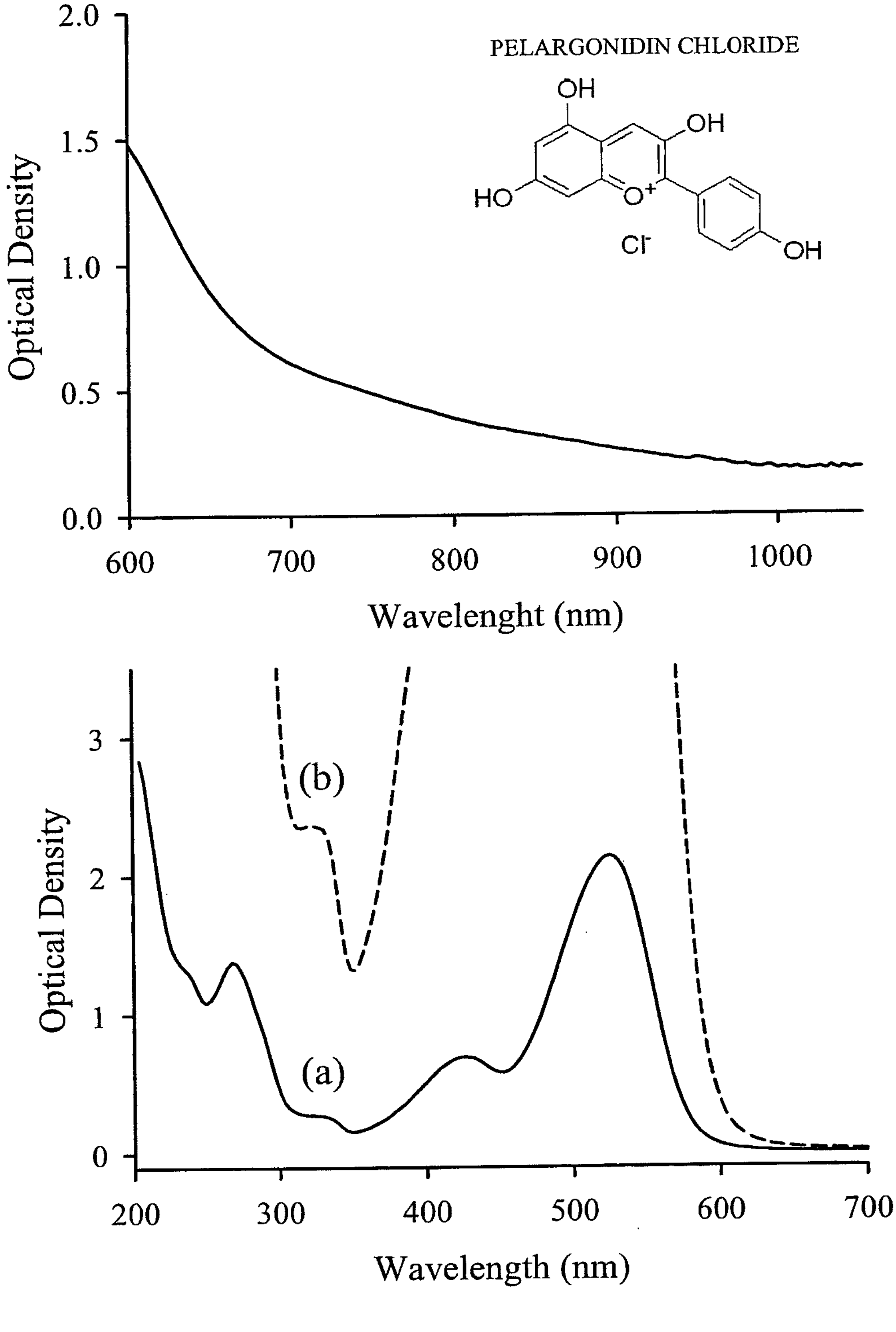

PELARGONIDIN CHLORIDE
OH
OH
HO
O+
Cl-
OH
2.0
1.5
1.0
0.5
0.0
Optical Density
600
700
800
900
1000
Wavelenght (nm)
3
2
1
0
Optical Density
(b)
(a)
200
300
400
500
600
700
Wavelength (nm)

Fig. 24

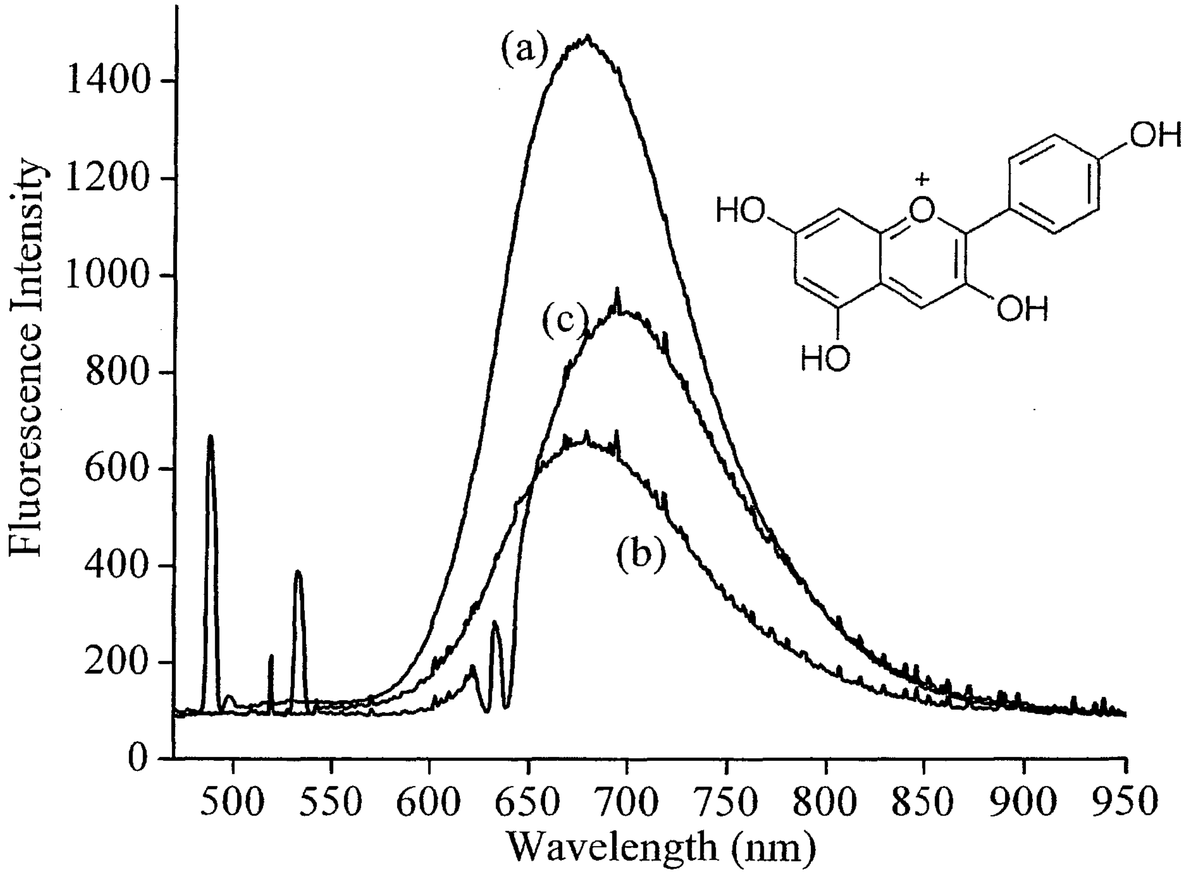

Fluorescence Intensity
0
200
400
600
800
1000
1200
1400
500
550
600
650
700
750
800
850
900
950
Wavelength (nm)
(a)
(b)
(c)
HO
OH
OH
HO
O
+

Fig. 25

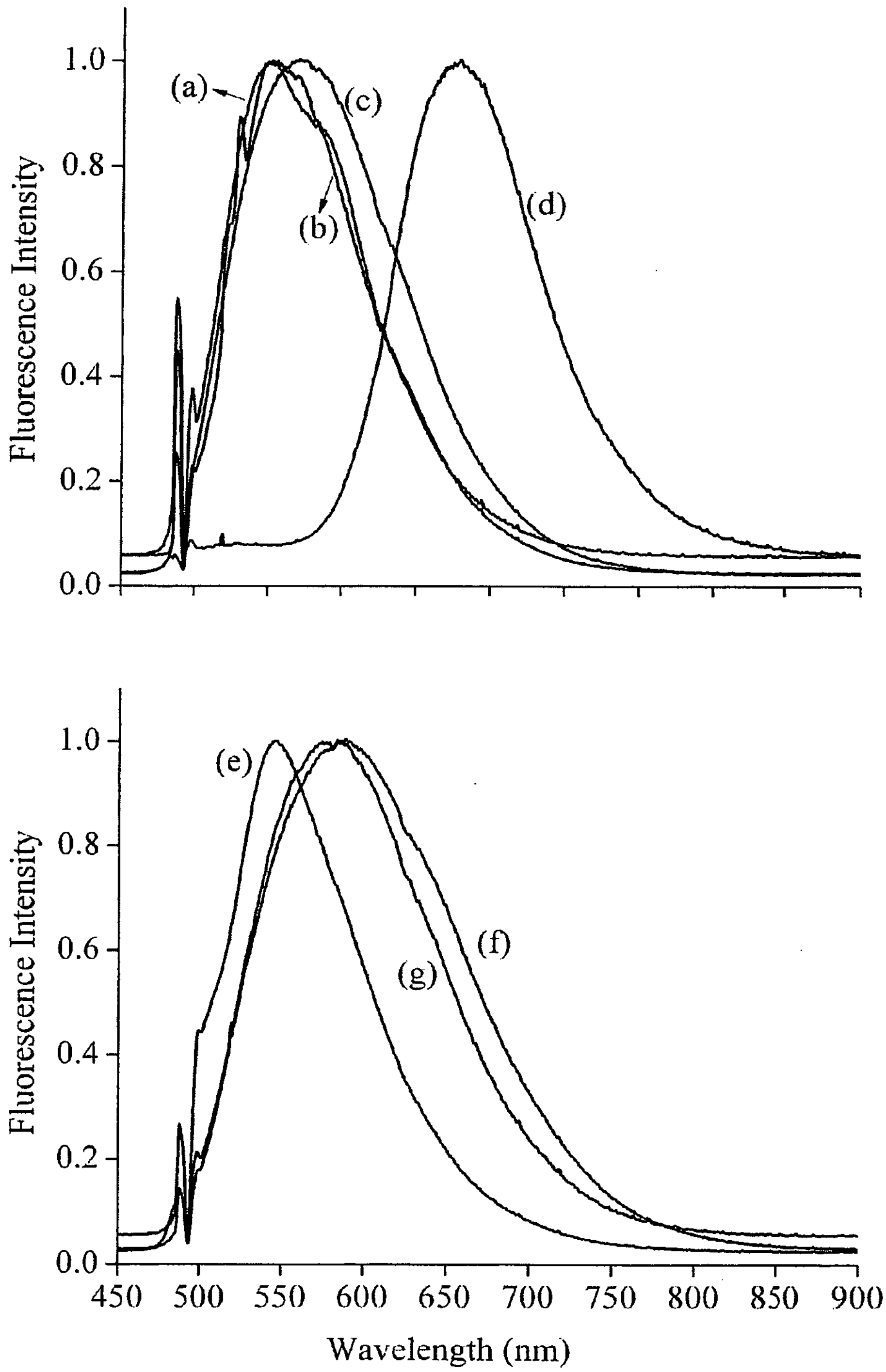


Fig. 26

(g) gallocatechin

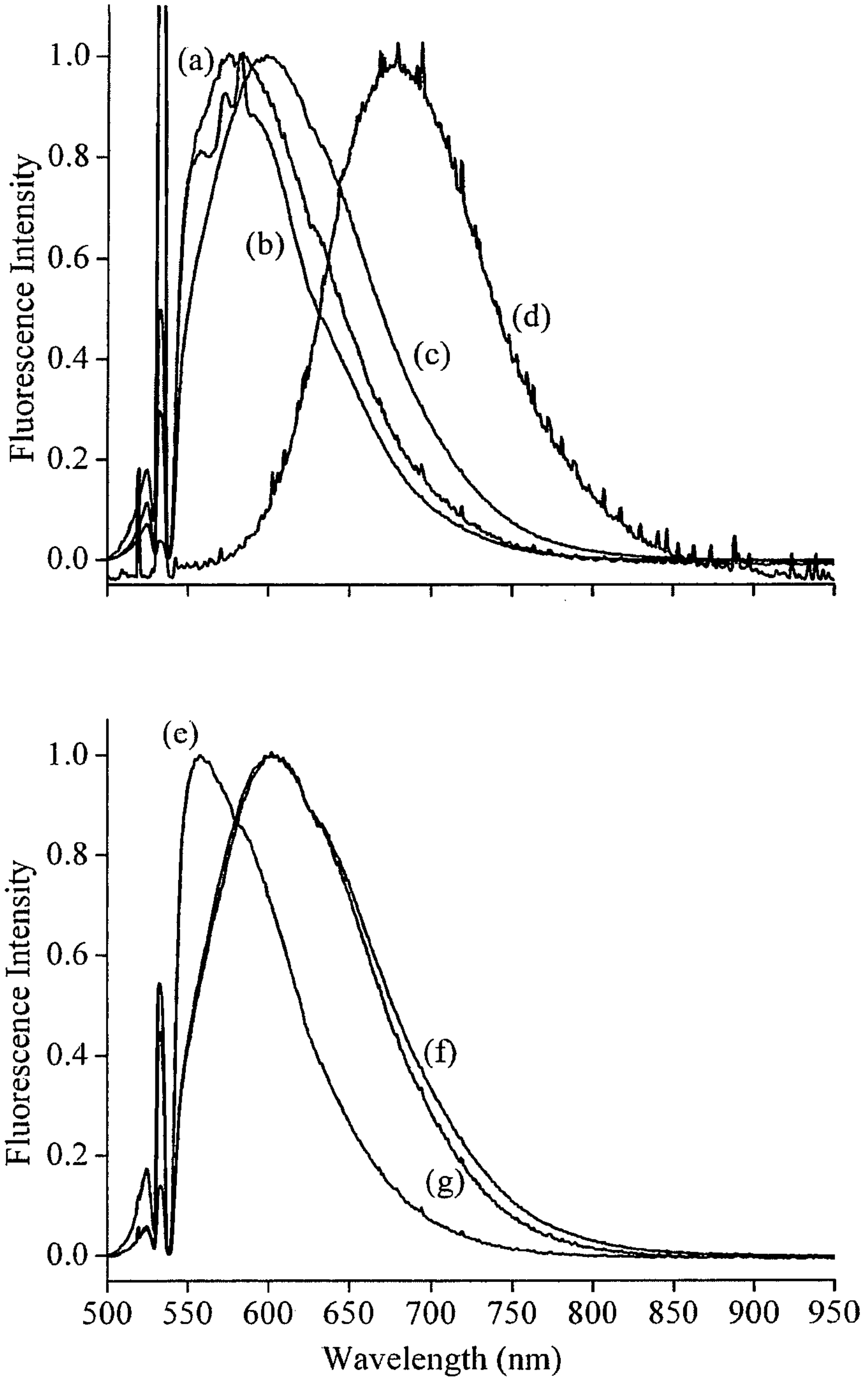

1.0
0.8
0.6
0.4
0.2
0.0
Fluorescence Intensity
(a)
(b)
(c)
(d)
1.0
0.8
0.6
0.4
0.2
0.0
Fluorescence Intensity
(e)
(f)
(g)
500 550 600 650 700 750 800 850 900 950
Wavelength (nm)

Fig. 27

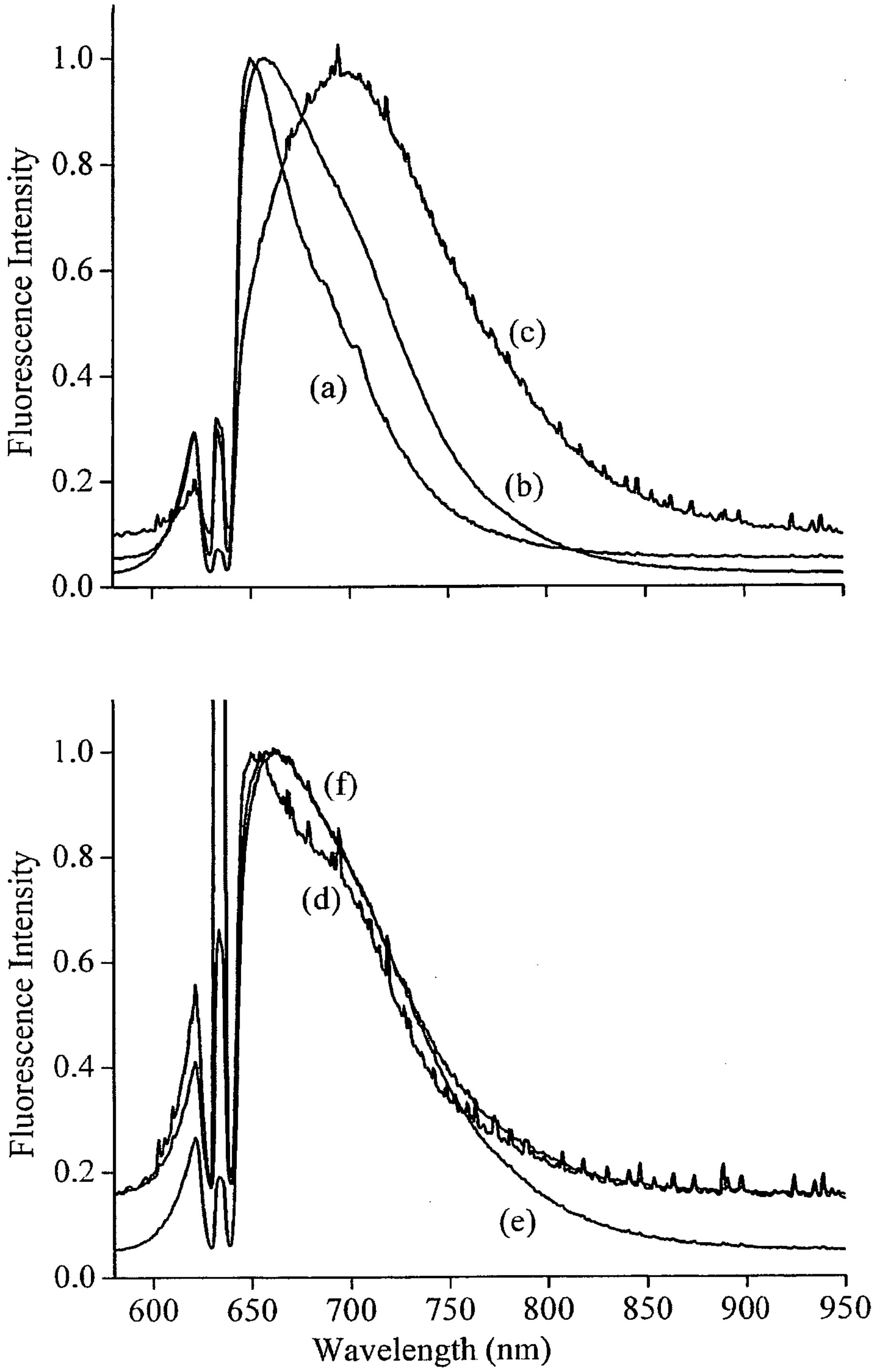

1.0
0.8
0.6
0.4
0.2
0.0
Fluorescence Intensity
(a)
(b)
(c)
(d)
(e)
(f)
600
650
700
750
800
850
900
950
Wavelength (nm)

Fig. 28

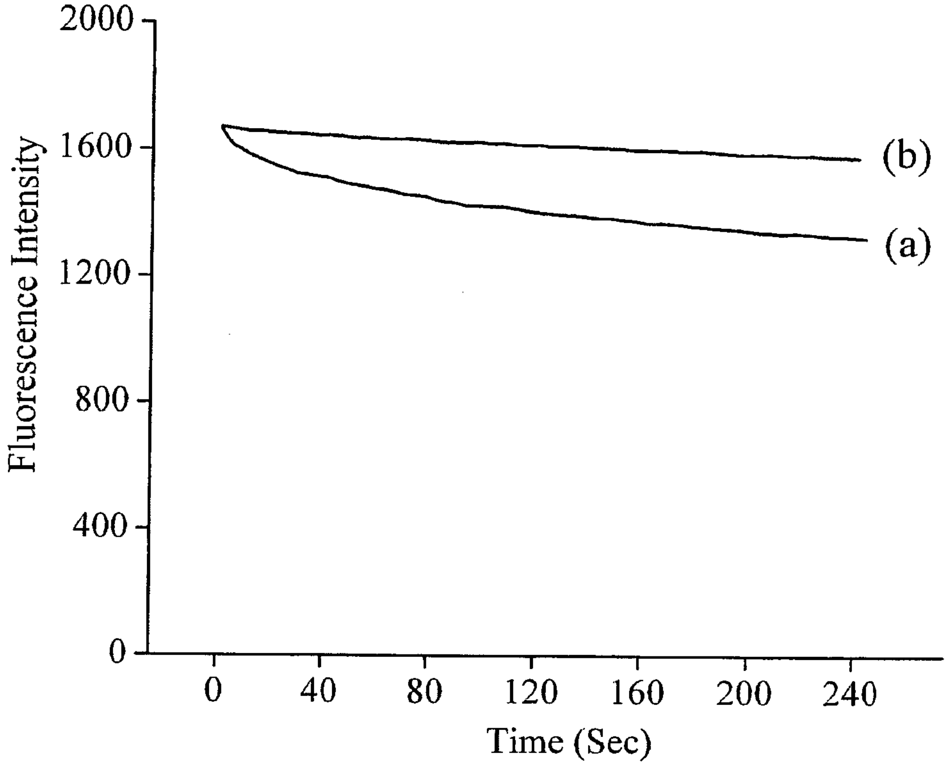

2000
1600
1200
800
400
0
Fluorescence Intensity
0
40
80
120
160
200
240
Time (Sec)
(b)
(a)

Fig. 29

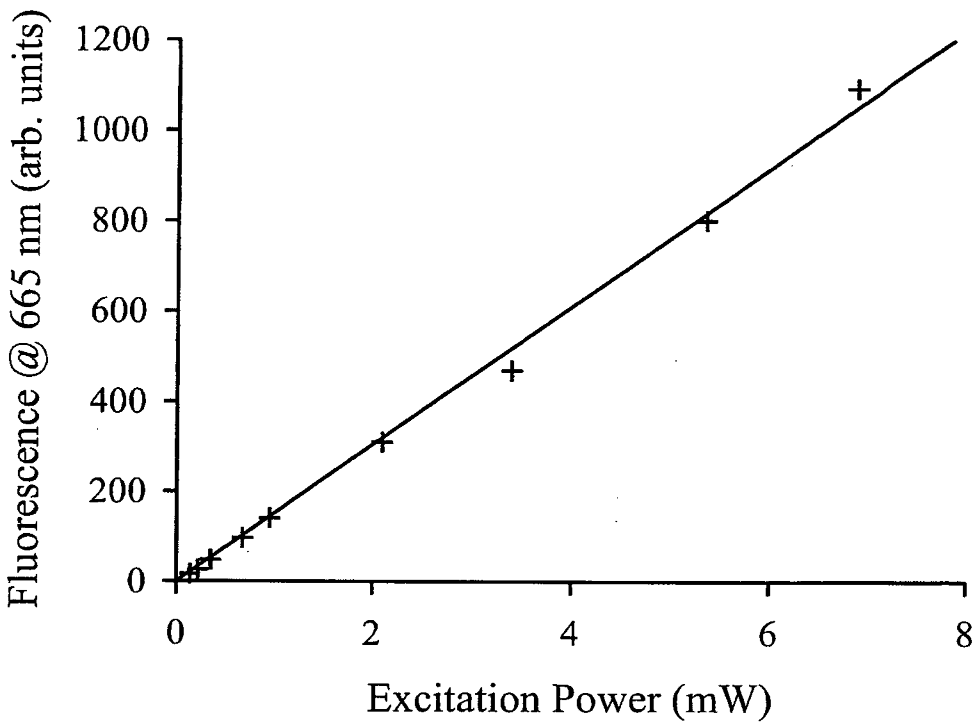
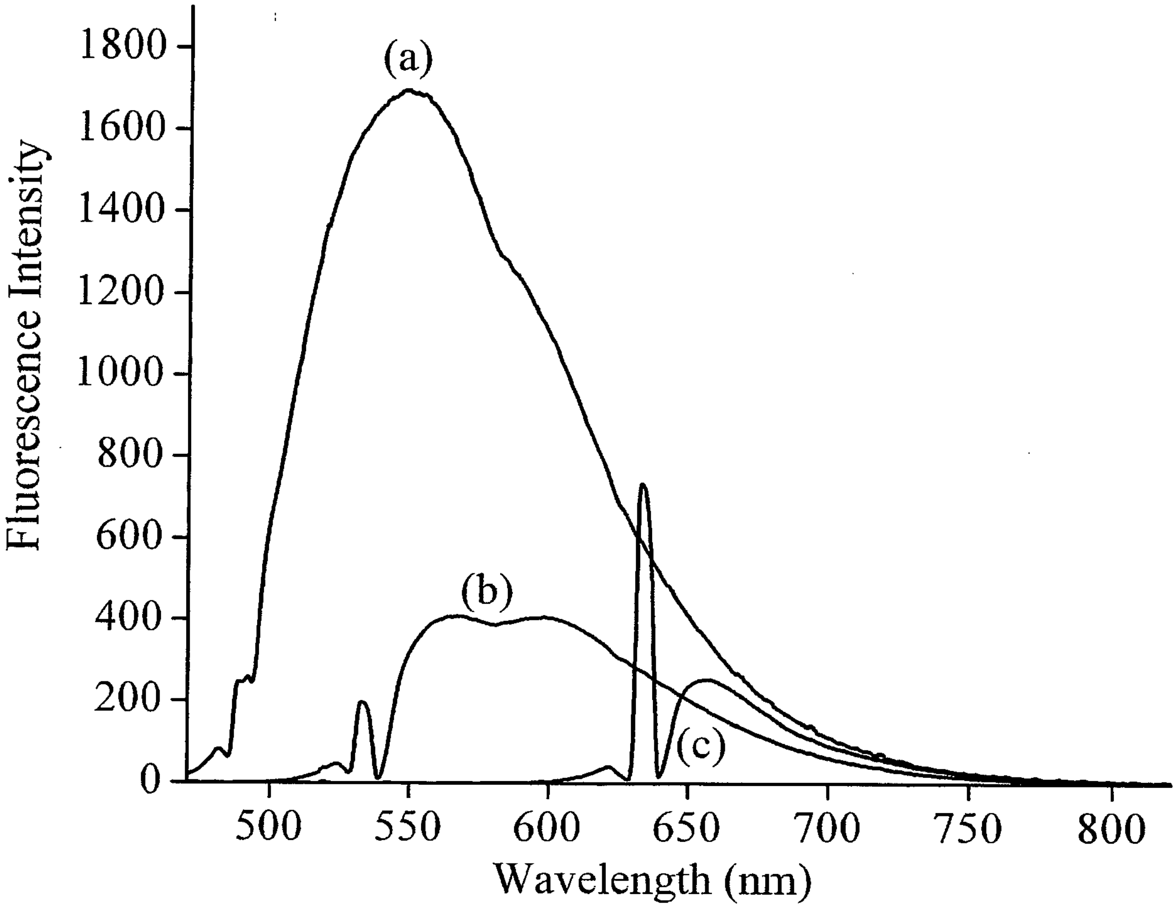

Fluorescence @ 665 nm (arb. units)
1200
1000
800
600
400
200
0
0
2
4
6
8
Excitation Power (mW)

Fig. 30



1800
1600
1400
1200
1000
800
600
400
200
0
Fluorescence Intensity
(a)
(b)
(c)
500
550
600
650
700
750
800
Wavelength (nm)

Fig. 31

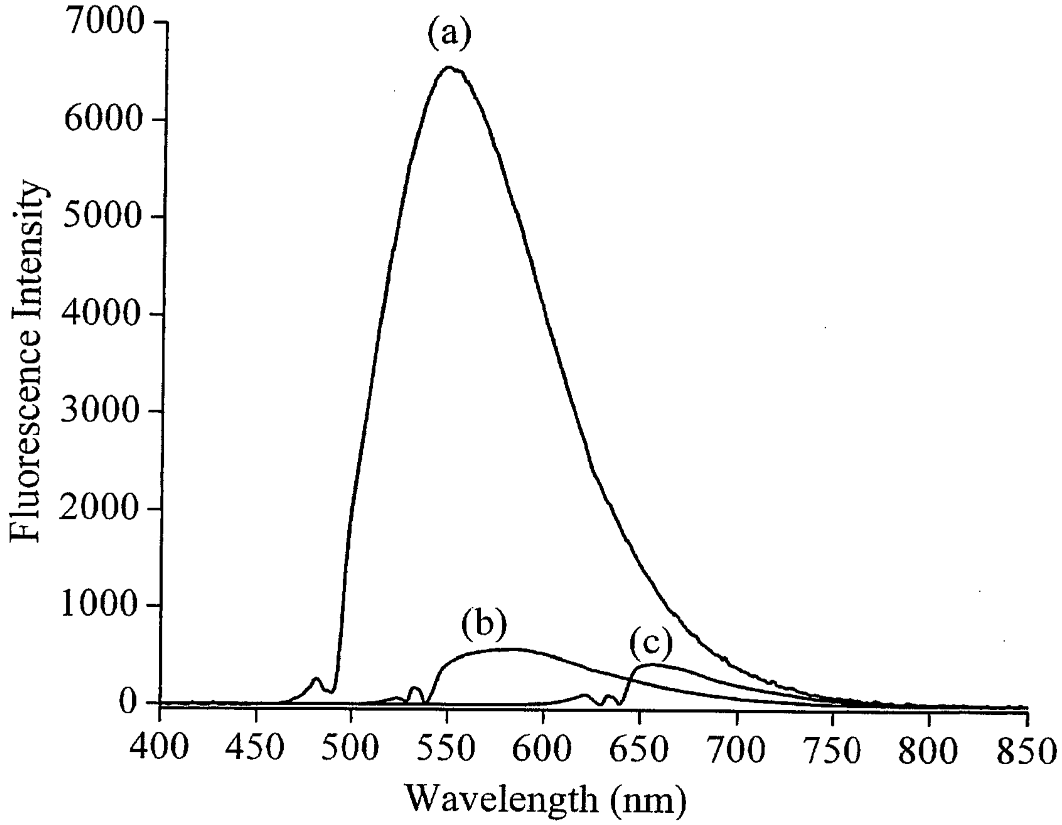

7000
6000
5000
4000
3000
2000
1000
0
Fluorescence Intensity
(a)
(b)
(c)
400
450
500
550
600
650
700
750
800
850
Wavelength (nm)

Fig. 32

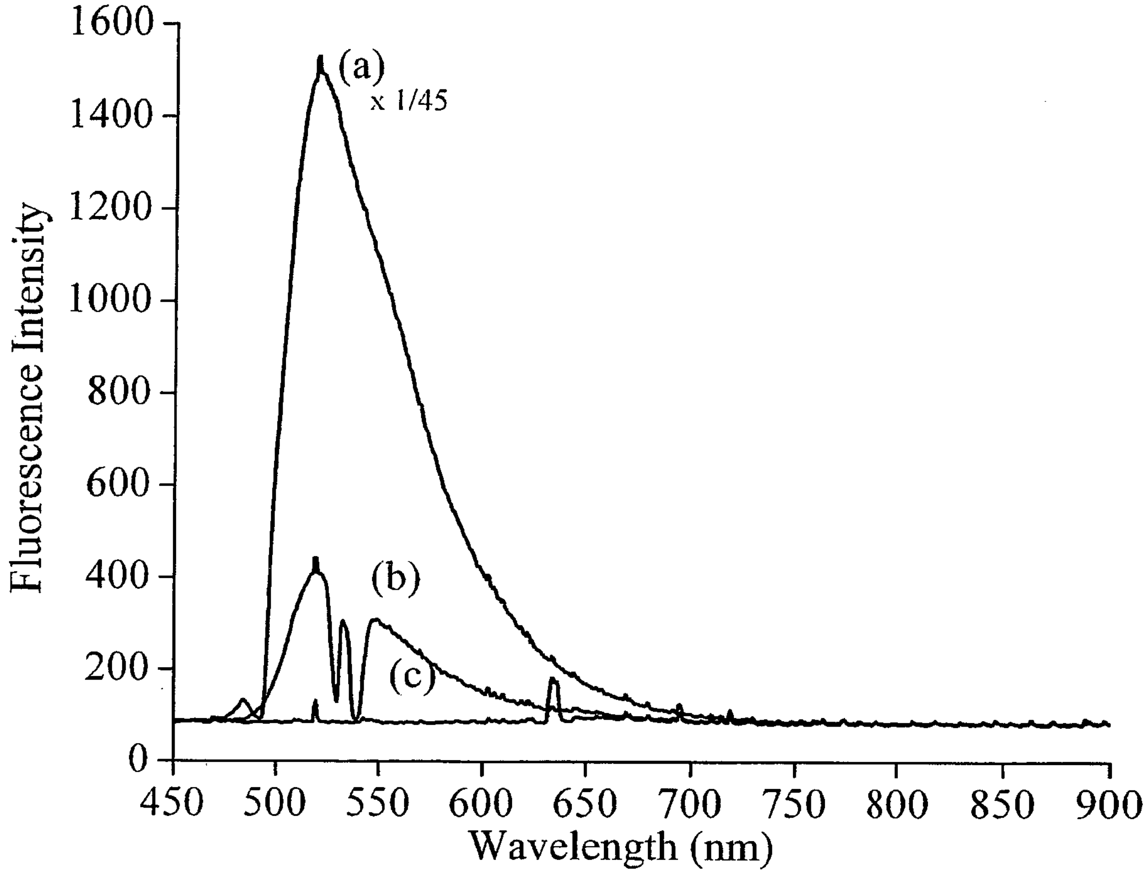

1600
1400
1200
1000
800
600
400
200
0
Fluorescence Intensity
(a)
x 1/45
(b)
(c)
450
500
550
600
650
700
750
800
850
900
Wavelength (nm)

Fig. 33

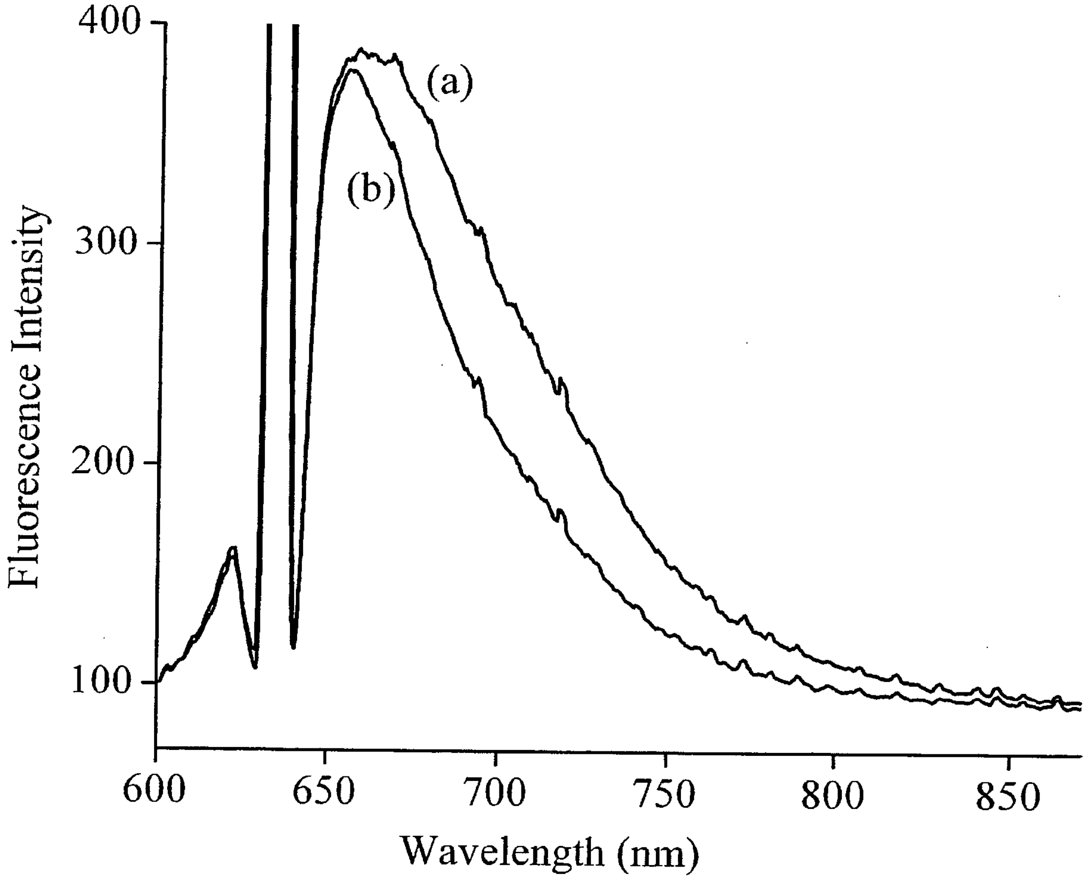
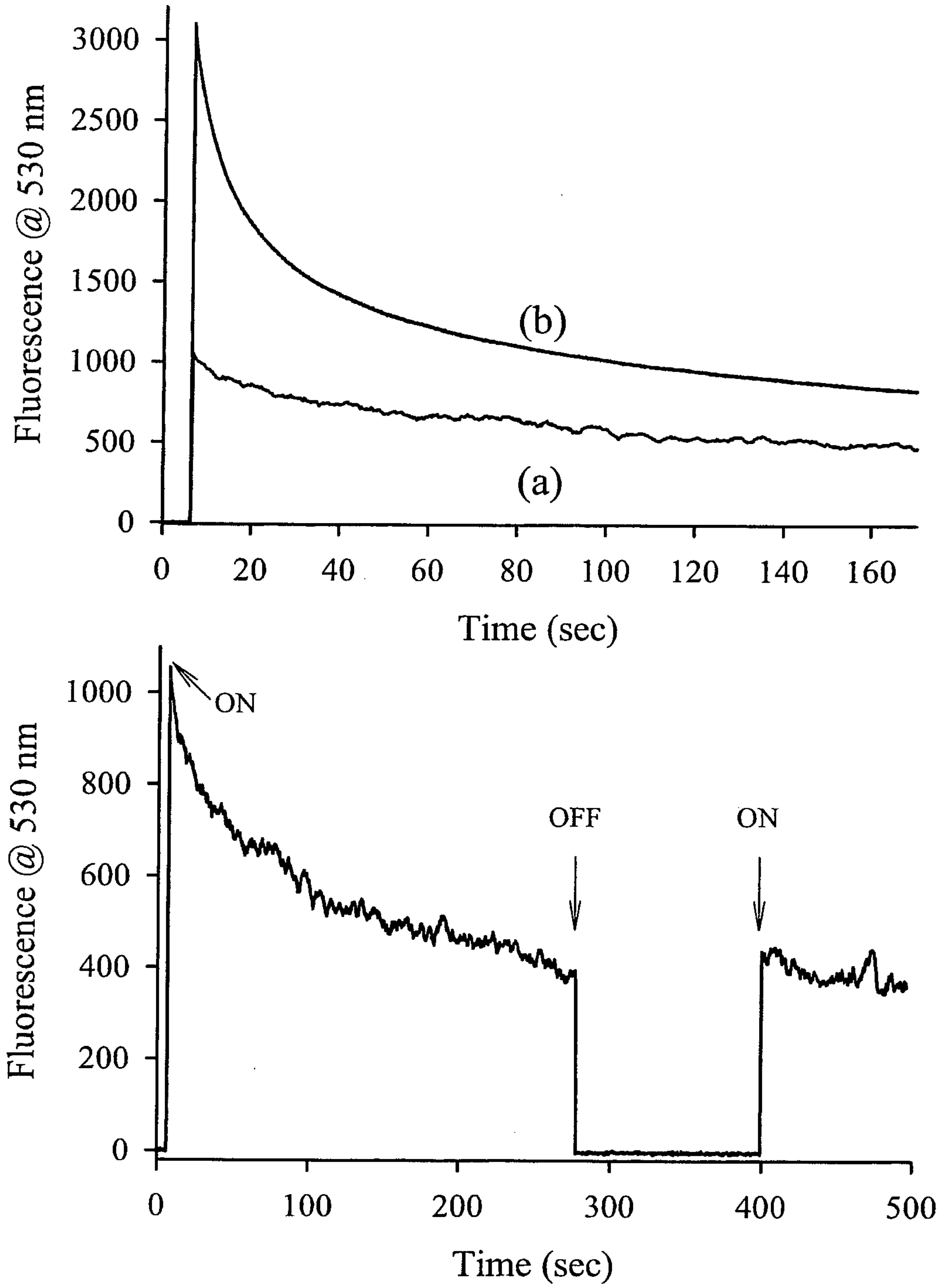

400
300
200
100
Fluorescence Intensity
600
650
700
750
800
850
Wavelength (nm)
(a)
(b)

Fig. 34



3000
2500
2000
1500
1000
500
0
Fluorescence @ 530 nm
(b)
(a)
0
20
40
60
80
100
120
140
160
Time (sec)
1000
800
600
400
200
0
Fluorescence @ 530 nm
ON
OFF
ON
0
100
200
300
400
500
Time (sec)

Fig. 35

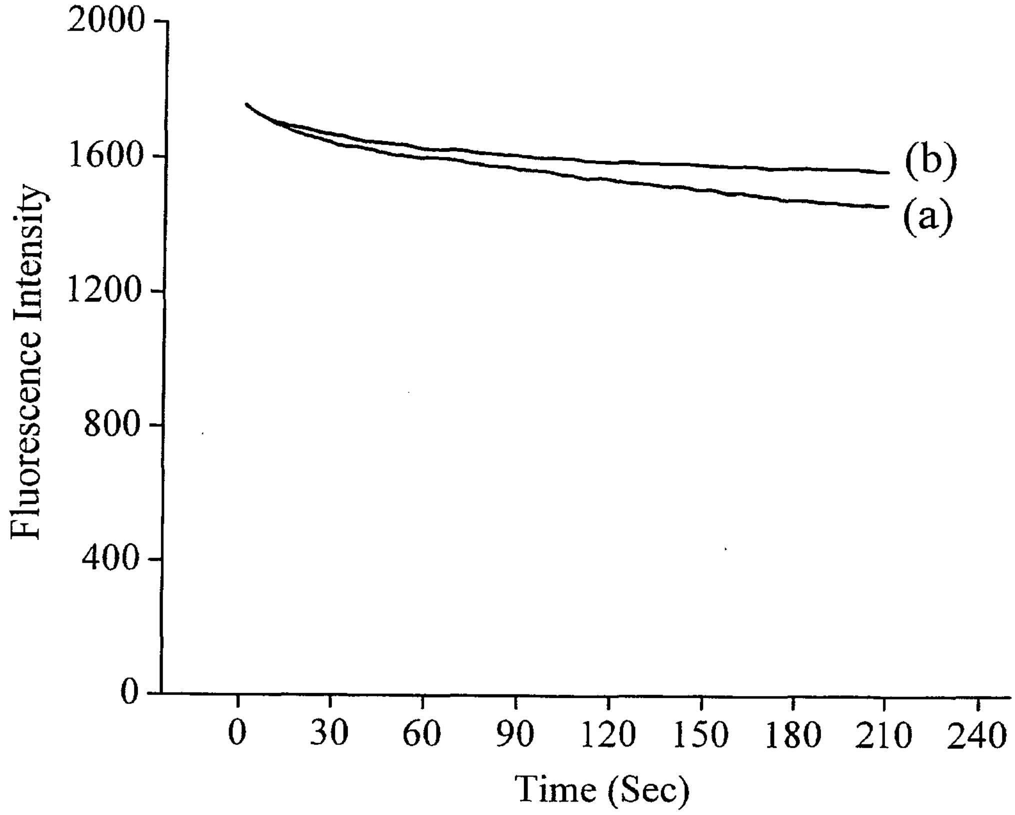

2000
1600
1200
800
400
0
Fluorescence Intensity
(b)
(a)
0
30
60
90
120
150
180
210
240
Time (Sec)

Fig. 36

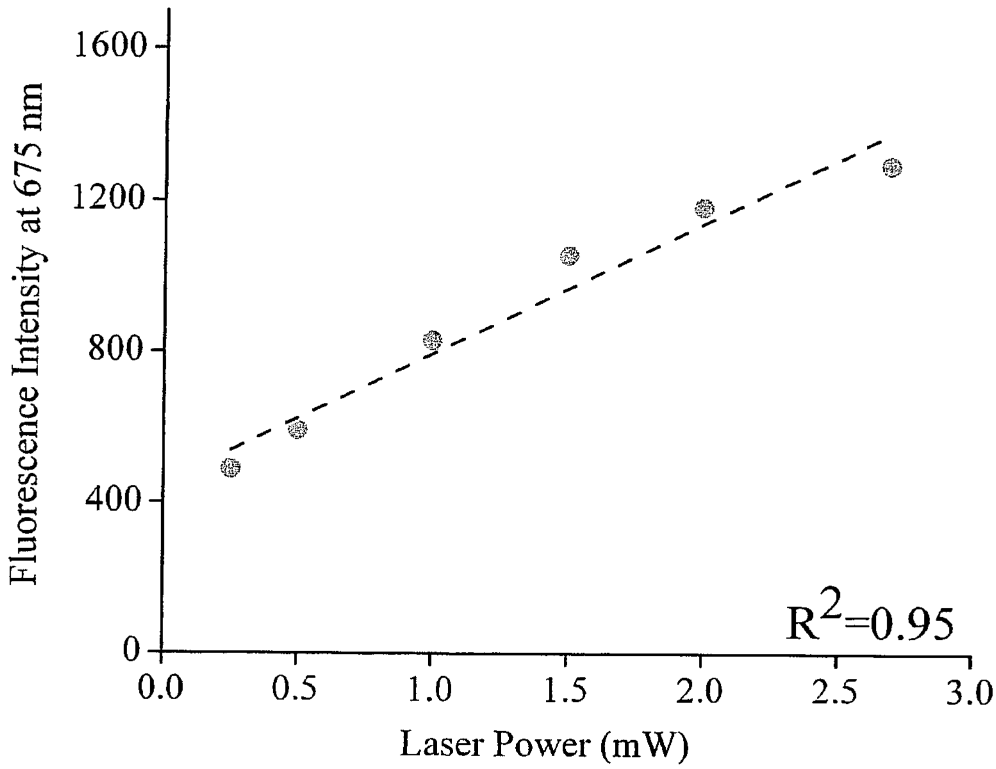

Fluorescence Intensity at 675 nm
1600
1200
800
400
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Laser Power (mW)
R2=0.95

Fig. 37

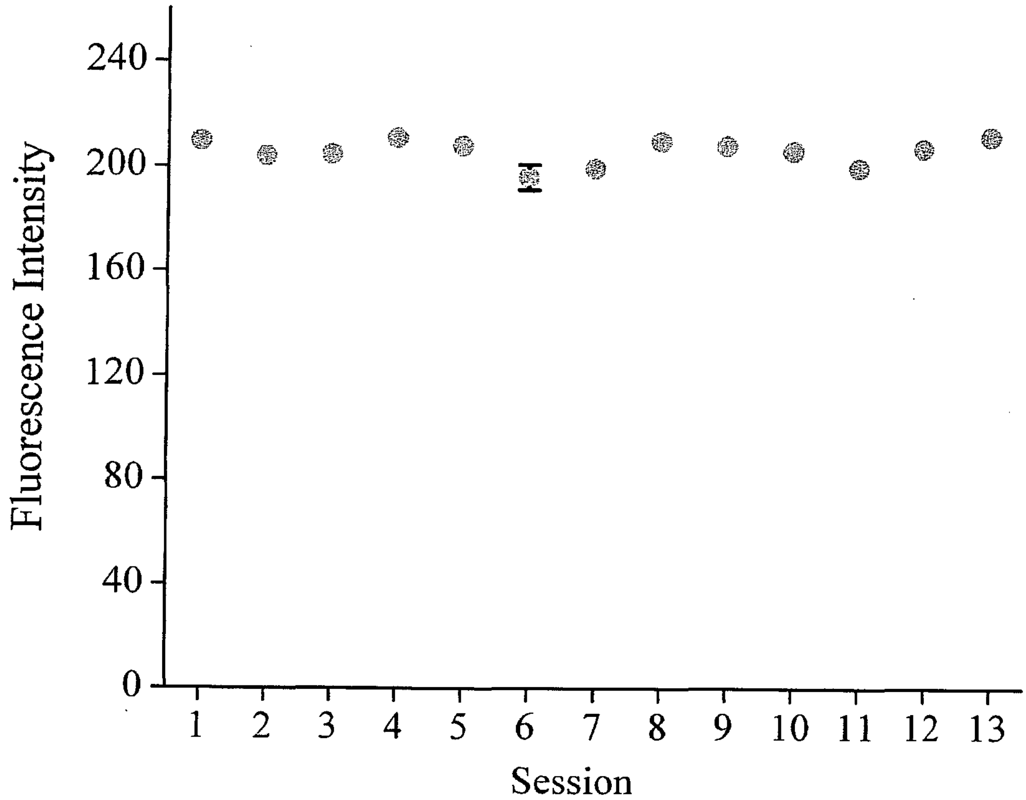
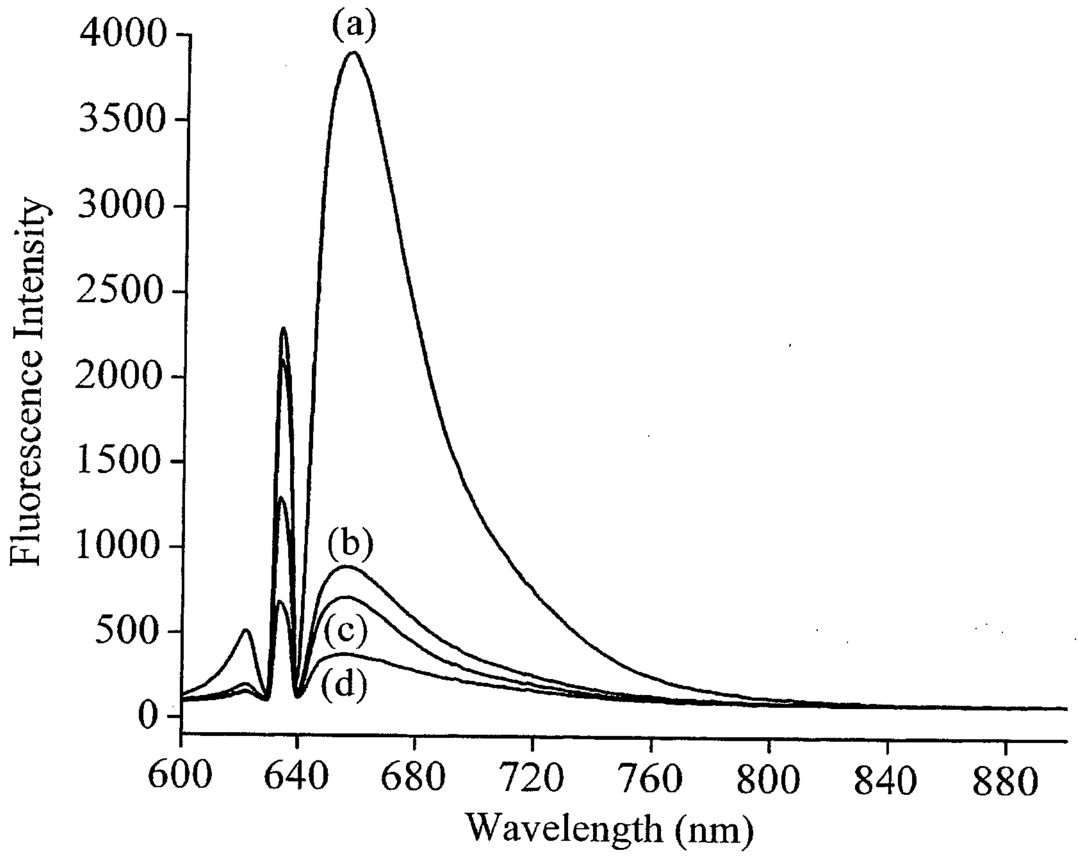

240
200
160
120
80
40
0
Fluorescence Intensity
1
2
3
4
5
6
7
8
9
10
11
12
13
Session

Fig. 38



(a)
(b)
(c)
(d)
4000
3500
3000
2500
2000
1500
1000
500
0
Fluorescence Intensity
600
640
680
720
760
800
840
880
Wavelength (nm)

Fig. 39

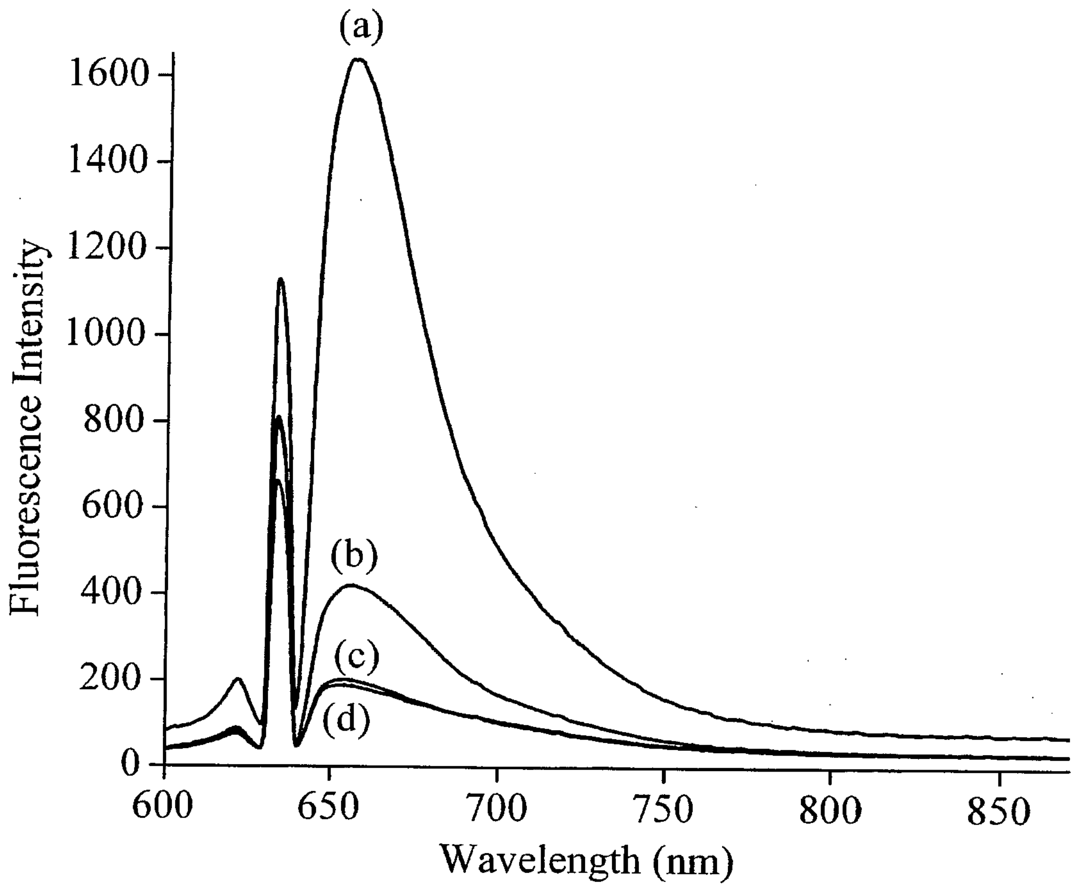

(a)
(b)
(c)
(d)
Fluorescence Intensity
0
200
400
600
800
1000
1200
1400
1600
600
650
700
750
800
850
Wavelength (nm)

Fig. 40

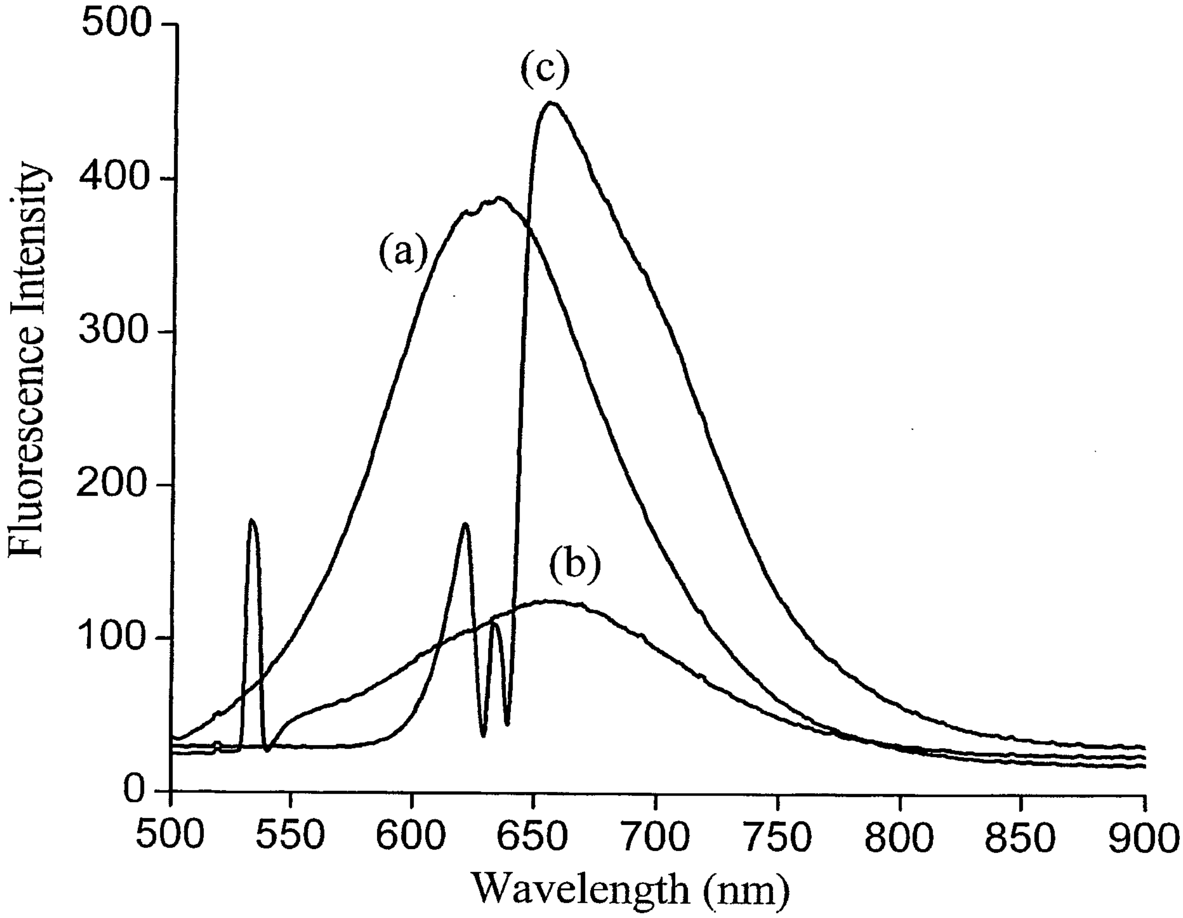

500
400
300
200
100
0
Fluorescence Intensity
(a)
(b)
(c)
500
550
600
650
700
750
800
850
900
Wavelength (nm)

Fig. 41

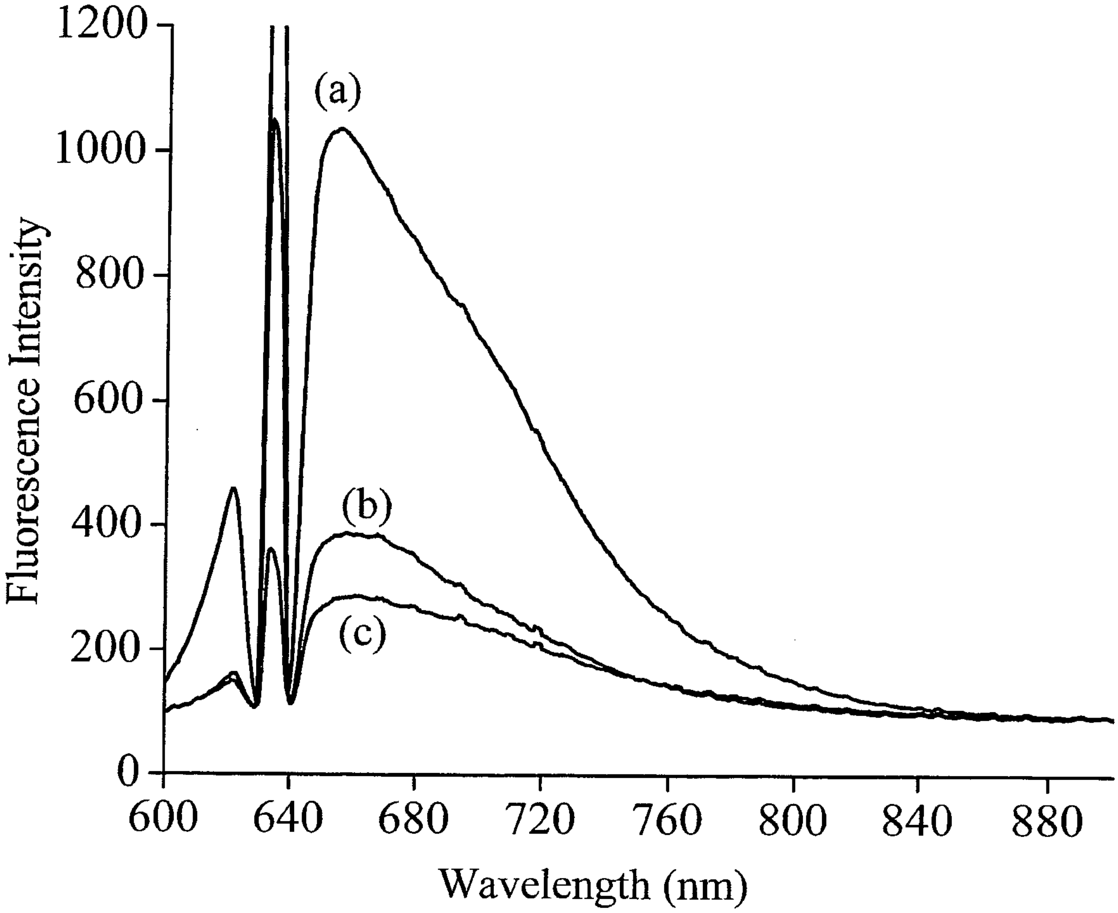

1200
1000
800
600
400
200
0
Fluorescence Intensity
(a)
(b)
(c)
600
640
680
720
760
800
840
880
Wavelength (nm)

Fig. 42

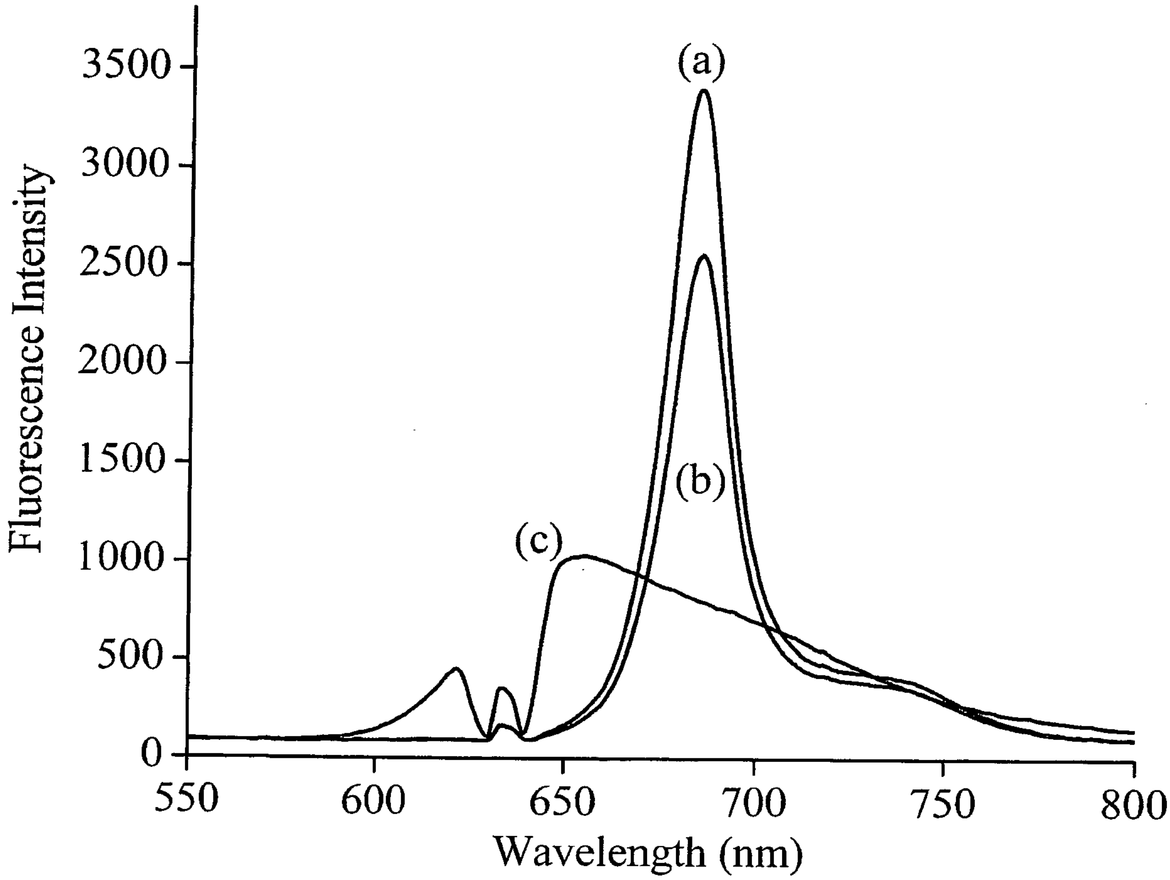

Fluorescence Intensity
3500
3000
2500
2000
1500
1000
500
0
550
600
650
700
750
800
Wavelength (nm)
(a)
(b)
(c)

Fig. 43

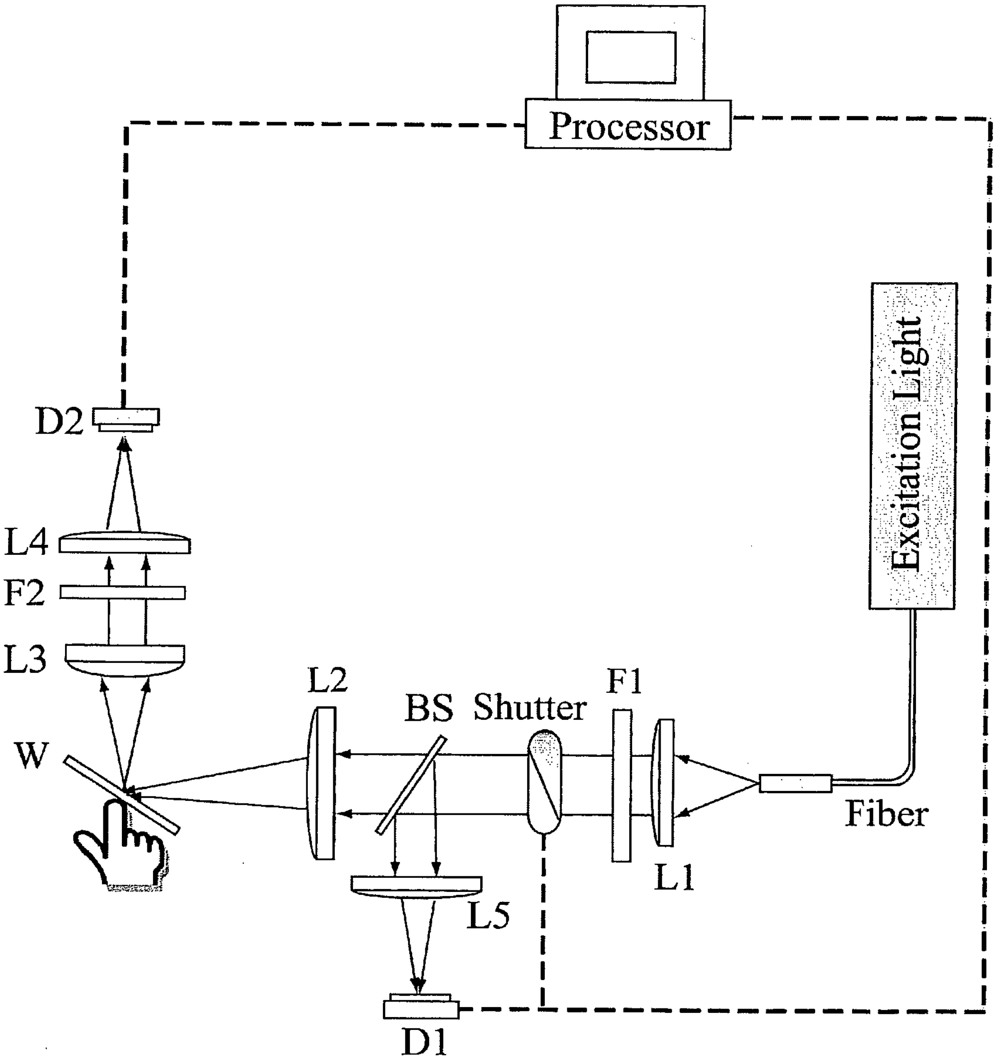

Processor
Excitation Light
D2
L4
F2
L3
W
L2
BS
Shutter
F1
Fiber
L1
L5
D1

Fig. 44

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 12134667 B **[0028]**
- US 6205354 B1, Gellermann W **[0051]**

Non-patent literature cited in the description

- **FORMICA JV ; REGELSON W.** Review of the biology of quercetin and related bioflavonoids. *Food Chem. Toxicol.,* 1995, vol. 33, 1061-80 **[0051]**
- Flavonoids: Chemistry, Biochemistry, and Applications. CRC Press, Taylor & Francis Group, LLC, 2006 **[0051]**
- **HANNEKEN A ; LIN F-F ; JOHNSON J ; MAHER P.** *Investigative Ophthalmology & Visual Science,* 2006, vol. 47, 3164-77 **[0051]**
- **RICE-EVANS CA ; MILLER NJ ; PAGANGA G.** *Free Radic. Biol. Med.,* 1996, vol. 20, 933 **[0051]**
- **BOHM BA.** Introduction to Flavonoids. Chemistry and Biochemistry of Organic Natural Products. Harwood Academic Publisher, 1998, 200 **[0051]**
- **JURASEKOVA Z ; GARCIA-RAMOS JV ; DOMINGO C ; SANCHEZ-CORTES S.** Surface-enhanced Raman scattering of flavonoids. *J. Raman Spectrosc.,* 2006, vol. 37, 1239-1241 **[0051]**
- **CORNARD JP ; DANGLETERRE, LAPOUGE C.** *J. Phys. Chem. A,* 2005, vol. 109, 10044-10051 **[0051]**
- **LOTITO SB ; FREI B.** *Free Radic. Biol. Med.,* 2006, vol. 41 (12), 1727-46 **[0051]**

专利名称(译)	无创测量生物组织中的黄酮类化合物
公开(公告)号	EP2387712B1
公开(公告)日	2014-06-04
申请号	EP2010732029
申请日	2010-01-13
[标]申请(专利权)人(译)	SHARIFZADEH MOHSEN Ermakov说IGOR V GELLERMAN WERNER
申请(专利权)人(译)	SHARIFZADEH，MOHSEN ERMAKOV，IGOR V. GELLERMAN，WERNER
当前申请(专利权)人(译)	SHARIFZADEH，MOHSEN ERMAKOV，IGOR V. GELLERMAN，WERNER
[标]发明人	SHARIFZADEH MOHSEN ERMAKOV IGOR V GELLERMAN WERNER
发明人	SHARIFZADEH, MOHSEN ERMAKOV, IGOR V. GELLERMAN, WERNER
IPC分类号	G01N33/483 G01N21/64 A61B5/00 G01N21/31
CPC分类号	A61B5/1455 A61B5/14546 G01N21/64 G01N2021/6421
优先权	12/352702 2009-01-13 US
其他公开文献	EP2387712A4 EP2387712A2
外部链接	Espacenet

摘要(译)

公开了有助于快速，非侵入性和定量测量生物组织例如人皮肤中的类黄酮化合物及其异构体和代谢产物的浓度的方法和设备。 完整组织的低强度可见光照明可提供较高的空间分辨率，并可精确定量组织中的类黄酮含量。 优选的实施方案利用了以前未知的，低振荡强度的类黄酮的光吸收跃迁。 这使得有可能在其他可能引起混淆的皮肤发色团的吸收范围之外，在活的人体组织中激发黄酮类化合物。 根据本发明构造的系统包括光源，该光源用与类黄酮化合物的吸收谱带重叠的光照射组织的局部区域；和 用于检测由照明导致的类黄酮化合物发射的荧光的装置； 处理器用于基于检测到的荧光确定类黄酮化合物的浓度水平。

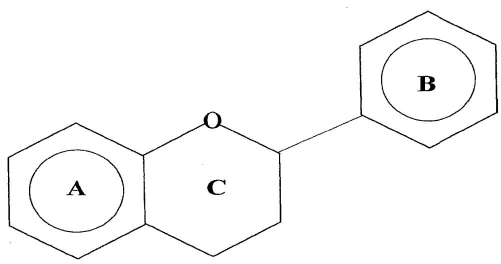


Fig. 1