

ARTÍCULO DE INVESTIGACIÓN ORIGINAL

A desk spectrofluorometer for *in-vivo* and *in-situ* biological material measurements

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ABSTRACT

Fluorescence methods are being widely used in lab for the measurement of species concentrations. Such measurements are normally expensive and time consuming because preparative procedures and they are not done under *in vivo* and *in situ* conditions. Here we present a portable fluorescence system based in a mini-spectrometer, led's as light sources, a bifurcated fiber optic and a laptop. The spectrofluorometer is useful to measure the substances fluorescence *in-vivo* and *in-situ*, such as human tissue and any other organic and non-organic material. The system is controlled by the laptop from a program written in the graphical language G of the LabVIEW 7.1, which measures and process the spectra in real time. We show measurements with a 200 mW UV led (365 nm).

Key Words: Fluorescence, spectroscopy, ultraviolet.

RESUMEN

Los métodos de fluorescencia son ampliamente usados en el laboratorio para la medición de concentraciones de especies. Tales mediciones son por tanto no realizadas ni *in vivo* ni *in situ*, además de que normalmente son costosas y consumen mucho tiempo debido a los procedimientos de preparación de las muestras. Aquí se presenta un sistema de fluorescencia portátil en base a un mini-espectrómetro, una fuente de luz en base a un led, una fibra óptica bifurcada y una laptop. El espectrofluorómetro es útil para medir *in vivo* e *in situ* sustancias fluorescentes tales como tejido humano y otros materiales orgánicos y no orgánicos. El sistema es controlado desde la laptop por medio de un programa escrito en el lenguaje gráfico G de LabVIEW 7.1, el cual mide y procesa los espectros en tiempo real. Se presentan mediciones realizadas con un led de 200 mW que emite a 365 nm.

Palabras clave: Fluorescencia, espectroscopia, ultravioleta.

INTRODUCTION

The absorption and subsequent emission of light by organic and inorganic specimens is typically the result of physical phenomena known as luminescence, which occurs from electronically excited states. Luminescence is formally divided into two categories:

fluorescence and phosphorescence. Fluorescence occurs in the order of nanoseconds to microseconds, phosphorescence in the order of millisecond to seconds¹⁻⁴.

The processes which occur between the absorption and emission of light are usually illustrated by a Jablonski diagram (Figure 1). S0, S1 and S2 denote

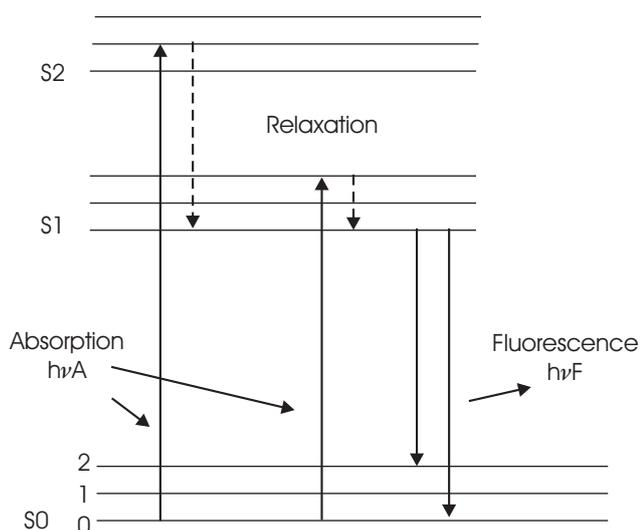


Figure 1. A simple Jablonski diagram.

ground, first, and second electronic states. At each of these electronic energy levels the electrons can exist in a number of vibration energy levels denoted by 0, 1, 2, etc. The transitions between states are depicted as vertical lines to illustrate the instantaneous nature of light absorption (10^{-15} s). An electron is usually excited to some higher vibration level of either S1 or S2 and rapidly relaxes (10^{-15} s) to the lowest vibration level of S1.

Examination of the Jablonski diagram reveals that the energy of emission is less than that of absorption. Hence, fluorescence occurs at lower energies or longer wavelength (Stokes shift). The amount of Stokes shift is a measure of the relaxation process occurring in the excited state, populated by absorption¹. Another property of fluorescence is that the same fluorescence emission spectrum is generally observed irrespective of the excitation wavelength. This is known as Kasha's rule¹. Although fluorescence measurements are more sophisticated than an absorption (transmission) experiment, they provide a wealth of the information about the molecular structure, interaction and dynamics of a species.

Fluorescence measurements can be broadly classified into two types of measurements: steady-state and time-resolved^{1-3,5}. Steady-state measurements are those performed with constant illumination and observation. This is the most common type of measurement. The sample is illuminated with a continuous beam of light and the intensity of the emission is usually recorded as a function of wavelength (fluorescence spectrum). When the sample is exposed to light, steady state is reached almost

immediately. Time-resolved measurements are used for measuring intensity decays⁵⁻⁷. For those measurements, the sample is exposed to a pulse of light, where the pulse width is typically shorter than the decay time of the sample. The intensity decay is recorded with a high speed detection system that permits the intensity to be measured on the nanoseconds timescale. There exists a rather simple relationship between steady-state and time-resolved measurements. The steady-state observation is simple an average of the time resolved phenomena over the intensity decay of the sample.

The fluorescence spectroscopy is being widely used now days for biomedical purposes and clinical analysis, among other many applications. The fluorescence emission from the skin can be used to monitor changes induced by the UV radiation. Most of the before mentioned studies are normally done in lab, such measurements are normally expensive and time consuming because preparative procedures and most of the time do not represent the in-vivo and in-situ of the reality one wants to know.

In vivo spectrofluorometry has emerged as a powerful technique for biomedical research covering a broad spectrum, from study of cellular and tissue structures, to biological function and early detection of cancer^{5,8-17}. Optical biopsy refers to detection of cancerous state of a tissue using optical methods. This is a new area, offering the potential to use noninvasive or minimally invasive *in vivo* optical spectroscopic methods, to identify a cancer at its various early stages and monitor its progression. The basic principle utilized for the method of optical biopsy is that the emission fluorescence is strongly influenced by the composition and the cellular structures of tissues. The changes in tissue from normal state to cancerous state have been shown to alter the fluorescence. Biomedical fluorescence spectroscopy is an extremely large and growing field of research. *In situ* fluorescence measurements are too very important in other areas as the study of water and soil contamination¹⁸⁻²⁰, the chlorophyll fluorescence in green plants to know their physiological and health status²¹⁻²³. Fluorescence spectroscopy is currently used for measuring chemical and physical changes in dairy products caused by processing and storage²⁴ or for adulteration assessment²⁵.

Here we present a fluorescence measurement system based in a mini-spectrometer, light sources based on LEDs, a bifurcated fiber optic and a laptop. The instrument is useful to measure the substances fluorescence in-vivo and in-situ, such as

human tissue, contaminated water, soils and any other organic and nonorganic material. The system is controlled by the laptop by means of a program written in the graphical language G of the LabVIEW 7.1. For the measurements reported here we use a 200 mW UV led (365 nm).

EXPERIMENTAL ARRANGEMENT

Figure 2 shows the experimental arrangement, to measure in this case the steady-state fluorescence spectra from skin. The system consists of an excitation light source with variable radiation power from 4 to 200 mW, a bifurcate optical fiber probe for delivery (six 200 μm channels) and collection (one 200 μm central channel) of light, a mini-spectrometer and a lap-top computer. The excitation light is direct coupled, with an efficiency of 18 (± 0.2) %, to the bifurcated fiber-optic probe (R200-UV/VIS manufactured by Ocean Optics). When a silver mirror is perpendicular located at 2 mm from the probe, a 2.2 (± 0.1) % of the incident light is captured by the central fiber. Following sample excitation, part of the emitted fluorescence light is captured by the central channel of the bifurcated fiber-optic probe and directed into the entrance slit of a HR4000CG-UV-NIR (200-1,100 nm bandwidth, 0.75 nm FWHM resolution, 130 photons/count sensitivity at 400 nm, and maximal amplitude counts of 16,000) or a USB4000-VIS-NIR spectrometer (325-1,000 nm bandwidth, 1.5 nm FWHM resolution, 130 photons/count sensitivity at 400 nm, and maximal amplitude counts of 64,000), both manufactured by Ocean Optics.

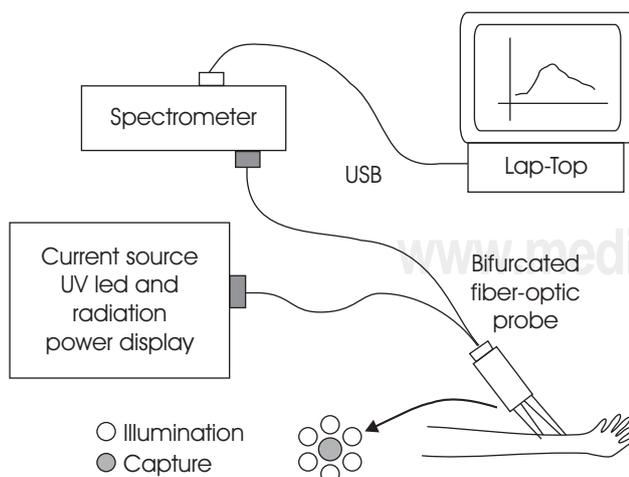


Figure 2. Experimental arrangement.

After the entrance slit, the fluorescence light in the spectrometer is separated in their different wavelengths and converted into electrical signals at the one dimensional photodiode array of the spectrometer. The electrical output from the spectrometer is sent to a laptop via a USB port to be analyzed. For this study we use a 200 mW NCSU033A led manufactured by Nichia Co., which shows a peak emission in the UV-A at 365 nm, an spectrum width of 9 nm and a radiation angle of 100° .

The led is supplied with a variable dc current from 10 to 500 mA to produce a variable radiation power from 4 to 200 mW. The current supply uses a basic design with the popular voltage regulator LM317 as a current regulator (Figure 3). To cover the wide range of the current, the regulator operates in one of two programmable ranges: one from 10 to 130 mA and the other from 130 to 500 mA. A microcontroller (Atmega 8 from Atmel Inc. Corp.), two BC547C transistors and two relays RAS-0510 were used to select the current range and therefore the radiation power ranges (4 to 70 mW and 70 to 200mW). At any range the radiation power emitted by the led is displayed in a LCD (2 X 16). The microcontroller performs the analog to digital conversion of a voltage, which is proportional to the current flowing trough the led, and resolves the transfer equation of the range being used. The displayed power was calibrated with a UV light meter YK-34UV from Digital Instruments. In figure 3, we are using a 12 V regulated DC power supply, which could be replaced by a battery to

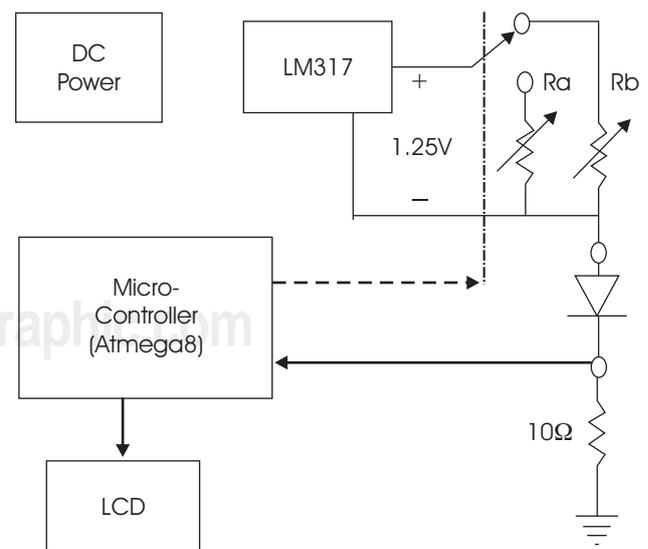


Figure 3. Current source circuit with power meter.

have a completely portable spectrofluorometer to make field measurements.

The system is controlled by the laptop from a program written in the graphical language G of the LabVIEW 7.1 which generate and process the spectra in real time. A LM7805 voltage regulator was used to polarize the microcontroller and the LCD.

MEASUREMENTS

The spectra reported here were measured with the HR4000CG-UV-NIR or with the USB4000-VIS-NIR spectrometers. For each group of measurements, the experiments were done under the same excitation and capture conditions. The fluorescence intensities are given in counts units as these are obtained from the spectrometers.

Figure 4 shows the fluorescence spectra of skin cancer biopsies fixed with formol at 10% and paraffin embedded. The biopsies were irradiated with 36 mW of the 365 nm light. The measurements shown clear differences between the spectra for different malignancies, which could be used to help in the biopsies diagnose. Figure 5 shows the fluorescence spectra of meningioma biopsies under the same condition as the biopsies in figure 4. The measurements shown no great differences between the fibroblastic and the mixed meningiomas, as a consequence of the dominant presence of the collagen tissue²⁵, which shows a similar fluores-

cence spectra²⁶, but the difference between both and the fluorescence of meningotelial meningioma is clear. Figure 6 shows the *in vivo* fluorescence of normal and irritated breast skin due to a confluent and reticulated papillomatosis in a 25 years old patient, irradiated with 10 mW of the 365 nm light on a spot of 3 mm diameter (it means an irradiance of 0.14 W/cm²) and a distance of 2mm between the probe and the skin. For 5 s of irradiation time that means a 700 mJ/cm² dose, well under the UV exposure limits at 365 nm wavelength, which has a value of 2.7×10^4 mJ/cm²²⁷. Figure 7 shows the fluorescence spectra of comestible sunflower and olive oils (the fiber optic probe was submersed into the oil, which was irradiated with 15 mW of the 365 nm light). Measurements of fluorescence spectra of mixtures of olive and sunflower oils are used to identify the adulteration of the first one²⁸. Figure 8 shows the fluorescence spectra virgin olive and sunflower oil mixtures. The measurements of the figures 4 to 6 were obtained with the HR4000CG-UV-NIR spectrometer and the showed in figures 7 and 8 with the USB4000-VIS-NIR.

CONCLUSIONS

A compact continuous wave spectrofluorometer for *in vivo* and *in situ* measurements has been developed. It consists of a miniature spectrometer, a variable 200 mW UV light source (365 nm), a bi-

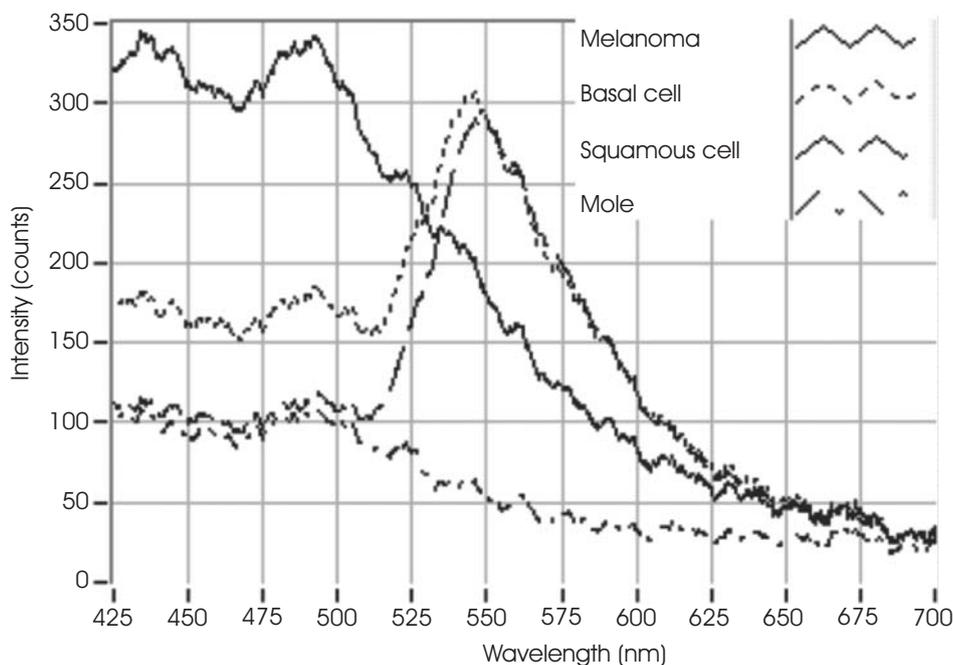


Figure 4. Fluorescence of skin cancer biopsies.

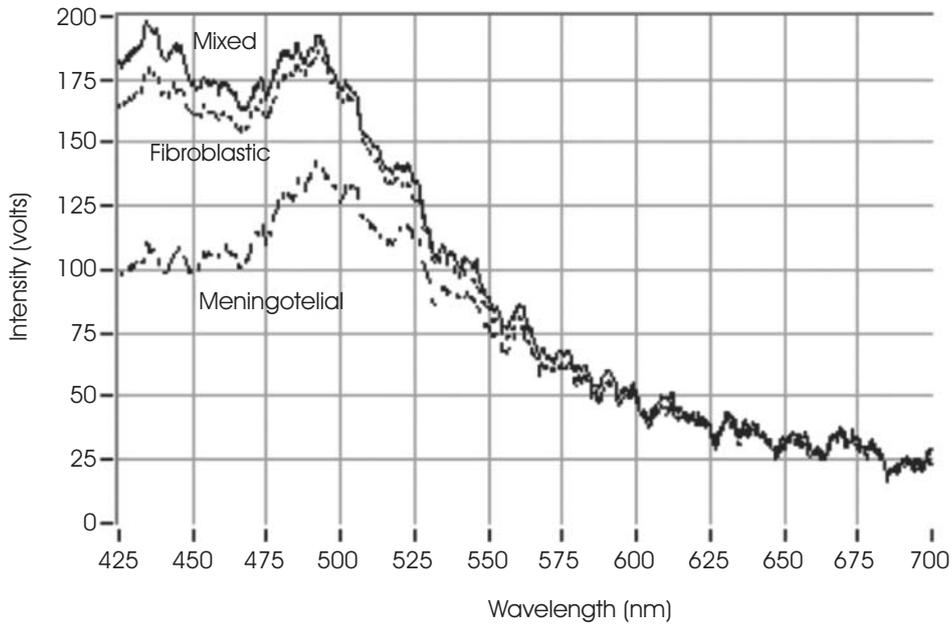


Figure 5. Fluorescence of meningioma biopsies.

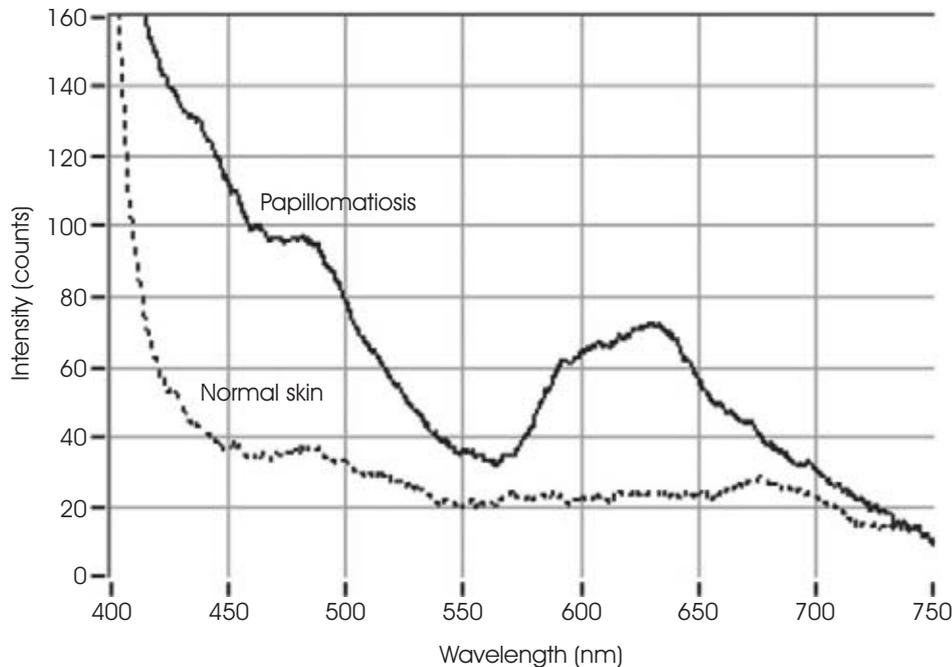


Figure 6. *In vivo* fluorescence of normal and irritated breast skin due to a confluent and reticulated papillomatosis.

furcated quartz fiber optic, and a laptop. The useful spectral range of the spectrometer is from 200 to 1,100 nm. So the system is also useful to measure the reflection at the excitation wavelength. Spectra can be obtained in less than 5 seconds. All the system can be transported in a portable aluminum briefcase (15 cm x 36 cm x 45 cm). Fluorescence measurements can be made from materials with a high quantum yield, as antifreeze

coolants or comestible oils, to *in vivo* or biopsies of biological tissue, which shows a lower quantum yields. The here reported measurements shown that the fluorescence spectroscopy could be used to support the detection of different cancers in biopsies and the possible adulteration of oils. The system could be also useful in the dermatological research, which is one of the principal objectives of our future research.

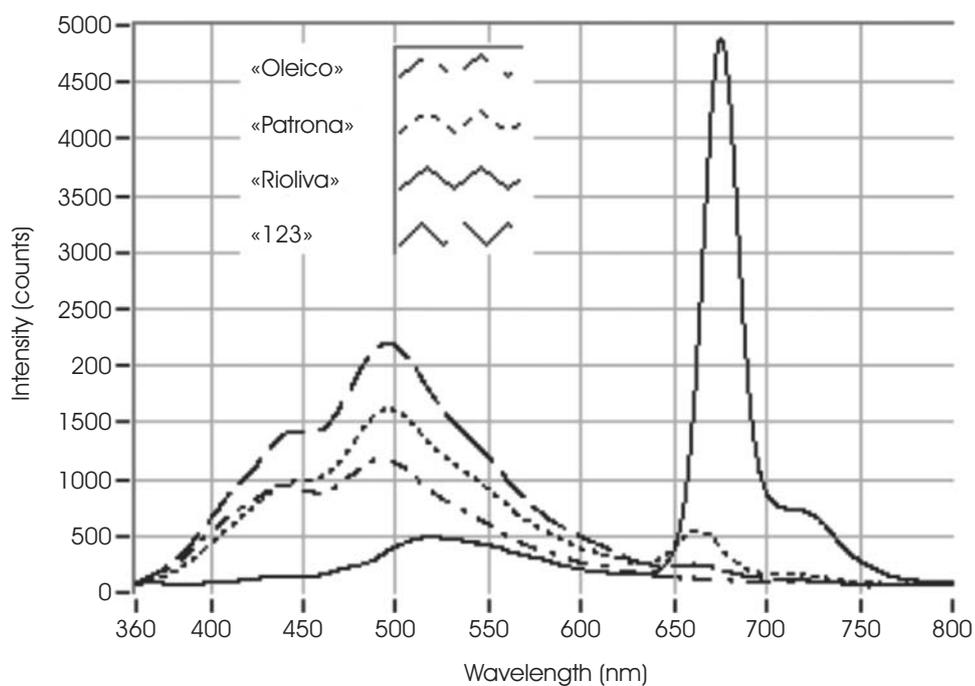


Figure 7. Fluorescence of commercial comestible oils: two sunflowers oils «Oleico» and «Patrona», an virgin olive oil «Reoliva» and a vegetable oil «123».

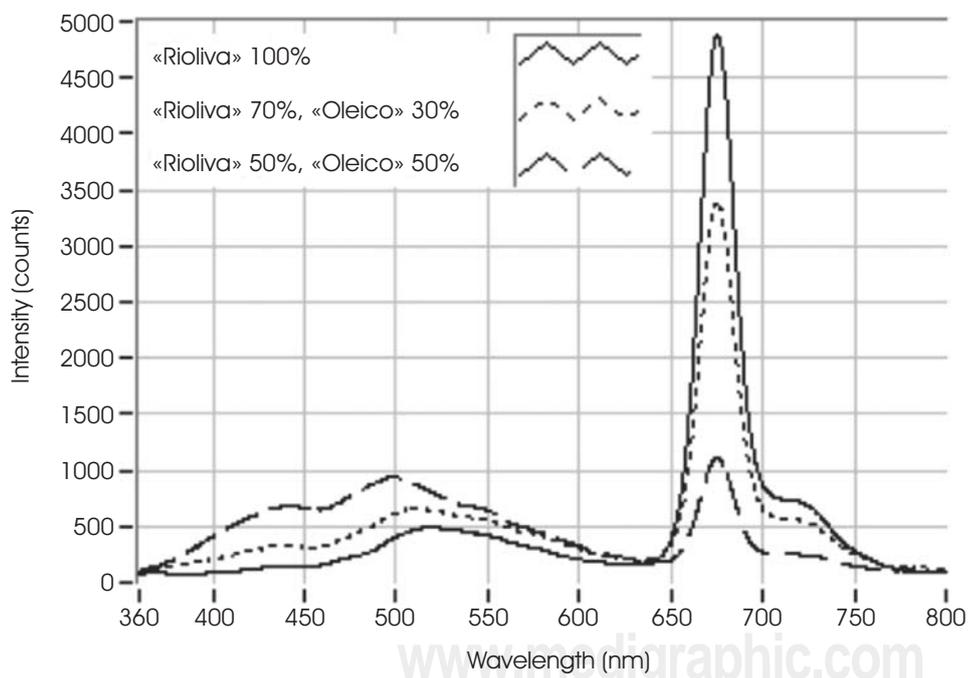


Figure 8. Fluorescence of mixtures of virgin olive «Reoliva» and sunflower oil «Oleico».

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