ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2014, *59*(6-7), 401-405

Dedicated to the memory of
Professor Eugen Segal (1933-2013)

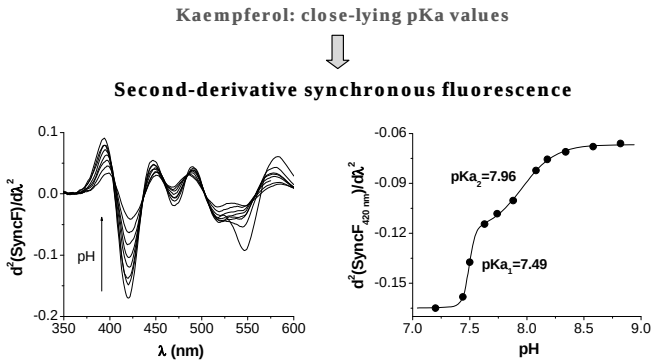
CLOSE-LYING pKa VALUES OF KAEMPFEROL DETERMINED BY SECOND-DERIVATIVE SYNCHRONOUS FLUORESCENCE

Iulia MATEI,[a,b] Cristina TABLET,[a,c] Sorana IONESCU[a] and Mihaela HILLEBRAND[a,*]

a Department of Physical Chemistry, University of Bucharest, 4-12 Bd. Regina Elisabeta, 030018 Bucharest, Roumania
[b] "Ilie Murgulescu" Institute of Physical Chemistry of the Roumanian Academy, 202 Spl. Independentei, Bucharest, 060021, Roumania
c Titu Maiorescu University, Faculty of Pharmacy, Gh. Şincai Bd. 16, Bucharest, Roumania

Received April 30, 2014

A precise knowledge of the pKa values of polyphenols is important in the discussion of the chemical species involved in different molecular interactions. However, in certain cases such as that of kaempferol, some acid-base equilibria are characterized by very close-lying pKa values, which hinders the possibility of obtaining reliable values. In the followings, a method based on the use of the second-derivative of the synchrounous fluorescence spectra recorded at different pH values is described and verified for the case of kaempferol. This method allows for the estimation of the two close-lying pKa values of kaempferol of 7.49 and 7.96, for which the classical spectral method fails. Concerning the third equilibrium in the pH range 10.0−11.5, the method leads to a pKa value of 11.05.



INTRODUCTION

Synchronous fluorescence (SyncF) is a spectroscopic technique widely used for identifying the constituents in a mixture, in cases when band overlapping prevents the use of the traditional, steady-state fluorescence (SSF) technique.[1,2] In the SyncF method, the excitation and emission wavelengths are scanned simultaneously while a constant interval, $\Delta\lambda$, is kept between them, which offers the advantages of spectral simplification and bandwidth reduction when this wavelength interval is set equal to the Stokes shift of one of the analytes.[3] This makes the technique very suitable to independently characterise a component from a mixture. Notably, SyncF has been applied to determine the pKa of some porphyrin[4] and fulvic acid[5] derivatives, and to identify the major metabolites of acetylsalicylic acid, on the basis of their SyncF emission dependence on the solution pH.[6]

An advancement of the SyncF method is the more sensitive second-derivative SyncF spectroscopy.[7] To date, this method has mostly been employed for the simultaneous determination of analytes from various mixtures,[8–10] *e.g.*, for the identification of ebastine in presence of its alkaline, acidic and UV degradation products.[11] A compound with more than one group prone to acid-base equilibria in solution is an example of such a mixture of many species, the neutral and the charged ones, which are in equilibrium with one

* Corresponding author: mihh@gw-chimie.math.unibuc.ro

another and thus their molecular ratio is dependent on the experimental conditions such as pH, the nature of the medium, other molecules present in solution (surfactants, proteins), etc. To add to the complexity of the problem, some of these acid-base equilibria may have very similar values of the acidity constants, making them extremely difficult to be determined, especially when the species have similar properties (*e.g.* overlapped bands in the electronic spectra). In these cases, the second-derivative SyncF method has the advantage of very sharp resolved bands and the versatility of choosing the wavelength interval, $\Delta\lambda$, corresponding to each component in the mixture. In the present study, we use second-derivative SyncF spectroscopy to differentiate between two close-lying pKa values of the flavonoid kaempferol.

Flavonoids are a class of natural polyphenolic compounds that have raised continuous interest due to their antioxidant and free radical scavenging properties.[12] Their antioxidant activity is closely related to the number of hydroxyl groups in the molecule, which ensures the existence of several charged species whose equilibrium is determined by the solution pH. This makes the accurate determination of the pKa values of the hydroxyl groups of paramount importance for understanding the *in vitro* and *in vivo* properties of flavonoids. For example, the correct identification of the species present in solution is essential for rationalizing flavonoid interaction with transport proteins. Previously, by using circular dichroism and quantum chemical calculations, we have shown that the interaction with the protein occurs *via* different species in the case of the flavonoids kaempferol,[13] fisetin,[14] and genistein.[15]

Kaempferol presents four ionisable hydroxyl groups in positions 3, 5, 7 and 4' (Fig. 1). According to literature data, their acidity decreases in the order 7 > 4' > 5 > 3, their pKa values spanning the range 7−14.[16] An experimental value of the fourth one, larger than 13, was not found in the literature, probably because strongly basic pHs cause degradation of kaempferol.[17] The first three pKa values from capillary electrophoresis have been reported[16] for the deprotonated of hydroxyls in positions 7 (A7), 4' (A7-A4') and 5 (A7-A4'-A5), namely pKa_1 = 7.05, pKa_2 = 9.04 and pKa_3 = 11.04, respectively. Other authors determined spectrophotometrically pKa = 7.89[18] as the first (and only) dissociation constant in the pH range 4−10 or pKa = 8.3 for kaempferol 3-o-galactoside, 7-o-rhamnoside,[19] which must correspond to the second dissociation step in kaempferol, *i.e.* the hydroxyl in position 4'. When using emission spectroscopy, the presence of an excited state proton transfer (ESIPT) process and formation of an excited state tautomer adds to the complexity of the system.[20] We consider that second-derivative SyncF is a suitable method for accurately determining these close-lying pKa values, as it allows minimisation of interferences that are inherent to the other techniques.

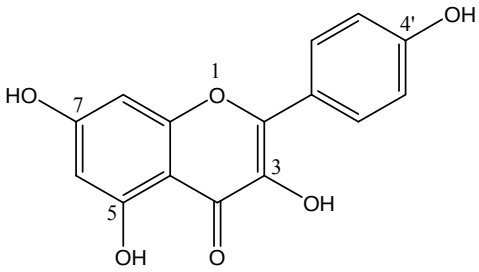


Fig. 1 – Molecular structure of kaempferol and numbering of ring positions.

RESULTS AND DISCUSSION

In aqueous solution at pH lower than 4, the UV-vis absorption spectrum of kaempferol corresponds exclusively to its neutral (N) form, with a band at 363 nm.[18] Upon increasing the pH, this band is bathochromically shifted (Fig. 2), evidencing the stepwise formation of anionic species.[13]

As concerns the emission spectrum of kaempferol, we will discuss its features and conduct the discussion of determining pKas from fluorescence data from simplest to more complex, from illustrating the concordance between the second-derivative method and the other two methods, SSF and SyncF, in the case when all yield good results to the case where two close-lying pKas are to be determined and the second-derivative method is the only reliable one.

In the pH range 10.0−11.5, the determination of the pKa_3 value of kaempferol, corresponding to the dianion−trianion equilibrium, can be made by either SSF or SyncF spectroscopy (Fig. 3). Both types of emission spectra are characterized by the presence of a single band, located at 550 nm and increasing in intensity with the pH value. This band is assigned to the third deprotonated form of kaempferol, the trianion A7-A4'-A5. Since in this spectral domain there are no interferences from other fluorescent species, both SSF and SyncF measurements can accurately estimate pKa_3 to 11.05±0.01. This value is in accordance to that obtained by Herrero-Martinez *et al.* by capillary electrophoresis.[16] The second-derivative is not necessary for such cases, but the pKa_3 value of 11.02±0.01 resulting from second-derivative SyncF data proves that it yields reliable results.

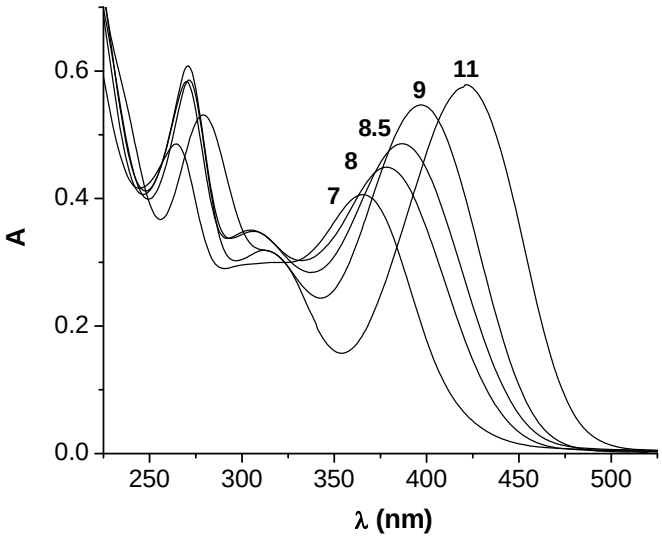


Fig. 2 – Absorption spectrum of kaempferol in aqueous solution at pH values (indicated on the figure) in the range 7–11.

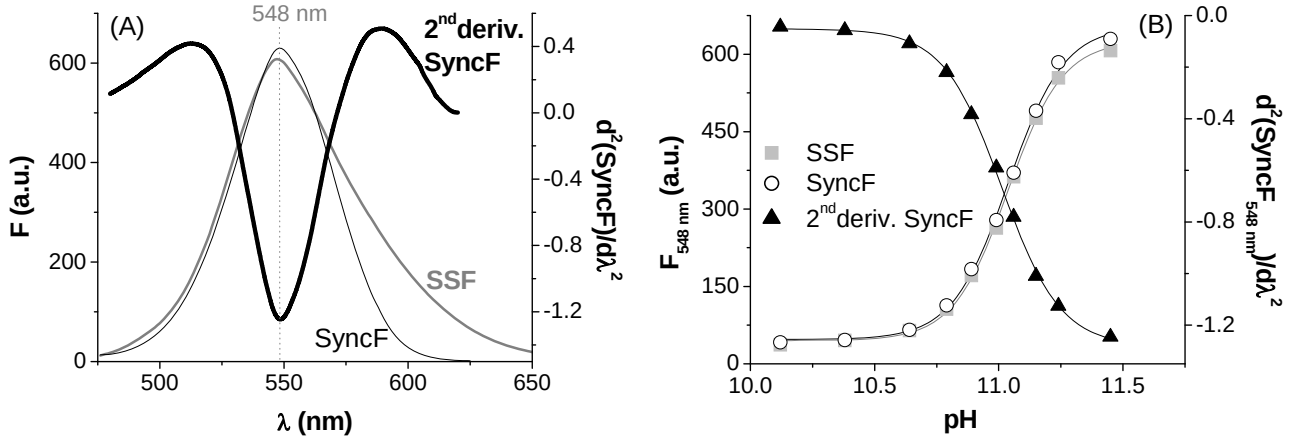


Fig. 3 – (A) The SSF (λ_{ex} = 423 nm), SyncF ($\Delta\lambda$ = 125 nm) and second-derivative SyncF spectra of kaempferol at pH = 11. (B) Determination of pKa_3 of kaempferol by SSF (R^2 = 0.996), SyncF (R^2 = 0.998) and second-derivative SyncF (R^2 = 0.999) spectroscopies. The solid lines represents the best fits of the data to sigmoid functions.

Problems arise when one wishes to determine the pKa_1 and pKa_2 values of kaempferol, which correspond to the formation of the monoanion A7 and dianion A7-A4', respectively. The SSF and SyncF spectra recorded in the pH range 6.5–9.0 are shown in Fig. 4. They both present three bands with maxima at 420, 480 and 530 nm and an isoemissive point, but the SSF bands are strongly overlapped and distorted by the presence of a Raman signal (sharp band at ~430 nm); by increasing the pH, the broad band with a maximum at about 530 and a shoulder at 480 nm increases in intensity (Fig. 4A). The SyncF spectra recorded in the same pH range display three well-separated bands, with the same maxima at 420, 480 and 530 nm, attesting the presence of several species in solution. The first two bands have been ascribed to the N (420 nm) and A7 (480 nm) species of kaempferol. The third band may have contributions from the dianion and kaempferol tautomer (T, 530 nm) resulting from an excited state proton transfer from the hydroxyl group in position 3 to the carbonyl group.[20]

In the attempt to determine pKa_1 and pKa_2, the SSF data at 420 nm cannot be used, as the neutral form is weakly emissive and overlapped with the Raman signal. The dependence of the fluorescence at the 530 nm maximum on the pH is represented in Fig. 4B and cannot be fitted. Although the SyncF spectra have the advantage of less overlapped bands, the experimental points can only be fitted by one sigmoid function, leading to the estimation of one pKa value. As it can be seen from Fig. 4B, the overlapped band at 480 nm cannot be used in this respect. This method fails to evidence the second acid-base equilibrium, as does the spectrophotometric method,[18] and the value of pKa_2 cannot be determined.

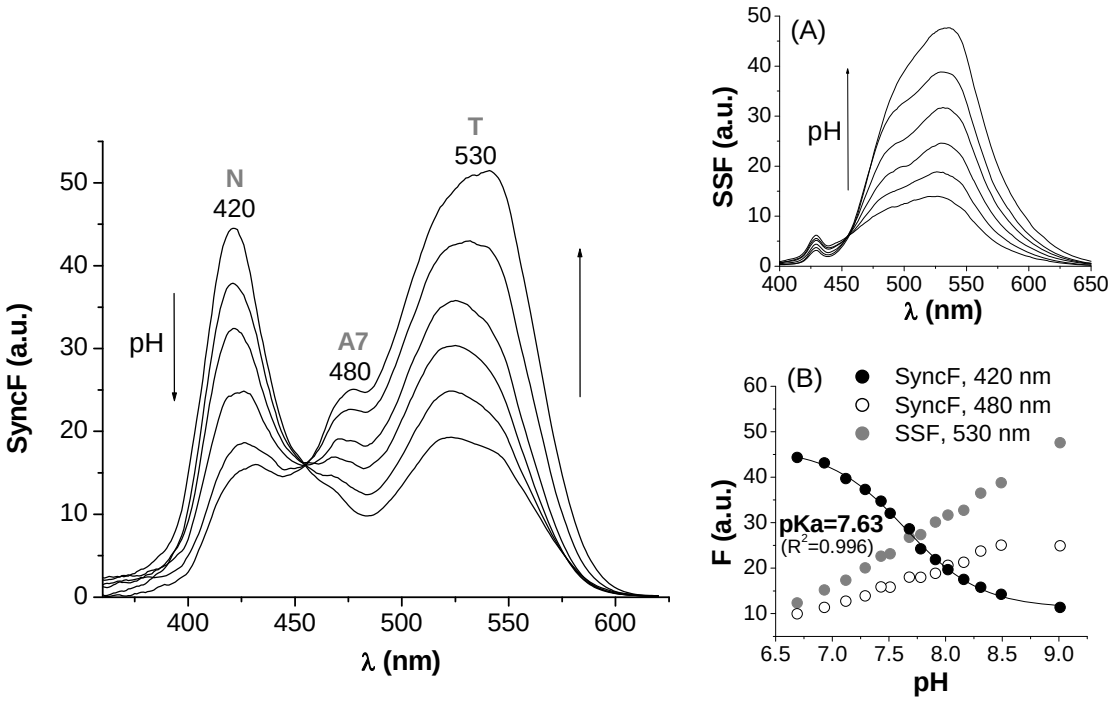


Fig. 4 – The SyncF ($\Delta\lambda$ = 130 nm) and SSF (λ_{ex} = 373 nm, inset A) spectra of kaempferol recorded at the same pH values in the range 6.5−9.0. Kaempferol species: N – neutral, A7 – anion at the hydroxyl group in position 7, T – tautomer. The SSF and SyncF data cannot be used for pKa_1 and pKa_2 differentiation (inset B).

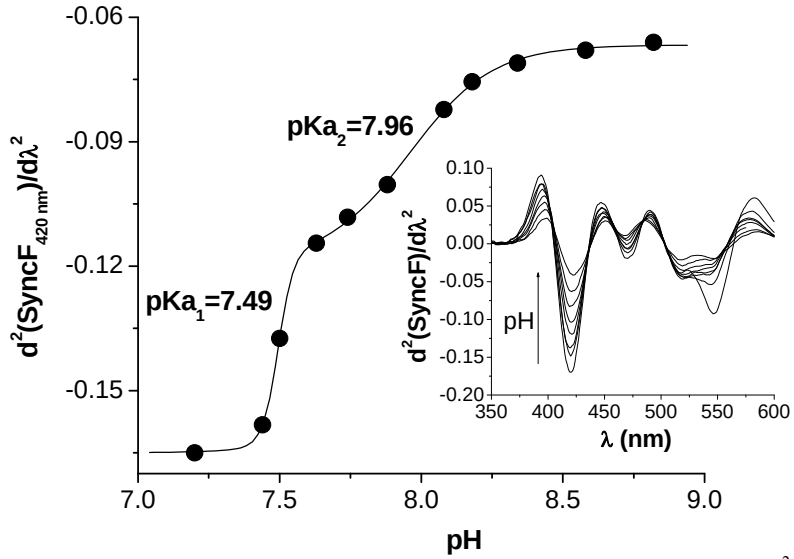


Fig. 5 – Determination of pKa_1 and pKa_2 of kaempferol by second-derivative SyncF spectroscopy (R^2 = 0.999). The solid line represents the best fit of the data to a double-sigmoid function. Inset: Second-derivative SyncF spectra of kaempferol ($\Delta\lambda$ = 130 nm).

In order to obtain two valid pKa values for the 6.5−9.0 pH range, the second-derivative of the SSF and SyncF spectra has been performed. Applying the derivatization process to SSF data results in a monotonous decrease of the second-derivative SSF intensity at 480 nm, without any inflexion point (data not shown), rendering the pKa determination impossible. Differently, the dependence of the second-derivative SyncF intensity at 420 nm on the pH value evidences the existence of two acid-base equilibria on this pH domain, and can be fitted to a double-sigmoid function (Fig. 5). One observes that the second-derivative SyncF method allows to differentiate between the two close-lying pKa values of kaempferol, yielding pKa_1 = 7.49±0.01 and pKa_2 = 7.96±0.02. These values are in good agreement with capillary electrophoresis data.[16] The advantage of this method is the better discrimination between the two steps in the

bisigmoidal graphical representation, which determines smaller errors.

EXPERIMENTAL

All chemicals were obtained from Sigma and used without further purification. 3×10^{-5} M kaempferol solutions at pH values covering the range 6.5–11.5 were freshly prepared by mixing the required volumes of ethanolic kaempferol stock solution and pH 2 or pH 11 Hydrion buffers. All experiments were conducted at room temperature.

UV-vis absorption and SSF/SyncF spectra were recorded on a V-560 Jasco UV-VIS spectrophotometer and a FP-6300 Jasco spectrofluorimeter, respectively. The solution pH was measured with a Radiometer PHM201 pH meter. Second-derivative SyncF spectra were obtained using the Spectra Analysis 1.53.04 software by Jasco.

CONCLUSIONS

The second-derivative of the synchronous fluorescence spectrum may prove the only viable solution to determine close-lying pKa values from emission data, especially for conjugated acid-base pairs with strongly overlapped fluorescence bands. We showed that all three acidity constants of kaempferol, situated in the pH range of 7−12, can be determined by this method, compared to only one determined by means of SSF or SyncF data. The values are in good agreement with data from capillary electrophoresis and have smaller errors.

Acknowledgements: This work was supported by the UEFISCSU grant PN II-ID-1914 no. 494/2009.

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