Journal of Luminescence 137 (2013) 138–142

Contents lists available at SciVerse ScienceDirect

Journal of Luminescence

journal homepage: www.elsevier.com/locate/jlumin

Synchronous fluorescence spectrometry: Conformational investigation or inner filter effect?

Maria Emilia Pacheco, Liliana Bruzzone *

División Química Analítica, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 47 y 115, 1900 La Plata, Argentina

ARTICLE INFO

Article history:
Received 14 July 2012
Received in revised form
12 December 2012
Accepted 28 December 2012
Available online 4 January 2013

Keywords:
Synchronous fluorescence spectrometry
Inner filter effect
Bovine serum albumin
Imazethapyr
Chlorimuron-ethyl

ABSTRACT

Synchronous fluorescence spectrometry has become a common tool to investigate conformational changes in proteins due to the presence of other molecules (quenchers). The same pattern is always followed, leaving aside the possibility of interfering effects that make conclusions incorrect. In this paper, we discuss the main cause of a wrong understanding of synchronous fluorescence spectrometry in a large number of papers published in many different journals. Based on the principles of synchronous fluorescence spectrometry, we perform a simple correction method of synchronous spectra taking into account inner filter effects. This criticism tries to demonstrate that the interpretation of synchronous fluorescence spectrometry is not as easy as it seems.

1. Introduction

Synchronous fluorescence spectrometry (SFS) has been extensively used in multicomponent analysis. The spectra are obtained through the simultaneous scanning of the excitation and the emission monochromators of a spectrofluorimeter, with a fixed wavelength difference ($\Delta\lambda$) between them. In the synchronous spectra, the sensitivity associated with fluorescence is maintained while offering several advantages: narrowing of spectral bands, an enhancement in selectivity by spectral simplification and a decrease of measurement time in multicomponent analysis [1].The idea of synchronous spectroscopy was first introduced by Lloyd [2] and the basic theory of it was formulated by Vo-Dinh [1]. The relation that represents the equation of synchronous spectrometry is:

$$I_s(\lambda,\lambda') = KcdE_X(\lambda')E_M(\lambda) \quad (1)$$

$$\lambda = \lambda' + \Delta\lambda \quad (2)$$

where I_s is the synchronous luminescence intensity, λ and λ' are the emission and excitation wavelength, respectively, K is a constant factor, c is the concentration of the analyte, d is the pathlength of the cuvette and E_X and E_M are the excitation and emission spectrum, respectively. In the synchronous technique, the luminescence intensity expression is an explicit function of λ as well as λ'. The improvement in sensitivity of this technique is indeed reflected in Eq. (1), which involves two functions instead of only one as in the conventional luminescence method. In addition, a new degree of selectivity is introduced by the parameter $\Delta\lambda$ which can be selected by the experimenter [1].

Miller [3] showed the application of this method to the resolution of tyrosine and tryptophan fluorescence. At small $\Delta\lambda$ values, the synchronous fluorescence of a tyrosine–tryptophan mixture is characteristic of tyrosine, whereas at large $\Delta\lambda$ values the spectra is similar to that of tryptophan.

Synchronous fluorescence spectra are frequently used to characterize the interaction between the molecule probe (quencher) and proteins since, as several articles report [4–42], it can provide information about the molecular microenvironment in the vicinity of the chromophore molecules. A general method to study the environment of amino acid residues is the measurement of the possible shift in synchronous maximum wavelength $\lambda_{SFS,max}$. As discussed in those papers, the shift in the position of the maximum corresponds to changes in polarity around the chromophore molecule: tyrosine ($\Delta\lambda$=15 nm) and tryptophan residues ($\Delta\lambda$=60 nm).

It is important to remark that fluorescence intensities are proportional to the concentration of the analyte over only a limited range of absorbances. In fluorescence instruments that use right-angle geometry (the cuvette is illuminated centrally and the observation is at a right-angle), the intensity of the exciting light at the centre of the cuvette is diminished due to the absorption of the sample. This effect may decrease the intensity of the excitation at the point of observation or decrease the observed fluorescence by absorption of the fluorescence. The

* Corresponding author. Tel.: +54 221 422 8328; fax: +54 221 425 4533.
E-mail address: bruzzone@quimica.unlp.edu.ar (L. Bruzzone).

0022-2313/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jlumin.2012.12.056

relative importance of each process depends upon the absorbance of the sample at the excitation and emission wavelengths. If the sample has a significant absorption at excitation (A_{ex}) and emission (A_{em}) wavelengths, the excitation and emission intensities are attenuated by $10^{-0.5A_{ex}}$ and $10^{-0.5A_{em}}$, respectively [43,44]. These effects define the so called "inner filter effect" (IFE). When the absorbance of the solution is lower than 0.3 [45] the following equation can be applied to correct the inner filter effects:

$$F_{corr} = F_{obs}\text{antilog}\,[(A_{ex}+A_{em})/2] \quad (3)$$

where F_{obs} and F_{corr} are the measured and corrected fluorescence intensities, respectively (in a 1.0 cm pathlength cuvette). A_{ex} and A_{em} are the absorbances at excitation and emission wavelengths, respectively in a 1.0 cm pathlength cuvette.

Excitation and emission spectra are altered by inner filter effects, distorting the synchronous maximum. As the synchronous spectra is obtained by multiplying the excitation and emission spectrum (Eq. (1)), it is necessary to correct them in order not to misunderstand the obtained results. In most articles, conclusions are derived from SFS without correcting for inner filter effects [4–42]. So, up to what extend is SFS a reliable tool in conformational investigation?

2. Experimental

2.1. Reagents and chemicals

Bovine serum albumin, BSA (Cohn-Analog ≥ 98%: Sigma-Aldrich), was dissolved in 0.10 mol L^{-1} Tris–HCl buffer solution (pH = 7.4), containing 0.15 mol L^{-1} NaCl, to prepare a stock solution (1.27×10^{-4} mol L^{-1}) which was then stored at 0–4 °C for a maximum period of 3 months.

Imazethapyr,5-ethyl-2-[(RS)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl] nicotinic acid, (IMA) and Chlorimuron-ethyl, ethyl 2-(4-chloro-6-methoxypyrimidin-2-ylcarbamoylsulfamoyl) benzoate, (CLO) were provided by a local industry. The IMA and CLO stock solutions, 1.00×10^{-3} mol L^{-1}, were prepared by dissolving the herbicide in buffer solution (pH = 7.4).

All chemicals were of analytical-grade reagent and all solutions were prepared using double-distilled water.

2.2. Apparatus

All recordings of fluorescence and synchronous spectra were carried out on a Perkin-Elmer LS-50B luminescence spectrometer (Beaconsfield, England) equipped with a pulsed xenon lamp (half peak height < 10 μs, 60 Hz), an R928 photomultiplier tube and a computer working with FL Winlab software. Excitation and emission bandwidths were set at 5 nm. All right-angle fluorescence measurements were performed in a 1.0 cm pathlength quartz cuvette and complementary front-face fluorescence measurements were performed using the front-face accessory and cylindrical quartz cuvettes of the same diameter of the powder holder.

Absorption spectra were measured on a Shimadzu UV-240 (Japan) recording spectrophotometer, equipped with a 1.0 cm pathlength quartz cuvette.

3. Results and discussions

In order to demonstrate if the observed shifts in SFS are caused by IFE, emission, excitation and synchronous fluorescence spectra of BSA in presence and absence of IMA or CLO were measured. Absorbance values corresponding to the wavelength range of excitation and emission fluorescence spectra were also measured. Absorption spectra are shown in Figs. 1 and 2.

The obtained fluorescence spectra were corrected for inner filter effects using Eq. (3). Figs. 3 and 4 show the uncorrected and corrected excitation spectra of BSA–IMA and BSA–CLO, respectively. As can be appreciated, the uncorrected excitation spectra show a red shift when the concentration of the herbicide is increased. After correcting for IFE, the red shift is no longer seen. Figs. 5 and 6 show the uncorrected and corrected emission spectra of BSA–IMA and BSA–CLO, respectively. There is no evident shift either in the uncorrected spectra or in the corrected spectra.

According to the basic SFS equation, the synchronous fluorescence spectra are a function of the excitation and emission spectra. This allows us to multiply the corrected excitation spectrum by the corrected emission spectrum. In doing so, we simulate the synchronous spectrum. For BSA-quencher systems, $\Delta\lambda = 60$ nm and $\Delta\lambda = 15$ nm are chosen in order to study the

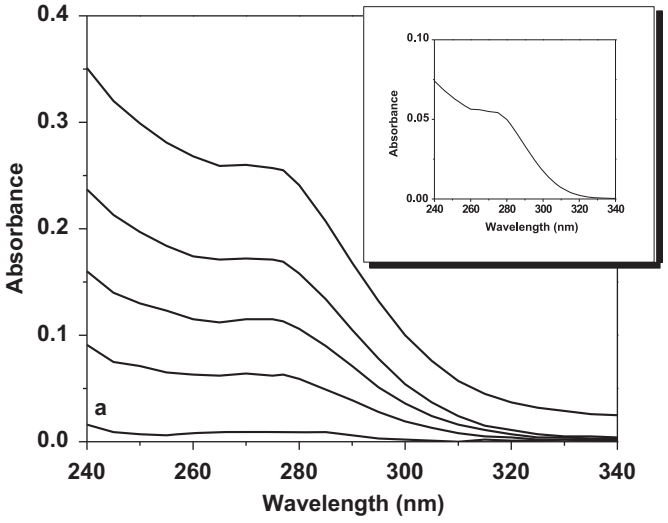

Fig. 1. Absorption spectra of BSA (a), IMA (inset, [IMA] = 7.81×10^{-6} mol L^{-1}) and BSA–IMA: [BSA] = 2.04×10^{-7} mol L^{-1}; [IMA] = 7.81×10^{-6}, 1.56×10^{-5}, 2.34×10^{-5} and 3.12×10^{-5} mol L^{-1}.

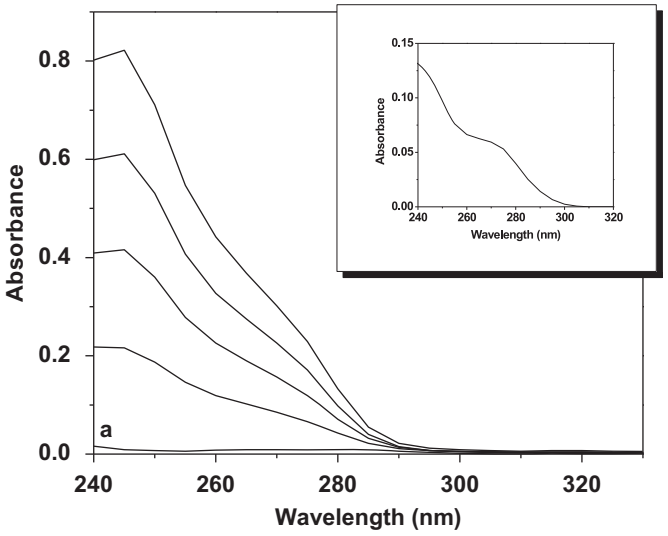

Fig. 2. Absorption spectra of BSA (a), CLO (inset, [CLO] = 7.71×10^{-6} mol L^{-1}) and BSA–CLO: [BSA] = 2.04×10^{-7} mol L^{-1}; [CLO] = 7.71×10^{-6}, 1.54×10^{-5}, 2.31×10^{-5} and 3.09×10^{-5} mol L^{-1}.

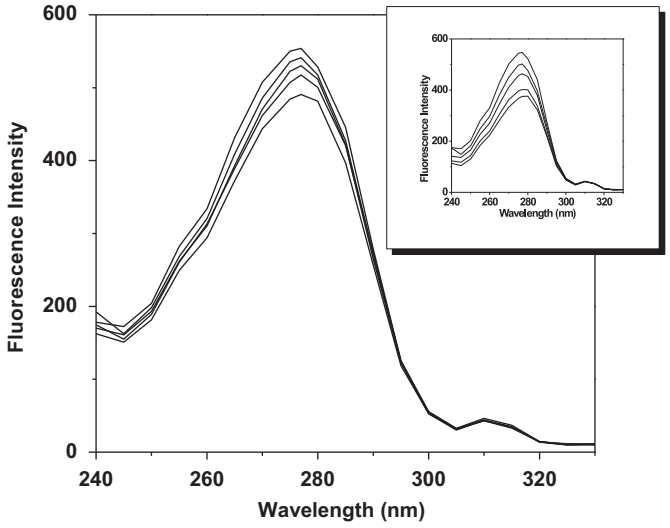

Fig. 3. Corrected and uncorrected (inset) fluorescence excitation spectra of BSA–IMA. λ_{em}=347 nm [BSA]=2.04×10^{-7} mol L^{-1}; [IMA]=7.81×10^{-6}, 1.56×10^{-5}, 2.34×10^{-5} and 3.12×10^{-5} mol L^{-1}.

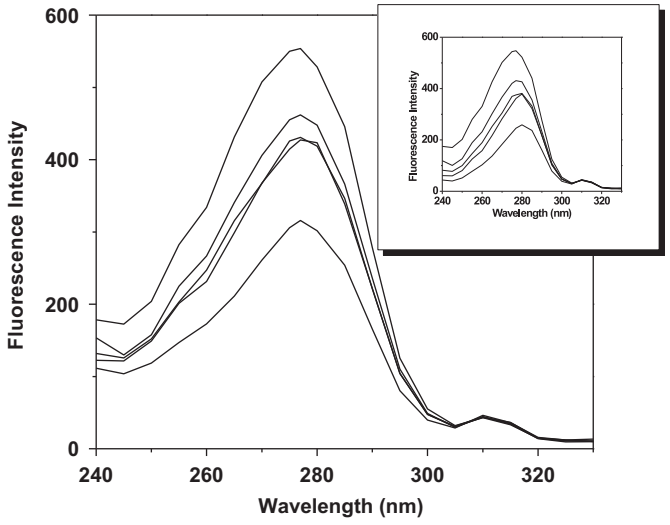

Fig. 4. Corrected and uncorrected (inset) fluorescence excitation spectra of BSA–CLO. λ_{em}= 347 nm [BSA]=2.04×10^{-7} mol L^{-1}; [CLO]=7.71×10^{-6}, 1.54×10^{-5}, 2.31×10^{-5} and 3.09×10^{-5} mol L^{-1}.

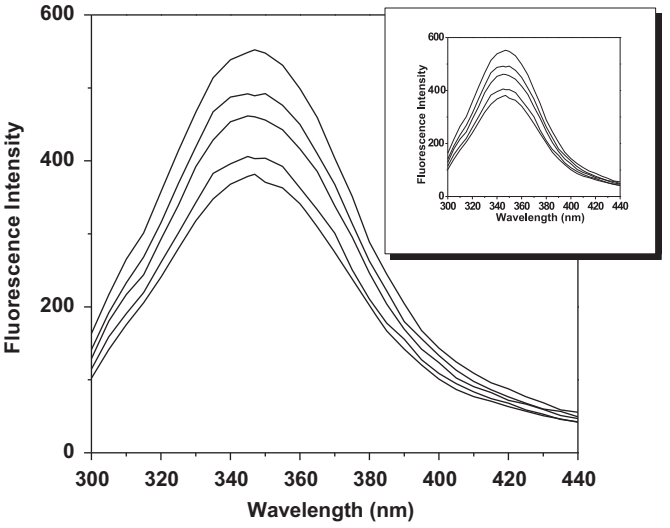

Fig. 5. Corrected and uncorrected (inset) fluorescence emission spectra of BSA–IMA. λ_{ex}=277 nm [BSA]=2.04×10^{-7} mol L^{-1}; [IMA]=7.81×10^{-6}, 1.56×10^{-5}, 2.34×10^{-5} and 3.12×10^{-5} mol L^{-1}.

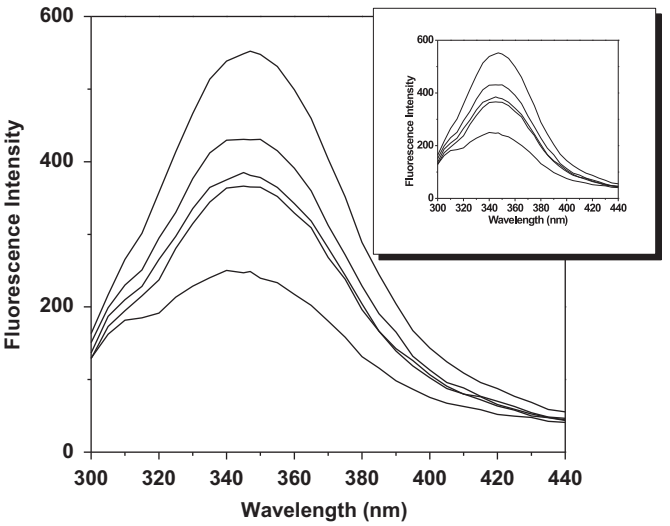

Fig. 6. Corrected and uncorrected (inset) fluorescence emission spectra of BSA–CLO. λ_{ex}=277 nm [BSA]=2.04×10^{-7} mol L^{-1}; [CLO]=7.71×10^{-6}, 1.54×10^{-5}, 2.31×10^{-5} and 3.09×10^{-5} mol L^{-1}.

conformational changes due to the presence of a quencher. We chose $\Delta\lambda$=60 nm because of the low fluorescence intensities obtained with $\Delta\lambda$=15 nm. The simulated and obtained (using the spectrofluorimeter) synchronous fluorescence spectra are shown in Figs. 7 and 8. Note that any possible shift was removed after correcting the spectra for IFE. Figs. 9 and. 10 show the synchronous fluorescence spectra obtained using front-face illumination geometry. The absence of an evident shift suggests that IFE is the major reason for the observed shifts in right-angle illumination geometry. At this point, our initial question is answered and the conclusions at which achieve most of the articles cited is questioned.

Victor and Crouch [46] described an instrument that measures synchronous fluorescence while simultaneously correcting for inner filter effects. They remarked that uncompensated inner filter effects can lead to unexpected and/or large quantitative errors. Lloyd [47] published a criticism where the use of SFS was subjected to inner filter effects, and there he specified that in fixed and synchronous luminescence techniques the spectra obtained are meaningless unless the corresponding absorption spectra are known and, preferably, sufficiently weak to be of negligible importance (absorbance of solutions should not exceed 0.02 per effective pathlength [44]). Divya and Mishra [48]explained the concentration-dependent red-shift of fluorescence in multi-fluorophoric solutions in terms of inner filter effects. They proposed a method which enables obtaining a $\Delta\lambda$ that can give maximum fluorescence at maximum synchronous wavelength ($\lambda_{SFS,max}$). Although an optimum $\Delta\lambda$ is not expected to obtain in this criticism, Ref. [48] is a good example of IFE. Consequently, taking into account these cites [46–48], conclusions derived from uncorrected spectra [4–42] are partly based on a wrong interpretation of SFS as well as basic fluorescence principles.

This criticism tries to be general, but we cite specific references from many journals published in the last four years in order to demonstrate that the discussed pitfall is common. Several articles perform a SFS study using protein (bovine serum albumin or human serum albumin) and quencher concentrations that have absorbance values at excitation and/or emission wavelength

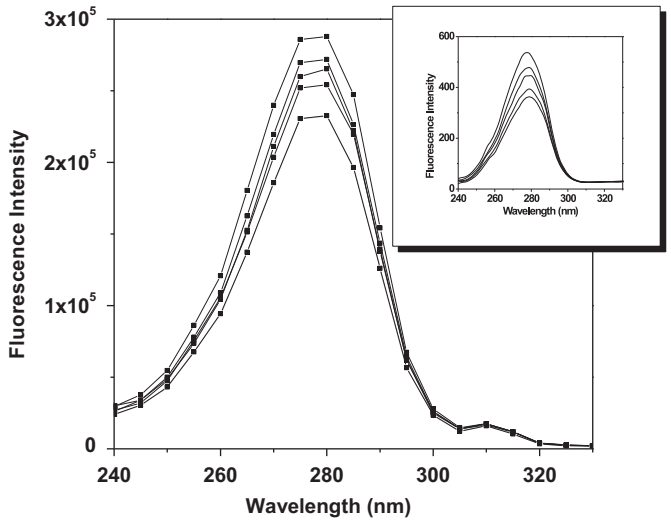

Fig. 7. Corrected and uncorrected (inset) synchronous fluorescence spectra of BSA–IMA. $\Delta\lambda=60$ nm [BSA]$=2.04\times10^{-7}$ mol L^{-1}; [IMA]$=7.81\times10^{-6}$, 1.56×10^{-5}, 2.34×10^{-5} and 3.12×10^{-5} mol L^{-1}.

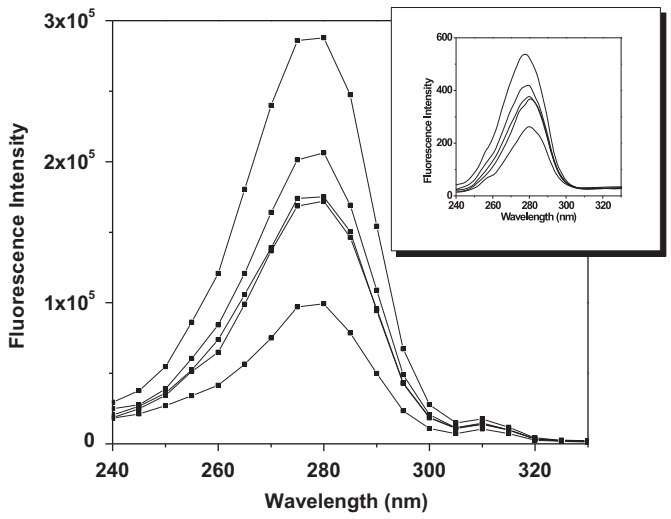

Fig. 8. Corrected and uncorrected (inset) synchronous fluorescence spectra of BSA–CLO. $\Delta\lambda=60$ nm [BSA]$=2.04\times10^{-7}$ mol L^{-1}; [CLO]$=7.71\times10^{-6}$, 1.54×10^{-5}, 2.31×10^{-5} and 3.09×10^{-5} mol L^{-1}.

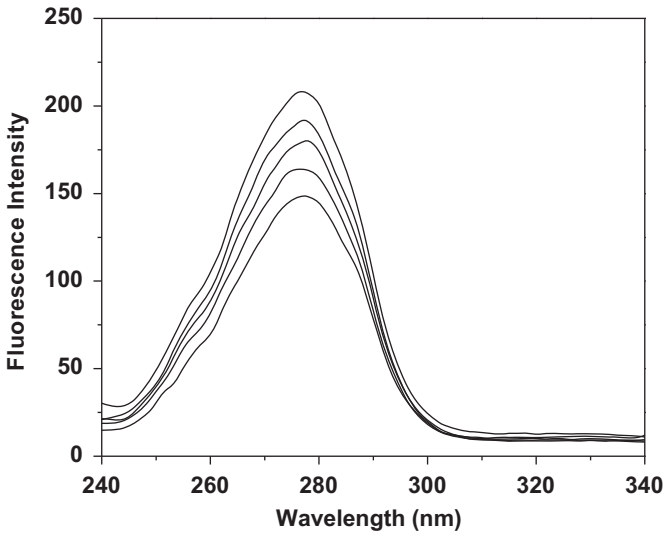

Fig. 9. Synchronous fluorescence spectra of BSA–IMA (front-face illumination geometry). $\Delta\lambda=60$ nm [BSA]$=2.04\times10^{-7}$ mol L^{-1}; [IMA]$=7.81\times10^{-6}$, 1.56×10^{-5}, 2.34×10^{-5} and 3.12×10^{-5} mol L^{-1}.

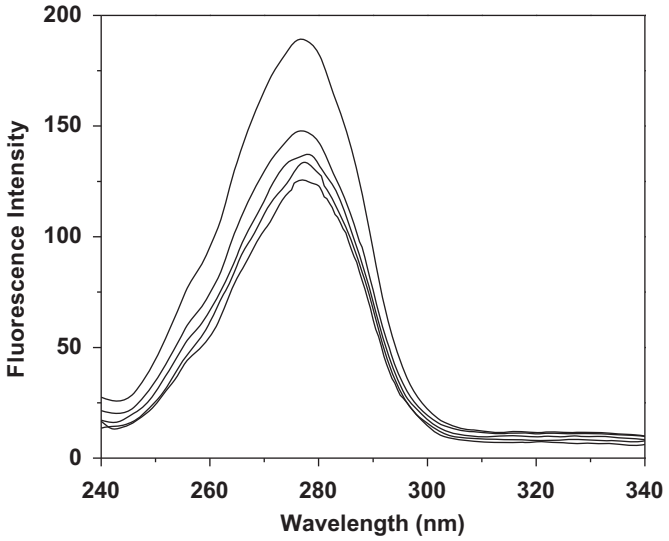

Fig. 10. Synchronous fluorescence spectra of BSA–CLO (front-face illumination geometry). $\Delta\lambda=60$ nm [BSA]$=2.04\times10^{-7}$ mol L^{-1}; [CLO]$=7.71\times10^{-6}$, 1.54×10^{-5}, 2.31×10^{-5} and 3.09×10^{-5} mol L^{-1}.

greater than 0.02 per effective pathlength and thus they are affected by IFE [4–42]. Although some articles [34] argue that fluorescence intensities have to be corrected for IFE (using Eq. (3)) in order to plot the Stern–Volmer equation, SFS is not corrected. The same is done by other articles [35–42], but using an incorrect equation. Inappropriately, all cited articles attribute the observed shift in SFS to conformational changes, when the main cause is the lack of correction for IFE.

4. Conclusions

In this paper we discussed a common error in articles which analyze the interaction between serum albumins (human and bovine) and a quencher molecule. The authors of the articles wrongly attribute the observed shift in SFS to conformational changes in the protein, without taking into account the deformation of the excitation and/or emission spectra due to IFE. Although absorption spectra show values of absorbance that decrease fluorescence intensity, and some of them correct the Stern–Volmer equation for IFE, no correction is done in SFS. Our study of the interaction of two herbicides with BSA is an example that demonstrates that IFE can lead to incorrect interpretations of the involved mechanism. The generally accepted methodology of SFS in protein investigation has diminished the basis of SFS and fluorescence spectrometry, at the extent of using concentrated solutions (high absorbance values) without realizing the non-linearity of fluorescence intensities under these conditions.

Acknowledgements

The authors thank Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina, for partial financial support.

References

[1] T. Vo-Dinh, Anal. Chem. 50 (1978) 396.
[2] J.B.F. Lloyd, Nature 231 (1971) 64.
[3] J.N. Miller, Proc. Anal. Div. Chem. Soc. 16 (1979) 203.
[4] F.-L. Cui, Y.-H. Yan, Q.-Z. Zhang, G.-R. Qu, J. Du, X.-J. Yao, J. Mol. Modeling 16 (2010) 255.
[5] N. Wang, L. Ye, F. Yan, R. Xu, Int. J. Pharm. 351 (2008) 55.
[6] Y. Ni, S. Su, S. Kokot, Spectrochim. Acta A 75 (2010) 547.
[7] H. Ye, B. Qiu, Z. Lin, G. Chen, Luminescence 26 (2011) 336.
[8] W. Zhang, B. Han, S. Zhao, F. Ge, X. Xiong, D. Chen, D. Liu, C. Chen, Anal. Lett. 43 (2010) 289.
[9] A. Varlan, M. Hillebrand, Molecules 15 (2010) 3905.
[10] A.S. Roy, D.R. Tripathy, A. Chatterjee, S. Dasgupta, J. Biophys. Chem. 1 (2010) 141.
[11] P. Qu, H. Lu, X. Ding, Y. Tao, Z. Lu, J. Mol. Struct. 920 (2009) 172.
[12] Y.-Q. Wang, H.-M. Zhang, Q.-H. Zhou, J. Mol. Struct. 932 (2009) 31.
[13] H.-M. Zhang, T.-T. Chen, Q.-H. Zhou, Y.-Q. Wang, J. Mol. Struct. 938 (2009) 221.
[14] Y.-Q. Wang, B.-P. Tang, H.-M. Zhang, Q.-H. Zhou, G.-C. Zhang, J. Photochem. Photobiol. B: Biol. 94 (2009) 183.
[15] X.-J. Guo, K. Jing, C. Guo, Y.-C. Jiang, J. Tong, X.-W. Han, J. Lumin. 130 (2010) 2281.
[16] X.-L. Han, P. Mei, Y. Liu, Q. Xiao, F.-L. Jiang, R. Li, Spectrochim. Acta A 74 (2009) 781.
[17] S. Bi, L. Yan, B. Pang, Y. Wang, J. Lumin. 132 (2012) 132.
[18] B. Pang, S. Bi, Y. Wang, L. Yan, T. Wang, J. Lumin. 132 (2012) 895.
[19] Y. Zhang, S. Shi, M. Peng, J. Lumin. 132 (2012) 1921.
[20] X. Yu, Y. Yang, X. Zou, H. Tao, Y. Ling, Q. Yao, H. Zhou, P. Yi, Spectrochim. Acta A 94 (2012) 23.
[21] D. Li, Y. Wang, J. Chen, B. Ji, Spectrochim. Acta A 79 (2011) 680.
[22] X. Yu, R. Liu, R. Yi, F. Yang, H. Huang, J. Chen, D. Ji, Y. Yang, X. Li, P. Yi, Spectrochim. Acta A 78 (2011) 1329.
[23] H. Jiang, R.-R. Chen, H.C. Wang, H.L. Pu, Chin. Chem. Lett. 23 (2012) 599.
[24] X. Yu, R. Liu, F. Yang, D. Ji, X. Li, J. Chen, H. Huang, P. Yi, J. Mol. Struct. 985 (2011) 407.
[25] H. Jiang, R. Chen, H. Pu, J. Lumin. 132 (2012) 592.
[26] R. Liu, X. Yu, W. Gao, D. Ji, F. Yang, X. Li, J. Chen, H. Tao, H. Huang, P. Yi, Spectrochim. Acta A 78 (2011) 1535.
[27] R.G. Machicote, M.E. Pacheco, L. Bruzzone, Spectrochim. Acta A 78 (2011) 1535.
[28] Z. Hou, N. Zhou, B. He, Y. Yang, X. Yu, J. Mol. Struct. 1004 (2011) 116.
[29] X. Yu, Y. Yang, R. Liu, H. Huang, J. Chen, D. Ji, X. Li, F. Yang, P. Yi, J. Lumin. 131 (2011) 1510.
[30] Z. Cheng, Spectrochim. Acta A 93 (2012) 321.
[31] X. Gao, Y. Tang, W. Rong, X. Zhang, W. Zhao, Y. Zi, Am. J. Anal. Chem. 2 (2011) 250.
[32] A.H. Hegde, B. Sandhya, J. Seetharamappa, Mol. Biol. Rep. 38 (2011) 4921.
[33] X.-J. Guo, A.-J. Hao, X.-W. Han, P.-L. Kang, Y.-C. Jiang, X.-J. Zhang, Mol. Biol. Rep. 38 (2011) 4185.
[34] A.D. Bani-Yaseen, J. Lumin. 131 (2011) 1042.
[35] X. Guo, L. Zhang, X. Sun, X. Han, C. Guo, P. Kang, J. Mol. Struct. 928 (2009) 114.
[36] F. Ding, W. Liu, L. Zhang, B. Yin, Y. Sun, J. Mol. Struct. 968 (2010) 59.
[37] F. Ding, W. Liu, Y. Li, L. Zhang, Y. Sun, J. Lumin. 130 (2010) 2013.
[38] L. He, X. Wang, B. Liu, J. Wang, Y. Sun, E. Gao, S. Xu, J. Lumin. 131 (2011) 285.
[39] F. Deng, Y. Liu, J. Lumin. 132 (2012) 443.
[40] F. Ding, G. Zhao, S. Chen, F. Liu, Y. Sun, L. Zhang, J. Mol. Struct. 929 (2009) 159.
[41] F. Samari, M. Shamsipur, B. Hemmateenejad, T. Khayamian, S. Gharaghani, Eur. J. Med. Chem 54 (2012) 255.
[42] F. Ding, W. Liu, N. Li, L. Zhang, Y. Sun, J. Mol. Struct. 975 (2010) 256.
[43] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, third ed., Springer Publisher, New York, 2006.
[44] C.A. Parker, Photoluminescence of Solutions, Elsevier Publishing Company, Amsterdan, 1968.
[45] E.P. Kirby, in: R.F. Steiner, L. Weinryb (Eds.), Excited States of Proteins and Nucleic Acids, Plenum Press, New York, 1971p. 31.
[46] M.A. Victor, S.R. Crouch, Appl. Spectrosc. 49 (1995) 1041.
[47] J.B.F. Lloyd, Anal. Chem. 52 (1980) 189.
[48] O. Divya, A.K. Mishra, Anal. Chim. Acta 630 (2008) 47.