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A COMBINATION OF SYNCHRONOUS FLUORESCENCE SPECTROSCOPY WITH CHEMOMETRIC TREATMENT AND INTERNAL STANDARDS IN NON-AQUEOUS POTENTIOMETRIC TITRATIONS OF FULVIC ACIDS

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Summary—The acid properties of a soil fulvic acid (sfua) were characterized by potentiometric titration with tetrabutylammonium hydroxide in two non-aqueous solvents with high acid–base resolution power, *N*,*N*-dimethylformamide (DMF) and acetonitrile. Synchronous fluorescence spectroscopy (SyF) was also used to monitor directly the sfua status during the potentiometric titration in DMF. The potentiometric titration curves showed no clear end-point and the analysis of the sets of spectra obtained at increasing neutralization degree, with a self-modeling curve resolution method (SIMPLISMA), revealed the existence of two components with featureless concentration profiles. Internal standards (maleic, salicylic and *p*-hydroxylbenzoic acids) were used to determine the amounts of acid groups with different acid strengths in the two non-aqueous solvents. It was shown that the variations observed in the SyF spectra sets of the internal standards are not correlated with those observed in the sfua data. The splitting of the sfua groups in the non-aqueous titration curves seems to be forced artificially depending on the standards used.

Non-aqueous solvents have been used in the study of the acid group composition of humic substances (HS) and related compounds to overcome the powerful acid leveling effect of water.[1–6] Indeed, although these substances are complex mixtures of acid functionalities containing carboxylic and hydroxylic structures, the shape of a titration curve in water (pH *vs.* volume of strong base) allows the identification of only one end point, situated at neutral pH. *N*,*N*-Dimethylformamide (DMF)[2,3] and dimethylsulphoxide (DMSO)[3–6] have been used as solvents for potentiometric titrations of HS with glass electrodes instead of water but, surprisingly, the expected enhancement of resolution of the titration curve was not observed. To overcome this situation, an experimental strategy based on the use of internal standards (known amounts of well defined substances) has been used[2–6] and up to three classes of acid groups could be titrated. The problem of validating this analytical procedure is very difficult because there are as yet no reliable model compounds of HS since little structural information is available about them.

HS molecules contain fluorescent fractions, which work as probes in their complex molecular environment, therefore molecular fluorescence spectroscopy can be used in studies about the interaction of these materials and metal cations[7–10] or organic compounds.[11,12] More recently, synchronous fluorescence (SyF) spectroscopy started to be used for the study of HS[10,13] and related products, like leaf litter extracts.[14] SyF opened new perspectives for the characterization of these substances, mainly because of the large enhancement of resolution and amount of information of the SyF spectra relatively to the more traditional fluorescence modes of analysis (emission and excitation). In our earlier studies,[15,16] it was concluded that from the variations observed in the SyF spectra of fulvic acids in aqueous solutions with pH, macroscopic acid–base constants can be determined from the analysis of the spectra with powerful self-modeling curve resolution techniques like evolving factor analysis[15] or SIMPLISMA.[16–19]

This paper reports the characterization of the acid properties of a soil fulvic acid (sfua) by potentiometric titrations in non-aqueous solvents. DMF and acetonitrile (AN) were used. AN was not used in previous studies[1–6] but, since

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it is a stronger acid than DMF, it was included to investigate if an increase of resolution relatively to DMF was observed in the titration curve. Internal standards were used in both solvents to improve the resolution of the acid groups of the sfua into a small number of classes. Also, a new approach to the use of non-aqueous solvents for the analysis of the acid properties of HS is presented and discussed in this paper. The procedure consists of using SyF, together with potentiometry and with spectral analysis by a self-modeling curve resolution method, SIMPLISMA, as a direct method for monitoring the sfua molecular status as function of the degree of neutralization. Since no study about the theoretical foundations of the use of internal standards in potentiometric titrations was detected in the literature, this direct analysis was also used to investigate if there exists any relation between the titration of the acid groups of the internal standards and the titration of any acid groups or classes of acid groups of sfua, with the purpose of finding a validation procedure for the internal standards methodology.

EXPERIMENTAL

Reagents

The sample of sfua was isolated from a Portuguese forest soil collected at Louros, Famalicão (30 km from Porto), by the procedure recommended by the IHSS.[20]

Analytical grade reagents were used to prepare all solutions. For the potentiometric titrations, the titrant was 0.015*M* tetrabutylammonium hydroxide (Bu_4NOH) solution in *i*-propanol, prepared from a commercial solution (*ca.* 0.1*M*) of the compound in methanol/*i*-propanol (Merck), standardized with a standard solution of 0.01*M* nitric acid (Titrisol Merck) and kept under nitrogen.

For the potentiometric titrations in DMF, solutions of salicylic acid (SAL) and *p*-hydroxylbenzoic acid (pHB) (1–3 m*M*) were prepared in this solvent to be used as internal standard. Sfua solutions (0.6 g/l.) were prepared by rigorous weighting and using the internal standard solution as solvent. Typical experiments consisted of the titration of 5.00 ml of these solutions with Bu_4NOH (using a titrant increment of 0.050 ml). The equivalent volumes corresponding both to each internal standard (to each protolysis for the diprotic acids) and to sfua were about 1 ml.

For the titrations in AN, a similar procedure was used but two internal standard solutions were prepared with binary mixtures of maleic acid (MAL) and SAL, and of SAL and pHB. The sfua sample was not completely soluble in AN, but dissolved after the first few additions of the strong base used as titrant.

For the potentiometric + fluorescence titrations in DMF, solutions of 0.06 g/l sfua in the presence of 0.7 m*M* perchloric acid were used. The titrant was the commercial solution of Bu_4NOH in methanol/*i*-propanol (Merck) diluted in DMF. Pure sfua solutions as well as mixtures of sfua and an internal standard were titrated. Three internal standards were used: 0.1 m*M* SAL; 2 m*M* pHB; and 2 m*M* catechol (CAT).

Apparatus for the potentiometric titrations

Potentiometric titrations were made with an automatic system (Metrohm Titroprocessor 636 coupled to a 5.000 ml Dosimat E635) with an Ingold U202 glass electrode and an Orion 90-02-00 (double junction) reference electrode (0.1*M* tetrabutylammonium chloride in *i*-propanol was used as outer solution). Titration end-points were calculated by derivative methods.

Apparatus for the potentiometric + fluorescence titrations

Burette control and potential readings were conducted with a PC controlled system assembled with a Crison MicropH 2002 pHmeter, a Crison MicroBu 2030 microburette, a Philips GAT 130 glass electrode and a Radiometer K711 (double junction) reference electrode (0.1*M* tetrabutylammonium chloride in DMF was used as outer solution).

Fluorescence measurements were made with a Perkin–Elmer LS-50 Luminescence Spectrometer with a flow cell. A Gilson Minipuls-2 peristaltic pump forced the displacement of the titrated solution (*ca.* 1 ml) into the flow cell after each addition of titrant (after measurement, this fraction was rejected). During potential and fluorescence intensity measurements, the pump was turned off. Synchronous fluorescence spectra were recorded with the following instrument settings: between 250 and 550 nm; 0.5 nm resolution; 7.5 nm excitation and emission slits width; wavelength difference of 20 nm; and scan rate of 200 nm/min. Data spectra were stored on disk and converted to ASCII format with LAB CALC software (Galactic Industries Co., U.S.A.).

CALCULATIONS

Determination of the number of equivalent acid groups

The sfua solutions were prepared in solution with known concentration of internal standards. Hence, the number of equivalents of acid groups in the molecules of sfua titrated at the i^{th} titration point (Neq_i, mmol H^+/g sfua) is given by:

$$Neq_i = (V_i^{sfua+IS} - V_i^{IS})C_B/C_{sfua}, \quad (1)$$

where, $V_i^{sfua+IS}$ is the volume of strong base added up the i^{th} end-point in the titration of a solution of sfua plus internal standards; V_i^{IS} is the volume of strong base added up to the same end-point, in the titration of a pure solution of internal standards; C_B is the concentration of the Bu_4NOH titrant solution is M; C_{sfua} is the concentration of the sfua solution in g/l.

SIMPLISMA analysis of the SyF spectra

SIMPLISMA[17-19], used as a spectroscopic self-modeling multiwavelength analysis technique for the SyF data reduction, is briefly described in this section. The first step of the SIMPLISMA procedure is the determination of the pure variables. In the present case, a pure variable is a wavelength of the SyF spectrum of which the intensity value is due to only one component of the mixture. In this process, the spectral set under analysis (represented by matrix **D**, size $n_v \times n_s$, where n_v is the number of variables—wavelengths—and n_s is the number of spectra) is reduced to three vectors or spectra named: mean spectrum, m; standard deviation (SD) spectrum, s; and purity spectrum, p. The elements of the mean spectrum are defined by

$$m_i = (1/n_s)\,\Sigma_{(j=1\ldots n_s)} d_{i,j} \quad (i = 1 \ldots n_v), \quad (2)$$

the elements of the SD spectrum by

$$s_i = ((1/n_s)\,\Sigma_{(j=1\ldots n_s)}(d_{i,j} - m_i)^2)^{1/2} \quad (i = 1 \ldots n_v) \quad (3)$$

and the elements of the purity spectrum by

$$p_i = s_i/m_i \quad (i = 1 \ldots n_v). \quad (4)$$

For small m_i values, where information is small and noise is relatively large, p_i values may become large. In order to remove this unwanted effect an offset is added to data.

The visualization of vectors s and p as spectra will be crucial for the detection of pure variables. The wavelength with the highest intensity in the purity spectrum is the first pure variable. The SD spectra can be used to check the validity of the selected pure variable. The variations that are associated to the selected pure variable are removed from the standard deviation and purity spectra and the second pure variable is determined. This process is repeated until these spectra show only noise, *i.e.* when all the useful information is removed. If the determination of the rank of the system (the number of independent species, n_p, that are provoking variance in data) is not visually sharp after determining j pure variables, the following two error functions can be helpful:

R_s, the relative total intensity of the SD spectra

$$R_{sj} = 100\,\Sigma_{(i=1\ldots n_v)} s_{i,j}/\,\Sigma_{(i=1\ldots n_v)} s_{i,1} \quad (5)$$

and R_r, the ratio of the relative total intensities of the j and the $j+1$ SD spectra

$$R_{rj} = R_{sj}/R_{s(j+1)}. \quad (6)$$

The second step of the SIMPLISMA procedure is the resolution of data into spectra (matrix **S**, size $n_p x n_v$) and concentration profiles (matrix **C**, size $n_s \times n_p$) for the number of detected species. Assuming that all the components have the same fluorescence efficiency, the concentration profiles are equal to the fluorescence intensities of the corresponding pure variables after normalization. If the data matrix is expressed as

$$\mathbf{D}^T = \mathbf{CS}, \quad (7)$$

the spectra and concentration profiles can be calculated by a least-squares procedure

$$\mathbf{S} = (\mathbf{C}^T\mathbf{C})^{-1}\mathbf{C}^T\mathbf{D}^T \quad (8)$$

$$\mathbf{C} = \mathbf{D}^T\mathbf{S}^T(\mathbf{S}\mathbf{S}^T)^{-1}. \quad (9)$$

In equation (8), the intensities of the pure variables in the **D** spectra matrix are used in **C**.

The components found in the analysis of the SyF spectra, obtained as function of the extent of neutralization of sfua, are related to the acid groups involved in that process, because the variations observed in the SyF spectra are a macroscopic view of that phenomena.

RESULTS AND DISCUSSION

Potentiometric titrations in aqueous solution

The shape of a titration curve with a strong base of an aqueous solution of the present sfua acid was that trivially described in the literature.[21-23] This curve is characterized by a plateau on the acid pH region, showing a relatively strong buffer capacity, and by a clear end-point at neutral pH. This shape of the titration curve is due to the acid–base leveling capacity of

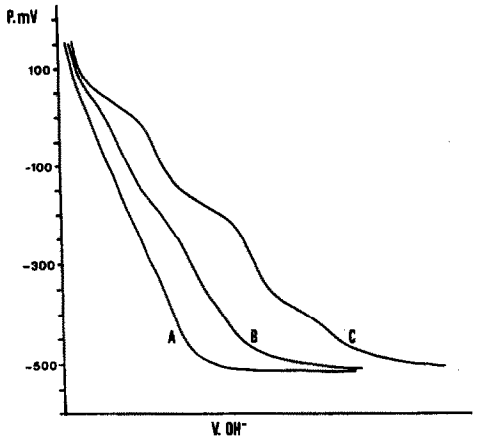

Fig. 1. Titration curve in DMF of a solution of (A) sfua, (B) sfua plus 0.1 m*M* of SAL and pHB and (C) sfua plus 0.3 m*M* of SAL and pHB.

water, which allows no clear differentiation of the acid–base strengths between acid groups unless their acidities are markedly different, and to its high dielectric constant, which minimizes the effect of charge accumulation on the polyelectrolyte sfua molecules on the acid–base properties of the acid groups.

The amount of acid groups that are titrated up to about pH 7 (the equivalent point determined by the methods of the first and the second derivative) will be called the total acidity directly titrated in water (T_w). For the present sample of sfua, this quantity was 5.74 mmol/g.

Potentiometric titrations in DMF

In contrast to what is observed in the titration curve in water, the acid–base titration curve in DMF of a sfua pure solution (Fig. 1, curve A) shows no sharp end-point and the buffering capacity found in water is no longer detected in the first part of the titration: the potential decreases with the consumption of strong base spent with the neutralization of acid groups. This result, normal for HS,[2-6] is not found for simple compounds. Indeed, the shape of titration curves of simple compounds shows the characteristic plateau due to the acid buffering capacity. The first derivative titration curves of sfua show several peaks, but their shapes and relative positions are not reproducible and they are unsuitable for analytical purposes.

To achieve resolution in the DMF titrations, an internal standard composed of a mixture of SAL ($pK_{a1} = 2.70$ in water[24]) and pHB ($pK_{a1} = 4.36$ and $pK_{a2} = 8.99$ in water[24]) in the same molar concentrations was used. Curve B in Fig. 1, shows the effect of this internal standard (1 m*M* concentration of each component) on the titration curve of sfua, where the appearance of three end-points became visible. Better resolution, with more clear end-points, is obtained with a higher concentration of internal standards as shown on Fig. 1, curve C (3 m*M* concentration of each component). The three end points correspond successively to the titration of the carboxylic group of SAL (between *ca.* 100 and −100 mV), carboxylic group of pHB (between *ca.* −150 and −350 mV) and phenolic group of pHB (between *ca.* −400 and −500 mV). Similar results have been reported in the literature.[4]

The amounts of acid groups in sfua directly titrated in DMF in the presence of internal standards are presented in Table 1. The total quantity of acid groups determined in DMF (5.2 mmol/g) was less than T_w (5.74 mmol/g). At first sight, this may appear surprising, because, in DMF, groups with an acid strength similar to phenols (like the phenolic group of pHB) are also titrated, in contrast to what happens in water. There are two factors affecting the acid character of the groups in sfua molecules: the intrinsic heterogeneity of acid–base structures and the accumulation of charge on the sfua molecules, which is highly solvent dependent. The interpretation of the acid–base properties of a substance, even for a pure substance, in a non-aqueous solvent like DMF, is much more

Table 1. Amount of equivalent acids (mmol/g sfua) obtained by the titration of sfua in DMF and AN, in the presence of internal standards, and in water*

Class of acid groups	DMF		AN		Water
	Cumulative	Δ	Cumulative	Δ	Cumulative
1			1.16 (6)		
2	2.30 (7)		2.25 (3)	1.09	
3	4.50 (9)	2.20	4.06 (9)	1.81	
4	5.2 (1)	0.69	4.81 (6)	0.75	5.74 (8)

*Average of five (DMF), four (AN) and five (water) titrations with standard deviations in parentheses.

difficult than for water. Indeed, the solvation capacity and hydrogen bonding of this non-aqueous solvent are quite different than those of water, chemical equilibria in it are much more involved[25] and, as its dielectric constant is nearly half of that of water, the effect of charge accumulation in the acid properties is non-negligible. For the sfua, interpretation of the acid–base properties in DMF is even more difficult because they are a mixture of unknown heterogeneous polyelectrolytes. Ignoring chemical heterogeneity, and considering only the charge accumulation at the sfua polyelectrolyte molecules, a possible explanation for the determination of less acid groups in DMF than in water is that a fraction of phenolic groups and a small fraction of carboxylic groups are too weak to be titrated in DMF.

A detailed analysis of the first part of the three titration curves of Fig. 1 suggests the existence of acid groups stronger than SAL because the titrations began above the 100 mV while the neutralization of SAL alone began below 100 mV. For a more detailed analysis of the part of the titration curve around 100 mV, which corresponds to the most acid groups, AN, a more acid solvent than DMF, was used.

Potentiometric titrations in AN

The titration curves of pure solution of sfua in AN (Fig. 2) showed an incipient resolution in three acid groups, but the inflection points were not marked enough to extract quantitative results. Relatively to the DMF titrations, a larger potential range was found (400 to −550 mV, while for DMF the range was between 150 and −550 mV).

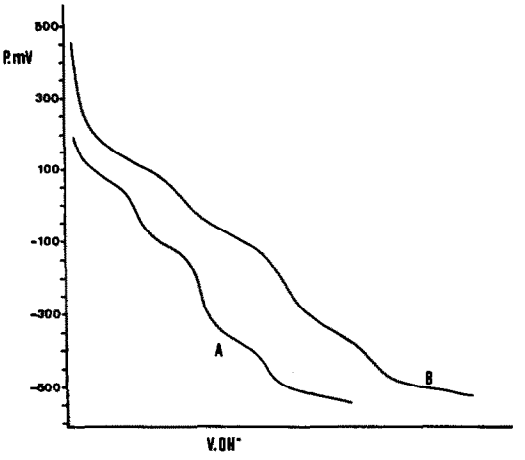

Fig. 3. Titration curve in AN of (A) a solution of SAL and pHB and (B) the same solution as in (A) plus sfua.

The titration curves of a mixture of SAL and pHB (curve A) and of a mixture of sfua with this solution (curve B) are compared in Fig. 3. An effect similar to that observed in the DMF titrations, with clear resolution of the titration curve of sfua into three titration points in the presence of the internal standards, is observed, and the existence of acid groups stronger than salicylic acid is now much more evident.

MAL ($pK_{a1} = 1.65$ in water[26]), which is stronger than SAL in water, was used as internal standard, together with SAL, to analyze the range of potential between 400 and 200 mV. Figure 4 shows titration curves of this internal standard in AN (curve A) and of a mixture of sfua with this same solution (curve B). The figure shows that the stronger acid groups of sfua fall into the first end point, which correspond to the first protolysis of MAL. The second end point of the titration corresponds to

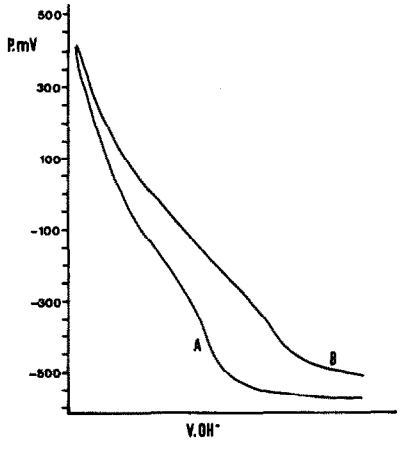

Fig. 2. Titration curve in AN of a solution of sfua with concentration (A) 0.6 and (B) 1.2 g/l.

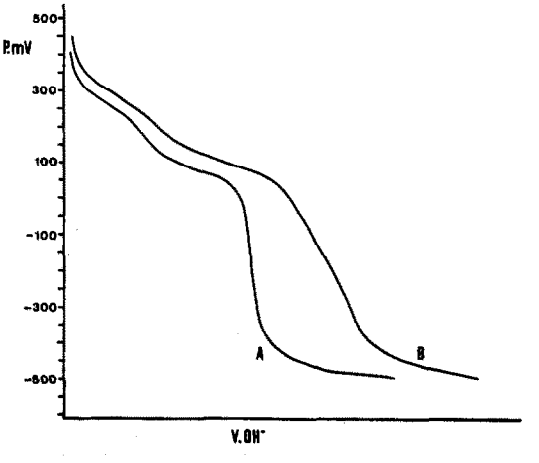

Fig. 4. Titration curve in AN of (A) a solution of MAL and SAL and (B) the same solution as in (A) plus sfua.

SAL. In conclusion, when dissolved in DMF and AN, the present sfua sample shows acid groups with strengths similar to those of MAL, SAL and pHB. In AN, the carboxylic acid that corresponds to the second protolysis of MAL was too weak to be titrated.[25]

The amounts of equivalent acid groups titrated in AN with the use of the two sets of internal standards are presented in Table 1. The sum of the quantities of the two first acid groups determined in AN, which have close values [1.16(6) + 1.09 = 2.25(3)], is similar to the amount of the first group determined in DMF [2.30(7)]. The total amount of acid groups detected in AN [4.81(6)] is somewhat smaller than for DMF [5.2(1)]. This may result from the much smaller dissociation constants of acid groups in AN than in DMF,[25] mainly due to the much weaker basicity of the first solvent, which means that some of the acid groups titrated in DMF are not titrated in AN.

The use of AN as a solvent for the titration of the sfua complemented the information collected from the titration in DMF because it has a high resolving power of the acid strength for the most acidic groups present in the sfua sample. Indeed, it allowed the unequivocal identification and quantitation of acid groups stronger than those of salicylic acid in these dipolar aprotic solvents.

Potentiometric + fluorescence titrations in DMF

Preliminary analysis. SyF spectra of the sfua as function of the volume of the titrant (Bu_4NOH) in DMF are shown in Fig. 5a. The spectra include four main bands at 345, 400, 450 and 500 nm, for which different variations with the increase of neutralization extent are observed. The effect of the presence of the internal standards can be seen in Fig. 5b–d. Some new bands in the lower wavelength spectral range show more abrupt variations, with increase of the neutralization degree, than the sfua bands.

To study the effect of the internal standards on the fluorescence properties of the sfua to investigate whether there exists any correlation between the variations observed in the SyF spectra of the sfua and those observed for the internal standards, further direct analysis of the four data sets represented in Fig. 5 is impracticable, due to the complexity of the situation (multiwavelength and multispectra data have to be compared). SIMPLISMA was used for data reduction to allow the comparison.

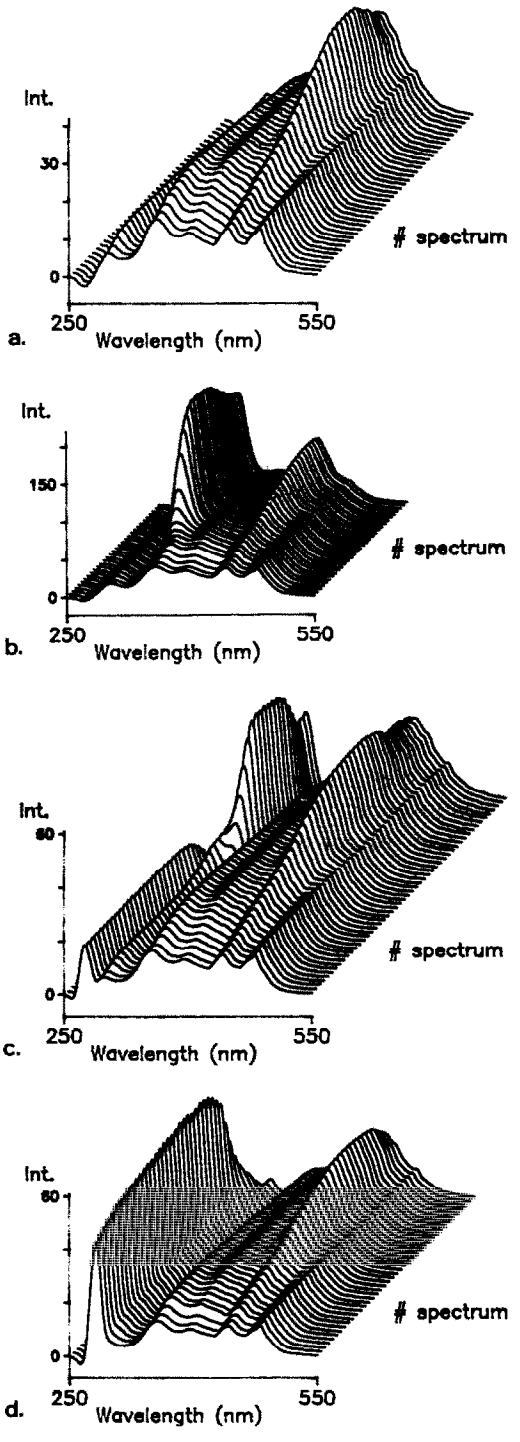

Fig. 5. SyF spectra as function of volume of titrant (Bu_4NOH) of (a) sfua without internal standard (b) sfua + SAL (c) sfua + pHB and (d) sfua + CAT.

SIMPLISMA analysis. The number of components that show different variations in the sfua spectra of Fig. 5a, *i.e.* that are not correlated, is two, as can be seen from the analysis of the SD and purity spectra shown in Fig. 6A and

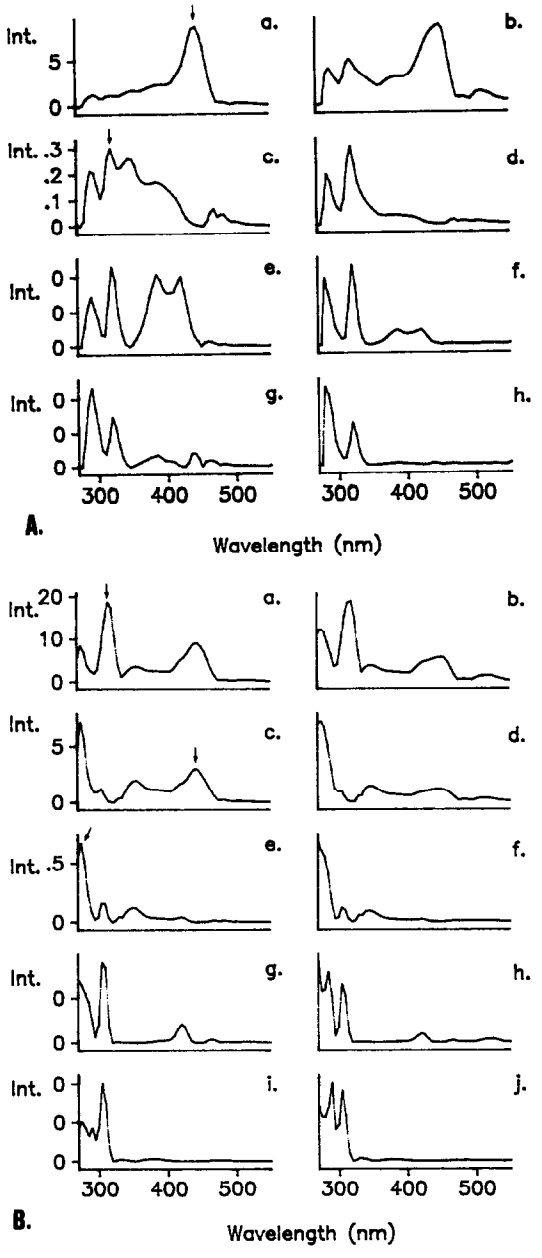

Fig. 6. Standard deviation (a, c, e, g, i) and purity (b, d, f, h, j) spectra resulting from the SIMPLISMA analysis of the spectra sets for (A) the sfua without internal standard and (B) sfua + CAT: first set (a, b); second set (c, d); third set (e, f); fourth set (g, h) and fifth set (i, j). Arrows indicate the pure variables. Maximum intensities of the spectra in Ae, Ag, Bg and Bi are 2×10^{-3}, 5×10^{-6}, 4×10^{-3} and 3×10^{-6}, respectively.

from the error functions shown in Table 2. In fact, the R_s error function (Table 2) showed a marked decrease ($R_r = 154$) when passing from the second to the third SD spectrum (from 5.8 to 0.04) (Fig. 6Ac and e) and the third SD and purity spectra (Fig. 6Ae and f) were almost zero with the main band in the purity spectrum in the highly noisy lower wavelength range (note the decreasing vertical scales from top to bottom in Fig. 6). The pure variables characteristic of the sfua are 450 and 350 nm, indicated by arrows in Fig. 6a and c.

The spectra and concentration profiles of the two components are shown in Figs 7a, b and 8a, b, respectively. The spectrum of the first component (Fig. 7a) was characterized by one band around 450 nm. The spectrum of the second (Fig. 7b) was more complex, being characterized by one main band around 350 nm. The CP of the first component (Fig. 8a) had a monotonous crescent shape with no marked variations, while the CP of the second component (Fig. 8b) showed a marked increase followed by a decrescent section. The overall featureless shape of the two CPs gives limited information, similarly to what happens with potentiometric titration curves without internal standards.

The detection of only two components in the SyF spectra set of sfua in DMF shows that the use of the non-aqueous solvent did not increase the resolution of the titration curves. Indeed similar studies in water as solvent[15,16] allowed the detection of three components, which correspond to three macroscopic acid–base systems. The comparison of the spectra of the two components detected in DMF (Fig. 7a, b) with those detected in water[15,16] shows the existence of some similarities between the spectrum of Fig. 7a and the spectrum for the strongest acid–base system detected in water, which suggests that in both solvents the corresponding acid structures are the same. The spectrum of the second component detected in DMF (Fig. 7b) roughly corresponds to a mixture of the spectra of the other two components detected in water.[15,16]

The effect of adding internal standards to sfua on the SyF spectra was an increase of the original two components due to sfua to three, as can be seen from the analysis of the SD and purity spectra (Fig. 6B shows those for the sfua + CAT experiment) and from the error functions shown in Table 2. For data obtained when pHB was used as internal standard, the existence of three or four components is difficult to decide, probably because pHB is diprotic.

The pure variables detected for the four types of experiences are presented in Table 3. The two pure variables characteristic of sfua (450 and 345 nm) are also present in the sfua plus internal standards data sets. The same information is obtained when comparing spectra of the two

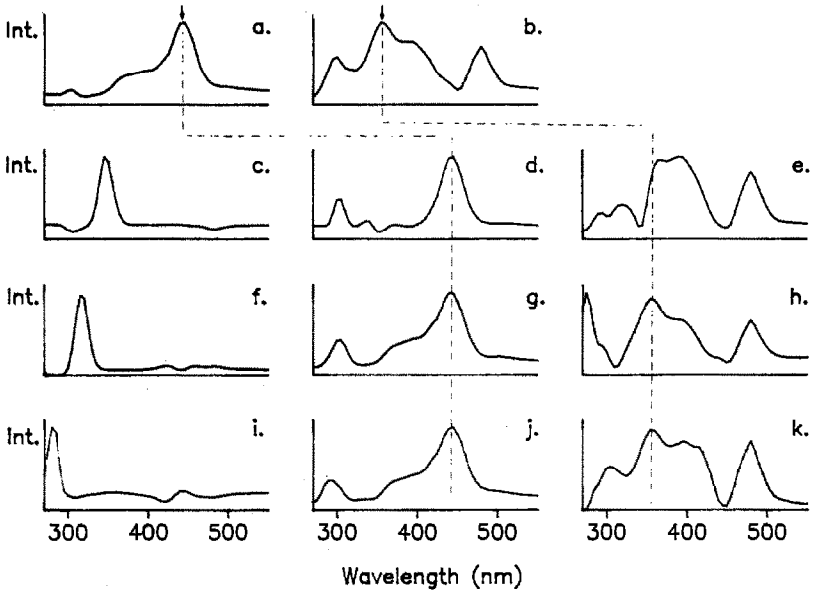

Fig. 7. Spectra of the pure variables extracted by SIMPLISMA from the spectra sets for: (a, b) sfua without internal standard; (c, d, e) sfua + SAL; (f, g, h) sfua + pHB; (i, j, k) sfua + CAT. Dashed lines indicate relationships between variables.

components of sfua (Fig. 7a, b) with the spectra of the second and third components for the experiments with the presence of internal standards. When SAL is used as internal standard, the first pure variable at 345 nm, that corresponds to the SyF spectrum of SAL, is similar to the second pure variable of sfua, which provoked the change of the pure variable (390 nm instead of the expected 350 nm) and the consequent distortion on the calculated spectra of the third component for this experiment (Fig. 7e).

The first CPs obtained for the experiments with internal standards (Fig. 8c, f, i) had a marked variation, which corresponds to the protolysis of the internal standard. SAL showed an increase of fluorescence in the 50 to −70 mV range; CAT showed a decrease of fluorescence in the −370 to −550 mV range; and pHB showed first an increase of fluorescence in the

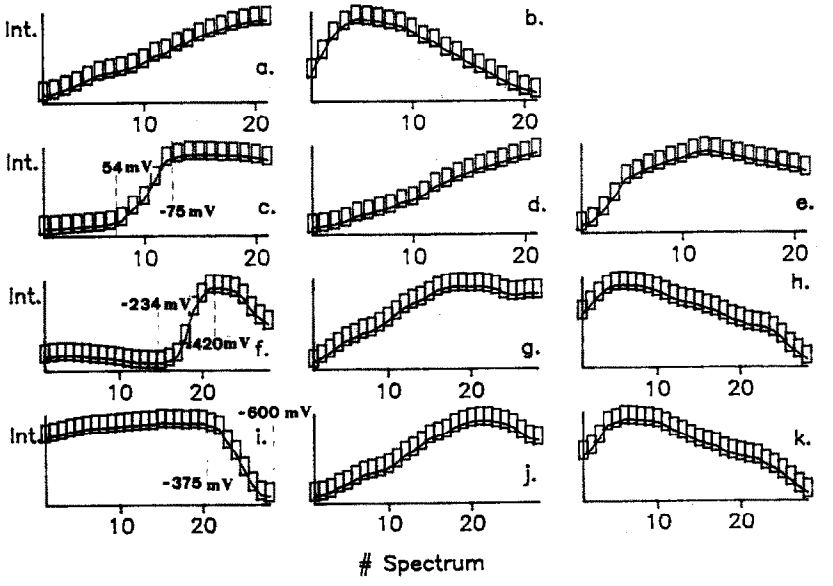

Fig. 8. CPs for the pure variables extracted by SIMPLISMA from the spectra sets for: (a, b) sfua without internal standard; (c, d, e) sfua + SAL (f, g, h) sfua + pHB; (i, j, k) sfua + CAT. Potential ranges are indicated by vertical dashed lines.

Table 2. Values of the error functions to determine the number of components in the SyF spectra sets

	sfua		sfua + SAL		sfua + pHb		sfua + CAT	
N*	R_s	R_r	R_s	R_r	R_s	R_r	R_s	R_r
1	100	15	100	19	100	3	100	7
2	5.8	154	5.2	55	35.2	19	13.9	30
3	0.04	1016	0.1	580	1.8	31	0.5	102
4	3.7e-5		1.6e-4	736	0.06	232	4.6e-3	398
5			2.2e-7		2.5e-4		1.1e-5	

*Number of the pure variable.

−230 to −400 mV range and then a decrease for values of potential lower than −400 mV. Two different variations were observed for pHB because it is a diprotic acid. The potential ranges where the protolysis of the internal standards occurred were similar to those observed in their potentiometric titration in DMF. The other two CPs for the experiments with internal standards were similar to those obtained with the sfua alone.

In conclusion, SIMPLISMA analysis separated the spectra and CPs of the internal standards and of the sfua and, therefore, the variations observed on the SyF spectra of the internal standards show no correlation with those for the components of sfua.

CONCLUSIONS

The use of dipolar aprotic solvents, like DMF and AN, in the study of the acid–base properties of HS, allows the direct observation of the complex mixture characteristics of their reactive structures in acid–base reactions. The strength of the acid groups found in the sfua molecules ranged from that of the first protolysis of maleic acid to that of phenol groups (in these non-aqueous solvents). This result was obtained with both indirect (potentiometry) and direct (molecular fluorescence) methods for the analysis of the chemical status of the HS molecules. The fluorescence results confirm the earlier potentiometric evidence[2–6] that macroscopic acid–base systems, defined for fulvic acids as being constituted by acid groups with similar acid strength when water is used as solvent,[15,16] are not resolved in a solvent with a lower leveling or higher acid–base resolution power, like DMF and AN.

The role of internal standards is to force the splitting of the titration curve into one or more end-points. For example, for the present sample, the potentiometric titration curves were split up into four end-points using suitable acids (MAL, SAL and pHB) as internal standards. The amounts of acid groups that correspond to those end-points are useful for comparison with other samples under the same experimental conditions (solvent and internal standards). However, the search for any structural relationship between the internal standards and the HS groups that were titrated with them in DMF proved impossible. This is probably a consequence that after the occurrence of the protolysis of the strongest acid groups, the acid–base equilibria become more complex than in water, due to charge accumulation and to the macromolecular structural rearrangements. Thus, any extrapolation of the results obtained in non-aqueous solvents to water should be taken with great care because, due to the HS complex structure, such an extrapolation may be a strongly non-linear process.

Table 3. Pure variables (wavelength, nm) detected by the SIMPLISMA analysis

N*	sfua	sfua + SAL	sfua + pHB	sfua + CAT
1	450	345	320	275
2	345	440	440	450
3		390	350	350

*Number of the pure variable.

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