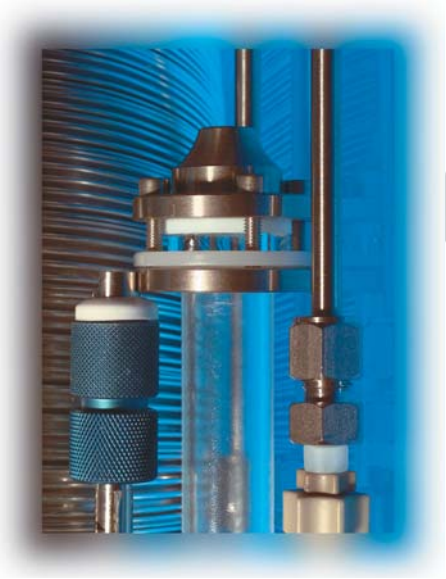
The Analysis of Fluorescent Whitening Agents Using Reversed-Phase HPLC and Mass Spectrometry



This article describes the separation of individual congeners of commonly used fluorescent whitening agents by reversed-phase high performance liquid chromatography and subsequent time-of-flight mass spectrometry analysis. The authors also compare the performance of a conventional C18 phase with phases possessing either nonpolar endcapping, polar endcapping, or polar-embedded groups.

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Fluorescent whitening agents, also known as brighteners or bluing agents, are used widely in the textile and paper industries and in household detergents (1–3). Fluorescent whitening agents are capable of absorbing light in the UV range (290–400 nm) and of emitting visible blue light (400–480 nm), thus enhancing the intensity of visual reflection and strengthening the optical impression of whiteness and brightness. This work focuses on fluorescent whitening agents used in paper and board materials, especially those that might be used in food packaging, and are thus intended to come into contact with foods.

Several fluorescent substances, belonging to various groups of compounds, are used as fluorescent whitening agents (for example, coumarin, quinolone, benzoxazole, and distyrylbiphenyl [4]), but approximately 80% of fluorescent whitening agents used in paper and board formulations are based upon stilbene derivatives (5), the most widely used being the disulfo, tetrasulfo, and hexasulfo congeners. The fluorescent whitening agents have low toxicity, with LD_{50} values ranging from 1000 to as high as 15,000 mg/kg (6–8). Nevertheless, not all of them are authorized for use as colorants by the U.S. Food and Drug Administration (FDA), and their inclusion in paper and board intended for food packaging in the European Union is under consideration due to their potential migration to the packed items.

So there is interest in developing a method that allows individual fluorescent whitening agents to be reliably identified, and subsequently quantified, within a reasonable timeframe. Determination of fluorescent whitening agents first was described by Judd in 1935 (9,10). Since then, a number of methods have been introduced, most of them involving thin-layer chromatography (11) or high performance liquid chromatography (HPLC) (12–18). None of these studies focused on fluorescent whitening agents found in paper and board formulations, and, to the best of our knowledge, only two methods specifically devoted to these compounds have been published, one involving capillary electrophoresis (19) and the other HPLC (20).

Reversed-phase HPLC is the most widely used chromatographic method for separating chemical mixtures. At present, approximately 90% of all HPLC separations are performed by reversed-phase HPLC (21), probably due to its applicability to a wide range of compounds and samples, which in turn is related directly to the vast number (approximately 600) of different stationary phases available. This facilitates the solution of many separation problems simply by selecting appropriate stationary phases, but on the other hand, identifying the most suitable column for a specific purpose can be far from straightforward. A further complicating factor is that large groups of nominally identical materials often show very different chromatographic properties (22,23).

Nonpolar alkyl-bonded phases (usually based upon octadecylsilane) are the most

widely used of the available stationary phases, but new alternatives have been introduced, the most popular being the embedded and endcapped phases. These are modifications of the traditional C18 phase, with the addition of a functional nonpolar or polar group, usually an amide or carbamate group, either within (embedded) or at the end (endcapped) of the alkyl chain, thus modifying selectivity in comparison to conventional alkyl-bonded phases (24–26).

The introduction and widespread implementation of liquid chromatography–mass spectrometry (LC–MS) techniques in the 1990s have increased the scope for characterizing polar compounds and elucidating their structure. This is of great interest in fluorescent whitening agent analysis because of the general lack of individual analytical-grade standards and structural information, due to patent protection. Since its inception, various excellent reviews and books have covered diverse aspects of the LC–MS (27–32), including some that are more specifically devoted to electrospray interface (33,34). With respect to the analytes, several chemical classes, including dyes (35) and sulfonated dyes (36), have been the subjects of specific reviews.

Studies correlating chromatographic data for selected analytes with fundamental chromatographic descriptors rarely are found in the literature, and to the best of our knowledge, no such studies have been devoted to fluorescent whitening agents or structurally related chemicals. Therefore, the study presented in this paper had several goals. First, to develop and optimize a reversed-phase HPLC method that allows the individual determination and quantitation of seven of the more commonly used fluorescent whitening agents in paper-and-board manufacture. Second, to explain the results obtained by correlating the data to column, analyte, and mobile-phase parameters, with special emphasis on embedding and endcapping characteristics. Third, to use MS detection to provide general information for identifying some unknown fluorescent whitening agents in a sample.

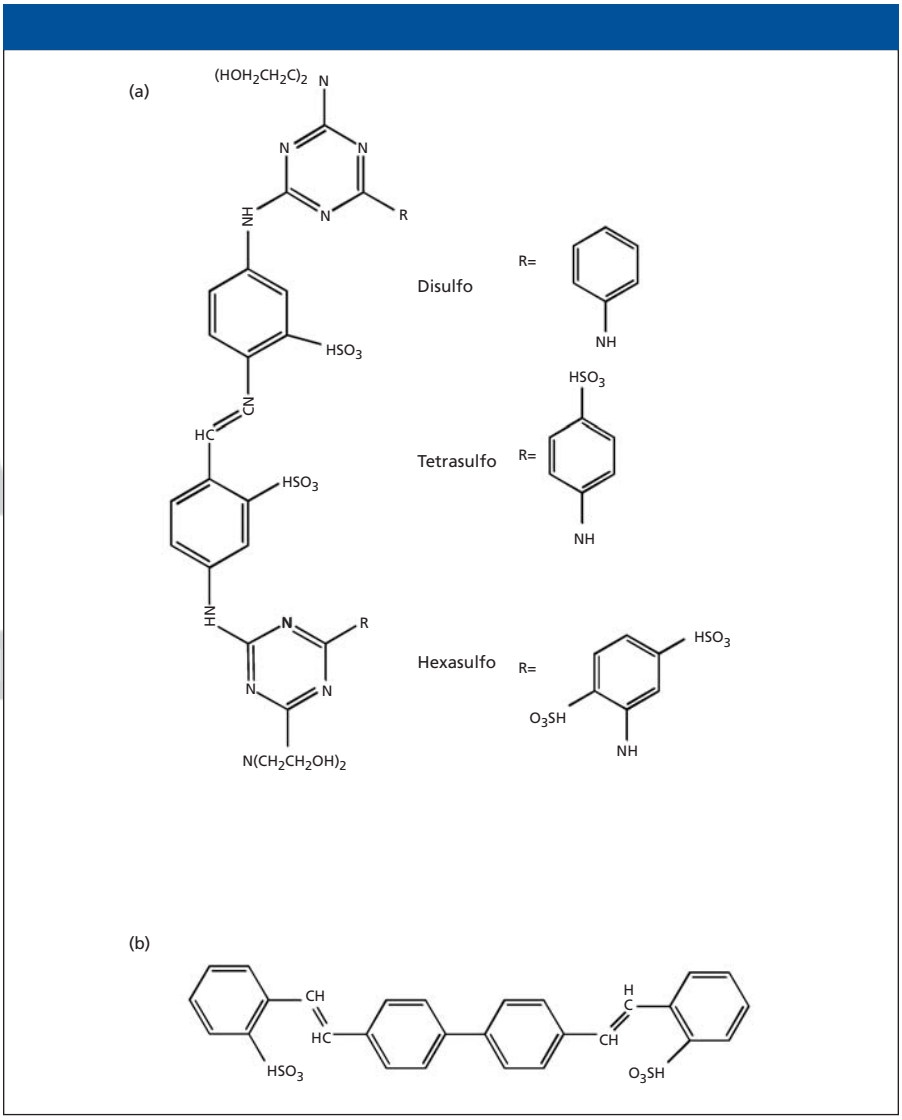

Figure 1: Formulas of fluorescent whitening agent. Shown are the structures of (a) di-, tetra-, and hexasulfo derivatives and (b) BS.

Experimental

Equipment: The HPLC system we used consisted of a Waters model 600 quaternary pump (Waters Corp., Milford, Massachusetts) and a Kontron model 360 autosampler (Kontron Instruments SpA, Milan, Italy) connected in series with a Waters model 474 scanning fluorescence detector and a Kontron model 332 variable-wavelength UV detector. A PC Integration Pack (Kontron Instruments) was used for data acquisition and analysis. Mobile phases of various compositions were evaluated, as discussed in the "Results and Discussion" section.

The LC–MS system included a Waters Alliance model 2795 separation system, which has separate temperature-adjustable compartments for the column and sample (held at 30 °C and 15 °C, respectively, throughout the analyses). This system was equipped with an autosampler and a reversed-phase C18 column (Prodigy 5-μm ODS 2, Phenomenex, Torrance, California). The absorbance of the eluent (flow rate, 1.0 mL/min) was monitored using a diode-array UV detector (Waters model 2996), and, after splitting (split ratio 1:0.6), it was directed to a Biotof II time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Billerica, Massachusetts) equipped with an Apollo orthogonal electrospray ionization (ESI) probe. Splitting of the eluent is needed to prevent failure of the acquisition due to source lens current overloading the system.

ESI MS was performed in negative mode, with the only exception being Blankophor ACR, which was analyzed by monitoring positive ions. The fragmentation of the analytes was optimized as follows: Each individual working standard was dissolved in 50:50 (v/v) methanol–water and directly infused into the MS system using a 74900 series syringe pump (Cole Palmer, Vernon Hills, Illinois) at a flow rate of 150 μL/h. The drying gas temperature then was varied between 150 °C and 250 °C and the capillary voltage between 50 V and 250 V. At low potentials, sensitivity is high because the protonated or deprotonated molecules are virtually the only fragments formed, whereas higher potentials favor extensive fragmentation, which is more suitable for identification and

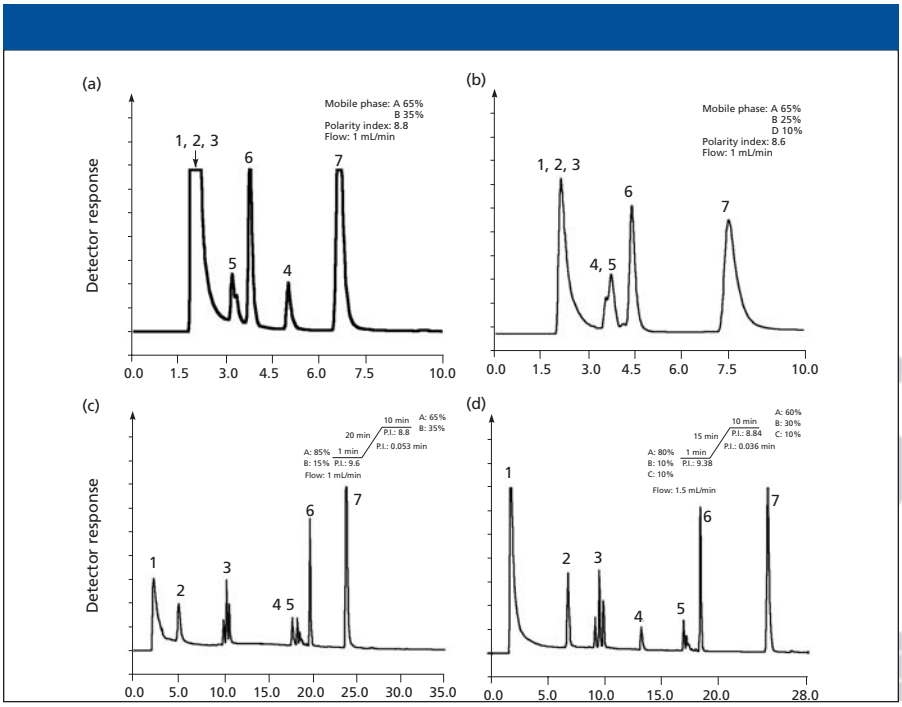

Figure 2: Chromatograms of fluorescent whitening agents obtained using (a,b) isocratic and (c–e) gradient operation with column 1. Mobile phases: A = Milli Q water (10 mM monobasic sodium phosphate, pH 5), B = acetonitrile, C = methanol, D = tetrahydrofuran. P.I.: polarity index; as an indication of gradient, the variation of P.I. per unit time (ΔP.I.) is also included. All chromatograms presented were obtained using fluorescence detection (l_{ex}: 350 nm, l_{em}: 450 nm). Peaks: 1 = RH, 2 = Tetra, 3 = RT*, 4 = ACR, 5 = AP, 6 = RD, 7 = BS.

the acquisition of structural information.

Chromatographic conditions were slightly different from those optimized for fluorescence experiments, as phosphate buffer is not compatible with ESI. Therefore, it was replaced by 5 mM ammonium acetate buffer, giving a final pH of 4.8. Solvent A was then Milli-Q water (5 mM ammonium acetate), while solvents B and C were acetonitrile and methanol, respectively. The initial composition was 80:10:10 (v/v) A–B–C, held isocratically for 1 min. The composition of the mobile phase was changed linearly to 60:30:10 over 15 min and held for a further 16 min. Then the mobile was returned to its initial composition over 3 min and the column was equilibrated with this phase for 10 min. No detrimental changes were observed in chromatographic performance when substituting with volatile ammonium acetate buffer for phosphate buffer.

Columns and Reagents

The columns used in this work were new and are summarized in Table I, together with some of their physicochemical properties. These parameters are useful in manu-

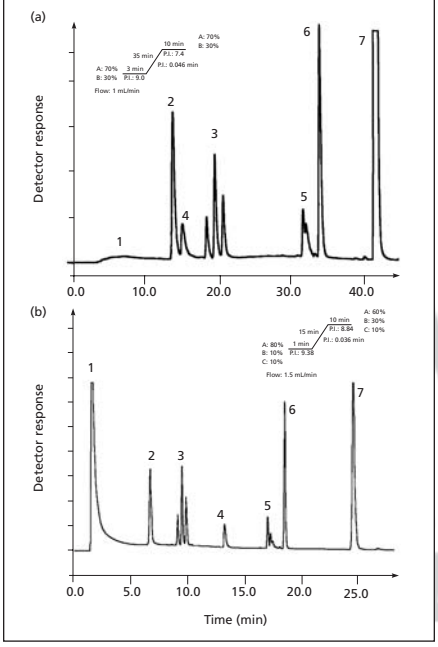

Figure 3: Separation of fluorescent whitening agents using (a) a methanol gradient and (b) optimum chromatographic conditions (l_{ex}: 350 nm, l_{em}: 450 nm). For a key to peaks and mobile phases, see Figure 2.

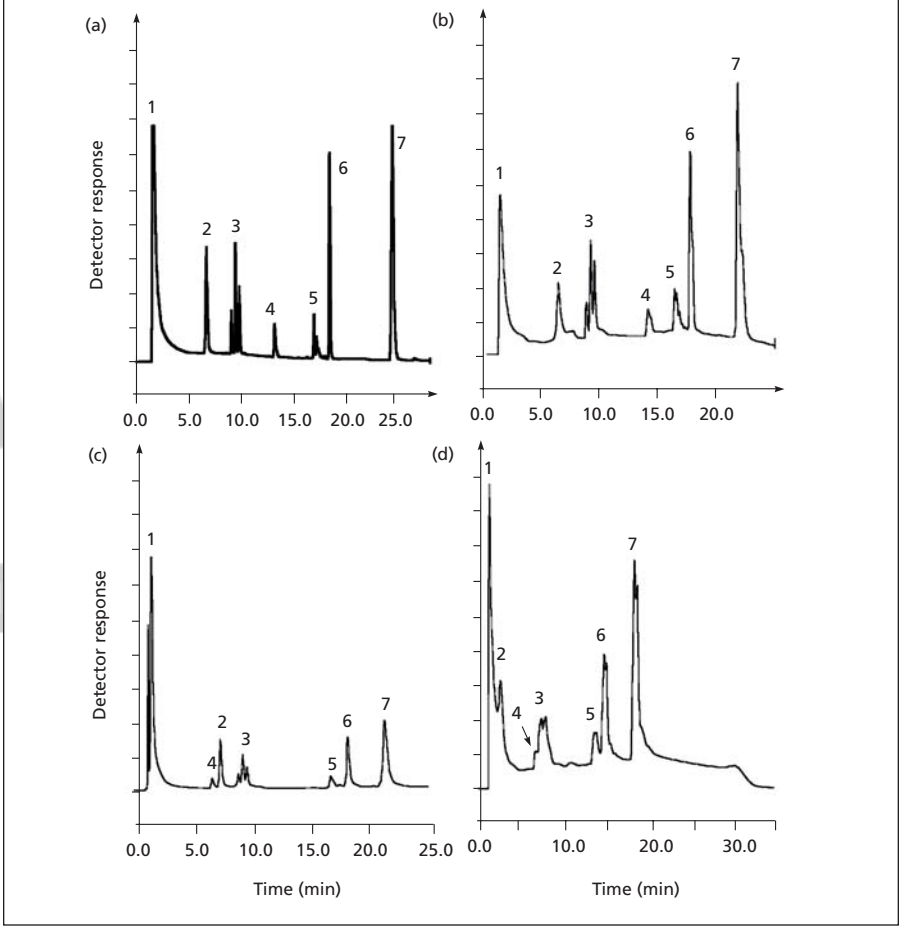

Figure 4: Chromatograms obtained using columns (a) 1, (b) 2, (c) 3, and (d) 4. For a key to peaks and mobile phases, see Figure 2, and for columns see Table I. Detection: fluorescence (lex: 350 nm, lem: 450 nm).

facturing quality control, but generally, they have little correlation with the true chromatographic performance of the phase (37). Except for the water, provided by a Milli-Q system (Millipore Ibérica S.A., Madrid, Spain), all solvents used were of at least HPLC grade and were supplied by Scharlab (Barcelona, Spain). Monobasic sodium phosphate (analytical grade) was obtained from Panreac (Barcelona, Spain), and ammonium acetate (analytical grade) was supplied by Merck (Darmstadt, Germany).

Fluorescent Whitening Agents

The fluorescent whitening agents selected for inclusion in this study are those most commonly used by European Union paper and board manufacturers. The following industrial whitening agents were investigated (no analytical-grade standards were available). Structures of these compounds (where known) are shown in Figure 1: bis(anilinodihydroxyethylaminotriazinylamino)stilbene tetrasulfonate (Tetra, Ciba-Geigy, Barcelona, Spain; Figure 1a); bis(anilinodihydroxyethyl-aminotriazinylamino)stilbene disulfonate (RD, Robama S.A., Barcelona, Spain; Figure 1a); bis(anilinodihydroxyethylaminotriazinylamino)stilbene hexasulfonate (RH, Robama S.A.; Figure 1a); Modified Tetra (RT*, Robama S.A.); Blankophor ACR (cationic benzimidazole derivative; Color Index Fluorescent Brightener 363) (ACR, Bayer Ibérica, Barcelona, Spain); Leucophor AP (anionic disulfonated stilbene brightener derivative, Clariant Ibérica, Barcelona, Spain); and bis(styrylsulfonate)biphenyl (BS, Ciba-Geigy; Figure 1b).

Stock solutions of the fluorescent whitening agents, as received, were prepared by dilution to 400–800 μg/g with 65:35 (v/v) water–acetonitrile. Working standards were prepared as needed by dilution with the appropriate volume of solvent. Once prepared, samples were stored in a refrigerator (4 °C) in the dark, to prevent the light-induced conversion of the *trans* isomers of fluorescent whitening agents (present in the commercial products) to the *cis* isomers.

Results and Discussion

Figure 2 shows the chromatograms obtained using the conventional endcapped octadecylsilane column (column 1 hereafter), together with the composition and strength (polarity indices) of the mobile phases used.

The chromatographic conditions listed in Figures 2c and 2d provide sufficient resolution and efficiency to allow determination of the individual fluorescent whitening agents under study. However, adding 10% methanol, and thus increasing the elution strength of the mobile phase (Figure 2d), reduced the retention times for all of the compounds but RT, for which it increased by about 1 min. The retention of ACR (the only cationic, acidic compound) especially was reduced, by more than 4 min, suggesting that additional factors, in addition to eluent strength, must be taken into account to explain the chromatographic behavior of the fluorescent whitening agents. When methanol was replaced in the mobile phase by tetrahydrofuran, retention of the analytes was further decreased (Figure 2e), which is consistent with theoretical expectations.

However, the retention of ACR again was reduced particularly (by about 10 min), so the changes in retention parameters clearly are not only correlated with the increase in elution strength of the mobile phase. More interestingly, when one compares the chromatograms depicting the isocratic elution profiles obtained using acetonitrile or acetonitrile–tetrahydrofuran as the organic modifiers (Figures 2a and 2b), giving rise to polarity indexes of 8.8 and 8.6, respectively, grouping and widening of the peaks is noted according to the general retention theory. So the presence of methanol clearly has a major influence on the retention.

A new set of experiments then was performed, using only methanol as the organic modifier. Figure 3a shows the results obtained. For comparative purposes, the chromatogram shown in Figure 2d also is included in the figure, labeled as Figure 3b. As we demonstrate, large differences in the chromatographic behavior were observed; notably, the retention of all the analytes increased (but less so for ACR than the other compounds).

There are two possible explanations for the effect of methanol. Apart from its obvious influence on the strength of the mobile phase, as illustrated in the figure, it can affect the performance of the stationary phase, thereby altering the system constants (38). Reversed-phase stationary phases have a fluid structure, volume, and composition that strongly depend on the composition of the mobile phase, among other factors. Because of its ability to form hydrogen bonds, methanol probably can drag water molecules into the stationary phase, which then can interact with free silanol groups, thus causing water molecules to be sorbed in the interface region, increasing the effective polarity of the bonded stationary phase. Polar interactions are then less favorable in the mobile phase, so retention of the more polar analytes is increased, as illustrated by the chromatographic behavior of RH (peak 1 in the figure). Another factor that should not be overlooked is that water is the most hydrogen-bond acidic solvent of the solvents that are commonly used in reversed-phase HPLC (Gutmann acceptor number of 54.8, compared with 41.3 for methanol or 18.9 for acetonitrile [39]), so its presence in the interphase increases the "effective" hydrogen-bond acidity of the stationary phase, thus increasing the retention of basic compounds while reducing its affinity for acidic compounds, as shown in Figure 3.

As for the elution order of fluorescent whitening agents for which the structure is known (Figure 1) with the optimum mobile-phase composition, it is worth noting that RH is the least retained, followed by Tetra, RD, and finally BS. These findings suggest that interactions between the largest molecules and the stationary phase are affected by steric hindrance, which prevents them from interacting with functional groups close to the surface of the stationary phase and thus reducing their retention fac-

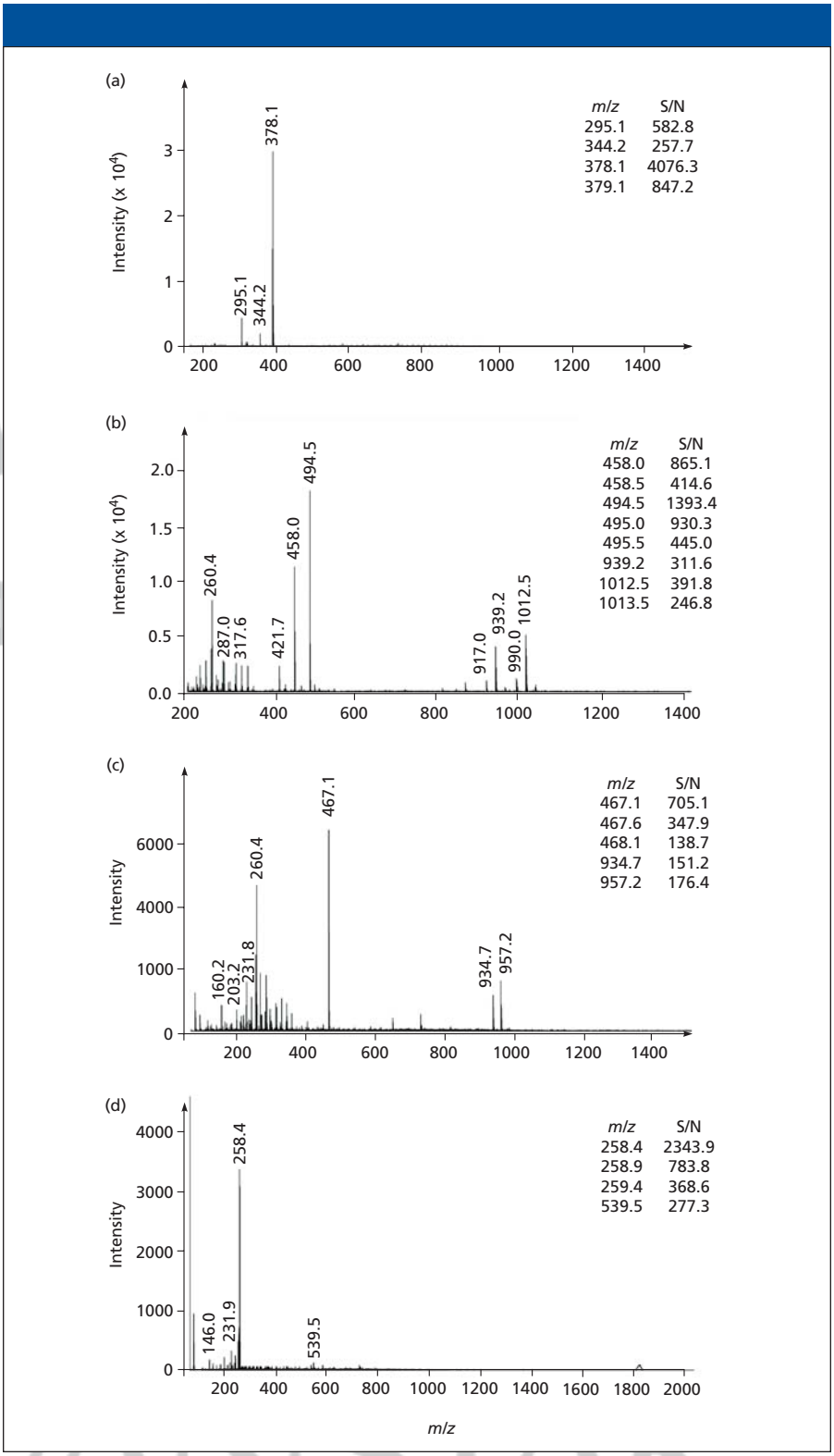

Figure 5: Mass spectra of fluorescent whitening agents (a) ACR, (b) AP, (c) RD, and (d) BS. For comparison purposes, all spectra depicted were obtained at a drying gas temperature of 200 °C and a capillary voltage of 120 V.

tor. Further confirmation of this hypothesis can be obtained by simple chemical calculations.

Among the several models that have been proposed for predicting chromatographic behavior in reversed-phase HPLC, probably the most widely accepted is the solvation parameter, also known as the linear solvation energy relationship model. In this model, a number of solute- and solvent-dependent factors are combined into a simple and intuitive mathematical expression of chromatographic retention (40–42). One of these solute descriptors, McGowan's characteristic volume (generally denoted V_x or V [cm^3 $mol^{-1}/100$]) generally is acknowledged to provide a correct estimation of solute size, and it has the advantage of being calculated easily for any molecule whose structure is known (43). For the analytes discussed here, the V_x values are 7.12, 6.31, 5.62, and 3.70, respectively, which are consistent with the rationale provided in the previous paragraph.

Figure 4 shows chromatograms obtained using the optimum mobile-phase composition, and the four different columns used in this study: conventional, polar-endcapped (Synergy, column 2 hereafter), polar-embedded (Symmetry, column 3), and nonpolar endcapped (XTerra, column 4). These chromatograms reveal several noteworthy points.

Among the several models that have been proposed for predicting chromatographic behavior in reversed-phase HPLC, probably the most widely accepted is the solvation parameter.

First, in line with expectations, nonpolar endcapped material (as in column 4, used to obtain the chromatogram shown in Fig 4d) is clearly inappropriate for the analysis of these polar compounds. Second, column 3 gives comparable retention times to the other two columns for almost all of the analytes, although it is just 10-cm long, while the others are 25-cm long. Third, retention of ACR again shows a different pattern from the other analytes. Its behavior on conventional and polar endcapped columns is very similar, in terms of both retention and peak width, but on polar embedded columns its retention time is reduced by more than 50% although its peak width is retained (Figure 4c).

The finding that basic compounds are related more strongly on column 3 seems to conflict with the pronounced basic character of the carbamate functional group built into the alkyl chain. Indeed, theory suggests that such a basic group should increase the retention of acidic analytes while weakening interactions with basic analytes. On the other hand, the distinctive chromatographic behavior of ACR is likely to be related to its cationic nature, which constitutes a major difference between this compound and the other analytes. We believe that the explanation for this apparent discrepancy is that the carbonyl oxygen of the carbamate group could be protonated in acidic conditions

Table I: Physico-chemical properties of the tested columns

Column	Prodigy 5 μm ODS (2)	Synergy 4 μm RP	Symmetry Shield C_{18} 5 μm	XTerra MS C_{18} 5 μm
Manufacturer	Phenomenex	Phenomenex	Waters	Waters
% Carbon load	18.5	19	19.8	15.4
Pore size (Å)	150	80	93	125
Pore volume (mL/g)	1.1	1.05	0.66	0.69
Surface area (m^2/g)	310	485	335	179
Surface coverage (m^2/g)	3.5	X	3.25	X
N (m^{-1})	60,500	113,300	82,700	41,500
Endcapping	Conventional	Polar	Polar-embedded	Nonpolar

and thus acquire a positive charge, causing a degree of ionic repulsion between the group and the cationic ACR analyte. This hypothesis, which also could explain the enhanced retention of anionic analytes, was first sketched by Layne (44), but no conclusive empirical evidence was then provided.

When comparing columns 1 and 2, interesting variations in peak shape between the columns were obtained. Best peak shape was obtained using column 1 in terms of both width and symmetry. Poor peak shape for many analytes, especially those with basic properties, previously has been reported as a major problem in reversed-phase HPLC analyses (45,46). As for its relation to stationary phases, it has been related with the presence of unreacted silanol groups attached to the surface of the phases. This might indicate, as illustrated by comparison of the chromatograms in Figures 4a and 4b, that more of these groups are available in column 2 than in column 1.

The validity of this hypothesis can be assessed using a number of models developed to identify factors that affect column performance and to establish appropriate methods for describing phase behavior (21,47,48). We selected a chromatographic approach (rather than a spectroscopy-based technique) for this purpose, in which several parameters accounting for different specific and discrete physicochemical interactions between the stationary phase and the analytes were included. The method involves constructing a model with six parameters, details, values, and explanation of which can be found elsewhere (47,49–51). Only the parameter related to silanol availability (and therefore the degree of endcapping) will be described here. This parameter, $\alpha_{C/P}$ or *hydrogen bonding capacity*, is a descriptor of the number of available silanol groups and thus is related inversely to the degree of endcapping. For columns 1 and 2, the $\alpha_{C/P}$ values are 0.37 and 0.58, respectively (49), indicating that more silanol groups are available in column 2 than in column 1. This supports the hypothesis presented earlier and indicates that the shielding procedure is less efficient for column 2.

LC–MS Study

As mentioned in the introductory section, one of the aims of this study was to develop a rapid LC–MS method for the identification of some fluorescent whitening agents commonly used in paper-and-board formulations. Because fluorescent whitening agents occur as ions in solution, ESI seemed to be the most suitable LC–MS interface for this study. In accordance with expectations, the anionic species were detected best in the negative ion mode, whereas positive ionization was found to be best for the only cationic analyte, ACR.

For ACR, positive-ion ESI yielded a very simple spectrum (see Fig. 5a) with ions of *m/z* 378, 344, and 295. Tentative explanation of the spectra could be as follows: *m/z* 378.1 corresponds to the protonated molecule, giving a molecular mass of 377, so, *m/z* 344.2 could correspond to $[M-2OH+H]^+$. Finally, *m/z* 295.1 will indicate the presence of the $[M-SO_3H+H]^+$ fragment, representing the loss of a sulfo group.

For AP, the other analyte for which no structural information is available, negative ESI yielded a very simple and clear spectrum, (Figure 5b) mainly composed of four high-mass ions, corresponding to *m/z* 1012.5, 990, 939.2, and 917, as well as two double-charged (indicated by the 0.5 *m/z* difference in their distribution, see Figure 5b), with *m/z* 494.5 and 458.0, respectively. We believe that this distribution of ions corresponds to two different congeners of the chemical in the standard, as indicated by the double peak obtained in the chromatograms (Figure 2d, peak 5). Tentative explanation is as follows: *m/z* 990.0 represents the $[M-H]^-$ deprotonated molecule, indicating molecular mass of 991, whereas *m/z* 1012.5 represents $[M+Na-2H]^-$ and 494.5 is the double-charged ion of *m/z* 991. On the other hand, *m/z* 917 is the deprotonated molecule for the second congener, then molecular mass should be 918. Then, in a way very similar to our earlier explanation, 939.2 should represent the sodiated adduct, according to $[M+Na-2H]^-$, and the double-charged ion should be 458.0 in this case.

As for the analysis of the compounds with known structures depicted in Figure 1, the disulfo derivative presented an interesting spectrum, as depicted in Figure 5c. The molecular mass of the chemical, obtained from simple calculation from its empirical formula ($C_{40}H_{42}O_{10}N_{12}S_2$) is 914. As we demonstrate, no ion corresponding to that mass-to-charge ratio is obtained, even in the less energetic conditions tested. Nevertheless, the mass-to-charge ratios corresponding to $[M+Na-2H]^-$, 934.7 and the disodiated adduct $[M+2Na-3H]^-$, 957.2 are obtained. This could be expected, as the structure of the chemical (see Figure 1a) includes two sulfonic acid groups with no sterical hindrance, where inclusion of sodium by substituting hydrogen can take place. So the first one corresponds to an "apparent" molecular mass of the compound of *m/z* 936, representing $[M+Na-H]$ (that is, substitution of one acidic hydrogen by sodium), whereas the other is related to the molecular mass of the compounds *m/z* 958, representing $[M+2Na-2H]$, the disubstituted molecule. Further confirmation is obtained by the presence of *m/z* 467.1, which is the double-charged molecule monosubstituted congener.

For BS, depicted in Figure 1b, the mass spectrum obtained is presented in Figure 5d. From the empirical formula of the compound, $C_{28}H_{22}O_6S_2$, a molecular mass of 518 can be calculated easily. Again, an mass-to-charge ratio of 517, which will represent the deprotonated molecula detected in negative ESI was not found in the spectra. As reported earlier, a mass-to-charge ratio of 539.5, corresponding to $[M+2Na+3H]^-$ was found. The disubstituted molecule was not found because in the structure depicted in Figure 1b, it is not easy to eliminate a third hydrogen to promote the negative charge needed for detection. As usual, the

double-charged molecule, corresponding to *m*/*z* 258.4, also was found.

Related to the other compounds, no conclusive information from their mass spectra could be obtained. As mentioned earlier, only industrial-grade standards were available, containing variable amounts of other fluorescent whitening agent congeners or reaction by-products, and very complex and nonreproducible spectra were found.

Conclusions

Chromatographic development usually is considered by most researchers to be a preliminary step in the development of analytical methodologies. In this study, we have shown that very interesting conclusions concerning not only the chromatography but also the general behavior of analytes can be obtained by correlating chromatographic data to well-established and simple models.

The presence of alcohol-type solvents in the mobile phase induces major changes in the stationary phase, altering both its effective polarity and its hydrogen-bond acidity. In addition, the results show that LC is not a simple process, and that general rules (for instance, acidic analytes will interact preferentially with basic phases and vice versa) must be treated cautiously to avoid unexpected and discouraging results. The findings demonstrate that ionic interactions (positive-charge repulsions) can overcome acidic–basic interactions resulting in chromatographic behavior that conflicts with expectations.

Simple MS patterns for some of the studied analytes are provided. These patterns can be used to achieve identification of some unknown fluorescent whitening agents in a sample. Semiquantitative results can be obtained by monitoring the attributed protonated or deprotonated molecules.

Acknowledgments

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