Separation, Purification, and Characterization of Analogues Components of a Commercial Sample of New Fuchsin

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Abstract

New Fuchsin (NF), also known as Magenta III, has potential applications in photodynamic therapy. The commercial product labeled NF contains two other dye components in different proportions, Magenta II and Magenta I (Rosaniline).
The proportions of NF, Magenta II, and Magenta I determined by reversed-phase high-performance liquid chromatography (RP-HPLC) in the commercial sample used were 71.6 ± 0.4%, 25.2 ± 0.2%, and 2.8 ± 0.1% ($n = 7$), respectively. The isolation, purification, and characterization of commercial NF dye components were carried out applying different techniques, such as preparative column liquid chromatography (PCLC), thin layer chromatography (TLC), RP-HPLC, absorption spectrophotometry, nuclear magnetic resonance spectroscopy (NMR), electrospray ionization mass spectrometry (ESI-MS), and tandem electrospray ionization mass spectrometry (ESI-MS–MS). After separation and isolation, the degree of purity obtained for NF compound was higher than 95% and 92% for Magenta II and Magenta I compounds, respectively. Therefore, it is essential to ensure a high degree of purity of these dyes as raw material to obtain new drugs intended for therapeutic treatments.

Introduction

The photochemistry of high-λ irradiation has been already addressed in relation to its efficacy in photodynamic therapy (PDT) (1–3). Modern phototherapy and photochemotherapy are now accepted for scientific treatment as a result of clinical studies and advances in basic sciences. PDT has gained increasing interest in medicine, representing an experimental tool for the detection and treatment of tumors in, for example, the lung, colon, eyes, and skin (4). The main advantages of PDT over other techniques such as oncotherapy include a rather significant degree of selectivity of drug accumulation in the tumor tissue, the absence of systemic toxicity of the drug alone, the ability to irradiate only tumors, the possibility of treating simultaneously multiple lesions, and the ability to retreat a tumor in order to improve this treatment (4,5).

Cationic dyes are being investigated because there is evidence suggesting that many tumor cells have a major capability to accumulate lipophilic cationic dyes attributable to the more negative mitochondrial membrane potential in comparison with normal cells (6–9).

The current and potential uses of dyes have led to further detailed studies on their chemical properties and their impact on the bioenvironment. Scientific progress has led to the development of numerous synthetic drugs; therefore, it is essential to deal with high purity dyes as raw material in order to obtain new drugs for medical applications. Several authors observed that not all the commercial dye batches have acceptable quality (10). For this reason, the purification, isolation, and characterization of analogues of New Fuchsin (NF) commercial sample was designed (Figure 1) because they were not previously described in the literature.

For many routine staining procedures, the composition and purity of NF (Magenta III) is probably of less importance.
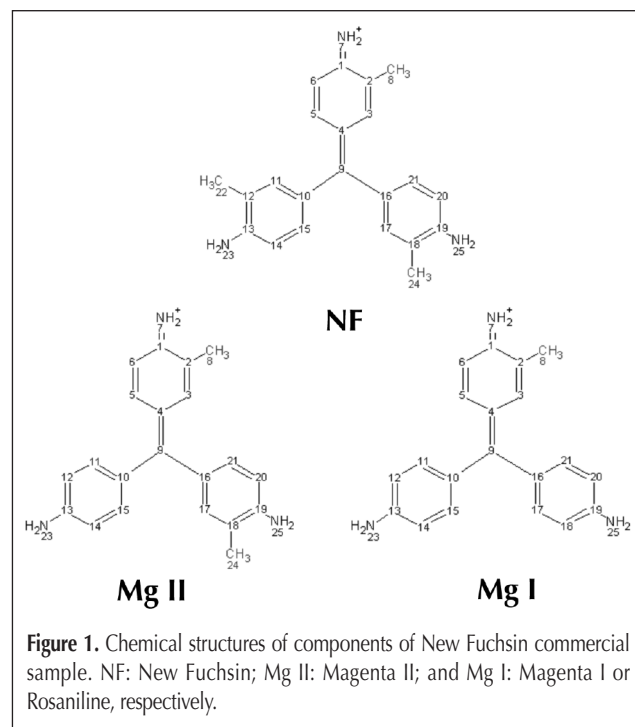

Figure 1. Chemical structures of components of New Fuchsin commercial sample. NF: New Fuchsin; Mg II: Magenta II; and Mg I: Magenta I or Rosaniline, respectively.

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Recently, however, it has been demonstrated that cationic tri-arylmethane dyes form a class of compounds that has a variety of therapeutical applications (11,12).

The work described here represents the initial stage in the study of a family of dyes. The pure components of NF will be used in future research to obtain new dye derivatives with better physicochemical properties than those of the precursor in view of the fact that an ideal photosensitizer should have a high degree of purity (3,13).

Experimental

Materials

All solvents and reagents were used without further purification. NF (Magenta III, Lot N 0638-25G) and silica gel (70–230 mesh, average pore diameter 60Å) were obtained from Sigma (St. Louis, MO). Ammonium chloride, chloroform, methylenechloride, and ethanol were analytical-grade and were all obtained from Laboratorios Cicarelli (San Lorenzo, Argentina). HPLC-grade acetonitrile (ACN) was provided by Mallinckrodt Baker (Mexico City, Mexico). Thin layer chromatography (TLC) plates used in this work were purchased from Macherey-Nagel Polygram (Dueren, Germany). Mobile phase was prepared from de-ionized water from a Millipore Milli-Q purification system (Billerica, MA) and was filtered through a 0.45-μm filter before use. Deuterated dimethyl sulfoxide (DMSO-d_6) and deuterium oxide (D_2O) were obtained from Sigma.

Purification by preparative column liquid chromatography

The components contained in the commercial NF were separated and purified by preparative column liquid chromatography (PCLC) using two differently sized glass tubes. One of them (750 mm × 23 mm) was used to get pure NF; the other (550 mm × 16 mm) was used to separate Magenta II (Mg II) and Magenta I (Mg I) (Figure 1). This mixture of compounds was obtained from the purification of commercial NF. In all cases, the silica gel was activated 35 min at 120°C and then slurry packed into the columns, which were protected from light.

The eluents were selected by TLC analysis, and a binary mixed solvent, methylenechloride–ethanol (8:2, v/v), was used for an effective separation. In the first stage of the purification, the commercial sample was dissolved in ethanol, adsorbed on silica gel, and then placed in column. A gradient mode elution was performed by A (methylenechloride) and B (ethanol) solvent system from 0 to 20% B with an increase of B estimated at 1% every 50 mL of the mobile phase.

The eluents were collected according to 150 fractions, each of them was of 5 mL. The fractions were analyzed by TLC and reverse phase-high performance liquid chromatography (RP-HPLC). Those containing isolated compounds NF, Mg II, and Mg I were combined together, and their solvent was removed under vacuum.

RP-HPLC and TLC

The RP-HPLC analyses were carried out using a liquid chromatograph Agilent 1100 Series apparatus equipped with an iso-cratic pump, a UV-Visible spectrophotometric detector, an autosampler, and a thermostatted column compartment (Santa Clara, CA). The optimization of RP-HPLC method was achieved by monitoring varying reversed-phase columns and mobile phases. The columns used in this study were as follows: 1, Waters Spherisorb ODS 2 (250 × 4.6 mm, 5 μm, Supelco, Bellefonte, PA); 2, Lichrosorb Hibar RP-C$_{18}$ (250 × 4 mm, 10 μm, Merck, Darmstadt, Germany); and 3, MICRO PAK mCH~5 N cap (150 × 4 mm, 5 μm, Wilmington, DE). The column temperature was set at 25°C in all cases, and the injection volume was 50 μL. The detection was performed at 280 and 550 nm, and the chromatographic system was controlled by an Agilent ChemStation software package.

An ascending TLC was performed on alumina plates coated with Silica Gel 60 F254 (20 cm × 20 cm) with a 0.25 mm thick layer. After TLC runs, plates were observed under UV light to detect the spots.

All these techniques have been applied simultaneously and successfully to assess the progress of purification and to confirm the purity and identity of isolated compounds.

UV–vis spectrophotometry, nuclear magnetic resonance, electrospray ionization mass spectrometry, and tandem electrospray ionization mass spectrometry

The spectrophotometric measurements were recorded on an Agilent 8453 spectrophotometer between 200 nm and 800 nm. The studies were carried out at 25 ± 1°C in quartz cuvettes with 1 cm optical path length.

NMR spectra were performed at room temperature and recorded in DMSO-d_6/D_2O solution on a Bruker AVANCE II 400 nuclear magnetic resonance spectrometer equipped with a 5 mm BBI 1H/D-BB ZGRD Z8202/0349 inverse probe and with a variable temperature unit (VTU) (Bruker BioSpin, Billerica, MA) for ^1H and ^{13}C. The spectra obtained were manually phased, baseline corrected, and calibrated to residual solvent of DMSO-d_6 at 2.504 ppm using the WINNMR 6.0 software as a data processor. The sample concentration used was 0.02 M.

Electrospray ionization mass spectrometry (ESI-MS) and tandem ESI-MS–MS experiments were performed with a Varian 1200L triple-quadrupole liquid chromatographic–mass spectrometer (Palo Alto, CA). The ESI-MS was operated in the positive ion mode. The data acquisition software Varian MS–MS workstation (version 6.6) was used for instrument control, data acquisition, and data handling. The samples were infused directly into the mass spectrometer, and the working concentration was close to 500 ng/mL.

For ESI-MS experiments the electrospray capillary voltage was set to 60 V. Nitrogen was used as a drying gas for solvent evaporation at 51 psi. The atmospheric pressure ionization (API) housing and drying gas temperatures were kept at 50 and 300°C, respectively. The needle was held at 5000 V and the shield at 600 V. The scan time was 1 s, and the detector multiplier voltage was set to 1500 V.

Stability Test

The stability analysis of NF in different solvents is important to ensure good quality during the entire process of separation, purification, and characterization. The stability test was carried
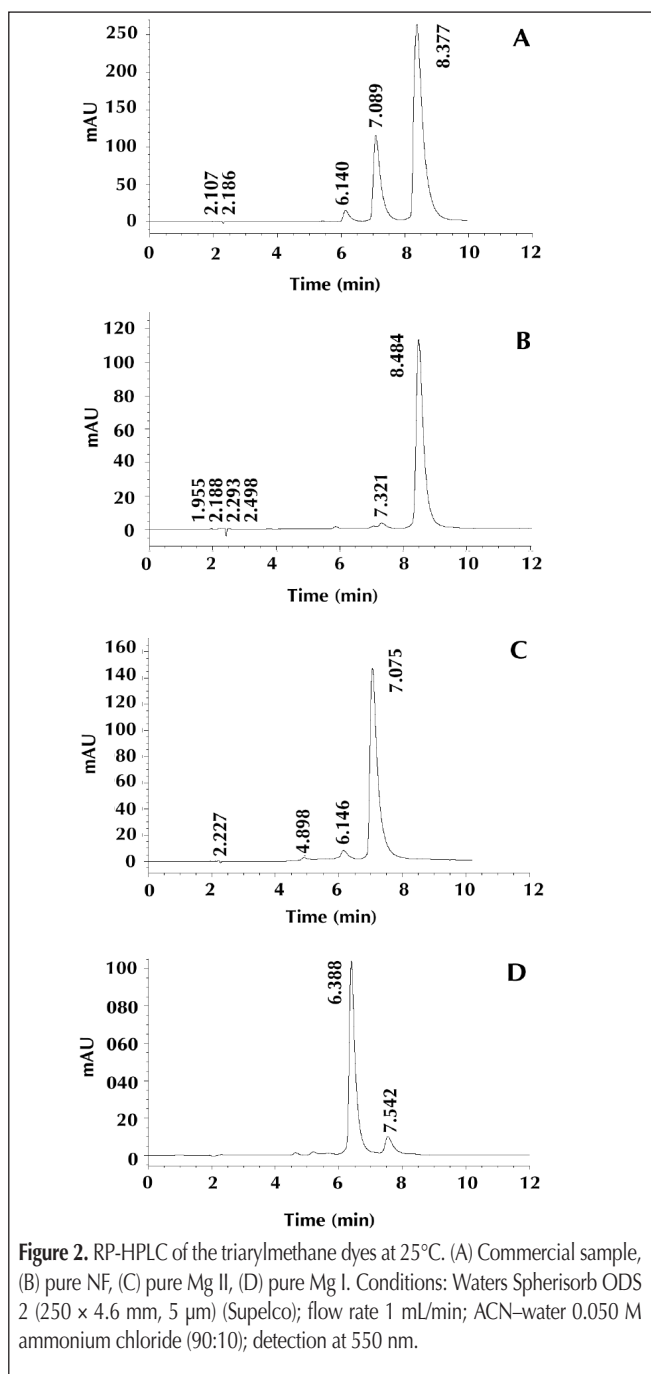
Figure 2. RP-HPLC of the triarylmethane dyes at 25°C. (A) Commercial sample, (B) pure NF, (C) pure Mg II, (D) pure Mg I. Conditions: Waters Spherisorb ODS 2 (250 × 4.6 mm, 5 μm) (Supelco); flow rate 1 mL/min; ACN–water 0.050 M ammonium chloride (90:10); detection at 550 nm.

out for 32 h in duplicate in hermetic quartz cells in the following solvents: methylenechloride, ethanol, acetonitrile, acetone, and the mobile phase used in RP-HPLC. The solutions, wrapped in aluminum foil to be protected from light, were maintained at 25 ± 1°C using a thermostatic bath Branson 1510; eighteen measurements were carried out by UV-visible spectrophotometry.

Results and Discussions

Purification of analogues components of the commercial NF

The purity of compounds obtained by PCLC was determined by RP-HPLC. Figure 2 shows a comparison of the RP-HPLC chromatogram of a commercial sample and the different pure constituents separated using column 1 and ACN–water 0.050 M ammonium chloride (90:10) as mobile phase. Recovery of the different constituents was expressed in percentage and determined by dividing the pure quantity of NF, Mg II, and Mg I obtained by the loading sample.

A preparative column of 750 mm × 23 mm was able to purify NF from 71.6% in the commercial sample to more than 90% with a recovery of 89% and to more than 94% of purity with a recovery of 58%. Mg II was purified from 25.2% in the commercial sample to more than 93% with a recovery of about 52%, and Mg I (found initially in approximately 2.8%) was obtained in more than 92% with a recovery of about 20%. The results were calculated considering that Mg II and Mg I were purified by the development of two preparative columns. All dyes, with a high degree of purity, were directly used for structure elucidation by ESI-MS and NMR analyses.

RP-HPLC analysis

During the development of an efficient method for the separation, identification, and quantification of fuchsin analogues, different RP-HPLC columns and mobile phases were evaluated.

First, a variety of mobile phases were used in reversed-phase column 1 in order to find out the optimum chromatographic conditions for the analysis of commercial NF dye. When we used ACN, methanol or ACN–water as mobile phase at a flow rate of 1 mL/min, a strong adsorption of this cationic dye on the stationary phase was found (14). A significant improvement was observed by adding an electrolyte as ammonium chloride to the mobile phase system (6).

Although it is known that column efficiency increases with the decrease in the particle size, the influence of the amount of electrolyte and ACN (organic modifier) was investigated using the commercial sample and the HPLC columns 1 and 2. As shown in Table I, the retention time of NF decreased as the addition of ammonium chloride increased. This phenomenon could be explained by the increased degree of ionization of the polar silanol groups that form the surface of the stationary phase as a consequence of the increasing pH that causes an increment of the retention of NF. Table I also

Table I. Effect of Ammonium Chloride and Acetonitrile Content in Mobile Phase on NF Retention Time New Fuchsin (NF) Chromatographic Parameters for Two HPLC Columns

Ammonium chloride (mM) (ACN–water)	pH MP mobile phase	Column 1						Column 2					
		t_R*	R*	α*	k'*	HETP*	As*	t_R	R	α	k'	HETP	As
10 (90:10)	5.50	24.0	3.98	1.21	10.25	0.04	2.47	34.9	1.72	1.22	20.35	0.23	2.09
20 (90:10)	5.38	16.3	3.68	1.19	7.59	0.05	1.89	17.9	1.62	1.21	9.03	0.20	2.17
30 (90:10)	5.25	12.5	3.49	1.21	4.85	0.04	1.50	17.3	2.04	1.23	4.94	0.12	1.38
50 (90:10)	4.90	8.4	2.77	1.25	3.32	0.06	1.75	11.3	1.53	1.22	4.23	0.14	1.67
50 (70:30)	5.48	15.2	2.47	1.33	6.41	0.12	2.80	20.1	2.30	1.36	9.04	0.26	1.85

* t_R = retention time; R = Resolution; α = column selectivity; k' = column capacity factor; HETP = Height Equivalent to Theoretical Plates; As = asymmetry of the peaks.

shows that when the amount of ACN decreases the retention time for NF increases.

In addition, it was observed that the resolution of the constituents also decreased as the addition of ammonium chloride increased.
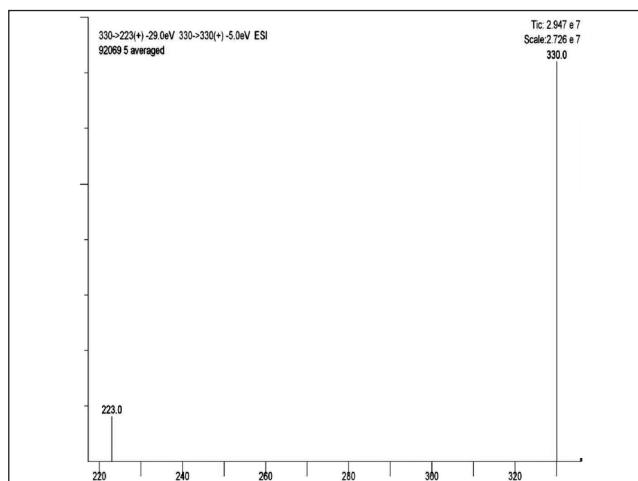

Figure 3. ESI-MS–MS product ion spectra spectrum of the (M+H)+ ion of NF (m/z 330).

Taking particular account of the chromatographic parameters shown in Table I, it can be concluded that a satisfactory and effective separation for column 1 was achieved using ACN–water 0.050 M ammonium chloride (90:10) as mobile phase (Table I) for which, as an average, a resolution higher than 2.77 was obtained. On the other hand, the resolution found for column 2 using the same mobile phase was 1.5. Although this resolution is lower than that observed in column 1, the variation among the degree of purity of the different components seen in Table II was not significantly different (variation < 1%). Because of this, column 2 can also be used for quantitative analysis under the same conditions.

In order to complete the column screening, column 3 was also analyzed using the mobile phase ACN–water 0.050 M ammonium chloride (90:10) (Table II). The resolution obtained was close to 1.0, which is not enough to get a desirable quantification of these dyes. This problem could be solved by adding a large amount of ammonium chloride; however, it could be harmful for chromatographic columns.

Therefore, we selected column 1 and mobile phase ACN–water 0.050 M ammonium chloride (90:10) to analyze the NF commercial sample and the purified fuchsin analogues due to the smaller retention time and the good resolution achieved. Figure 2 shows the chromatographic runs for NF commercial sample, composed of a combination of triarylmethanes NF, Mg II and Mg I dyes, and the pure samples. The assignation of peaks was corroborated by NMR and mass spectrometry analyses.

The retention time ($n = 7$, Table II) of these compounds depends on their polarity, in which a peak at t_R = 6.16 min corresponding to Mg I was found to be the more polar compound. The second peak at t_R = 7.11 min was identified as Mg II, and the third peak at t_R = 8.40 min was related to NF, the less polar compound.

Finally, the composition of NF commercial was 71.6 ± 0.4% of NF, 25.2 ± 0.2% of Mg II, and 2.8 ± 0.1% of Mg I (Table II).

TLC analysis

The solvent system consisted of chloroform and methanol in a 4:1 (v:v) ratio. Solutions of the testing samples were applied to the TLC plates as spots. After having been developed, the TLC plates were dried with warm air for 5 min, and the sample spots were then analyzed under 254 nm UV light; the R_f values of NF, Mg II, and Mg I dyes ($n = 7$) are shown in Table III.

NMR, ESI-MS, and ESI-MS–MS

Table III also list ^{1}H and ^{13}C NMR (DMSO-d_6/D_2O) data of compounds NF, Mg II, and Mg I. Exchange of the amine proton of these dyes with deuterium of deuterium oxide allows assignation and integration of aromatic signals. The ^{1}H NMR of the three compounds matched with the NMR data reported in the literature (15). All spectra were corroborated with those

Table II. t_R and Purity Percentage of NF, Mg II, and Mg I in Commercial Sample Using Different HPLC Columns (n = 7)

Column Particle size-length	Commercial Sample*					
	NF		Mg I		Mg II	
	t_R (min)	(%)	t_R (min)	(%)	t_R (min)	(%)
(1) 5 µm-25 cm	8.40 ± 0.05	71.6 ± 0.4	7.11 ± 0.03	25.2 ± 0.2	6.16 ± 0.02	2.8 ± 0.1
(2) 10 µm-25 cm	11.3 ± 0. 2	72.48 ± 0.08	9.7 ± 0.2	24.66 ± 0.08	8.6 ± 0.1	2.85 ± 0.03
(3) 5 µm-15 cm	2.40 ± 0.01	76.2 ± 0.5	2.12 ± 0.01	21.0 ± 0.4	1.91 ± 0.01	2.7 ± 0.3

* HPLC mobile phase: acetonitrile–water 0.050 M of ammonium chloride 90:10.

Table III. Rf Values, 1H NMR, and 13C NMR chemical shifts of NF, Mg II, and Mg I*

NF 0.66 ± 0.01	Mg II 0.60 ± 0.01	Mg I 0.55 ± 0.01

Rf (n = 7) Chemical shift δ (ppm) 1H NMR (400.15 MHz)

2.111 (s; 9H; H_8, H_{22}, H_{24})	2.143 (s; 6H; H_8, H_{24})	2.143 (s; 3H; H_8)
6.853 (m; 3H; H_6, H_{14}, H_{20})	6.850 (m; 4H; H_6, H_{12}, H_{14}, H_{20})	6.826 (m; 5H; H_6, H_{12}, H_{14}, H_{18}, H_{20})
7.031 (m; 3H; H_3, H_{11}, H_{17})	7.065 (m; 4H; H_3, H_5, H_{17}, H_{21})	7.082 (m; 2H; H_3, H_5)
7.072 (m; 3H; H5, H15, H21)	7.201 (m; 2H; H_{11}, H_{15})	7.176 (m; 4H; H_{11}, H_{15}, H_{17}, H_{21})

Rf (n = 7) Chemical shift δ (ppm) 13C NMR (100.62 MHz)

17.330 (C_8, C_{22}, C_{24});	–	–
114.384 (C_6, C_{14}, C_{20}),		
121.688 (C_2, C_{12}, C_{18});		
126.567 (C_4, C_{10}, C16),		
138.237 (C_5, C_{15}, C_{21});		
139.827 (C_3, C_{11}, C17),		
155.945 (C_1, C_{13}, C_{19});		
177.749 (C_9)		

* Corresponds with Figure 1.

obtained from the Spectral Database for Organic Compounds, SDBS, organized by the National Institute of Advanced Industrial Science and Technology and those obtained from the ACD Labs program.

A low degree of fragmentation produced by ESI, especially to stable molecular ions as aromatic compounds, allowed us to determine the molecular weight and the purity of the different dyes obtained by column chromatography. The sensitive detection of this technique is adequate for the determination of minor components in complex mixtures. The mass spectrum of commercial NF presented three peaks that were assigned to their components while the mass spectrum of pure samples corroborated the results obtained by RP-HPLC.

The identity of pure compounds NF, Mg II, and Mg I was confirmed in the positive ion mode, and the molecular ion species $(M +H)^+$ were detected at m/z 330, 316, and 302 in the linear scan mode (MS); their molecular weights were determined as 329, 315, and 301 Da, attributed to NF, Mg II, and Mg I, respectively.

For tandem ESI-MS–MS experiments, diverse conditions were studied. Only little fragmentation of NF was recorded using a drying gas temperature of 200°C, a needle and a shield of 5850 V and 325 V, respectively. The other two dyes were stable in all the conditions assayed. Figure 3 shows the ESI-MS–MS mass spectrum of the product ion of m/z 330 of a pure sample of NF dye. The fragment ion of m/z 223 results from the loss of a methylaniline group.

Spectrophotometric analysis

Figure 4 shows the electronic absorption spectra of NF, Mg II, and Mg I in ACN at 25°C. The absorption spectra of these dyes display a maximum of absorption close to 545 nm and a shoulder near 500 nm. Like other extensively studied triarylmethanes, for example crystal violet, this characteristic spectrum appears to be composed of two overlapped bands, and different theories account for this. Lewis and co-workers proposed the existence of two ground state rotational isomers in rapid equilibrium with each other (16,17). Other authors suggested the presence of a symmetry-breaking process due to electronic interaction between central cationic carbon and dipole moment of solvent molecules or the interaction between the central atom with a counter-ion near it or with one of the amino groups (18,19). The existence of solvated isomers was also stated (20–22).

The maximum absorption of Mg I, Mg II, and NF in ethanol, ACN, mobile phase used in RP-HPLC and methylenechloride is shown in Table IV. In several cases, there is a bathochromic effect which varies between 1 and 3 nm due to an increment in the degree of methylation. This effect could be attributed to the elec-

tron-donor capacity of the methyl group that increases the number of electrons in the π system and decreases the energy required to promote one of those electrons to the excited state. In consequence, the λ_{max} increased as shown for ACN and methylenchloride (23).

NF, Mg II, and Mg I show absorption characteristics that are largely dependent on the polarity of the solvent (solvatochromic effects). In general, the shape and λ_{max} (Table IV) of these triarylmethane dyes absorbance spectra present differences among them dependent on solvent effects. It was observed that λ_{max} increases with the increment of the dielectric constant (λ_{max} = mobile phase HPLC > acetonitrile > methylenechloride). The changes in the λ_{max} might be attributed to the proton donor capability of the solvent or by the formation of species in excited states that have different chemical or polarity characteristics compared with those in the ground state. Dipole-dipole interactions reduce the energy of the more polar excited state because in most π to π* transitions, the excited states are more polar than their ground states due to a larger load separation in the excited state (24). However, we observed that NF, MgII and MgI in ethanol present a larger λ_{max} due to the capacity of this solvent for acting as a donor of protons in H bond interactions with the solute (25).
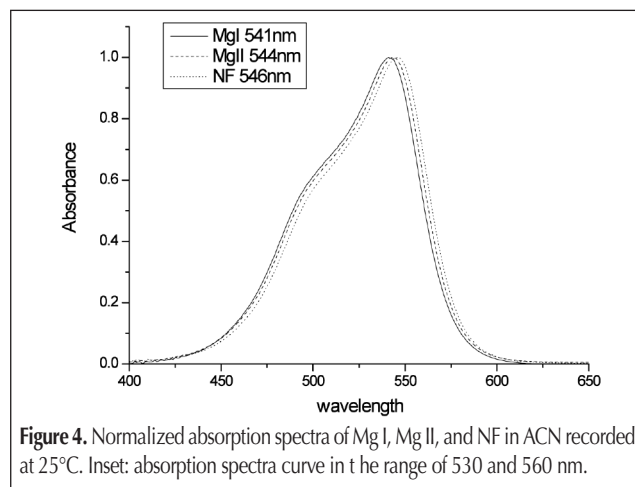

Figure 4. Normalized absorption spectra of Mg I, Mg II, and NF in ACN recorded at 25°C. Inset: absorption spectra curve in t he range of 530 and 560 nm.
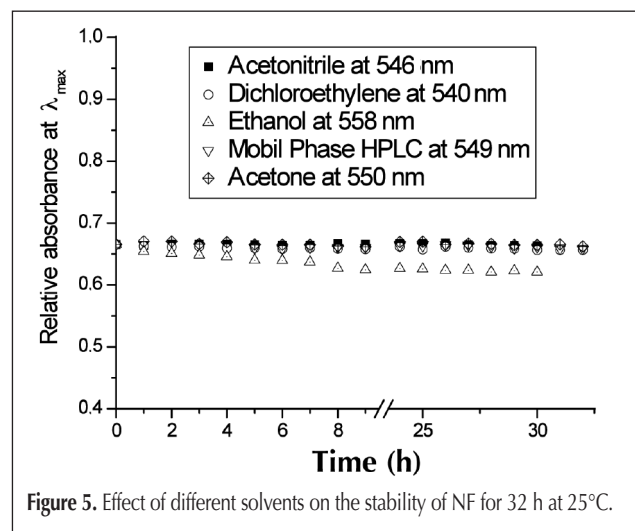

Figure 5. Effect of different solvents on the stability of NF for 32 h at 25°C.

Table IV. Maximum Absorption of NF, Mg II, and Mg I			
	Maximum absorption (nm)		
	NF	Mg II	Mg I
Acetonitrile HPLC	546	544	541
Mobile phase HPLC	549	547	548
Methylenechloride PA	540	539	537
Ethanol PA	558	557	566

Stability test

The stability of purified NF in different solvents was followed by UV-vis spectrophotometry at λ_{max}. The results are shown in Figure 5. It can be seen that the UV-Vis spectra of this dye did not evidence a significant degradation up to 32 h at 25ºC.

Conclusions

For many staining procedures where NF is most generally applied to, in textiles for instance, the composition of the dye mixture is probably of less importance and used without further purification. However, at present, the purity of the dyes is essential for their therapeutic application as drugs. Therefore, this work proposes adequate methods for separation, purification, and quantification of the three components contained in commercial NF.

A PCLC gradient eluent process was successfully developed to isolate and purify NF commercial samples. This methodology provides a good efficiency and high recovery percentage of purity, exhibiting a great simplicity and low cost. The separation conditions by PCLC were optimized by using only two organic eluents.

A simple and useful identification of the different constituents of this commercial sample was successfully carried out by TLC. In comparison with the RP-HPLC method, the TLC method appears to be equally suitable for routine testing. Among its advantages, we can include short run time and the need of a small quantity of solvents required by this technique.

RP-HPLC and positive ion electrospray mass spectrometry techniques were suitable tools for quality control and characterization of these related compounds with high resolution. NF demonstrated to be stable in all the solvents evaluated, and no significant difference of decomposition at 25°C for 32 h was observed.

Acknowledgement

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