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Analyses of four ball point pen inks and two dyes by thin-layer chromatography with fluorescence detection and matrix assisted laser desorption time-of-flight and electrospray ionization mass spectrometry

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ABSTRACT

Ball point pen inks contain one or more dyes. Characterization and identification of dyes of ball point pen inks is very important in forensic document examination. Some problems may arise because mixtures of dyes give complex instrumental responses especially when dyes are not pure substances. It is true that rhodamine 6G shows three different color bands on a thin layer chromatographic (TLC) plate after separation. Fluorescent and mass spectrometric analyses after TLC and high performance liquid chromatographic (HPLC) separation provide specific results of different components in dyes. Matrix assisted laser desorption time-of-flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometric analyses in this work cross-examined the molecular weights of different components in dye. The methods used in this work show strong potential for the characterization and identification of dyes, especially when further analysis such as ink dating is desirable.

Keywords: Ball Point Pen Inks, Dyes, Rhodamine 6G, Rhodamine B, Thin-layer Chromatography, Fluorescent Analysis, Matrix Assisted Laser Desorption Time-of-Flight Mass Spectrometry, Electrospray Ionization Mass Spectrometry

Introduction

Fluorescence is a sensitive and selective method. The spectral distribution of the fluorescence radiation is a physical and absolute characteristic of a given substance and is useful for qualitative considerations [1]. Moreover, the emission intensity of fluorescence at a given wavelength is useful for quantitative analysis if careful standardization is made. Mass spectrometry is an even more powerful qualitative detection method. From the mass spectrum, a wealth of information can be obtained concerning the composition of mixtures of organic compounds and the elemental analysis of solid state samples [2]. MALDI-TOF mass spectrometer produces charged ions consisting of mainly the parent ion and few fragmented ions of the original molecule [3]. The electrospray ionization mass spectrometry (ESI-MS) is a widely used method of analysis combining both the universality and the selectivity of MS with liquid chromatographic separation in order to solve many complex analytical problems [4,5,6]. Pseudo-molecular ions are produced with little or no fragmentation [7,8] Dye components present in ball point pen inks separated by thin layer chromatography can be characterized by the analysis of these three methods in this work [9,10,11,12, 13,14].

Experimental

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Materials and apparatus

Four red ballpoint pens, Staedtler 430 M, Corvina 81, Bic red and Micron red were bought in Great Britain. rhodamine 6G and rhodamine B dyes of reagent grade were purchased from TCI, Tokyo, Kaisei Kogyo, Co., Ltd. All the thin layer chromatography (TLC) plates used were aluminum backed silica gel 60 purchased from Merck, Taiwan. Methanol, ethyl acetate, acetonitrile and ethyl alcohol of LC grade and triflouroacetic acid were purchased from Taiwan Alps Chem. Co., Ltd., and used throughout this work. Water used to prepare the solvent mixture was filtered and deionized using Milli-Q-Plus.

Fluorescence spectra were recorded on a Shimadzu RF-5000 spectrofluorophotometer. A Hewlet-Packard G 2025 A Laser Desorption-Time of Flight system was used for mass analysis which employed N_2 Laser (337 nm) as the light source with energy ranging from 0 to 2.5 mJoule/cm^2. An HP Prep Accessory system was used to prepare the sample for MALDI-TOF detection while α-Cyano-4-hydroxycinnamic acid (CHC), purchased from Hewlet Packard, USA, was used as the matrix. The CHC matrix was prepared by weighing 6.24g of CHC into a 1000 mL volumetric flask and diluting to volume with methanol.

A Waters Alliance system with a platform LC mass detector (Qed 1640) was used for mass analysis. Mobile phase used to elute the ink and dye samples was 0.05N trifluoroacetic acid: acentonitrile: H2O (20:40:40 v/v). Separation column was a Waters symmetry column (0.5 mm). Injection volume of the samples was 0.5 μL. Ion source for mass detection was produced using electrospray ionizer and the mass range was from 80 Da to 1360 Da.

Preparation of ink and dye samples.

Each of the four different ballpoint pens, Staedtler 430 M, Corvina 81, Bic red and Micron red was used to draw as a square (2*2 cm) on a sheet of Chapman carbonless paper. The line was cut out of the paper in small squares and placed in a clean microvial. An aliquot of 100 μL HPLC grade methanol was added into the microvial and subjected to extraction for 6 minutes. The methanol extract in the microvial was transferred with a Gilson micropipet to another clean microvial for the procedures detailed below.

Thin-layer chromatography (TLC)

A TLC plate was prepared (6 * 6 cm) with a pencil baseline drawn 1 cm from the bottom of the plate. Each methanolic extract of ink and dye samples obtained as above described in sample preparation was separately applied onto the plate using a capillary tube. Each spot was 1.5 cm apart from it neighbor(s). The plate was developed with a solvent mixture of ethyl acetate / ethanol / H_20 (7:3:2 v/v). When the solvent front reached the line 1 cm from the baseline, the plate was taken out and dried. When the plate was completely dry, it was then returned to the development tank again for another development over a distance of 5 cm. The plate was taken out and dried. The separated color spots of the sample on the TLC plate were measured and their Rf values recorded. The plate was also observed under ultraviolet wavelengths of 254 and 366 nm, the fluorescent areas on the plate were framed with a pencil for the following fluoresence detection.

Fluorescent examination of inks and dyes

The thin layer of a fluorescing area on the TLC plate was scraped off, put into an Eppendorf vial and extracted with an aliquot of 400- μL methanol for 6 minutes by placing it in an ultrasonic bath. After extraction, the vial was centrifuged at 1000 G and the methanolic extract moved to another clean Eppendorf tube. The extraction was repeated using a further aliquot of 400 μL of methanol and the two extracts combined. The combined extracts (approximately 800 μL) were then placed in a 5-mL spectrometric cuvette and diluted with methanol to a volume of 3 ml for the fluorescence analysis. The fluorescence spectrum was acquired by scanning over 400-700 nm for emission and 200-500 nm for excitation. The same procedure was applied to all the other fluorescing areas on the plate and the results recorded.

Matrix Assisted Laser Desorption Time-of-Flight mass analysis

An amount of 6.24 g of CHC was weighed and dissolved in a volume of 1000 mL of methanol. The excitation scanning wavelength ranged from 300 to 500 nm. An N_2 laser used as the energy ionization source had an energy output of about 0-2.5 mJoule/cm^2. An aliquot of 3 to 5 μL of each of the methanolic extracts of the ink and dye components was added to an equivalent

amount of matrix and thoroughly mixed. An aliquot of 0.7 μ L of this mixture was placed on the sample mesa (table). The solvent in the mixture was evaporated under vacuum, and the analyte and matrix co-crystallized. The co-crystallized particles were placed in the MALDI-TOF mass spectrometer that started to detect the generated ions once the vacuum level reached 10^{-6} torr. The molecular weight scan ranged from 100 to 500 Da. The molecular weights of all of the colored ink components and the dyes were identified from their mass spectra.

Electrospray Ionization mass analysis

An aliquot of 0.5 μ L of each of the methanolic extracts of the ink and dye components obtained from TLC development were separately injected into an ESI LC-MS system for analysis. Separation column was a Waters symmetry column. The mobile phase was trifluoroacetic acid / acetonitrile / H_2O (2:4:4 v/v) at a flow rate of 1.0 ml/min. For mass analysis, ion source was produced by an electrospray ionizer and its needle voltage was set to 3.50 kVolts. The molecular weight scan ranged from 80 to 1360 Da. All results for the methanolic extracts of the ink and dye components were recorded and compared with the results obtained from the MALDI-TOF analysis.

Identification of ink dyes

The Rhodamine 6G dye, the Staedtler 430 M and Corvina 81 inks were processed through the TLC separation process according to the procedures described for TLC development.

Dye components of rhodamine 6G dye and Staedtler 430 M and Corvina 81 inks demonstrating equivalent Rf values on the TLC plate were scraped off, extracted according to the procedures described above. The fluorescence excitation and emission maxima and the MALDI-TOF and ESI mass spectra were obtained for all components. Results from all analytical stages and detection were recorded and compared.

Dye components in rhodamine B dye and Bic red and Micron red inks were separated by TLC, scraped off the plate, extracted into methanol and analyzed by fluorescence detection. MALDI-TOF and ESI analyses were performed on samples showing the same analytical traces and were performed according to the procedures described before. Results from all analytical stages and detection were recorded, tabulated and compared with each other.

Results and Discussion

Fluorescence detection of the components of Staedtler 430 M, Corvina 81, Bic Red and Micron Red inks, and rhodamine 6G and rhodamine B dyes

All the components of the inks and the dyes separated by TLC were fluorescent under UV at 366 nm except three spots associated with Micron red ink. All of those fluorescing components were eligible for fluorescence detection using wavelengths of maximum excitation and emission. The results are listed in Table 1.

For Staedtler 430 M, Corvina 81 inks and rhodamine 6G dye, excitation wavelength maxima fell in the range of 345.6 to 348.8 nm and emission wavelength maxima 544 to 550.5 nm. The distance between the excitation and emission maxima permitted both excitation and emission slits of the detector to be set to 5 nm. For Bic red, Micron red inks and rhodamine B dye, the excitation wavelength maxima fell in the range of 528 to 550 nm and emission wavelength maxima 549 to 568 nm. Because of the proximity of the excitation to the emission wavelength, the detector slits had to be narrowed to 3 nm to prevent the overlapping of the fluorescence spectra.

All spots of rhodamine 6G dye and Staedtler 430 M and Corvina 81 inks separated by TLC showed fluorescence when irradiated with UV light at 366 nm. Spots 1, 2 and 3, respectively, of rhodamine 6G had equivalent Rf values to those of spots 1, 2 and 3 of both Staedtler 430 M and Corvina 81 inks. A comparison among these three inks or dye with respect to the fluorescence excitation and emission wavelength maxima of the three sets of matched spots showed that the corresponding components were identical. Thus, the fluorescence excitation and emission maxima were 347.2 and 550.4 nm, respectively, for the first spots, 345 and 547 nm for the second, and 347.2 and 547 nm for the third. All spots of rhodamine B dye and Bic red ink separated by TLC were fluorescent under UV radiation at 366 nm. Two of the five spots of Micron ink were fluorescence under 366 nm, their Rf values being the same as those of the components of rhodamine B dye and Bic red ink, and their fluorescence excitation and emission maxima being 550 and 568 nm for the first spots and 528 and 549 nm for the second.

Table 1 Excitation wavelengths and emission maxima of different components of ink and dye samples

Ink name	Rf value[a]	Excitation wavelength maximum (nm)	Emission wavelength maximum (nm)
Staedtler 430M	0.57± 0.02	347.2	550.4
	0.62± 0.02	347.2	547.2
	0.66± 0.01	345.6	547.2
	0.73± 0.01	348.8	544.0
Corvina 81	0.57± 0.01	347.2	550.4
	0.63± 0.02	347.2	547.2
	0.68± 0.02	345.6	547.2
	0.74± 0.01	348.8	544.0
Rhodamine 6G	0.57± 0.01	347.2	550.4
	0.63± 0.01	347.2	547.2
	0.67± 0.01	345.6	547.2
Bic red	0.74± 0.01	550	568
	0.77± 0.01	528	549
Micron red	0.74± 0.01	550	568
	0.77± 0.01	528	549
Rhodamine B	0.74± 0.01	550	568
	0.77± 0.01	528	549

[a] N=5

Matrix assisted laser desorption time-of-flight and electrospray mass spectrometric analyses

The energy of the laser beam used in the MALDI-TOF analyser was about 0-2.5 mJoule/cm^2. The setting mostly resulted in the components being detected in their mass spectra as their molecular ions with few fragmentations. The matrix used in this work was CHC (α-cyano-4-hydroxycinnamic acid) which is suitable for analytes whose molecular weight are less than 1000. A good matrix should meet the following conditions. The first condition, solubility, is necessary so that the analyte and the matrix material can be dissolved in the same solution. This condition can be expanded to include any solvent system in which the analyte of interest will co-dissolve with the matrix. The second condition, absorption, allows the energy to be deposited in the matrix, not the analyte. The third condition, reactivity, is required for obtaining useful analytical results. CHC is the one chosen as the matrix in many researches. The molecular weights of rhodamine 6G and rhodamine B dyes are the same at 443, so using CHC as the matrix was appropriate. The major matrix ions of CHC are m/z 190 [(M+H)$^+$], 212 [(M+Na)$^+$], 228 [(M+K)$^+$], 235 [(M+2Na)$^+$], 251 [(M+N+K)$^+$] and 379 [(2M+H)$^+$], where M is the molecular ion. A mass spectrum of CHC is shown in Fig. 1.

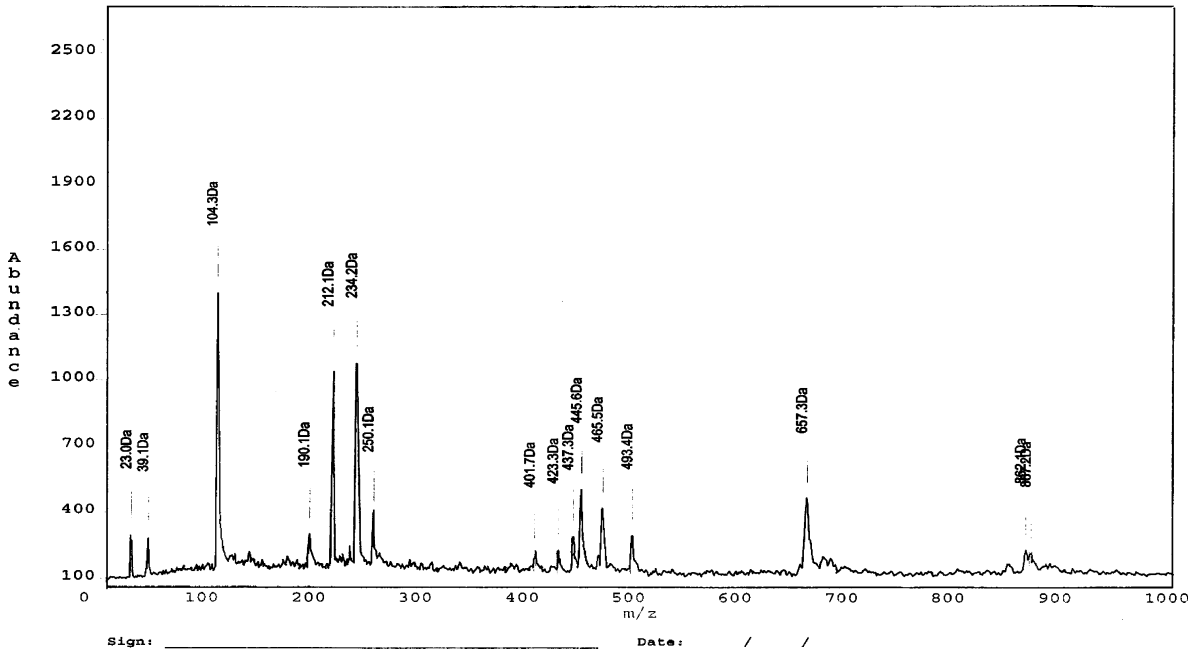

Fig.1 MALDI-TOF mass spectrum of the matrix, CHC.

At first, the methanolic extract of Staedtler 430 M ink from Chapman carbonless paper without any other treatment was analyzed by MALDI-TOF. Then the TLC spots 1, 2, 3 and 4 of Staedtler 430 M ink were scraped off, extracted and dissolved in methanol. The four methanolic extracts were analyzed by MALDI-TOF. The same procedures are also applied to Corvina 81 ink, rhodamine 6 G and another group of inks and dye which includes Bic red, Micron red and rhodamine B. In undertaking the MALDI-TOF analyses of the inks and dyes, there might be some interferences from some sources other than the analytes, such as paper, TLC absorbent, and even the extracting methanol and the CHC matrix depending upon the sample preparation procedures. It therefore became necessary to analyze also those background materials as controls. It turns out to be that all interfering ions in these mass spectra can be readily subtracted from the raw mass spectra of the inks and dyes. The mass spectra of methanolic extracts of blank paper, and TLC layer and the methanol itself are shown in Figs. 2, 3 and 4. The background (paper, CHC, and methanol)-subtracted mass spectra of Staedtler 430 M and Corvina 81 inks are shown in Figs. 5 and 6. The four mass spectra obtained for the methanolic extracts of the four TLC layers of Staedtler 430 M ink are shown in Figs. 7 through 10. Four distinctive ions, m/z 415, 443, 473 and 535 are present with both inks.

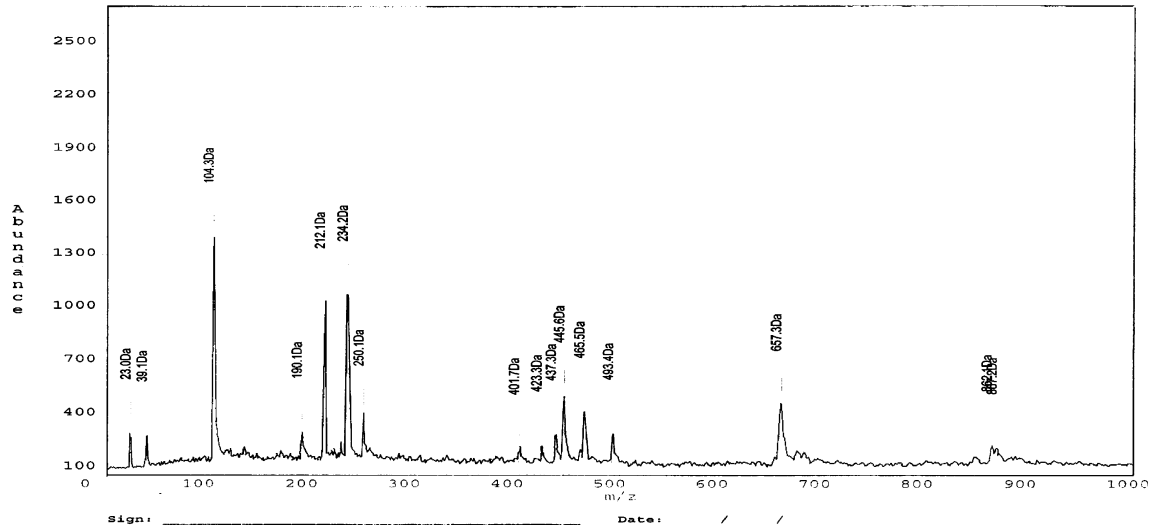

Fig.2 MALDI-TOF mass spectrum obtained for the methanolic extract of blank carbonless paper

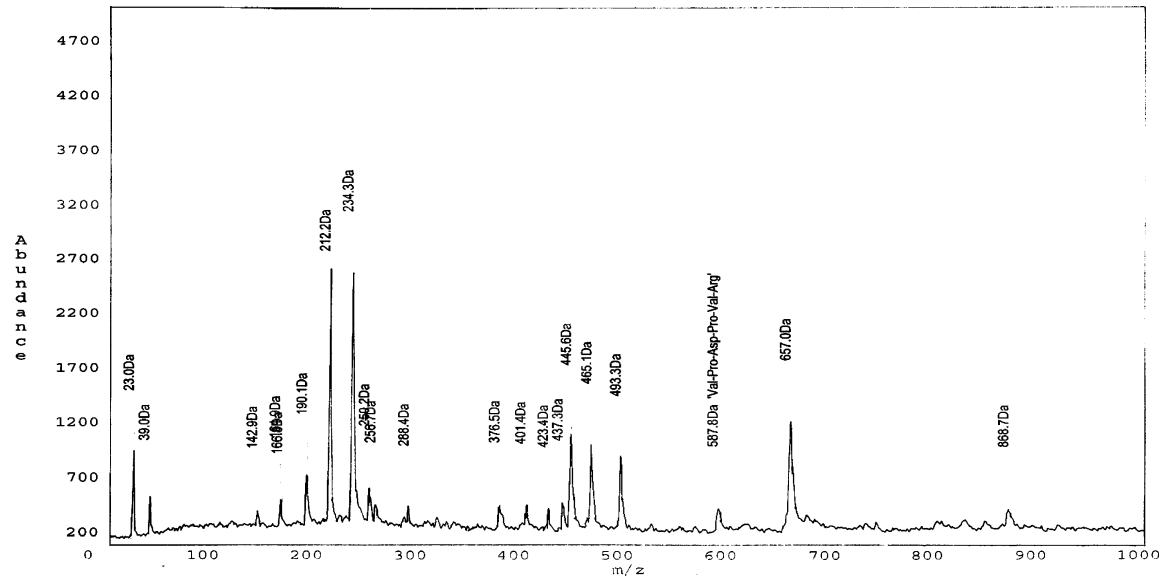

Fig.3 MALDI-TOF mass spectrum obtained for the methanolic extract of blank TLC layer

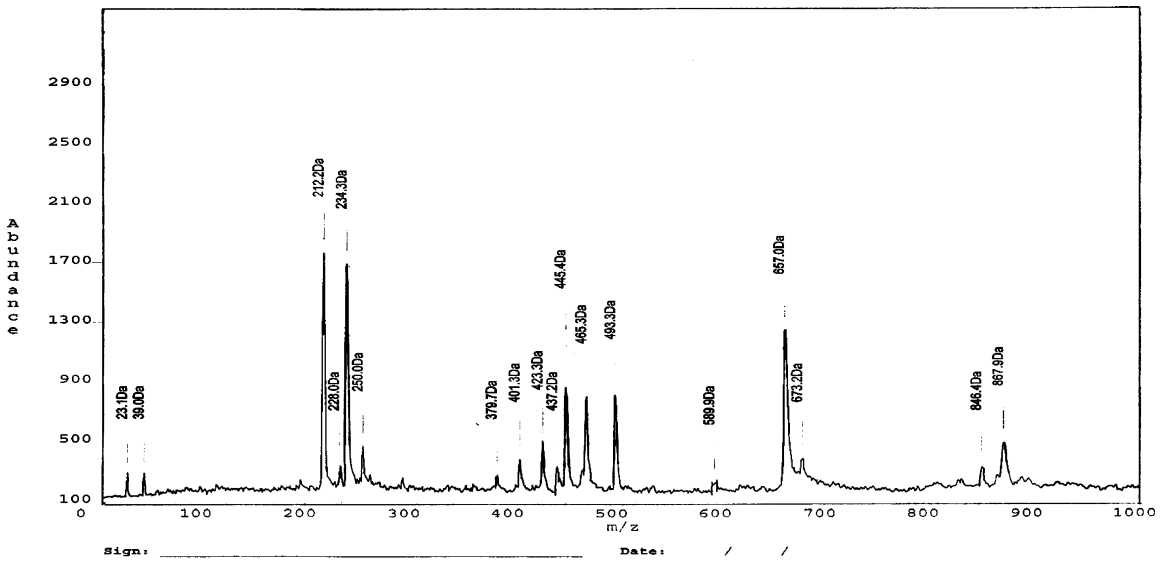

Fig.4 MALDI-TOF mass spectrum of methanol

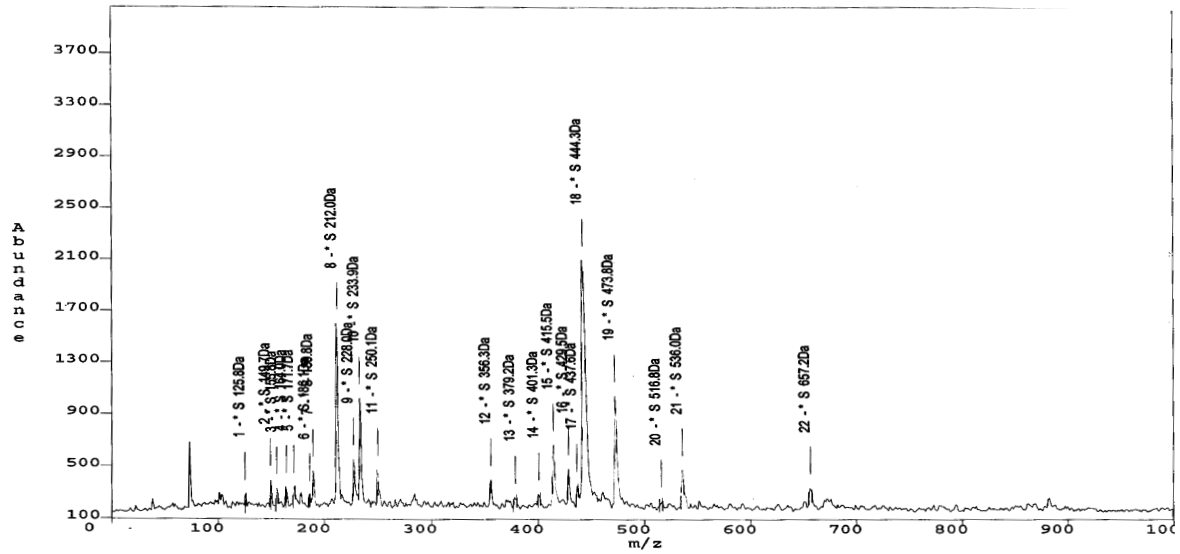

Fig.5 MALDI-TOF mass spectrum of Staedtler 430 M

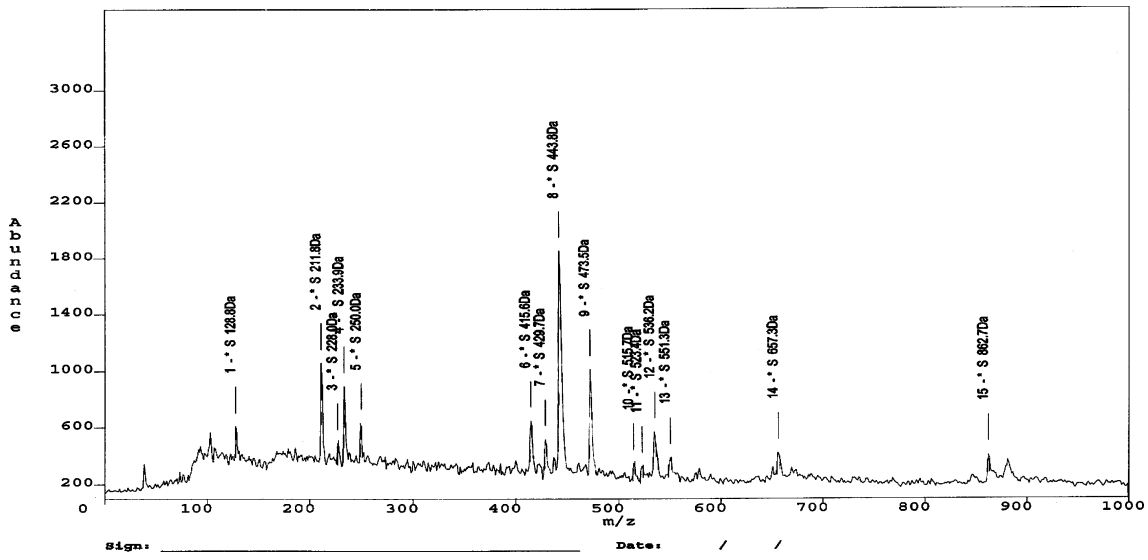

Fig.6 MALDI-TOF mass spectrum of Corvina 81 ink.

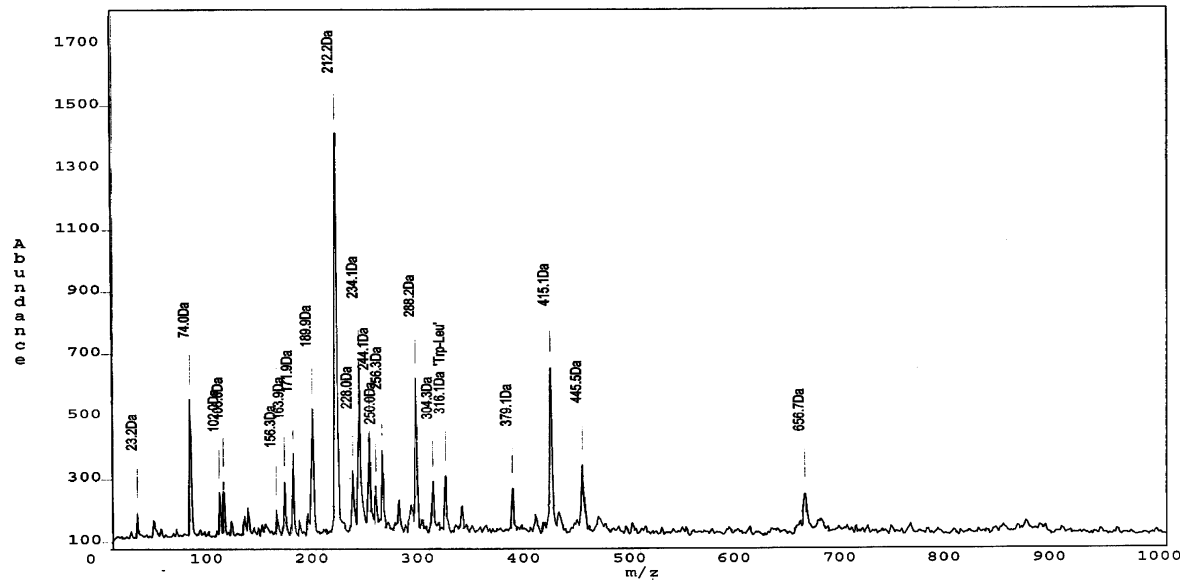

Fig.7 MALDI-TOF mass spectrum obtained for the methanolic extract of the TLC spot No. 1 of Staedtler 430 M ink.

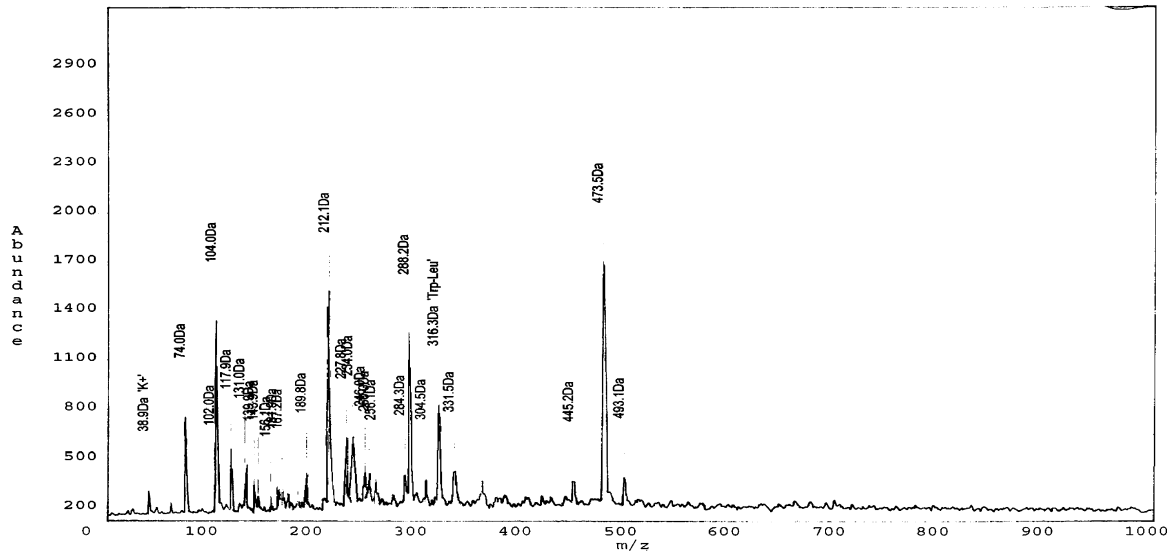

Fig.8 MALDI-TOF mass spectrum obtained for the methanolic extract of the TLC spot No.2 of Staedtler 430 M ink.

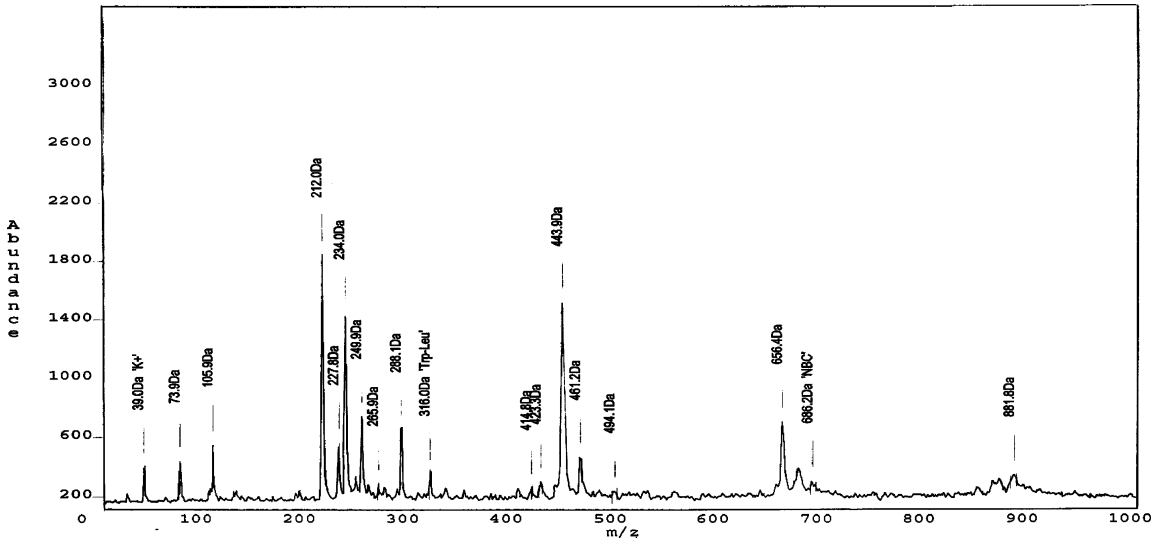

Fig.9 MALDI-TOF mass spectrum obtained for the methanolic extract of the TLC spot No.3 of Staedtler 430 M ink.

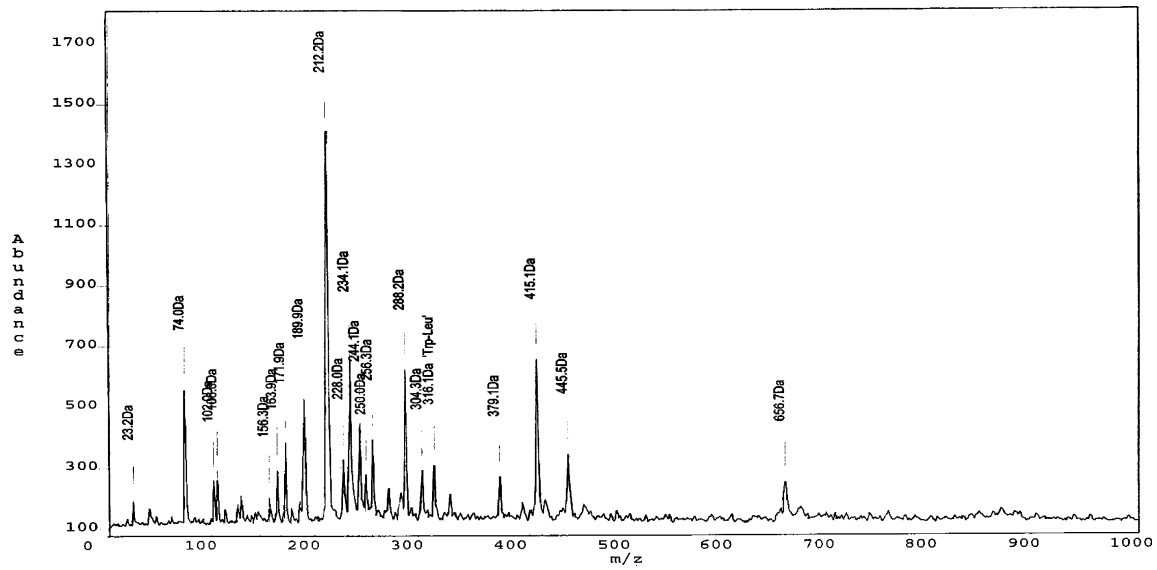

Fig.10 MALDI-TOF mass spectrum obtained for the methanolic extract of the TLC spot No.4 of Staedtler 430 M ink.

A comparison was made between the mass spectra of a methanolic extract of Staedtler 430 M ink on Chapman carbonless paper before and after TLC separation. While the chromatographed Staedtler 430 M ink gave four distinctive ion peaks of m/z 415, 473, 443, and 535, the TLC spot No. 1 for Staedtler 430 M produced a corresponding m/z 415, and spots 2 to 4, m/z 473, 443, 535, respectively.

To further verify that the four ions are the respective molecular or quasi-molecular ions of the corresponding separated components, another instrument, Waters LC mass detector employing electrospray ionization (ESI-MS), was used. The mass spectra obtained for the methonolic extracts of the TLC spots 1 through 4 of Staedtler 430 M are shown in Fig. 11. Apparently, the soft ionization technique gives specific proof of the case. Samples of Corvina 81, rhodamine 6g, Bic red , Micron red and rhodamine B were all subjected to the same analytical procedures as for Staedtler 430 M ink, and the results are listed in Table 2. Also, exemplary MALDI-TOF and ESI mass spectra of TLC spots 1 through 3 of rhodamine 6G and spots of 1 through 4 of Corvina 81 are

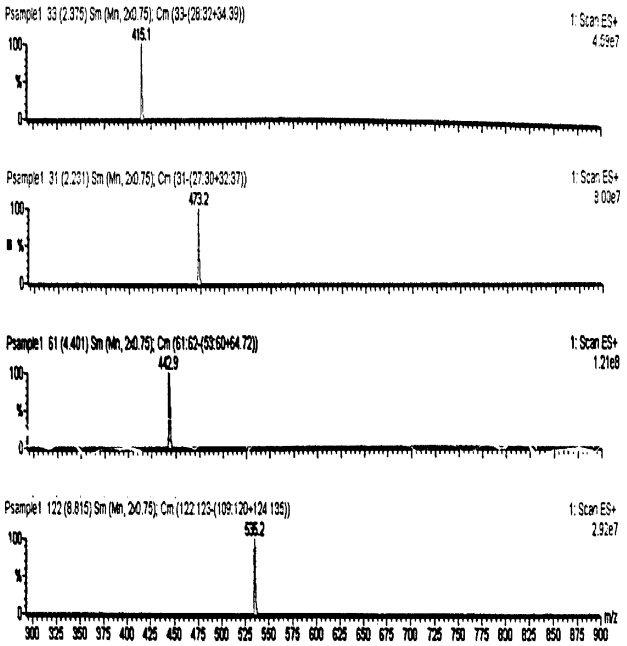

Fig.11 ESI mass spectra obtained for the methanolic extracts of the TLC spots 1, 2, 3 and 4 of Staedtler 430 M ink.

Table 2 Ions produced by MALDI-TOF mass spectrometric analyses of methanolic extracts of TLC spots of Staedtler 430 M, Corvina 81, Bic red and Micron red inks and rhodamine 6G and rhodamine B dyes

Ink or dye	Spot No. on TLC	Ions
Staedtler 430 M	1	415
	2	473
	3	443
	4	535
Corvina 81	1	415
	2	473
	3	443
	4	535
Rhodamine 6g	1	415
	2	473
	3	443
Bic red	1	415
	2	443
Micron red	1	415
	2	443
Rhodamine B	1	415
	2	443

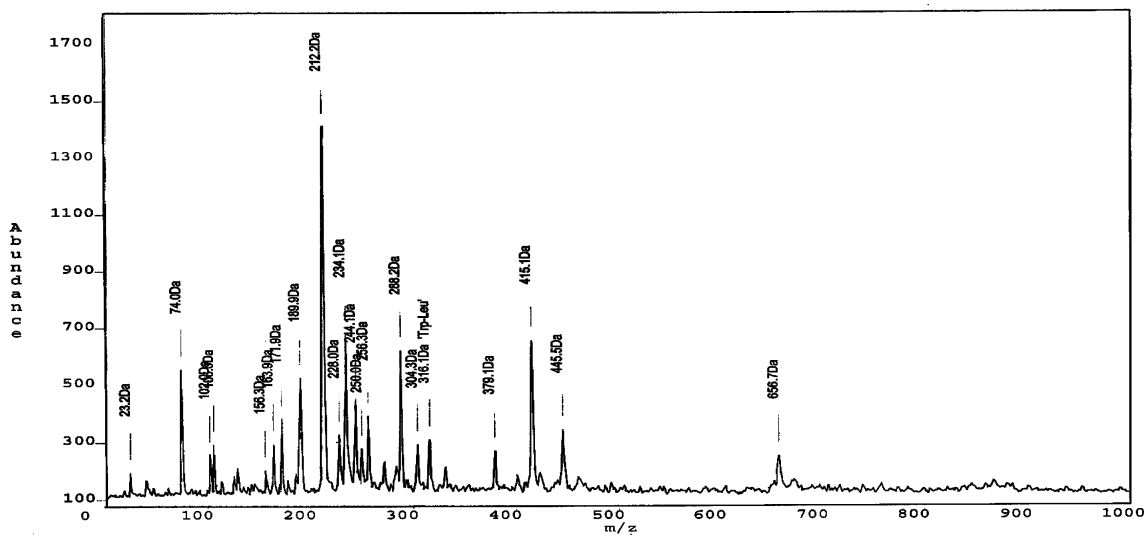

Fig.12 MALDI-TOF mass spectrum obtained for the methanolic extract of the TLC spot No.1 of rhodamine 6G dye

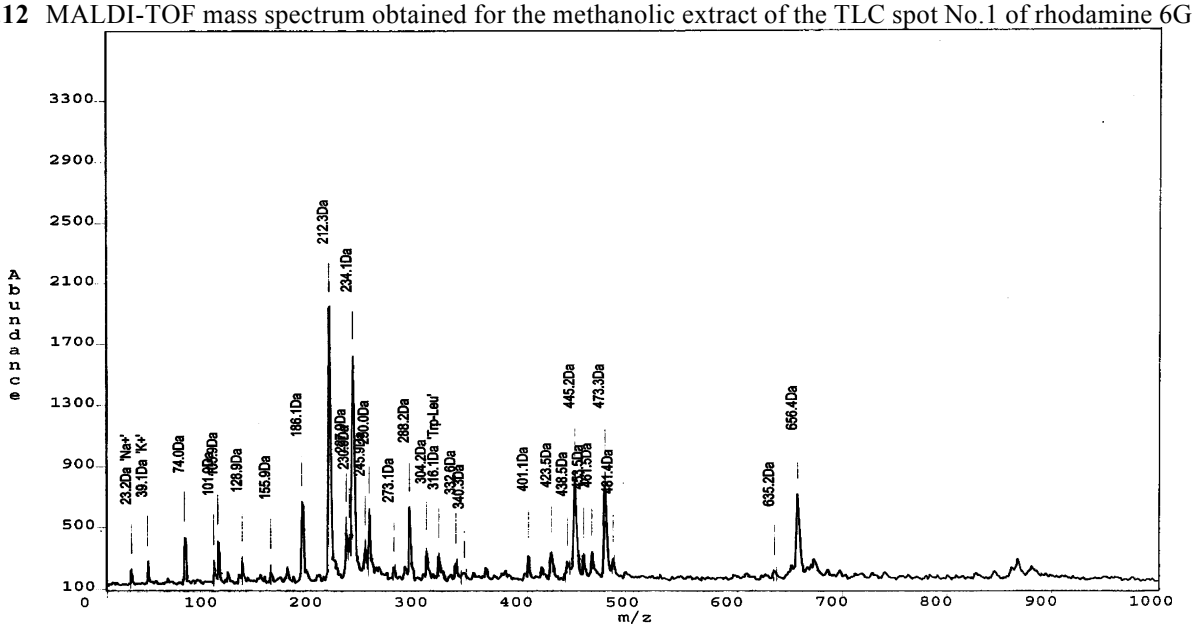

Fig.13 MALDI-TOF mass spectrum obtained for a methanolic extract of TLC spot No.2 of rhodamine 6G dye

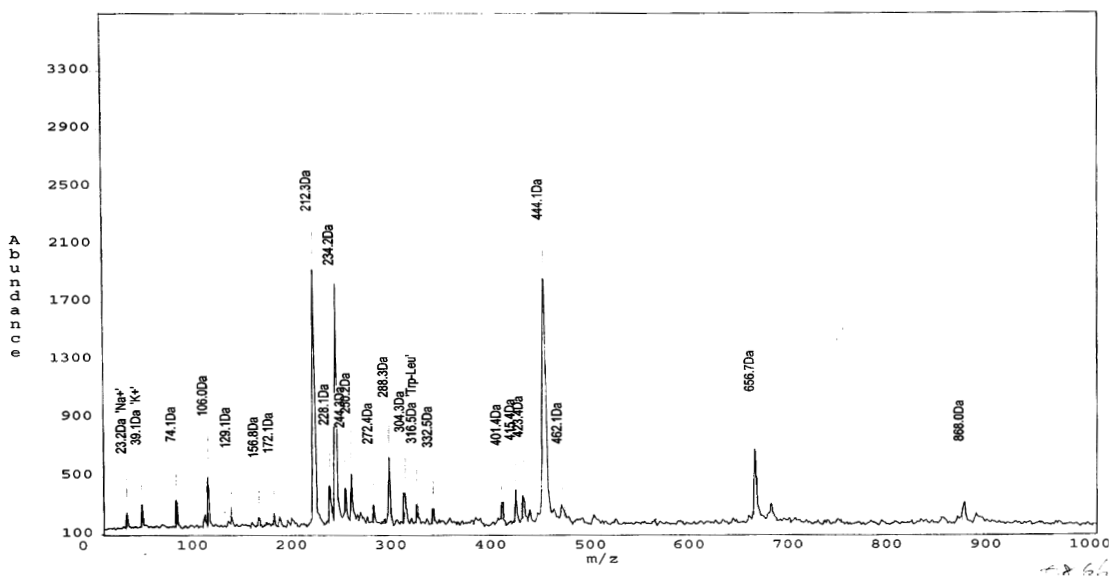

Fig.14 MALDI-TOF mass spectrum obtained for the methanolic extract of the TLC spot No.3 of rhodamine 6G dye

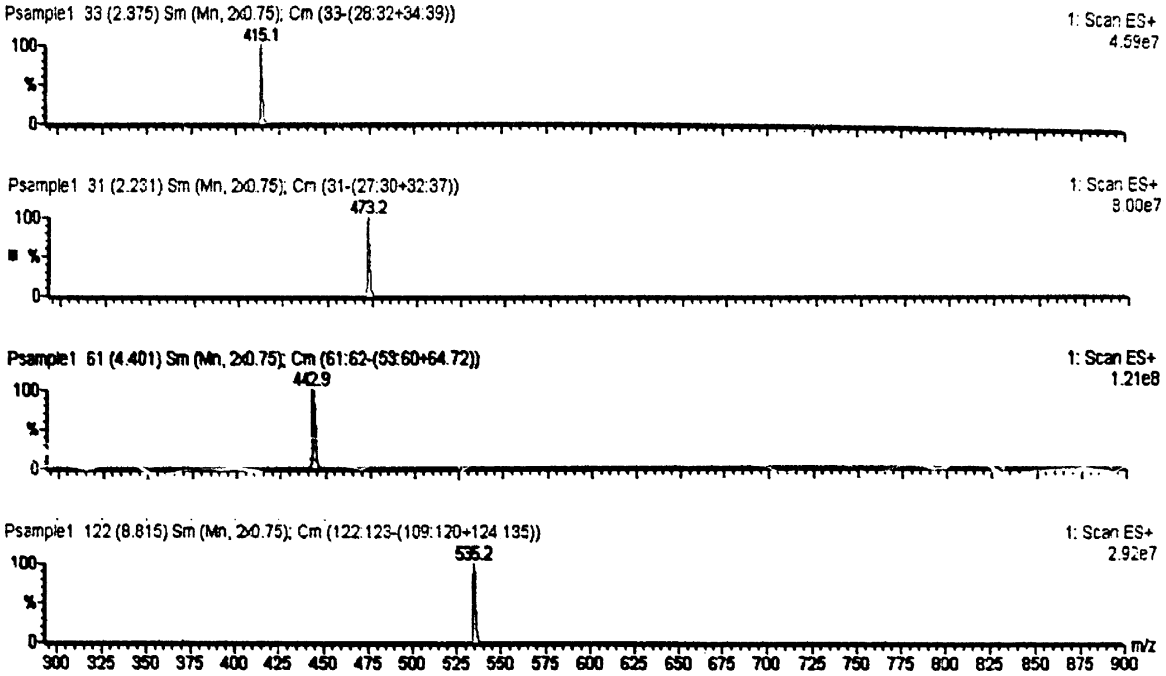

Fig.15 ESI mass spectra obtained for the methanolic extracts of the TLC spots 1, 2, 3 and 4 of Corvina 81 ink.

displayed in Figs. 12, 13, 14, 15.

The component corresponding to spot No.1 of Staedtler 430 M ink has a distinctive ion of m/z 415 [(M $-C_2H_5)^+$] where M = molecular weight of rhodamine 6G. This ion should have arised from an unalkylated component of the original rhodamine 6G. Likewise, spots 2, 3 and 4 are fully characterized by the molecular or quasi-molecular m/z 443 [$(M+H)^+$], 473 [$M+(C_2H_5)^+$], and 535 [$(M+(C_2H_5)2+Cl)^+$], respectively. For rhodamine 6G, there are three ions, m/z 415 [$(M-C_2H_5)^+$], 473 [$M+(C_2H_5)^+$] and 443 [$(M+H)+$], for spots 1, 2 and 3, respectively (M = molecular weight of rhodamine 6G). For each of Bic red, Micron red and rhodamine B, there are ions of m/z 415 [$(M-C_2H_5)^+$] and 443 [$(M+H)+$] for spots 1 and 2 respectively. The ESI mass spectra of Bic red ink and rhodamine B dye are shown in Figs. 16 and 17.

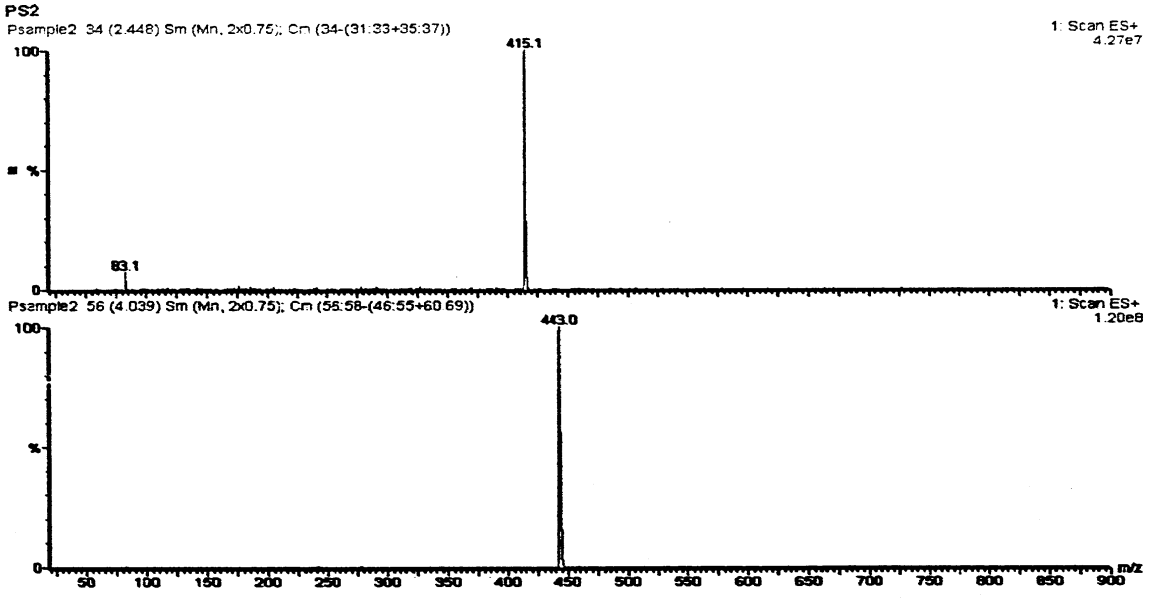

Fig.16 ESI mass spectra obtained for the methanolic extracts of the TLC spots No.1 and No. 2 of Bic red ink.

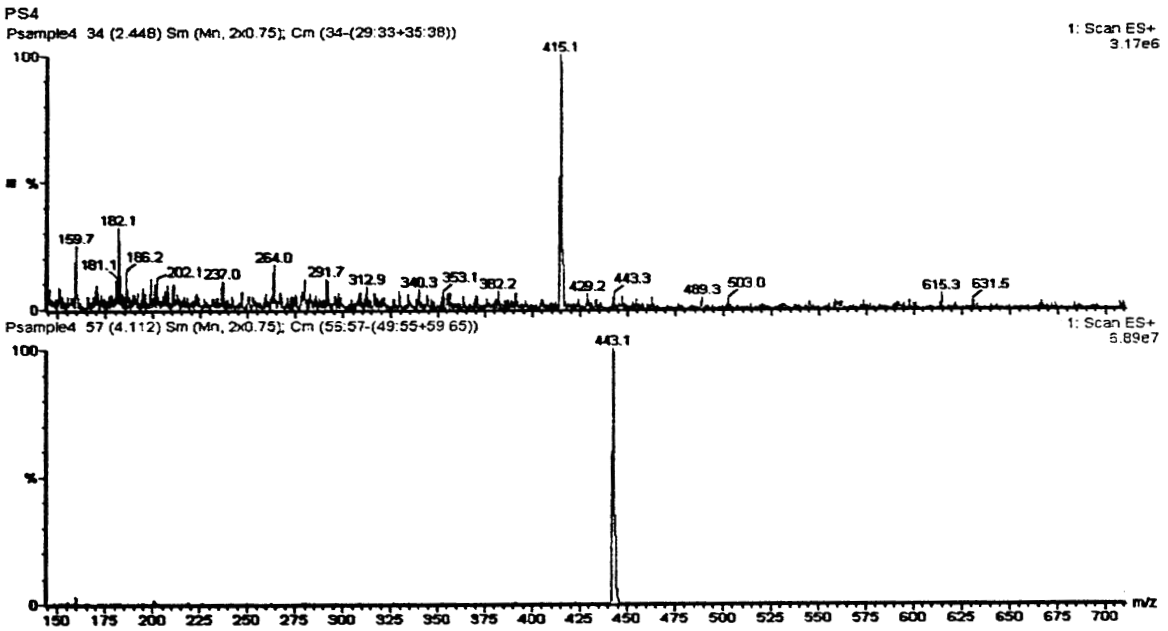

Fig.17 ESI mass spectra obtained for the methanolic extracts of the TLC spots No.1 and No.2 of rhodamine B dye

Conclusions

Based on the comparative examination of the four inks and two dyes, it is clear that all the TLC spots of equivalent Rf values are of the same compounds. Furthermore, Staedtler 430 M and Corvina 81 inks contain the same four components. Thus, the results of this study have demonstrated that TLC coupled with fluorescence spectroscopy and MALDI-TOF mass spectrometry followed by confirmatory ESI mass spectrometry is a useful and reliable analytical scheme for the evidential characterization and identification of ballpoint pen inks and dyes.

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