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Article

Fragmentation Reactions of Rhodamine B and 6G as Revealed by High Accuracy Orbitrap Tandem Mass Spectrometry

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Correct interpretation of mass spectra is essential to our understanding of the ion chemistry occurring in the gas phase inside mass spectrometers and for constructing a solid knowledge from which mass spectrometry (MS) data of novel molecules will be interpreted. Assignments of product ions leading to incorrect dissociation mechanisms can also be dangerous in several disciplines such as in forensic chemistry and clinical diagnostics. Main fragmentation routes for rhodamines B and 6G were investigated by high accuracy Orbitrap tandem mass spectrometry (MS/MS). Isobars were properly resolved and molecular formulas were correctly attributed to all major product ions. This proper evaluation of ions composition and formula allowed us to propose a detailed mechanism for their dissociation. A comprehensive mechanistic fragmentation is proposed for rhodamine B and 6G using product ion assignments resulting from high resolution and accuracy tandem MS data, which should serve as a guide for MS and MS/MS data interpretation of homologues molecules.

Keywords: xanthene dyes, reaction pathway, ESI mass spectra

Introduction

Mass spectrometry (MS) is a technique mainly based on the measurement of the masses of gaseous ions. If this measurement is done with sufficient accuracy, the ion molecular formula can be unequivocally attributed, but the connectivity of such ions cannot be determined from the molecular mass alone. To get information for the actual ion structure, MS investigations must rely on the dissociation chemistry of selected ions via tandem mass spectrometry (MS/MS). When this type of dissociation is investigated, it is therefore essential to use proper resolution and accuracy to be able to resolve isobars and to correctly assign masses for all fragment ions. Unfortunately, low resolution and accuracy tandem MS data had been often used to interpret MS and MS/MS data. Incorrect product ion composition and formulas are therefore sometimes obtained and used to construct dissociation mechanisms. These assignments can be particularly disastrous in some areas where false negatives or positives should not be tolerated such as in forensic chemistry and clinical analysis. An incorrect dissociation scheme can also be propagated leading to improper interpretations of the dissociation chemistry of similar or homologues molecules.[1]

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Xanthene dyes represent a class of important organic compounds and are extensively employed.[2] Rhodamines (Figure 1), such as rhodamine B and 6G and their derivatives, due to their unique photophysical[3] and photostability[4] properties, are among the oldest and most commonly used synthetic dyes.[5] Rhodamine B is used widely as a colorant in textiles and in the plastic industries, and is also a well-known fluorescent dye applied in organic chemistry and biological studies.[6] Rhodamine 6G is also widely used as a fluorescence tracer[7] and as a lasing[8] medium. These commercially available rhodamines are also used as a security feature in automatic teller machines (ATM's) to deter bank robberies.[9] These dyes are also illegally used by sweet markets or bakers for coloring different confectionery.[10]

MS is commonly used for rhodamine detection particularly due to its high sensitivity, selectivity and speed. Unfortunately, many contrasting interpretations for the ion chemistry of the cationic rhodamine B have been proposed.[11] Lipson and co-workers[12] have studied the rhodamine 610's fragmentation using visible matrix-assisted laser desorption ionization, suggesting that the degree of fragmentation is inversely proportional to the strength of the contact ion pair in the solid state. Similar results were also reported for salts of rhodamine 6G. In 1984, Brown *et al.*[13] reported the first investigation on the

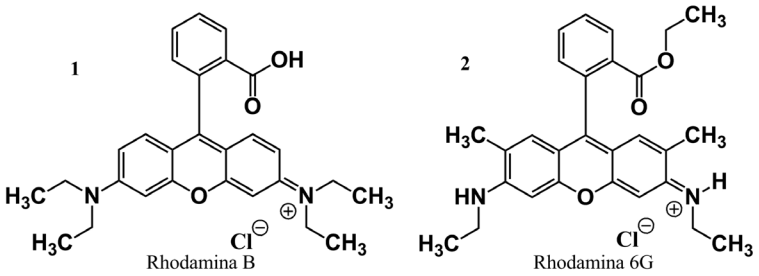


Figure 1. Chemical structures of rhodamine B and 6G.

fragmentation behavior of rhodamine B by means of fast atom bombardment (FAB) MS. Two years later, Ballard and Betowski[14] reported the MS/MS collision-induced dissociation (CID) of the cationic rhodamine B using thermospray triple quadrupole MS. The authors found a double loss of 44 Da as the main dissociation channel and attributed the first 44 Da loss to propane (C_3H_8) and the second to CO_2. However, it should be noted that the loss of 44 Da from the rhodamine B cation structure could also be attributed to either elimination of CO_2 or C_2H_6N molecules. A mechanism for rhodamine B dissociation was not discussed. In 2011, Peters and Grotemeyer[15] studied the fragmentation of xanthene dyes by means of Fourier transform ion cyclotron resonance MS (FT-ICR-MS). Fragmentation of the electrosprayed cations of rhodamine B was induced by laser photodissociation (laser PD) or SORI CID (sustained off-resonance irradiation collision-induced dissociation). But now the two 44 Da losses were both attributed to propane (C_3H_8) via either a concerted or a radicalar mechanism (Scheme 1).

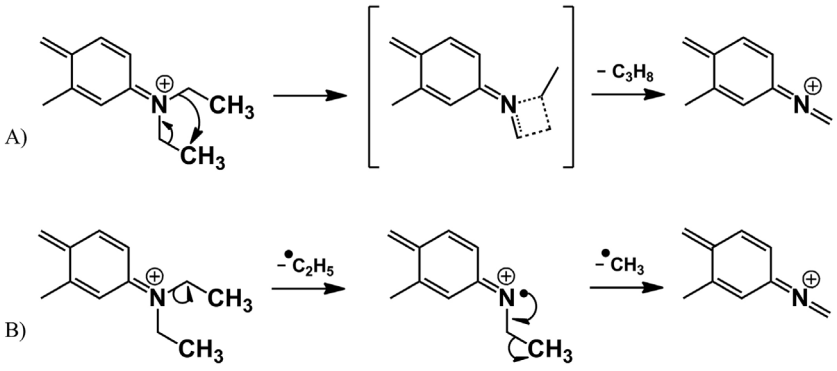
Peters *et al.*[16] investigated the dissociation of rhodamine B using laser and collisional activation in an FT-ICR-MS. By D-labeling, they determined that the double 44 Da loss involves indeed the exocyclic NEt_2 group of rhodamine B. From high accuracy *m/z* measurements, they attributed the two sequential 44 Da losses to propane, favoring a concerted mechanism since no radical loss fragment ions are detected. Furthermore, using different isotopologues, they observed lack of fragment ions which incorporate both ethylated amine groups. Wang *et al.*[17] also investigated the fragmentation of the cationic rhodamine B by electrospray ionization-ion trap MS (ESI-MS) and also interpreted the double 44 Da loss to propane, whereas a third sequential 44 Da loss was attributed to CO_2.

In view of these contrasting interpretations, we have therefore performed a comprehensive study to settle the dissociation chemistry for both cationic rhodamine B and its isomer protonated rhodamine 6G molecule using 140k of resolution and 1 ppm accuracy tandem mass spectrometry in an orbitrap mass analyzer. A revised MS/MS fragmentation mechanism for these dyes is therefore proposed.

Experimental

Materials and samples

High performance liquid chromatography (HPLC)-grade methanol was purchased from Burdick & Jackson (Muskegon, MI). Rhodamine B and 6G were purchased from Sigma-Aldrich, Brazil Ltda, at purities of 95%. Rhodamine B and 6G were diluted in methanol and injected with 3.0 μL min^{-1} of flow rate on Q-Exactive™ (Thermo Scientific, Germany), mass spectrometer with orbitrap analyzer, via ESI, both experiments were performed in the positive mode. For collision was applied high collision dissociation (HCD) 45.00-55.00 of energy.



Scheme 1. Mechanistic proposal: (A) concerted step; (B) radical step (adapted from reference 15).

Results and Discussion

Figure 2 shows the MS/MS data for the precursor ion of rhodamine B cation of *m/z* 443. Note the most abundant ion of *m/z* 399 and a series of other characteristic fragment ions at medium abundances.

Although isobars have never been considered or detected, enlargements have shown that indeed two main fragment ions (Figure 3) of cationic rhodamine B are in fact composed of isobaric mixtures.

Table 1 shows the accurate *m/z* values and the correct assignments for the losses and fragment ions of the rhodamine B cation. Note that the first 44 Da loss is indeed found to correspond exclusively to propane. But for the second 44 Da loss leads to the product ion of nominal *m/z* 355, an isobaric mixture composed of two product ions at *m/z* 355.10684 and 355.17929 (Figure 3b). This second loss was attributed in fact to either loss of CO_2 and/or C_3H_8 units. These two losses correspond to an *m/z* difference of only 0.07245 Da, and indeed MS data obtained with resolutions lower than 70k would not resolve these isobars, which explains the failure of some previous MS studies to resolve the mixture. Another isobaric mixture was found for the product ion having a nominal value *m/z* 371 and it was composed of product ion being formed by losses of a $CH_2N^{\cdot}$ radical (*m/z* 371.15054) or an ethylene (C_2H_4) molecule (*m/z* 371.13821) (Figure 3a).

Figure 4 now shows the ESI(+)-HCD-MS/MS of protonated rhodamine 6G, whereas Table 2 summarizes the high accuracy data and interpretation of its major product ions. Please note that as the protonated rhodamine 6G in which the exocyclic N contains a single ethyl group, therefore it is not possible loss of propane, but that of ethane. Accordingly, the product ion with a nominal mass *m/z* 415 is an isobaric mixture containing two product ions formed by loss of either OEt and/or NEt groups, or both (Scheme 2).

Although of low abundance, a typical fragment ion for rhodamine 6G demonstrates the imperious need to

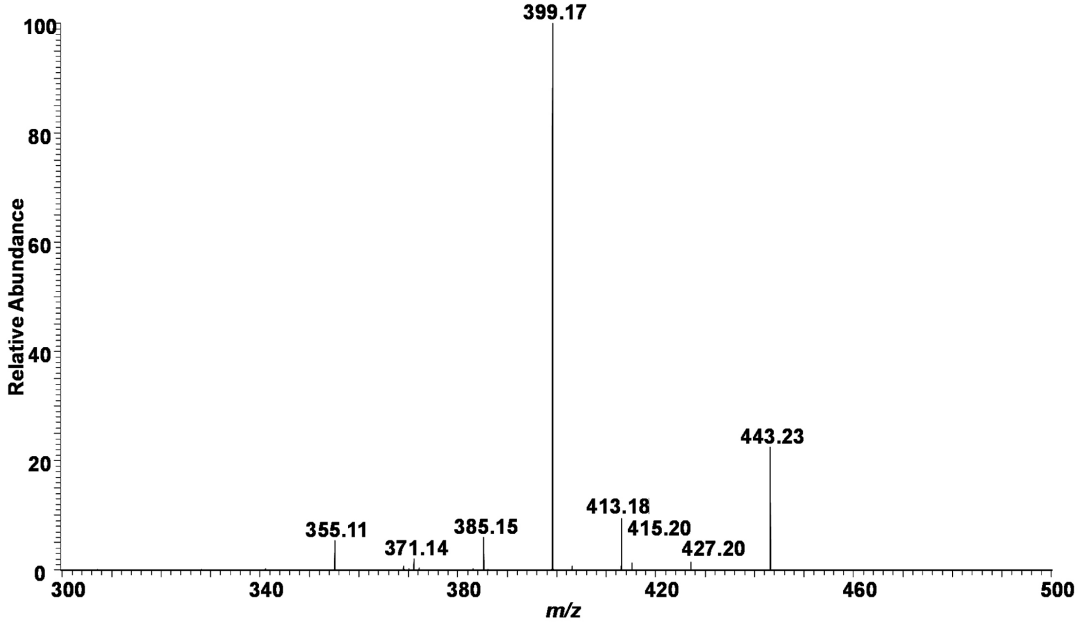


Figure 2. ESI(+)-MS/MS of the cationic rhodamine B.

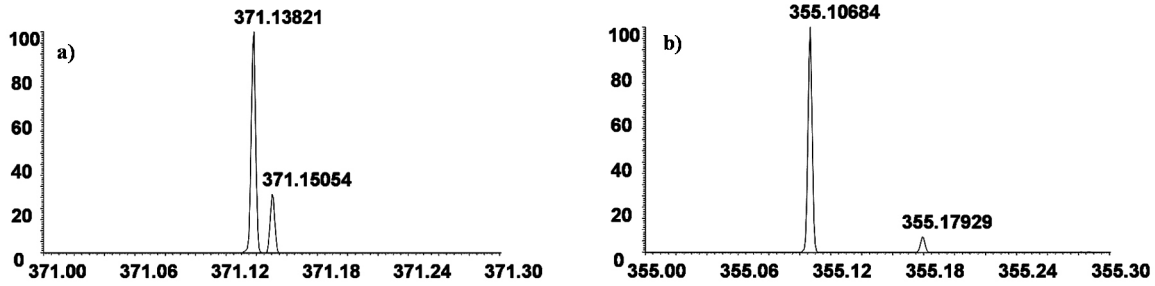


Figure 3. Expanded MS/MS data showing the isobaric composition of the rhodamine B fragment ions of nominal *m/z* 371 and 355.

Table 1. High accuracy data and interpretation for the fragment ions of cationic rhodamine B

Precursor ion	Product ion	Intensity (TIC)	Mass difference	Loss	Mechanism fragmentation	Theoretical mass	Error (Da)
443.23190	442.22420	2.00e3	1.00770	H	radical	1.00783	0.00013
443.23190	428.20842	4.16e3	15.02333	CH_3	radical	15.02348	0.00015
443.23190	427.20050	7.54e5	16.03140	CH_4	radical	16.03121	–0.00019
443.23190	415.20038	7.73e5	28.03152	C_2H_4	neutral loss	28.03130	–0.00022
443.23190	414.19299	2.54e3	29.03891	C_2H_5	radical	29.03913	0.00022
443.23190	413.18478	4.72e6	30.04712	C_2H_6	radical	30.04695	–0.00017
443.23190	399.16940	4.78e7	44.06250	C_3H_8	nadical	44.06260	0.00010
413.18478	385.15344	2.58e6	28.03134	C_2H_4	neutral loss	28.03130	–0.00004
399.16919	371.15054	2.78e5	28.01865	CH_2N	radical	28.01872	0.00007
399.16940	371.13821	5.97e5	28.03119	C_2H_4	neutral loss	28.03130	0.00011
399.16940	355.17929	1.33e5	43.99011	CO_2	neutral loss	43.98983	–0.00028
399.16940	355.10684	1.87e6	44.06256	C_3H_8	radical	44.06260	0.00004

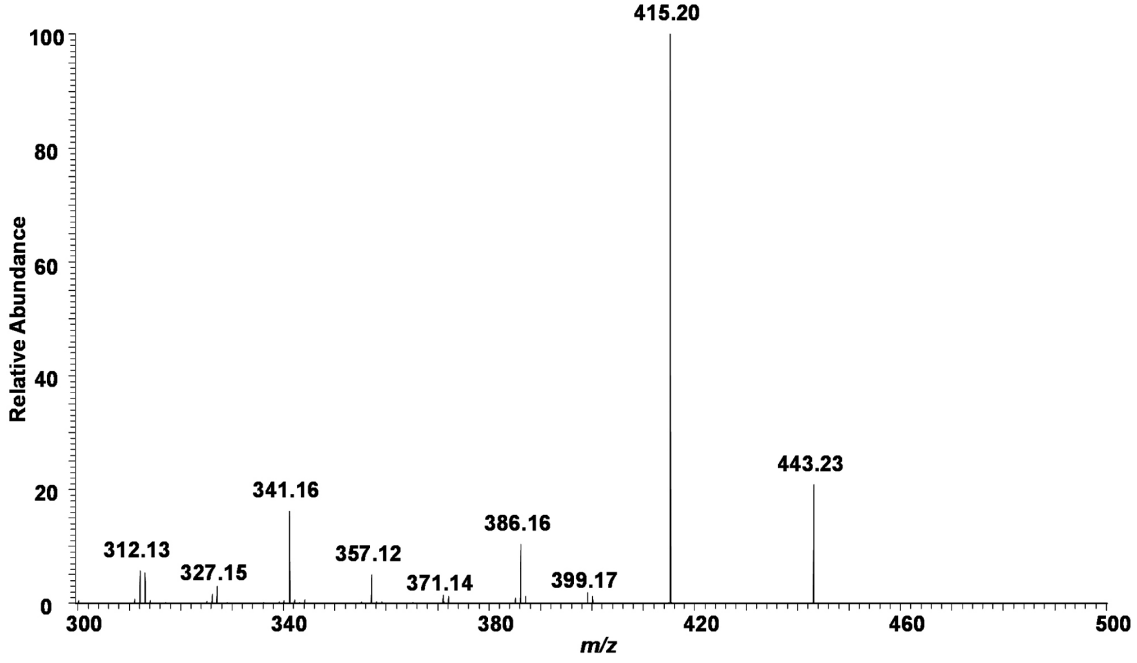


Figure 4. ESI(+)-MS/MS of rhodamine 6G.

have enough accuracy and resolution to properly interpret fragmentation routes in that of nominal *m/z* 371 (Figure 5). Note that, in fact, it is composed of three isobars of *m/z* 371.13880, 371.15080 and 371.21131, which leads to different compositions: $C_{25}H_{27}N_2O$, $C_{24}H_{21}NO_3$, and $C_{23}H_{19}N_2O_3$, respectively. Therefore, three different mechanisms of fragmentation starting from the parent ion must be involved. These isobaric ions should therefore be formed by initial losses of either CH_3· or CH_2N· radicals or a neutral CO_2 (Table 2 and Schemes 2 and 3).

In all, the high resolution and accuracy Orbitrap MS/MS data summarized in Tables 1 and 2 allowed us to propose

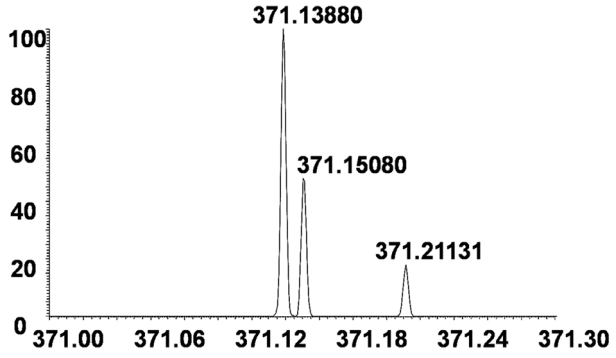


Figure 5. Expanded MS/MS for the ion of *m/z* 317 from protonated rhodamine 6G.

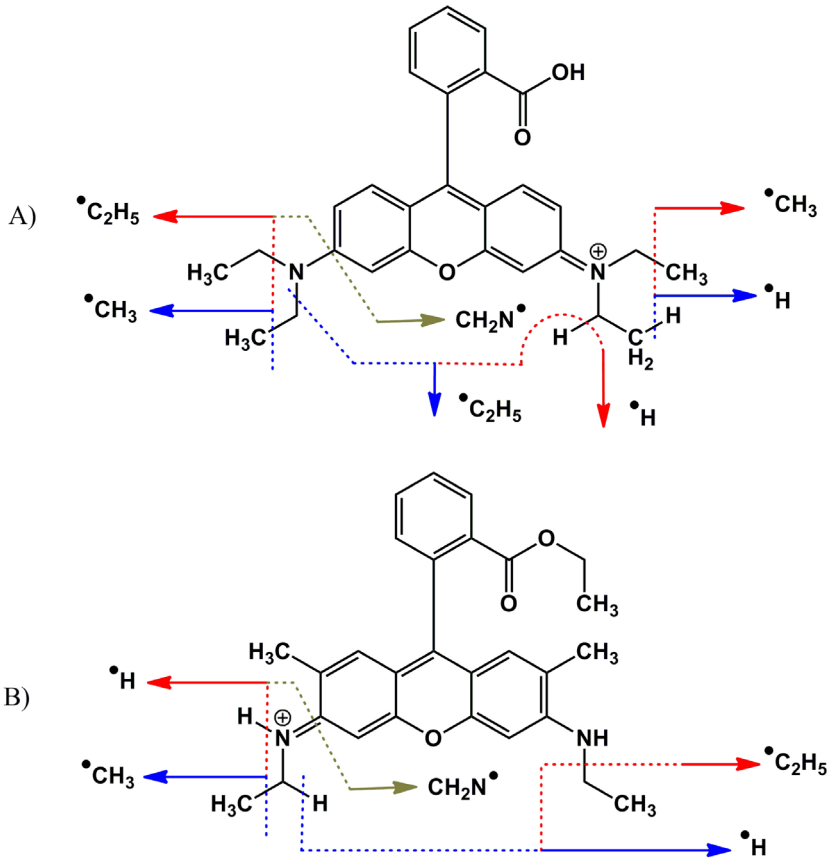
Table 2. High accuracy data and interpretation for the fragment ions of cationic rhodamine 6G

Precursor ion	Product ion	Intensity (TIC)	Mass difference	Loss	Mechanism fragmentation	Theoretical mass	Error
443.23223	415.20109	7.96e8	28.03114	C_2H_4	neutral loss	28.03130	0.00016
415.20109	400.17779	9.69e6	15.02330	CH_3	radical	15.02348	0.00018
415.20109	399.16980	1.53e7	16.03129	CH_4	radical	16.03121	–0.00008
415.20109	386.16200	8.31e7	29.03909	C_2H_5	radical	29.03913	0.00004
415.20109	385.15423	7.58e6	30.04686	C_2H_6	radical	30.04695	0.00009
415.20109	371.21131	2.09e6	43.98978	CO_2	neutral loss	43.98983	0.00005
399.16980	371.15080	5.61e6	28.01900	CH_2N	radical	28.01872	–0.00028
386.16200	371.13880	1.17e7	15.02320	CH_3	radical	15.02348	0.00028
385.15423	357.12295	4.05e7	28.03128	C_2H_4	neutral loss	28.03130	0.00002
385.15423	341.16454	1.30e8	43.98969	CO_2	neutral loss	43.98983	0.00014
371.13880	327.14885	2.44e7	43.98995	CO_2	neutral loss	43.98983	–0.00012
341.16454	312.12558	4.55e7	29.03896	C_2H_5	radical	29.03913	0.00017

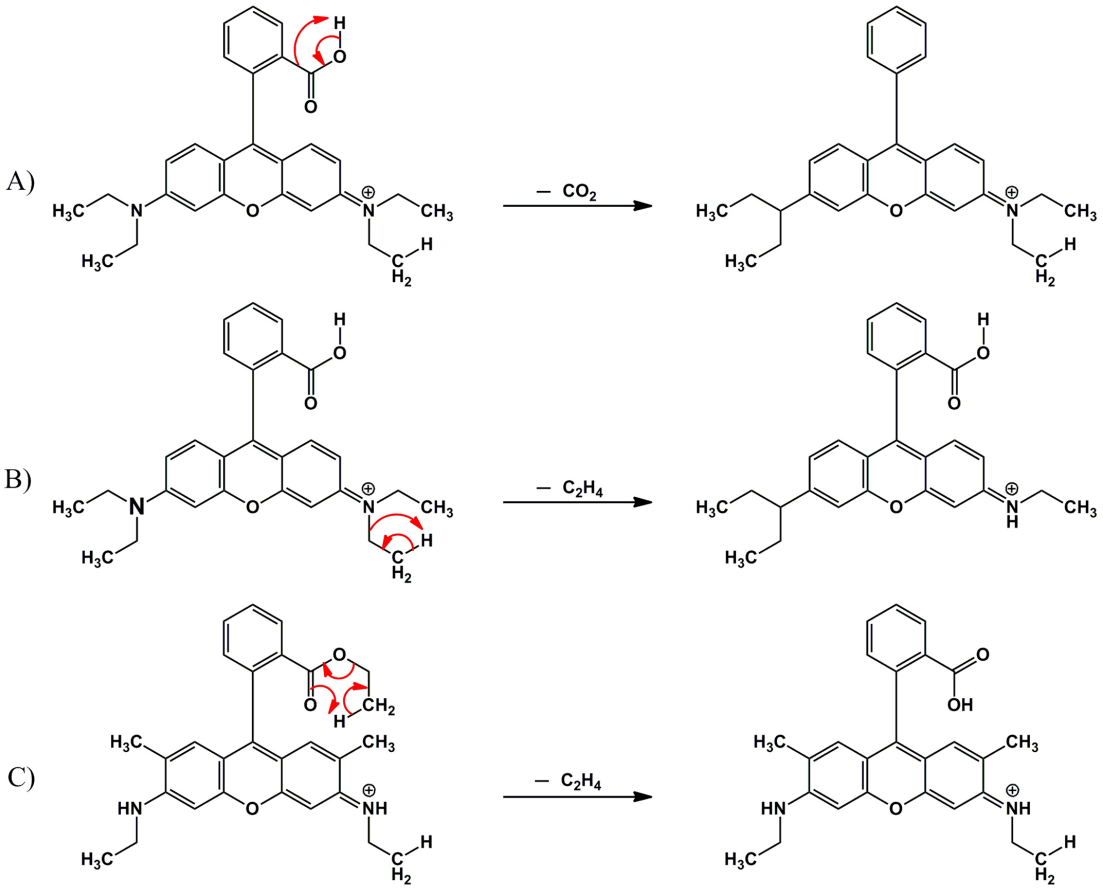
detailed fragmentation mechanisms for both isomers, summarized in terms of bond breaking and radical species in Scheme 2. For the neutral losses, the mechanisms are proposed in Scheme 3.

Conclusions

Using therefore proper high resolution and high accuracy MS/MS data, correct assignments of all fragment ions



Scheme 2. Fragmentation routes eliminating radical species for (A) cationic rhodamine B; and (B) protonated rhodamine 6G. The color blue represented the first fragmentation, red second fragmentation and green third fragmentation sequential.



Scheme 3. Proposed mechanisms for the neutral losses for (A,B) cationic rhodamine B and (B,C) protonated rhodamine 6G.

from cationic rhodamine B and its isomeric protonated rhodamine 6G were made. Several fragmentation ions were found to be composed in fact by a mixture of isobaric ions, from which previous work based on low resolution or low accuracy MS/MS data failed to resolve and therefore to correctly interpret. But when the correct isobaric composition is determined and correct formula are assigned, previous misinterpretations could be corrected and a detailed fragmentation mechanism could be proposed for two important molecules widely used in forensic and food chemistry.

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