
Reduction of Cationic Free-Base *meso*-Tris-*N*-Methylpyridinium-4-yl Porphyrins in Positive Mode Electrospray Ionization Mass Spectrometry

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Reductions involving more than one electron with formation of the M^+ and $[M+2H]^+$ ions were observed for electrosprayed *meso*-tris(*N*-methylpyridinium-4-yl)porphyrin iodides, MI_3 . These reductions were studied by using different solvents and flow rates. Formation of the $[M+2H]^+$ ions occurred only for protic solvents and to a larger extent at lower flow rates. The type of the fourth substituent does not seem to affect the reduction processes. Formation of the two reduced species, M^+ and $[M+2H]^+$ ions, may occur through the participation of counter ion/solvent clusters. Reduction of multiply charged, non-metallated species with formation of $[M+nH]^+$ ions ($n > 1$) was not observed before in positive mode electrospray mass spectrometry. (J Am Soc Mass Spectrom 2007, 18, 762–768) © 2007 American Society for Mass Spectrometry

The electrolytic nature of some processes occurring in electrospray interfaces was postulated a decade ago [1, 2] and, notwithstanding some controversy [3], it is generally accepted that redox reactions do occur in the capillary sprayers and that they are responsible for charge balance [1, 2]. Redox processes in electrospray mass spectrometry were comprehensively investigated by Van Berkel and collaborators [4–8] and, more recently, electrochemical aspects and applications of electrospray ionization were the focus of reviews [9, 10].

Ions formed by one electron reduction from non-metallated dicationic species in solution were previously observed in positive-mode electrospray mass spectrometry for ionic compounds such as diquaternary pyridinium salts [11–13] and free-base *N*-alkylpyridiniumyl porphyrin salts [14]. One- and two-electron reductions of the cations of metallated *N*-alkylpyridiniumyl porphyrin salts were also reported, when electrospray was used in the positive mode [15–17].

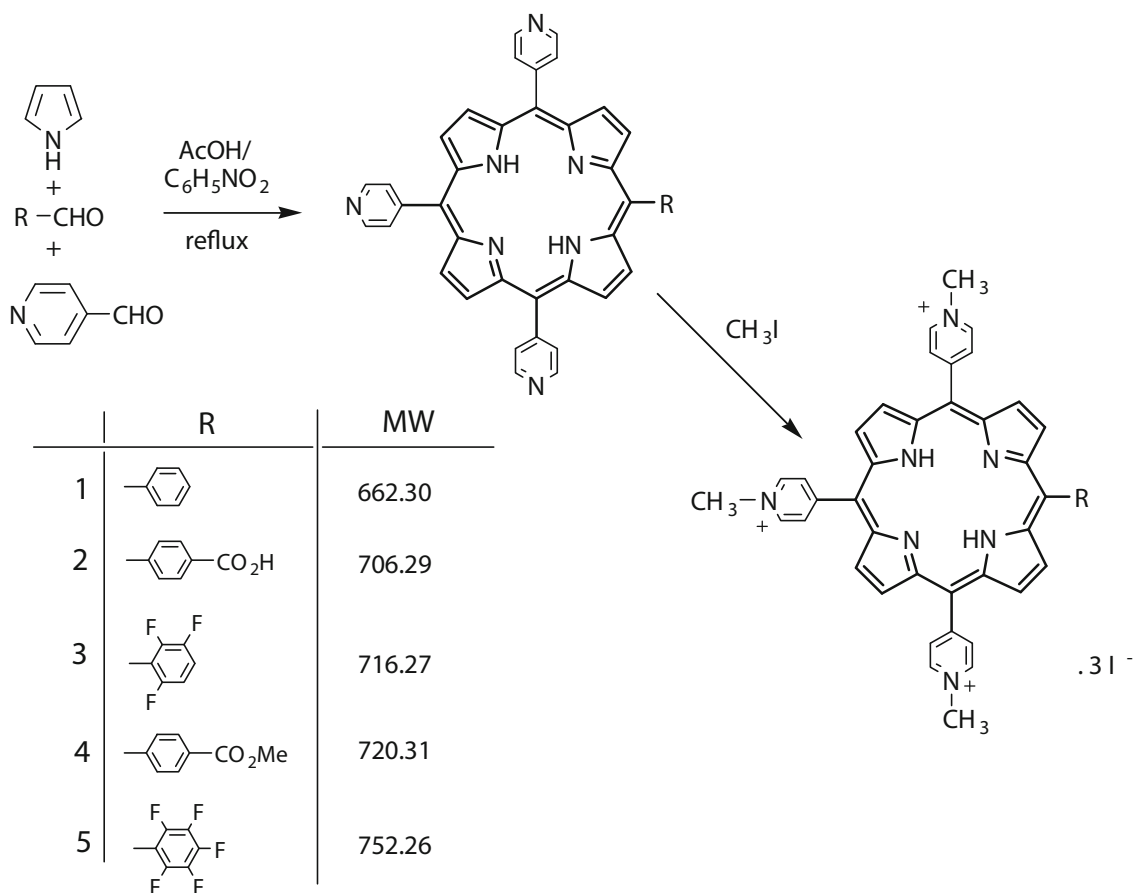
When acquiring the electrospray mass spectra of 5,10,15-*tris*(*N*-methyl-pyridinium-4-yl)-20-phenylporphyrin iodide, MI_3 , we observed, besides the expected M^{3+} ion, ions such as M^{2+} , M^+ , and $[M+2H]^+$, which may be the result of reductive processes. Reductions involving more than one electron were never reported before for electrosprayed free-base cationic porphyrins and, although formation of $[M+nH]^+$ ions ($n > 1$) in desorption ionization (fast atom or ion bombardment) [18–20] and matrix assisted laser desorption (MALDI) [21] has been described, it was never reported as occurring in electrospray mass spectrometry (ESI-MS) until now. These facts prompted us to use ESI-MS to investigate a group of *meso*-tris(*N*-methylpyridinium-4-yl)porphyrin iodides, MI_3 , with a different fourth *meso* substituent (see Scheme 1) to gather more information on the species formed with respect to their formation reactions and to their relation with the ionization processes occurring in the electrospray interface.

Experimental

The key neutral porphyrin derivatives were obtained from crossed-Rothemund reactions using the appropriate benzaldehydes and pyrrole in refluxing acetic acid and nitrobenzene [22–24]. The alkylation with methyl

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Scheme 1

iodide in dry DMF (dimethylformamide) gave the expected tris(*N*-methylpyridinium)porphyrin salts **1–5** in roughly 95% yield (Scheme 1) [23].

The porphyrin iodides were characterized by nuclear magnetic resonance (NMR) ^1H , ^{19}F , and ^{13}C , elemental analysis, and ultraviolet–visible (UV–Vis) spectroscopy.

UV–Vis absorption spectra were obtained using a Uvikon 922 spectrophotometer (Kontron Instruments Ltd., Hertfordshire, UK) and scanning from 350 to 800 nm; a 1-cm path length cuvette was used in all experiments. For the UV–Vis measurements, solutions about 10^{-5} M of the porphyrins in methanol were used.

ESI mass spectra were acquired with a Micromass Q-ToF 2 (Micromass, Manchester, UK), operating in the positive mode, equipped with a Z-spray source, an electrospray probe, and a syringe pump. Source and desolvation temperatures were 80 and 150 °C, respectively. Capillary voltage was 3000 V. The spectra were acquired at a nominal resolution of 9000 and at cone voltages between 30 and 50 V (mostly at 30 V). Nebulization and collision gases were N_2 and Ar, respectively. Porphyrin solutions were prepared at about 0.86, 1.7, and 3.5 μM concentrations in a variety of solvents (methanol, 1% vol/vol *N,N* dimethylaniline in methanol, acetonitrile, and D_4 -methanol). Sample flow rates were 5, 7.5, and 10 $\mu\text{L min}^{-1}$, as indicated. Percentages

of the total ion current (%TIC) for selected ions of the spectra were determined by averaging the spectra for 0.8 to 2 min of the acquisition time, after sample injection. The pusher frequency recorded was 22,727 Hz.

Product-ion spectra were acquired by selecting the precursor ions with the quadrupole and using the hexapole as collision cell with energies from 20 to 50 eV.

Results and Discussion

Electrospray Mass Spectrometry Using Methanol

ESI mass spectra. In the ESI mass spectra of all the *meso*-tris(*N*-methylpyridinium-4-yl)porphyrin iodide, MI_3 , peaks corresponding to the M^{3+} , M^{2+} , and $[\text{M}-\text{H}]^{2+}$ ions are observed. For higher concentrations and higher flow rates the base peaks correspond to the M^{3+} ions; the second most abundant ions are the M^{2+} ions. Singly charged ions corresponding to M^+ and $[\text{M}+2\text{H}]^+$ are also observed. Peaks corresponding to the formation of ion clusters, such as $[\text{M}^{3+}+2\text{I}]^+$ and $[\text{M}^{3+}+\text{I}]^+$, are not observed. In Figure 1 the mass spectrum of compound **1** is shown (concentration 3.5×10^{-6} M, flow rate 10 $\mu\text{L min}^{-1}$). The experimental and calculated isotopic patterns of the M^+ and $[\text{M}+2\text{H}]^+$

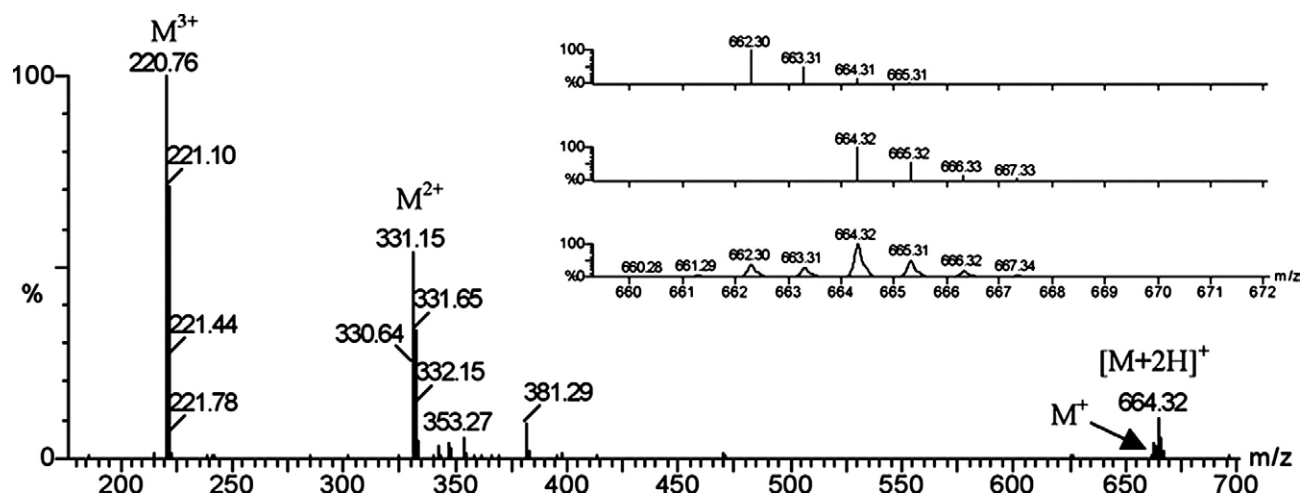


Figure 1. Mass spectrum of compound 1: (a) calculated isotopic pattern of the M^{3+} ion; (b) calculated isotopic pattern of the M^{2+} ion; (c) observed experimental pattern of the M^+ and $[M+2H]^+$ ions.

ions are also shown. It is apparent from the figure that, in the singly charged experimental cluster, the most abundant peak may be attributed to the $[M+2H]^+$ ion.

The relative abundance of the $[M+2H]^+$ ions increases dramatically at lower flow rates for the solutions with higher and intermediate concentrations. In Table 1 the abundances of the M^+ and $[M+2H]^+$ ions (expressed by the average of triplicate measurements of the percentage of the TIC) for several concentrations and flow rates, for compound 1, using methanol as a solvent, are presented. The spectra were acquired at a cone voltage of 30 V.

The abundance of both ions increases with increasing concentration, especially the abundance of the $[M+2H]^+$ ions. The substitution of a phenyl group by aryl groups with electron-withdrawing substituents (2,3,6-trifluorophenyl, pentafluorophenyl, 4-carboxyphenyl, and 4-methoxycarbonylphenyl) does not significantly affect the abundance of the reduced species M^{2+} , M^+ , and $[M+2H]^+$.

Product-ion spectra. Product-ion spectra were acquired for selected ions using methanol as the eluent, for solutions 3.5×10^{-6} M and flow rates of $10 \mu\text{L min}^{-1}$. When the precursor ions were the M^{3+} , M^{2+} , and M^+ ions, the cone voltage and collision energy used were, respectively, 30 V and 25 eV. When the precursor ions were the $[M+2H]^+$ ions, the cone voltage and collision energy used were, respectively, 30 V and 45 eV. In Table 2 the product-ion spectra of the M^{3+} and M^{2+} ions are summarized.

The fragmentation of the M^{3+} and M^{2+} ions for all the porphyrins studied occurs predominantly through radical losses from the substituent groups of the macrocycle, with formation of triply and doubly charged product ions, respectively. For the same experimental parameters the relative abundances of the M^{3+} precursor ions are higher than the relative abundance of the corresponding M^{2+} precursor ions. The M^{2+} ions frag-

ment mostly through one single channel—loss of one methyl radical, leading to the $[M-\text{CH}_3]^{2+}$ ions. The formation of doubly charged product ions from the M^{3+} precursors, and of singly charged product ions from the M^{2+} precursors, may be ascribed to charge separation, through either loss of the elements of one methylpyridinium cation plus loss of other radicals (H, CH_3) or loss of one methyl cation plus the elements of one methylpyridinium radical, plus other radicals.

Similarly to the M^{2+} ions, the M^+ ions fragment mostly through one single channel, losing a 30-Da neutral fragment, with formation of species to which the $[M-2\text{CH}_3]^+$ composition may be ascribed. The second most abundant species are formed by loss of one methyl radical.

The M^{2+} ions may be formed by one electron reduction of the M^{3+} ions and are probably hypervalent radicals. Detection of similar hypervalent radicals formed by one electron reduction of quaternary ammonium (and phosphonium) salts in the gas phase, by using several mass spectrometric techniques, has been known since 1980 [25] and is extensively documented

Table 1. Abundances of the M^+ and $[M+2H]^+$ ions (%TIC)

Concentration	Flow ($\mu\text{L}/\text{min}^{-1}$)	Average (%SD)	
		M^+ (%TIC)	$[M+2H]^+$ (%TIC)
8.6×10^{-7} M	5	0.1(9)	3 (14)
	7.5	0.1(6)	3 (14)
	10	0.2(6)	3 (24)
1.7×10^{-6} M	5	0.8(12)	19 (5)
	7.5	1 (13)	11 (15)
	10	1 (7)	8 (15)
3.5×10^{-6} M	5	2 (15)	37 (9)
	7.5	3 (7)	33 (8)
	10	3 (9)	14 (14)

Note: %SD, percentage standard deviation; %TIC, percentage total ion current.

Table 2. Product-ion spectra of the M^{3+} and M^{2+} ions (m/z , %RA)

Fragment composition	Compound				
	1	2	3	4	5
$[M]^{3+}$ (precursor ion)	220.76 (100)	235.44 (100)	238.74 (100)	240.11 (100)	250.75 (100)
$[M-CH_3]^{3+}$	215.83 (50)	230.40 (14)	233.74 (33)	235.11 (20)	245.75 (45)
$[M-R-CH_3]^{3+}$	190.08 (20)	190.08 (11)	190.07 (19)	190.08 (14)	190.08 (44)
$[M-R]^{3+}$	195.09 (14)	195.09 (9)	195.07 (6)	195.09 (26)	195.09 (32)
$[M-C_6H_7N-CH_3]^{2+}$	277.11 (42)	299.10 (15)	304.08 (19)	306.12 (42)	322.09 (57)
$[M-C_6H_7N]^{2+}$	284.64 (50)	306.66 (13)	311.61 (10)	313.66 (27)	329.62 (40)
$[M]^{2+}$ (precursor ion)	331.15 (0.8)	353.09 (8.8)	358.12 (0.9)	360.14 (3.8)	376.12 (1.7)
$[M-CH_3]^{2+}$	323.64 (100)	345.57 (100)	350.61 (100)	352.63 (100)	368.62 (100)
$[M-2CH_3]^{2+}$	316.15 (7.0)	338.09 (5.3)	343.12 (5.0)	345.14 (4.0)	361.12 (5.0)
$[M-2H-C_6H_7N]^+$	567.23 (0.3)	613.25 (1.9)	621.17 (0.2)	625.21 (0.4)	657.19 (0.9)
$[M-H-C_6H_7N-CH_3]^+$	553.22 (3.0)	597.21 (3.3)	607.16 (1.2)	611.2 (1.3)	643.18 (4.0)
$[M-H-C_6H_7N-2CH_3]^+$	538.19 (1.7)	582.17 (1.7)	592.14 (0.7)	596.17 (0.5)	628.16 (1.4)

[11,12,26–32]. Contrary to aliphatic ammonium hypervalent radicals that are transient species, aromatic ammonium hypervalent radicals are stabilized by partial delocalization of the electronic density throughout the macrocycle. Loss of one methyl group from the M^{2+} ions may lead to the formation of an even-electron doubly charged species with formation of a neutral pyridine substituent group.

Similarly, formation of the M^+ ions may occur by two electron reduction of M^{3+} and the former are probably a cationic porphyrin with two neutral hypervalent nitrogens. Two electron reductions were never reported before for free-base cationic porphyrins or other organic cations when using electrospray mass spectrometry.

The most abundant product ions of the $[M+2H]^+$ ions are, as in the case of the M^+ ions, formed by a 30-Da neutral loss, arising from the loss of two methyl radicals, with formation of the $[(M+2H)-2CH_3]^+$ ions. The second most abundant product ions are the $[(M+2H)-CH_3]^+$ ions. Losses of the elements of one methylpyridiniumyl substituent and losses of the elements of one methylpyridiniumyl plus one and two

methyl groups are also observed. At lower m/z values, low abundant doubly and triply charged species are observed, respectively: the $[M-CH_3]^{2+}$ and M^{3+} ions. In Figure 2 the partial product-ion spectra of the $[M+2H]^+$ ions of compounds 2, 3, and 4, acquired at the same cone voltages and collision energies, are presented for m/z values >580 .

Reduction of the cationic M^{3+} porphyrins to the $[M+2H]^+$ species must involve hydrogen addition and multielectron reduction, leading to the corresponding dihydroporphyrins (also known as chlorins). If the $[M+2H]^+$ ions (and the M^+ ions) are hypervalent diradical species, the predominant loss of 30 Da observed for both could lead to the formation of two neutral pyridine functions (see Scheme 2 for the $[M+2H]^+$ ions).

Observation of the $[M+2H]^+$ ions could be attributable either to chlorin contamination of the porphyrin samples or to an unlikely solution reduction (instantaneous upon dissolution, or overnight). These hypotheses were ruled out through acquisition of the UV-visible spectra of methanol solutions of the porphyrins, freshly prepared, and after 24 h. No significant alter-

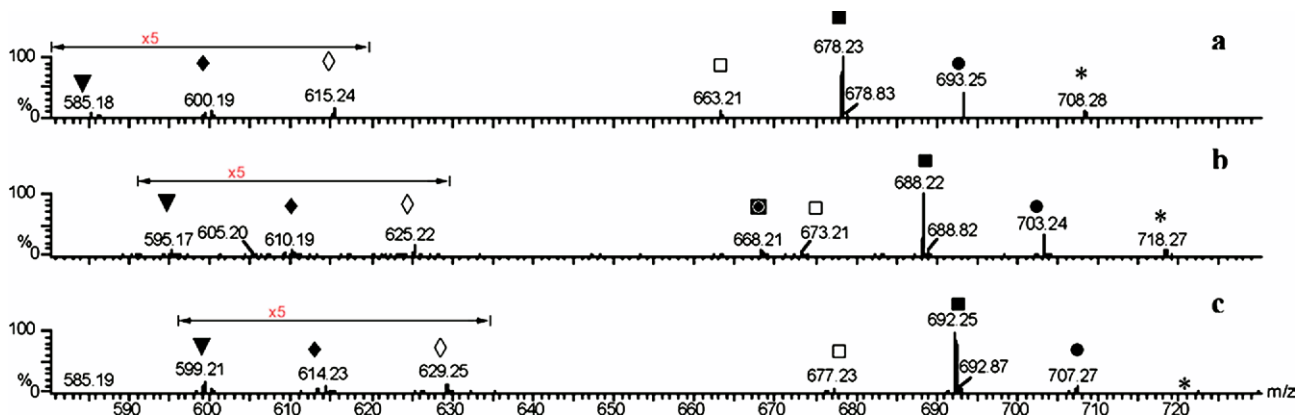
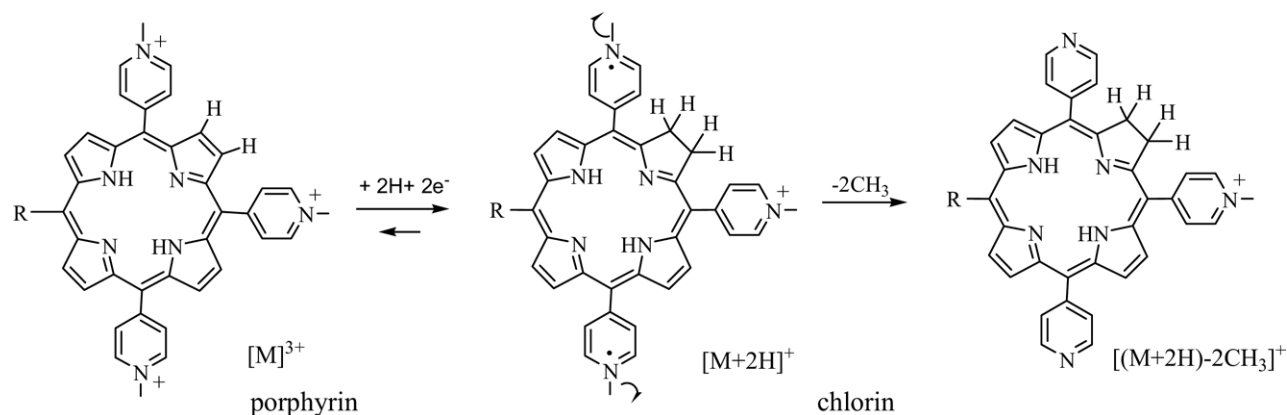


Figure 2. Product ion spectra of the $[M+2H]^+$ ions: (a) of compound 2; (b) of compound 3; (c) of compound 4. Legend: *, precursor ion $[M+2H]^+$; ●, $[(M+2H)-CH_3]^+$; ■, $[(M+2H)-2CH_3]^+$; ◐, $[(M+2H)-HF-2CH_3]^+$; □, $[(M+2H)-3CH_3]^+$; ◇, $[(M+2H)-C_6H_7N]^+$; ◆, $[(M+2H)-C_6H_7N-CH_3]^+$; ▼, $[(M+2H)-C_6H_7N-2CH_3]^+$.



Scheme 2

ations in the fourth Q band (fourth satellite band of the Soret or B band) [33,34] were detected that could be ascribed to the presence of the chlorins.

Reduction reactions in the metal capillary sprayers of mass spectrometers operating in the negative mode are an important step in the overall ionization process, being responsible for charge balance, but in the positive mode this balance is achieved through oxidation [1,2]. Thus the reductions that we observed in positive-mode electrospray, for the analytes studied, cannot be direct needle-induced redox reactions of the analytes, and the formation of the M^+ and $[M+2H]^+$ ions must occur, either previously in the solution, or after the migration through the needle. Nevertheless, direct solvent oxidation in the needle may be a plausible contributor to the overall redox process.

ESI Mass Spectra and Product Ion Spectra in Other Solvents

Additional information was gathered by acquiring the spectra using other solvents: a mixture of methanol and an electron donor, *N,N* dimethylaniline; an aprotic solvent, acetonitrile; and deuterated methanol. When methanol with added dimethylaniline was used, no significant differences were detected in the ESI mass spectra. When using acetonitrile CH_3CN , although the M^{2+} and M^{3+} ions were observed, the $[M+2H]^+$ ions were not.

The absence of the $[M+2H]^+$ ions when acetonitrile

was used is indicative of the participation of methanol (a protic solvent) on their formation; thus the experiments were repeated using deuterated methanol CD_3OD . When the latter was used peaks corresponding to the triply charged $[M-2H+2D]^{3+}$ ions; the doubly charged M^{2+} and $[M-2H+2D]^{2+}$ ions; and the singly charged M^+ , $[M-H+D]^+$, and $[(M-2H+2D)+2D]^+$ ions, whose formation may be ascribed to exchange of the central hydrogens for deuterium atoms and to a reductive process in which two deuterium atoms are added.

The product-ion spectra of the $[M+2H]^+$ and $[(M-2H+2D)+2D]^+$ ions, acquired in the same experimental conditions, are similar for all the compounds. In Table 3 the main product ions of the precursor ions $[M+2H]^+$ and $[(M-2H+2D)+2D]^+$ ions, for compound 5, are presented. The data were acquired using methanol and deuterated methanol as the eluents, for solutions with concentrations 3.5×10^{-6} M and flow rates $5 \mu L \text{ min}^{-1}$, at a cone voltage of 30 V and collision energy of 45 eV.

As was described above the most abundant product ions are formed by the loss of two methyl groups and the second most abundant are the ions formed by loss of one methyl group. Ions formed by loss of two methyl groups and a HF molecule are also present. The presence of the triply charged species at m/z 250.75 and 251.46, which are the M^{3+} and $[M-2H+2D]^{3+}$ ions, respectively, reinforces the similarity of the fragmentation processes of the $[M+2H]^+$ and $[(M-2H+2D)+2D]^+$ ions and, consequently, of their structures.

For approximately the same concentrations the for-

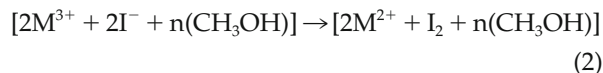
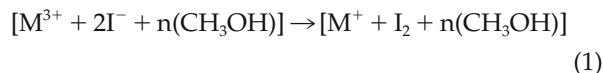
Table 3. Product-ion spectra of the $[M+2H]^+$ and $[(M-2H+2D)+2D]^+$ ions of compound 5

Peak Identification	Compound 5 in CH_3OH		Compound 5 in CD_3OD	
	m/z	R-A (%)	m/z	R-A (%)
Precursor ions $[M+2H]^+ / [(M-2H+2D)+2D]^+$	754.28	3	758.41	15
$[(M+2H)-CH_3]^+ / [(M-2H+2D)+2D-CH_3]^+$	739.27	15	743.38	52
$[(M+2H)-2CH_3]^+ / [(M-2H+2D)+2D-2CH_3]^+$	724.24	100	728.36	100
$[(M+2H)-3CH_3]^+ / [(M-2H+2D)+2D-3CH_3]^+$	709.21	6	713.33	4
$[(M+2H)-2CH_3]^+ / [(M-2H+2D)+2D-HF-2CH_3]^+$	704.23	16	708.35	10
$M^{3+} / [M-2H+2D]^{3+}$	250.75	5	251.46	4

mation of the reduced species does not increase significantly with the addition of an electron-donating compound such as *N,N* dimethylaniline, and remains also approximately the same for the solvents used (except for the absence of the $[M+2H]^+$ ions in the case of acetonitrile). Taking these facts into account the sources of the electrons responsible for the reduction of the M^{3+} to the M^{2+} and M^+ ions are probably the iodide (I^-) counter ions. Although in the experimental conditions used, the formation of aggregates such as $[M^{3+}+2I^-]$ and $[M^{3+}+I^-]$ was not detected, the M^+ and M^{2+} ions may result of electron transfer, in counter ion/solvent clusters, from the iodide anions.

If we assume that such clusters are formed upon solvation, in a similar way as was described for dicationic analytes^o [35], subsequent electron transfer from the iodides would lead to the formation of the reduced species M^+ and M^{2+} . These clusters would exist in the droplets as tight solvated ion pairs [11], $[M^{3+}+2I^-+n(CH_3OH)]$ and $[M^{3+}+I^-+n(CH_3OH)]$ and the reduction may occur while these aggregates are still in the relatively high-pressure regions of the source [36].

It is thus probable that the formation of the M^+ and M^{2+} ions may occur within the cluster, without change of the overall charge, as indicated below:



The increase of the abundance of the $[M+2H]^+$ for lower flow rates and the formation of the $[(M-2H+2D)+2D]^+$ ions in deuterated methanol confirms the important role of the solvent in the formation of the chlorin cations. Possibly their formation occurs within loose ion pair clusters [11] such as $[M^{3+}+n(CH_3OH)+2I^-]$ and $[M^{3+}+n(CH_3OH)+I^-]$, in which the ions are separated by solvent molecules. Transfer of electrons, molecular fragments, and even protons within cluster ions have been reported for clusters of metal cations and aprotic solvents [37]. The occurrence of proton transfer is thus much more probable in the present case, given that methanol is a protic solvent. If loose clusters of large organic cations, such as porphyrins, are formed when methanol is used, both proton and electron transfer from the solvent may occur.

The fact that the reduced species $[M+2H]^+$ is formed with higher abundances when lower solvent flow rates are used is consistent with the first step of the overall reduction process being a protonation reaction. Decrease in the solution pH, in positive-mode electrospray mass spectrometry, caused by solvent oxidation reactions in the needle, was reported, this decrease being higher when flow rates were lower [38]. Thus, if we assume that methanol can be oxidized in the needle,

protons, formed as products of this oxidation, would be available in solution. More protons would then be produced when lower flow rates would be used and the extent of chlorin formation would be higher. The source of the electrons may be either the iodide counter ions or methoxide ions formed in the clusters by deprotonation of methanol.

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