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Analysis of metal complex azo dyes by high-performance liquid chromatography/ electrospray ionization mass spectrometry and multistage mass spectrometry

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Five metal complex azo compounds were analyzed using negative-ion electrospray ionization mass spectrometry (ESI-MS). Mass spectra of all compounds yield intense peaks corresponding to $[M - H]^-$ ions without any fragmentation, where M denotes the neutral compound with a proton as the counterion. Under collision induced dissociation (CID) conditions, structurally important fragment ions were studied using the ion trap analyzer with a multistage mass spectrometry (MS^n facility. Synthesized compounds with ^{15}N atoms in the azo group facilitated the fragmentation pattern recognition. A reversed-phase high-performance liquid chromatography (HPLC) method using 5 mM ammonium acetate in 70% aqueous acetonitrile as mobile phase was developed making possible the separation of all complex compounds tested. The lower detection limits of the ESI-MS method are in the range 10–20 ng of each compound. The HPLC/ESI-MS method makes possible the monitoring of ligand exchange in aqueous solutions of metal complex azo dyes, and also investigation of the stabilities of the complexes in solution. Copyright © 2000 John Wiley & Sons, Ltd.

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Electrospray ionization mass spectrometry (ESI-MS) is a soft ionization technique which has been widely applied in the analysis of biopolymers,[1–3] but it also offers interesting possibilities for the analysis of low molecular weight (MW) organic compounds. ESI-MS enables the investigation of organometallic complexes without breaking coordination bonds during the ionization process. ESI-MS analysis of organometallic and inorganic compounds has been reviewed in the last few years.[4,5]

Azo dyes have found widespread applications in the textile and food industries and elsewhere. To improve the solubility in water, azo dyes often contain one or more sulphonic acid groups. Previously, thermospray ionization mass spectrometry was used for their analysis, but this technique was limited to compounds with a maximum of two sulphonic groups in the molecule.[6,7] Negative-ion ESI-MS has no limitations with respect to the number of sulphonic groups in the analyte molecule (at least up to eight sulphonic groups per molecule[8]) and offers better sensitivity and an easy MW determination for anionic azo dyes.[9,10]

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Trivalent metal ions form deeply coloured and stable metal complexes with o,o′-dihydroxy-substituted azo benzenes or similar azo compounds. Such complexes are six-coordinated[11] and carry a negative charge. Some ESI-MS measurements of sulphonated metal complex azo dyes have been reported[12,13] but, to our knowledge, mass spectrometric analysis has not been applied to non-sulphonated metal azo complexes thus far.

In our previous work,[14] we also studied five sulphonated metal complexes of Cr(III) and Co(III). Their ESI mass spectra contain only peaks due to the deprotonated molecules without any fragmentation. The background noise was very low and we did not observe increased contamination of the ion source during the analysis of metal complex compounds. In this work, we have investigated possibilities of electrospray ionization of non-sulphonated complexes of Al(III) and Co(III) following HPLC separation. Using the ion trap analyzer and ^{15}N labelled standards, more detailed structural information could be obtained.

EXPERIMENTAL

Materials

Acetonitrile for HPLC (Baker, Deventer, The Netherlands) and methanol for HPLC (Merck, Prague, Czech Republic) were used as obtained. Water was double-distilled in glass with addition of potassium permanganate. Ammonium

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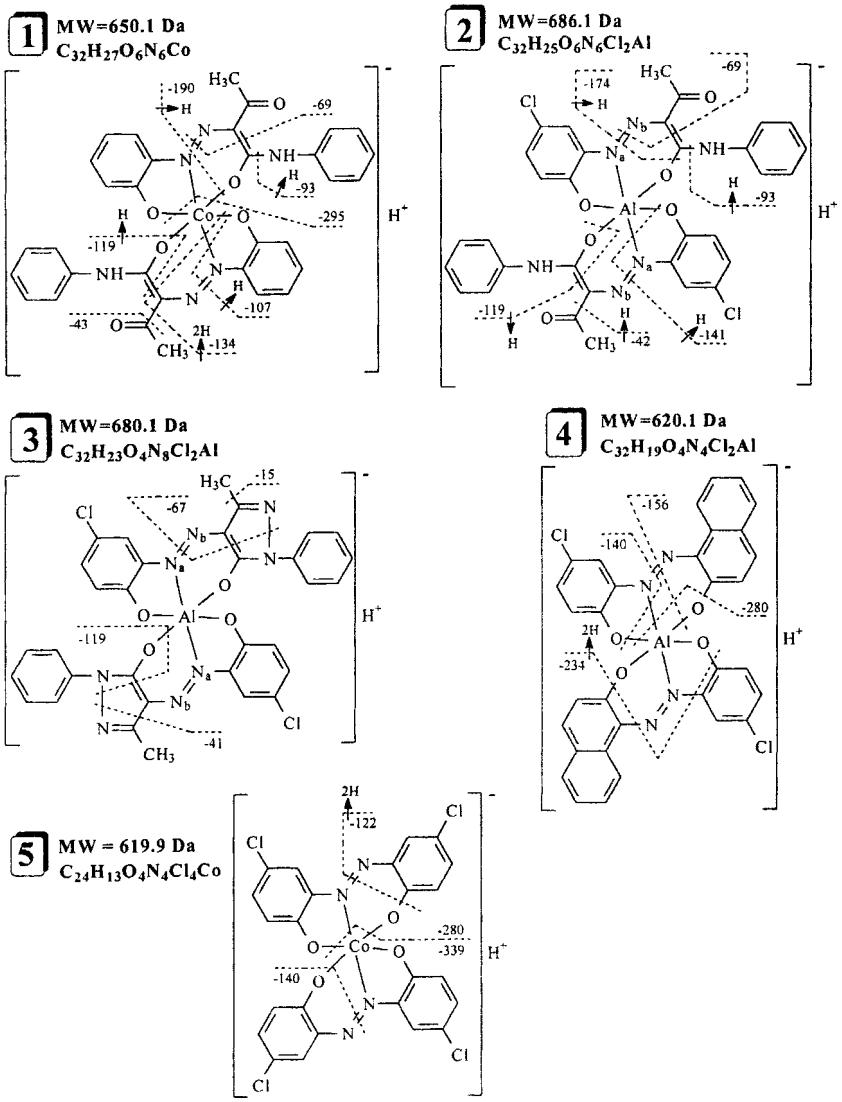

Figure 1. Structures of metal complex compounds with basic fragmentation paths (cations are shown schematically as protons, see Discussion).

acetate was purchased from Sigma-Aldrich (Prague, Czech Republic). All sample compounds were synthesized in the laboratory and their structures (Fig. 1) were confirmed by ^{27}Al, ^{15}N, ^{13}C and ^{1}H nuclear magnetic resonance spectra.[11,15] Compounds No. **2** and **3** were also synthesized with labelled ^{15}N isotopes in the N_b position (see Fig. 1).

High-performance liquid chromatography

The chromatographic apparatus consisted of a Waters 616 pump, a Waters 996 diode-array detector and a Waters 717+ autosampler (all from Waters, Milford, MA, USA). The separation was performed on a Luna C18, 5 μm column, 250 × 4.6 mm i.d., (Phenomenex, Torrance, CA, USA) using a pre-mixed mobile phase comprised of 5 mM ammonium acetate in 70% aqueous acetonitrile. The mobile phase was filtered through a 0.45 μm Millipore filter prior to use and degassed by continuous stripping with a stream of helium. The flow rate of the mobile phase was kept at 1 mL/min, the temperature was 40 °C, the wavelength of the UV detection was 254 nm and the injection volume was 20 μL in all experiments. The samples were dissolved in the mobile phase. The HPLC effluent was split approximately at the ratio 1:50, so that the flow at the inlet of the ESI-MS ion source was 20 μL/min.

Electrospray ionization mass spectrometry with a quadrupole analyzer

For the measurement of mass spectra by flow injection analysis, the individual samples were dissolved in 50% acetonitrile/50% water, injected into the solvent stream at a flow rate of 20 μL/min and analyzed on a Platform quadrupole mass spectrometer (Micromass, Manchester, UK) using negative-ion electrospray ionization. The ion source temperature was set to 100 °C, the cone voltage was 20 V for the molecular weight determination and 60–120 V for the in-source CID mass spectra.

Electrospray ionization mass spectrometry with an ion trap analyzer

The measurements were performed in negative-ion mode using an LCQ ion trap mass spectrometer (Finnigan-MAT, San Jose, CA, USA) equipped with an electrospray ion source. Sample solution (approximately 10 μg/mL in

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Table 1. Theoretical (theor.) and experimental (exp.) abundances of isotopic peaks of $[M - H]^-$ ions (the numbers correspond to Fig. 1) measured with quadrupole^{*q} and ion trap^{*it} analyzers

1	*m/z*	649	650	651	652				
	theor.	100	38.4	8.4	1.3				
	exp.^{*q}	100	35.1	7.3	1.3				
	exp.^{*it}	100	34.7	6.8	0.9				
2	*m/z*	685	686	687	688	689	690		
	theor.	100	38.4	72.3	25.9	15.7	4.8		
	exp.^{*q}	100	35.8	70.7	23.6	14.7	4.1		
	exp.^{*it}	100	32.6	68.4	21.4	13.7	4.4		
3	*m/z*	679	680	681	682	683	684		
	theor.	100	39	72.1	26.1	15.6	4.8		
	exp.^{*q}	100	37.6	70	24.2	15.5	4.1		
	exp.^{*it}	100	35.6	73.7	24.7	13.8	4.4		
4	*m/z*	619	620	621	622	623	624		
	theor.	100	38.4	72.3	25.9	15.7	4.8		
	exp.^{*q}	100	35.6	69.3	23.3	14.8	4.1		
	exp.^{*it}	100	34.3	74.4	25.4	14.5	4.3		
5	*m/z*	619	620	621	622	623	624	625	626
	theor.	75.4	21.5	100	27.9	50.8	13.7	12.1	3.1
	exp.^{*q}	72.4	12.6	100	23.5	45.1	11.8	10.5	2.6
	exp.^{*it}	63.9	15.5	100	27.2	50.8	13.7	9.5	3.4

methanol or acetonitrile) was directly infused into the ion source at a flow rate of 5 μL/min of 100% methanol or acetonitrile. The spectrometer was tuned to obtain maximum response for the sample compounds. The source parameters were set to the following values: spray voltage −4.8 kV, sheath gas flow rate 30 in arbitrary units, capillary temperature 280 °C and capillary voltage −4.0 V. The sample compounds were fragmented by collision induced dissociation after isolation of their $[M - H]^-$ ions in the ion trap. Central mass (CM, *m/z*) and isolation width (IW, *m/z*) were selected to achieve fragmentation of the $[M - H]^-$ peaks, including the isotopic ones: complex No. **1**—CM = 650 and IW = 6; No. **2**—CM = 687 and IW = 8; No. **2** (with two isotopes ^{15}N) - CM = 689 and IW = 8; No. **3**—CM = 681 and IW = 8; No. **3** (with two isotopes ^{15}N)—CM = 683 and IW = 8; No. **4**—CM = 621 and IW = 8; No. **5**—CM = 622 and IW = 10. Collision energy was 35% in arbitrary units in all experiments, except for compound No. **4** (40%). For the measurement of MS^n spectra, the parameters were modified to enable the study of the fragmentation of daughter ions from MS^2 spectra.

RESULTS AND DISCUSSION

In solution, all metal complexes studied are present as anions and the counter-cations do not affect their ESI mass spectra in the negative-ion mode. Hence the counter-cations are schematically shown as protons in the formulas in Fig. 1, and the $[M - H]^-$ ions are the complex anions. The spectra were measured using flow injection analysis (with the quadrupole analyzer) or direct infusion of sample solution (with ion trap analyzer). Under mild ionization conditions, all spectra contain deprotonated molecules $[M - H]^-$ only without any fragmentation. These ions can be used for quantitation purposes as well as for structure elucidation using MS^n experiments in the ion trap. The relative abundances of the isotopic ions are in acceptable agreement with ratios calculated from the natural abundances of the individual elements (see Table 1). The experimental isotopic abundances determined with the quadrupole analyzer are slightly lower than the theoretical values. The abundances of two of the isotopic peaks measured with the ion trap analyzer are higher than the theoretical values, but the abundances of most isotopic peaks are lower than the calculated values. All compounds studied yield intense signals without any background noise. To induce the in-source CID, it is necessary to increase the cone voltage to unusually high values, because of the high stability of these $[M - H]^-$ ions.

To gain better insight into the fragmentation pathways and to facilitate the structure elucidation, we measured the MS^n spectra using an ion trap analyzer. The MS/MS spectra measured with the ion trap analyzer were in good agreement with the mass spectra obtained using in-source CID with the quadrupole analyzer, but with different relative abundances of the individual fragment ions (Fig. 2). The MS/MS measurements allow better control of the collision energy, which thus yields mass spectra with intense fragment ions over a broader mass range than in the in-source CID mass spectra. The CID mass spectra of complexes **2** and **3** with labelled ^{15}N atoms in known positions facilitated the recognition of the fragmentation paths.

Interpretation of mass spectra measured with the ion trap analyzer

Isolated ions $[M - H]^-$ as well as some fragment ions were collisionally dissociated in the ion trap. CID MS/MS spectra of individual $[M - H]^-$ ions measured with the ion trap are shown in Figs 2–6, except for Fig. 2(b) (in-source CID mass spectrum measured with the quadrupole analyzer). It should be mentioned that some of the fragment ions can be produced in different ways, not only via the paths shown in the figures.

Fragmentation of compound 1

The most abundant fragment ion (*m/z* 530) in the MS/MS spectrum of compound **1** is formed by the loss of phenylisocyanate (neutral loss of 119 Da) from the $[M - H]^-$ ion (Fig. 2(a)). Other important cleavages correspond to the elimination of the neutral fragments with

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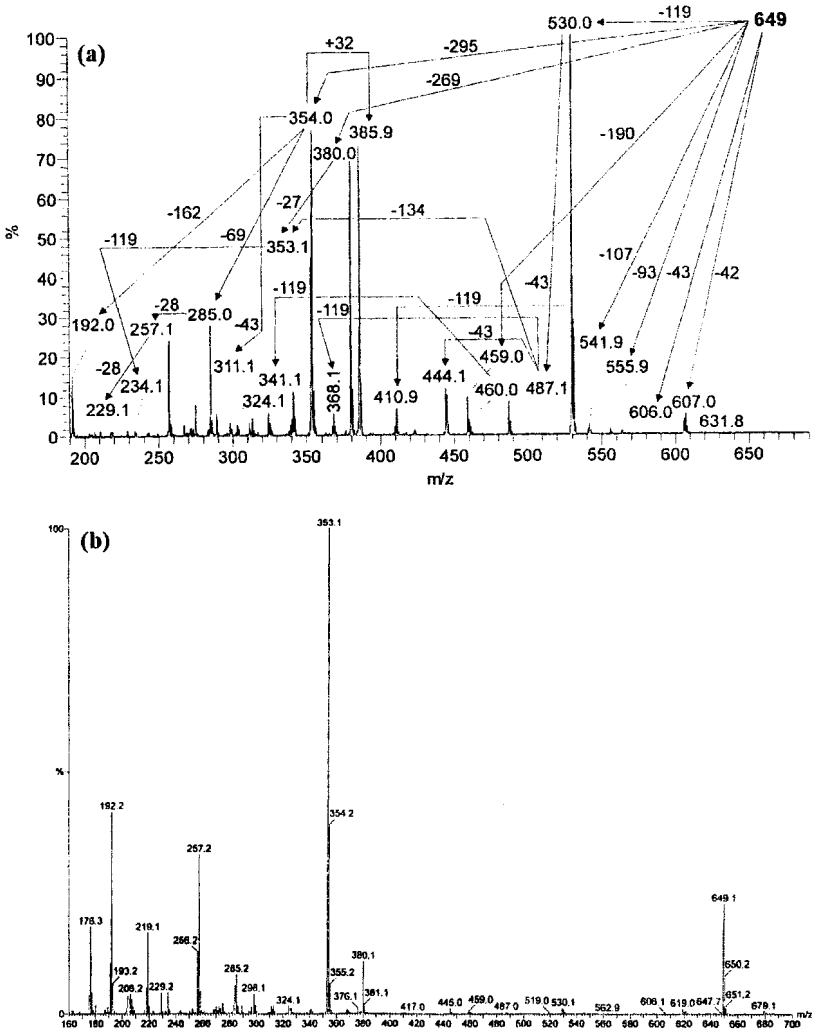

Figure 2. (a) CID MS/MS spectrum of $[M - H]^-$ ion of compound No. **1** (for the structure, see Fig. 1), (b) in-source CID spectrum of compound No. **1** measured with the quadrupole analyzer.

mass 295 Da (mass of the organic ligand) and 269 Da (possible simultaneous loss of three neutral fragments with masses 119, 107 and 43 Da, Figs 1 and 2). The ion at *m/z* 192 is produced by the loss of masses 119 and 43 Da (total 162 Da, Fig. 2) from the ion at *m/z* 354. The third most abundant fragment ion with *m/z* 386 (Fig. 2(a)) is formed (surprisingly enough) by the addition of 32 Da to the ion with *m/z* 354. This was confirmed by the MS/MS spectrum of the parent ion at *m/z* 354 and observed both in aqueous methanol and acetonitrile. This phenomenon also occurs for compound **2** (*m/z* 354 = 322 + 32, Fig. 3(a)) and for compound **5** (*m/z* 371 = 339 + 32, Fig. 6) and will be further investigated. We suppose that these adducts are formed by ion-molecule reactions with the traces of oxygen molecules in the ion trap. This finding was confirmed by measurements using another ion trap (Esquire from Bruker Daltonics) but with lower relative abundances of oxygen adducts. These adducts are not observed in the in-source CID mass spectra measured with the quadrupole analyzer (Fig. 2(b)). The observed losses of small neutral fragments probably correspond to HCN (27 Da), CO (28 Da), CH_2CO (42 Da) and CH_3CO (43 Da). Other cleavages are schematically shown in Fig. 1. Repeated losses of the same masses (e.g. 43 and 119 Da) suggest successive cleavages of two identical ligands in the complexes. Figure 2(b) illustrates that in-source CID mass spectra measured with the quadrupole analyzer are similar to CID MS/MS spectra measured with the ion trap analyzer, but with different relative abundances.

Fragmentation of compound 2

Similarly to compound **1**, the loss of phenylisocyanate (119 Da) from the $[M - H]^-$ ion was observed, as shown in Figs 3(a) and 3(b); the mass spectrum in Fig. 3(b) corresponds to the complex with two ^{15}N isotopes in N_b positions (see Fig. 1). This cleavage is followed (Fig. 3(a)) by the competing eliminations of 70 Da (C_3H_4NO) and 69 Da (C_3H_3NO). The MS^n experiments with separate isolation of the corresponding product ions with *m/z* 496 (=566 − 69) and *m/z* 497 (=566 − 70) confirmed their different structures. The loss of 174 Da from *m/z* 496 can be explained as the sum of neutral fragments with masses 119, 28 and 27 Da (confirmed by MS^n experiments), or in terms of the cleavage shown in Fig. 1. The losses of low mass neutral fragments can be attributed to the following formulas: HCN (27 Da), N_2 or CO (28 Da), CH_3N (29 Da) and HCl (36 Da). Other cleavages are shown in Fig. 1. The spectrum of the ^{15}N labelled compound in Fig. 3(b) shows that the neutral fragments with masses 27, 28, 29, 69 and

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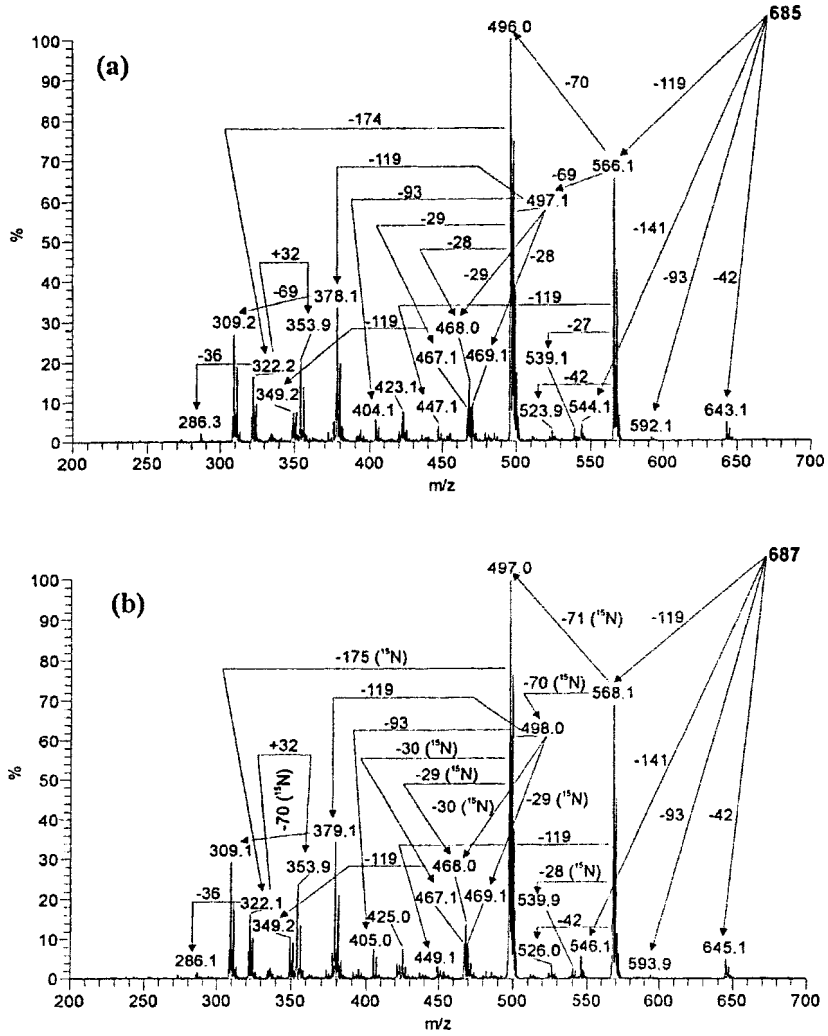

Figure 3. CID MS/MS spectra of $[M - H]^-$ ion of (a) compound No. **2**, (b) compound No. **2** with two ^{15}N atoms in positions N_b (for the structure, see Fig. 1).

70 Da contain the nitrogen atom originating from the azo group (compare Figs 3(a) and 3(b)). The fragment ions containing chlorine atoms show characteristic isotope distributions. (This applies also to compounds **3, 4** and **5**). Pairs of ions from losses of the neutral fragments corresponding to the same fragmentation patterns of both ligands are observed (Fig. 3(a)), e.g. ions *m/z* 566 = 685 − 119 and *m/z* 447 = 685 − 2 × 119; *m/z* 497 = 685−(119 + 69) and *m/z* 309 = 685 − 2× (119 + 69); and other losses of 69, 93 and 119 Da.

Fragmentation of compound 3

The MS/MS spectrum of compound **3** is depicted in Fig. 4(a), and in Fig. 4(b) for the ^{15}N labelled compound. The most abundant fragment ion (*m/z* 560) is formed by the elimination of phenylisocyanate (*m/z* 119) from the $[M - H]^-$ ion. The second most abundant ion (*m/z* 519) is produced from the ion *m/z* 560 by the neutral loss of 41 Da (CH_3CN). The other neutral fragments correspond to the following simple species: CH_3 (15 Da), HCN (27 Da), CH_3N (29 Da) and $C_3H_3N_2$ (67 Da). The last two of these fragments contain a nitrogen atom originating from the azo group. The HCN neutral fragment contains the ^{15}N isotope in some but not all cases (compare Figs 4(a) and 4(b)). The elimination of the fragment with mass 69 Da is attributed to the simultaneous losses of CH_3CN (41 Da) and N_2 (28 Da). The spectra indicate that the same kinds of fragmentation occur for both ligands. As for compounds **1** and **2**, we can locate the pairs of ions and the neutral losses that reflect the 'symmetric' fragmentations with the same neutral losses for both ligands, such as the masses 27, 41, 67 and 119 Da, or the ions with *m/z* 560 (=679 − 119) and *m/z* 441 (=679 − 2 × 119); *m/z* 519 (=679−(119 + 41)) and *m/z* 359 (=679 − 2× (119 + 41)).

Fragmentation of compound 4

The base peak (*m/z* 563) in the MS/MS spectrum of compound **4** (Fig. 5) is formed by the successive elimination of two neutral fragments (2 × 28 Da, N_2 or CO) from the $[M - H]^-$ ion (*m/z* 619). The losses of the small fragments with 28, 36 (HCl) and (less abundantly) 27 Da (HCN) give rise to series of multiplet peaks of low intensity. The losses of higher masses (280, 234, 156 and 140 Da) are shown schematically in Fig. 1.

Fragmentation of compound 5

The second most abundant fragment ion (*m/z* 339)

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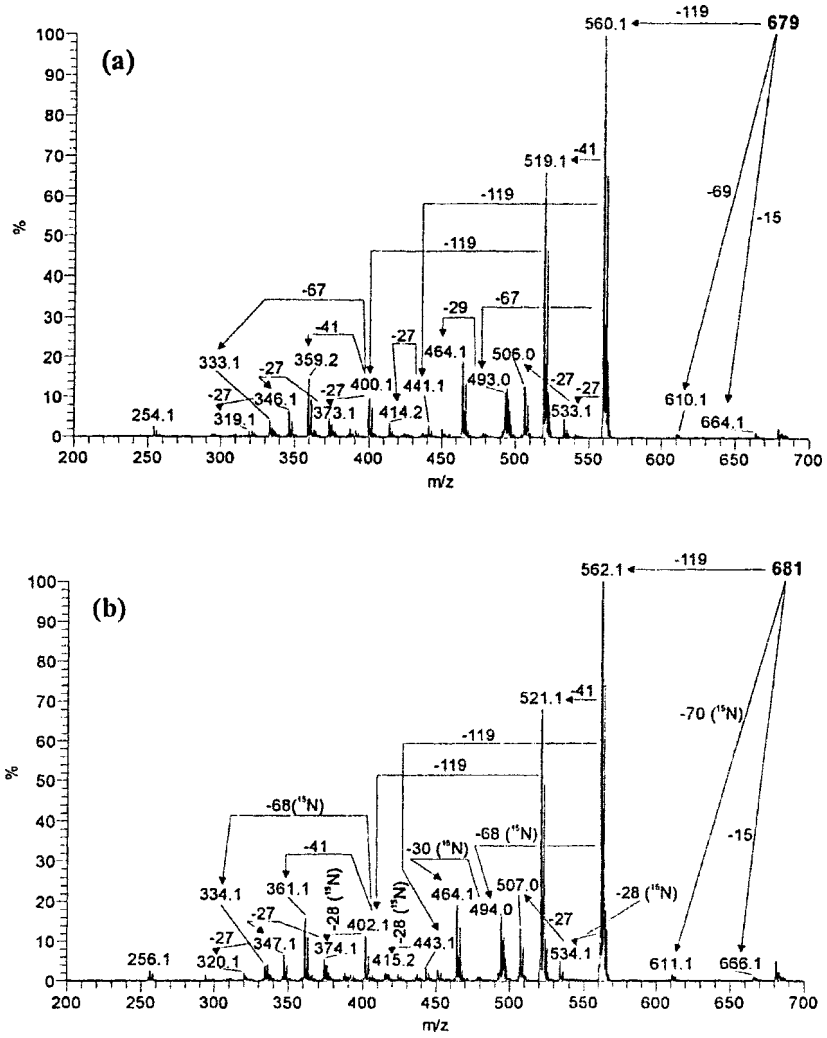

Figure 4. CID MS/MS spectra of $[M - H]^-$ ion of (a) compound No. **3**, (b) compound No. **3** with two ^{15}N atoms in positions N_b (for the structure, see Fig. 1).

corresponds to the loss of one entire ligand (Figs 1 and 6). The base peak of the MS/MS spectrum of compound **5** is assigned to the oxygen adduct ion at *m/z* 371 (=339 + 32), as discussed above. In addition to the losses of small neutral fragments with masses 28 (CO or N_2), 29 (CHO) and 36 (HCl) Da, the mass spectrum (Fig. 6) reveals the elimination of neutral fragments with masses 64 (35 (Cl) + 29), 122 and 157 Da. The loss of 122 Da from the ion at *m/z* 339 is

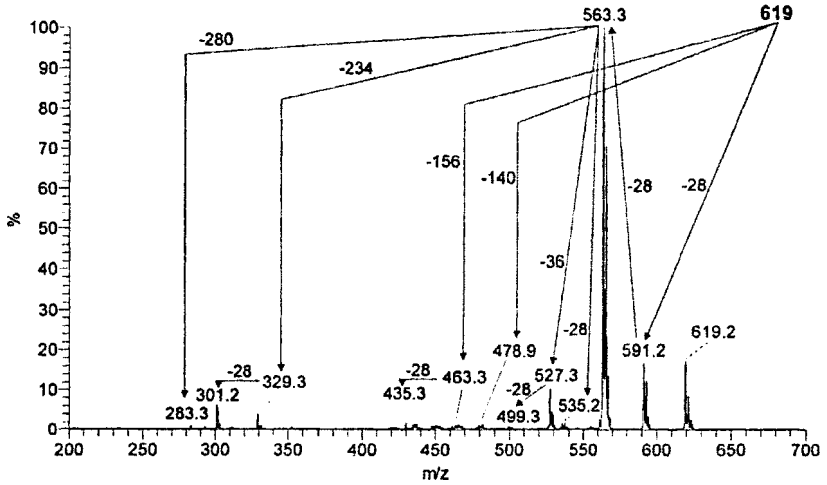

Figure 5. CID MS/MS spectrum of $[M - H]^-$ ion of compound No. **4** (for the structure, see Fig. 1).

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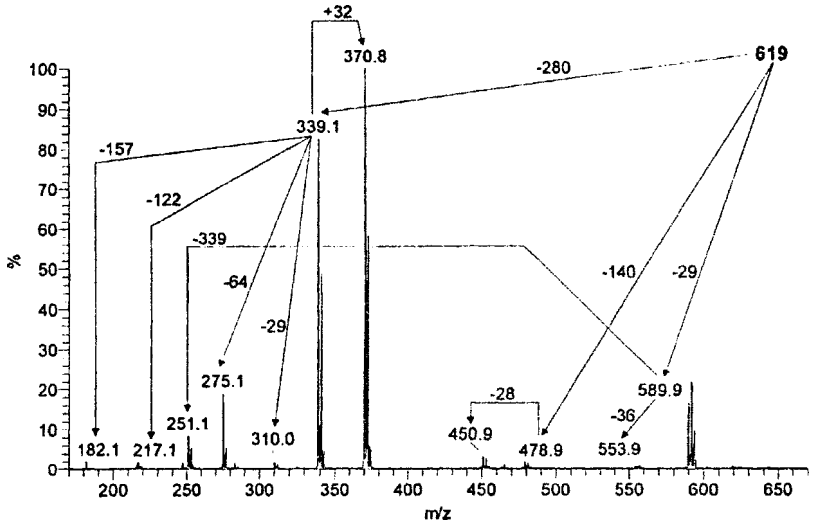

Figure 6. CID MS/MS spectrum of $[M - H]^-$ ion of compound No. **5** (for the structure, see Fig. 1).

explained in Fig. 1, and the neutral fragment of 157 Da contains one additional chlorine atom in comparison with the 122 Da fragment. The interpretation of the loss of 140 Da is shown in Fig. 1. The ion *m/z* 251 does not contain the central metal ion, in contrast to most ions discussed in this work, and is formed by the loss (339 Da, see Fig. 1) of an entire organic ligand, together with the central cobalt atom, from the ion $[M - H - CHO]^-$ at *m/z* 590.

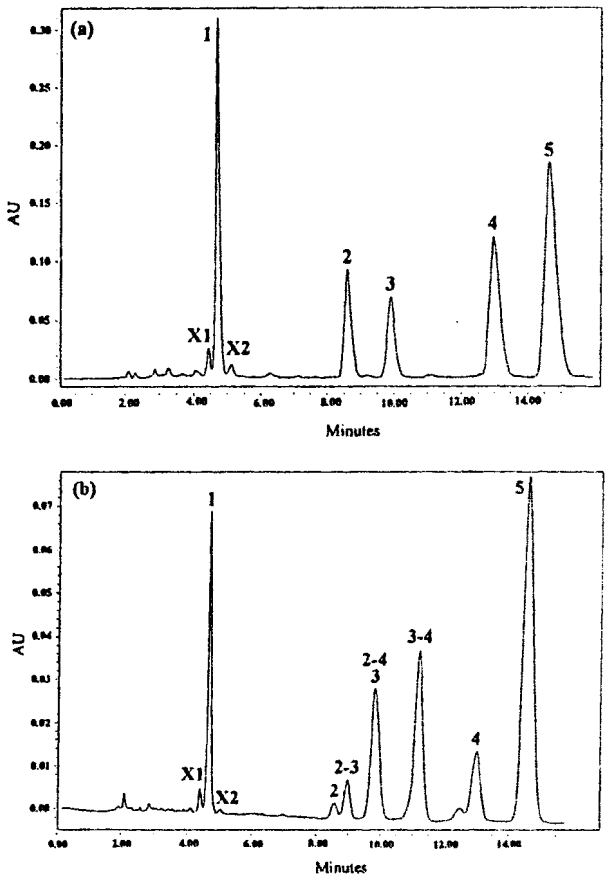

Figure 7. Chromatograms of compounds **1–5** with UV detection at 254 nm, HPLC conditions as in the Experimental part, the numbers of the peaks correspond to the numbers of compounds in Figs 1 and 8. X1 and X2 are unknown compounds with the same MW as compound No. **1**. (a) chromatogram of a fresh solution, (b) the chromatogram of a solution left overnight at ambient temperature.

HPLC separation and ligand exchange between the complexes in aqueous solutions

The HPLC separation of all complexes studied here was accomplished in 16 min on a Luna C18 column with a mobile phase containing 5 mM ammonium acetate in 70% aqueous acetonitrile. Without the addition of ionic modifier to the mobile phase, the compounds are eluted close to the column hold-up volume. Ammonium acetate has no influence on the sensitivity and the appearance of the mass spectra at least up to a concentration of 10 mM in the mobile phase, which provides good separation without sacrificing mass spectrometric performance. Figure 7(a) shows the UV chromatogram of a fresh solution of the five compounds in the sample solvent. The limits of detection at S/N = 5, determined using selected ion monitoring of the $[M - H]^-$ ions, were between 10–20 ng of each compound. Figure 7(b) shows the chromatogram of a sample mixture left overnight in the solution at ambient temperature. Two small peaks marked as X1 and X2 are impurities present in compound No. **1**, with the same molecular weight. In the chromatogram shown in Fig. 7(b), the relative intensities of peaks 2 to 4 diminished and new peaks appeared, which were identified as mixed complexes of Al(III) with the organic ligands of compounds **2** to **4** interchanged. The structures of the mixed complexes (Fig. 8) were confirmed by the agreement of the calculated *m/z* values and abundances of the isotopic peaks with the experimental data: MW (**2**–**3**) = 683 Da, MW (**2**–**4**) = 653 Da and MW (**3**–**4**) = 650 Da. The notation **2**–**3** denotes an Al(III) complex containing the organic ligands derived from compounds **2** and **3**, etc. The mixed complexes of Co(III) were not observed even after heating the solution at 80 °C for 20 min, which suggests better stability of these Co(III) complexes in solution in comparison to the corresponding Al(III) complexes.

CONCLUSIONS

The HPLC/ESI-MS technique with ammonium acetate in

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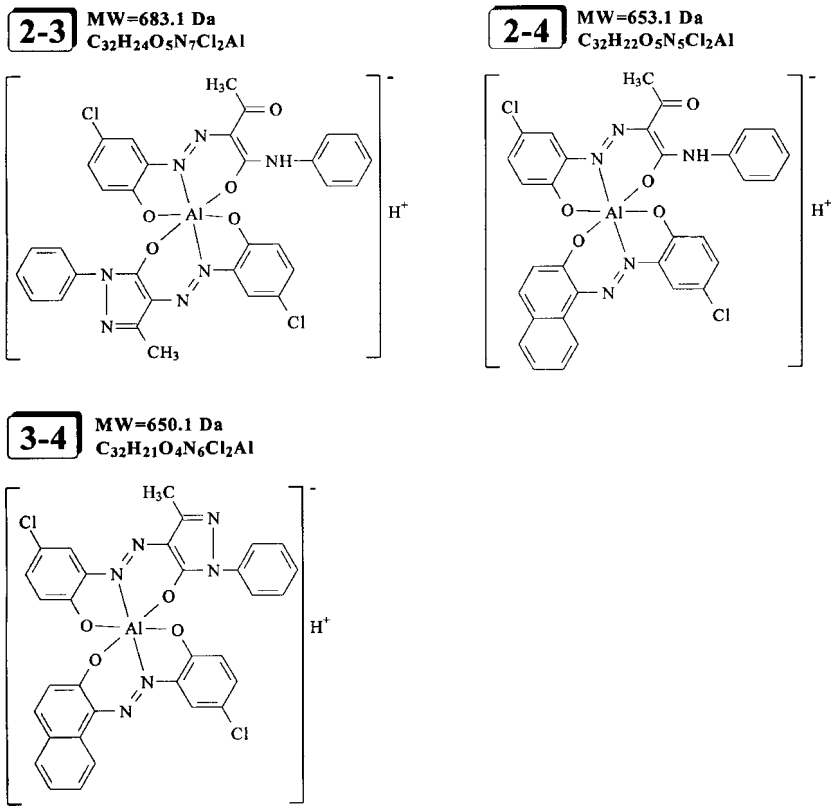

Figure 8. Suggested structures of the compounds formed by ligand exchange in solution corresponding to peaks 2-3, 2-4 and 3-4 in Fig. 7(b).

the aqueous/organic mobile phase makes possible the analysis of mixtures of anionic metal complex dyes with good sensitivity, and also facilitates the study of ligand exchange occurring in aqueous solutions of Al(III) complexes. The isotopic abundances of $[M - H]^-$ ions of trivalent metal complexes of azo dyes in the negative-ion ESI mass spectra may be used for partial confirmation of the structures suggested. Use of an ion trap analyzer made possible the recognition of fragmentation paths under CID conditions. Some similarities are evident between the fragmentation paths of the complexes **2** to **4** with an aluminium central ion on the one hand, and complexes **1** and **5** with cobalt as the central atom on the other. In the mass spectra of the complexes **2** to **4**, the cleavages of bonds within the ligands predominate, in contrast to **1** and **5**, for which the most important cleavage corresponds to the loss of entire ligands. This finding is in contrast to the stabilities of these complexes in aqueous solutions, where ligand exchange has been observed for aluminium complexes but not for cobalt complexes. This discrepancy presumably reflects the fact that the fragmentation is a gas phase process, but the ligand exchange takes place in solution. The characteristic feature of the mass spectra of compounds **1**, **2** and **3** is the loss of a neutral phenylisocyanate molecule. According to the suggested structures, nearly all fragment ions contain a central metal ion. The complexity of the CID mass spectra appears to be related to the numbers of oxygen and nitrogen heteroatoms in the ligands (compare spectra of complexes **1, 2, 3** with those of **4** and **5**).

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