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Ion-pairing high-performance liquid chromatography-mass spectrometry of impurities and reduction products of sulphonated azodyes

An HPLC separation method with triethylammonium acetate mobile phase additive developed for the analysis of impurities in polysulphonated azo dyes provides good separation selectivity and compatibility with electrospray ionisation (ESI) mass spectrometry. The negative-ion ESI mass spectra containing only peaks of deprotonated molecules $[M-H]^-$ for monosulphonic acids, $[M-xH]^{x-}$, and sodiated adducts $[M-(x+y)H+yNa]^{x-}$ for polysulphonic acids allow easy molecular mass determination of unknown impurities. Based on the knowledge of the molecular masses and of the fragment ions in the MS/MS spectra, probable structures of trace impurities in commercial dye samples are proposed. To assist in the interpretation of the mass spectra of complex polysulphonated azodyes, additional information can be obtained after chemical reduction of azodyes to aromatic amines. The structures of the non-sulphonated reduction products can be determined by reversed-phase HPLC/MS with positive-ion atmospheric pressure chemical ionisation and of the sulphonated products by ion-pairing HPLC/MS with negative-ion ESI.

Key Words: Sulphonic acid; Dye; HPLC/MS; Electrospray; Ion-pairing

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1 Introduction

Organic dyes are annually produced in huge quantities worldwide. Some dyes, intermediates, and their degradation products, especially those containing amino groups and metal complexes, are of great environmental and toxicological concern and therefore reliable methods for the identification and monitoring of dyestuff products are needed. Due to continuous development of new products with improved dyeing and ecological properties, the range of dyes is very broad and information about those of primary interest is updated in accordance with the latest knowledge about their toxicity and biodegradability. Usually, the dye classification is based on their chemical structures (such as azodyes, metal complex dyes, anthraquinone dyes, phthalocyanine dyes) or their applications (reactive dyes, textile dyes, pigments, etc.) [1]. Each dye has a unique Color Index name (e.g., C.I. Acid Violet 7) and one or more commercial names (e.g., Egacid Red 6B). Various types of water-soluble dyes such as (poly)-azodyes, anthraquinone dyes, metal complex azodyes, etc. contain one or more sulphonic acid groups.

Samples containing complex mixtures of ionic dyes can be separated either by high-performance liquid chromatography (HPLC) or by capillary electrophoresis; the first technique being more elaborated and more widely used. HPLC separations of strongly acid anionic (poly)sulphonated dyes and intermediates require ionic additives to the mobile phase – either ion-pairing reagents, most frequently tetraalkylammonium salts [2, 3], or high concentrations (0.1 – 1 mol/L) of inorganic salts such as sodium sulphate [2 – 4], ammonium sulphate [5], or another salt [6], providing salting-out effects assuring satisfactory retention and separation. Unfortunately, these additives are usually not volatile enough to be compatible with mass spectrometric detection. To prevent the tetraalkylammonium ions from entering the ion source of the mass spectrometer, an ion-suppressor column can be inserted between the UV and mass spectrometric detectors [7]; however, the additional extra-column volumes can impair chromatographic resolution, especially when micro-columns are used. Hence, HPLC/MS with volatile di- and trialkylammonium ion-pairing additives are often selected as a reasonable compromise between separation selectivity and mass-spectrometer performance for the analysis of dyes and intermediates [8 – 12].

Aromatic acids and dyes with a single sulphonic acid group often can be separated in mobile phases with

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Abbreviations: APCI, Atmospheric Pressure Chemical Ionisation; TBAA, TriButylAmmonium Acetate; DHAA, DiHexylAmmonium Acetate; TEAA TriEthylAmmonium Acetate; RIC, Reconstructed Ion Current.

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ammonium acetate [10, 13, 14], but compounds with two and more sulphonic acid groups are usually very weakly retained. Triethylammonium acetate (TEAA) in the mobile phase often improves their retention and separation [9, 15]. The retention and separation selectivity for di- and trisulphonic dyes and intermediates usually increase when alkylammonium acetates with longer alkyls are used as mobile phase additives, such as tributylammonium acetate (TBAA) [9, 11], or dihexylammonium acetate (DHAA) [8]. However, the selection of a generally suitable mobile phase for the analysis of polysulphonated dyes with three and more sulphonic acid groups is still an open issue.

Negative-ion ESI is the only generally useful ionisation technique for the analysis of anionic dyes [8, 13, 16–18]. In addition to the determination of the molecular masses and of the number of sulphonic acid groups, it can also yield additional structural information, especially with MS/MS or MS^n techniques [11, 14, 19–22]. Negative-ion atmospheric pressure chemical ionisation (APCI) is restricted to mono- and disulphonic acids [7, 10, 13] and the sensitivity is lower than with ESI. The negative-ion thermospray ionisation [10, 23] used in the early work before the introduction of ESI-MS is hardly applied any more. Matrix-assisted laser desorption/ionisation (MALDI) [20, 21, 24] can be also used for MS of dyes, but the coupling with HPLC is less common.

The first objective of our work was to develop a suitable method for the HPLC/MS analysis of (poly)sulphonated azodyes in commercial samples, allowing identification of by-products and impurities, which can influence the dyeing properties, even if present at trace levels, on the basis of molecular masses, fragment ions in the MS/MS spectra, and a knowledge of the dye production technology. In the second part of this work, chemical reduction of azo dyes to amines is combined with HPLC/MS analysis of the reduction products to acquire additional structural information on complex sulphonated azodyes.

2 Experimental

2.1 Chemicals and reagents

Methanol for HPLC and acetonitrile for HPLC were purchased from Merck (Darmstadt, Germany). Water was doubly distilled in glass in the presence of potassium permanganate. The solvents were filtered through a 0.45 mm Millipore filter prior to use and degassed by continuous stripping by a stream of helium. The best available purity (>99%) ion-pairing reagents triethylamine and dihexylamine were obtained from Aldrich (Milwaukee, WI, USA) and acetic acid from Sigma (St. Louis, MO, USA). Triethylammonium acetate (TEAA) was prepared by mixing equimolar amounts of triethylamine and acetic acid and dihexylammonium acetate (DHAA) by mixing equimolar amounts of dihexylamine and acetic acid. Titanium(III) chloride and perchloric acid were purchased from Lachema (Brno, Czech Republic). The samples of sulphonated dyes were obtained from Alliachem (Pardubice, Czech Republic): 1) Saturn Blue L4G (C.I. Direct Blue 78), 2) Saturn Green LB (C.I. Direct Green 26), 3) Egacid Yellow M (C.I. Acid Yellow 36), 4) Egacid Blue A2G (C.I. Acid Blue 40), 5) Midlon Red E (C.I. Acid Red 118), 6) Rylan Red 3G (C.I. Acid Red 357), 7) Rylan Orange R (C.I. Acid Orange 142), 8) Rylan Yellow 3R (C.I. Acid Yellow 194), 9) Rylan Bordeaux B (C.I. Acid Violet 90), 10) C.I. Acid Orange 5, 11) C.I. Direct Red 7, 12) C.I. Mordant Black 1, 13) Saturn Orange L7G (C.I. Direct Orange 46), 14) Ostazin Olive HG (C.I. Reactive Green 8) and 15) Ostazin Blue S-26 (C.I. Reactive Blue 109).

2.2 Instrumentation

Two HPLC/MS systems were used: The HPLC/MS system 1 consisted of a Waters Assoc. (Milford, MA, USA) liquid chromatograph assembled from a Model 616 Pump, a Model 996 diode-array UV detector, a Model 717+ autosampler and a Platform quadrupole mass spectrometer equipped with ESI and APCI probes was from Micromass (Manchester, UK). The mass spectra were acquired in the range of *m/z* 35–1 000. In the positive-ion APCI mode, the cone voltage was 10 V, the APCI ion source temperature was set at 100°C and the APCI probe temperature at 400°C. In the negative-ion ESI mode, the ion source temperature was set at 100°C and the cone voltage was 30 V.

The HPLC/MS system 2 consisted of a HP 1090 liquid chromatograph with a three-solvent gradient pump, a UV detector (Hewlett-Packard Co., Palo Alto, CA, USA), and an Esquire 3000 mass spectrometer with an ion trap analyser (Bruker Daltonics, Bremen, Germany). A nitrogen flow rate of 10 L/min, a nitrogen temperature of 365°C, a nebuliser pressure of 345 kPa, an *m/z* range of 50–800 in the negative-ion ESI mode, an automatic measurement of MS/MS spectra with an isolation width of $\Delta m/z = 4$ and the collision amplitude of 1 V were used.

LiChrospher C18 columns (125 × 4 mm ID, 5 μm particle size) purchased from Merck (Darmstadt, Germany) were used for all HPLC separations.

2.3 Procedures

2.3.1 HPLC/MS analysis of dyes and impurities

Suitable separation conditions for the dye samples were investigated using HPLC with UV detection under isocratic conditions with mobile phases containing 2.5 mM TEAA and 40–70% methanol in water. The column hold-up volume was determined as the elution volume of uracil, unretained in the mobile phases used.

The ESI mass spectra of the original dyes and impurities were obtained by injecting 20 μL dye samples, dissolved in methanol/water (50/50, *v/v*), into the HPLC/MS system 2 using gradient elution from 40% to 70% B in A in 30 min, with 2.5 mM TEAA in water as the solvent A and 2.5 mM TEAA in methanol as the solvent B at a column temperature of 40°C and a flow rate of 1 mL/min. The HPLC effluent was split at a ratio of 1:50, yielding a flow rate of 20 μL/min to the mass spectrometer. For direct infusion measurements, 10 μL samples of dyes dissolved in methanol/water (50/50, *v/v*) were introduced directly into the mass spectrometer in a stream of methanol pumped at a flow rate of 20 μL/min.

2.3.2 Reduction of dyes and identification of the reduction products

2 mg of the dye sample was dissolved in 1 mL of water, 3 mL of 0.02 M titanium(III) chloride was added and the reaction mixture was shaken for 5 to 10 minutes.

An octadecyl silica cartridge column Separon SGX C18 (20 × 9 mm ID, 60 μm particle size) purchased from Tessek (Prague, Czech Republic) was used for SPE of non-sulphonated reduction products. The cartridge was first conditioned by washing with 2 mL of methanol and 2 mL of water, then 1 mL of the reaction mixture was passed through the cartridge, followed by washing with 2 mL of water and by elution with 2 mL of methanol (extract 1).

A 0.5 mL volume of the reaction mixture after the dye reduction was passed through a cation-exchange cartridge column (Supelclean LC-SCX, 3 mL, Supelco, Bellefonte, USA, conditioned by washing subsequently with 6 mL of 0.05 M $HClO_4$, 10 mL of water, 2 mL of methanol and finally 2 mL of water) to remove the metal cations before the HPLC/MS analysis. After washing the cartridge with 1 mL of water, sulphonated products were eluted with 2 mL of 50% aqueous acetonitrile (extract 2). Both extracts were evaporated to 0.5 mL at room temperature under a stream of nitrogen and stored in closed glass flasks in a refrigerator for no longer than two days before the analysis. A 10 μL volume of the concentrated extracts was injected into the appropriate HPLC/MS system.

HPLC/MS system 1 was used for LC/MS analysis of non-sulphonated products in extract 1, with the mobile phase consisting of acetonitrile/water (70/30, *v/v*); the flow rate was 0.5 mL/min and the column temperature 40°C.

HPLC system 2 was used for LC/MS analysis of sulphonated reduction products in extract 2, with gradient elution from 30% B to 80% B in A in 20 min, with the solvent A: 2.5 mM DHAA in water and the solvent B: 2.5 mM DHAA in acetonitrile; the flow rate was 0.5 mL/min and the column temperature 40°C.

3 Results and discussion

3.1 Ion-pairing HPLC and ESI MS of (poly)sulphonated dyes

Theoretically, the ESI mass spectra obtained by direct infusion into a mass spectrometer can provide sufficient information on the individual dyes. However, high concentrations of salts usually present in both technological and environmental dye samples can cause problems. First, in the presence of high concentrations of sodium ions in the ion source, abundant adducts with sodium ions with a general formula $[M-(x+y)H+yNa]^{x-}$ are observed in the direct infusion mass spectra, in agreement with earlier reports [8, 25]. Because the total signal intensity is distributed among all molecular, fragment, and adduct ions with single or multiple charges, the sensitivity (the signal-to-noise ratio) is reduced. In reversed-phase HPLC, the inorganic salts elute close to the column hold-up volume and are separated from the dyes, in the mass spectra of which the relative abundances of the sodium ion adducts are significantly decreased. Further, in the ESI mass spectra recorded by the HPLC/MS technique, the relative abundances of multiply charged anions are suppressed with respect to the direct infusion mass spectra, probably due to the presence of dye adducts with trialkylammonium ions at the time of ionisation. Similar behaviour as with Saturn Green LB was observed in the spectra of all studied sulphonated dyes, in agreement with the results published earlier by Ballantine et al. [25]. Hence, the interpretation of the mass spectra is easier with the HPLC/MS technique than with direct infusion. Last but not least, the signal-to-noise ratio is improved.

DHAA or TBAA cations (both containing 12 carbon atoms) usually provide adequate selectivity for successful separation of dyes with two or more sulphonic acid groups, but their higher masses (*m/z* 186) can interfere in the mass spectra more seriously than the TEAA cations with 8 carbon atoms (*m/z* 102). We studied the retention and the separation selectivity of selected Ostazin, Saturn, Midlon, and Rylan dyes containing 1–4 sulphonic acid groups with the TEAA ion-pairing additive instead of DHAA used earlier [8]. Mobile phases with 2.5 mM TEAA provided a reasonable compromise between the retention selectivity and the MS signal suppression and were therefore used in all experiments. The retention of the organic dyes in ion-pair HPLC systems decreases with increasing concentration of the organic solvent in the mobile phase and the slope of this change strongly depends both on the ion-pairing reagent and on the structure of the dye. For large polysulphonated dyes, the slope is very steep, so that even a minor change in the concentration of the organic solvent by 1–2% may affect the retention very significantly. This has an important practical impact, as only a narrow organic solvent

Table 1. Molecular masses, M_r, of sulphonated dyes (No. 1–5) and their impurities, t_R are the retention times in gradient-elution HPLC/MS with TEAA (see Experimental part).

No.	Dye	Component	M_r	t_R [min]	Acid groups	Fragment ions[a] (*m/z* proposed structures)	Proposed molecular structure
1	Saturn Blue L4G	Dye No. 1	967	4.1	4	468.5–$[M—2H–N_2]^{2-}$, 428.5–$[M–2H–N_2–SO_3]^{2-}$, 313, 252, 187	Initial dye, Fig. 4
		Impurity 1a	967	2.5	4	468.5–$[M–2H–N_2]^{2-}$, 326, 313, 232, 187	Positional isomer of initial dye
		Impurity 1b	967	4.9	4	468.5–$[M–2H–N_2]^{2-}$, 428.5–$[M–2H–N_2–SO_3]^{2-}$, 313, 252, 187	Positional isomer of initial dye
2	Saturn Green LB	Dye No. 2	1 222	5.7	5	601–$[M–2H–H_2O]^{2-}$, 588–$[M–2H–CO_2]^{2-}$	Initial dye, Fig. 4
		Impurity 2a	158	2.1	1	$[SO_3]^-$	Benzene sulphonic acid
		Impurity 2b	219	2.1	1	–	No proposal
		Impurity 2c	512	15.5	2	–	No proposal
3	Egacid Yellow M	Dye No. 3	353	9.1	1	324–$[M–H–N_2]^-$, 288–$[M–H–SO_2]^-$, 272–$[M–H–SO_3]^-$, 260–$[M–H–C_6H_5NH]^-$, 156–$[C_6H_4SO_3]^-$	Initial dye, Fig. 4
		Impurity 3a	353	13.6	1	322–$[M–H–HN{=}NH]^-$,171–$[NHC_6H_4SO_3]^-$,156–$[C_6H_4SO_3]^-$	Positional isomer of initial dye
		Impurity 3b	264	6.1	1	234–$[M–H–HN_2]^-$, 233–$[M–H–HN{=}NH]^-$, 223, 199–$[M–H–SO_2]^-$, 97–$[HSO_4]^-$	$C_6H_5NHNHC_6H_4SO_3H$
		Impurity 3c	320	16.5	1	–	No proposal
		Impurity 3d	320	17.3	1	290–$[M–H–HN_2]^{-\cdot}$, 275, 261, 255–$[M–H–SO_2]^-$,157–$[C_6H_5SO_3]^-$	No proposal
		Impurity 3e	325	6.5	1	–	$C_6H_5NHC_6H_4C_6H_4SO_3H$
		Impurity 3f	336	5.3	1	–	No proposal
		Impurity 3g	336	6.0	1	–	No proposal
		Impurity 3h	387	11.7	1	322–$[M–H–SO_2]^-$, 310–$[M–H–C_6H_4]^-$, 302, 294–$[M–H–NHC_6H_5]^-$, 190–$[ClC_6H_3SO_3]^-$	$C_6H_5NHC_6H_4N{=}N(Cl)C_6H_3SO_3H$
		Impurity 3i	493	16.1	1	–	No proposal
		Impurity 3j	509	3.6	2	480–$[M–H–N_2]^-$, 428–$[M–H–SO_3]^-$, 416–$[M–H–NHC_6H_5]^-$, 400–$[M–H–N_2–SO_3]^-$, 322–$[M–H–N_2–SO_3–C_6H_6]^-$, 246–$[M–H–N_2–SO_3–C_6H_6–C_6H_4]^-$, 156–$[C_6H_4SO_3]^{-\cdot}$	$(HO_3SC_6H_4)C_6H_4NHC_6H_4N{=}NC_6H_4SO_3H$ or $C_6H_5N(HO_3SC_6H_4)C_6H_4N{=}NC_6H_4SO_3H$ or $C_6H_5NH(HO_3SC_6H_4)C_6H_3N{=}NC_6H_4SO_3H$ or $C_6H_5NHC_6H_4N{=}N(HO_3SC_6H_4)C_6H_3SO_3H$
		Impurity 3k	509	15.3	2	The same as for No. 3j	The same as for No. 3j
		Impurity 3l	509	19.2	2	The same as for No. 3j	The same as for No. 3j
4	Egacid Blue A2G	Dye No. 4	451	9.8	1	407–$[M–H–CH_3CO]^-$, 386–$[M–H–SO_2]^-$, 368–$[M–H–SO_2–H_2O]^-$, 343–$[M–H–SO_2–CH_3CO]^-$, 316–$[M–H–CH_3CONHC_6H_4]^-$, 252–$[M–H–CH_3CONHC_6H_4–SO_2]^-$	Initial dye, Fig. 4
		Impurity 4a	303	4.0	1	284–$[M–H–H_2O]^-$, 238–$[M–H–SO_2]^-$,	Fig. 5
		Impurity 4b	371	24.0	1	355–$[M–H–CH_3]^-$, 337–$[M–H–CH_3–H_2O]^-$, 328–$[M–CH_3CO]^-$, 311–$[M–H–NH_2COCH_3]^-$	Fig. 5
		Impurity 4c	376	26.4	1	346–$[M–H–HN_2]^-$, 345–$[M–H–HN{=}NH]^-$, 318, 311–$[M–H–SO_2]^-$, 283–$[M–H–SO_2–N_2]^-$	No proposal

Table 1. Continued ...

No.	Dye	Component	M_r	t_R [min]	Acid groups	Fragment ions[a] (*m/z* proposed structures)	Proposed molecular structure
5	Midlon Red E	Dye No. 5	540	13.0	1	475–[M–H–SO_2]$^-$, 459–[M–H–SO_3]$^-$, 355–[M–H–$C_6H_5N(C_2H_5)SO_2$]$^-$, 327–[M–H–$C_6H_5N(C_2H_5)SO_2$–N_2]$^-$, 275–[M–H–$C_6H_5N(C_2H_5)SO_2$–SO_3]$^-$, 250–[M–H–$C_6H_5N(C_2H_5)SO_2$–$CH_3C_6H_3NH$]$^-$, 237–[M–H–$C_6H_5N(C_2H_5)SO_2$–$CH_3C_6H_4N_2$]$^-$, 186–[M–H–$C_6H_5N(C_2H_5)SO_2$–SO_2]$^-$	Initial dye, Fig. 2
		Impurity 5a	540	6.9	1	511–[M–H–N_2]$^-$, 355–[M–H–$C_6H_5N(C_2H_5)SO_2$]$^-$, 327–[M–H–$C_6H_5N(C_2H_5)SO_2$–N_2]$^-$, 250–[M–H–$C_6H_5N(C_2H_5)SO_2$–$CH_3C_6H_3NH$]$^-$, 237–[M–H–$C_6H_5N(C_2H_5)SO_2$–$CH_3C_6H_4N_2$]$^-$, 173–[M–H–$C_6H_5N(C_2H_5)SO_2$–$CH_3C_6H_4N_2$–SO_2]$^-$	Positional isomer of initial dye
		Impurity 5b	540	16.5	1	459–[M–H–SO_3]$^-$, 355–[M–H–$C_6H_5N(C_2H_5)SO_2$]$^-$, 275–[M–H–$C_6H_5N(C_2H_5)SO_2$–SO_3]$^-$, 250–[M–H–$C_6H_5N(C_2H_5)SO_2$–$CH_3C_6H_3NH$]$^-$, 237–[M–H–$C_6H_5N(C_2H_5)SO_2$–$CH_3C_6H_4N_2$]$^-$, 186–[M–H–$C_6H_5N(C_2H_5)SO_2$–SO_2]$^-$	Positional isomer of initial dye
		Impurity 5c	291	14.6	1	–	$C_6H_5N(CH_2CH_3)SO_2(CH_3)C_6H_3(OH)$
		Impurity 5d	512	8.3	1	483–[M–H–N_2]$^-$, 447–[M–H–SO_2]$^-$, 431–[M–H–SO_3]$^-$, 355–[M–H–$C_6H_5NHSO_2$]$^-$, 327–[M–H–$C_6H_5NHSO_2$–N_2]$^-$, 291–[M–H–$C_6H_5NHSO_2$–SO_2]$^-$, 275–[M–H–$C_6H_5NHSO_2$–SO_3]$^-$ 250–[M–H–$C_6H_5NHSO_2$–$CH_3C_6H_3NH$]$^-$, 186–[M–H–$C_6H_5NHSO_2$–SO_2]$^-$	Fig. 2
		Impurity 5e	524	11.2	1	–	Hydroxyl group is missing compared to initial dye
		Impurity 5f	525	12.7	1	496–[M–H–N_2]$^-$, 444–[M–H–SO_2]$^-$, 416–[M–H–SO_3–N_2]$^-$, 340–[M–H–$C_6H_5N(C_2H_5)SO_2$]$^-$, 312–[M–H–$C_6H_5N(C_2H_5)SO_2$–N_2]$^-$, 222–[M–H–$C_6H_5N(C_2H_5)SO_2$–$CH_3C_6H_3N_2$]$^-$	Amino group is missing compared to initial dye
		Impurity 5g	526	13.6	1	445–[M–H–SO_3]$^-$, 341–[M–H–$C_6H_5N(C_2H_5)SO_2$]$^-$, 249–[M–H–$C_6H_5N(C_2H_5)SO_2$–C_6H_5NH]$^-$	Methyl group is missing compared to initial dye

[a] Observed in MS/MS spectra of deprotonated molecules.

concentration range (often narrower than 5–8%) is available for the separation between the "full retention" and "no retention". This behaviour is typical for more or less polar non-ionic high-molecular compounds [26], but to our best knowledge it has not been reported earlier for ion pairs of large sulphonated dyes. Hence, the mobile phase should be carefully optimised when developing appropriate HPLC/MS methods for large-molecular sulphonated dyes. We will publish the results concerning the effect of the mobile phase additives on the separation selectivity in more detail elsewhere.

3.2 HPLC/MS analysis of impurities in (poly)sulphonated azodyes

The retention times (see the Experimental section for conditions) and the molecular masses, M_r, of the main compounds and impurities in commercial samples of five sulphonated azodyes (Saturn Blue L4G, Saturn Green LB, Egacid Yellow M, Egacid Blue A2G, and Midlon Red E) and of three metal complex sulphonated azodyes (Rylan Red 3G, Rylan Orange R, and Rylan Yellow 3R) are listed in **Table 1** and **Table 2**. The structures of the original dyes

Table 2. Molecular masses, M_r, of sulphonated metal complex dyes (No. 6–8) and their impurities, t_R are the retention times in gradient-elution HPLC/MS with TEAA (see Experimental part).

No.	Dye	Component	M_r	t_R [min]	Fragment ions[a] (*m/z*, proposed structures)	Proposed molecular structure[b]
6	Rylan Red 3G	Dye No. 6	887	3.3	402.5–$[M-2H-SO_3]^{2-}$, 387.5–$[M-2H-SO_3-NO]^{2-}$, 372.5–$[M-2H-SO_3-2NO]^{2-}$	Initial dye, Fig. 4
		Impurity 6a	887	9.5	–	Positional isomer of initial dye
		Impurity 6b	345	6.4	210, 198, 183, 169	No proposal
		Impurity 6c	358	12.9	297, 238, 219, 197, 183	No proposal
		Impurity 6d	422	28.3	405, 403, 362, 337, 307, 291, 227	No proposal
		Impurity 6e	426	20.3	–	No proposal
		Impurity 6f	1104	2.0	–	Triply charged ions
7	Rylan Orange R	Dye No. 7	887	9.7	806–$[M-H-SO_3]^-$, 726–$[M-H-2SO_3]^-$	Initial dye, Fig. 4
		Impurity 7a	219	2.0	–	$(OH)(NO_2)C_6H_3SO_3H$
		Impurity 7b	345	6.6	210, 198, 183, 169	No proposal
		Impurity 7c	358	13.0	339, 315, 299, 238, 222, 197, 168	No proposal
		Impurity 7d	404	19.6	–	No proposal
		Impurity 7e	422	28.5	405, 403, 362, 337, 307, 291, 227	No proposal
		Impurity 7f	903	6.0	–	Initial dye plus one oxygen atom
		Impurity 7g	963	13.2	–	Initial dye plus one additional phenyl group
		Impurity 7h	1024	15.6	–	Quadruply charged ions
8	Rylan Yellow 3R	Dye No. 8	900	9.1	856–$[M-H-CH_3CO]^-$, 819–$[M-H-SO_3]^-$, 780–$[M-H-C_6H_5NCO]^-$, 737–$[M-H-C_6H_5NCO-CH_3CO]^-$	Initial dye, Fig. 4
		Impurity 8a	900	5.3	The same as for No. 8	Positional isomer of initial dye
		Impurity 8b	900	6.8	The same as for No. 8	Positional isomer of initial dye
		Impurity 8c	177	4.0	–	$C_6H_5NHCOCH=C(OH)CH_3$
		Impurity 8d	177	10.9	–	Positional isomer of 8c
		Impurity 8e	219	2.0	–	$(OH)(NO_2)C_6H_3SO_3H$
		Impurity 8f	887	3.7	–	Triply charged ions

[a] Observed in MS/MS spectra of deprotonated molecules.

[b] Doubly charged ions: 1) either two acid groups, or 2) one acid group and a single negative charge on a metal complex (similar explanation for triply and quadruply charged ions).

are shown in **Figure 1** and **Figure 2**. For the identification of the impurities in the dye samples, we first determined the M_r from the peaks of $[M-xH]^{x-}$ ions in the negative-ion scan ESI mass spectra with the absence of fragment ions. Then, MS/MS spectra of $[M—H]^-$ were recorded to obtain structural information from the fragment ions. This approach is illustrated for the example of the impurity 5d ($M_r = 512$) in Midlon Red E dye (compound No. 5) in Figure 1, showing the quality of the MS spectra of trace impurities. The difference in molecular masses of the Midlon Red E and the impurity 5d, $\Delta M_r = 28$, suggests that the impurity does not contain either an ethyl substituent or an azo group, as compared to the structure of the original dye.

The MS/MS spectrum of the impurity 5d in the Midlon Red E dye sample in **Figure 3** represents a typical example of the MS/MS spectra of impurities obtained in this work. The comparison of the MS/MS spectra of the dye (not shown) and 5d (Figure 3) allows us to distinguish between the two possibilities, because the same base peak with $m/z = 355$ appearing in the MS/MS spectra of both compounds should be attributed to the cleavage of an substituent on the benzene ring in *m*-position to the azo bond. Instead of the typical neutral loss $\Delta m/z = 184$ ($C_6H_5N(C_2H_5)SO_2$) observed in the MS/MS spectra of compounds 5, 5a, 5b, and 5g, the loss $\Delta m/z = 156$ corresponds to $C_6H_5NHSO_2$. The structure proposal of the impurity, probably formed by the diazotisation of the active component without *N*-ethyl functionality, is further supported by other logical neutral losses shown in Figure 3.

The same approach was used for the identification of the impurities in the other dye samples (Nos. 1–3, 5–8). Because of limited space, the interpretation of all MS/MS spectra cannot be discussed here in detail. The detected fragment ions in the MS/MS spectra of sulphonated azodyes and of metal complex azodyes are listed in Table 1 and Table 2, respectively, except for a few detected impurities, whose concentrations were too low to allow the

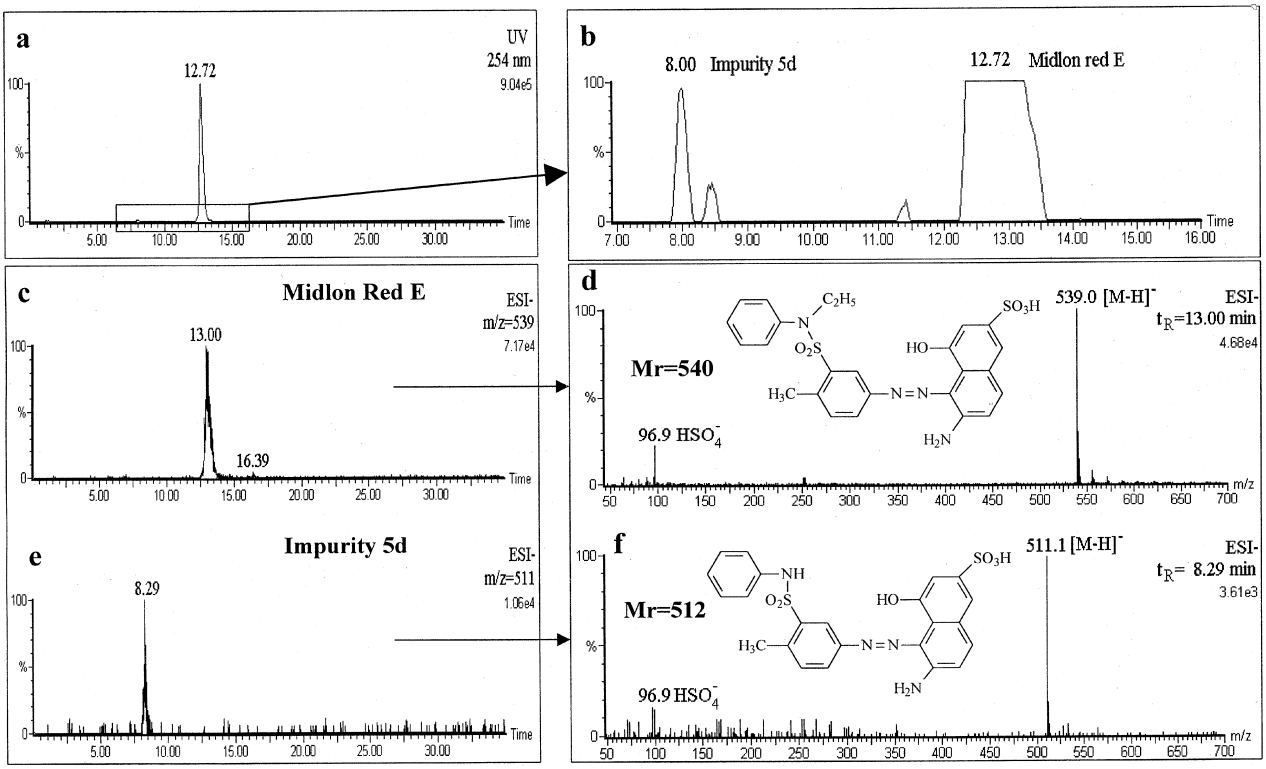

Figure 1. HPLC/MS analysis of the Midlon Red E dye (No. 5). (a) Chromatogram recorded with UV detection at 254 nm, (b) detail of the separation of impurities, (c, e) reconstructed ion current chromatograms of the main component and of the impurity 5d using negative-ion ESI-MS detection, (d, f) negative-ion ESI mass spectra of the main compound and of the impurity 5d.

measurement of the MS/MS spectra. Some impurities are attributed to positional isomers (such as the impurities 1a, 3a, 5a, 5b, 6a, 8a, and 8b) in the initial dyes, others to the by-products formed during the diazotisation and coupling reactions or to the reduction products of the azo group (the impurities 3b, 6b, 7b), to other technological impurities (the impurities 2a, 3e, 3h, 4a, 4b, 5c–5g, 7a, 7f, 7g, 8e), or, finally, to free ligands in metal complex Rylan dyes and their decomposition products (the impurities 6b, 6c, 7b, 7c, 8c, 8d). In agreement with previous work [27, 28], the fragmentation behaviour of sulphonated azo dyes shows some common features, such as the neutral losses of SO_2 or SO_3 from the sulphonic acid groups, the re-arrangement loss of N_2 from azo group, the losses of C_6H_4 or C_6H_6 from phenyl substituents. Mostly but not exclusively, even-electron fragment ions are observed in the MS/MS spectra. Further research in this direction is in progress.

3.3 HPLC/MS of the reduction products of azodyes

The interpretation of the mass spectra of large azodye molecules, which may contain several azo-bonds and aromatic ring systems, can be facilitated using additional information from the mass spectra of the products obtained by reduction cleavage of the azo group. In acidic medium, a dye molecule is reduced to two smaller molecules of aromatic amines, as illustrated in **Figure 4**. The dyes with two or more azo groups yield more than one different aromatic amines (in general, the number of the reduction products is equal to the number of the azo bonds plus one, except for symmetrical azo-compounds, such as Direct Red 7 in Figure 4). Reducible groups in the dye molecules, such as a nitro group in the Mordant Black 1 dye, are also reduced to an amino group (Figure 4). The molecular masses of the reduction products can be compared with the M_r of the original dye to provide information about the number of azo bonds, nitro groups, etc. If an azo dye does not contain other reducible groups, the sum of the M_r of the reduction products equals the M_r of the dye plus four times the number of azo bonds; a nitro group in a dye with a single azo bond such as Mordant Black 1 can be detected by the difference $\Delta m/z = 26$ between the molecular mass of the original dye and the sum of M_r of the reduction products, etc. (see examples in Figure 4). Generally, the number of azo bonds, n_{azo}, and of nitro groups, n_{nitro}, can be calculated from Eq. (1):

$$M_r(\text{azodye}) = \Sigma M_r(\text{reduction products}) - 4x(n_{azo}) + 30x(n_{nitro}) \quad (1)$$

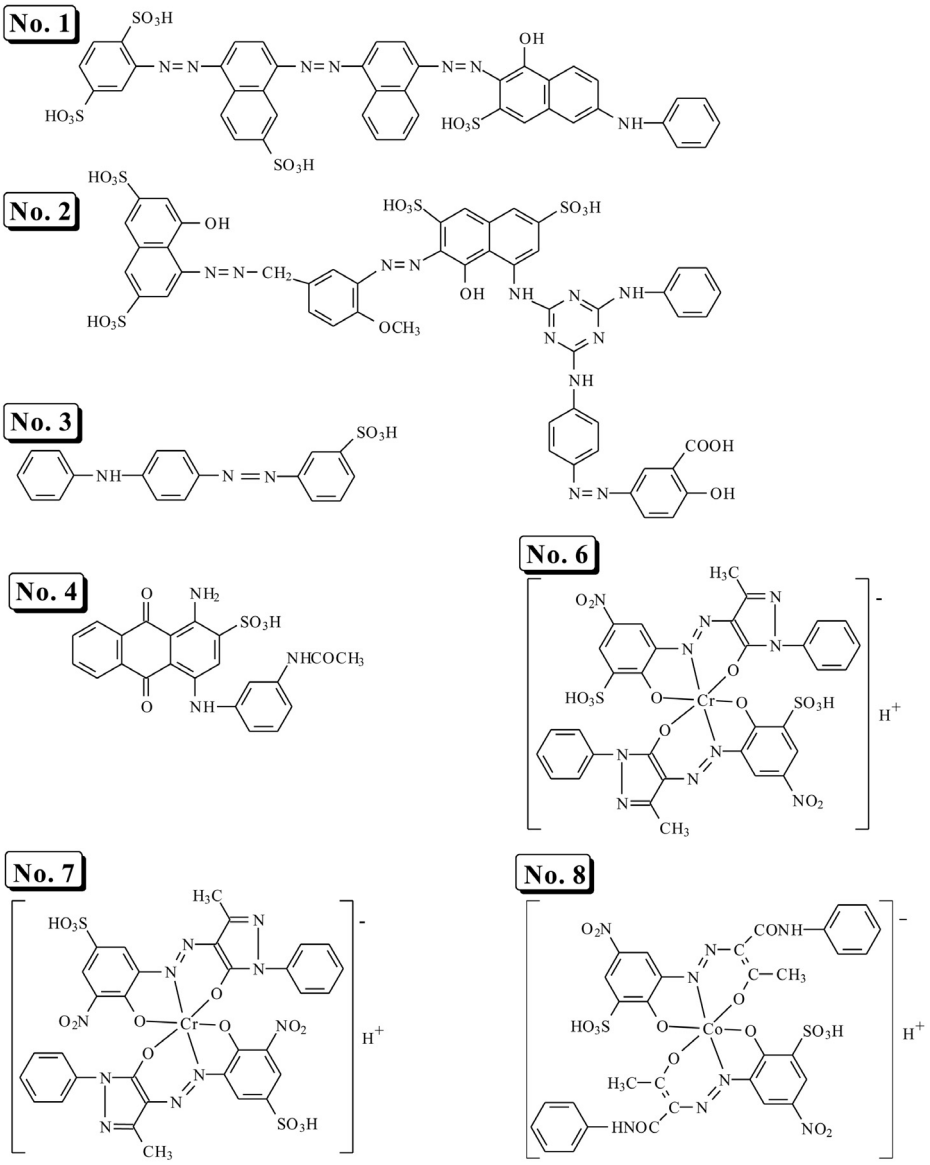

Figure 2. The structures of the sulphonated azo dyes (No. 1–4) and metal complex azo dyes (No. 6–8).

Several approaches can be applied for the reduction of azo dyes. The reduction method should be simple and robust. This is the case with the reduction by the hydrogen gas catalysed by Pd, which is rapid and does not require additional treatment of pure dye samples [29]. We employed another, more traditional method used for many years for volumetric quantitative determination of azo dyes, namely the reduction with titanium(III) chloride [30]. The reduction products were isolated by solid-phase extraction. This simple two-step procedure can be carried out within a few minutes.

Generally, the excess of the inorganic salts in the sample can suppress the mass spectrometric response and may cause the corrosion of the instrument and hence should be removed before injecting the sample into a HPLC/MS system. For this purpose, we employed solid-phase extraction of non-ionic reduction products on a C18 cartridge and sorption of metal cations on a small cation-exchange column, as described in detail in the Experimental section.

The mass spectra of the sulphonated and non-sulphonated reduction products require different ionisation techniques, hence different HPLC/MS methods were used for each type of compounds. Non-sulphonated products were analysed by reversed-phase HPLC in the HPLC/MS system 1 with positive-ion APCI, while the sulphonated products were analysed by ion-pairing HPLC in the HPLC/MS system 2 with negative-ion ESI-MS (see the Experimental section for details). Figure 4 illustrates the reduction scheme by three examples:

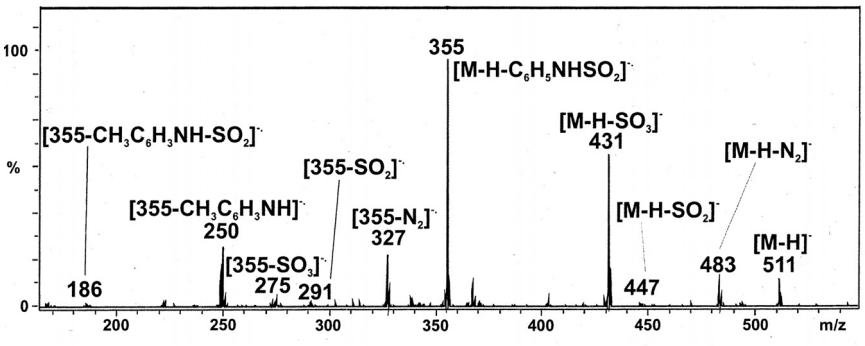

Figure 3. MS/MS spectrum of deprotonated molecule of the Midlon Red E impurity 5d with $M_r = 512$.

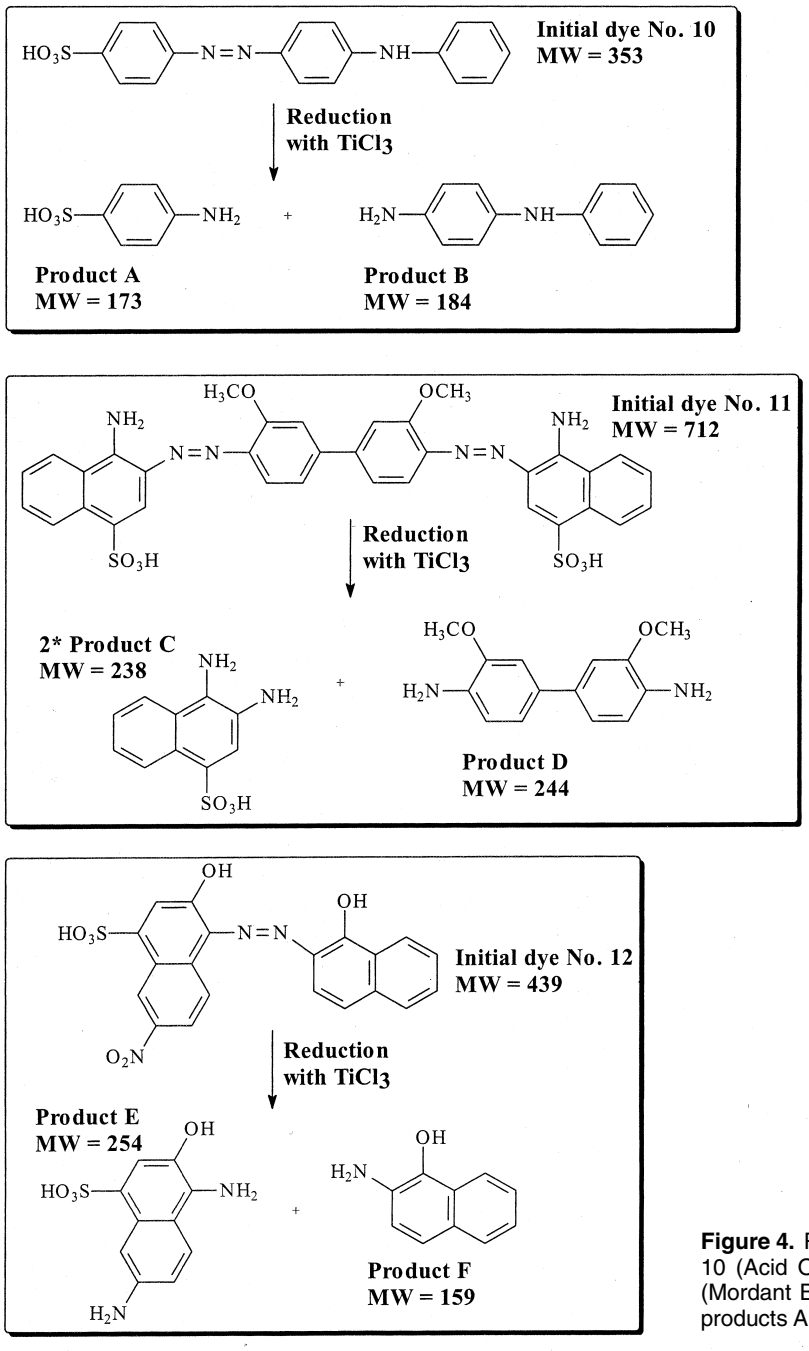

Figure 4. Reaction scheme of the reduction of dyes No. 10 (Acid Orange 5), No. 11 (Direct Red 7), and No. 12 (Mordant Black 1) showing the structures of the reaction products A–F (Table 3).

Table 3. Observed ions of sulphonated dyes (No. 10–12) and their reduction products in the negative-ion ESI mass spectra and positive-ion APCI mass spectra, with molecular masses determined from the (de)protonated molecules.

No.	Dye	Component	*m/z*	Significant ions	M_r	t_R [min]	S[a]	N[b]
10	C.I. Acid Orange 5	Initial dye	352	$[M-H]^-$	353	10.20[c]	1	1
		Product A	172	$[M-H]^-$	173	3.60[c]	1	–
		Product B	185	$[M+H]^+$	184	3.14[d]	0	–
			226	$[M+H+ACN]^+$				
11	C.I. Direct Red 7	Initial dye	711	$[M-H]^-$	712		2	2
			355	$[M-2H]^{2-}$				
		Product C	237	$[M-H]^-$	238	4.00[c]	1	–
		Product D	245	$[M+H]^+$	244	2.94[d]	0	–
			286	$[M+H+ACN]^+$				
12	C.I. Mordant Black 1	Initial dye	438	$[M-H]^-$	439		1	1
		Product E	253	$[M-H]^-$	254	4.00[c]	1	–
		Product F	160	$[M+H]^+$	159	2.56[d]	0	–

[a] Number of sulphonic acid groups in the molecule.
[b] Number of azo bonds in the initial dye.
[c] Ion-pairing HPLC, see Experimental part.
[d] Reversed-phase HPLC, see Experimental part.

1. The azo dye Acid Orange 5 with a single azo bond yields the sulphonated product A and a non-sulphonated amine B.

2. The symmetrically substituted azo dye Direct Red 7 with two azo bonds yields the reduction products C (sulphonated) and D (non-sulphonated).

3. The reduction of Mordant Black 1 with one nitro group yields a sulphonated diamine E and a non-sulphonated amine F.

The retention times in the two HPLC modes, the molecular masses determined on the basis of the $[M-H]^-$ ions in the negative-ion ESI mass spectra for the sulphonated products (A, C, and E) and from the $[M + H]^+$ and [M + H + acetonitrile]$^+$ ions in the positive-ion APCI mass spectra for the non-sulphonated products (B, D, and F) are listed in **Table 3**. The mass spectra of the products fully agree with the structures of the reduction products expected on the basis of the reduction scheme of the original dyes in Figure 4. No other significant peaks were found in the mass spectra of any of the three dyes tested (Nos. 10–12), which means that no reaction by-products are formed in significant concentrations during the reduction, unlike in the earlier work published by Kudlich et al. [29], who detected the auto-oxidation of the reduction products. However, they exposed the reduction products (*o*-aminohydroxynaphthalenes) to air at neutral pH for several hours, to investigate the progress of the oxidation, whereas we tried to avoid the contact of the reduction products with air by keeping the solutions in sealed vials for a short time before the HPLC/MS analysis.

We believe that our results prove the potential utility of HPLC/MS after previous chemical reduction for the structure elucidation of water soluble azo dyes. Of course, with unknown dyes or impurities, the MS/MS spectra of the reduction products containing peaks of the fragment ions will be necessary for complete structure determination. Further research is continuing in this direction, involving more complex dyes and HPLC/MS/MS methods.

4 Concluding remarks

HPLC/MS with triethylammonium acetate ion-pairing additive in the mobile phase was employed for the analysis of impurities in commercial (poly)sulphonated azodyes. For the first time, the impurities in polysulphonated dyes were analysed by HPLC/MS at a concentration level of tenths of a per cent. The structures of some impurities could be proposed on the basis of the molecular masses attributed to the ions observed in the negative-ion ESI mass spectra. The fragment ions detected in the MS/MS spectra are useful for structure confirmation. Combination of chemical reduction with HPLC/MS can be used for the structure determination of complex dyes, with separate experiments for sulphonated and non-sulphonated products.

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References

[1] R.M. Christie, *Colour Chemistry*, The Royal Society of Chemistry, Cambridge, UK 2001.

[2] P. Jandera, J. Churáček, B. Taraba, *J. Chromatogr.* **1983**, *262*, 121–140.

[3] P. Jandera, J. Fischer, V. Staněk, M. Kučerová, P. Zvoníček, *J. Chromatogr. A* **1996**, *738*, 201–213.

[4] P. Jandera, J. Churáček, *J. Chromatogr.* **1980**, *197*, 181–187.

[5] R.N. Rao, S.N. Alvi, S. Husain, *J. High Resol. Chromatogr.* **2000**, *23*, 329–332.

[6] P. Jandera, J. Churáček, J. Bartošová, *Chromatographia* **1980**, *13*, 485–492.

[7] G. Socher, R. Nussbaum, K. Rissler, E. Lankmayr, *Chromatographia* **2001**, *54*, 65–70.

[8] M. Holčapek, P. Jandera, P. Zderadička, *J. Chromatogr. A* **2001**, *926*, 175–186.

[9] T. Storm, T. Reemtsma, M. Jekel, *J. Chromatogr. A* **1999**, *854*, 175–185.

[10] C. Ràfols, D. Barceló, *J. Chromatogr. A* **1997**, *777*, 177–192.

[11] T. Reemtsma, *J. Chromatogr. A* **2001**, *919*, 289–297.

[12] T. Reemtsma, *Trends Anal. Chem.* **2001**, *20*, 500–517.

[13] M. Holčapek, P. Jandera, J. Přikryl, *Dyes Pigm.* **1999**, *43*, 127–137.

[14] C. Baiocchi, M.C. Brussino, E. Pramauro, A.B. Prevot, L. Palmisano, G. Marci, *Int. J. Mass Spectrom.* **2002**, *214*, 247–256.

[15] M.C. Alonso, M. Castillo, D. Barceló, *Anal. Chem.* **1999**, *71*, 2586–2593.

[16] K. Lemr, M. Holčapek, P. Jandera, A. Lyčka, *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1881–1888.

[17] K. Lemr, M. Holčapek, P. Jandera, *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1878–1879.

[18] A. Lyčka, D. Luštinec, J. Holeček, M. Nádvorník, M. Holčapek, *Dyes Pigm.* **2001**, *50*, 203–209.

[19] M.R. Fuh, K.J. Chia, *Talanta* **2002**, *56*, 663–671.

[20] A.G. Sullivan, R. Garner, S.J. Gaskell, *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1207–1215.

[21] A. Conneely, S. McClean, W.F. Smyth, G. McMullan, *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2076–2084.

[22] A. Weisz, D. Andrzejewski, H.M. Fales, A. Mandelbaum, *J. Mass Spectrom.* **2002**, *37*, 1025–1033.

[23] J. Yinon, T.L. Jones, L.D. Betowski, *Biomed. Environ. Mass Spectrom.* **1989**, *18*, 445–449.

[24] H. Chromá-Keull, J. Havliš, J. Havel, *Rapid Commun. Mass Spectrom.* **2000**, *14*, 40–43.

[25] A. Ballantine, D.E. Games, P.S. Slater, *Rapid Commun. Mass Spectrom.* **1997**, *11*, 630–637.

[26] P. Jandera, *J. Chromatogr.* **1984**, *314*, 3–36.

[27] A.P. Bruins, L.O.G. Weidolf, J.D. Henion, W.L. Budde, *Anal. Chem.* **1987**, *59*, 2647–2652.

[28] P.O. Edlund, E.D. Lee, J.D. Henion, W.L. Budde, *Biomed. Environ. Mass Spectrom.* **1989**, *18*, 233–240.

[29] M. Kudlich, M.J. Hetheridge, H.-J. Knackmuss, A. Stolz, *Environ. Sci. Technol.* **1999**, *33*, 896–901.

[30] E. Knecht, E. Hibbert, *Ber. Dtsch. Chem. Ges.* **1903**, *36*, 1549.