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Effects of functional groups on the fragmentation of dyes in electrospray and atmospheric pressure chemical ionization mass spectra

Michal Holčapek*, Kateřina Volná, Dana Vaněrková

University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, nám. Čs. legií 565, 532 10 Pardubice, Czech Republic

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Abstract

Electrospray ionization (ESI) is most suitable for dyes with an ionic or very polar character, while atmospheric pressure chemical ionization (APCI) suits better those dyes and pigments with low to medium polarity. The negative-ion mode of ESI is used for anionic dyes such as (poly)-sulfonated and/or (poly)sulfated variants, where the series of deprotonated molecules enables an easy determination of molecular weights and the number of acid groups. The positive-ion APCI or ESI mass spectra of non-ionic and cationic dyes and pigments provide typical molecular adducts $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ used for the molecular assignment. The first-order mass spectra often suffer from the lack of fragment ions, which can be solved by using multistage tandem mass spectra (MS^n) measured with an ion trap analyzer. The effects of various functional groups on the fragmentation behavior are described herein. The detailed understanding of fragmentation paths enables the correlation among particular functional groups and the fragmentation patterns. The presented approach is applicable for the structural analysis of complex mixtures using HPLC/MS/MS techniques with volatile ion-pairing agents.

Keywords: Mass spectrometry; Electrospray ionization; Atmospheric pressure chemical ionization; Dye; Azo; Sulfonic; Sulfate

1. Introduction

Dyes are important industrial products with widespread applications and are produced annually in huge quantities. Their classification is based on chemical structure (Color Index names) or their application field (food dyes, textile dyes, spectral sensitizers, etc.). Due to the continuous development of new products with improved dyeing properties and lower environmental impact, it is important to have suitable analytical tools for the structural confirmation or elucidation of new dyes, their byproducts, synthetic impurities and degradation products.

Mass spectrometry (MS) with atmospheric pressure ionization has become a standard, very powerful technique for the identification of dyes [1]. MS is a superior spectral technique for metal-complex dyes, polysulfonated dyes and in general for dye mixtures, for which nuclear magnetic resonance (NMR) may have troubles because of paramagnetic impurities or the complexity of such mixtures. Electrospray ionization (ESI) is the most suitable for dyes with an ionic or very polar character, while atmospheric pressure chemical ionization (APCI) gives better results for dyes and pigments with low to medium polarity [1]. New ionization technique, atmospheric pressure photoionization (APPI), has a similar application field to APCI, but it extends the polarity range a little bit towards non-polar compounds [2]. The negative-ion mode of ESI is used for anionic dyes such as (poly)sulfonated and/or (poly)sulfated dyes [3–7], where the series of deprotonated molecules [7–9] enables an easy molecular weight (MW) determination. Moreover, the total number of acid groups may be determined on the basis of the ion with a maximum charge or

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* Corresponding author.
E-mail address: michal.holcapek@upce.cz (M. Holčapek).

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a maximum number of protons replaceable by sodium ions [7]. Matrix-assisted laser desorption/ionization (MALDI) has been demonstrated [9,10] as a suitable alternative for the MW determination of dyes containing one to six sulfonic acid groups. On the other hand, the positive-ion mode of APCI or ESI is suitable for non-ionic and cationic dyes and pigments, where the typical molecular adducts $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ are formed [11,12]. In addition to ESI, APCI and MALDI, fast atom bombardment (FAB) and secondary ion mass spectrometry (SIMS) coupled to time of flight (TOF) mass analyzer can be used [13,14], but atmospheric pressure ionization techniques are prevailing in the current mass spectrometric practice due to the several advantages, such as easy coupling to liquid-separation techniques, better sensitivity, signal stability and reproducibility.

Recently, the combination of liquid-phase separation techniques with mass spectrometry has been established in the separation of complex mixtures as well as the identification and structural elucidation of dyes [7,15–20], for example HPLC/MS identification of dye degradation products [16,21–27] in food or environmental samples attracts a lot of attention nowadays. In connection with soft ionization techniques, tandem mass spectrometry (MS/MS) or multiple-stage tandem mass spectrometry (MS^n) is essential, because of the lack of fragment ions in the first-order mass spectra [16,21,25]. The HPLC methods for the analysis of medium and low polar dyes employ the common reversed-phase C18 column packing and mobile phases consisted of aqueous methanol or acetonitrile. Unfortunately, such chromatographic systems are not appropriate for ionic dyes (e.g., sulfonic acids and sulfates), where the ionic additives in the mobile phase have to be used to increase the retention. Conventional non-volatile additives, such as high concentrations of inorganic salts for salting-out chromatography or tetrabutylammonium hydrogensulfate as a non-volatile ion-pairing agent [28], are not compatible with mass spectrometric detection. For HPLC/MS coupling, more volatile ion-pairing agents (di- and trialkylammonium acetates) can successfully replace conventional non-volatile agents [7,15–27] as a reasonable compromise between the separation selectivity and mass spectrometric performance, but there are still some problems with signal suppression and memory effects [29]. The past few years have seen a demand for the development of analytical methods for the identification and quantification of the 'Sudan' type of azo dyes in foodstuffs due to the contamination of hot chilli products originating from Asia and some southern European countries by these banned dyes. A method based on capillary HPLC/MS/MS for the simultaneous identification and determination of Sudan I, II, III and IV in chilli tomato and cheese sauce has been reported [30] and later an accurate mass measurement for the confirmation of these Sudan dyes using tandem quadrupole orthogonal-acceleration TOF mass spectrometry is used [31]. The use of high-resolution MS techniques such as TOF analyzer improves the identification of the analytes of interest by providing the elemental composition. In addition, accurate mass measurements of product ions in MS/MS mode help to elucidate unknown and structural rearrangements of fragment ions.

The main goal of this current work is a generalization of the fragmentation behavior of various dye classes with respect to the relation between the fragmentation and the presence of individual functional groups. This generalization is based on the measurement of more than 400 various dyes, dyestuffs' impurities and byproducts measured by direct infusion or using HPLC/MS with ESI and APCI ionization techniques during last 8 years.

2. Experimental

2.1. Materials

Acetonitrile and methanol for HPLC were purchased from Merck (Darmstadt, Germany). Water was deionised using Demiwa 5-roi purifyng system (Watek, Ledeč nad Sázavou, Czech Republic). The solvents were filtered through 0.45 μm Millipore filter prior to use and degassed by continuous stripping by a stream of helium. The samples of dyes were obtained from Synthesia (Pardubice, Czech Republic) or from the cooperation with the Institute of Polymeric Materials and with the Department of Technology of Organic Compounds, both at the University of Pardubice. Triethylamine and dihexylamine (purity > 99%) were purchased from Aldrich (Milwaukee, WI, USA) and acetic acid from Sigma (St. Louis, MO, USA). Triethylammonium acetate (TEAA) or dihexylammonium acetate (DHAA) was prepared by mixing of equimolar amounts of triethylamine (or dihexylamine) and acetic acid and used as an ion-pairing reagent in HPLC/MS experiments [7,16,27,30].

2.2. Solid-phase extraction of ionic dyes

Three different solid-phase extraction principles were used for the pretreatment (salt removal) of dye solutions, reversed-phase, anion-exchange and cation-exchange columns. For nonpolar treatment mechanism, octadecyl silica cartridge columns Separon SGX C18 from Tessek (Prague, Czech Republic), Zorbax C18 from Agilent Technologies (Waldbronn, Germany) and polymer columns LiChrolut EN (Merck, Darmstadt, Germany) were tested. The sorbent conditioning was accomplished by passing 2 ml of methanol and 2 ml of water through the cartridge. One milliliter of the dye solution was passed through, then the cartridge was washed with 2 ml of water and the elution step was done with 2 ml of methanol. Sulfonated dyes were not retained under such conditions, thus ion-pairing mechanism with TEAA or DHAA was used; 2.5 mmol/l of the ion-pairing reagent was added in the conditioning and loading step. Separon SGX NH2 from Tessek (Prague, Czech Republic) were used as anion-exchange columns. Conditioning of the sorbents was accomplished by passing 2 ml of methanol, 2 ml of water and 1 ml of 0.2% (v/v) formic acid through the cartridge. One milliliter of the dye solution was passed through, then the cartridge was washed with 2 ml of water and the elution step was done with 2 ml of 50% (v/v) methanol in water with 5% (v/v) ammonium acetate. The cation-exchange column Supelclean LC-SCX (3 ml) from

Supelco (Bellefonte, USA) was used for the removal of alkali metal ions. The sorbent conditioning was carried out by passing 6 ml of 0.05 mol/l perchloric acid, 10 ml of water, and 1 ml of the dye solution was introduced and the effluent was immediately collected to get the desalted solution used for MS measurements.

2.3. Instrumentation

An Esquire 3000 ion trap mass analyzer (Bruker Daltonics, Bremen, Germany) was used for all experiments either with ESI or APCI probe. The individual samples were dissolved in 50% aqueous acetonitrile or methanol (less polar dyes in 100% acetonitrile) and introduced into the ion source by the infusion pump (COLE Palmer Instrument Company, Vernon Hills, IL, USA) at the flow rate 1–5 μL/min. The mass spectrometer was tuned to obtain a maximum response for sample compounds. The source parameters for ESI were as follows: flow rate of drying gas 4 l/min, pressure of nebulizing gas 10 psi, and temperature of drying gas 300 °C. The source parameters for APCI were as follows: flow rate of drying gas 4 l/min, pressure of nebulizing gas 10 psi, and temperature of drying gas 330 °C. The target mass was set according to expected masses of studied dyes, the isolation width for tandem mass spectrometric experiments was $\Delta m/z = 4$. The detailed conditions for dyes measured by HPLC/MS technique were described in our previous works [5,7,16,27].

3. Results and discussion

3.1. Molecular weight (MW) determination

The MW determination is the first and the basic information obtained from the interpretation of mass spectra. The right choice of the ionization technique is an important step in the procedure of mass spectra interpretation. In the past, the conventional electron ionization (EI) was the most widespread ionization technique, but its application is rather limited only for compounds with a sufficient volatility, low to medium polarity and MWs at maximum about 800 Da. The MW with EI is determined from the presence of radical-cation $M^{+\bullet}$, but this ion is missing for about 10% of compounds amenable to EI-MS due to the extensive fragmentation. The advent of atmospheric pressure ionization techniques (ESI, APCI and APPI) has opened a new era in the mass spectrometry of dyes, because it enables the analysis of dyes over the full range of polarities and MWs.

Non-polar and cationic dyes are measured in the positive-ion mode, where they provide the characteristic molecular adducts $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$. The protonated molecule $[M+H]^+$ is a typical base peak (i.e., 100% relative abundance) in the first-order mass spectra accompanied by less abundant adduct ions with alkali metals with relative abundances depending on the salt concentration in the solution. If ammonium ions are also present in the solution, then the $[M+NH_4]^+$ adduct ions can be expected as well.

The negative-ion ESI mass spectrometry is routinely applied for the MW determination of polar and ionic dyestuffs and the total number of acid groups. Alternatively, negative-ion APCI can be used for that purpose, but the sensitivity is worse, the fragmentation is increased and this technique is limited to compounds containing two sulfonic acid groups at maximum unlike ESI, where no limitation is found concerning the total number of sulfonic acid groups. The detailed procedure for the determination of the total number of sulfonic and carboxylic acid groups from the series of $[M-xH]^{x-}$ ions and their sodiated adducts $[M-(x+y)H+yNa]^{x-}$ was described previously [5]. Briefly, the MW is determined from the $[M-xH]^{x-}$ series and the number of acid groups is equal to the highest observed charge or the highest number of protons replaceable by sodium ions in $[M-(x+y)H+yNa]^{x-}$ ions. For 2:1 *o*,*o*′-dihydroxyazodye complexes with three valent metal ions (Al^{3+}, Cr^{3+}, Co^{3+}, Fe^{3+}, etc.), the total number of acidic groups is increased by one because these complexes carry one negative charge themselves [32–36], i.e., non-sulfonated metal-complexes provide only $[M-H]^-$ ions [32–34] and sulfonated/sulfated metal-complexes [36] provide the series of multiply charged ions increased by one charge.

3.2. Correlation between the fragmentation and individual functional groups

The fragment ions usually have only low relative abundances in the first-order spectra or they are totally absent. This drawback may be overcome by tandem mass spectrometric (MS/MS) experiments, where the selected precursor ion is isolated first by the mass analyzer, then the collision activation of this ion is performed yielding the fragment ions and obtained fragment ions are recorded in the so-called product-ion scan (i.e., MS/MS spectrum). It is important to understand the relation between the fragmentation behavior and the presence of individual functional groups and structural features in dye molecules, because it is essential for the interpretation of MS/MS spectra of unknowns.

On the basis of mass spectrometric measurements of more than 400 various dyes and related compounds of various chemical classes, the typical neutral losses have been correlated with individual functional groups and summarized in Table 1. This list is not comprehensive, but rather focuses on functionalities with a strong effect on the ionization and fragmentation behavior. The functional groups are listed in the order of their influence on the extent of the fragmentation: sulfate group, sulfonic group, carboxylic group, nitro group, etc. This order may be roughly applied for the interpretation of mass spectra of compounds (not only dyestuffs) containing multiple functional groups, but of course different functional groups may exhibit a competitive behavior during the fragmentation, so this order should not be applied too strictly. For a better understanding of basic fragmentation mechanisms of individual functional groups, this table is supported by Fig. 1, where the characteristic neutral losses are schematically drawn with *m*/*z* values of corresponding

Table 1
Typical neutral losses observed in ESI mass spectra for individual functional groups

Functional group	Observed ions	Comments
$-OSO_3H$	$[M-H-H_2SO_4]^-$, $[M-H-SO_3]^-$, $[M-H-SO_2]^-$, $[HSO_4]^-$	Very low abundance of $[M-H]^-$, $[M-H-H_2SO_4]^-$ is usually the base peak in the spectrum, subsequent losses for polysulfates, no retention in reversed-phase HPLC without ion-pairing agents
$-SO_3H$	$[M-H-SO_3]^-$, $[M-H-SO_2]^-$, $[SO_3]^{-\bullet}$, rarely $[SO_2]^{-\bullet}$	Subsequent losses for polysulfonated acids, adducts with sodium ions $[M-(x+y)H+yNa]^{x-}$, no retention in reversed-phase HPLC without ion-pairing agents
$-COOH$	$[M-H-CO_2]^-$	CO_2 is a typical first cleavage, when SO_3H and OSO_3H groups are absent, low retention in reversed-phase HPLC
$-NO_2$	$[M-H-NO]^-$, $[M-H-NO_2]^-$, low abundant $[M-H-O]^-$	Radical-ion $M^{-\bullet}$ may be present instead of $[M-H]^-$ in negative-ion APCI
$-N{=}N-$	$[M-H-N_2]^-$	Rearrangement loss of N_2 from an azo bond, cleavages of or next to the azo bond
$-OH$	$[M-H-H_2O]^-$ or $[M-H-CO]^-$	Loss of water or shortening of an aromatic ring by one carbon atom for phenols
$-Cl$	$[M-H-HCl]^-$, $[Cl]^-$	For polychlorinated compounds, subsequent losses of HCl and decreased relative abundance of $[M-H]^-$, characteristic isotopic ratio $^{35}Cl{:}^{37}Cl=3{:}1$
$-Br$	$[M-H-HBr]^-$, $[Br]^-$	For polybrominated compounds, subsequent losses of HBr and decreased relative abundance of $[M-H]^-$, characteristic isotopic ratio $^{79}Br{:}^{81}Br=1{:}1$
$-CO-$	$[M-H-CO]^-$	Shortening of an aromatic ring by one carbon atom for anthraquinone dyes

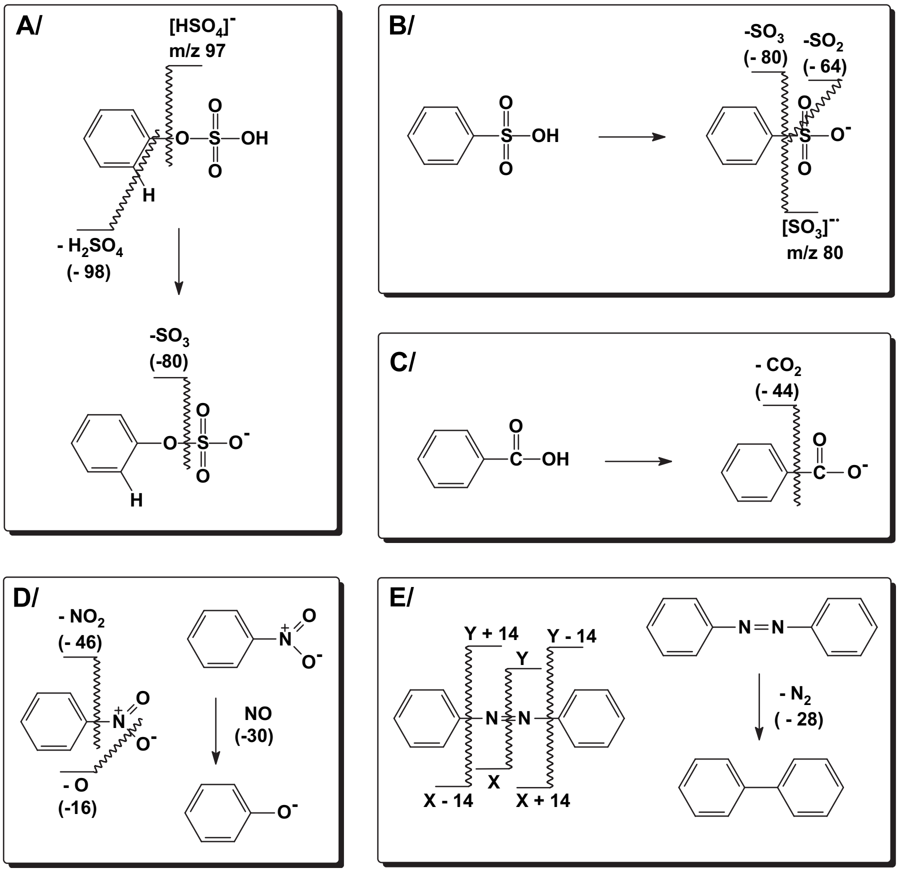

Fig. 1. Characteristic fragmentation behavior and logical neutral losses for selected functional groups: (A) Sulfate, (B) sulfonic acid, (C) carboxylic acid, (D) nitro group, (E) azo group.

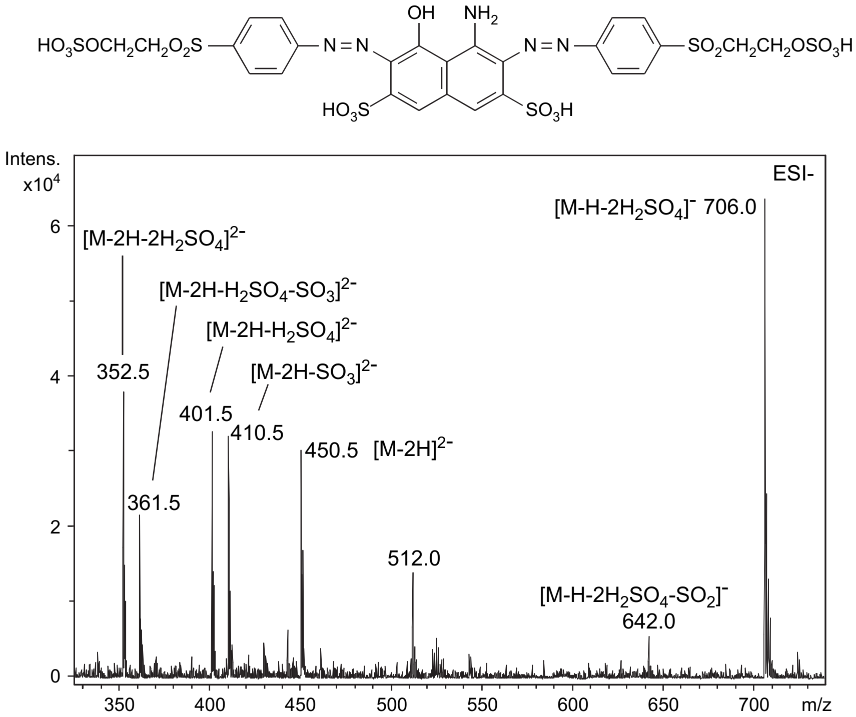

Fig. 2. Negative-ion ESI mass spectrum of C.I. Reactive Black 5 (MW = 903).

neutral losses. The characteristic examples of mass spectra of dyes with various functional groups are shown in Figs. 2–6 to illustrate the correlation between the structure and typical neutral losses.

3.2.1. Sulfates and sulfonates

Generally, the presence of sulfate group(s) causes the reduced stability of quasimolecular ions resulting in lower relative abundances of peaks of deprotonated molecules $[M-H]^-$, which may cause troubles with the MW assignment for polysulfated compounds. Moreover, the sulfate group is usually present in the form of sodium salt (similarly as for sulfonic acids), so the mass spectra also show the abundant adducts with sodium ions. The mass spectrometric convention [5–7,9,10,14–16,21,24,27,37] for labeling the observed ions starts from the neutral molecule (for example $ROSO_3H$ or RSO_3H) regardless the fact that these ionic compounds are usually present as salts in the solid state (i.e., $ROSO_3Na$, RSO_3Na). In the solution, these salts are dissociated into $ROSO_3^-$ and Na^+ ions (respectively RSO_3^- and Na^+ ions), which is also reflected in measured mass spectra. For polysulfated and/or polysulfonated dyes, $[M-H]^-$ ions are observed together with sodium adducts, such as $[M-2H+Na]^-$, $[M-3H+2Na]^-$, etc., in general $[M-(x+y)H+yNa]^{x-}$ ions [5,7]. When HPLC/MS technique is used, then the relative abundances of sodium adducts are significantly reduced or they disappear completely [16,27]. Occasionally, different labeling based on the sodium salt form is used, which may cause some confusion for readers. In our opinion, the ion labeling should reflect the form in the liquid-phase state, because ESI is a liquid-phase ionization technique.

The most suitable ionization technique for sulfates and sulfonates is the negative-ion ESI. For monosulfated compounds, the typical ions are $[M-H]^-$, $[M-H{-}H_2SO_4]^-$ and $[HSO_4]^-$. Unlike polysulfated compounds, the intensity of $[M-H]^-$ is always sufficient for unambiguous determination of MW. An example of the first-order ESI mass spectrum of commercial dye C.I. Reactive Black 5 with two sulfate and two sulfonic acid groups is shown in Fig. 2. At a first glance, this spectrum shows an increased level of background noise and surprisingly a high extent of fragmentation for soft ionization technique, but both are typical for polysulfated compounds. No ions are observed in the region of molecular weight MW = 903. Two observed ions are singly charged (*m/z* 706 and 642), while the others are doubly charged (*m/z* 450.5, 410.5, 401.5, 361.5 and 352.5). The charge of individual ion can be determined on the basis of mass difference between isotopic peaks, which is one for singly charged ions, 1/2 for doubly charged ions, 1/3 for triply charged ions or generally $1/x$ for ions with x charges. It may be seen after zooming of individual doubly charged ions in Fig. 2 (not shown) that the isotopic differences are 0.5 *m/z* units. The MW is determined from $[M-2H]^{2-}$ ion. Following subsequent losses of SO_3 and H_2SO_4 confirm the presence of at least two sulfate/sulfonic groups. The singly charged fragment ion at *m/z* 642 $[M-H{-}2H_2SO_4{-}SO_2]^-$ shows that at least three sulfate/sulfonic groups are in the structure and finally the doubly charged ion $[M-2H{-}2H_2SO_4]^{2-}$ at *m/z* 352.5 confirm the presence of two sulfates plus two other acid groups, which is in agreement with the structure of C.I. Reactive Black 5 (Fig. 2). High salt content in this sample caused not only the increased noise and decreased signal stability, but also

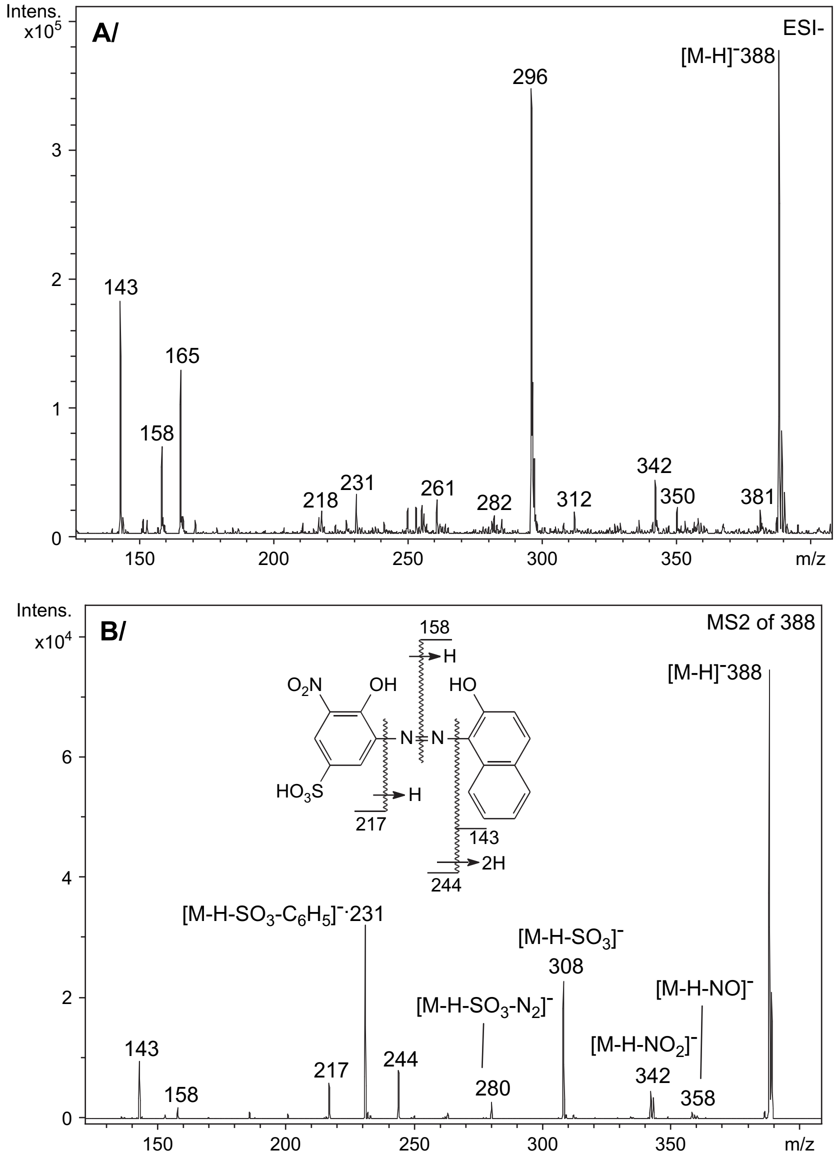

Fig. 3. Negative-ion ESI mass spectra of C.I. Mordant Black 15 (MW = 389): (A) first-order spectrum, (B) MS/MS spectrum of *m*/*z* 388.

disabled the measurement of tandem mass spectra. This spectrum is a typical representative spectrum of polysulfated compound.

The negative-ion ESI mass spectrum of relatively simple monosulfonated monoazo dye C.I. Mordant Black 15 (MW = 389) containing one nitro group, two hydroxyl groups, one benzene and one naphthalene aromatic system is shown in Fig. 3. The deprotonated molecule $[M - H]^-$ is the base peak in the first-order spectrum (Fig. 3A), but there are also observed some fragment ions (i.e., *m*/*z* 342, 231, 158 and 143) and other unexplained ions corresponding most probably to sample impurities, such as *m*/*z* 381, 350, 296, 165, etc. The first-order spectrum does not enable to distinguish, which peaks are the fragment ions and which ones belong to the impurities, but fortunately there are two ways how to differentiate them: MS/MS of deprotonated molecule $[M - H]^-$ at *m*/*z* 388 or HPLC/MS. Coupling to HPLC is time-consuming and needs some optimization of chromatographic conditions to achieve acceptable separation selectivity and in case of ionic dyes, there are additional problems due to the use of ion-pairing agents. MS/MS experiment without HPLC separation enables to distinguish easily the fragment ions from sample impurities (Fig. 3B). The MS/MS spectrum yields the characteristic fragment ions corresponding to the nitro group (i.e., $[M - H{-}NO]^-$ ion at *m*/*z* 358, $[M - H{-}NO_2]^-$ ion at *m*/*z* 342), the sulfonic group (i.e., $[M - H{-}SO_3]^-$ ion at *m*/*z* 308), the rearrangement loss of N_2 from the azo group (i.e., $[M - H{-}SO_3{-}N_2]^-$ ion at *m*/*z* 280) and other fragments arising from the cleavages of or next to the azo bond (*m*/*z* 143,158, 217 and 244). In a low-mass region, the radical anion $[SO_3]^{-\bullet}$ (*m*/*z* 80) as a typical marker for the presence of sulfonic acid may be observed. Rarely, the loss of radicals $HSO_3^{-\bullet}$ for sulfonates or $HSO_4^{-\bullet}$ for sulfates may occur instead of usual neutral losses of SO_3 or H_2SO_4 [37].

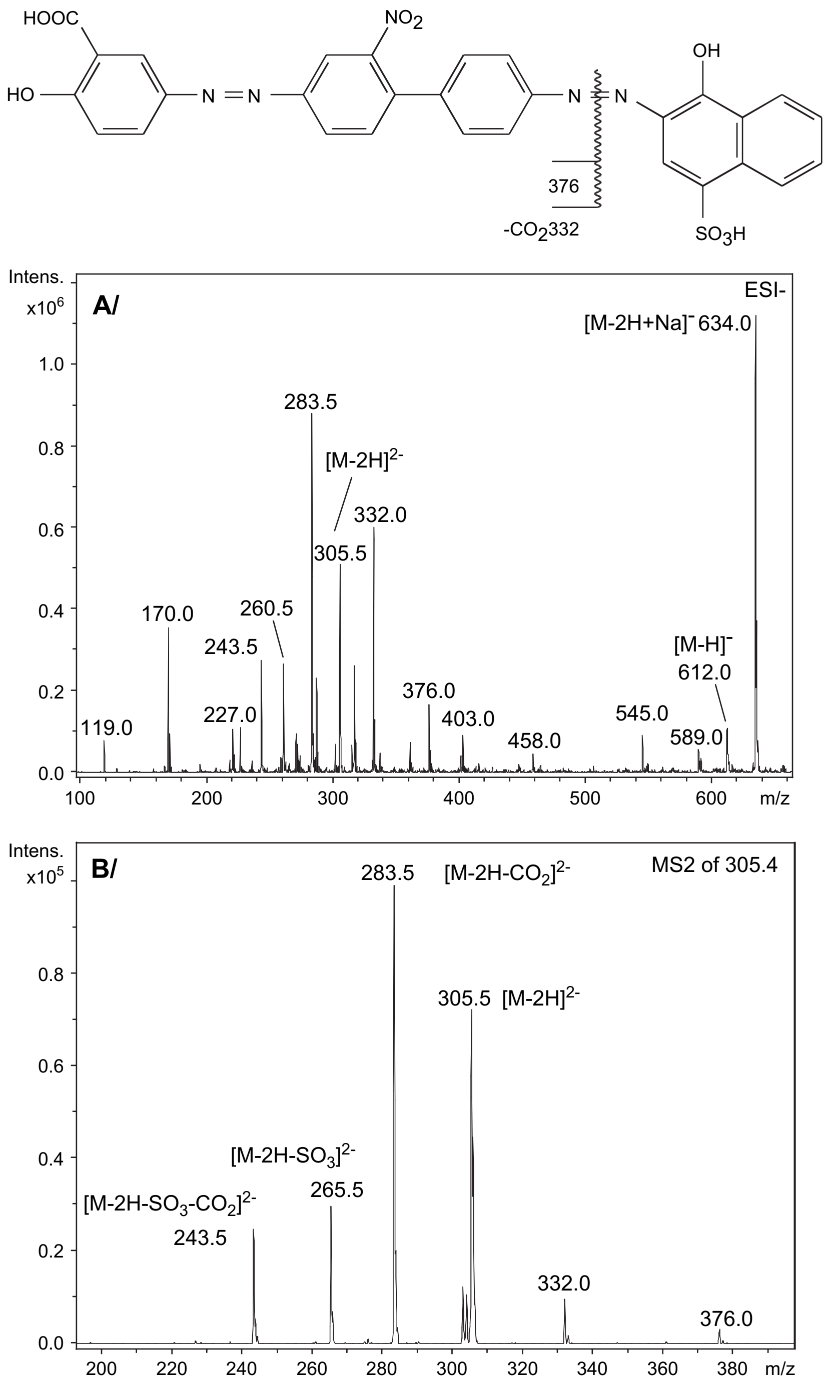

Fig. 4. Negative-ion ESI mass spectra of C.I. Mordant Red 8 (MW = 613): (A) first-order spectrum, (B) MS/MS spectrum of *m/z* 305.5.

3.2.2. Carboxylic acids

When sulfate and sulfonic acid groups are absent, then the neutral loss of CO_2 ($\Delta m/z$ 44) is the first typical fragmentation path for carboxylic acids. This neutral loss is very characteristic for carboxylic acids and it is usually observed already in the first-order APCI or ESI mass spectra with lower relative abundances. Both ESI and APCI in the negative-ion mode are suitable for the measurement of carboxylic acids, the ionization and fragmentation behavior is the same. Fig. 4 depicts the ESI mass spectra of C.I. Mordant Red 8 (MW = 613) containing one sulfo group, one carboxylic acid group, one nitro group, two hydroxyl groups and two azo bonds. In addition to the ions important for MW determination (i.e., $[M-2H+Na]^-$ ion at *m/z* 634, $[M-H]^-$ ion at *m/z* 612 and $[M-2H]^{2-}$ ion at *m/z* 305.5), the first-order spectrum (Fig. 4A) exhibits many other ions corresponding either to fragment ions arising from the target molecule or impurities in the sample. The tandem mass spectrum (Fig. 4B) is much easier for the interpretation and the logical neutral losses enable the identification of some functionalities, e.g., carboxyl and sulfonic acid groups ($[M-2H{-}CO_2]^{2-}$ ion at *m/z* 283.5, $[M-2H{-}SO_3]^{2-}$ ion at *m/z* 265.5 and $[M-2H{-}SO_3{-}CO_2]^{2-}$ ion at

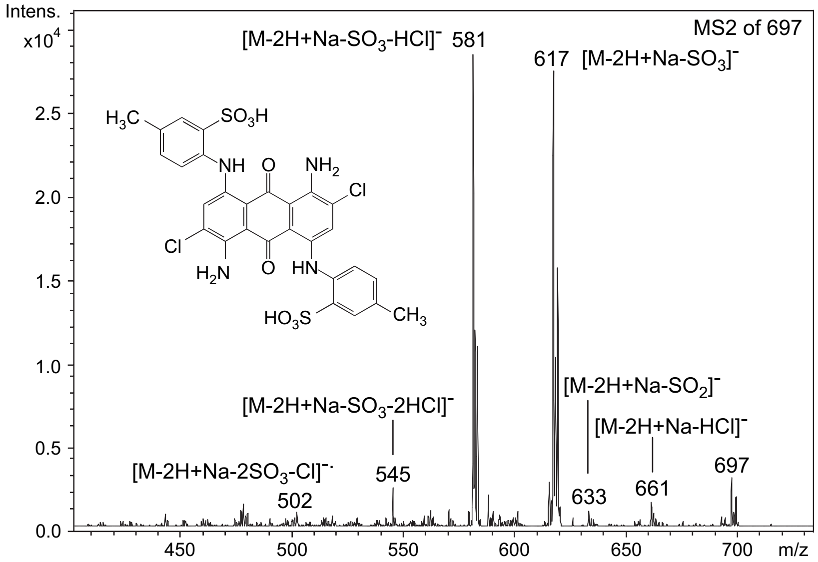

Fig. 5. Negative-ion ESI-MS/MS spectrum of *m/z* 697 of C.I. Acid Blue 81 (MW = 676).

m/z 243.5). The other observed ions correspond to the cleavage of the azo bond (*m/z* 376) and the following loss of CO_2 from this ion (*m/z* 332). When sulfate/sulfonic groups are absent, then the compound with one carboxylic acid and some functionality with sufficient proton affinity can provide a signal also in the positive-ion mode, but the sensitivity is worse in comparison to the negative-ion mode.

3.2.3. Nitrogen containing functional groups

Basically, all nitrogen containing compounds must follow the so-called nitrogen rule, which is very useful for the interpretation of mass spectra. It says that odd MW automatically means odd number of nitrogen atoms in the structure, while even MW means even number or absence of nitrogen atoms. This rule is valid for all compounds made from common organic elements and in general for all elements with either both odd atomic mass and odd valence or both even atomic mass and even valence. Nitrogen is the only element having even atomic mass (14) and odd valence (3). The presented structures in Figs. 2–6 may be used as examples, where odd MWs correspond to odd nitrogen number (MW = 903 in Fig. 2 for five nitrogen atoms, MW = 389 in Fig. 3 for three nitrogen atoms, MW = 613 in Fig. 4 for five nitrogen atoms and MW = 379 in Fig. 6 for one nitrogen atom), while even MW means even number or absence of nitrogens (MW = 676 in Fig. 5 for four nitrogen atoms). The effect of nitro group on the fragmentation is clearly demonstrated in Fig. 3B, where the logical neutral losses of NO_2 (ion at *m/z* 342) and of NO (ion at *m/z* 358) from deprotonated molecule $[M-H]^-$ are observed. The same figure illustrates all possible

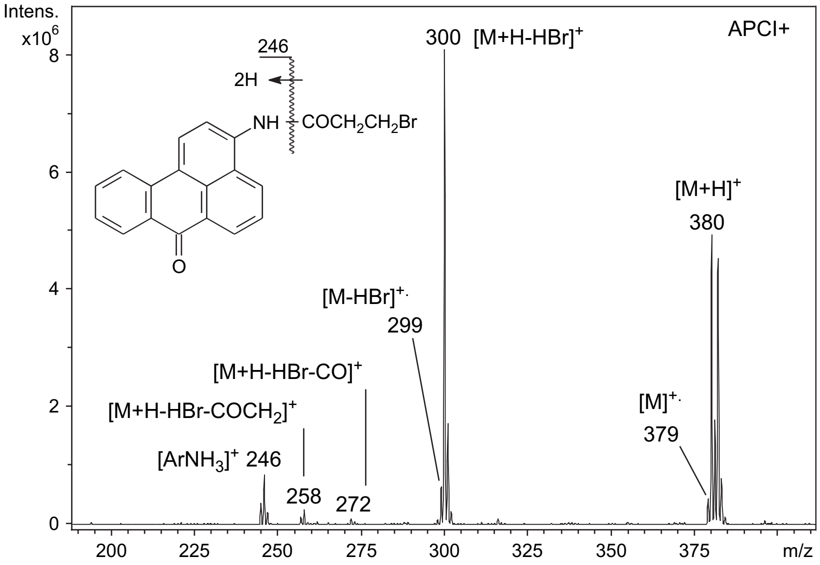

Fig. 6. Positive-ion APCI mass spectrum of *N*-(7*H*-benz[*de*]anthracen-7-on-3-yl)-3-brompropanamide (MW = 379).

cleavages associated with the azo bond including the rearrangement loss of N_2 (the transition from m/z 308 $[M-H-SO_3]^-$ to m/z 280 $[M-H-SO_3-N_2]^-$), the cleavage of the azo bond (ion at m/z 158) and next to the azo bond (ions at m/z 143, 217 and 244). Mentioned losses are schematically drawn in Fig. 3B for a better understanding together with the numbers of rearranged hydrogen atoms.

The primary amino group usually does not provide a neutral loss of NH_3, as one may expect. For secondary and tertiary amines, the corresponding ammonium ions are formed, for example the ion at m/z 246 in Fig. 6.

3.2.4. Halogen atoms

The chlorine and bromine atoms have characteristic pattern of isotopic peaks (^{35}Cl:$^{37}Cl = 3:1$ for chlorine and ^{79}Br:$^{81}Br = 1:1$ for bromine), which may be used for an easy identification of the presence and also the number of chlorine and bromine atoms in particular ions in mass spectra including both quasimolecular and fragment ions. When more chlorine or bromine atoms are present, then their isotopic ratios can be calculated by binomial equation $(3a+b)^n$ for chlorine or $(a+b)^n$ for bromine, where n is the number of chlorine or bromine atoms. When both chlorine and bromine atoms are present, then the equation is $(3a+b)^{n1}(a+b)^{n2}$, where $n1$ is the number of chlorine atoms and $n2$ is the number of bromine atoms. Simple examples of mass spectra of compounds containing chlorine (Fig. 5) or bromine (Fig. 6) are shown to illustrate this explanation.

The first-order negative-ion ESI mass spectrum (not shown) of C.I. Acid Blue 81 (MW = 676, Fig. 5) contains two important ions for MW determination, i.e., $[M-2H+Na]^-$ ion at m/z 697 and $[M-2H]^{2-}$ ion at m/z 337. This molecule contains two chlorine atoms, which results in characteristic isotopic clusters 9:6:1 differing by two mass units, for example ions at m/z 697 and 617 (Fig. 5). When one chlorine atom is lost during the fragmentation process, then the isotopic ratio is 3:1 for one chlorine atom (m/z 581). For ions without chlorine (i.e., both chlorine atoms are lost during the fragmentation), the $M+2$ isotopic peak is negligible (m/z 545). The other logical neutral losses are observed in Fig. 5, for example one or two neutral losses of SO_2/SO_3 for two sulfonic acid groups.

The similar behavior may be described for bromine containing compound shown in Fig. 6. One bromine atom present in particular ion in the spectrum exhibits the isotopic ratio approximately 1:1 for $M:M+2$ isotopic peaks (m/z 380), while the absence of bromine after the neutral loss of HBr is reflected by the absence of abundant $M+2$ isotopic peak.

3.2.5. Others

The last example (Fig. 6) shows the positive-ion APCI mass spectrum unlike all previous spectra (Figs. 2–5) recorded in the negative-ion mode with electrospray ionization. The benzanthrone derivative containing one bromine atom (MW = 379) yields an intensive peak of the protonated molecule $[M+H]^+$ accompanied by less abundant radical-cation $M^{+\bullet}$ at m/z 379, which is in general unusual for atmospheric pressure mass spectra, but it is not so rare for polyaromatic compounds because of possible delocalization of unpaired electron on the conjugated polyaromatic system. The radical ion is also observed next to the fragment ion $[M+H-HBr]^+$, where small peak of $[M-HBr]^{+\bullet}$ radical ion is observed as well. The subsequent neutral losses correspond to carbon monoxide (m/z 272), which is typical for anthraquinones and sometimes also for phenols. The ion $[ArNH_3]^+$ (m/z 246) formed by the transfer of two hydrogen atoms represents the characteristic ion for the secondary or tertiary amines. These types of compounds do not provide any signal in the negative-ion mode in accordance with the structure.

For alkyl substitution on the aromatic or in general any cyclic structure, the typical neutral losses are alkenes or less frequently alkanes, but it should not be alkyl radical because of contradiction of even-electron rule. For phenyl substitution, the neutral loss is C_6H_6 or C_6H_4 but rarely C_6H_5 radical. The cleavages related to alkyl/aryl substituents are usually not the primary fragmentation path and the corresponding fragments are observed after the neutral losses of polar functional groups, for example sulfate, sulfonic acid, carboxylic acid, etc.

4. Conclusions

Mass spectrometry with atmospheric pressure ionization techniques (ESI and APCI) is ideally suited for the structure confirmation of synthetized dyes and the identification of impurities and byproducts during the dyestuffs' production. Both ESI and APCI can be easily coupled with the liquid-phase separation techniques, which make them appropriate also for the analysis of complex dye mixtures instead of time-consuming preparative isolation of impurities in mixtures. The first information for the structure elucidation based on mass spectra interpretation is always the molecular weight determination, which should be followed by detailed interpretation of tandem mass spectra. This article summarizes the correlation between the observed ionization and fragmentation behavior versus the individual functional groups typical for dye molecules ($-OSO_3H$, $-SO_3H$, $-COOH$, $-NO_2$, $-N_2-$, $-OH$, $-CO-$, $-Cl$, $-Br$, etc.). It is illustrated by selected examples and can be applied for the identification of these structural features in the mass spectra of unknown dyestuffs.

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