ELSEVIER

Journal of Chromatography A, 825 (1998) 37–44

JOURNAL OF
CHROMATOGRAPHY A

Purification and identification of several sulphonated azo dyes using reversed-phase preparative high-performance liquid chromatography

Min Chen, David Moir*, Frank M. Benoit, Cariton Kubwabo

Bureau of Chemical Hazards, Health Protection Branch, Department of Health, P.L. 0803B, Tunney's Pasture, Ottawa K1A 0L2, Canada

Received 28 April 1998; received in revised form 14 August 1998; accepted 17 August 1998

Abstract

Reversed-phase preparative HPLC has been successfully used to isolate several sulphonated azo dyes (Acid Red 1, Acid Red 8, Acid Red 106, Acid Violet 5, Chromotrope 2R, Reactive Orange 16 and Cibacron Brilliant Red 3B-A) from their impurities. The separations were achieved using mobile phases of methanol and ammonium acetate. The major components of these azo dyes were collected and then identified by electrospray mass spectrometry using the characteristic ions $[M-2Na]^{2-}$, $[M-2Na+H]^{-}$ and $[M-Na]^{-}$. The degree of purification, as measured by electrospray mass spectroscopy, was variable. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Preparative chromatography; Dyes; Azo dyes

1. Introduction

Azo dyes are characterized by the presence of one or more R-N=N-R groups, whose nitrogen atoms are linked to sp^2-hybridized carbon atoms. At least one of these carbon atoms belongs to an aromatic ring (usually a benzene or naphthalene derivative). Commercially, the dyes are synthesized by coupling the aromatic derivatives in an aqueous medium. Given the almost unlimited choice of starting materials, an extremely wide variety of azo dyes is possible. Of the commercial dyes listed in the Color Index (1971) [1], more than 2200 are azo dyes, which are used in the food, cosmetic, textile and printing industries.

Commercially prepared azo dyes are rarely pure initially, because of the presence of impurities in the reactants and the occurrence of side reactions during manufacture. Azo dyes used in the food and cosmetic industries must be purified because ingestion brings with it the possibility of biotransformation. The toxicity of the likely metabolites must be considered. The required purity is dye-specific and ranges from 85% for tartarazine to 96% for Citrus Red Number 2 [2]. Limits are also put on percentages of allowed insoluble matter, ether soluble matter, subsidiary dyes and intermediates. For azo dyes used in the textile and printing industries, where ingestion is less likely, purity is secondary to colour and there is no incentive for manufacturers to further purify these azo dye formulations. The ultimate goal of this project is to investigate the effect of azo dye structure on the ease of bioreduction by intestinal bacteria. Such a study requires that the test compounds be as free from contamination as possible, to allow accurate measurement of the kinetics of reduction. As the eight azo dyes used in this study had

*Corresponding author.

0021-9673/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(98)00695-5

stated purities ranging between 40 to 75%, substantial purification was required for each dye. This paper describes these purifications. In the literature, high-performance liquid chromatography (HPLC) has been used to analyze mixtures of dyes in food [3,4] and in cosmetics [5,6]. Most of these studies employed reversed-phase ion-pair chromatography. A quaternary ammonium salt, such as tetrabutylammonium chloride, was required as the counterion. Alternative separation strategies have been developed in the examination of dye intermediates by Jandera et al. in 1980 [7]. Aromatic sulphonic and carboxylic acids were successfully separated by reversed-phase chromatography in the presence of inorganic electrolytes, such as sodium sulphate. Chaytor and Heal [3] adopted this approach to the separation of some common synthetic food dyes. For preparative work, the addition of an inorganic electrolyte to the mobile phase can complicate the recovery of the analyte, especially if the analyte is water soluble. Ammonium acetate has been used as an electrolyte in the mobile phase in the separation of food dyes [8,9], and can be removed by lyophilization. This paper describes the purification of sulphonated azo dyes, used in the textile industry, by reversed-phase HPLC employing ammonium acetate as a mobile phase modifier.

2. Experimental

2.1. Chemicals

Eight sulphonated azo dyes were obtained from Aldrich (Milwaukee, WI, USA). The structures of these dyes are shown in Table 1. The stated purities of the azo dyes ranged from 40% for Acid Red 8 to 75% for Chromotrope 2R. Ammonium acetate (97%) was also obtained from Aldrich. HPLC grade methanol (BDH, Toronto, Canada) and deionized water were used for HPLC. The concentrations of the aqueous azo dye solutions were about 0.15 mg/ml and 10 mg/ml for analytical and preparative purposes, respectively.

2.2. Preliminary analysis

Aqueous solutions of each dye were extracted with hexane, chloroform and ethyl acetate. Any appreciable colour in the organic layer would be indicative of non-sulphonated azo dyes impurities.

Aqueous solutions of the dyes were analyzed using thin-layer chromatography (TLC) employing silica gel and C_{18} adsorbents. Solvent systems were various ratios of methanol with chloroform, acetone, acetonitrile or isopropanol for silica gel and various ratios of methanol with water or 0.1 *M* sodium sulphate in methanol–water (1:3) for C_{18}.

2.3. Instrumentation

The HPLC system consisted of two Waters 510 HPLC pumps (Waters, Milford, MA, USA). A Waters 486 Tunable Absorbance detector, and a Waters 712 WISP autosampler integrated through a System Interface Module (Waters) to a data station running Waters Baseline software.

Mass spectra were acquired using a Micromass Quattro II triple stage quadrupole mass spectrometer (Micromass, Altrincham, UK) equipped with an electrospray ion source and controlled by MassLynx 2.0 operating system using a DECpc XL 560 (Digital Equipment, MA, USA) data system.

2.4. Chromatography

2.4.1. Analytical HPLC

Analytical HPLC was performed with one of two columns, either a Waters Nova-Pak C_{18}, 4 μm (150×3.9 mm), or a Supelcosil LC-18-DB, 5 μm (250×4.6 mm) (Supelco, Bellefonte, PA, USA) with detection at 254 nm. Two pumps were used to deliver the mobile phase. One component of the mobile phase was 100% methanol and the other was an aqueous ammonium acetate solution, optimized at 0.05 *M*. The total flow-rate was 0.9 ml/min. The ratios of methanol and ammonium acetate solution were varied for different azo dyes (see Table 2). Both methanol and the ammonium acetate solution were degassed prior to use.

2.4.2. Preparative HPLC

Preparative HPLC was performed on the same HPLC system as above except a preparative column [Supelco PLC-18 (250×21.2 mm)] was used. All connecting tubing was changed to wide bore to

Table 1
The names, molecular masses, Colour Index numbers and structures of the azo dyes used in this study

R1 OH N=N—R2 NaO3S SO3Na

Name (MW)	R1	R2
Acid Red 1 (509) C.I. 18050	O ‖ H3C—C—NH—	—(phenyl)
Acid Red 8 (480) C.I. 14900	H	H3C —(phenyl)—CH3
Chromotrope 2R (468) C.I. 16570 (Acid Red 29)	HO	—(phenyl)
Acid Red 106 (621) C.I. 18110	H3C—(phenyl)—S(O)(O)—NH—	—(phenyl)
Acid Violet 5 (678) C.I 18125	H3C—(phenyl)—S(O)(O)—NH—	—(phenyl)—NHCOCH3
Procion Red MX-5B (615) (Reactive Red 2)	Cl, N, N, N, Cl (dichlorotriazinyl)—NH—	—(phenyl)
Cibacron Brilliant Red 3B-A (995) C.I. 18105 (Reactive Red 4)	(phenyl)—C(O)—NH—	—(phenyl, NaO3S)—NH—(triazinyl: N, N, N, Cl)—NH—(phenyl)—SO3Na

Reactive Orange 16 (617) C.I. 17757: H3CCONH, OH, N=N—(phenyl)—S(O)(O)—CH2CH2—O—SO3Na, SO3Na

reduce back pressure. The total flow-rate was 13.5 ml/min. The ratio of methanol and ammonium acetate solution was varied for different dyes (see Table 2). Between 10 and 15 separate injections were made for each dye. The pooled fractions were concentrated to a small volume (about 10 ml) by

Table 2
The percentages of methanol in the aqueous ammonium acetate buffer (0.05 *M*) used in the analytical and preparative mobile phases for each dye

Dye	Analytical HPLC	Preparative HPLC
Acid Red 1	30% methanol	32% methanol for 20 min and linear gradient to 50% methanol at 30 min and kept at 50% methanol
Acid Red 8	40% methanol	40% methanol
Acid Red 106	35% methanol	38% methanol
Acid Violet 5	35% methanol	40% methanol for 20 min and linear gradient to 50% methanol at 30 min and kept at 50% methanol
Chromotrope 2R	30% methanol	35% methanol for 20 min and linear gradient to 45% methanol at 30 min and kept at 45% methanol
Reactive Orange 16	30% methanol	35% methanol
Procion Red MX-5B	50% methanol	40% methanol for 10 min and linear gradient to 55% methanol at 15 min and kept at 55% methanol
Cibacron Brilliant Red 3B-A	35% methanol	38% methanol for 20 min and linear gradient to 45% methanol at 25 min and kept at 45% methanol

rotary evaporator, then frozen and lyophilized to remove the ammonium acetate. Each fraction was analyzed by electrospray mass spectrometry (ES-MS) to verify the authentic material and to determine the purity of the fraction [10].

2.4.3. ES-MS

Aqueous samples (~100 ng/μl) were introduced into the mass spectrometer by continuous infusion using a syringe pump (Harvard Apparatus, MA, USA) at a flow-rate of 10 μl/min. The mass spectra were acquired in negative ion mode over the mass range 50 to 1000 Daltons using the multi channel analysis function. For tandem ES-MS experiments, argon was used as the collision gas. The fragmentation of the precursor ion was performed at a collision induced dissociation energy of 25 eV and a cone voltage of −15 V.

3. Results and discussion

3.1. Preliminary analysis

3.1.1. Organic solvent extraction

Acid Red 8 gave a slight yellow colour to chloroform and ethyl acetate but all other dyes showed no colour in any of the organic phases, indicating that these azo dyes contained no non-sulphonated impurities.

3.1.2. TLC

Although reversed-phase TLC has been used for the analysis of several food dyes [11], no separation was attained with the various solvent systems used here. It is possible that the efficiency of this technique was not great enough to separate the azo dyes from their impurities.

3.2. HPLC analysis

Analytical HPLC was used to determine the proper separation conditions for preparative HPLC. There are two useful ways to recover purified material after preparative HPLC: (1) extraction into an organic solvent or (2) concentration of the eluent. The first requires a suitable partition coefficient, the second a volatile mobile phase. The azo dyes studied here are all sulphonated and are very soluble in water, thus eliminating extraction as a recovery means. The ideal mobile phase, in terms of ease of removal, would be an unbuffered mixture of water and some volatile organic solvent, which could be removed by evaporation. To this end, various concentrations of methanol in water were used as mobile phases with a Waters NovaPak C_{18} column. Each

mobile phase mixture resulted in either poor resolution or severely tailing peaks or both. It seemed evident that using an organic solvent with unbuffered water would be insufficient to purify these sulphonated azo dyes. It is known that the addition of an inorganic electrolyte to the mobile phase can substantially improve the separation of ionizable species [3,7,9].

Jandera et al. [7] investigated the separation of aromatic sulphonic acids by reversed-phase chromatography in the presence of various inorganic electrolytes including sodium nitrate, potassium phosphate and sodium sulphate. They found that the retention volume of a sulphonic acid slightly increased in the order: formate<nitrate<acetate< hydrogenphosphate<sulphate, for cations $Li^+ < Na^+ < K^+$ used in the mobile phase. Ammonium acetate has also been used as an electrolyte in the separation of food dyes [8] and in the purification of a food dye [9] and since ammonium acetate can be removed by freeze drying, this compound is an ideal inorganic electrolyte for use in the mobile phase in this study.

Using Acid Red 1 as a test compound, the effect of concentration of ammonium acetate on plate number, asymmetry factor and retention time was investigated (Fig. 1). The plate number increased with increasing concentration of ammonium acetate in the mobile phase until reaching a plateau at 0.1 *M*. Peak symmetry also improved with increasing concentration of ammonium acetate. Unfortunately, retention times also increased with higher concentrations of ammonium acetate. These effects mediated by ammonium acetate are likely due to the electrolyte's capacity to minimize chromatographic interference by the free silanol groups which are invariably present in C_{18} packing material [12] and so enhance the interaction of the analyte with the stationary phase. The optimum concentration of ammonium acetate in terms of plate number and peak symmetry was determined to be around 0.1 *M*. For preparative HPLC, however, the need for reasonable separation times can outweigh the desire for optimum peak symmetry and plate number. Therefore, the compromise concentration of 0.05 *M* ammonium acetate was used in preparative HPLC.

Other factors can have an effect on the chromatography as well and were briefly investigated. Decreasing the pH of the aqueous portion of the mobile phase using either 0.05 or 0.005 *M* acetic acid resulted in no improvement in the chromatography of these azo dyes, nor did a switch from methanol to

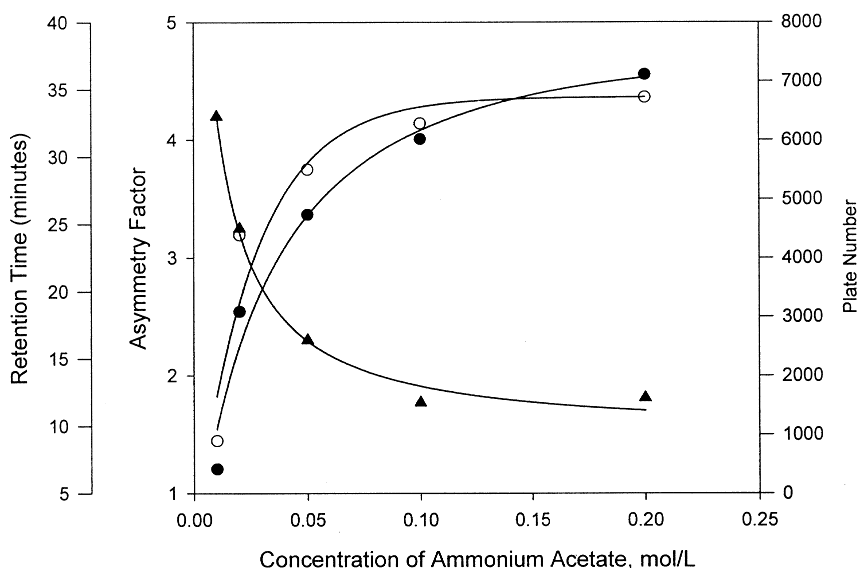

Fig. 1. Retention time (●) of Acid Red 1, asymmetry factor (▲) at 10% baseline and plate number (○) as a function of the molar concentration of ammonium acetate.

acetonitrile. Not surprisingly, a change in flow-rate from 0.9 to 0.45 ml/min caused an increase in plate number from 4700 to 5600, using Acid Red 1. Two brands of analytical columns were investigated. A Supelco LC-18-DB was finally selected over a Waters NovaPak C_{18} on the basis of better resolution, likely due to its slightly larger dimensions. An example chromatogram of Reactive Orange 16 is shown in Fig. 2.

3.3. Preparative HPLC

The dimensions of the preparative C_{18} column (250×21.2 mm) are much greater than those of the analytical column. If the analytical conditions were to be directly converted to preparative use, the flow-rate would have to be 19.1 ml/min which gives too great a back pressure. The maximum flow-rate used in this system was 13.5 ml/min. This means that the linear flow-rate was less than that with the analytical column and the azo dyes would take a much longer time to elute from the column. For preparative purposes, it is desirable to take the shortest time possible while maintaining a reasonable separation in order to maximize output. Therefore, in order to make the total run time less than 1 h, the methanol content was slightly increased relative to the analytical conditions. Good separations were still achieved. A preparative chromatogram for Reactive Orange 16 is shown in Fig. 3 and the collected regions are indicated. The times of the collected fractions and the colours (in parentheses) for each azo dye are listed in Table 3. Only some of the earliest part of the eluent was colourless; this contained the solvent front and some small molecules. All other fractions were coloured. Some were a different colour from the major dye component while others had the same colour such as Procion Red MX-5B. These secondary dyes were either byproducts or isomers.

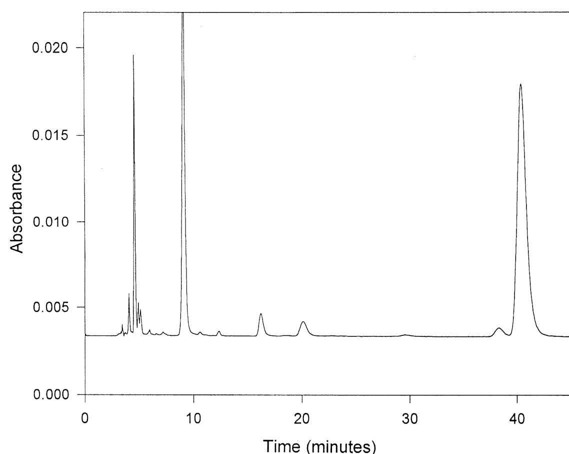

Fig. 2. Analytical HPLC chromatogram of crude Reactive Orange 16 with detection at 254 nm. The mobile phase was methanol–0.05 *M* ammonium acetate (30:70, v/v) with a flow-rate of 0.9 ml/min. The column was a Supelco LC-18-DB, 5 μm (250×4.6 mm).

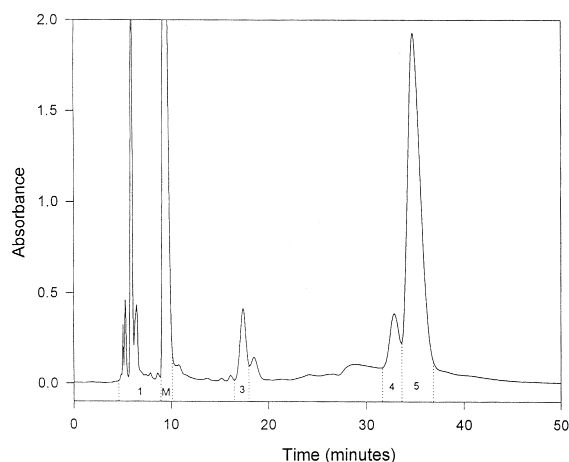

Fig. 3. Preparative HPLC chromatogram of Reactive Orange 16 with a mobile phase of methanol–0.05 *M* ammonium acetate (35:65, v/v) at 13.5 ml/min and detection at 254 nm. Collected region M is major component. The column was a Supelco PLC-18 (250×21.2 mm).

After collection and evaporation in vacuo to remove the methanol, the fractions were frozen and lyophilized to remove the ammonium acetate. It was anticipated that the major peaks would be the authentic, pure azo dyes. To verify this, ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy were initially used for the two major components of Procion Red MX-5B. Although sulphonated azo dyes are highly water soluble, a high enough concentration could not be reached to obtain a reasonable ^{13}C-NMR spectrum. A ^{1}H-NMR spectrum by itself could not provide enough information to identify the azo dyes. Therefore, ES-MS was used

Table 3
The time (min) of collection of fractions for each dye, the colours of those fractions in parentheses and the fraction containing the dye (Dye)

Azo dyes	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Acid Red 1	35–39 (pink)	39–45 (red) (Dye)			
Chromotrope 2R	3.5–12 (light orange)	15–18 (red) (Dye)	18–25 (pink)	36–38 (purple)	
Acid Violet 5	3–13 (no colour)	16–18 (fuchsia) (Dye)	18–25 (light pink)	31–32 (pink)	
Acid Red 8	3–13 (no colour)	24–28 (orange) (Dye)	28–40 (pink)	41–45 (pink)	
Acid Red 106	3–14 (no colour)	45–49 (red) (Dye)	49–62 (pink)	62–67 (pink)	
Cibacron Brilliant Red 3B-A	3–18 (fuchsia)	18–24 (fuchsia) (Dye)	24–33 (fuchsia)	34–37 (fuchsia)	
Reactive Orange	3–8.5 (orange)	8.5–10 (orange) (Dye)	17–18 (orange)	32–34 (orange)	34–37 (orange)
Procion red MX-5B	9–10 (red)	15.5–17 (red)	20–21 (red)	26–28 (red) (Dye)	34–36 (red)

to identify these eight azo dyes and to estimate the degree of purification attained by preparative chromatography [10].

3.4. ES-MS

Five components of the azo dyes were confirmed as consistent with the structure of the authentic material. The ES-MS of the major fractions of two dyes, Acid Red 106 and Acid Violet 5, provided evidence of a molecular mass that was different from that of the authentic dye structures. The ES-MS of the dyes present three ions ($[M-2Na]^{2-}$, $[M-2Na+H]^{-}$ and $[M-Na]^{-}$) that point to the molecular mass. In the case of Acid Red 106 (observed 607; authentic 621) and Acid Violet 5 (observed 664; authentic 678) molecular masses of 14 μ lower than the authentic materials were observed. Tandem MS was

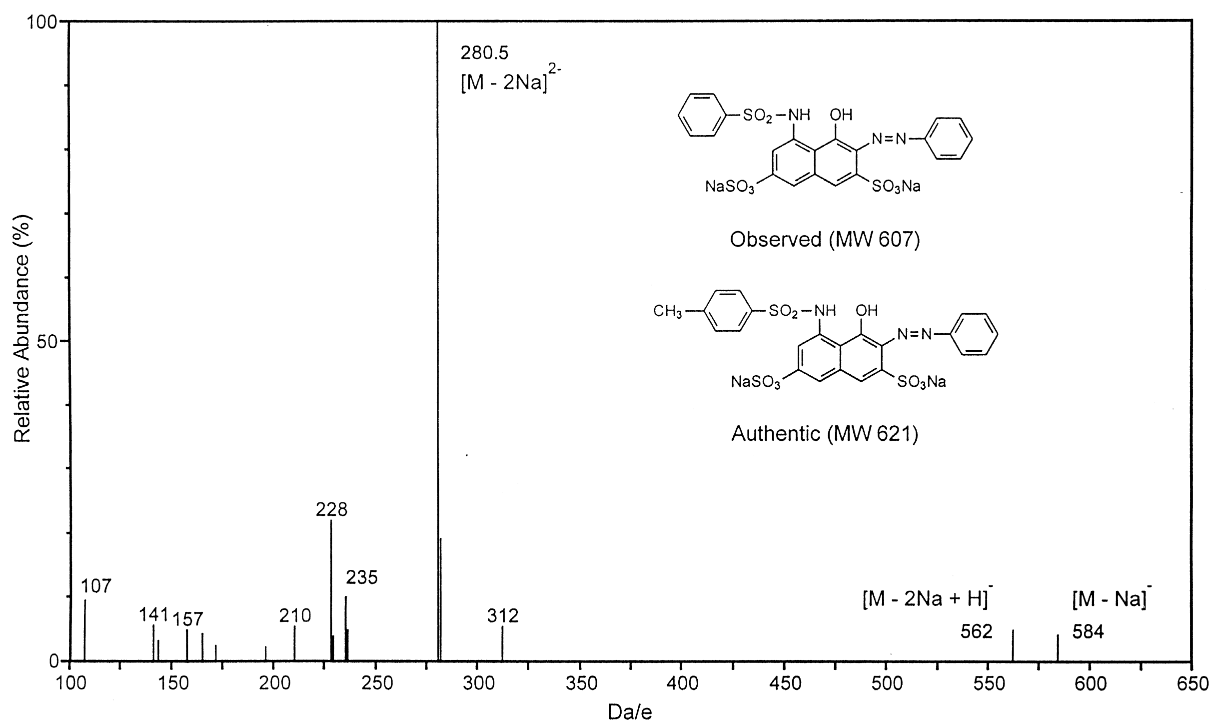

Fig. 4. Negative ion electrospray mass spectrum of the primary dye isolated from the purification of Acid Red 106. MW=Molecular mass.

used to confirm that the mass differential was due to the absence of the methyl group in the *para*-phenylsulphonyl group at the C-8 naphthalene position in each dye. The mass spectrum of Acid Red 106 is shown in Fig. 4. The ES-MS–MS of these two azo dyes also supported this explanation.

The ES-MS of the major fraction from the preparative HPLC of Procion Red MX-5B indicated that this fraction was contaminated with a second chlorinated azo dye which was not resolved using the HPLC methods developed here. Therefore, only seven of the purified azo dyes were identified as being sufficiently pure for subsequent degradation studies.

In the absence of certified reference standards of these dyes, it was not possible to use HPLC to quantitate the degree of purification of the preparative HPLC process. Therefore, the degree of purification of this procedure was assessed by summing all ions in the mass spectrum of each purified dye, then dividing by the sum of all ions in the mass spectrum of the corresponding crude dye. The resulting ratios, reported as percentages, are shown in Table 4. The lower the number, the fewer extraneous ions, and the greater the degree of purification.

Table 4
Ratios of the sum of ions from the mass spectra of the major liquid chromatography fraction divided by the sum of ions from the corresponding crude dye mass spectra, expressed as percentages

Dye	Percent ions (purified/crude)
Acid Red 106	73
Acid Red 8	107
Acid Red 1	125
Acid Violet 5	81
Reactive Orange 16	94
Chromotrope 2R	78
Cibacron Brilliant 3B-A	47

4. Conclusions

Seven sulphonated azo dyes used in the textile industry have been purified by using reversed-phase preparative HPLC with a C_{18} column and methanol and aqueous ammonium acetate as a mobile phase. Confirmation studies by ES-MS showed that the reversed-phase preparative HPLC was successful in the purification of five of these sulphonated azo dyes and of those five, two had structures which were different from the purported structures. These methods could be applicable to the analysis and purification of a wide range of water soluble di-, tri- and even tetrasulphonated azo dyes.

References

[1] Color Index, Society of Dyers and Colorists, 3rd ed., 1971.
[2] Government of Canada, Food and Drugs Act and Regulations, Part B, Div. 6, 1984, p. 33.
[3] J.P. Chaytor, R.L. Heal, J. Chromatogr. 368 (1986) 450.
[4] J.F. Lawrence, F.E. Lancaster, H.B.S. Conacher, J. Chromatogr. 218 (1981) 168.
[5] J.W.M. Wegener, H.J.M. Grünbauer, R.J. Fordham, W. Karcher, J. Liq. Chromatogr. 7 (1984) 809.
[6] J.W. Wegener, J.C. Klamer, H. Govers, U.A.Th. Brinkman, Chromatographia 24 (1987) 865.
[7] P. Jandera, J. Churacek, J. Bartosova, Chromatographia 13 (1980) 485.
[8] T. Tonogai, Y. Ito, M. Harada, J. Food Hyg. Soc. Jpn. 25 (1984) 10.
[9] Y. Takeda, Y. Goda, H. Noguchi, T. Yamada, K. Yoshihira, M. Takeda, Food Addit. Contam. 11 (1994) 97.
[10] F.M. Benoit, C. Kubwabo, M. Chen, presented at the 9th Lake Louise Workshop on Tandem Mass Spectrometry, December, 1996.
[11] H. Oka, Y. Ikai, N. Kawamura, M. Yamada, H. Inoue, T. Ohno, K. Inagaki, A. Kuno, N. Yamamoto, J. Chromatogr. 411 (1987) 437.
[12] K. Jones, J. Chromatogr. 392 (1987) 1.