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DETERMINATION OF SULPHONATED AZO DYESTUFFS AND THEIR BACTERIAL METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HUGO GROSSENBACHER, THOMAS THURNHEER, DANIEL ZÜRRER and ALASDAIR M. COOK*

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, CH-8092 Zürich (Switzerland)

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SUMMARY

Mixtures of single- and multiple-substituted benzene and naphthalene sulphonic acids and azo dyestuffs were separated by high-performance liquid chromatography using gradient elution on reversed-phase columns. A UV detector was used. The mobile phase was 100 m*M* potassium phosphate buffer (pH 6.7) and the polarity was modified by the addition of methanol. Multiple acidic substituents required the buffer to be pH 2.

INTRODUCTION

High-performance liquid chromatography (HPLC) is recognized as the method of choice for the routine determination of ionic aromatic sulphonates[1–6], but published methods enable separations of only narrow ranges of compounds. Our research is aimed at the microbial degradation of azo dyestuffs in the wastes from their syntheses and application[7–10], so we require a simple and reliable analysis in a single chromatogram of dyestuffs, their precursors and their microbial products in microbial cultures.

Separations on ion exchangers[11] are unsatisfactory because of poor recovery of analytes[2,3]. The use of a silica column with an aqueous phase leads to non-reproducible retention times[1] and unstable baselines[12]. Reversed-phase columns, in contrast, give reproducible separations and stable baselines[2–6]. Several separations are known in the ion-pair mode[5,6,13], but separations do not seem to be predictable and use of the pairing agent seems to shorten column life[2,3]. Better, predictable, and more economical separations are observed using reversed-phase columns with inorganic electrolytes as the mobile phase[2,3,10,14]. We have extended this idea to determine individual sulphonates (Table I) in complex mixtures by gradient elution from reversed-phase columns.

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TABLE I

ARYL SULPHONATE DYESTUFFS, PRECURSORS AND METABOLITES

Substance (Common name)	*Coding in Fig. 1*	*CAS Registry Number*	*Retention time in routine gradient (min)*
Benzene sulphonic acid		98-11-3	18.1
Orthanilic acid	IV	88-21-1	13.1
Metanilic acid	III	121-47-1	7.8
Sulphanilic acid	I	121-57-3	6.0
Sulphanilamide	V	63-74-1	16.9
p-Hydroxybenzene sulphonic acid	II	98-67-9	6.9
p-Toluene sulphonic acid		6192-52-5	25.0
p-Sulphobenzoic acid*		636-78-2	ND**
Naphthalene-1-sulphonic acid		130-14-3	30.3
Naphthalene-2-sulphonic acid	XII	532-02-5	31.5
Peri acid***	XI	82-75-7	30.3
Laurent acid***	VI	84-89-9	18.0
Tobias acid***	IX	81-16-3	27.0
J acid***	VIII	87-02-5	21.3
Gamma acid***	VII	90-51-7	20.3
C acid***		131-27-1	ND**
H acid***		90-20-0	8.6
Orange I	XV	523-44-4	39.4
Orange II	XVII	633-96-5	42.8
Orange III		547-58-0	41.6
Orange IV		554-73-4	ND§
Tropaeolin 0	X	547-57-9	29.5
Acid red I	XIII	3734-67-6	33.0
Trypan blue	XIV	72-57-1	36.4
Croceine acid red	XVI	5413-75-2	40.9
Congo red		573-58-0	41-6
Benzyl orange		589-02-6	ND§

* Material synthesized by the Department of Chemical Engineering and Industrial Chemistry.

** Little or no interaction with the stationary phase; no satisfactory separation under these conditions.

*** Material supplied by Ciba-Geigy AG, Basel, Switzerland.

§ Samples contained many impurities and the authentic material was not located.

EXPERIMENTAL

Materials

The compounds that were analysed (Table I) were obtained from Fluka (Buchs, Switzerland) unless otherwise indicated (Table I). Standard solutions were prepared in 10 m*M* potassium phosphate buffer (pH 7.2).

Glass double-distilled water was used throughout. Water for use in mobile phases for gradient-elution HPLC was collected in an all-glass apparatus and used with minimum exposure to plasticizers in order to avoid ghost peaks in chromatograms. The methanol used was HPLC grade (Romil, Loughborough, U.K.) and the analytical reagents used were of the highest quality available commercially.

Sample preparation

Samples from bacterial cultures were filtered (0.2 μm pore diameter) or centrifuged (23 000 *g* for 20 min at 4°C) before chromatography. Samples from enzyme assays were treated with perchloric acid (0.5 *M* final concentration) and the precipitated protein was removed by centrifugation (12 000 *g* for 20 min at 4°C). The supernatant fluid was neutralized with 1 *M* potassium hydroxide and the precipitate of potassium perchlorate was removed by centrifugation (12 000 *g* for 20 min at 4°C) before analysis of the supernatant fluid. Samples to be stored were frozen to avoid growth of microorganisms.

Apparatus and assay conditions

HPLC was done at room temperature (*ca.* 20°C) using stainless-steel columns (250 × 4.6 mm I.D. with a 40 × 4.6 mm I.D. precolumn) that contained a reversed-phase packing material of 7.5 μm mean particle diameter (Nucleosil 7 C_{18}; Macherey und Nagel, Düren, F.R.G.). The mobile phase was delivered through a high-pressure mixer by two pumps which were controlled by a gradient programmer (Altex)[15]. The eluate from the column passed through a UV detector (Kontron)[15] set at 220 nm. Isocratic separations requiring routine, automated collection of peaks were done with another system (DuPont/Pharmacia)[16].

In the routine assay, the filtered (0.2 μm), degassed mobile phase was maintained at a flow-rate of 0.7 ml/min. The column was equilibrated with 100 m*M* potassium phosphate buffer (pH 6.7). A sample (20–250 μl) was injected on the column and after 10 min the linear gradient (0–100%; 30-min duration) was started by addition of the second solvent [70% (v/v) methanol in 10 m*M* potassium phosphate, pH 6.7]: the chromatogram was complete in 50 min, at which time the gradient was reversed (0.1-min duration) and 60 min after injection the starting conditions had been re-established.

An alternative assay, usually used for compounds carrying multiple acidic functions, was used similarly, but in this case the mobile phase was 100 m*M* potassium phosphate buffer (pH 2.0).

RESULTS AND DISCUSSION

It was known that ionic aryl sulphonates could be separated by HPLC on reversed-phase columns (see Introduction). We have confirmed this observation and shown that not only can simple phenyl and naphthyl derivatives be measured but that complex azo dyestuffs can be easily determined and separated from their educts and metabolic products (Fig. 1, Table I).

We chose the Nucleosil packing material because of good peak symmetry, high selectivity and long column life[16]. The selectivity of the column, especially for the more polar compounds, was a direct function of the ionic strength of the mobile phase. Where less selectivity was required, wear in the pump-seal could be reduced by reducing the buffer concentration. Increased selectivity could be obtained by lowering the column temperature[15]: if room temperature fluctuated markedly, retention times were unstable.

Fig. 1 shows the separation of dyestuffs and compounds with a single sulphono substituent. Multiple acidic substituents (*e.g.* sulphonobenzoate) lead to a high net

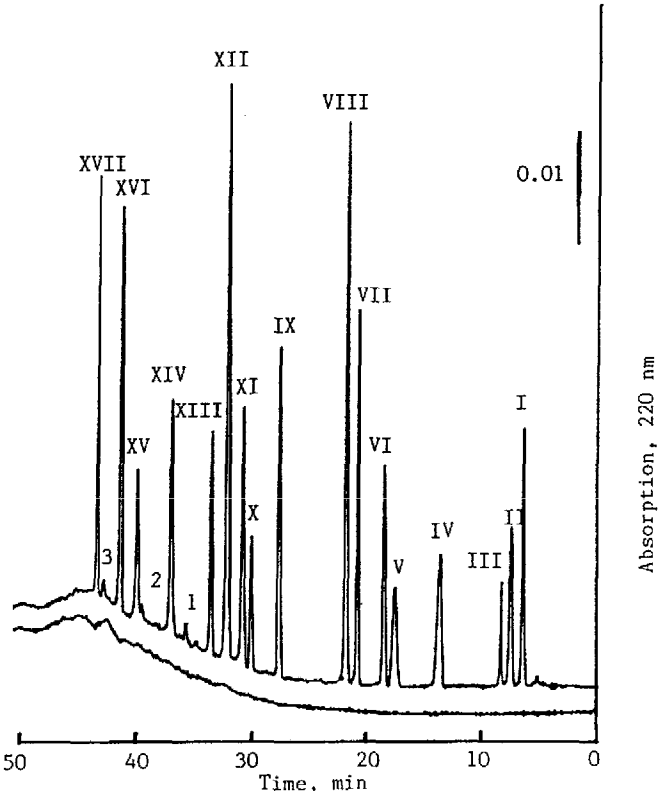

Fig. 1. Typical chromatogram of a mixture of aryl sulphonates. The compounds corresponding to the roman numerals are given in Table I: 1, 2 and 3 are unknown impurities. The concentrations of analyte were *ca.* 0.1 m*M* except for I, IV, X and XIV (*ca.* 0.3 m*M*). The lower chromatogram is the baseline, which rises due to the absorption of the methanol.

charge on the molecule and to elution with the void volume of the column. This problem was eliminated in isocratic separations of, *e.g.* C acid (t_R 7.5 min) and *p*-sulphobenzoic acid (t_R 19.4 min) with a mobile phase of low pH in which at least one of the acidic substituents was protonated.

Our method gave stable retention times (*e.g.* ± 1.7% S.D., $n = 10$, for orthanilic acid) and reproducible peak heights (or areas) (*e.g.* ± 2.6% S.D., $n = 10$, for orthanilic acid). Calibration curves were linear to at least 1 m*M* for sulphanilic acid and benzene sulphonic acid, for example. The sensitivity of the assay was a function of the wavelength chosen and the absorption spectrum of the analyte: naphthalene-2-sulphonic acid could be detected at 0.5 μM when 250-μl samples were used. A diode-array detector would appear to be the detector of choice for these widely different compounds.

The method enabled us to check the purity of many dyestuffs. Our preparations of Acid red I, Croceine acid red, Orange II and Tropaeolin 0 were chromatographically pure. Trypan blue and Orange I were sufficiently pure so that the corresponding material could be isolated and its identity checked by UV-spectrometry. Some so-

lutions (*e.g.* Benzyl orange and Orange IV) contained many components of similar peak height. In addition to analytical applications, we routinely used the method, usually in the isocratic mode, to purify components of mixtures for biological experiments. This separated out not only the other aromatic sulphonates but also contaminant SO_4^{2-}, which we observed to elute with the void volume of the column.

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