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Strategies for continuous on-line high performance liquid chromatography coupled with diode array detection and electrospray tandem mass spectrometry for process monitoring of sulphonated azo dyes and their intermediates in anaerobic–aerobic bioreactors

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Abstract

On-line HPLC with diode array detection (DAD) coupled to electrospray tandem mass spectrometry (ESI-MS/MS) is presented as an integrated quality control and process integrated optimisation tool for the continuous monitoring of sulphonated azo dyes (SADs) and their intermediates in anaerobic and aerobic bioprocesses. Ion-pair RP-HPLC is found out to be more suitable for simultaneous monitoring of aromatic amines (AAs), sulphonated aromatic amines (SAAs) and sulphonated azo dyes in comparison to RP-HPLC with polar embedded phases. Monitoring of the anaerobic degradation of the diazo Reactive Black 5 displays the dependency of a two stage azo group reduction mechanism on the redox potential of the bioreactor. An autoxidation sensitive intermediate released from the anaerobic reduction is characterised by ESI-MS/MS for the first time. The functionality of the method is demonstrated by the control and evaluation of selective adaptation of bacteria to certain pollutants and the identification of unknown intermediates causing re-gaining colour released from azo dye treatment.

Keywords: Sulphonic acids; Azo dyes; Aromatic amines; On-line LC–MS/MS; Reactive Black 5

1. Introduction

Complementary information from various analytical techniques is often required for the unambiguous identification and quantification of organic dyes [1]. Dyes have always been the objective of analytical interest of scientists concerning the identification of natural dyes as well as synthetic dyes produced by oneself or by competitors. Brunner in 1929 recommended a technique called capillary analysis which was first described by Goppelsröder for the purity analysis of natural or synthetic dye mixtures [2–4]. In the 1920s, the analytical task of the separation and analysis of more than 1000 azo dyes encouraged the use of chromatographic techniques as described by Tswett [5] and Fierz-David [6]. About 30 years after Tswett's invention of the chromatographic adsorption method, dye chemists assessed the new analytical method as useful. However, this method can only be applied to simple systems, which only consist of few dyes [7–9]. Since this time, an approach has been made to separate azo dyes by liquid chromatography. Today, the number of known azo dyes and other commercial dyes exceeds 10,000 [10]. In 1979 Sträule and von Wattenwyl [11] developed a RP-HPLC method to separate anionic azo dyes and intermediates. However, dye manufacturers and end users are often more focused on performance and intensity of dyes rather than on a comprehensive characterisation of the content of their products. Therefore, the required chromatographic conditions to effectively resolve the organic impurities in dyes are not readily available from manufactures [12]. Following the studies of the carcinogenic potential of benzidine derived dyes initiated by National Institute for Occupational Safety and Health (NIOSH) in 1978 and the health hazard alert for

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benzidine, *o*-toluidine and *o*-dianisidine based dyes in 1980 by NIOSH analytical methods for the identification and quantification of azo dyes became more and more important in the field of environmental and toxicological analytics [13,14]. The combination of HPLC with UV–vis detection has been found to be a useful method for the determination of benzidine impurities in azo dyes and for the analysis of structurally similar dyes [12,15,16]. In 1986 the proposed US Environmental Protection Agency (EPA) regulatory program for the dye industry required the development of mass spectrometry (MS) methods for the qualitative analysis of sulphonated azo dyes (SADs) that are easily adaptable to inexpensive routine analysis procedures [17]. LC–MS appears to offer good performance for the analysis of dyes in industrial wastes and treated effluents since the complex nature of these wastes and effluents will require HPLC separation for unambiguous mass spectrometric identification [18]. The detection of azo dyes (azo benzene) by mass spectrometry was performed first by Bowie et al. in 1967 [19]. Coupling of liquid flow injection with thermospray ionisation tandem mass spectrometry without previous chromatographic separation was introduced for the analysis of azo dyes, driven by the concern over the discharge of wastes into the aquatic environment from the industries manufacturing and using dyes [18,20,21]. Improvements in the LC–MS interfacing technology lowered detection limits for dyes and increased the potential for LC–MS based structural elucidation analysis by fragmentation experiments [22–25]. An overview about the recent development of mass spectrometry for azo dye analysis and its different applications is given by Richardson [26]. Reemtsma [27] recently reviewed analytical LC–MS methods suitable for the analysis of sulphonated compounds. Effects of ion-pairing agents on ESI signal suppression were investigated by Holčapek et al. [28]. Due to an increasing use of biological wastewater treatment concepts for synthetic compounds such as sulphonated azo dyes in industrial wastewater, analytical interest is focused on the degradation of these compounds. Textile dye producers and textile colouration plants contribute to the release of sulphonates in the aquatic environment which represents a major problem in drinking water plants in south-eastern Spain [29]. Analysis of industrial wastewater from dye producers and colouring plants showed the demand for selective analytical methods for the monitoring of sulphonated aromatics (SAs), sulphonated aromatic amines (SAAs) and aromatic amines (AAs) in water [30–38].

Textile dyes of various categories, i.e. acid, direct, mordant and reactive dyes, are sulphonated in order to induce high water solubility, which is important for the dyeing process. Water solubility results from full dissociation of the dyes in aqueous solutions as anionic solutes. The analytical strategies used for the determination of SAs, SAAs and SADs include a wide range of classical and modern techniques. Many of these techniques make use of the negative charge of the dye. Small SAs and SAAs such as sulphanilic acid are not retained on RP phases because they are rejected from the pores by the Donnan exclusion effect originated by negative-charged free residual silanol on the particle surface.

Jandera et al. [32] investigated the separation of SAs by reverse-phase chromatography in the presence of various inorganic electrolytes including sodium nitrate, potassium phosphate and sodium sulphate in order to suppress the Donnan exclusion effect on the solutes. Jandera et al. [33] found that isomeric naphthalene mono- to tetrasulphonic acids are best separated with aqueous-methanolic mobile phases containing sodium sulphate and various aromatic aminosulphonic acids by ion-pair reversed-phased chromatography with mobile phase containing tetrabutylammonium ions. Several researchers [30,34,35] investigated the use of ammonium acetate (5–30 mM) as an electrolyte in the HPLC separation of dyes because of its weak ion-paring (IP) properties and volatility which allows coupling to LC–MS. Ammonium formate was used for RP-HPLC separation of benzene and stilbene sulphonic acids coupled to diode array detection (DAD) with electrospray mass spectrometry (ESI-MS) [36]. The use of amine bases was found to enhance the sensitivity of ESI-MS towards complex polysulphonated azo dyes [37]. It was observed that the use of triethylamine (TEA) and diethylamine (DEA) as organic modifiers in LC–MS follows the concept of total cation removal to combine individual ions from a series into a single intensive peak. The base gas-phase proton and sodium affinity is considered to be responsible for the cation removal mechanism and also to effect the observed sensitisation.

The most commonly used IP agents for the separation of anionic solutes are tetraalkylammonium salts, such as tetrabutylammonium salts (TBA) which allow separation of very strong acidic SAAs (for example benzene and naphthalene sulphonic acids). However, non-volatile TBA salts obstruct the interface if IP-RP-HPLC is coupled with ESI or APCI to MS. Socher et al. [38] developed a method to determine sulphonated compounds by RP-IP-HPLC–MS with on-line removal of non-volatile TBA ion-paring agent. Based on the same coupling method Bauer et al. [39] combined ion chromatography (IC) with ESI-MS for the analysis of polar organic micropollutants, such as EDTA, belonging to the group of polar persistent pollutants (P^3).

Azo dyes, SAs and SAAs are known to be poorly degradable in communal biological wastewater treatment plants. An approach has been made to adapt micro-organisms to these compounds whereby a biodegradation could be achieved in industrial wastewater treatment plants [40]. However, many SAs and SAAs still belong to the group of recalcitrant synthetic compounds, which are known to pass aerobic biological wastewater treatment without degradation. Knowledge about the degradation mechanism and pathway of SAs and SAAs such as azo dyes is necessary for a process optimisation of biological treatment processes of such compounds and analytical control of the process, respectively. SAAs can be found in the untreated production and colouring wastewater of the dye and dyeing industry [27,29,41,42]. In addition

to SAAs toxic AAs are released from azo dyes due to the cleavage of the azo bonds initiated by anaerobic reduction in municipal biological treatment plants and sediments of rivers. In 2004 Pinheiro et al. [43] reviewed the formation of aromatic amines from azo dye reduction. Furthermore, SAAs, e.g. *ortho*-aminohydroxynaphthalene sulphonic acids, play an important role in the autoxidation initiated regaining colour of biologically treated azo dye containing textile effluents [44]. The remaining colouration in treated effluents of textile wastewater treatment plants cannot be distinguished from the autoxidised intermediates by use of standardised measurement of the spectral absorption coefficients. Thus, a process integrated optimisation and continuous process control must be based on highly selective detection methods adapted to the requirements for the understanding of complex degradation reactions in a problematic matrix such as sludge.

In its ambitious form, bioprocess monitoring means effortless access to continuous, real-time information about all variables relevant to a given process [45]. The monitoring and structural elucidation of both known and unknown compounds in complex wastewater samples requires the combination of hyphenated analytical techniques which are not yet commercially provided at the process analytical market by means of automated analytical systems [46]. On-line HPLC monitoring of bioprocesses is critically discussed in the literature [45,47,48] as it appears not to be appropriate for process control, due to its long analysis time and difficult sampling. Yet it has been established in the biological and medical monitoring [49] and approaches have been made in the development of a selective HPLC based on-line monitoring in waste waters [42,50,51] and bioreactors [52–56].

In our work a partially automated process analytical system is developed in order to achieve a 'process integrated quality control' [57], i.e. a quality control of the biodegradation process of dyes and intermediates. A microfiltration based continuous on-line sampling method which has been established for HPLC–DAD monitoring of letter-acids and sulphonated reactive azo dyes in anaerobic/aerobic bioreactors [55,56] and a RP-HPLC–DAD systems was combined with a Qtrap hybrid mass spectrometer for the monitoring and structural elucidation in a biological wastewater treatment process of azo dyes.

The analytical system was optimised by the use of a test-mixture comprising several basic AAs and anionic SAAs as well as hydrolysed reactive azo dyes. It was the objective of this work to identify a suitable chromatographic method that allows the analysis of these samples by one chromatographic method in order to use it for a continuous on-line HPLC–DAD–ESI-MS/MS. The functionality of the on-line analytical system is therefore primarily connected to the choice of the liquid chromatographic method and its performance with respect to the analytical specifications. Two chromatographic techniques were investigated for the development of the analytical system: novel LC-phases with polar selectivity and classical TBA-ion-pair-chromatography on C8 and C18-phases combined with ion suppression next in line with an ESI hybrid MS. Functionality of the analytical system is demonstrated by monitoring of biological treatment of synthetic dyebath concentrate comprising hydrolysed azo dye Reactive Black 5. Complementary information derived from mass spectromic analyses with different scan modes and spectrometric analyses by DAD formed the basis for the structural elucidation of several dye intermediates.

2. Experimental

2.1. Chemicals

Ammonium acetate analytical-reagent grade, HPLC-grade LiChrosolv® water and LiChrosolv® gradient grade acetonitrile were obtained from Merck (Darmstadt, Germany). The ion pairing agent tetrabutylammonium acetate (TBAAc) was purchased puriss. (>99%) from Fluka (Buchs, Switzerland). All AAs, SAAs and SADs listed in Fig. 1 were kindly provided from DyStar (Leverkusen, Germany), except $TAHNDS_{DP2}$ which was isolated from the bioreactor in our laboratory. Name, abbreviation (purity, concentration in test-mixture (μM)): 1—metanilic acid, M-acid (98%, 100), 2—H-acid (82,5%, 10), 3—p-Base, PB (60%, 100), 4—RB-OH (60%, 50), 5—RB-NH_2 (60%, 40), 6—Reactive Black 5 hydrolysed, RB5-H (73%, 50), 7—Reactive Orange 16 hydrolysed, RO16-H (40%, 50), 8—Reactive Orange 107 hydrolysed, RO107-H (40%, 50), 9—4,6-dihydroxy-3,5-diimino-naphthalene-2,7-disulphonic acid derived from 2,3,5-triamino-4-hydroxy-naphthalene-2,7-disulphonic acid (Diamino-H-acid), $TAHNDS_{DP2}$ (50%, 100).

2.2. Equipment—analytical set-up

A schematic drawing of the monitoring system is shown in Fig. 2. In situ sampling modules of the type ESIP 5441 and ESIP 5442 are fixed in each of the bioreactors and were purchased from Trace Biotech (Braunschweig, Germany). Sampling details have been published recently [56]. Prior to use the membrane was preconditioned by pumping 1 mL min^{-1} of propanol/water (v/v 70/30) for 2 h. Pump 1 is a 2-channel hose pump from Ismatec (Wertheim-Mondfeld, Germany) which was connected to debubblers from Trace Biotech. Two 75-meter-bypass circuits were connected with the debubblers. The liquid in the by-pass circuits was transported by two isocratic pumps (K-120, 0.1–10 mL) from Knauer (Berlin, Germany) and continuously flushed with sample (pumps 2 and 3) to valve 1. The electrical six-way switching valve (valve 1) was connected with the by-pass circuits, the HPLC-pump and the HPLC. It was controlled by the Analyst 1.4 software from Applied Biosystems (Darmstadt, Germany) which allowed the automatic switching between anaerobic or aerobic bioreactor in-situ sampling. LC analyses were performed with an Agilent 1100 from Agi-

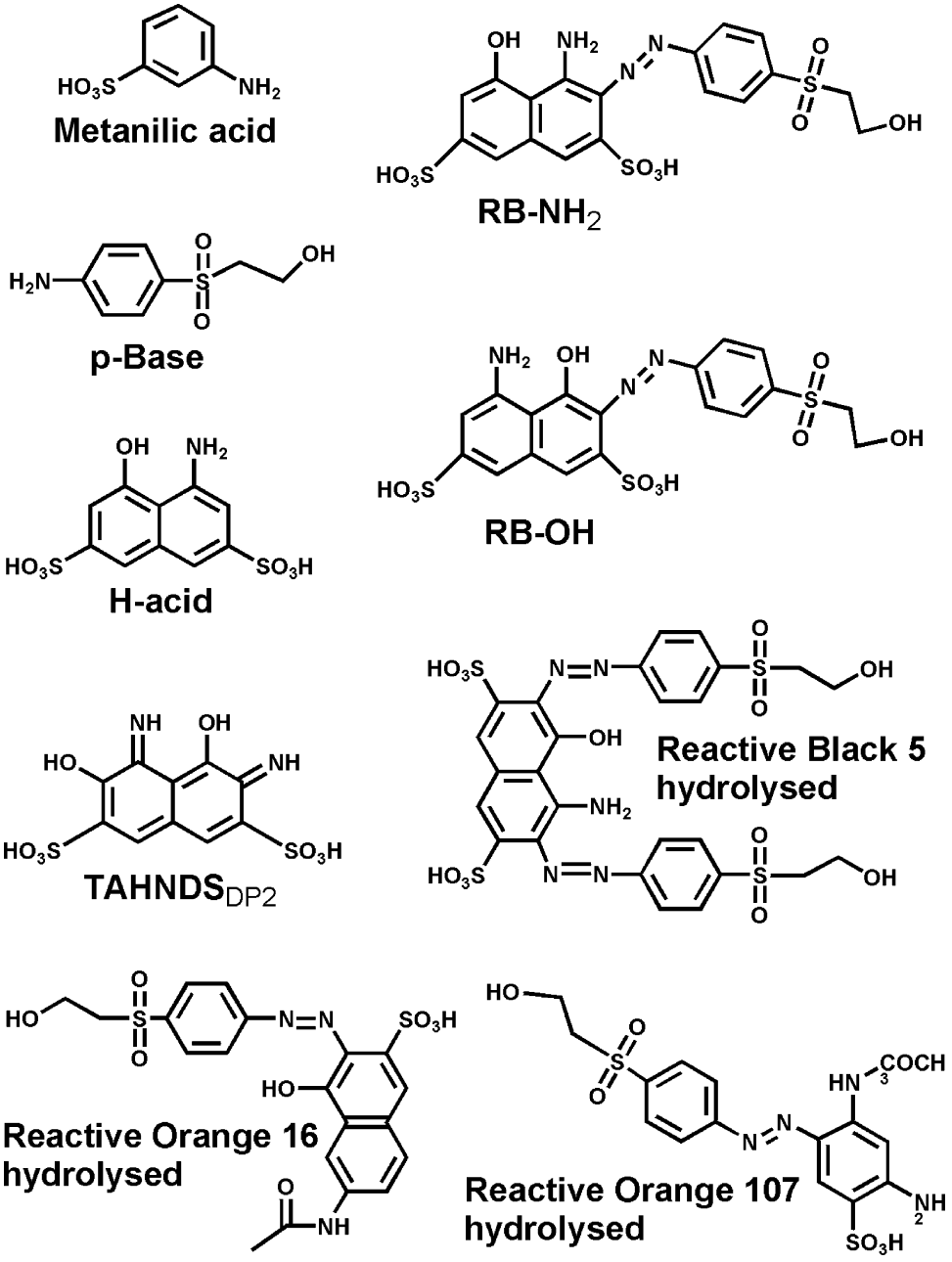

Fig. 1. Chemical structure of AAs, SAAs and SADs. Azo dyes containing hydroxy groups ortho to the azo linkage exhibit azo/hydrazone tautomerism in aqueous solution.

lent (Waldbronn, Germany) consisting of an Agilent 1100 degasser, Agilent 1100 G1312A binary pump (pump 4), Agilent 1100 G1313A autosampler (20 μL off-line injection), Agilent 1100 G1316A column oven with an electrical six-way switching valve (valve 2) with 4.6 μL injection loop, Agilent 1100 Diode Array Detector (slit width 4, sampling rate 1.25 Hz) and data processing with the Analyst 1.4 software under Windows 2000.

The cationic suppressor was a 753 Suppressor Module from Metrohm (Herisau, Switzerland) and controlled by Analyst 1.4 software. The cationic suppressor rotor was regenerated with a 0.2 mol L^{-1} H_2SO_4 water–acetone (80:20, v/v) and flushed with HPLC gradient grade water.

2.3. Column liquid chromatography

The analytical columns listed in Table 1 were purchased from the specified distributors. A pre-column was used for phase SB (4 mm × 4 mm select B), AQ (10 mm × 4 mm) and NHD (8 mm × 4 mm). Different methods were used for the evaluation of the HPLC phases and method development. They are listed in Table 2.

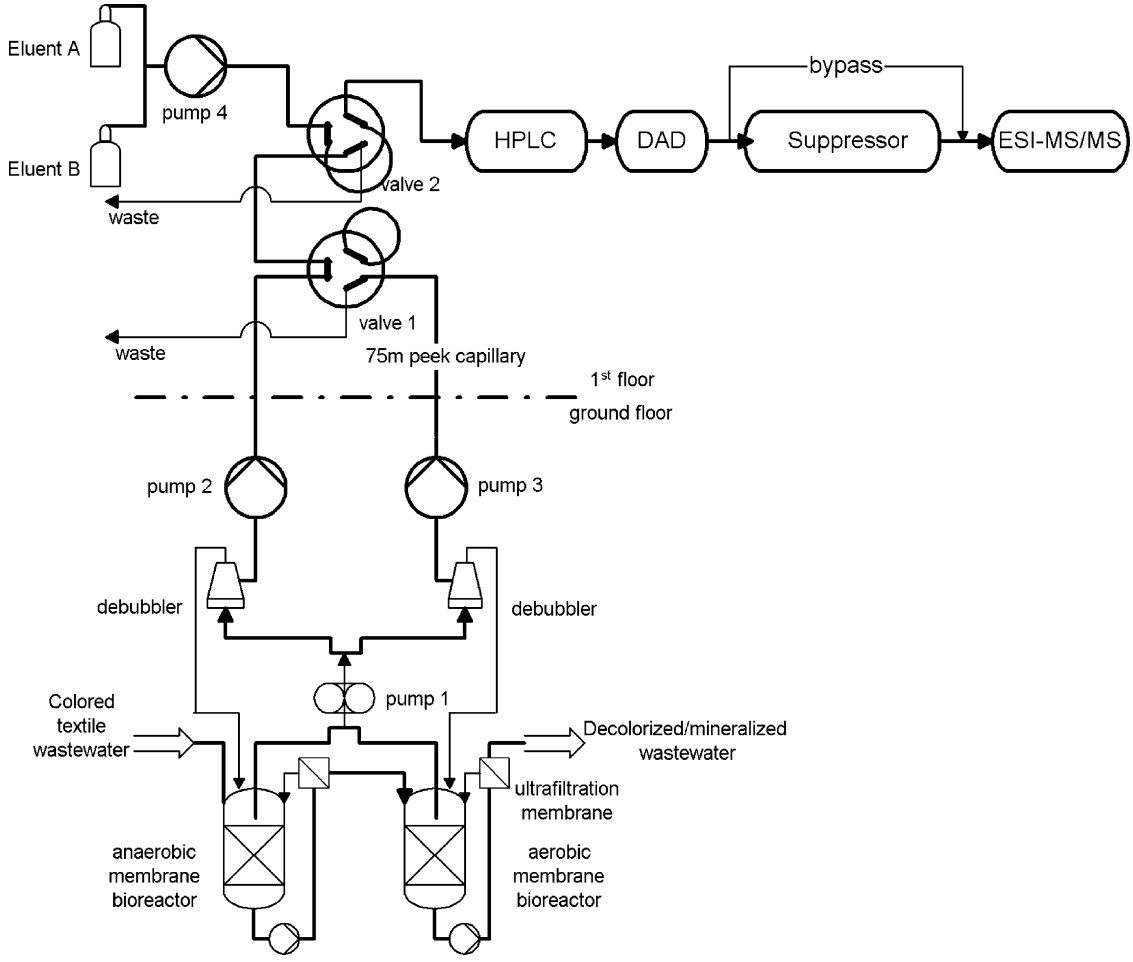

Fig. 2. Set-up of the wastewater treatment and process analytical system.

2.4. Mass spectrometry

A Qtrap mass spectrometer (linear ion trap quadrupole LC/MS/MS mass spectrometer) from Applied Biosystems/MDS Sciex (Ontario, Canada) was used for LC–MS and LC–MS/MS detection. The ESI-MS/MS was operated with a split ratio of 1:1 and a TurboIonSpray Ion Source in the negative ion mode at a Vacuum Gauge of (1.33 mPa) 3.7, Source temperature (at setpoint) 400 °C, curtain gas: 276 kPa, CAD: 34.5 kPa, ion spray voltage: −4500 V, nebuliser gas (GS1):

Table 1
Classical and novel polar embedded/endcapped RP-phases

Abbreviation	Type	Modification	$L \times d$ (mm)	d_p (μm)	Manufacturer (all Germany)
SB	LiChrospher 60, RP-select B	C18 ep[a]	125 × 4	5	Merck, Darmstadt
ZE	ZORBAX Eclipse XDB-C8	C8 dep[b], hd[c]	150 × 4.6	5	Agilent, Waldbronn
AQ	ProntoSIL AQ	C18 pep[d]	53 × 4	3	Bischoff, Leonberg
NHD	Nucleosil HD	C18 hd	70 × 4	3	M&N, Düren
SR	Chromolith SpeedROD	C18 ep	50 × 4.6		Merck, Damstadt
SH	Synergi Hydro	C18 pep	150 × 4.6	4	Phenomenex, Aschaffenburg
PA5u	ProntoSIL ace-EPS	C18 pebp[e]	125 × 4	5	Bischoff, Leonberg
PA3u	ProntoSIL ace-EPS	C18 pebp	125 × 4	3	Bischoff, Leonberg
SS	Symmetry Shield	C18 pebp	150 × 4.6	5	Waters, Eschborn
PP	ProntoSIL Phenyl	propyl-phenyl	150 × 4.6	5	Bischoff, Leonberg

[a] ep: endcapped phase.
[b] dep: double endcapped.
[c] hd: high density.
[d] pep: polar endcapped phase.
[e] pebp: polar embedded phase.

Table 2
Different methods and conditions for the evaluation of RP-columns

Method no. (v/v)	Isocratic (min)	% gradient B	Equilibration (min)	Total time (min)
Ion pair RP-HPLC				
Eluent A: 5 mM TBAAc water–acetonitrile (v/v see first column)				
Eluent B: acetonitrile				
I (100:0)	5	12 (5′) 40 (1′), 2′ hold	3	15
II (95:5)	5	11 (5′) 40 (1′), 2′ hold	3	15
III (85:15)	5	9 (5′) 40 (1′), 2′ hold	3	15
Eluent A: 5 mM TBAAc water–acetonitrile (v/v see first column)				
Eluent B: acetonitrile				
IV (98:2)	5	2 (25′), 40 (1′)	10	45
V (90:10)	1	6 (7′), 2 (4′), 10 (3′)	10	28
RP-HPLC on polar modified/embedded C18 phases				
Eluent A: 10 mM NH_4Ac water (pH adjusted with formic acid see first column)				
Eluent B: acetonitrile				
VI (pH 2.5)	5	10 (10′), 2′ hold	3	20
VII (pH 6,6)	5	10 (10′), 2′ hold	3	20
Eluent A: water (pH adjusted with formic acid see first column)				
Eluent B: acetonitrile				
VIII (pH 2.5)	5	10 (10′), 2′ hold	3	20

310 kPa, turbo gas (GS2): 552 kPa. All gases consisted of nitrogen (purity 99.999%) produced from gas generator from PEAK Science (Scotland). The MS was operated in different modes described in the following:

(1) Multiple reaction monitoring mode (MRM): resolution Q1: unit, resolution Q3: unit, detector parameters (negative): CEM 2300 V, MRM transients see Table 3.
(2) Enhanced MS (EMS).
(3) Enhanced product ion (EPI): Q1 is operated as a standard quadrupole mass spectrometer, Q3 is operated as a LIT mass spectrometer under the following conditions: scan rate: 4000 μs^{-1}, Q0 trapping: yes, LIT fill time: 20, dynamic fill time: on (2–150 ms), Q3 entry barrier: 8 V, scan range: 100–1000 u, declustering potential (DP): −90 V, entrance potential (EP): −10 V, collision energy (CE): −20 V. The DP, EP and CE of EPI mode were changing. Details are given if necessary in the text.

The Analyst software allows to create an information dependent acquisition (IDA) with up to two survey scans in a single experiment. IDA was used to analyse unknown

Table 3
Overview of solutes, DAD, MS and calibration data

$c = \mu M$, $n = 5$	PB	RB5-H	RB-NH_2	RB-OH	TAHNDS DP_2	RO16-H	RO107-H	H-acid	M-acid
MW	201	743	532	532	348	492	442	319	173
RT (min)	2.2	8.0	8.3	7.8	7.3	8.4	7.1	7.5	5.4
Q1 (u)	199.95	370.54	530.01	264.55	346.87	490.65	440.95	317.79	171.82
Q3 (u)	106.85	370.55	316.85	79.95	187.05	278.95	186.05	237.83	79.95
DP (V)	−56	−41	−91	−31	−61	−71	−81	−151	−51
EP (V)	−10	−7	−9	−8.5	−9.5	−7	−7	−7.5	−10
CEP (V)	−13.52	−18	−38	−14	−18	−24	−22	−16	−12
CE (V)	−28	−8	−54	−48	−44	−48	−66	−36	−34
CXP (V)	0	−4	−4	0	−4	−4	−4	−4	0
LOD (MRM)	n.d.[a]	0.012	0.019	0.003	0.001	0.509	0.001	0.010	0.59
LLOD	–	1.22	1.22	4.88	0.153	9.77	0.610	0.122	4.88
ULOD	–	313	625	313	78	325	625	7.81	78.1
r^2 [b,c]	–	0.9982[b]	0.9994[b]	0.9989[b]	0.9985[b]	0.9982[b]	0.9977[b]	0.9927[b]	0.9953[b]
RSD (%)	–	4.7	4.3	6.2	7.0	4.6	6.1	6.8	7.8
Wavelength	270	585	500	500	585	500	n.d.	300	n.d.
LOD	1.33	0.15	0.32	0.26	0.33	0.32	–	0.26	–
LLOD	9.77	0.61	9.77	2.44	4.88	4.88	–	15.6	–
ULOD	2500	625	625	625	625	625	–	500	–
r^2 [b,c]	0.9997[b]	0.9991[b]	0.9963[b]	0.9993[b]	0.9996[b]	0.9987[c]	–	0.9997[b]	–
RSD (%)	3.1	7.0	6.5	4.1	3.6	10.8	–	8	–

[a] n.d., not determined.
[b] Quadratic regression, x^{-1} weighting.
[c] Linear regression, x^{-1} weighting.

intermediates released from azo dye Reactive Black 5 under anaerobic–aerobic treatment. Based on the EMS scan masses which exceed a certain cps (counts per second) threshold were determined in order to perform EPI scans, automatically.

2.5. Bioreactor

Anaerobic bacterial sludge was obtained from a local municipal sewage treatment plant and filled in the anaerobic 30-L-bioreactor (see Fig. 2). The aerobic bioreactor was operated with activated sludge from an industrial wastewater treatment plant from Bayer AG (Leverkusen, Germany) The sludge was immobilised on 1 cm^3 polyurethane foamed carriers coated with activated carbon obtained from Biosys (Leverkusen, Germany) after sieving (2 mm) in both bioreactors. The sludge was conditioned 14 days (anaerobic: under nitrogen atmosphere) until an immobilisation rate of >95% could be observed. Redox potential electrode was a PFGR-2 and pH-electrode a HGK-Gel A/AKB, both purchased from Dosatronic GmbH (Immenstaad, Germany) and installed in a flow-through-cell flushed by fluid of the bioreactor. The pH was held constant at 7.0 ± 0.2 in both bioreactors. The temperature of the anaerobic bioreactors was maintained at $39 \pm 0.5\,^{\circ}C$. The redox-potential (against hydrogen potential) was continuously measured during the experiment. The content of both reactors was continuously pumped through the ultrafiltration membrane cells in cross flow mode (320 L h^{-1} at 0.5 MPa).

3. Results and discussion

The adaptation of an IP-RP-HPLC method developed for on-line monitoring with diode array detection [55,56] to LC–MS based monitoring was not applicable due to the requirements of the cation suppressor coupled with ESI-MS. Different reverse phases with 3 μm particle size and short columns were chosen in order to reduce analysis time and loading of the cation suppressor due to its limited capacity and to realise faster analyses for the monitoring of both bioreactors. In comparison to RP phases with 5 μm particles, a higher detection intensity was observed with 3 μm particles for the analysis of the same sample due to smaller peak volumes [58]. Thus, an additional peak broadening outer the column by suppressor module had to be taken in consideration with regards to the negative effect of a decreasing detection intensity. Reverse phases with polar functionality such as polar embedded phases were investigated as an alternative to the established IP-RP-HPLC. A standard mixture containing nine compounds representative for production and reactive colouration wastewater was used to compare the performance of all tested phases.

3.1. Ion-pair RP liquid chromatography

The retention of anionic solutes such as SAs, SAAs, and SADs can be obtained by use of amphiphilic modifiers like tetrabutylammonium acetate which was used in this work. The coupling to LC–MS required a subsequent cation suppressor for ESI-MS coupling. Retention of sulphonated solutes was achieved with a 5 mM TBBAc aqueous buffer which showed a comparable separation performance as 1 mM tetrabutylammoniumhydrogen sulphate which was used for IP-HPLC–DAD monitoring in previous works [55,56]. The column capacity ratios k' of four solutes (AAs, SAAs) on different columns are presented in Fig. 3A. All tested columns showed good separation capacity for the sulphonated solutes, but metanilic acid was insufficiently retained on the SB column. A decrease of organic content in the mobile phase is associated with a higher retention of all sulphonates solutes (see AQ column with 15, 5 and 0% ACN). Columns as AQ and NHD with a particle size of 3 μm show very similar k'-values for the later retaining solutes H-acid and $TAHNDS_{DP2}$. The quality of the separation was therefore evaluated by selectivity (α-values) and resolution (R_S) of the isomer pairs sulphanilic/metanilic acid and RB-OH/NH_2 (structure see Fig. 1). The α-values and R_S values are shown in Fig. 4 and were calculated on basis of the mass spectrometric detection in the MRM mode. High selectivity and resolution of the isomeric pair sulphanilic/metanilic acid is achieved by a mobile phase content between 0 and 15% ACN for all columns (NHD, AQ, SR). The later retaining pair RB-

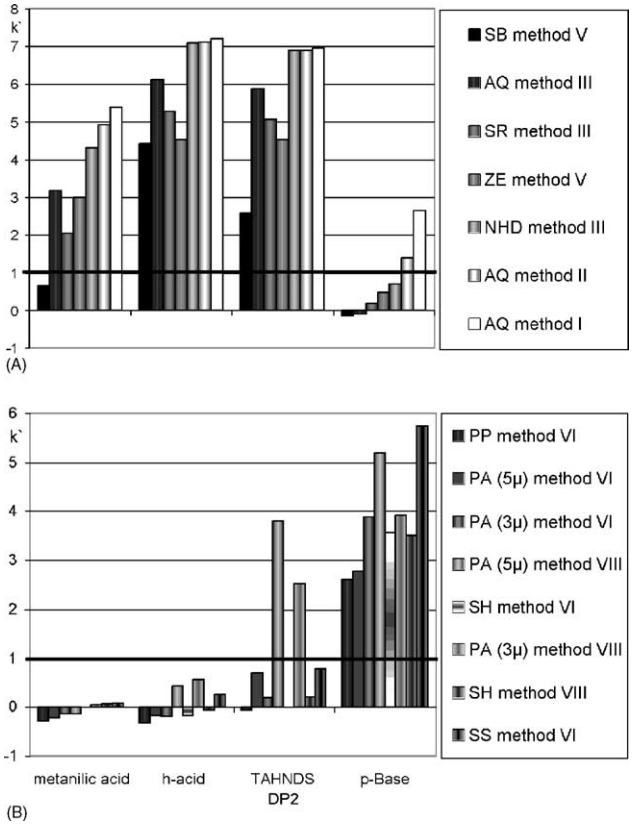

Fig. 3. k'-values of retention problematic compounds (A) different RP with IP (B) different RP with polar functionality. Hold-up time (t_0) was determined by injection of acetonitrile.

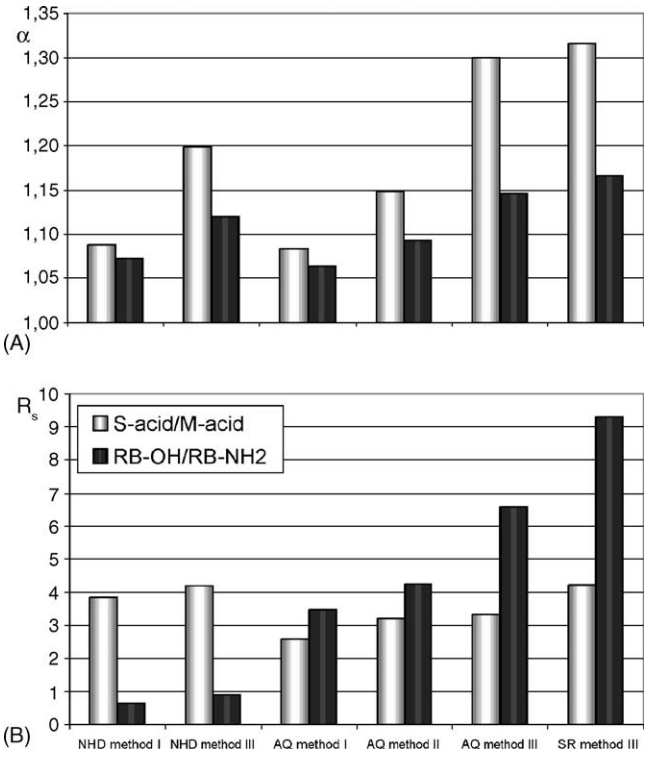

Fig. 4. α-values (A) and resolution values R_S (B) of isomeric solutes: sulphanilic/metanilic acid and RB-OH/NH_2.

OH/NH_2 is insufficiently separated on NHD column whether with or without a content of ACN in the mobile phase.

The separation of the AA p-Base required low content of organic mobile phase (ACN) at the beginning of the separation run which always started with 1–5 min isocratic elution (see Table 2 for details). With highly aqueous mobile phases (>95%), conventional RP columns often exhibit a visible deterioration of column performance after a certain period of time. The non-polar alkyl chains lose their brush-type structure for maintaining the hydrophobic interactions between the stationary phase and the analytes. The result of this phase collapse is a drastic decrease in retention time and poor resolution. Insufficient retention of p-Base i.e. $k' > 1$ was observed on several RP columns with a ACN content of 10–15%. Even though a temporary operation with a lower organic content is possible the performance of columns such as SB, ZE SR and NHD is limited with respect to highly aqueous mobile phases as they are not designed for these conditions in terms of a standard application for monitoring. A special modification of the silica surface allows the use of C18-phase under the required chromatographic conditions. A polar function increases the hydrophilic properties and interactions (H-bonding, dipole–dipole-interactions) of the stationary phase, which results in a higher wettability of the original non-polar surface. This can be achieved by the treatment of silica with a hydrophilic silane (hydrophilic end-capping) or by embedding a polar group on the alkyl chain. The surface chemistry of AQ-phases is designed to allow alkyl phase to remain fully accessible in highly aqueous mobile phases. The AQ column retained p-Base with k'-values >1 with a content of 0–5% ACN in the mobile phase. Therefore the AQ column was preferred as it allowed reproducible separation even with highly aqueous mobile phases. Chromatographic method time could be reduced by use of a short 53 mm × 4 mm column in combination with 3 μm particles. A rapid re-equilibration to initial aqueous conditions was observed on the AQ column. Re-equilibration was reduced from 10 min on the SB column to 3 min on the AQ column.

3.2. Liquid chromatography on RP phases with polar functionality

As mentioned above stationary phases with polar embedded groups consisting of amide or carbamate groups were designed to improve the analysis of strongly basic, acidic and polar solutes and allow the use of highly aqueous mobile phases. The improvement is attributed to the weakening of the interaction between non-bonded silanols and the analytes as a result of the embedded group [59]. A higher retention of phenolic and other polar groups was observed on phases with polar functionality due to their hydrogen-bonding potential. The separation of the test mixture was started with a 5′ isocratic elution using 100% aqueous buffer with and without 10 mM NH_4Ac in order to compare the separation performance of very polar anionic solutes on different columns with polar functionality (see Fig. 3B). Retention and separation of metanilic acid and H-acid was not observed if 100% aqueous buffer containing 10 mM NH_4Ac at pH 2.5 was used as well as by use of 100% aqueous buffer at pH 2.5 containing no NH_4Ac. It is likely that this effect can be attributed to residual silanol activity which caused Donnan exclusion of the anionic solutes [32]. It is an indication that the charge repulsion of anionic sulphonic acids can not be fully eliminated by the polar groups embedded in the bonded silane in order to shield the residual surface silanols. Even though a higher retention was observed for all of three sulphonated solutes by use of an aqueous buffer without NH_4Ac it is difficult to explain this effect because interactions of sulphonated solutes causing retention on polar embedded phases are not fully understood. On the one hand a higher retention of basic solutes can be attributed to the reduced hydrophobic properties of polar embedded phases and an increased potential of hydrogen-bonding interaction [59]. Thus, the use of NH_4Ac increases the ionic strength of the buffer and decreases the non-specific polar interaction of the acidic solutes resulting in lower retention. On the other hand adsorption of acetate on the stationary phase can strengthen the repulsion of the anionic solutes. The significant increase of retention of H-acid and even more of $TAHNDS_{DP2}$ can be attributed to these effects observed on the PA3u phases.

Chen et al. [35] investigated the effect of concentration of NH_4Ac on plate number, asymmetry factor and retention time on RP phases. The plate number increased with increasing concentration of NH_4Ac in the mobile phase until reaching a plateau at 100 mM. Peak symmetry and retention also

improved with increasing concentration of NH_4Ac. These effects mediated by NH_4Ac are likely due to the electrolyte's capacity to minimise chromatographic interference by free silanol groups which are invariably present in C18 packing material [60] and so enhance the interaction of the analyte with the stationary phase. The contradiction between the reduction of silanol activity by NH_4Ac which should cause a higher retention of acidic solutes on polar embedded phases and the increase in retention by polar interaction without use of NH_4Ac should be objective of further investigations.

The retention of the standard mixtures on PA3u with and without NH_4Ac is compared (data not shown). The influence of NH_4Ac on the retention, selectivity and the peak symmetry is demonstrated on the tailing and peak shape of $TAHNDS_{DP2}$ which was improved by use of NH_4Ac accompanied by a shorter retention. Selectivity α and resolution R_S of the isomeric pair RB-OH/NH_2 remained constant with $\alpha = 1.05$, $R = 3.9$ (without NH_4Ac) and $\alpha = 1.05$, $R = 4.3$ (with NH_4Ac). It was observed that the detection intensity of the ESI-MS was decreased as a result of the addition of NH_4Ac. In summary, polar embedded phases such as PA and SS and polar endcapped phase as SH showed better retention in comparison to modified C18 phases, e.g. phenyl phases PP and classical RP columns under the same conditions (data not shown). The PP column showed insufficient retention for all of the strong anionic solutes due to the above mentioned charge repulsion of the anionic solutes which exceeded the possible π–π-interaction of the aromatic ring.

Based on of the results derived from the column comparison of IP-RP-HPLC and RP-HPLC with polar selectivity we decided to focus further effort on the establishing of an IP-RP-HPLC based DAD-MS monitoring. Although basic solutes such as p-Base underlie an ion-exchange when they were passing the suppressor module this method showed better analytical performance than the RP-HPLC with polar selectivity.

3.3. Quantitative calibration by DAD and MS/MS (MRM)

The analytical system was calibrated with nine solutes in a concentration range between 0.01 and 5000 mM with diode array detection and with MS/MS in the MRM mode on an AQ column with mobile phase of water–acetonitrile (95:5, v/v) comprising 5 mM TBAAc (detailed conditions see Table 2). The MS/MS and DAD adjustment parameters as well as calibration data and results are presented in Table 3. DAD detection covered the upper concentration range between 5 and 2500 μM. MS/MS detection was more suitable for the lower detection range between 0.1 and 300 μM. Dynamic ranges of three to four decades could be performed with both detectors. The main advantage of MS/MS detection is its high selectivity derived from the mass spectrometric determination of transients in the MRM mode.

The relative average detection intensities of nine solutes related to the maximum detection intensity were determined (data not shown). The order of intensity of the standard mixture can be described in the following: phases with polar selectivity and a NH_4Ac-eluent with pH 2.5 (very low intensities), phases with polar selectivity and a NH_4Ac-eluent with pH 6.5 (low intensities), classical RP with short isocratic part and high gradient (middle intensities) and classical RP with long isocratic part and low gradient (highest intensities). Robustness of the analytical procedure was tested by addition of NaCl and Na_2SO_4 to the standard solutions (for details see Section 2). Textile wastewater from colouring process contains a high amount of inorganic salts such as NaCl and Na_2SO_4 which are added to the dyebath in order enhance the dyeing process and to reduce the loss of hydrolysed dyes especially in reactive dyebathes. The loss of reactive dye can exceed 20 wt.% and more. Different concentrations of NaCl (0.02, 0.1, 1, 5 M) and Na_2SO_4 (0.02, 0.1, 0.5, 1 M) were added to the stock solution which consists of varying concentrations of the selected solutes from 10 to 100 mM (see Section 2.1). The solutions were tested on several columns under different conditions in order to determine the effect of high salt content in samples on the electrospray mass detection. An overview of some of these tests is presented in Fig. 5 as the average recovery of all tested solutes related to the non-spiked solution. The AQ column shows a very high and reproducible recovery even at high salt concentrations of 5 M NaCl which was not observed on other columns. This is an indication for the influence of chromatographic separation conditions on the performance and sensitivity of the mass spectrometric detection. However, Na_2SO_4 has a much higher ionisation suppression potential than NaCl. In this case

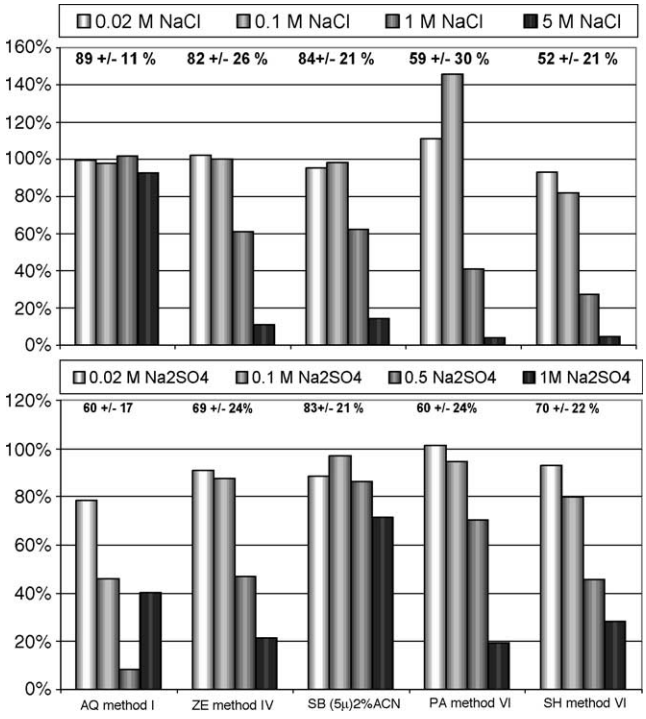

Fig. 5. Salt induced matrix effect on MRM-detection concerning different RP columns (average recovery and RSD, $n = 3$).

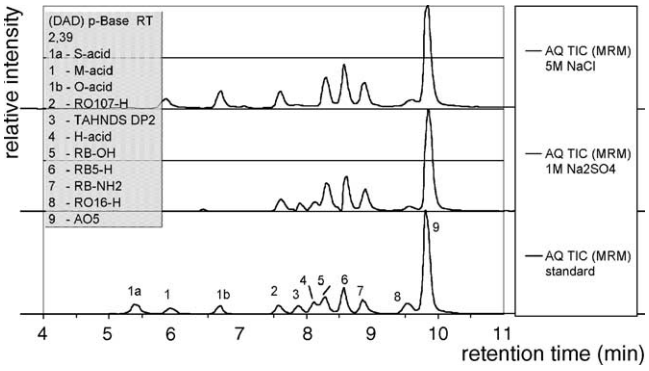

Fig. 6. Influence of 1 M Na_2SO_4 and 5 M NaCl on Total Ion Chromatogram (MRM detection) of standard mixture (RP-IP on AQ method II; 9—additional azo dye Acid Orange 5—AO5).

classical RP columns and RPs with polar selectivity show a better performance than the AQ column.

The highest suppression rate was observed on the detection of early eluting solutes such as M-acid (isomers: sulphanilic acid—S-acid, orthanilic acid—O-acid), H-acid and $TAHNDS_{DP2}$ (see Fig. 6). In comparison to measurements on SB with a low gradient, these SAAs eluted later and showed a decreased suppression. High aqueous mobile phases and low gradients have a diluting effect on the salt-containing samples whereby the analysis time is increased but ionisation suppression effects by salts can be reduced. Published approaches to minimise the ion suppression effects are discussed by Matuszewski et al. [61] and include: providing more chromatographic retention of analytes, using more selective extraction procedure for matrix cleanup and further techniques. An influence of salt injections on the chromatographic peak shape was not observed. However, typical salt concentrations of undiluted textile dye bathes are in the area of 50 g L^{-1} NaCl and Na_2SO_4 10 g L^{-1}. The ionisation suppressive effect of salt has to be observed if concentrated dye bathes or dye solutions are analysed.

3.4. On-line monitoring of anaerobic/aerobic degradation of RB5-H

A dye solution comprising hydrolysed Reactive Black 5 (RB5-H), NaCl and traces of Na_2SO_4 was fed into the anaerobic–aerobic bioreactor system. The wastewater treatment system was operated with a volume load of 5 $mM_{RB5\text{-}H}$ d^{-1} L^{-1} and 128 mM_{NaCl} d^{-1} L^{-1} for 8 days. The volume load related to the spectral absorption of the dye solution was 26,500 $m_{585nm}{}^{-1}$ d^{-1} L^{-1}. After stopping the feed, the bioreactors were run in cycle operation, i.e. no in- or export of any liquid, in order to study the degradation processes in both bioreactors. The analytical monitoring procedure was as follows (AE, aerobic bioreactor; QC, quality control consisting of solution of nine standard solutes; AN, anaerobic bioreactor): Blank – MRM (AE) – EMS (AE) – IDA (AE) – (QC) – MRM (AN) – EMS (AE) – IDA (AN) – (QC). An except of the monitoring data is presented in Fig. 7 for the anaerobic bioreactor and in Fig. 8 for the aerobic bioreactor. Fig. 7 shows the relative concentrations of three analytes and the development of the redox potential. The beginning of feeding of the anaerobic bioreactor caused an increase of the redox potential from below −500 up to −250 mV. Between 0.1 and 1 mL Ethanol per litre of the reactor volume were added daily as an auxiliary substrate to the anaerobic reactor in order to stimulation the sulphate reducing bacteria which enhance the decrease of the redox potential. The effect derived from feed of auxiliary substrate can be seen in the plot as a partial drop of the potential (days 1–5). However, a sufficient and continuous decrease of the redox potential was not observed during the feed-period. The concentrations of the hydrolysed dye (RB5-H) and a partially hydrolysed dye with a non-hydrolysed vinyl group (RB5-HV) vary within day 1 and 2 of operation and decrease to zero on days 3–5 (structures of dyes and pathway of intermediates are presented in Figs. 9 and 10). The increase of the concentration is always observed when the redox potential exceeds a barrier of −250 mV. The decrease of the hydrolysed dyes is followed by an increase of the concentration of the partial reduced diazo dye, i.e. RB5-H-PB. The azo bond in ortho-position to

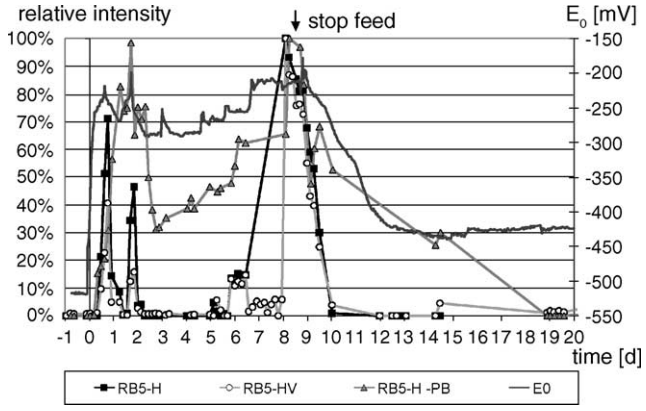

Fig. 7. Monitoring of the reduction process of Reactive Black 5 hydrolysed in the anaerobic reactor and redox potential E_0 (RB5-H, Reactive Black 5 hydrolysed; RB5-HV, RB5-H with vinyl group; RB5-H-PB, partial reduced RB5-H).

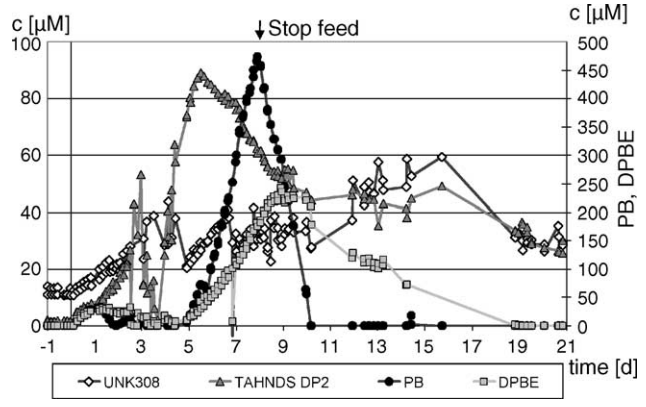

Fig. 8. Monitoring of selected intermediates from anaerobic reduction released to the aerobic bioreactor.

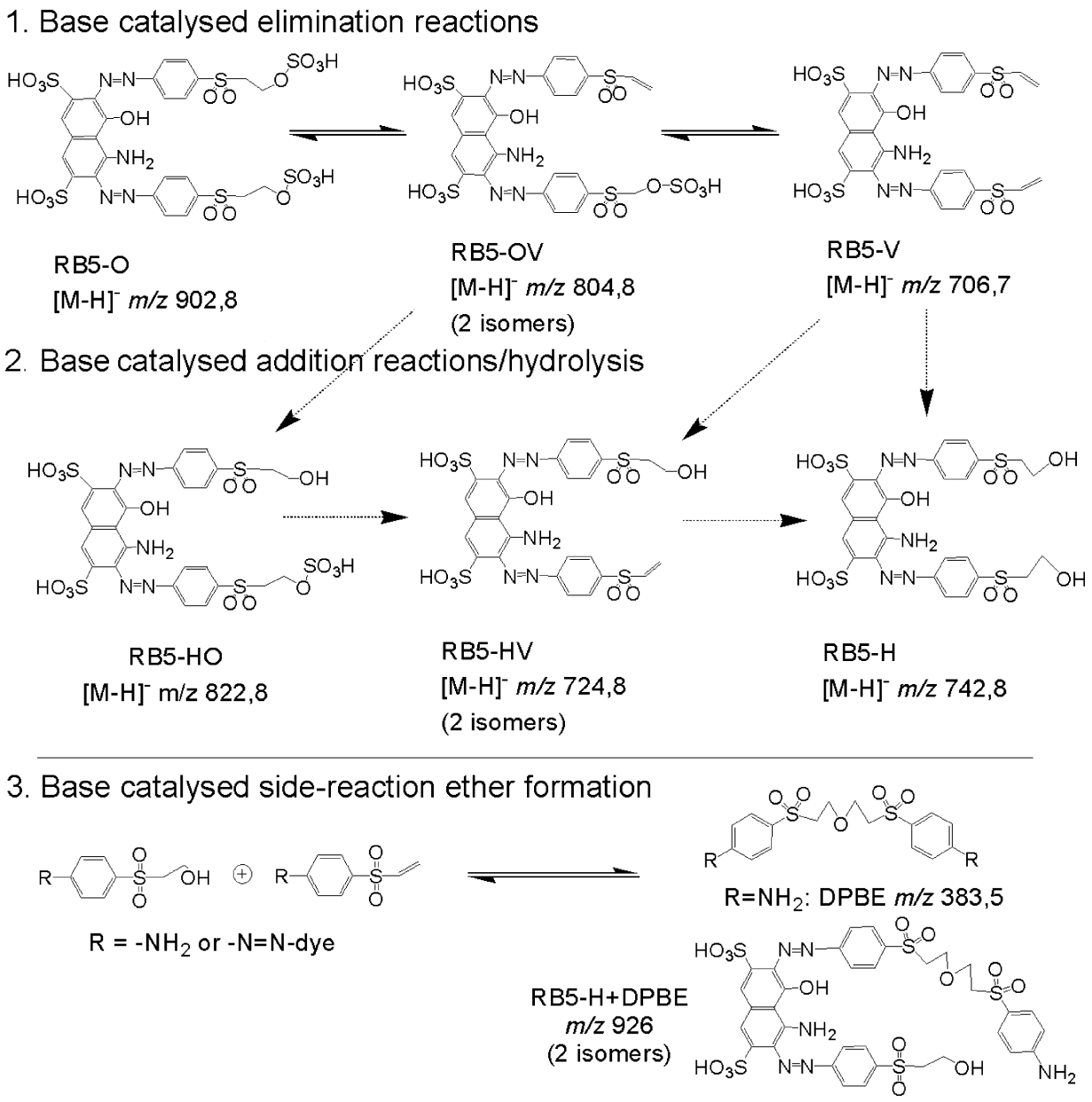

Fig. 9. Base catalysed elimination, hydrolysis and ether formation of Reactive Black 5 in the colouration process: structures and pathway [10,68].

the amino group is supposed to be reduced at first while the azo bond in ortho-position is tautomerised. Tautomerisation of RB5-H and a detailed description of additional compounds of this commercial dye are presented by Pham et al. [62]. During the zero-concentration period of RB5-H and RB5-HV, RB5-H-PB (2nd–3rd day) slowly increased to its maximum on the 8th day as the redox potential exceeded the border value again. Thereby RB5-H and RB5-HV increased to maximum concentration (7th–8th day). A continuous decrease of the redox potential was observed after the stopping the feed to a stable value of −430 mV. It is accompanied by a drop of the concentration of both hydrolysed dyes. The complete anaerobic reduction of the partially reduced dye was noticed after the redox potential significantly decreased below −250 mV. Most models of the biological reduction have in common that as long as no reaction partners with redox potential higher than the dyes, e.g. no O_2 or NO_3^-, are present, the electrons and protons gained by oxidation of the auxiliary substrate (ethanol in this work) will be transferred to the dye [63,64]. The observed relation between redox potential, concentration of hydrolysed and partial reduced dyes indicates that diazo dyes undergo a two stage reduction. It is expected that this effect can also be attributed to tri- and tetraazo dyes. This is important for the control of the anaerobic reduction of azo dyes in wastewater treatment by an open or closed loop controller. The redox potential can be controlled by feeding an auxiliary substrate such as ethanol or glucose to the process.

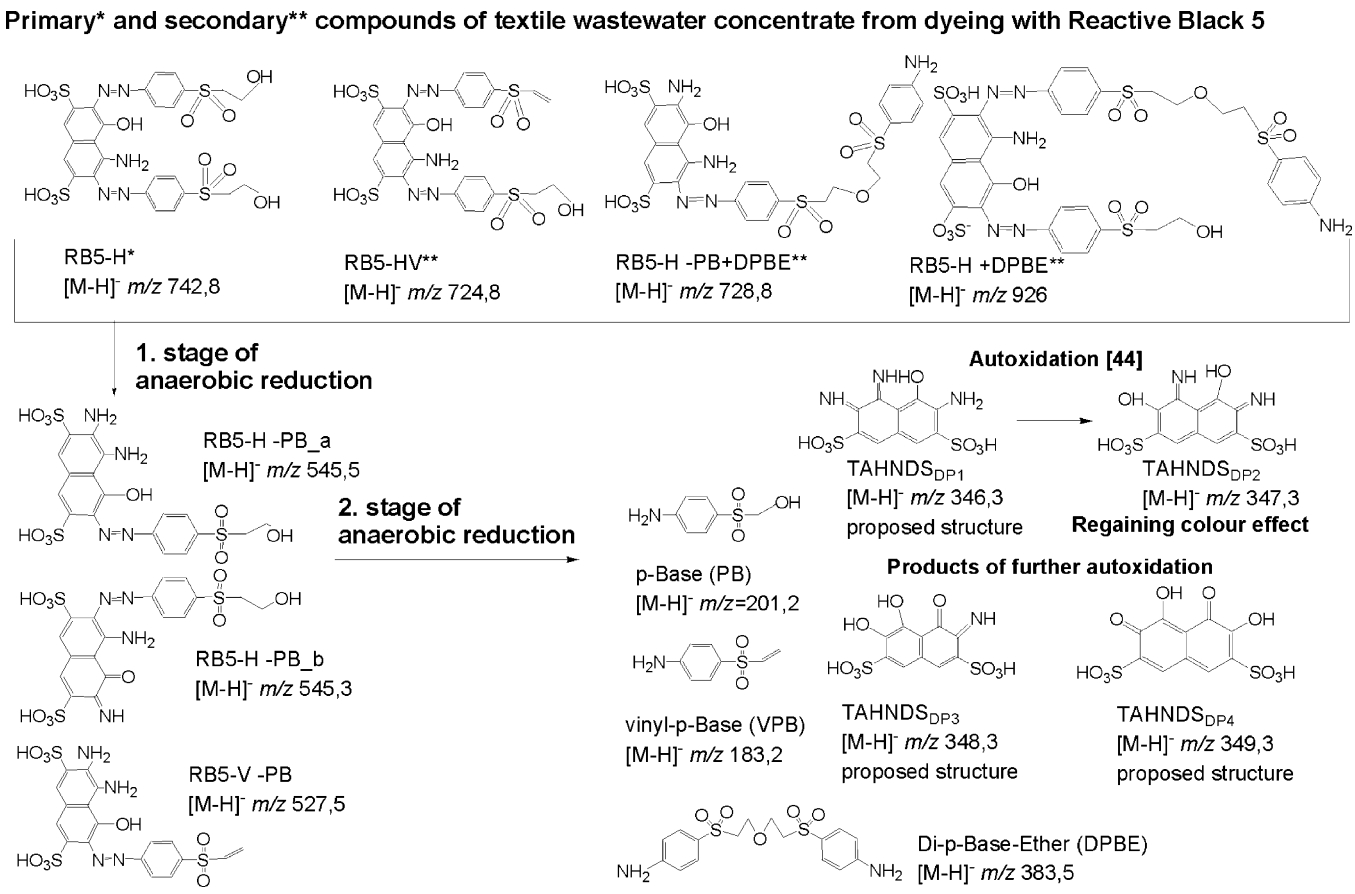

Fig. 10. Major pathway of the anaerobic degradation of Reactive Black 5 and intermediates.

The goal of aerobic mineralization of intermediates released from anaerobic reduction was monitored in the same way as the anaerobic reduction. Hereby, it was observed that the intermediate $TAHNDS_{DP1}$, which is released under the total reduction of RB5-H is autoxidised to $TAHNDS_{DP2}$ (see Fig. 10). The autoxidation reaction is proposed in analogy to the autoxidation reactions of *o*-aminohydroxynaphthalenes described by Kudlich et al. [44]. Both analytes have a deep blue colour which is origin of the so called "regaining colour" after complete reduction of an azo dye.

The proposed analyte $TAHNDS_{DP1}$ could be detected and characterised by on-line LC–MS/MS for the first time from the anaerobic bioreactor. A confirmation of the proposed structure could not be presented because of insufficient information derived from fragmentation. Nevertheless, two further intermediates ($TAHNDS_{DP3}$ *m/z* 348 and $TAHNDS_{DP4}$ *m/z* 349) were observed, one of which only under aerobic conditions ($TAHNDS_{DP4}$). It is assumed that $TAHNDS_{DP2}$ underlies further oxidation reactions in the aerobic reactor. The degradation fate of selected intermediates in the aerobic bioreactor is presented in Fig. 8: AAs (PB, VBP, DPBE) were found to be degraded after a short adaptation time. Biological inhibition or an overload was observed at the 5th day of operation as the concentration of all analytes increased. p-Base was degraded within 2 days and DPBE within 11 days after the stop of feed (8th day). The degradation of SAAs such as UNK308 and $TAHNDS_{DP2}$ was much slower than of the observed AAs. Therefore, the degradation is not necessarily attributed to a biological degradation which should be continuous after an adaptation phase of micro-organisms. Azo dyes (RB5-H, RB-HV, RB5-H-PB) were not degraded in the aerobic bioreactor. The structure of UNK308 (*m/z* 308) is not yet confirmed but supposed to be a derivative of PB: 2-[2-(4-amino-benzenesulphonyl)-ethoxy]-ethane sulphonic acid. Thus, sulphonation of AAs such as PB cause inhibition of the biological degradation. Further sulphonated derivatives of PB such as 2-(4-amino-benzenesulphonyl)-ethanesulphonic acid (*m/z* 264) were found to be not or poorly degraded in the aerobic bioreactor. Proofs of a total biological degradation and mineralisation of the most popular reactive dye Reactive Black 5 are so far reported by Supaka et al. [64] for low dye concentration using bacteria isolated from dye effluent-contaminated soil. Sakalis et al. [65] found Reactive Black 5 and additional reactive dyes to be fully degraded and decolourised up to 99.9% by an electrochemical treatment method. A regaining colour effect of the treated dyes after exposure to air was not investigated. The full biological degradation of textile effluent concentrates has not yet been reported. Most investigations dealing with the biological degradation of dyes use lumping parameters derived from

spectral absorption analysis for the description of the observed degradation rate of low concentrated synthetic dye effluents. It has to be kept mind that the reduction of an azo bond causes a reduction of spectral absorption, but not always fully decolourisation. Thus, the concentration of pollutants is kept constant while the spectral adsorption is decreased. Therefore, decolourisation and degradation, i.e. mineralisation has to be well distinguished.

An approach has been made by the use of the far more selective monitoring of degradation processes by use of HPLC coupled with DAD whether used on-line [55,56] or off-line as reported by Libra et al. [66], who observed an overall dye removal of ∼65% for the fully and the partially hydrolysed dye RB5 in a two stage rotating disc reactor. $TAHNDS_{DP2}$ was described to cause the effluent to be coloured although no RB5-H was present on basis of off-line LC–MS analyses.

3.5. On-line monitoring of anaerobic/aerobic degradation of RB5-H and structural elucidation of intermediates

EMS and EPI scan modes were used for structural elucidation purpose in line with the on-line LC–DAD–ESI-MS/MS. The information dependent acquisition mode allowed the automated combination of EMS and EPI for on-line MS/MS experiments of peaks which exceeded a certain intensity threshold. Several intermediates e.g. $TAHNDS_{DP1–4}$, UNK308 of the azo dye Reactive Black 5 could be detected and characterised on the basis of their *m*/*z* ratio and specific fragmentation. The molecular weight of the autoxidation sensitive compound $TAHNDS_{DP1}$ (structure see Fig. 10) was confirmed by on-line LC–MS determination of the *m*/*z* ratio. A confirmation of the proposed structure was difficult because only low fragmentation was observed at different collision energies (20, 35, 50, 80 V) in the EPI mode: *m*/*z* 346 → (−80 u) *m*/*z* 266 → (−80 u) *m*/*z* 186 → (−27 u) *m*/*z* 159. However, further intermediates of the autoxidation of $TAHNDS_{DP1}$ such as $TAHNDS_{DP2–4}$ show similar fragmentation patterns. $TAHNDS_{DP3}$ could be released from reduction of a side product of Reactive Black 5 which was described in the literature (the dye is based on 4,5-dihydroxy-naphthalene-2,7-disulphonic acid instead of 4-amino-5-hydroxy-naphthalene-2,7-disulphonic acid (H-acid) [63]) or could be product of further autoxidation of $TAHNDS_{DP2}$. The fragmentation pattern (*m*/*z* 348 → −80 u → −80 u → −28 u → −16 u) indicated a exchange of another NH_2-group against an OH-group (autoxidation mechanism). Investigations on the fragmentation pattern of $TAHNDS_{DP4}$ showed the same mechanism with an additional loss of 28 u (*m*/*z* 349 → −80 u → −80 u → −28 u → −16 u → −28 u). $TAHNDS_{DP4}$ is supposed to be the final autoxidation product of $TAHNDS_{DP1}$. Nevertheless, the main pathway from hydrolysis of the reactive dye in the colouration process, reduction in the anaerobic treatment stage and partial oxidation in the aerobic bioreactor could be revealed and is presented in Figs. 9 and 10. The pathway of hydrolysis was similar to the matrix-assisted laser desorption/ionisation MS determinations of hydrolysis by Chromá-Keull et al. [67]. An ether form of RB5-H and PB was observed throughout the monitoring of both bioreactors and could also be determined in the hydrolysed dye solution which had been fed to the bioreactors (DPBE, RB5-H–PB + DPBE, RB5-H + DPBE). It is a product of a competing reaction of the reactive dyeing process under alkaline conditions which was described by Rys and Stamm [68]. There are still several unknown intermediates at lower concentration levels whether sulphonated or not sulphonated in addition to the described intermediates of the treatment of dye Reactive Black 5. Thus, the intermediates of real textile wastewaters containing several azo dyes are expected to be even more complex than those derived from one dye.

4. Conclusions

Ion-pair chromatography showed sufficient selectivity and resolution for separation of a test mixture comprising nine aromatic amines, sulphonated aromatic amines and sulphonated reactive azo dyes by use of a short polar endcapped RP phase with 3 μm particles. Good analytical performance was observed for the quantification of the test mixture on both, a diode array detection and a ESI-MS/MS detector. The use of tetraalkylammonium ion-paring agent chromatography required a cationic suppression before the analytes were introduced to the ESI-MS/MS. Robustness of the analytical systems was demonstrated on the test mixture which contained different loads of NaCl and Na_2SO_4, whereby sulphate concentrations of $> 0.5\,M_{Na_2SO_4}$ showed a high ionisation suppression potential on the ESI-MS/MS. An ion exchange of basic and cationic solutes such as p-Base was observed as a secondary effect. Polar embedded reverse phase columns were found to be not usable for the separation of small mono- and disulphonated aromatic amines. Nevertheless, they are suitable for the separation of sulphonated azo dyes as an alternative to ion-pair chromatography.

On-line HPLC–DAD–ESI-MS/MS used as a process diagnostic tool helped to reveal information about the relation between sum parameters such as the redox potential and the biological induced mechanisms of azo dye reduction. The stage reduction mechanism of polyazo dyes can be controlled by the feed of auxiliary substrate and the consequent lowering of the redox potential. An approach was made by using sample devices which were directly inserted into the process and coupled to LC–DAD–MS. Autoxidation sensitive analytes such as *o*-aminohydroxynaphthalene sulphonic acids released from anaerobic reduction of azo dyes can be characterised by mass spectrometric methods. Monitoring of aerobic wastewater treatment process helps to characterise the biodegradability of specific analytes and to control and evaluate the selective adaptation of bacteria to a certain chemical pollutant such as p-Base even in complex wastewaters. Selective monitoring of pollutants which are known to inhibit

the biodegradation process can be detected even at low concentration levels. However, the remaining colour of water discharged from the biological treatment system could be attributed to non-reduced azo dye, partial reduced azo dyes or poorly degradable autoxidised intermediates released from totally reduced azo dyes. Process optimisation as well as the necessity of the application of further treatment methods can be derived from selective monitoring. The determination of an ether form of the widely used dye Reactive Black 5 suggests that on-line LC–MS/MS can be applied to further chemical reactions such as dyeing processes in order to use it as a process integrated optimisation tool to minimise undesired side-effect reactions.

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