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Harmful azo colorants in leather Determination based on their cleavage and extraction of corresponding carcinogenic aromatic amines using modern extraction techniques

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Abstract

This study concerns the possibilities of using microwave-assisted extraction (MAE) or supercritical fluid extraction (SFE) for detection of harmful azo colorants in leather. After degreasing of the leather sample with SFE there follows a reductive cleavage of the azo colorants to their corresponding aromatic amines in the MAE or SFE equipment. The aromatic amines are subsequently extracted using either MAE or SFE and then finally determined by liquid chromatography with diode-array detection. The results have been compared with recoveries obtained using the German DIN method 53316. This standard method, based on conventional solvent extraction, is used in several European countries. Overall much better recoveries were obtained using MAE or SFE. With both MAE and SFE the amine recoveries of spiked leather samples were generally above 50%. The average recoveries were 62% for MAE and 60% for SFE (solvent collection) compared to 24% with the DIN method. For genuine leather samples the recoveries decreased, especially for benzidine. In this case the average values for MAE, SFE and DIN were 54, 38 and 19%, respectively. The quantification limits in leather samples using MAE or SFE were below 1 mg/kg for all amines investigated. The within-laboratory precision was generally better than 10%, varying somewhat with the analyte considered. With the proposed methodology, the amount of hazardous organic solvents used could be decreased and the sample throughput increased with at least a factor of two with less manual handling compared to the DIN method.

Keywords: Supercritical fluid extraction; Microwave-assisted extraction; Extraction methods; Amines, aromatic; Azo dyes

1. Introduction

Proven or suspect carcinogenic amines may occur as components in azo colorants, used to colour textiles and leather. Although the intact dye molecule is biologically inactive, the living organism is able to cleave the azo bonds releasing the amines [1–3]. In view of these findings some member states of the European Union (EU) have introduced a ban on azo colorants used in consumer goods, which might release any of 20 listed harmful aromatic amines.

At present the German method DIN 53316 is the most accepted analytical method for determination of azo colorants in leather [4]. This method was de-

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veloped within a restricted time period in 1997 after the German ban on certain azo colorants and has some drawbacks leading to relatively low accuracy. Additionally, the method is time consuming and uses hazardous organic solvents such as *n*-hexane and methyl *tert*.-butyl ether.

Microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE) are well established techniques for the determination of different pollutants from solid samples, providing faster extractions and less usage of organic solvents than conventional solvent extraction. No SFE studies on aromatic amines, which are formed during the reductive step for azo dye determination have been reported. However, Garrigós et al. recently reported determination of free aromatic amines such as benzidine in fingerpaints for children using SFE [5,6]. Similarly, SFE has been applied to the extraction of carcinogenic, aromatic amines from soil, sand and rubber samples [7–10]. To our knowledge no MAE paper on this subject has hitherto appeared. In publications concerning extractions of amines from textiles and leather, released from azo dyes, solid-phase microextraction (SPME) [11] and traditional liquid–liquid extraction procedures [12] have been used.

In this paper we have studied the possibilities of using a totally new approach utilising MAE or SFE for the determination of harmful azo colorants in leather. It is based on determination of corresponding aromatic amines by high-performance liquid chromatography (HPLC) after reduction of the azo colorants.

2. Experimental

2.1. Samples

Cattle hide leather samples were prepared at the Research Center for Leather and Artificial Leather (FILK, Freiberg, Germany). After dyeing and waterproofing, all samples were ground and stored in sealed beakers at 8 °C before they were extracted. For the degreasing and spiking experiments, a leather sample dyed with Acid Black 210 was used. This azo dye does not form after reduction any of the aromatic amines investigated in this study. For the native experiments, leather samples were dyed with Acid Red 035, Acid Black 209, Acid Orange 031, Acid Black 077, Direct Red 061 and Direct Blue 015. The carcinogenic amines released after reductive cleavage of these azo dyes are *o*-toluidine, 3,3-dimethylbenzidine, 4-chloroaniline, benzidine, 3,3-dichlorobenzidine and 3,3-dimethoxybenzidine, respectively. Taking into account the amount of azo dye used for dying, the content of pure azo dye in the dye sample and the assumption that 90% of the azo dye is fixed on the leather, the theoretical amount of amines for the leather sample was calculated. The samples were prepared to give amine concentrations in the range of 100–600 mg/kg leather, depending on which dye used. These values were considered as the true values for genuine samples. All recovery values obtained with the different methods refer to these values.

Fig. 1 shows the structures of the dyes together with the released carcinogenic amines investigated.

2.2. Chemicals and materials

Solvents such as methanol, acetonitrile, cyclohexane and methyl *tert*.-butyl ether of HPLC grade, and buffer solution (citrate–sodium hydroxide) with pH 6.00 at 20 °C, were purchased from Merck (Darmstadt, Germany). Benzidine, 3,3-dimethylbenzidine and 3,3-dimethoxybenzidine were delivered by Fluka (Buchs, Switzerland), *o*-toluidine and 3,3-dichlorobenzidine from Sigma–Adrich (Steinheim, Germany) and finally 4-chloroaniline from Riedel-de Haën (Seelze, Germany).

Stock standard solutions of the amines were separately prepared in methanol at a concentration of 1.0 mg/ml. Standard solutions of all amines were then diluted to appropriate concentrations in methanol. All solutions were stored in darkness at 8 °C.

The reducing agent used was mainly sodium dithionite (85% from Acros Organics, Geel, Belgium) prepared in distilled water, but also tin(II) chloride dihydrate (98% from Sigma–Aldrich), 0.6 g/ml in conc. HCl, was used in initial experiments. These solutions were always freshly prepared.

Carbon dioxide, 4.8 grade (AGA Gas, Sundbyberg, Sweden) was used as extraction medium throughout the SFE experiments. Support materials used in the SFE experiments were stainless steel beads (300–385 μm, Anval, Torshälla,

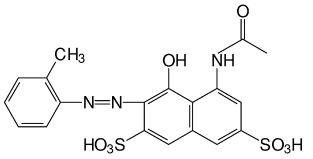
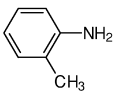
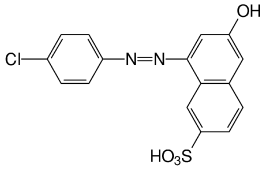
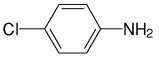
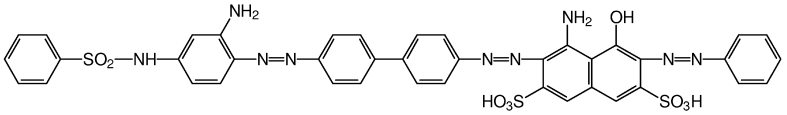
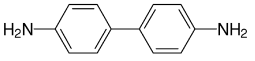
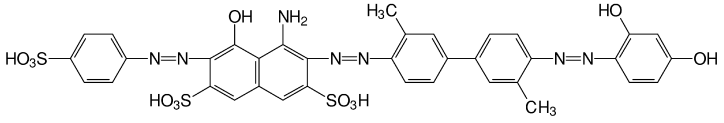
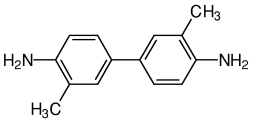
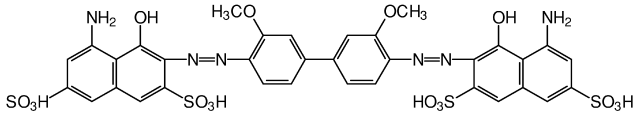
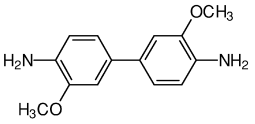
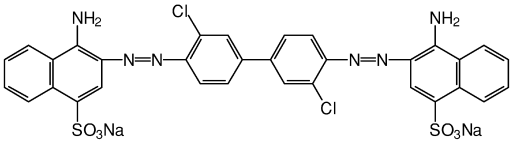
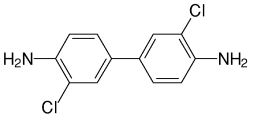
Azo dye	Amine
Acid Red 035 	*o*-Toluidine 
Acid Orange 031 	*4*-Chloroaniline 
Acid Black 077 	Benzidine 
Acid Black 209 	*3,3′*-Dimethylbenzidine 
Direct Blue 015 	*3,3′*-Dimethoxybenzidine 
Direct Red 061 	*3,3′*-Dichlorobenzidine 

Fig. 1. Chemical structures of the azo colorants together with the released carcinogenic amines investigated in this study.

Sweden), glass beads (200 μm, Kebo Lab, Spånga, Sweden), Hydromatrix (Sorbent, V. Frölunda, Sweden) and Florisil (Supelco, Bellefonte, PA, USA).

2.3. Analytical procedures using SFE equipment for reduction/extraction of leather samples

Two SFE equipments differing in the trapping step (solid-phase trap or solvent collection) were utilised for the extractions. The SFE system with a solid-phase trap was a Hewlett-Packard (HP) 7680T extraction unit (Wilmington, DE, USA) equipped with a Hewlett-Packard 1090 LC pump for addition of modifier. Standard 7-ml extraction vessels were used with two inserts each containing a hydrophobic membrane (0.2 μm Fluoropore, Millipore, Bedford, MA, USA) inside to prevent water leakage, described elsewhere [13]. Hewlett-Packard standard traps filled with octadecyl silica (ODS) were used throughout the experiments and the extracted compounds were eluted from the trap with 1.5 ml portions of methanol.

The SFE system with solvent collection was an Isco SFX 3560 (Isco, Lincoln, NE, USA) with 10-ml extraction thimbles. The extracted compounds were collected in 20 ml of methanol containing 100 μl of 1 *M* HCl. The acid was added to the collecting solvent to decrease the risk for losses of amines in a subsequent evaporation step.

2.3.1. Solubility experiments

The solubilities of the target amines in supercritical carbon dioxide and in methanol modified carbon dioxide were investigated using the HP equipment, by extracting 100 μl of amine standard solutions (1.0 mg/ml in methanol) added onto support materials of stainless steel beads, glass beads, Hydromatrix or Florisil. The support material volume was 5 ml, filling up the extraction vessel to ca. 2/3. The 100 μl sample solvent was evaporated before the extraction. These experiments were carried out at conditions; 40 °C, 138 bar (0.75 g/ml) or 276 bar (0.90 g/ml) for 5, 10, 20 or 30 min dynamic extraction with flow-rate set at 4 ml/min. Neat carbon dioxide or methanol-modified (2 or 5%) carbon dioxide was used as extracting solvent.

2.3.2. Experiments with leather samples spiked with target amines

All samples (dyed with Acid Black 210, which does not release any of the investigated amines) considered had previously been degreased using SFE (neat CO_2 at 138 bar, 40 °C, 4 ml/min for 30 min). Using the HP equipment, the degreased sample (ca. 0.3 g) was spiked with 100 μl of a standard solution (1.0 mg/ml of each amine). Using the Isco equipment, the degreased leather sample (0.3 g) was spiked similarly and mixed with 0.5 g of Hydromatrix in the extraction vessel. After this step, the analytical procedure followed the scheme, as from the decolourising step, depicted below in Fig. 2. Optimisation was done by varying the time for the decolourising step and the conditions in the following extraction steps. HPLC analysis was carried out on the fractions separately.

2.3.3. Experiments with genuine leather samples

Ca. 0.3 g of leather sample was placed between the membrane inserts (HP) or mixed with Hydromatrix (Isco). The samples were treated according to the flow scheme described in Fig. 2.

The fractions were all analysed separately. The procedure was carried out in duplicate for each sample.

2.4. Analytical procedures using MAE equipment for reduction/extraction of leather samples

Prior to any MAE experiment, the leather samples were degreased using SFE (neat CO_2 at 138 bar, 40 °C, 4 ml/min for 30 min). The MAE experiments were then performed with an MES-1000 closed vessel system (CEM, Matthews, NC, USA) equipped with a 12-sample tray with temperature and pressure control.

2.4.1. Experiments with leather samples spiked with target amines

Degreased leather samples dyed with Acid Black 210 (ca. 0.3 g), which does not release any of the investigated amines, were transferred to MAE vessels and spiked with 100 μl of a standard solution (1.0 mg/ml of each amine). To each sample, 8 ml of buffer (pH 6) and 1.5 ml of freshly prepared sodium dithionite solution (0.2 g/ml) were added. The

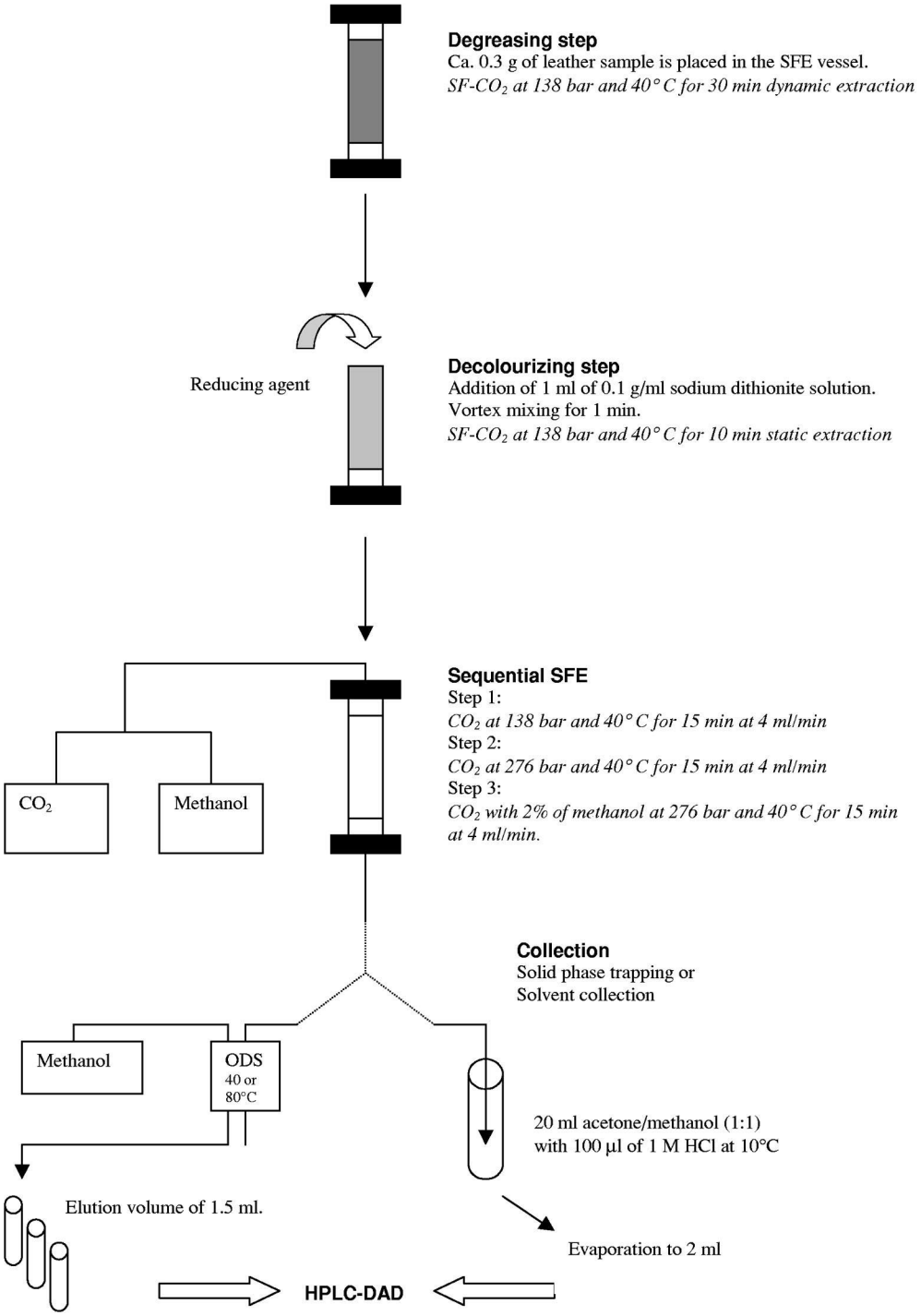


Fig. 2. Flow scheme for the analytical procedure based on supercritical fluid extraction (SFE).

samples were placed into the microwave oven and, after completion of the reduction/extraction step, the extracts were filtered and made up to 10 ml with methanol. The leather matrix was then re-extracted under the same extraction conditions with 8 ml of methanol (without addition of reducing agent). Again the extract was filtered and made up to 10 ml with methanol. HPLC analysis was carried out on the extracts separately. This procedure was carried out in duplicate for each sample, and was repeated varying the extraction temperature, the reduction/extraction time, the solvent composition, the amount reducing agent as well as the addition of acid to the matrix.

2.4.2. Experiments with genuine leather samples

After taking into account experiences from the optimisation procedure above, a four-step fractionated extraction procedure was performed, where the first step included the reduction procedure. Ca. 0.3 g of degreased leather sample was transferred to a MAE vessel and treated according to the flow scheme described in Fig. 3. All fractions were analysed separately and the procedure was carried out in duplicate for each sample.

2.5. Standard method DIN 53316

Briefly described, the DIN method [4] starts with a degreasing step using *n*-hexane for 20 min at 40 °C in an ultrasonic bath. After evaporation overnight, the leather sample is treated with sodium dithionite (up to three times with 1.5 ml of a 0.2 g/ml solution) in an aqueous buffer solution (pH 6) at 70 °C for 30 min in a closed vessel. The amines released are transferred to a methyl *tert*.-butyl ether (MTBE) phase by means of liquid–liquid extraction using kiselguhr columns. The MTBE (totally 40 ml) is then concentrated under mild conditions and the residue is dissolved in a suitable solvent for final analysis, in our case HPLC–diode array detection (DAD).

2.6. Analysis conditions

The Isco extracts were evaporated under a gentle stream of nitrogen to a final volume of 2 ml and then analysed with HPLC while HP extracts could be analysed directly.

Spectophotometric assays of co-extracted dye during the degreasing procedure were carried out by measurement of the absorbance at 610 nm using an Ultrospec II spectrophotometer (LKB Biochrom, Cambridge, UK). Determination of amines was performed with a HPLC system consisting of a Hewlett-Packard series 1050 high-pressure gradient pump, a Kontron autosampler 460 (Kontron Instruments, Milan, Italy), and a photodiode-array detector (PDA 996, Waters, Milford, MA, USA). The amines were separated on a Purospher RP-18e column, 250×3 mm (5 μm) from Merck kept at 30 °C with a column oven (Croco-cil, Sainte-Foy-La Grand, France) and equipped with a guard column LiChrospher 60, RP-select B, 4×4 mm (Merck). The mobile phase was composed of acetonitrile (eluent A) and 3 m*M* phosphate buffer (pH 7) (eluent B). Separations were accomplished at a flow-rate of 0.3 ml/min using the following gradient sequence. The column was first eluted with 15% of eluent A for 2 min. Then a gradient elution was performed from 15 to 60% eluent A in 50 min. With this gradient elution conditions the analytes were separated within 50 min. Millennium 2.15 (Waters) chromatographic data system software was used for data processing. A standard chromatogram as well as a chromatogram obtained for a genuine leather sample are shown in Fig. 4.

In the chromatograms the amine 4-aminobiphenyl is also included since this amine is sometimes detected as a false positive in leather samples.

3. Results and discussion

3.1. Degreasing

By visual inspection, the fat extracts from the DIN method were found to be coloured and therefore an investigation of the amount co-extracted colorant was performed comparing the DIN sonication procedure with other sonication procedures as well as MAE and SFE. Ca. 0.5 g of a leather sample dyed with Acid Black 210 was treated using the different methods and the amount of extracted fat and amount of co-extracted dye are shown in Table 1.

Obviously SFE gives extracted amounts of fat, similar to the other methods and much better selec-

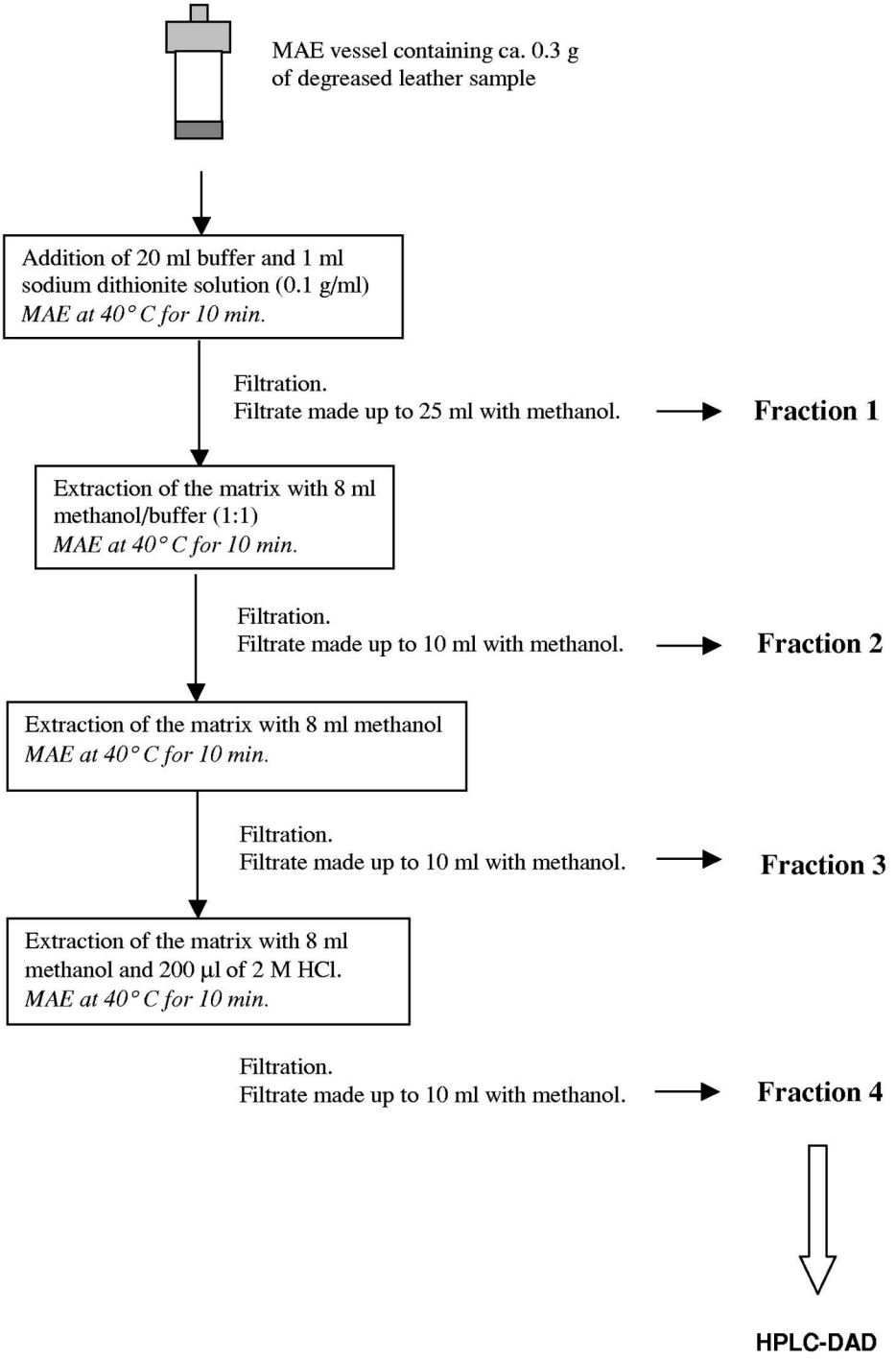


Fig. 3. Flow scheme for the analytical procedure based on microwave-assisted extraction (MAE).

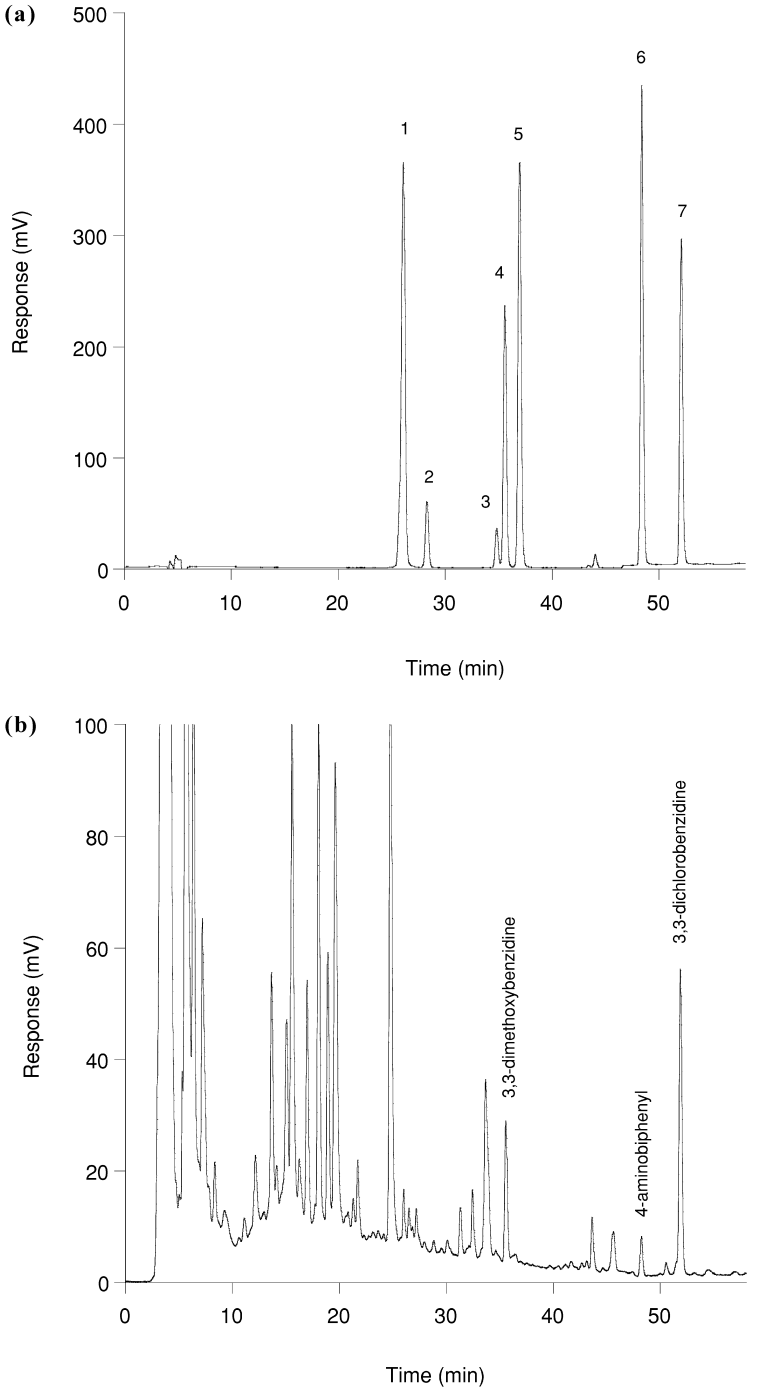


Fig. 4. (a) Standard HPLC chromatogram. (1) Benzidine, (2) *o*-toluidine, (3) 4-chloroaniline, (4) 3,3-dimethoxybenzidine, (5) 3,3-dimethylbenzidine, (6) 4-aminobiphenyl, (7) 3,3-dichlorobenzidine. Detection wavelength: 280 nm. Amine concentration: ca. 50 μg/ml. HPLC conditions as described in Section 2.6. (b) Chromatogram of a genuine leather sample treated with the MAE procedure.

Table 1
Determination of fat and co-extracted azo dye using different methods

Method	Amount extracted fat (mass %)[a]	Amount co-extracted azo dye (μg/g leather)[b]
Sonication		
n-Hexane, 2×10 ml, 2×20 min, 40 °C (as in DIN 53316)	11.9	241
Cyclohexane, 2×10 ml, 2×20 min, 40 °C	11.4	370
MTBE, 2×10 ml, 2×20 min, 40 °C	12.6	539
MAE		
Cyclohexane–acetone (95:5), 2×10 ml, 2×20 min, 40 °C	12.0	450
Cyclohexane–isopropanol (95:5), 2×10 ml, 2×20 min, 40 °C	12.2	626
SFE (solvent collection)[1]		
276 bar, 40 °C (0.90 g/ml), 30 min	7.9	3.2
276 bar, 60 °C (0.81 g/ml), 30 min	10.5	2.6
SFE (solid-phase trap)[2]		
276 bar, 40 °C (0.90 g/ml), 30 min	11.3	5.6
276 bar, 60 °C (0.81 g/ml), 30 min	12.1	7.6
138 bar, 40 °C (0.75 g/ml), 30 min[3]	11.9	5.4

Each value given is the average of two experiments.
[a] Determined gravimetrically.
[b] Determined by spectrophotometry. [1] Cyclohexane (20 ml) at 20 °C. [2] ODS at 40 °C. Elution of the trap with 2×1.5 ml cyclohexane and 2×1.5 ml methanol. [3] Final degreasing conditions chosen.

tivity in terms of co-extracted azo dye. Thus, SFE with a solid-phase trap at 138 bar and 40 °C for 30 min was used in all further experiments. These conditions are similar to those published recently by Manganiello et al. [14].

3.2. Solubility experiments using SFE

An investigation of the solubility of the target amines in supercritical carbon dioxide and in methanol modified carbon dioxide was performed as described in Section 2.3.1. No differences in recoveries were obtained between the different support materials (e.g., stainless steel beads, glass beads, Hydromatrix or Florisil), which means that it is possible to avoid dead volume in the extraction cell by filling up the vessel with a support material. Full recovery was obtained for *o*-toluidine, 4-chloroaniline and 3,3-dichlorobenzidine at conditions 40 °C, 138 bar (0.75 g/ml), 4 ml/min within 10 min dynamic extraction. Adding 2% methanol as modifier to neat carbon dioxide gave recoveries in the range of 85–100% also for benzidine, 3,3-dimethylbenzidine and 3,3-dimethoxybenzidine. These results confirm that with extraction conditions as outlined above, losses in genuine leather samples would largely depend on amine–matrix interactions.

3.3. Decolourising

3.3.1. Choice of reducing agent

Two reducing agents, namely sodium dithionite and tin(II) chloride, are the ones mostly utilised in the determinations of amines originated from azo dyes in a variety of products as candy, soft drinks and commodities. Accordingly these agents were tested for decolourisation of leather samples as well. The reduction step was studied using MAE at conditions; 70 °C for 30 min using 1.5 ml of 0.2 g/ml sodium dithionite or 0.6 g tin (II)chloride in 1.5 ml HCl for the reduction with an additional extraction of the leather matrix with methanol. Compared to the DIN method, both of the MAE conditions gave higher recoveries. All methods gave similar RSD values (in the range of 2–20%; $n=3$). For some amines, 3,3-dimethylbenzidine, 3,3-dimethoxybenzidine and 3,3-dichlorobenzidine, higher recoveries could be achieved using tin(II) chloride,

but this reducing agent increased the number of peaks in the chromatograms, sometimes making the amine determinations unreliable. Sodium dithionite was therefore used in all further experiments.

3.3.2. Optimisation of the reduction step (temperature and time) on spiked sample

Using MAE, the reduction step was performed at different temperatures (40, 60 and 80 °C) and times (10, 30 and 60 min). The results are shown in Fig. 5.

As seen in Fig. 5, relatively low temperature and short time for the reduction step is beneficial. (This should be compared to the DIN method where the azo dyes are reduced at 70 °C for 30 min). For all amines except benzidine the recoveries were better than 50% and for *o*-toluidine as high as 80%.

3.4. Method development for MAE and SFE

Using the MAE equipment on spiked samples, the effects of different parameters as solvent composition, added amount of reducing agent and the addition of a small amount of acid to the matrix were

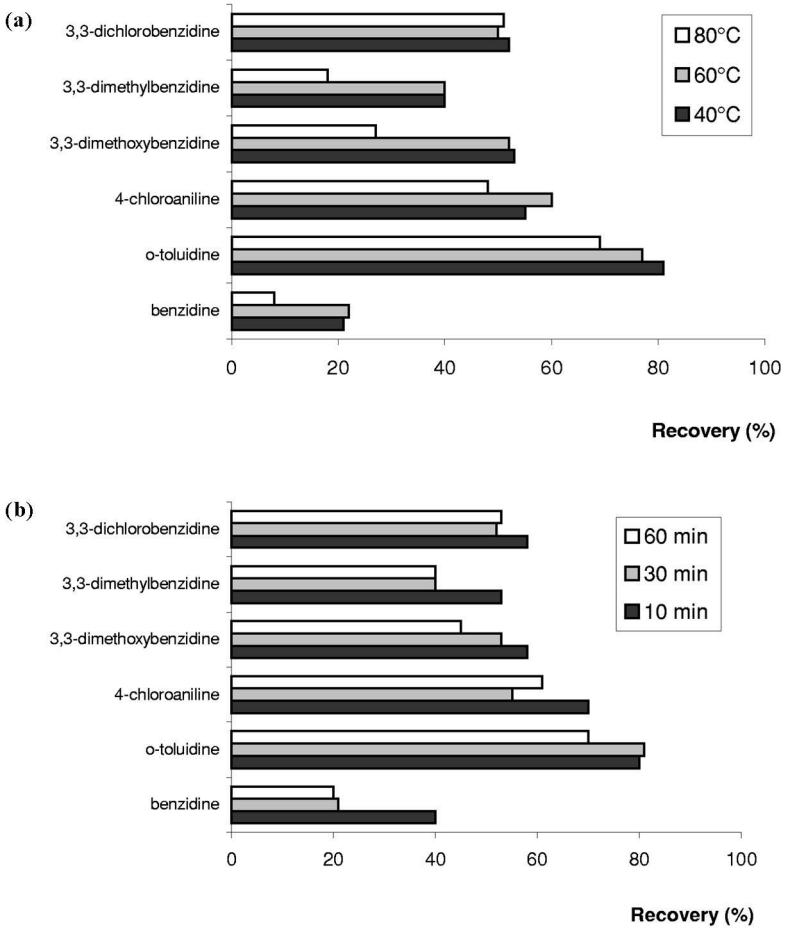


Fig. 5. (a) Recovery (%) of amines extracted from spiked leather samples using MAE for 30 min at different temperatures (40, 60 and 80 °C). (b) Recovery (%) of amines extracted from spiked leather samples using MAE at 40 °C for different times (10, 20 and 30 min). Each value given is the average of two experiments.

investigated. The results showed that by increasing amount of methanol in buffer the recovery decreased for the smaller amines but increased for the larger ones. It was also found that when increasing the amount of reducing agent (sodium dithionite), from 0.1 to 0.6 g reducing agent/g leather sample, the recoveries decreased. To investigate this further, buffer was spiked with amines and treated with or without sodium dithionite under MAE conditions. It was found that without the reducing agent, 100% recovery was achieved but when this agent was added the recoveries were reduced to 30–70%. It thus seems reasonable that the decrease in recovery is attributed to side reactions between target compounds and sodium dithionite. By the addition of acid (200 μl of 2 *M* HCl) to the matrix, the recoveries of all amines improved, except for 3,3-dichlorobenzidine, which was unaffected. These results were implemented in the fractionated MAE procedure (Fig. 3) that was used for the genuine leather samples.

In the case of SFE, it was stated that a static time of 10 min for the decolourising step at 40 °C and 138 bar was to be preferred. When increasing this time, the recoveries decreased slightly. Different parameters in the sequential extractions of the amines from the leather matrix were then investigated. The results showed that by increasing the pressure and by adding methanol as modifier (2%) the recoveries were increased. Additionally, the amount of reducing agent (sodium dithionite) should be as low as possible. In Fig. 6 it can be seen that the amines behave differently in the different extraction steps as well as if the sample is spiked or genuine.

3.5. Comparison between results obtained for spiked and genuine samples for different methods

Spiked and genuine leather samples were treated with the DIN method, the SFE procedure (Fig. 2) and the MAE procedure (Fig. 3). The recoveries obtained are shown in Table 2.

Overall better recoveries are achieved with MAE and SFE than the DIN method. The recoveries using MAE are very similar for spiked and genuine leather samples in the case of *o*-toluidine, 4-chloroaniline, 3,3-dimethylbenzidine and 3,3-dichlorobenzidine, which reflects that the reductive step here is less important. Only for benzidine and to a lesser extent 3,3-dimethoxybenzidine, the spiked samples give higher recoveries indicating problems in the reduction step. In the case of SFE, higher recoveries are obtained for spiked samples for most of the amines compared to genuine samples. Adsorption or ion exchange of target analytes in the leather matrix and/or restricted diffusion might be responsible for the low recoveries of amines. Further studies on the modifier composition may improve this situation and give recoveries similar to MAE.

3.6. Concluding remarks

The quantification limits in genuine leather samples using the SFE or MAE procedure were below 1 mg/kg for all amines considered. The within laboratory precision was in the range of 5–10% ($n=5$) for *o*-toluidine and the substituted benzidines. Somewhat higher RSD values were obtained for 4-chloroaniline and benzidine, 16 and 18% ($n=5$), respectively.

The major problem in the methods seems to be the extraction step. Further development should be firstly directed towards improving the recoveries in the extraction step. Complexation of interfering substances and/or derivatisation of the target analytes may be an accessible way to go.

4. Conclusions

Using MAE or SFE it is possible to increase the sample throughput in the determination of azo colorants in leather with less manual steps and with less usage of organic solvents compared to the DIN method. Remarkably higher recoveries are generally achieved with MAE and SFE. However, an increase in recovery for especially benzidine and 3,3-dimethoxybenzidine would be advantageous.

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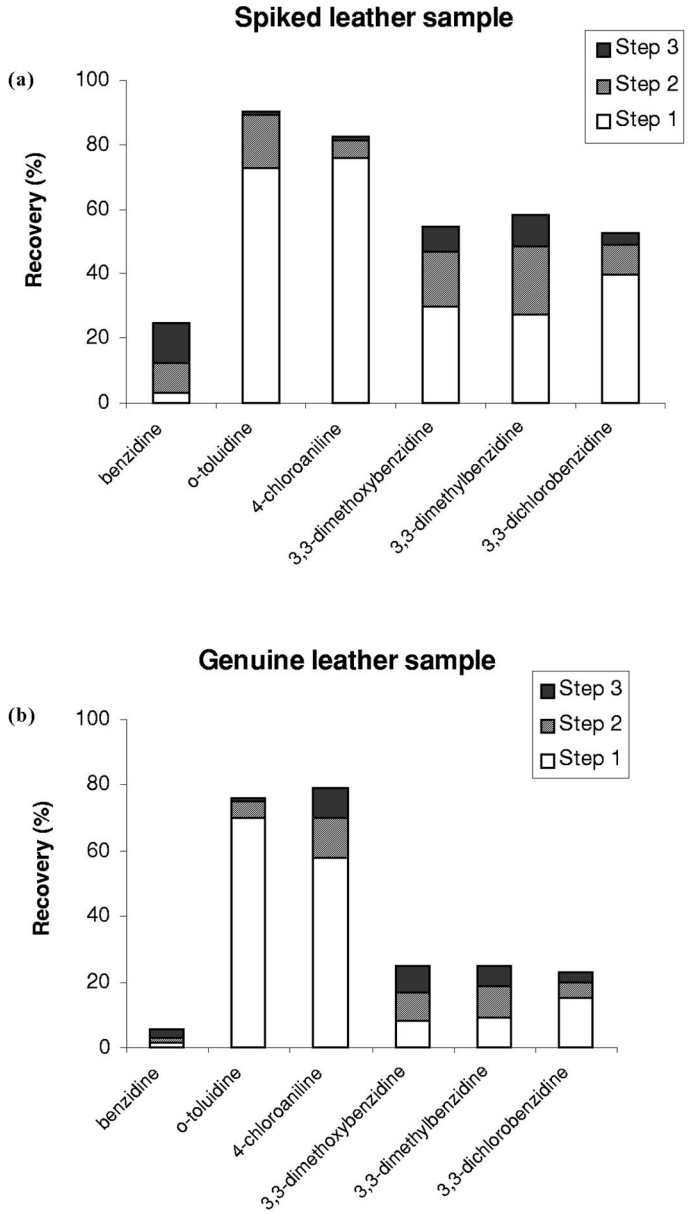

Fig. 6. Recoveries (%) of amines extracted, using the Isco equipment, from (a) spiked leather sample and (b) genuine leather samples performing sequential SFE. Decolourising at 40 °C and 138 bar for 10 min static extraction time. Thereafter sequential extractions with step 1: 40 °C and 138 bar for 30 min dynamic extraction at 2 ml/min; step 2: 40 °C and 276 bar for 30 min dynamic extraction at 2 ml/min and step 3: 2% of methanol as modifier at 40 °C and 276 bar for 30 min dynamic extraction at 2 ml/min. Each value given is the average of two experiments.

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Table 2
Recovery (%) for different methods in the determination of aromatic amines from spiked and genuine leather samples

Amine	DIN standard 53316		MAE		SFE (solvent collection)		SFE (solid-phase trap)	
	Spiked	Genuine	Spiked	Genuine	Spiked	Genuine	Spiked	Genuine
Benzidine	6.2	9.8	42	18	25	5.5	37	8.1
o-Toluidine	44	26	82	79	89	74	46	59
4-Chloroaniline	48	20	71	78	81	78	44	51
3,3-Dimethoxybenzidine	9.8	19	55	35	55	26	49	24
3,3-Dimethylbenzidine	11	28	57	56	58	24	50	20
3,3-Dichlorobenzidine	24	13	63	55	52	23	62	31

Each value given is the average of two experiments.

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