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Abstract

A method for molecularly imprinted solid-phase extraction of banned Sudan azo-dyes from food samples was investigated. The molecularly imprinted polymer was obtained by suspension polymerization using 1-(4-chlorophenyl)azonaphthalen-2-ol as the mimic template. The molecular recognition properties of imprinted beads were evaluated for use as a solid-phase extraction sorbent, in order to develop a selective extraction protocol for the Sudan class of dyes. The optimised extraction protocol resulted in a reliable MISPE method suitable for HPLC analysis. It was selective for the main analyte, Sudan I and the related azo-dyes Sudan II, III, IV, Sudan Red B and Sudan Red 7B, while the permitted azo-dyes Allura Red AC, Neococcin and Sunset Yellow FCF were not extracted. The method was tested for Sudan I, II, III and IV in five different food samples (hot chilli pepper, hot chilli tomato sauce, sausage, tomato sauce and hard boiled egg yolk) at three concentration levels (15 – 100 - 300 μg/g). It demonstrated itself to be insensitive to the presence of different complex matrices, precise, accurate and with good recovery rates (85-101%). The limit of detection and limit of quantification were satisfactory for most analytical determinations.

1 - Introduction

Sudan dyes (figure 1) are a class of lipophylic synthetic organic colorants that are widely used in industry because of their colourfastness and low price. These azo-dyes are known to be biologically active through their metabolites [1], and they represent a potential risk to public health if they enter the food chain. In fact, they have been associated with increased occurrence of bladder cancer in textile and leather dyers, painters and hairdressers [2,3]. Sudan I is considered a possible human carcinogen and mutagen [4], and it is classified as a category 3 carcinogen by the International Agency for Research on Cancer (IARC) [5]. Moreover, Sudan II has been shown to cause a high frequency of bladder carcinomas in mice [6].

As a consequence of their dangerous properties, Sudan dyes have been banned in products for human consumption in the European Union and several other countries. Notwithstanding, these dyes are often illegally used to enhance the appearance of products such as chilli, tomato sauces, salami, olive oil and many other frequently eaten foodstuffs. In fact, repeated notifications about the detection of these illegal additives in imported foods have been issued by the European Union Rapid Alert System for Food and Feed since 2003. In particular, the discovery of Sudan I in products from India prompted the European Commission to make a decision on emergency measures concerning Sudan I in food products intended for human consumption [7]. Later on, these recommendations were extended to other dyes, such as Sudan II, Sudan III and Sudan IV, setting the detection limit for these substances at 0.5-1.0 mg/kg [8,9].

As the illegal use of Sudan dyes has significant economic consequences for most European Union food industries as well as an impact on public health, suitable analytical screening methods are required for the fast identification and quantification of such banned additives. In the last five years several analytical methods based on gas chromatography [10], liquid chromatography [11,12], or capillary electrophoresis [13] have been reported in literature. However, except when very expensive HPLC-MS/MS instrumentation is used [14,15], the direct detection of the target analytes in complex food matrices is a difficult task, and some sample clean-up treatment is mandatory before performing the analysis.

Recent years have seen a significant increase in the use of highly selective solid phases for the extraction of analytes in complex samples. Beside the largely diffused immunoaffinity sorbents [16], extraction based on molecularly imprinted polymers — the so called “molecularly imprinted solid phase extraction” (MISPE) technique — has been proposed as a very efficient and innovative approach in food contaminant analysis [17].

In fact, this technique is particularly suitable for clean-up applications where analyte selectivity in the presence of very complex samples represents the main problem. Sudan I has been the target molecule in some papers dealing with molecular imprinting published in the recent years, but no efforts have been reported to prepare polymers with molecular recognition properties towards the whole class of Sudan dyes [18,19].

In this work we report the preparation of a molecularly imprinted polymer with selectivity towards several Sudan dyes. To avoid the interference of residual template molecules in the solid phase extraction of the analytes, the polymer was obtained through a mimic template approach, by using a halogenated analogue of Sudan I, 1-(4-chlorophenyl)azonaphthalen-2-ol (chloro-Sudan, **1**) as a template molecule. After the characterization of its binding properties, the imprinted polymer was successfully applied as a sorbent for the solid phase extraction and successive HPLC determination of trace Sudan dyes in several food products.

2 - Experimental

2.1 - Materials

Acetic acid, acetone, acetonitrile 2,2′-azobis-(2-isobutyronitrile), 4-chloroaniline, chloroform, N,N-dimethylaminoethylmethacrylate, ethylene dimethacrylate, naphtalen-2-ol, polyvinylalcohol, sodium nitrite, sodium sulphate (anhydrous), tetrahydrofurane and triethylamine were from VWR International (Milan, Italy).

Allura Red AC ([4-[(2-hydroxy-6-sodiooxysulfonyl-1-naphthyl)azo]-5-methoxy-2-methyl phenyl]sulfonyloxysodium), Neococcin ([4-[(2-hydroxy-6-sodiooxysulfonyl-1-naphthyl) azo]-1-naphthyl]sulfonyloxysodium), Sudan I (1-phenylazonaphthalen-2-ol), Sudan II (1-(2,4-dimethylphenyl)azonaphthalen-2-ol), Sudan III (1-(4-phenylazophenyl)azo naphthalen-2-ol), Sudan IV (1-[2-methyl-4-(o-tolylazo)phenyl]azonaphthalen-2-ol), Sudan Red B (1-[3-methyl-4-(m-tolylazo)phenyl]azonaphthalen-2-ol), Sudan Red 7B (N-ethyl-1-(4-phenyl azo phenyl)azo-naphthalen-2-amine) and Sunset Yellow FCF ([4-[(2-hydroxy-6-sodiooxysulfonyl-1-naphthyl)azo]phenyl] sulfonyloxysodium) were from Sigma–Aldrich–Fluka (Milan, Italy). Dye stock solutions were prepared dissolving 50.0 mg of substance in 25.00 ml of chloroform and storing in the dark at −20 °C.

Chloroform was distilled before the use to eliminate the stabiliser (ethanol). Polymerisation inhibitors in vinyl monomers were removed by clean-up on activated alumina columns.

Food samples were purchased in a local supermarket, transferred in polypropylene

containers and stored in the dark at -20 °C. The absence of contamination by banned dyes was confirmed by using a dedicated enzyme linked immunosorbent assay (ELISA) previously developed in our laboratory [20].

The Elite Lachrom HPLC apparatus consists of an L-2130 constant-flow quaternary pump, an L-2400 UV-Vis detector, an L-2200 autosampler and a data acquisition system EZChrom Elite 3.1 from VWR-Hitachi (Milan, Italy). The HPLC analytical column was an Onyx Monolithic reversed-phase C18 (100×4.6 mm) from Phenomenex (Chemtek, Anzola Emilia, Italy).

2.2 – Synthesis of 1-(4-chlorophenyl)azonaphthalen-2-ol (chloro-Sudan, 1)

In a 250 ml round-bottomed flask, 10 g (78.4 mmoles) of 4-chloroaniline were dissolved in 36 ml of cold (ice bath) 6 M aqueous hydrogen chloride and diazotized by slowly adding 25 ml (84.6 mmoles) of 3.84 M aqueous sodium nitrite under continuous stirring. The solution containing the diazonium salt was added drop by drop to a cold (ice-bath) solution of 11.3 g (78.4 mmoles) of naphtalen-2-ol in 65 ml of aqueous sodium hydroxide (10% w/v) and the reaction mixture was stirred for 3 hours. Then, the raw product was separated by filtration in a G4 Buchner, washed with hot water and dried in an oven at 35 °C. Pure 1-(4-chlorophenyl)azonaphthalen-2-ol (18 g, 92% yield) was obtained as an orange powder by flash chromatography on silica (mobile phase ethylacetate – hexane 3+7 v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.93-7.97 (dd, 1H, $C_{10}H_6$, J=9.2), δ 7.79 (dd, 1H, $C_{10}H_6$, J=7.2), δ 8.55 (dd, 1H, $C_{10}H_6$, J=8), δ 7.62 (t, 1H, $C_{10}H_6$, J=7.2), δ 7.59-7.91 (d, 2H, C_6H_4, J=8.8), δ 7.47 (t, 1H, C_6H_4, J=7.2). Mass spectrum ESI (m/z): 281 (M+, 100%), 283 (M+2, 33%).

2.3 – Preparation of imprinted beads by suspension polymerization

An organic phase was prepared by dissolving 140 mg (0.495 mmoles) of chloro-Sudan, 0.59 ml (3.49 mmoles) of N,N-dimethylaminoethyl methacrylate, 4.70 ml (24.9 mmoles) of ethylene dimethacrylate and 100 mg (0.61 mmoles) of 2,2′-azobis-(2-isobutyronitrile) in 7.5 ml of chloroform. The solution was sonicated, purged with nitrogen and stored in the dark at −20 °C for 30 min. Meanwhile, in a thermostated 250 ml three-necked flask provided with a magnetic stirrer, nitrogen inlet and Liebig condenser, 6.0 g of polyvinylalcohol were added to 150 ml of pre-heated (95 °C) ultrapure water and dissolved under rapid stirring. The clean solution was allowed to slowly cool to 60 °C. The organic phase was slowly added with a glass syringe to the aqueous solution while stirring at 400 rpm under a gentle stream of nitrogen. The polymerization process was maintained at 60 °C under nitrogen for 30 h.

The beads formed were then filtered through a glass filter, suspended in hot water and

boiled for 20 min. This step was repeated three times to dissolve and wash away all of the residual polyvinylalcohol. The cleaned red beads were suspended in chloroform, sonicated and filtered, and this procedure was repeated until the template could not be detected in the washing solvent, leaving beads with a very pale reddish colour. No efforts were made to measure the amount of template recovered. A non-imprinted polymer was prepared and processed in the same manner, but with the template omitted.

Beads morphology was observed by scanning electron microscopy (SEM) on a Philips 525M. Samples were prepared by dispersing the beads with acetone and placing one drop each on a cover glass. The drops were dried at room temperature and then coated under vacuum with approximately 4 nm of gold. The parameters used were as following: magnification ×300, voltage 10 kV, current 200 pA and distance 25 mm.

2.4 – Chromatographic characterization of the imprinted beads

An adequate amount of polymeric beads was suspended in acetonitrile and the slurry was packed in a 100 mm stainless-steel HPLC column (I.D. 3.9 mm, geometrical volume 1.19 cm^3). The packing of the stationary phase was performed by gradually adding the slurry of the polymer to the column and eluting it with acetonitrile at a constant pressure of 10 MPa. The packed column was washed at 1.0 ml/min until a stable baseline was reached (476 nm). After equilibration, the pressure in the column was 5 MPa at a flow-rate of 1.0 ml/min.

The packed and stabilized columns were equilibrated at a flow rate of 1.0 ml/min with 40 ml of proper mobile phase; then, 5 µl of a stock solution of dye diluted with mobile phase (1+99, v/v) were injected and eluted at 1.0 ml/min, and the absorbance was recorded at 476 nm, corresponding to the maximum absorbance in the visible region of the UV-Vis spectrum for Sudan I. Each elution was repeated three times to assure the reproducibility of the chromatogram. Column void volumes were measured for each mobile phase formulation by eluting 5 µl of acetone 0.1% v/v in the mobile phase, and the absorbance was recorded at 295 nm.

The capacity factor (k) was calculated as $(t - t_0) / t_0$, where t is the retention time of the eluted substance, and t_0 the retention time corresponding to the column void volume. The selectivity factor (α) is defined as an index of the imprinted polymer selectivity towards analogues of the template dye. It was calculated as $k_{analogue} / k_{template}$.

2.5 - Reverse-phase liquid chromatography

RP-HPLC analysis was used for quantification of the azo-dyes after solid-phase

extraction. Sample injection was 10 μl. The detection wavelength was recorded at 476 nm. The mobile phase consisted of acetonitrile–water (4+1, v/v) with a flow-rate of 1.0 ml/min. With Allura Red AC and Sunset Yellow FCF the mobile phase was changed to methanol-water (1+1, v/v) at a flow-rate 0.7 ml/min, while Neococcin was eluted with methanol-water (3+7, v/v) at a flow-rate 0.5 ml/min. Reference standard solutions of analytes at a concentration of 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1 and 2 μg/ml were analysed three times consecutively and peak areas were plotted against concentration. Calibration curves were drawn using weighted linear regression (weight = 1/conc.).

2.6 - Preparation of the MISPE cartridges

Adequate amounts of dry imprinted polymer (0.200 g) were suspended in acetonitrile, sonicated in a water-bath for 10 min and packed in 5-ml empty polypropylene SPE cartridges provided with frits to secure the packing and outlet stopcocks. The columns were connected to a vacuum manifold and washed extensively with chloroform, and then dried under vacuum. Immediately before any use, the cartridges were activated with 3×1 ml of acetonitrile-water (1+2, v/v). When necessary, the columns were cleaned and regenerated by washing with 5×2 ml of chloroform and 3x1 ml of acetonitrile-acetic acid (9+1, v/v).

2.7 - Optimisation of MISPE method

In order to optimise the procedure of azo-dyes extraction, different protocols were applied during the washing and elution steps. In the subsequent experiments each extraction was repeated three times and analyte recovery was evaluated as the average of the single values measured.

To investigate the effect of different washing solutions 1.0 ml of Sudan I (1 μg/ml) in acetonitrile-water (1+2, v/v) was loaded by applying a vacuum to facilitate the passage of the sample through the cartridge bed. After sample loading, air was passed through the column for 10 min to remove all the residual traces of solvent. Then, the cartridge was sequentially washed with 0.5 ml of water containing increasing amounts (0-100%, v/v) of acetone, acetonitrile, methanol or tetrahydrofurane, measuring the unretained Sudan I by HPLC.

To investigate the effect of different elution conditions, 1.0 ml of Sudan I (1 μg/ml) in acetonitrile-water (1+2, v/v) was loaded by applying a vacuum to facilitate the passage of the sample through the cartridge bed. After sample loading, air was passed through the column for 10 min to remove all the residual traces of solvent. Then, the cartridge was sequentially washed with 3x0.5 ml of acetonitrile-water (1+1, v/v). Sudan I was

recovered by eluting the cartridge with 6x0.5 ml of tetrahydrofurane mixed with increasing amounts (1-2-5-10%, v/v) of acetic acid or triethylamine as displacing agents.

2.8 - MISPE selectivity

In order to investigate the selectivity of the optimised extraction protocol in the loading solution Sudan I was substituted with other banned (Sudan II, Sudan III, Sudan IV, Sudan Red B and Sudan Red 7B) or permitted azo-dyes (Allura Red AC, Neococcin and Sunset Yellow FCF). For each of these analytes, 1.0 ml of 1 μg/ml dye solution in acetonitrile-water (1+2, v/v) was loaded, applying a vacuum to facilitate the passage of the sample through the cartridge bed. After sample loading, air was passed through the column for 10 min to remove all the residual traces of solvent. Then, the cartridge was sequentially washed with 3x0.5 ml of acetonitrile-water (1+1, v/v) and the azo-dye was recovered by eluting the cartridge with 2x0.5 ml of 2% (v/v) triethylamine in tetrahydrofurane.

2.9 - MISPE of real samples

A portion of solid or semisolid sample (about 1 g) was introduced into a 25 ml glass centrifuge tube, mixed with 2 g of anhydrous sodium sulphate, weighted, spiked with known amounts of Sudan I, II, III and IV ranging from 15 to 300 μg and suspended in 5.0 ml of acetonitrile. The mixture was sonicated for 30 minutes at room temperature and then centrifuged at 8000 rpm for 15 minutes. A known volume of supernatant was diluted 1+2 (v/v) with water, vortexed, filtered through a 0.22 μm polypropylene membrane and immediately extracted with the optimised protocol reported in section 2.8. To evaluate the reproducibility of the MISPE protocol, each extraction was repeated five times and analyte recovery was evaluated as the average of the single values measured. Sudan I preconcentration was investigated by loading on the MISPE cartridge acetonitrile-water 1+2 (v/v) solutions obtained by extracting increasing amounts (1-2-5-10-20 g) of hot chilli pepper samples spiked with a fixed amount (15 μg) of Sudan I.

3 - Results and fdiscussion

3.1 - Molecular recognition properties of the imprinted beads

A preliminary study performed with Sudan I as a template showed a significant release of the residual template during batch-binding experiments. Thus, to avoid interference with the extraction of samples containing Sudan I, it was necessary to develop and use a mimic template. It was identified in chloro-Sudan, namely 1-(4-chlorophenyl)azo naphthalen-2-ol, whose chlorine atom makes this molecule very similar to Sudan I and

Sudan II. In fact, as chlorine represents an isostere of the methyl substituent [21], chloro-Sudan can be seen as a “methyl”-Sudan I or a “des-methyl”-Sudan II.

Before developing a MISPE for target dyes, the molecular recognition properties of the chloro-Sudan-imprinted polymer were evaluated by liquid chromatography to study the effect of the mobile phase polarity on the binding capacity of the beads. Chloro-Sudan was eluted in acetonitrile containing increasing amounts of water as a polar mobile-phase modifier.

It is apparent from figure 2 that the template molecule shows a significant increase in its capacity factor when the increase in the amount of water the mobile phase becomes more polar. This result can be easily explained by considering a partition mechanism mostly based on hydrophobic interactions between dye molecules and the stationary phase. In this manner, the dye–mobile phase/stationary phase system operates in reverse phase conditions, where increasing the more polar component in the mobile phase (water) causes an increase in the dye capacity factor due to the shift of the partition equilibrium towards the stationary phase. It is also clear that the chromatographic behaviour of the imprinted column is different to that of the non-imprinted one. In fact, over the whole range of mobile phase compositions, capacity factors measured on the imprinted column are larger than the corresponding values measured on the non-imprinted column one. This indicates clearly that during the polymerization stage chloro-Sudan effectively acted as a template, producing imprinted binding sites in the polymeric matrix. It should be considered that, as it is quite difficult to see any differences in shape and size distribution between different typologies of beads reported in SEM images (figure 3), the hypothesis that different binding capacity will be related to morphological differences in the surface morphology should be ruled out.

The same approach was used to evaluate the selectivity of the imprinted beads towards several Sudan Dyes. The analytes were eluted with a mobile phase constituted of acetonitrile containing increasing amounts of water as a polar modifier. From the experimental results reported in figure 4, it is possible to see that all that azo-dyes are recognized well by the imprinted beads and that molecular recognition is clearly conditioned by the polarity of the mobile phase. In fact, in the presence of limited amounts of water the selectivity sequence is Sudan I > Sudan II < chloro Sudan (template) < Sudan III > Sudan IV > Sudan Red B > Sudan Red 7B. Thus, molecular shape and dimensions of the analyte molecules control the interaction between the azo-dye and the stationary phase. Contrarily, when water predominates in the mobile phase composition, the selectivity sequence changes completely and becomes Sudan I < Sudan

II < chloro Sudan (template) < Sudan III < Sudan IV < Sudan Red B < Sudan Red 7B, showing that in a more polar environment the molecular recognition can be directly related to the azo-dye hydrophobicity, through a partition mechanism mainly controlled by hydrophobic interactions between dye molecules and the stationary phase.

3.2 - Optimisation of MISPE protocol

To develop an extraction method for the isolation of Sudan dyes from food samples it was not possible to directly load the MISPE cartridge with an organic solution containing the analytes because of the limited capacity of the imprinted polymer to retain azo-dyes when eluted in pure organic solutions, as reported in figure 4. Thus, a "selective desorption" mode performed on aqueous samples was chosen as the most appropriate. In this extraction modality the analyte and any other interfering substance are retained by the hydrophobic polymeric matrix, which acts as a reversed-phase material without any apparent specificity towards the target analyte. The elution of the interfering substances is obtained by increasing the hydrophobicity of the mobile phase, while the target analyte is not eluted because of its ability to bind the imprinted binding sites. Its recovery is obtained by eluting the column with a mobile phase able to interfere with these selective non-covalent interactions [22].

As no significant release of Sudan I was found in the eluate obtained in a preliminary experiment using water as eluent, water was considered a suitable start-up solvent to remove the more hydrophilic interferents before more hydrophobic solvents were used to wash the cartridge. Subsequently, water containing increasing amounts of acetonitrile, methanol, acetone and tetrahydrofurane was tested (see figure 5). When methanol – the most polar organic solvent considered – was used, a very limited release of azo-dye was observed for solvent-rich washing solutions. On the contrary, increasing loss of Sudan I from the cartridge was observed when more hydrophobic solvents were used. Thus, as a compromise between washing strength and solvent hydrophobicity, acetonitrile-water 1+2 (v/v) and acetonitrile-water 1+1 (v/v) were respectively adopted to load and wash a cartridge without any loss of analyte. For the same reason, in order to quantitatively recover the azo-dye with a reduced volume of eluent, consideration was given to using tetrahydrofurane as the solvent of choice, studying the effect of increasing amounts of displacing additives such as acetic acid and triethylamine. Increasing recovery rates for Sudan I from the cartridge was observed when the amount of both the additives were progressively increased from 1% to 10% (v/v) (figure 6). Anyway, high recovery rates (>95%) in a limited amount of eluent (0.5x2 ml) were not obtained when acetic acid was used, while the same amount of eluent containing 2%, 5%

or 10% (v/v) of triethylamine produced recovery rates of up to 96%. Thus, a solution of 2% (v/v) triethylamine in tetrahydrofurane was selected as elution medium.

It should be considerd that in the experimental conditions here reported, a cartridge prepared with the non-imprinted polymer showed a complete retention of Sudan I in the loading step, while washing with acetonitrile-water 1+1 (v/v) caused a substantial loss of analyte (85%), due to the very low binding capacity of polymer prepared in absence of a template molecule. Of consequence, NIP polymer was deemed not suitable for the extraction of food samples.

3.3 - MISPE selectivity

Besides Sudan I, 5 related Sudan dyes (Sudan II, Sudan III, Sudan IV, Sudan Red B and Sudan Red 7B) and 3 azo-dyes legally used in the food industry (Allura Red AC, Neococcin and Sunset Yellow FCF) were selected to evaluate the selectivity of the MISPE protocol. From table 1 it can be seen that almost all of the Sudan dyes were completely retained by the cartridge when loaded into an acetonitrile-water solution (1+2, v/v), while the more polar Allura Red AC, Neococcin and Sunset Yellow FCF showed quite limited retention. The washing step enhanced the preferential retention of the Sudan dyes compared to the other dyes, which were washed away almost completely. After the elution step, the recoveries of the retained compounds were higher than 90% for the considered Sudan dyes, except for Sudan Red 7B, that was only partially recovered, due to the significant loss observed during the washing step. This is not unexpected, as Sudan Red 7B is an aliphatic-aromatic secondary amine, and it lacks the naphtoic hydroxyl moiety typical of the other Sudan dyes considered in this study.

3.3 - MISPE of real samples

Satisfactory sample clean-up was achieved by the MISPE protocol when performed on several food products spiked with mixtures of Sudan I, II, III and IV. An example of successful clean-up is reported in figure 7, where two different chromatograms of acetonitrile-water 1+2 (v/v) extracts of hot chilli sausage spiked at 15 μg/g level with a mixture of the named azo-dyes are reported. It can be seen that azo-dyes can be detected with difficulty when a food sample is separated directly by RP-HPLC without any solid-phase extraction, while the same sample analysed after solid-phase extraction shows a very clean chromatographic trace, where peaks corresponding to azo-dyes can be easily detected and, as a consequence, quantified. The same chromatogram shows a very small peak (peak #2 in figure 7) corresponding to the retention time of chloro-Sudan, indicating the presence of a bleeding effect (i.e., the release of the residual template from the imprinted polymeric matrix) at the level of about 0.01–0.05 μg/ml.

Such bleeding does not anyway hamper the use of this MISPE method to detect azo-dyes at significant levels in food samples. In fact, chloro-Sudan shows a retention time of 4.72 min., significantly different from the other azo-dyes (Sudan I, 3.58 min.; Sudan II, 6.32; Sudan III, 10.15; Sudan IV, 18.63) and, as a consequence, it cannot be confused with the other analytes.

The recovery of the MISPE extraction was determined by comparing the detector response of extracted food samples (hot chilli pepper, hot chilli tomato sauce, sausage, tomato sauce and hard boiled egg yolk) with that of directly injected standards prepared in acetonitrile-water 1+2 (v/v). Recovery rates, reported in table 2, were determined at three concentration levels (15, 100 and 300 μg/g) and came out at between 85 and 101%. An analysis of variance (ANOVA) performed comparing the recovery rate obtained at the different concentration levels for each of the analyzed azo-dyes showed no relevant statistical differences between groups of measures, with the few exceptions of Sudan I in hot chilli tomato sauce samples ($F = 4.759$, $P = 0.030$) and Sudan III in hot chilli tomato sauce and sausage ($F = 7.490$, $P = 0.008$; $F = 6.205$, $P = 0.014$) where the 15 μg/g level came out different (underestimated) compared to the 100 and 300 μg/g levels. Also the same statistical analysis performed comparing different samples at the same concentration level showed no statistical differences between matrices, with the few exceptions of Sudan I, at 100 μg/g, where hot chilli pepper came out significantly different campared to tomato sauce and hard boiled egg yolk ($F = 4.063$, $P = 0.014$ at level of 100 μg/g) and Sudan II, at 300 μg/g, where hot chilli pepper came out significantly different compared to tomato sauce ($F = 3.462$, $P = 0.026$). Thus, the extraction protocol performed quite well, with good recovery rates for all the considered analytes and a substantial insensitivity to the matrix composition.

The possibility of detecting and quantifying small amounts of Sudan dyes in food samples was investigated by extracting some hot chilli pepper samples spiked with decreasing amounts of Sudan I. As can be seen from figure 8, in the dilution range considered, the analyte recovery was acceptable, with values between 84 and 101%. Thus, with this extraction technique it seems possible to measure, without difficulty, low amounts of Sudan dyes down to 0.75 μg/g in food samples.

4 - Conclusions

In this work we show that a molecularly imprinted polymer prepared by emulsion polymerization in the presence of a mimic template recognizes the main target analyte

(Sudan I) and several other analogs well, whereas there is no recognition for analytes different from Sudan-type azo-dyes. This polymer has been used as a selective sorbent in an MISPE-HPLC format for the determination of Sudan dyes in food samples. The method tested on five different samples at three concentration levels demonstrated itself to be insensitive to the presence of complex matrices, fairly precise, accurate and with good recovery rate (85-101%). Moreover, LOD and LOQ were satisfactory for most analytical determinations, while preconcentration and quantitative extraction of Sudan I from hot chilli pepper samples was shown to be feasible down to 0.75 μg/g.

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Tables

Table 1. Recovery rate of 9 selected azo-dyes on MISPE cartridge after loading of 1.0 ml of 1 μg/ml dye solution in acetonitrile-water 1+2 (v/v); n.d. = not detected. The reported values were the results of three independent measurements.

Azo-dye	% Recovery		
	Loading	Washing	Elution
Sudan I	n.d.	n.d.	92±7.3
Sudan II	n.d.	n.d.	98±4.5
Sudan III	n.d.	n.d.	92±8.3
Sudan IV	n.d.	n.d.	98±2.6
Sudan Red B	n.d.	n.d.	95±6.2
Sudan Red 7B	n.d	n.d.	72±3.7
Allura Red AC	88±1.8	18±2.5	n.d.
Neococcin	65±2.2	24±1.9	6.8±1.5
Sunset Yellow FCF	59±1.2	27±1.3	9.7±3.6

Table 2. Recovery rate on MISPE cartridge after loading of 1.0 ml of acetonitrile-water 1+2 (v/v) extracts from differtent food samples containing 15 – 100 - 300 μg/g of azo-dyes. The reported values were the results of three independent measurements.

Azo-dye	Food sample	% Recovery		
		15 μg/g	100 μg/g	300 μg/g
Sudan I	Hot chilli pepper	85 ± 8.3	94 ± 1.5	93 ± 5.9
	Hot chilli tomato sauce	91 ± 3.9	101 ± 1.5	99 ± 1.0
	Sausage	90 ± 6.9	100 ± 0.8	100 ± 0.5
	Tomato sauce	94 ± 6.3	100 ± 2.2	101 ± 2.5
	Hard boiled egg yolk	99 ± 1.0	102 ± 1.7	100 ± 0.8
Sudan II	Hot chilli pepper	92 ± 7.7	95 ± 3.5	94 ± 1.4
	Hot chilli tomato sauce	94 ± 6.3	102 ± 3.0	100 ± 0.8
	Sausage	93 ± 5.3	99 ± 1.0	99 ± 0.2
	Tomato sauce	96 ± 6.8	100 ± 1.0	101 ± 2.8
	Hard boiled egg yolk	96 ± 3.3	101 ± 1.7	100 ± 0.8
Sudan III	Hot chilli pepper	100 ± 7.1	96 ± 4.2	94 ± 8.8
	Hot chilli tomato sauce	92 ± 2.4	100 ± 1.3	99 ± 0.4
	Sausage	94 ± 2.3	101 ± 1.0	100 ± 0.8
	Tomato sauce	97 ± 7.6	100 ± 1.7	100 ± 0.7
	Hard boiled egg yolk	95 ± 6.2	98 ± 1.0	99 ± 0.8
Sudan IV	Hot chilli pepper	97 ± 13	101 ± 7.9	96 ± 2.8
	Hot chilli tomato sauce	95 ± 8.2	101 ± 1.7	99 ± 1.1
	Sausage	94 ± 7.9	100 ± 1.3	99 ± 0.6
	Tomato sauce	95 ± 8.8	101 ± 1.4	102 ± 6.8
	Hard boiled egg yolk	97 ± 3.8	101 ± 1.6	99 ± 1.1

Figures

Figure 1. Azo-dyes used in this work: **1**, chloro-Sudan; **2**, Sudan I; **3**, Sudan II; **4,** Sudan III; **5**, Sudan IV; **6**, Sudan Red B; **7**, Sudan Red 7B; **8**, Allura Red AC; **9**, Neococcin; **10**, Sunset Yellow FCF.

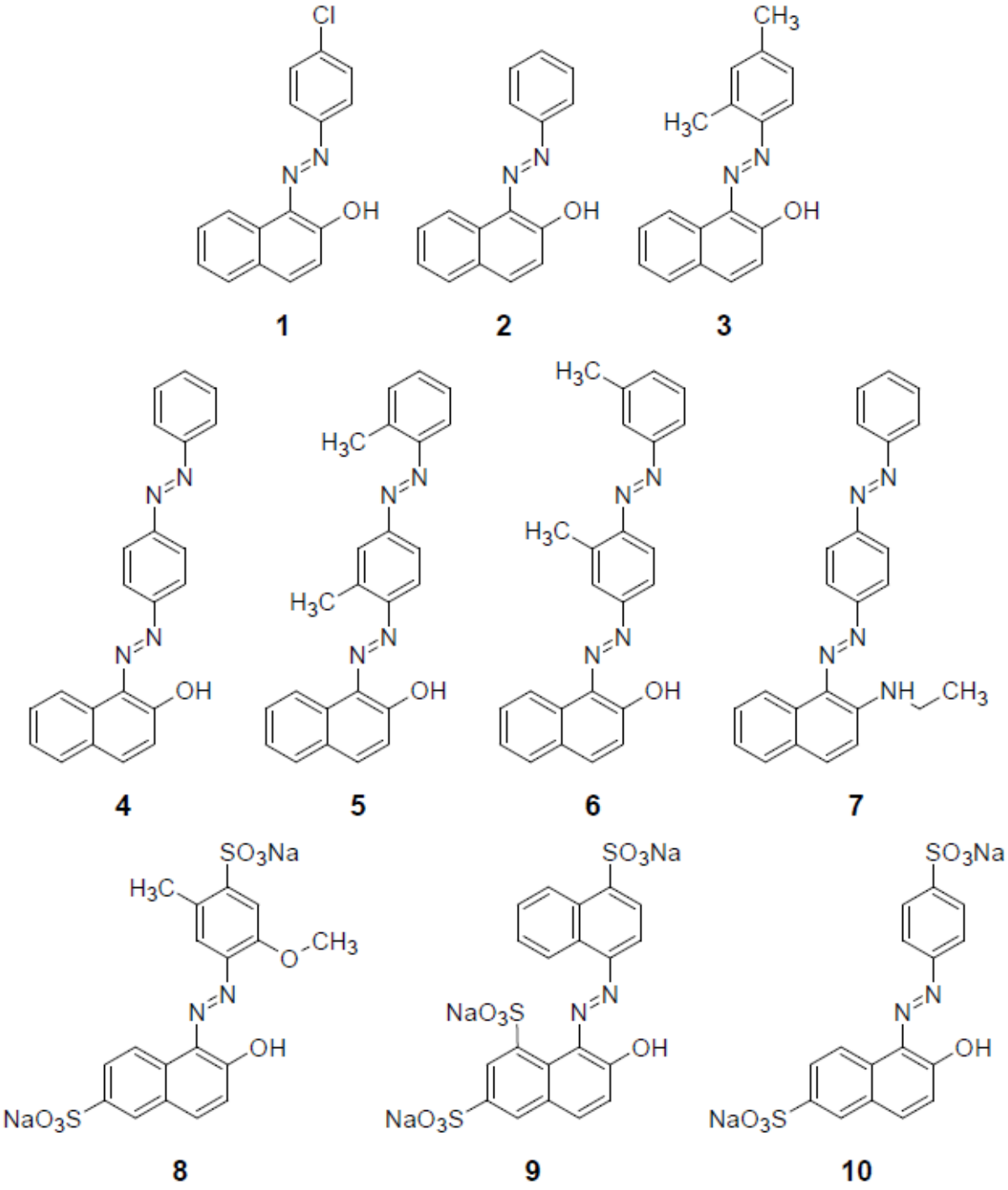

Figure 2. Capacity factors (k) measured for chloro-Sudan with acetonitrile-water mobile phases on imprinted (closed circles) and not-imprinted (open circles) columns.

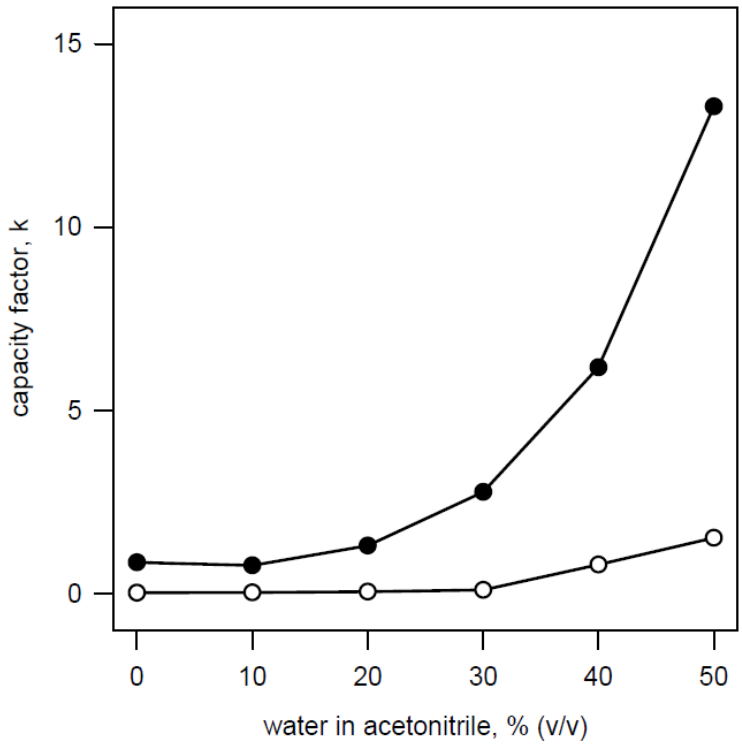

Figure 3. SEM images of imprinted (left) and not-imprinted (right) beads.

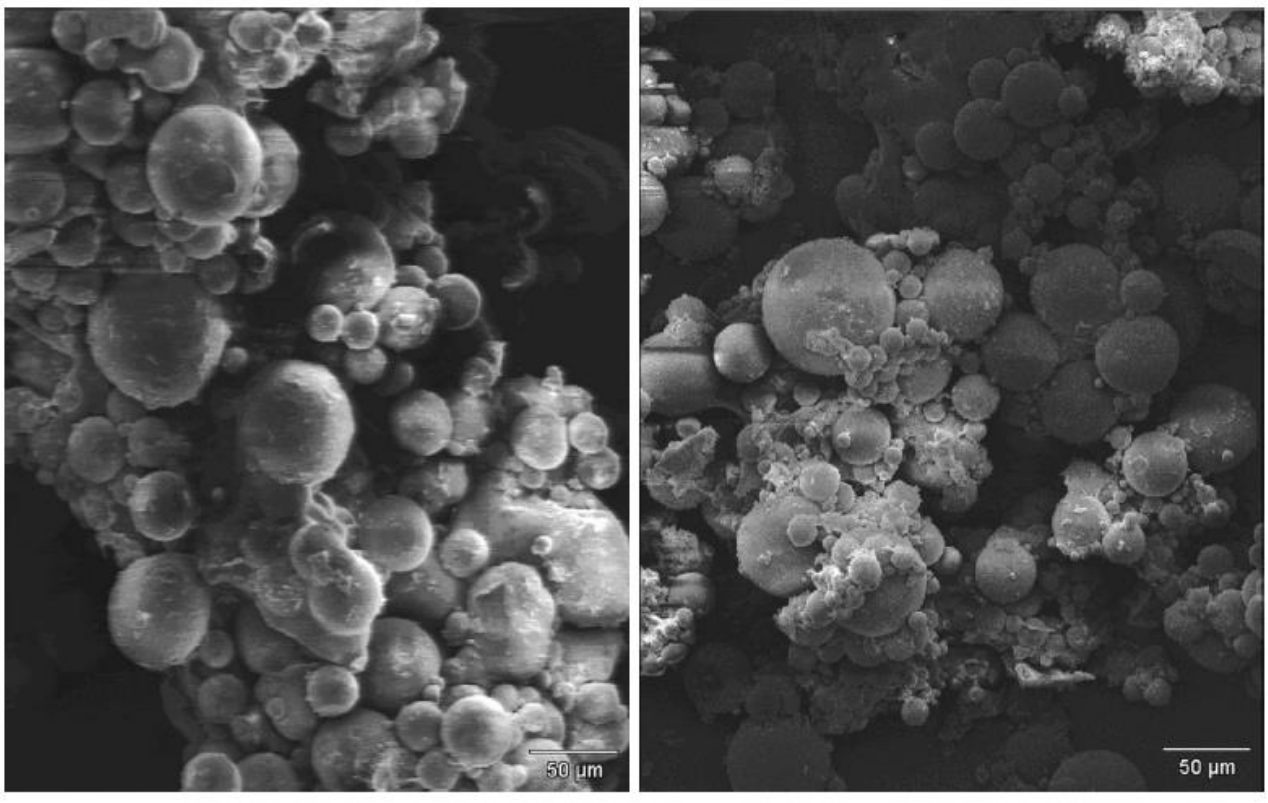

Figure 4. Selectivity factors (α) measured for Sudan dyes with acetonitrile-water mobile phases (chloro-Sudan, α = 1). Circles: Sudan I; lower triangles: Sudan II; squares: Sudan III; upper triangles: Sudan IV; diamonds: Sudan Red B; hexagons: Sudan Red 7B.

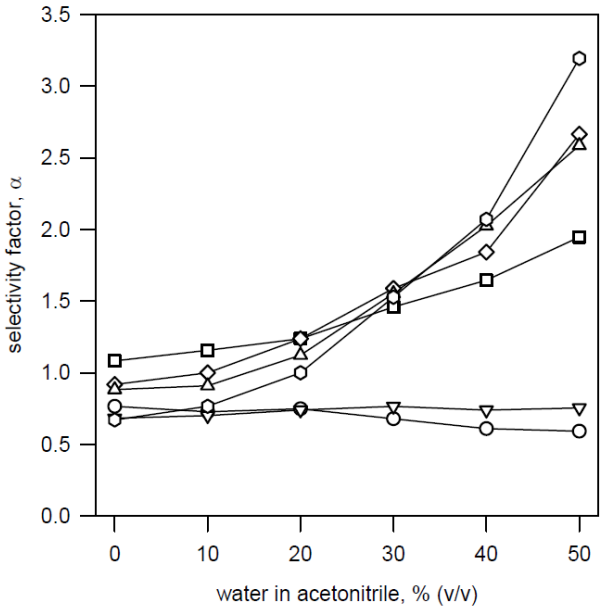

Figure 5. Cartridge washing performed on MISPE columns previously loaded with 1.0 ml of Sudan I (1 μg/ml) in acetonitrile-water 1+2 (v/v). Data expressed as the mean of three separate samplings. Open circles: acetonitrile; closed circles: acetone; open squares: tetrahydrofurane; closed squares: methanol.

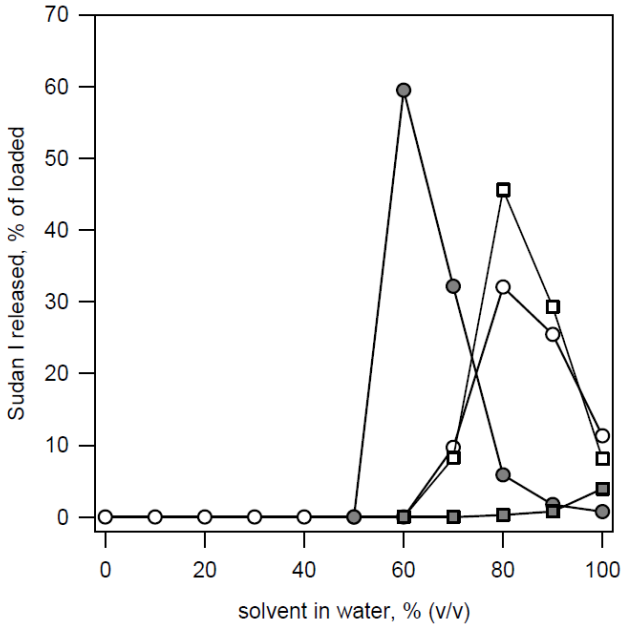

Figure 6. Cartridge elution performed on MISPE columns previously loaded with 1.0 ml of Sudan I (1 μg/ml) in acetonitrile-water 1+2 (v/v) and washed with 3x0.5 ml of water-acetonitrile 1+1 (v/v). Data expressed as the mean of three separate samplings. Open symbols: acetic acid; closed symbols: triethylamine. Circles: 10% (v/v); squares: 5% (v/v); upper triangles: 2% (v/v); lower triangles: 1% (v/v).

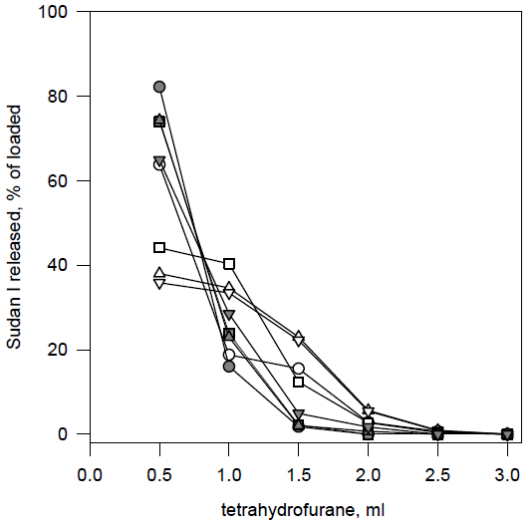

Figure 7. RP-HPLC of acetonitrile-water extracts of sausage spiked at 15 μg/g with a mixture of Sudan I, II, III and IV. Chromatogram A: direct injection of extract without performing MISPE. Chromatogram B: injection of extract after MISPE. Peaks identification: 1, Sudan I; 2, chloro-Sudan; 3, Sudan II; 4, unknown; 5, Sudan III; 6, Sudan IV.

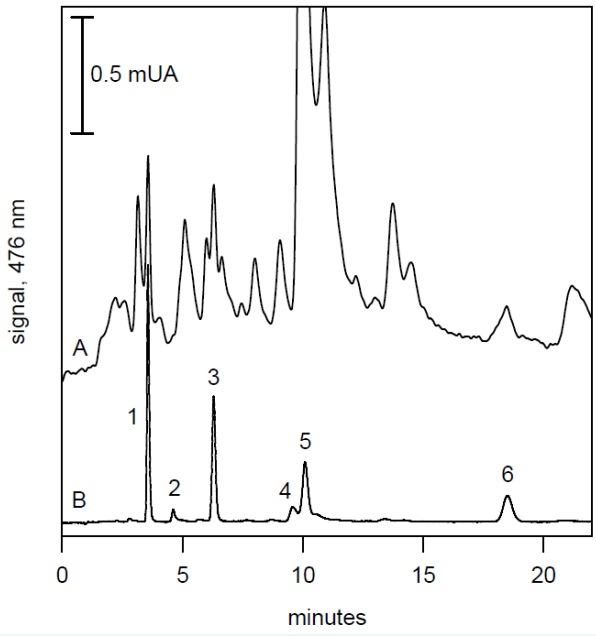

Figure 8. Preconcentration of hot chilli pepper samples containing Sudan I, in the range 0.75–15 μg/g. Data expressed as the mean of three separate samplings ± 1 standard deviation.