VILNIUS UNIVERSITY

Giedrė Kesiūnaitė

MATRIX SOLID PHASE DISPERSION-ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF ORGANIC COMPOUNDS IN SOLID MATRICES

Summary of doctoral dissertation

Physical sciences, chemistry (03 P)

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The dissertation is available at the library of Vilnius University and at the library of Institute of Chemistry.

VILNIAUS UNIVERSITETAS

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KIETAFAZĖ DISPERGUOJAMOJI EKSTRAKCIJA-ULTRAEFEKTYVIOJI SKYSČIŲ CHROMATOGRAFIJA ORGANINIŲ JUNGINIŲ NUSTATYMUI KIETOSE MATRICOSE

Daktaro disertacijos santrauka

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1. INTRODUCTION

In the past 10 years chromatography together with its various techniques has won a firm place amongst the instrumental methods of analysis. The popularity of this method was determined by the fact that it enables simultaneous separation and quantitative determination of analytes. High-performance liquid chromatography (HPLC) has become the technique of choice for the analysis of organic compounds, which are nonvolatile and in some cases thermally labile. A wide variety of the HPLC modes enable effective separation and quantitative determination of charged, highly polar uncharged, mediately polar and even completely unpolar compounds only by the proper choice of the sorbent and the mobile phase. In the recent years the on-line combination of HPLC with modern diode array spectrophotometric and mass spectrometric detectors has developed into a widely applied and routinely applicable separation, on-line identification and quantitative determination approach for complex environmental, biological and biomedical samples. However, the chromatographic part of this technique is responsible for some limitations as well. For example, the multi-component analysis of complex samples often requires separation times of 30 min and more. Recent advances in HPLC instrumentation have enabled the development of a new liquid chromatography technique termed ultra performance liquid chromatography (UPLC). A high speed of analysis, greater resolution, higher peak capacity and sensitivity are obtained due to the novel technology that utilizes a new generation of columns packed with pressure stable 1.7 μm hybrid material (ethylsiloxane/silica) particles and novel low dead volume, very high pressure (1000 bar) equipment.

Despite an exceptional selectivity of the chromatography even for this technique an additional sample pretreatment procedures for the isolation of the analytes from sample matrix and/or for their preconcentration are often required. Thus, sample preparation remains the key step determining the sample throughput and performance of most analytical procedures, particularly when applied to the analysis of solid samples. Most protocols dealing with the determination of organic compounds in solid samples involve liquid-liquid extraction followed by clean-up step, which make them time consuming and expensive to perform when many samples must be analyzed. In this context matrix solid-phase dispersion (MSPD) technique that involves blending of a small amount of

the solid or semi-solid sample with a solid support, followed by desorption with a small amount of appropriate solvent seems to be very attractive. Although MSPD was patented in 1993, only since 2000 an increased interest in this technique was observed. MSPD can be regarded as a valuable alternative to the conventional extraction methods because of its simplicity and robustness. In addition, depending on the nature of the solid support selected, a simultaneous clean-up of the extract occurs, which allows the direct analysis of the extracts. Finally, MSPD is well compatible with common chromatographic techniques. Consequently, the combination of MSPD extraction and modern UPLC analysis would offer a number of advantages for the analysis of organic compounds in solid matrices.

The aim of this work was investigation and application of matrix solid-phase dispersion and ultra performance liquid chromatography techniques for the rapid and effective determination of some organic compounds in solid matrices. For this purpose two analytically important analyte/sample matrix systems were selected: carbadox and olaquindox in animal feed and Sudan dyes in chili powder.

The main tasks set to achieve the aim were as follows:

1. Systematic investigation of the chromatographic behaviour of carbadox and olaquindox in UPLC under hydrophilic interaction chromatographic (HILIC) conditions and comparison with their separation in reversed phase HPLC.
2. Optimization of UPLC conditions for the separation of Sudan dyes.
3. Development of MSPD techniques for the extraction of carbadox and olaquindox from feed and Sudan dyes from chili powder samples.
4. Evaluation of the analytical performance of MSPD-UPLC methods and application of the developed methods in the analysis of real samples.

Statements for defence:

1. UPLC separation of carbadox/olaquindox and Sudan dyes is more effective, more selective and faster in comparison to the conventional HPLC method.
2. HILIC mode enables very selective and efficient determination of highly polar compounds without gradient elution. Introduction of HILIC principle for MSPD significantly accelerates the extraction of hydrophobic compounds from solid samples and improves extraction selectivity.

3. Developed new MSPD procedures are faster, simpler and more effective than liquid-liquid and solid-phase extraction techniques currently used for solid samples.
4. MSPD-UPLC techniques completely fulfill the EU requirements for the methods designed to control the veterinary drug residues and other banned compounds in food and related products.

2. EXPERIMENTAL

HILIC-UPLC separations were performed on the Waters Acquity UPLC system (Waters, Milford MA) equipped with an Acquity UPLC PDA detector. Acquity UPLC BEH HILIC (100 mm×2.1 mm i.d., 1.7 μm) and Acquity BEH C18 (100 mm×2.1 mm, i.d., 1.7 μm) columns, maintained at 30 °C were used for the analysis. Separations were performed at a flow rate of 0.5 mL/min using mobile phases composed of acetonitrile-water. The injection volume was 10 μL using a partial loop with needle overfill injection mode. Absorbance data were collected at 307 nm for carbadox, at 384 nm for olaquindox and at 500 nm for Sudan dyes. Data collection and management was performed by Empower 2 build 2154 software (Waters).

HPLC separations were performed on the Waters Alliance 2695 Separations Module equipped with a quaternary solvent delivery system, autosampler, and column heater. LichroCART Purosphere RP-18 (250 mm×4.6 mm, i.d., 5 μm particle size), Waters Symmetry Shield RP-8 (250 mm×4.6 mm, i.d., 5 μm particle size) and Zorbax Phenyl SDB (150 mm×4.6 mm, i.d., 5 μm particle size) columns, maintained at 20°C with a mobile phase flow rate of 1.0 mL/min were used. The injection volume was 20 μL. Absorbance detection was performed at 373 nm using a Waters 2487 Absorbance detector. AccuBond Alumina N (500 mg/5 mL) cartridges for sample clean-up were obtained from Agilent (Milford, MA, USA).

The solid-phases used for MSPD were silica gel (40-63 μm), aminopropylsilica (40-63 μm), octadecylsilica (40-63 μm) and florisil (80-120 μm) from Sigma-Aldrich. Polypropylene SPE syringe barrels (15 mL capacity) fitted with a single bottom frit and additional polyethylene frits were obtained from International Sorbent Technology (Mid Glamorgan, UK). Acetonitrile, methanol, ethanol, acetone, ethylacetate, acetic acid and ammonium acetate were HPLC grade and used as received (Merck, Darmstadt,

Germany). Standards of carbadox and olaquindox were purchased from Sigma-Aldrich. Stock solutions of carbadox and olaquindox at the concentration of 0.1 mg/mL were prepared in acetonitrile-methanol (6:4, v/v) and stored at 4 ºC, protected from light. Standards of Sudan I, Sudan II, Sudan III, and Sudan IV were purchased from Sigma-Aldrich. Stock solutions (0.1 mg/mL) of Sudan I and II were prepared in acetonitrile, whereas stock solutions (0.1 mg/mL) of Sudan III and IV were prepared in acetone and stored at 4 ºC, protected from light. All working standard solutions were prepared fresh daily by diluting the stock solution with acetonitrile.

For the preparation of spiked samples, the appropriate volume of the standard solution was added to 0.25 g of sample. Then they were allowed to stand at room temperature in the dark for 24 h.

3. RESULTS AND DISCUSSION

3.1. MSPD-HILIC-UPLC determination of carbadox and olaquindox

Carbadox and olaquindox are the most known members of quinoxaline-1,4-dioxides, a group of synthetic antibacterial drugs (Fig. 1) which are often used as a growth promoters as well as to prevent a number of diseases in animals. They are administered orally or mixed with animal feed. The European Commission prohibited the use of carbadox and olaquindox in animal feeds as a feed antibiotic from the beginning of 1999 due to their carcinogenic, mutagenic and photoallergenic effects. To control the compliance of the ban of carbadox and olquindox, a simple and effective analytical technique for monitoring of these drugs in animal feeds is of great significance. In this part of the present thesis a simple and fast MSPD procedure for the extraction of carbadox and olaquindox from feed followed by hydrophilic interaction UPLC analysis was developed.

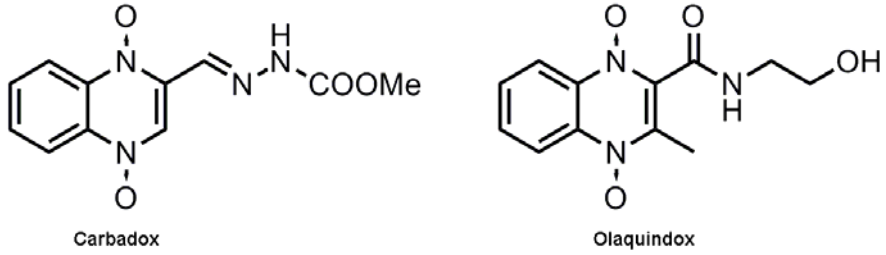

Fig. 1. Chemical structures of the analytes investigated in the present study.

Chromatographic behaviour. Similar to reserved-phase separation, HILIC separation commonly employs water and acetonitrile (ACN) as the mobile phase, but requires much higher organic content (>60%) to ensure significant hydrophilic interaction. The level of organic solvent in the mobile phase is probably the factor that has the largest influence on retention. Fig. 2 demonstrates the influence of acetonitrile concentration on the retention of analytes. As can be seen, both carbadox and olaquindox exhibited typical HILIC behavior of decreasing retention with increasing water content in the mobile phase. Its retention decreased significantly as the acetonitrile content decreased from 95 to about 80%, but leveled off when the acetonitrile content further decreased to 60%. Using pure ACN as the mobile phase retention times dramatically decreased and peaks were broadened. This indicates that the retention mechanism was changed from partition in the presence of water to adsorption with pure ACN.

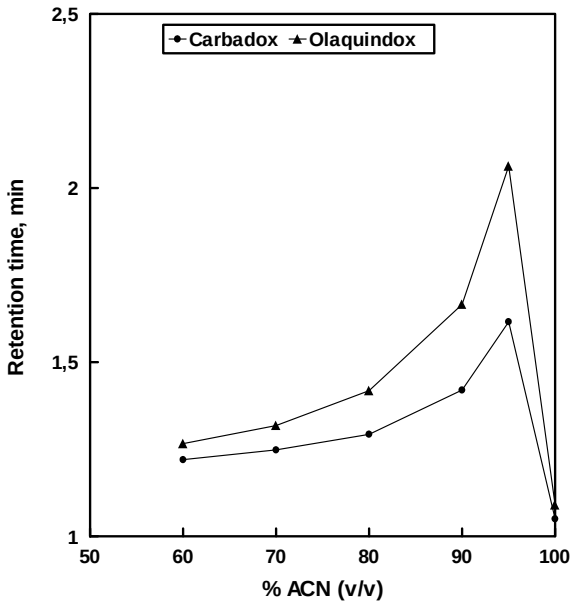

Fig. 2. Effect of acetonitrile content in the mobile phase on the retention of carbadox and olaquindox. Mobile phase flow rate, 0.25 mL/min.

Replacing water in the mobile phase with another protonic solvent was also investigated. Methanol and ethanol were tested as possible organic modifiers. The chromatograms obtained for a standard solution are compared in Fig. 3. Since water is the strongest elution solvent in HILIC, both analytes were stronger retained with the use of alcohol instead of water and their retention increased when the carbon chain of the alcohol increased. The peaks also exhibited significant tailing when alcohols were used

as strong solvent. Consequently, additional work was focused on acetonitrile-aqueous mobile phases.

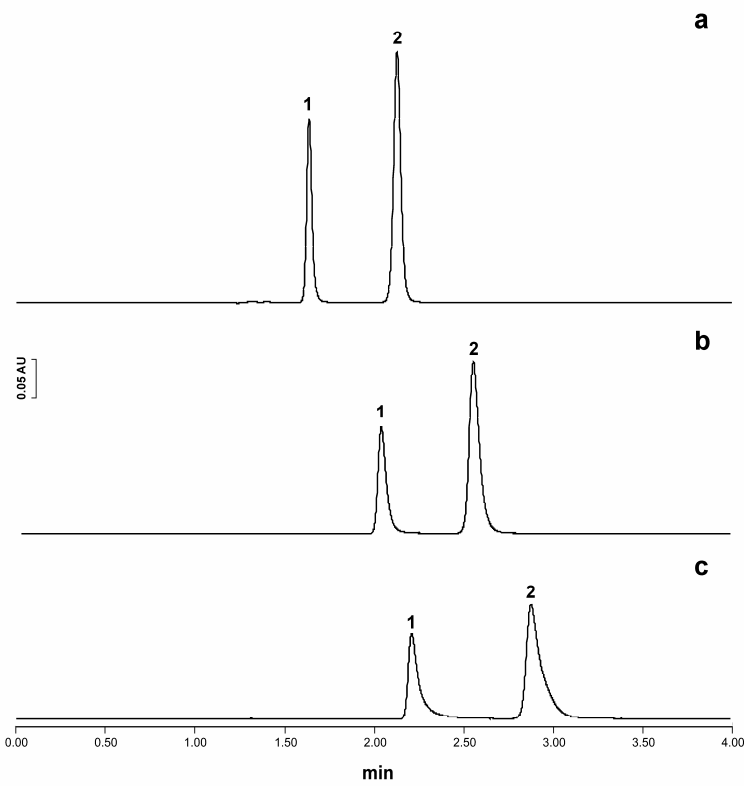

Fig. 3. Effect of protonic solvent on the separation performance. Mobile phase, (a) acetonitrile-water, (b) acetonitrile-methanol and (c) acetonitrile-ethanol (95:5, v/v); flow rate, 0.25 mL/min. Peaks: (1) carbadox, (2) olaquindox.

Mobile phase pH also can have significant impact on retention and selectivity in HILIC by influencing analyte ionization in the mobile phase. The effect of the mobile phase pH was studied by changing the pH of aqueous phase before mixing with acetonitrile. For both analytes the retention time and peak efficiency only fluctuated slightly with pH ranged from 3.5 to 7.0 most likely due to the lack of change in the ionization state of the analytes in the examined pH range.

A similar effect on retention and peak shapes was observed as the ammonium acetate concentration in the mobile phase was increased from 5 to 50 mmol/L. Thus, such low impact of the mobile phase pH and salt concentration on the retentivity of carbadox and olaquindox indicates considerable stability of the elution conditions for these analytes.

As a further parameter the effect of column temperature on retention of the analytes was investigated in the range 20–50 °C and it was found to be slight. In particular,

retention times of carbadox and olaquindox gradually decreased (in about 8 and 13% for carbadox and olaquindox, respectively) as a function of the temperature. A temperature of 30 °C was selected as optimum for retention and peak shape.

Finally, the effect of mobile phase flow rate (from 0.1 to 1.0 mL/min) upon efficiency was briefly studied (Fig. 4). For both compounds the HILIC column exhibited almost constant theoretical plate height values at the mobile phase flow rates ranged from 0.2 to 0.7 mL/min. However, there was approximately a 15-40% increase in theoretical plate height as the flow rate was further increased from 0.7 to 1 mL/min. Further work was carried out at 0.5 mL/min mobile phase flow rate which provided the separation of both analytes in about 1 min with adequate resolution and efficiency.

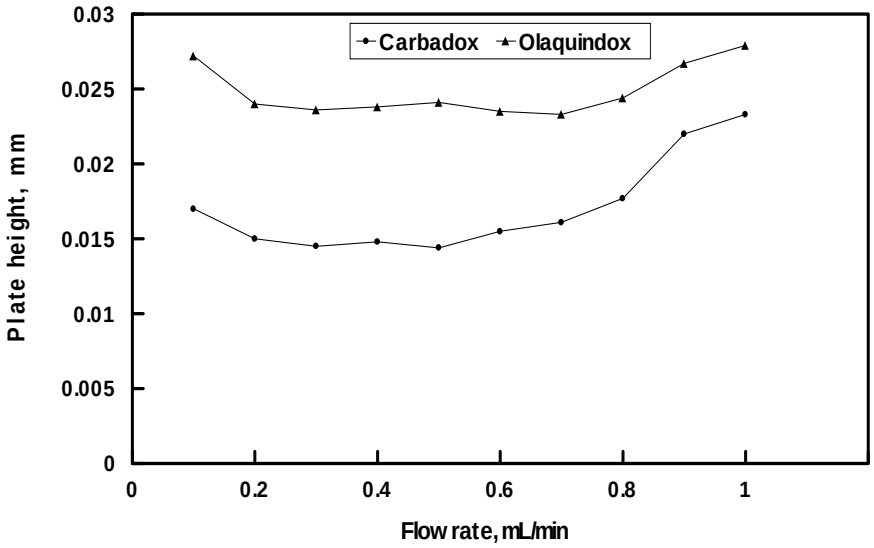

Fig. 4. Effect of mobile phase flow rate on the peak efficiency. Mobile phase, acetonitrile-water (95:5, v/v).

On the basis of all of the above findings, the best results were obtained using the Asquity UPLC BEH HILIC column maintained at 30 °C and eluted isocratically at 0.5 mL/min with acetonitrile-water (95:5, v/v) mobile phase containing 10 mmol/L ammonium acetate. The UPLC chromatogram obtained under optimum conditions for a standard solution is shown in Fig. 5. For comparison purposes, the chromatogram showing the reversed-phase HPLC separation of both drugs is given in Fig. 6. Using HILIC-UPLC the separation time was shortened in about 10 times reducing the run time from 11 to 1.1 min and a better peak shape was achieved compared to reversed-phase HPLC.

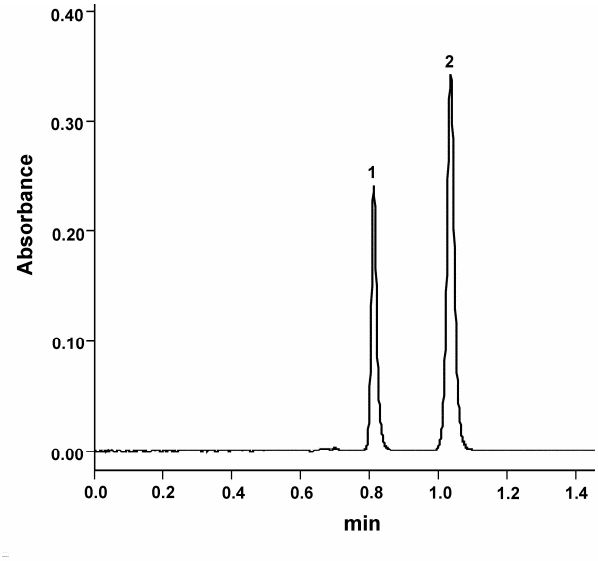

Fig. 5. HILIC-UPLC chromatogram of carbadox and olaquindox standard solution under optimized conditions. Mobile phase, 10 mmol/L CH_3COONH_4 in acetonitrile-water (95:5, v/v); flow rate, 0.5 mL/min; column temperature, 30 °C. Peaks: (1) carbadox, (2) olaquindox.

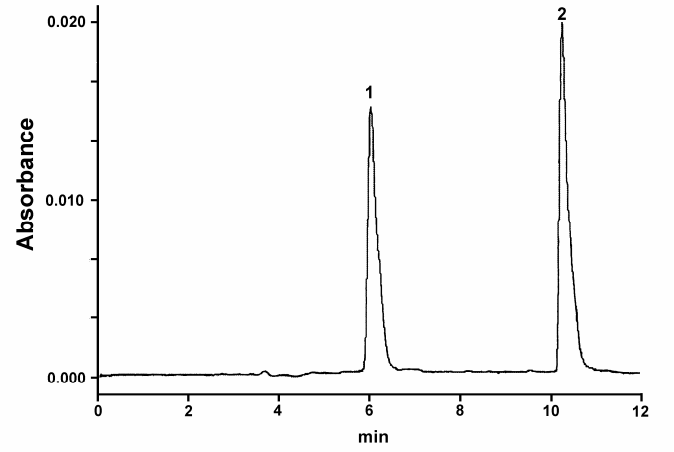

Fig. 6. Reversed-phase HPLC chromatogram of carbadox and olaquindox standard solution under optimized conditions. Gradient elution with ACN/H_2O mobile phase; flow rate, 1.0 mL/min. Peaks: (1) olaquindox, (2) carbadox.

MSPD extraction. The first step in the method development was the evaluation of a suitable sorbent to allow selective and complete isolation of the analytes from feed matrix. Three sorbents with quite different properties, octadecylsilica (C_{18}), aminopropylsilica (Si-NH_2) and silica gel (Si-OH), were investigated as solid supports for matrix dispersion. Recovery studies were carried out by spiking feed blanks (0.25 g) with the analyte standard at a 10 mg/kg fortification level and subsequent blending with 0.5 g of appropriate sorbent. Acetonitrile (10 mL) was selected as the extraction solvent for preliminary experiments because it is the major component of our HILIC mobile phase and therefore allows direct injection of the extract onto the separation column. The results presented in Fig. 7 show that no significant differencies in terms of recoveries were found for the tested sorbents.

However, hydrophobic octadecylsilica sorbent produced the cleanest extracts as evidenced by the visual inspection of the extracts and by the number and intensity of

peaks in the chromatographic profiles measured in 200-500 nm wavelength range with the PDA detector.

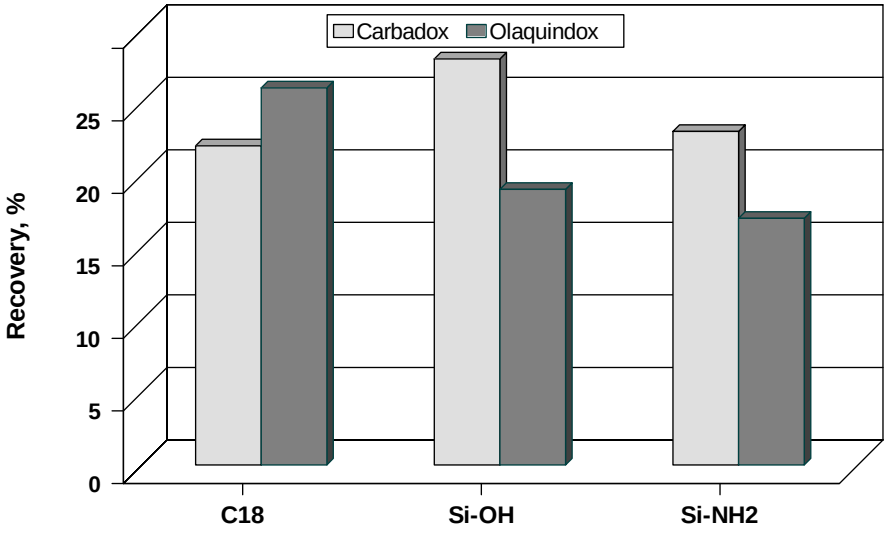

Fig. 7. Average recoveries (%) obtained from different sorbents by MSPD extraction of blank feed samples spiked at 10 mg/kg (n=3). Extraction solvent – 10 mL ACN.

The second step in the MSPD method setup was the evaluation of a suitable elution solvent. As already mentioned, acetonitrile is the best choice since it is the major component of our HILIC mobile phase. One and perhaps the simplest way to enhance the extraction efficiency is to increase the acetonitrile volume. However, the extraction with higher acetonitrile volumes (up to 25 mL) produced not only an enhancement of recoveries (up to about 75-80%), but also more interfering peaks.

A second possible way of increasing the performance of MSPD extraction involves the increase in solvent elution/extraction strength by evaluation of different eluting solvents. Primarily, the selected solvents should have a good solubility for the target analytes. According to this principle, water, methanol and ethanol were selected. Results are shown in Fig. 8. As expected, the use of protonic solvents significantly enhanced the MSPD extraction efficiency, but the recoveries found using the water were significantly lower than those obtained with methanol or with ethanol. In MSPD the eluting solvent plays at least two roles: one for dissolution/extraction of the analytes and the other for their elution from the solid dispersant. Thus, using water as elution solvent the lower recoveries obtained for both analytes can be attributed to the very low eluting strength of

the water causing the incomplete elution of the analytes from hydrophobic octadecylsilica surface.

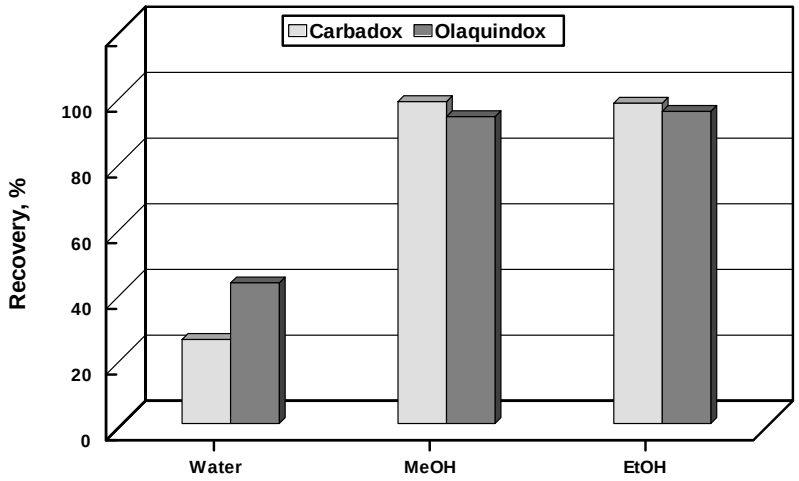

Fig. 8. Effect of elution solvent on the recovery of carbadox and olaquindox by MSPD extraction of blank feed samples spiked at 10 mg/kg (n=3).

Despite the fact that recoveries were similar when either ethanol or methanol was used as elution solvent, the use of methanol was preferred because it gave cleaner extracts and exhibited better volatility. Nonetheless, the extracts obtained using pure methanol were not clear enough as compared with acetonitrile extracts. For this reason, the extraction efficiency from spiked blank samples was examined using acetonitrile-methanol mixtures (10 mL) at different volume ratios (9:1, 8:2, 7:3 and 6:4, v/v). Table 1 summarizes the recoveries obtained using four mixtures as eluting solvents. As can be seen, the average recoveries of the analytes were significantly improved by adding even small amounts of methanol to the acetonitrile. Satisfactory recoveries (≥90%) were observed with a methanol content of ≥20%, v/v.

Finally, to establish the volume of elution solvent (acetonitrile-methanol, 8:2, v/v) required, feed blanks containing analytes at two concentration levels (2.0 and 10 mg/kg) were investigated. During MSPD extraction, 1 mL fractions were collected, evaporated to dryness, re-dissolved with 0.5 mL of acetonitrile, filtered and analyzed. At both concentration levels, the total solvent volume required to extract ≥90% of the analytes was about 8 mL. To ensure robustness of the method, we used 10 mL in further method development. Based on the obtained results, the optimal conditions selected for MSPD extraction were: 0.25 g of feed sample, 0.5 g of octadecylsilica as solid sorbent and 10 mL of acetonitrile-methanol (8:2, v/v) as eluting solvent. With this MSPD extraction

procedure, the extracts were generally clear enough to allow for direct hydrophilic interaction UPLC analysis.

Table 1. Average recoveries obtained with different elution solvents by MSPD extraction of blank feed samples spiked at 10 mg/kg (n=3; solvent volume 10 mL)

Acetonitrile-methanol, v/v	Recovery, %	
	Carbadox	Olaquindox
9:1	91	76
8:2	99	94
7:3	95	94
6:4	97	93

Analytical performance. The linearity of the method was tested by preparing calibration curves for both analytes at seven concentration levels in the range of 0.1–10 mg/kg. For both analytes the calibration curves were linear in the tested concentrations range with acceptable correlation coefficients of more than 0.998. The limits of detection defined as the concentration which produced a signal equal to three times the background noise level, were 0.02 and 0.03 mg/kg for carbadox and olaquindox, respectively.

Table 2 reports the recoveries together with the precision results for feed samples fortified at two concentration levels. For carbadox the recoveries ranged between 95 and 98% whereas values from 89 to 96% were attained for olaquindox. Considering that these data were not corrected with the use of internal standard, they are still high enough to allow practical analysis. In both fortification levels, the RSD values ranged from 5 to 10% and can be called satisfactory.

Fig. 9 shows the HILIC-UPLC chromatograms, obtained following the MSPD extraction procedure, for a non-fortified porcine feed sample (a) ant the same sample fortified at 0.25 mg/kg of carbadox and olaquindox (b).

Table 2. Average recoveries (%) and relative standard deviations (% in parenthesis) obtained by MSPD extraction of fortified feed samples (n=3)

Sample	Analyte	Fortified, mg/kg	Recovery (RSD), %
Porcine feed	Carbadox	1.0	98 (7)
		5.0	95 (5)
	Olaquindox	1.0	89 (10)
		5.0	94 (8)
Broiler feed	Carbadox	1.0	96 (6)
		5.0	95 (7)
	Olaquindox	1.0	92 (8)
		5.0	96 (7)

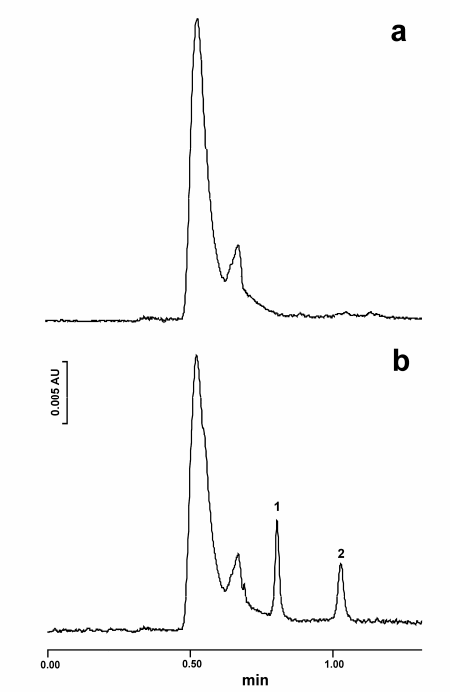

Fig. 9. Hydrophilic interaction UPLC chromatograms, obtained following the MSPD extraction procedure, for a non-fortified porcine feed sample (a) ant the same sample fortified at 0.25 mg/kg of carbadox and olaquindox (b). Peaks: (1) carbadox, (2) olaquindox.

The MSPD-HILIC-UPLC method was compared with conventional liquid-liquid extraction (LLE) followed by HPLC analysis. The main performance characteristics are reported in Table 3. From these data it can be seen that proposed technique generally provides extraction yields and precision comparable to those achieved by LLE-HPLC with the advantages of being simpler, significantly faster and more sensitive.

Table 3. Comparison of MSPD extraction-hydrophilic interaction UPLC analysis with conventional liquid-liquid extraction-HPLC method

Characteristic	MSPD-HILIC-UPLC	LLE-HPLC
Recovery[a], %	89-98	91-97
RSD[a], %	6-10	5-7
Limit of detection, mg/kg	0.02; 0.03	0.3; 0.2
Sample, g	0.25	1.0
Solvent, ml	10	10
Sample preparation time, min	15	90
Separation time (with reequilibration), min	2	20

[a] At 1.0 mg/kg fortification level (n=3).

3.2. MSPD-UPLC determination of Sudan dyes

Sudans I, II, III, and IV (Fig. 10) are synthetic phenyl-azoic derivatives widely used in chemical industries for coloring materials such as oils, plastics, waxes, printing inks, etc. For many years they had been also employed as an additive in foods, particularly in

those containing chili powder, because of their intense red-orange color. However, now its use as food additives is prohibited worldwide due to potential carcinogenic effects. Consequently, all chili-containing food products coming into any EU state are certified to be free of Sudan dyes. To control the compliance of the ban of Sudan dyes, a simple and effective analytical technique for monitoring of these compounds in chili-containing food products is of great significance.

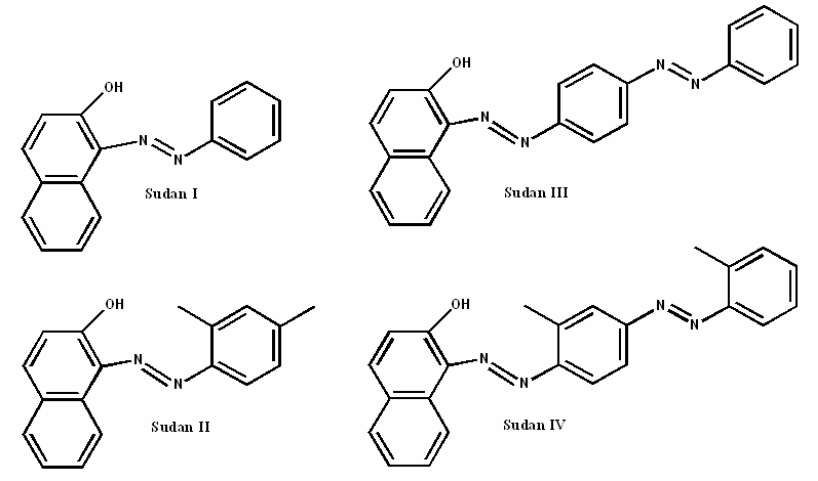

Fig. 10. Chemical structures of Sudan dyes studied.

UPLC separation. One of the objectives of this study was to evaluate the potential of the UPLC technique in the analysis of Sudan dyes. In general, HPLC separations of Sudan dyes are performed on conventional reversed-phase columns using isocratic or gradient elution with water/acetonitrile or water/methanol mobile phases. Three reversed-phase UPLC stationary phases with slightly different hydrophobicity (C18, C8 and phenyl) were compared. The results showed that the best separation performance was obtained on the C18 stationary phase. The chromatogram showing optimized UPLC separation of Sudan dyes is given in Fig. 11. It can be observed, the selected gradient resulted in a good resolution with reasonable retention times and good peak shapes. The total run time including a 1 min re-equilibration step for UPLC separation was under 3 min, *i.e.*, about 5-10 times shorter in comparison to the conventional HPLC method.

MSPD extraction. The first step in the method development was the choice of a suitable solid sorbent for the selective and complete isolation of the analytes from sample matrix. Sudan dyes are strongly hydrophobic compounds. Consequently, for their effective isolation from the sample matrix the dispersion of the sample with polar solid sorbent seems to be very attractive. Three polar sorbents with slightly different

properties, aminopropylsilica, silica gel and florisil, were investigated as solid supports for matrix dispersion. For comparison purposes, octadecylsilica, a reversed-phase sorbent commonly used to retain non-polar and moderately polar compounds was also tested.

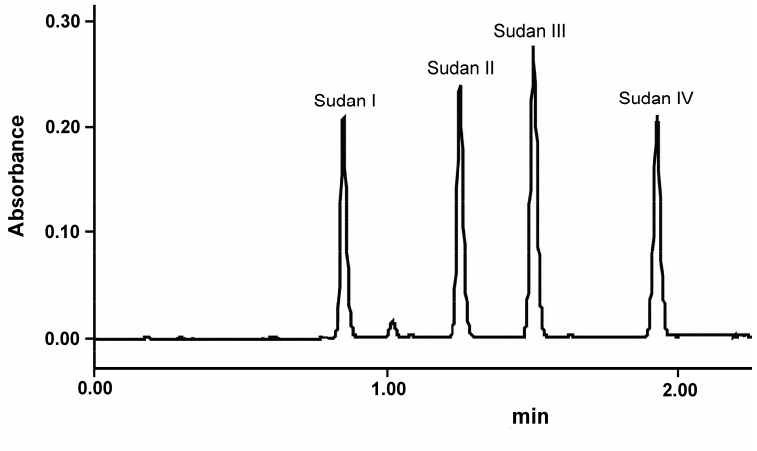

Fig. 11. UPLC chromatogram of Sudan dyes (10 mg/L) obtained under optimized conditions using C18 stationary phase and gradient elution with ACN/H_2O mobile phase.

Recovery studies were carried out by spiking chili powder blanks (0.25 g) with the analyte standard at a 10 mg/kg fortification level and subsequent blending with 0.5 g of appropriate sorbent. Acetonitrile (5 mL) was selected as the extraction solvent for preliminary experiments. As expected, the recoveries of Sudan dyes obtained after dispersing with octadecylsilica (18-34%) were significantly lower than those obtained by the use of polar sorbents (45-58%). Moreover, aminopropylsilica and silica gel produced cleaner extracts as evidenced by the visual inspection of the extracts and by the number and intensity of peaks in the chromatographic profiles measured in 200-500 nm wavelength range with the PDA detector. Although no significant differences in terms of recoveries were found for silica gel and aminopropylsilica phases, silica gel was considered the optimum choice for the subsequent MSPD experiments because it is less expensive.

The second step in the MSPD method setup was the evaluation of a suitable elution solvent. As already mentioned, acetonitrile is the best choice since it is the major component of our UPLC mobile phase. Perhaps the simplest way to enhance the extraction efficiency is to increase the acetonitrile volume. However, the extraction with higher acetonitrile volumes (up to 20 mL) produced not only an enhancement of

recoveries, but also more interfering peaks. In addition, larger volumes of the elution solvent reduce detection sensitivity (without preconcentration by evaporation of the extract) or prolong the sample preparation procedure (with evaporation).

Another way of improving of the performance of MSPD extraction involves the increase of the solvent elution/extraction strength by proper choice of eluting solvents. MeOH, ACN, EtOH, acetone and ethylacetate were compared. Results are shown in Fig. 12. As expected, the use of less polar solvents (EtOH, acetone and ethylacetate) enhanced the MSPD extraction efficiency, but the recoveries found using these eluting solvents were significantly higher than 100. This indicates that some interfering compounds are co-eluted together with the analytes.

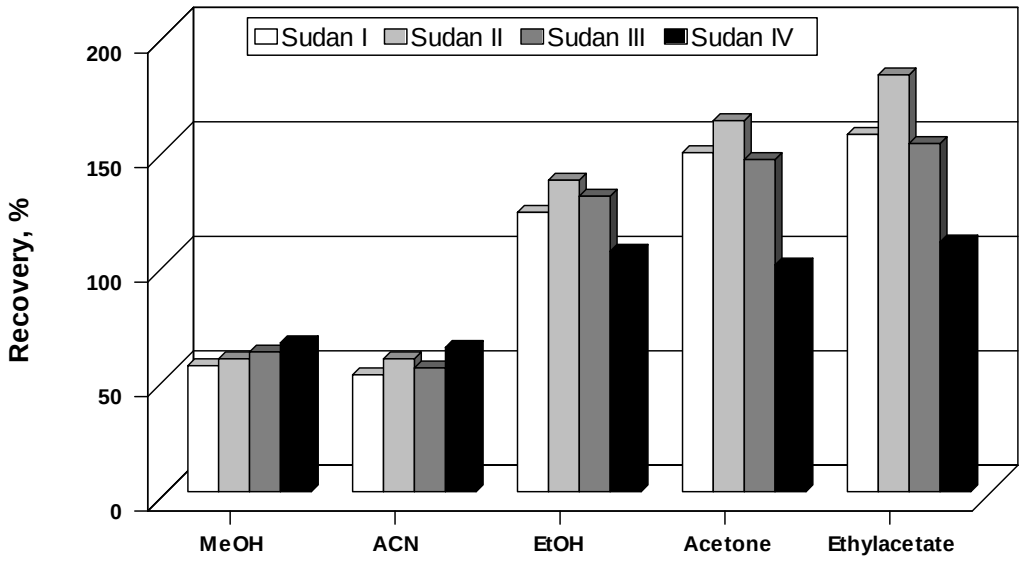

Fig. 12. Effect of elution solvent on the recovery of Sudan dyes by MSPD extraction of blank chili powder samples spiked at 10 mg/kg (n=3). Solid sorbent – Si-OH. Solvent volume – 5 mL.

In order to enhance MSPD selectivity, the extraction of Sudan dyes from spiked blank samples was examined using acetonitrile-water, acetonitrile-methanol and acetonitrile-ethanol mixtures (5 mL) at 9:1 volume ratios. Such solvent mixtures combined with polar sorbent represent the typical mobile phases for hydrophilic interaction chromatography with extremely high eluting strength for hydrophobic compounds and poor eluting strength for hydrophilic ones. As can be seen (Fig. 13), the average recoveries of the analytes were improved by adding a small amount of alcohol to the acetonitrile, whereas the recoveries using acetonitrile-water remain slightly lower

than those obtained with pure acetonitrile (51-56%). In MSPD the eluting solvent plays at least two roles: one for dissolution/extraction of the analytes from the disrupted sample and the other for their elution from the solid dispersant. Thus, the lower recoveries obtained with acetonitrile-water as elution solvent can be attributed to the lower solubility of the non-polar analytes in the water-containing solvent. Despite the fact that recoveries were similar when either acetonitrile-ethanol or acetonitrile-methanol elution solvents were used, acetonitrile-methanol mixture has been chosen due to its higher volatility.

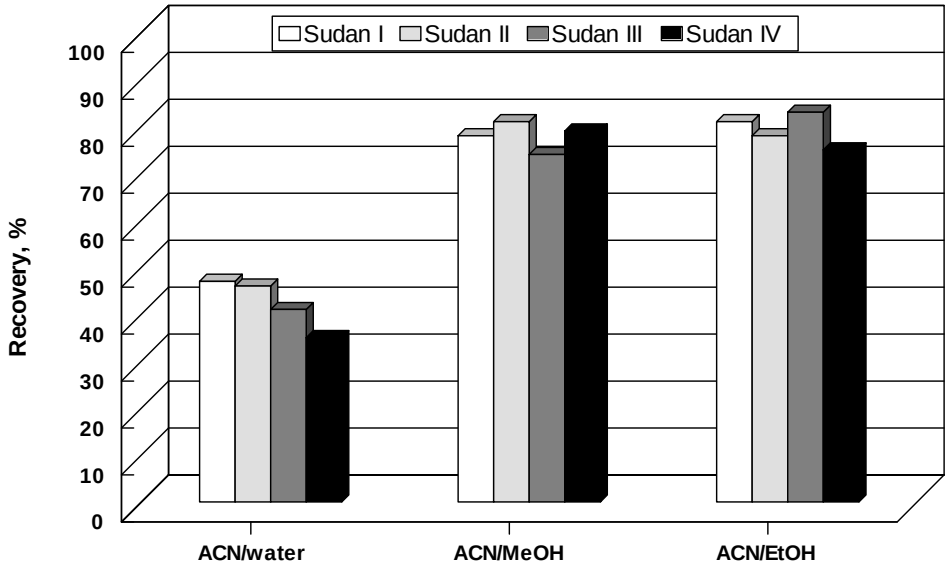

Fig. 13. Effect of elution solvent (5 mL) on the recovery of Sudan dyes by MSPD extraction of blank chili powder samples spiked at 10 mg/kg (n=3). Elution solvent: ACN containing 10% (v/v) of appropriate solvent.

Finally, to establish the optimal volume of elution solvent (acetonitrile-methanol, 9:1, *v/v*) required, chili powder blanks spiked at 10 mg/kg were investigated. During MSPD extraction, 1 mL fractions were collected, evaporated to dryness, re-dissolved with 0.5 mL of acetonitrile, filtered and analyzed. As can be seen (Fig. 14), the total solvent volume required to extract ≥90% of the analytes was about 6 mL. To ensure robustness of the method, we used 7 mL in further method development.

Based on the obtained results, the optimal conditions selected for MSPD extraction were: 0.25 g of sample, 0.5 g of silica gel as solid sorbent and 7 mL of acetonitrile-methanol (9:1, *v/v*) as eluting solvent. With this MSPD extraction procedure, the extracts were generally clear enough to allow the direct UPLC analysis.

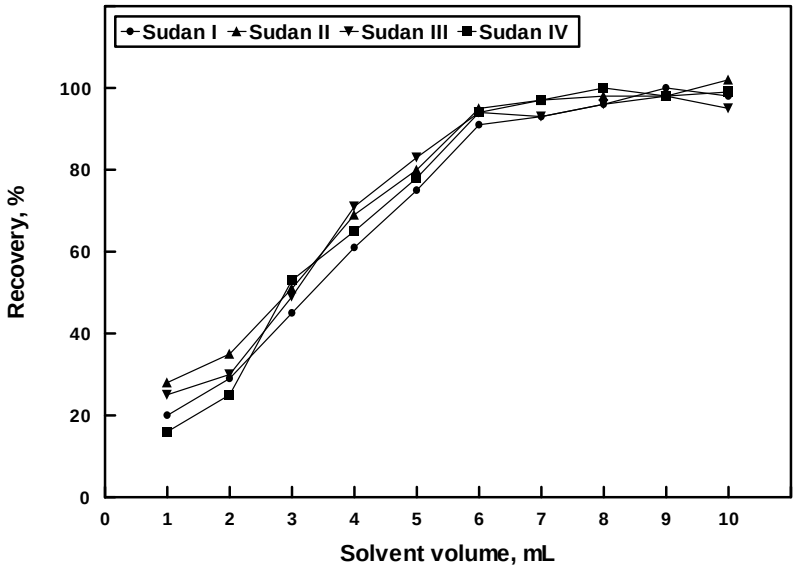

Fig. 14. Effect of elution solvent volume on the recovery of Sudan dyes by MSPD extraction of blank chili powder samples spiked at 10 mg/kg (n=3). Solvent – ACN/MeOH (9:1, v/v). Sorbent – Si-OH.

Analytical performance. The linearity of the method was tested by preparing matrix-matched calibration curves at seven concentration levels in the range of 1-25 mg/kg. For all analytes the calibration curves were linear in the tested concentrations range with acceptable correlation coefficients (r^2) higher than 0.997. The limit of detection was estimated from spiked blank samples based on 3:1 signal-to-noise ratios. The results are summarized in Table 4.

Table 4. Main analytical characteristics for Sudan dyes (*n*=3)

Analyte	Linearity range, mg/kg	r^2	Detection limit, mg/kg
Sudan I	1.0 - 25	0.997	0.25
Sudan II	1.0 - 25	0.999	0.25
Sudan III	1.0 - 25	0.998	0.30
Sudan IV	1.0 - 25	0.998	0.40

Finally, the developed method was applied to ten chili powder samples obtained from local supermarkets. For comparison purposes four samples procured from Turkish and Indian bazaars were also analyzed. Fig.15 shows the UPLC chromatograms obtained following the MSPD extraction procedure for a non-fortified chili powder sample and the same sample fortified at 1.0 mg/kg of Sudan dyes. It can be seen, excellent separation of the four dyes with no interference from the matrix was obtained. An unidentified peak observed in all sample extracts at 1.38 min can arise from one of the

carotenoids (most likely from beta-carotene) present in the chili pepper, which have a similar absorbance range to Sudan dyes. Among the fourteen samples tested, thirteen were found negative for dyes studied. Only one sample coming from Turkish bazaar was found to contain 760 mg/kg Sudan I and 340 mg/kg Sudan IV (Fig. 16).

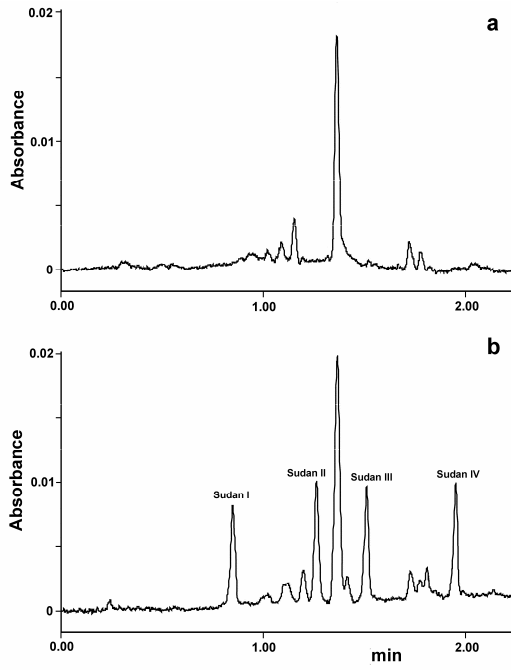

Fig. 15. UPLC chromatograms obtained following the MSPD extraction procedure for a non-fortified chili powder sample (a) and the same sample fortified at 1.0 mg/kg of Sudan dyes (b).

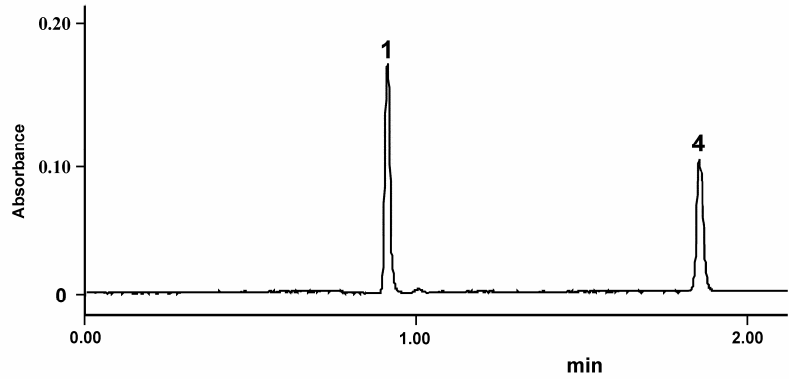

Fig. 16. UPLC chromatogram obtained following the MSPD extraction procedure for a non-fortified chili powder sample coming from Turkish bazaar.

Table 5 reports the obtained results and recoveries together with the precision data for non-fortified and fortified chili powder samples. Generally, the recoveries obtained for all analytes were within the range of 83-106%. Considering that these data were not corrected with the use of internal standard, they are still high enough to allow practical

analysis. In all fortification levels, the RSD values ranged from 5 to 15% and can be called satisfactory.

Table 5. Average recoveries (%) and relative standard deviations (%) obtained by MSPD-UPLC analysis of fortified chili powder samples (n=3)

Purchasing country	Analyte	Found, mg/kg	Fortified, mg/kg	Recovery, %	RSD, %
Lithuania	Sudan I	-	1.0	89	9
	Sudan II	-	1.0	95	11
	Sudan III	-	1.0	85	15
	Sudan IV	-	1.0	83	13
Turkey*	Sudan I	760	50	96	5
	Sudan II	-	50	96	6
	Sudan III	-	50	96	6
	Sudan IV	340	50	106	5
India	Sudan I	-	5.0	94	7
	Sudan II	-	5.0	99	7
	Sudan III	-	5.0	91	9
	Sudan IV	-	5.0	84	8

*MSPD extract was diluted with ACN to 25 mL.

CONCLUSIONS

1. Under HILIC-UPLC conditions the elution strength of protonic solvents decreases in the order: water > methanol > ethanol. The nature of the protonic solvent strongly affects the peak efficiency: by replacing water with ethanol about twice broader peaks for both analytes were observed. The effect of mobile phase pH and ionic strength on the retention of the drugs was negligible. Using HILIC-UPLC the separation time was 10-times shortened and about 3-4-times higher efficiency with better resolution was achieved compared to conventional reversed-phase HPLC.
2. Influence of the nature of sample solvent on the peak efficiency and separation selectivity decreases in the order: water > methanol > ethanol > acetonitrile. By injection of the analytes diluted in acetonitrile/water mixtures, their peak efficiency remains unchanged up to 15% v/v concentration of H_2O in the acetonitrile, for the methanol – up to 25% v/v, and for the ethanol – up to 40% v/v.
3. Three solid phases (octadecylsilica, silica gel and aminopropylsilica) as solid supports for MSPD of carbadox and olaquindox from feeds were compared and the cleanest extracts were obtained with hydrophobic octadecylsilica. Protonic solvents (methanol and ethanol) exhibited higher eluting strength, whereas acetonitrile was more selective. Complete and selective desorption/elution of the drugs from feed matrix was achieved with 8 mL of acetonitrile-methanol (8:2 v/v) at 2 mL/min flow rate.
4. MSPD-HILIC-UPLC technique generally provides extraction yields (89-98%) and precision (6-10 %) comparable to those achieved by the liquid-liquid extraction-HPLC (90-97% and 5-7%, respectively) with the advantages of being simpler, about 6-7 times faster and 10 times more sensitive.
5. Three stationary phases (C18, C8 and phenyl) for the UPLC separation of Sudan dyes were compared and highest efficiency and best peak shapes were observed using C18 phase. Using gradient elution with water/acetonitrile mobile phase four dyes were completely resolved in 2 min, i.e., about 10 times faster in comparison to the conventional HPLC method.
6. Cleanest extracts of Sudan dyes from chili powder were obtained with polar (silica gel and aminopropylsilica) solid sorbents. With both phases the eluting/desorption

strength of solvents increases in the order: methanol ≈ acetonitrile < ethanol < acetone < ethylacetate. In the same order MSPD extraction selectivity decreases.

7. MSPD extraction selectivity was significantly improved by using acetonitrile with small amount (10% v/v) of methanol as eluting solvent. Such solvent mixture combined with polar sorbent represents the typical mobile phases for HILIC with extremely high eluting strength for hydrophobic compounds and poor eluting strength for hydrophilic ones. Complete and selective desorption/elution of the dyes from chili powder was achieved with 7 mL of acetonitrile-methanol (9:1 v/v) at 2 mL/min flow rate.
8. MSPD-UPLC method provides extraction yields (83-106%) and precision (≤15 %) which completely fulfill the EU requirements for the methods designed to control the foods. The procedure allows extraction and clean-up to be carried out into a single step, without additional purification of the extracts, is very simple and about 6-times faster compared to existing techniques.

The List of Original Publications by the Author

Articles in journals

1. **G. Kesiūnaitė**, A. Padarauskas. Development of high-performance liquid chromatography for the determination of carbadox and olaquindox in animal feed. *Chemija*, Vol. 18, No 3 (2007) 30-34.
2. **G. Kesiūnaitė**, E. Naujalis, A. Padarauskas. Matrix solid-phase dispersion extraction of carbadox and olaquindox in feed followed by hydrophilic interaction ultra-high pressure liquid chromatographic analysis. *Journal of Chromatography A*, Vol. 1209 (2008) 83-87.
3. **G. Kesiūnaitė**, A. Linkevičiūtė, E. Naujalis, A. Padarauskas. Matrix solid-phase dispersion extraction and UPLC determination of Sudan dyes in chili powder. *Chromatographia*, (2009) in press.

Published contributions to academic conferences

1. A. Linkevičiūtė, **G. Kesiūnaitė**, E. Naujalis, A. Padarauskas. Comparison of liquid-liquid extraction and matrix solid phase dispersion for the extraction of sudan dyes from chilli powder. Proceedings of the conference "Chemistry and Chemical Technology", Kaunas, Lithuania, 2008, p. 81-82.
2. **G. Kesiūnaitė**, A. Čiuberkytė, A. Padarauskas. Investigation of the matrix solid-phase dispersion method for the extraction of carbadox and olaquindox from animal feed. Proceedings of the conference "Chemistry and Chemical Technology", Kaunas, Lithuania, 2008, p. 79-80.
3. **G. Kesiūnaitė**, A. Linkevičiūtė, E. Naujalis, A. Padarauskas. Matrix solid-phase dispersion extraction and ultra-performance liquid chromatographic determination of Sudan dyes. Thesis of the 5th Nordic Separation Science Society International Conference, Tallinn, Estonia, 26-29 August 2009, p. 101.

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1999-2003 Bachelor studies at Vilnius University – Bachelor degree in chemistry.

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KIETAFAZĖ DISPERGUOJAMOJI EKSTRAKCIJA–ULTRAEFEKTYVIOJI SKYSČIŲ CHROMATOGRAFIJA ORGANINIŲ JUNGINIŲ NUSTATYMUI KIETOSE MATRICOSE

SANTRAUKA

Šioje daktaro disertacijoje apibendrintų mokslinių tyrimų tikslas – nuodugniai ištirti ir pritaikyti kietafazės disperguojamosios ekstrakcijos (KDE) ir ultraefektyviosios skysčių chromatografijos (UESCh) metodus greitam ir efektyviam organinių junginių nustatymui kietose matricose. Tyrimams buvo pasirinktos dvi pakankamai aktualios analičių/mėginio matricos sistemos: karbadoksas ir olakvindoksas pašaruose bei Sudano dažikliai čili pipirų milteliuose.

Ištyrus karbadokso ir olakvindokso atskyrimo UESCh metodu hidrofilinės sąveikos chromatografijos (HILIC) sąlygomis ypatumus nustatyta, kad protoninių tirpiklių išstūmimo jėga silpnėja tokia tvarka: vanduo > metanolis > etanolis. Protoninio tirpiklio prigimtis stipriai įtakoja ir analičių smailių efektyvumą: pakeitus vandenį etanoliu, smailės išsiplečia apie 2 kartus bei pablogėja jų simetriškumas. Judrios fazės pH, joninės jėgos ir kolonėlės temperatūros įtaka analičių sulaikymui nežymi. Tyrimų rezultatai parodė, kad karbadokso ir olakvindokso atskyrimas HILIC-UESCh metodu yra 3-4 kartus efektyvesnis ir maždaug 10 kartų greitesnis už jų atskyrimą atvirkščių fazių efektyviosios skysčių chromatografijos metodu. Mėginio tirpiklio prigimties įtaka analičių smailių efektyvumui bei jų atskyrimo atrankumui silpnėja tokia tvarka: vanduo > metanolis > etanolis > acetonitrilas. Analizuojant analičių tirpalus acetonitrilo/vandens mišinyje, jų smailių efektyvumas išlieka nepakitęs pridėjus iki 15% v/v vandens. Metanoliui maksimali neįtakojanti efektyvumo jo koncentracija acetonitrile siekia maždaug 25% v/v, o etanoliui - 40% v/v.

Palyginus skirtingos prigimties (oktadecilsilikagelį, silikagelį ir aminopropilsilikagelį) sorbentus karbadokso ir olakvindokso išskyrimui iš pašarų KDE metodu nustatyta, kad gryniausi ekstraktai gaunami disperguojant mėginius su oktadecilsilikageliu. Efektyviau analitės desorbuojamos poliniais protoniniais tirpikliais (MeOH, EtOH), tuo tarpu ACN yra atrankesnis.

Pilna ir atranki analičių ekstrakcija/desorbcija pasiekiama eliuuojant 8 ml ACN-MeOH mišinio (80:20 v/v, greitis ~2 ml/min).Karbadokso ir olakvindokso nustatymo KDE-HILIC-UESCh metodu charakteristikos palygintos su alternatyvaus skysčių-skysčių ekstrakcijos-ESCh metodo charakteristikomis. Išgavos (89-98 ir 90-97%) bei analizės rezultatų atsikartojamumas (6-10 ir 5-7%) yra panašūs abiem metodams. Pagrindiniai KDE-HILIC-UESCh metodo privalumai: 10 kartų didesnis nustatymo jautris bei maždaug 6-7 kartus greitesnė analizės procedūra.

Palyginus tris sorbentus (C18, C8 ir fenilo) Sudano dažiklių atskyrimui UESCh metodu nustatyta, kad truputį mažiau hidrofobinėse C8 ir fenilo kolonėlėse jie yra sulaikomi silpniau. Geriausiu efektyvumu bei smailių simetriškumu pasižymi C18 sorbentas. Naudojant H_2O/acetonitrilo judrią fazę ir gradientinę eliuciją keturi dažikliai puikiai atskiriami per 2 min, t.y. apie 10 kartų greičiau nei tradiciniu ESCh metodu. Gryniausi Sudanų ekstraktai gaunami disperguojant su poliniais silikagelio ir aminopropilsilikagelio sorbentais. Su abiem sorbentais tirtų tirpiklių ekstrakcinės/desorbcinės savybės stiprėja tokia seka: metanolis ≈ acetonitrilas < etanolis < acetonas < etilacetatas. Tokia pačia seka didėja ir ekstraktų užterštumas pašaliniais junginiais. Pridėjus į ACN nedidelį kiekį protoninio tirpiklio (MeOH), t.y. pakeitus analičių sorbcijos mechanizmą iš adsorbcinio į HILIC, papildomai diferencijuojama skirtingo poliškumo junginių sąveiką su sorbentu: labai hidrofobinių junginių sąveika susilpninama, mažiau hidrofobinių – nesikeičia arba netgi sustiprinama. HILIC principo panaudojimas KDE metode pagreitino hidrofobinių junginių ekstrakciją bei pagerino ekstrakcijos atrankumą. Pilna (išgavos >90%) analičių ekstrakcija/desorbcija pasiekiama su 7 ml ACN/MeOH (9:1, v/v; greitis ~2 ml/min). Įvertinus KDE-UESCh metodo analizines charakteristikas nustatyta, kad visiems dažikliams gaunami ES reikalavimus tenkinantys išgavų (83–106%) ir glaudumo (SSN≤15%) rezultatai. Pagrindinis metodo privalumas lyginant su iki šiol publikuotais metodais – nereikalingas papildomas mėginių valymas po ekstrakcijos, žymiai mažesnė (~6 kartus) analizės trukmė ir paprastesnis mėginio paruošimas.