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On-Line Coupling of a Flow-Through Sensor to a Supercritical Fluid Extractor

M. T. Tena, M. D. Luque de Castro,* and M. Valcárcel

Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, E-14004 Córdoba, Spain

A new approach to detection in supercritical fluid extraction (SFE) by use of a continuous-flow manifold including a flow-through sensor connected to the SFE collector is presented. The coupled system allows continuous derivatization and monitoring of extracted analytes and was used to develop a method for the determination of sulfoquinoxaline in solid foodstuffs. The proposed method features a linear determination range from 10 to 1000 ng of the analyte and an RSD smaller than 5%. It was applied to the analyses of spiked feedstuffs, lyophilized milk, corn, wheat, and oats samples with excellent results in all instances (mean recovery and RSD of 95.3% and 7.5%, respectively).

Preliminary operations remain a pending goal in today's analytical chemistry.[1] Most are difficult or even impossible to automate, particularly for handling solid samples, which are highly diverse and complex. Automatic continuous-flow systems have been conceived essentially for liquid samples,[2,3] so few of them permit direct introduction of solid samples.[4–7]

The nature of supercritical fluid extraction (SFE) makes it an effective tool for significantly facilitating implementation of preliminary operations on solid samples.[8–13] Physical and (bio)-chemical sensors[14,15] also offer very promising prospects for the automation of preliminary operations. Flow-through sensors integrating reaction and/or retention by means of a suitable support packed in the flow cell of a conventional, nondestructive optical detector[16,17] have proved to be powerful tools for enhancing such capital analytical properties as sensitivity and selectivity, as well as for circumventing problems arising from occasional gas bubbling in flow systems.[18]

Sulfonamides are broad-spectrum antibacterials and, as such, are frequently included in feedstuffs as either prophylactics or growth promoters in order to boost animal production. The nature and proportion of any such compounds added to feedstuffs must be certified by the manufacturer, so nominal contents require analytical checking. Withdrawal periods are set to minimize their effects on the human diet (high residual levels may be encountered in meat products unless such periods are adhered to). Sulfonamide residues in food are monitored to ensure that withdrawal periods are observed and that any hazardous effects on consumers are avoided. The tolerance level for these substances in food for human consumption has been set at 0.1 μg/mL. Sulfonamides in food are usually screened by thin-layer chromatography[19] or enzyme immunoassay,[20] positive samples being confirmed by HPLC or GC/MS.[21–23]

A sensor based on integrated retention and photometric detection of the product of the Bratton–Marshall reaction for determination of sulfonamides was previously reported.[24] This reaction has also been used for on-line postcolumn derivatization in HPLC.[25] Flow-through sensor-based methods offer two major advantages over other automated and nonautomated alternatives: enhanced sensitivity resulting from in situ concentration and the fact that no debubbler is needed to remove any N_2 formed in the flowing system.[24] Cross et al.[26] have reported a SFE for sulfonamides from inert matrices and animal meat products. While flow-through sensors have already been coupled to chromatographic[27,28] and nonchromatographic continuous separation systems,[29] no such devices had so far been coupled on-line to a

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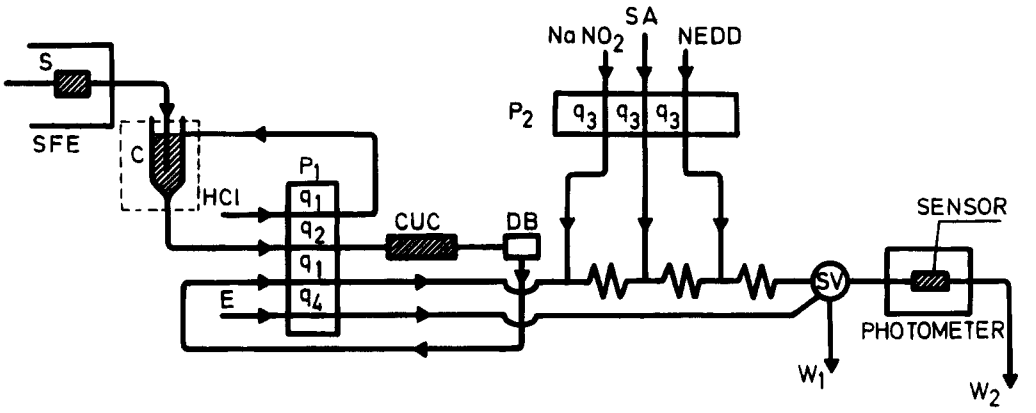

Figure 1. On-line coupling of a flow-through chemical sensor to a supercritical fluid extractor for the determination of sulfonamides in food samples. SFE, supercritical fluid extractor; S, sample; C, collector; *q*, flow rate; P, low-pressure peristaltic pump; CUC, cleanup column; E, eluent; DB, debubbler; R, reactor; SV, switching valve; W, waste; D, detector.

supercritical fluid extractor for continuous monitoring of extracted analytes. SFE was combined with flow injection analysis (FIA) by using a membrane phase separator to remove CO_2 from extracted samples. The SFE–FIA system was employed for the analysis of chloramphenicol and penicillin G.[30]

FOUNDATION

The basis for the proposed methodology is the on-line coupling of a supercritical fluid extractor to a continuous unsegmented flow manifold including a flow-through sensor via the collector unit of the leaching module. The combined assembly affords separation of the analytes by the extractor, cleanup and derivatization by the continuous system, and retention (in situ concentration) of the reaction product and detection by the flow-through sensor.

Figure 1 depicts the composite setup. The analyte is first removed from the solid matrix in the extractor (SFE in the figure) using a supercritical solvent and driven to the collector (C), where it is transferred to an aqueous solution, into which it is selectively dissolved [other extracted sample components are excluded and remain in the collector or are retained in a cleanup column (CUC)]. The analyte is then derivatized along the flow manifold by mixing with suitable reagents. Finally, the resulting reaction product is retained on the support packed in the flow cell, which has an in situ concentration effect that results in appreciably enhanced sensitivity.

Concentration of the monitored product over a small area in the flow-through sensor is the key to proper performance, since coupling the extractor to a conventional flow manifold lowers the sensitivity through dilution of the analyte during extraction; in conventional flow systems, dilution increases with increasing retention of the analyte by the sample matrix. The kinetics of the extraction process in the proposed approach are reflected only as a change in the rising slope of the analytical signal, which reaches a plateau at a variable time dependent on the rate of analyte feeding to the acceptor carrier. This phenomenon is apparent from Figure 2, which shows the analytical signal provided by the same amount of sulfoquinoxaline in a standard solution added to the collector (A), to a feedstuff (B), and to a milk sample (C).

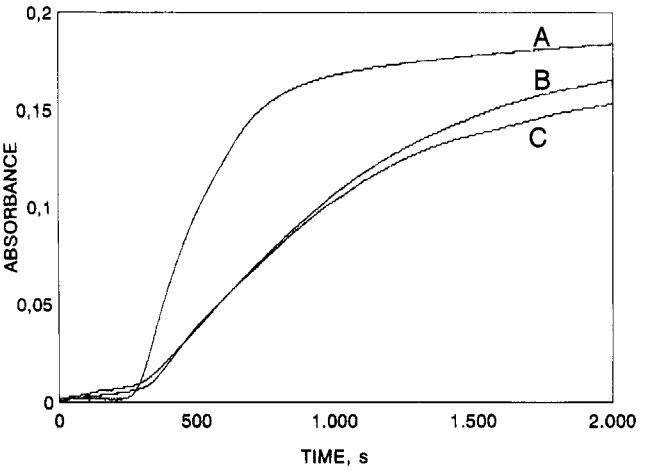

Figure 2. Analytical signals obtained for 200 ng of SQX added to the collector solution (A) and added before SFE to 1 g of feedstuffs (B) and for 0.5 g of lyophilized milk (C).

EXPERIMENTAL SECTION

Instruments and Apparatus. A Fisons supercritical fluid extractor consisting of an SFC 300 double syringe pump, an SFE 30 analytical extraction unit furnished with a single 3-mL extraction cell, an SFE 30 collector unit, an SFE 300 control system, a Haake K20 cooling circulator (ethanol-filled), and a VR100 variable restrictor from CCS Instrument Systems was used. The flow manifold was built from two Gilson Minipuls-3 low-pressure peristaltic pumps, a Rheodyne 5041 low-pressure injection valve acting as a switching valve, and a Unicam 8625 UV–visible spectrophotometer furnished with a laboratory-made glass flow cell of 4 cm × 1.5 mm i.d. (MSI, Emeryville, CA) described elsewhere,[24] both of which were connected to a PC computer and a Knauer recorder. An air thermostat was used to avoid freezing of the collector solution. A Hetosicc CD53-1 laboratory lyophilizer was used to prepare the milk samples. The collector was laboratory made from a Teflon test tube (9.5 cm length, 1.5 cm i.d.), fitted with an outlet tube at the bottom and an inlet 1 cm above the outlet.

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Waste from the flow system was gathered and collected by the safety unit of our research center. The extractor collector was placed under a fume hood essentially to avoid exposure to methanol vapor. Liquid CO_2 containers were handled and their pressure was controlled as per the supplier's instructions.

Materials. Aqueous solutions containing 0.1 mol/L HCl (Probus, Spain), 0.2 g/L $NaNO_2$ (Merck), 10 g/L sulfamic acid (SA, Sigma Chemical Co.), 0.01 g/L *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDD, Merck), and 40:60 ethanol–water were used. A stock standard solution of sodium sulfoquinoxaline (SQX, Guinama, Spain), containing 1 g/L SQX in HPLC-grade methanol (Romil Chemicals), was used in the preparation of more dilute solutions by addition of methanol. The stock solutions were stored at −4 °C in the dark. C_{18} bonded silica of 60–100 μm from Sep-Pak cartridges supplied by Waters was used as solid support for packing the flow cell. Silica and C_{18} Sep-Pak cartridges from Waters were used for on-line cleanup of extracts.

Liquid CO_2 (99.998%) supplied by SEO (Spain) in a deep-tube cylinder was used as the extraction fluid and HPLC-grade methanol (Romil Chemicals) as the SC-CO_2 modifier.

Diatomaceous earth from Sigma and milk, feedstuff, wheat, corn, and oat samples purchased at a local supermarket were also used.

Sample Treatment. The milk samples were lyophilized before extraction. They lost 87.82% of their weight (0.44% RSD) as a result; hence, the preconcentration factor was 8.2. The corn, wheat, and oat samples were ground and dried at 120 °C. The feedstuff was ground and homogenized.

A volume of 100 μL of a methanol solution of SQX was added to the samples in the flow cell prior to their SFE.

Procedure. The supercritical fluid (CO_2–10% methanol at 30 MPa and 70 °C) was passed at a flow rate of 1.0 mL/min through the 3-mL extraction cell holding the solid sample (0.5–1.0 g) to extract the analyte. The extracted SQX was driven to the restrictor, where the CO_2 expanded, and the analyte was transferred to the continuously renewed solution (0.1 M HCl) in the collector. This carrier solution was passed through a silica cleanup column (2 cm length, × 1 cm i.d.) that retained insoluble extracted compounds so as to avoid turbidity in the circulating stream.

If the restrictor was plugged owing to a high load of extractable materials (e.g., fat), the flow rate of the supercritical fluid was increased in order to flush the restrictor and then reset to the appropriate, preset flow rate.

A debubbler was used to remove CO_2 prior to derivatization. The main channel was then merged with a 0.2 g/L $NaNO_2$ stream to effect diazotization along reactor R_1. Excess nitrous acid was decomposed by reaction with sulfamic acid along R_2. The dye (monitored product) was formed along reactor R_3 by coupling NEDD and the diazotized sulfoquinoxaline channel after merging with the R_2 NEDD stream. On reaching the detector, the reaction product was retained on the C_{18} bonded silica packed in the flow cell and monitored spectrophotometrically at 540 nm. After the recording was obtained, valve SV was switched to elute the reaction product from the support, which was thus made ready for a fresh sample. The cleanup column was flushed with 10 mL of methanol and 10 mL of carrier between samples. Some samples required removing built-up fat from the collector walls.

Table 1. Optimization of Variables

variable	range studied	optimum value
chemical		
concn $NaNO_2$, g/L	0.01–2	0.2
concn HCl, mol/L	0–1	0.1
concn SA, g/L	2–20	10
concn NEDD, g/L	0.01–2	0.01
ethanol (eluent), %	5–50	40
temperature, °C	20–60	RT[a]
hydrodynamic system		
q_1, mL/min	0.41–1.64	0.82
q_3, mL/min	0.06–0.45	0.32
length of R_1, cm	100–300	100
length of R_2, cm	50–200	50
length of R_3, cm	50–350	50
vol in collector, mL	1–5	1
SFE		
pressure, MPa	10–30	30
temperature, °C	40–100	70
flow rate, mL/min	0.5–2.0	1.0
methanol, %	0–10	10

[a] Room temperature.

RESULTS AND DISCUSSION

Optimization of Variables. Experimental variables were optimized both to concentrate the reaction product on the the flow cell packing and to maximize extraction in a short time. The univariate method was used for this purpose. The ranges over which the effect of variables was investigated and the optimum values of the variables are given in Table 1.

The influence of some variables was studied in previous work.[24] Hydrodynamic variables were initially investigated by adding the analyte solution to the collector and performing no extraction. SFE variables were studied by extracting the analyte added to an inert support filling the extraction cell. Finally, the influence of the methanol in the supercritical mixture on the analytical signal and the need for on-line cleanup of the carrier stream to avoid interferences from the sample components extracted with the analyte were also studied and optimized.

(a) SFE Variables. Increasing pressures and decreasing temperatures resulted in increased recovery throughout the ranges studied. The highest recovery achieved with pure SC-CO_2 (53%) was obtained at 30 MPa and 40 °C. The extraction efficiency was increased by using a polar modifier such as methanol mixed with the CO_2. Quantitative extraction (102%) was achieved in 30 min using as the extraction fluid CO_2–10% methanol at 30 MPa and 70 °C. This higher operating temperature was imposed by the critical temperature for the mixture (ca. 65 °C).

(b) Chemical and Flow-Through Variables. The influence of chemical variables and the features of the sensing device were previously established.[24]

The longer measurement times (ca. 30 min) used in this systems relative to previously reported coupled configurations[24] resulted in a higher blank signal and a gradual baseline rise. Decreasing NEED concentrations yielded a sustained decrease in baseline drift, so this variable must be reoptimized sustained. The blank signal was reduced by a factor of 7 by diluting the NEDD stream 10-fold (from 0.1 to 0.01 g/L), with no effect on

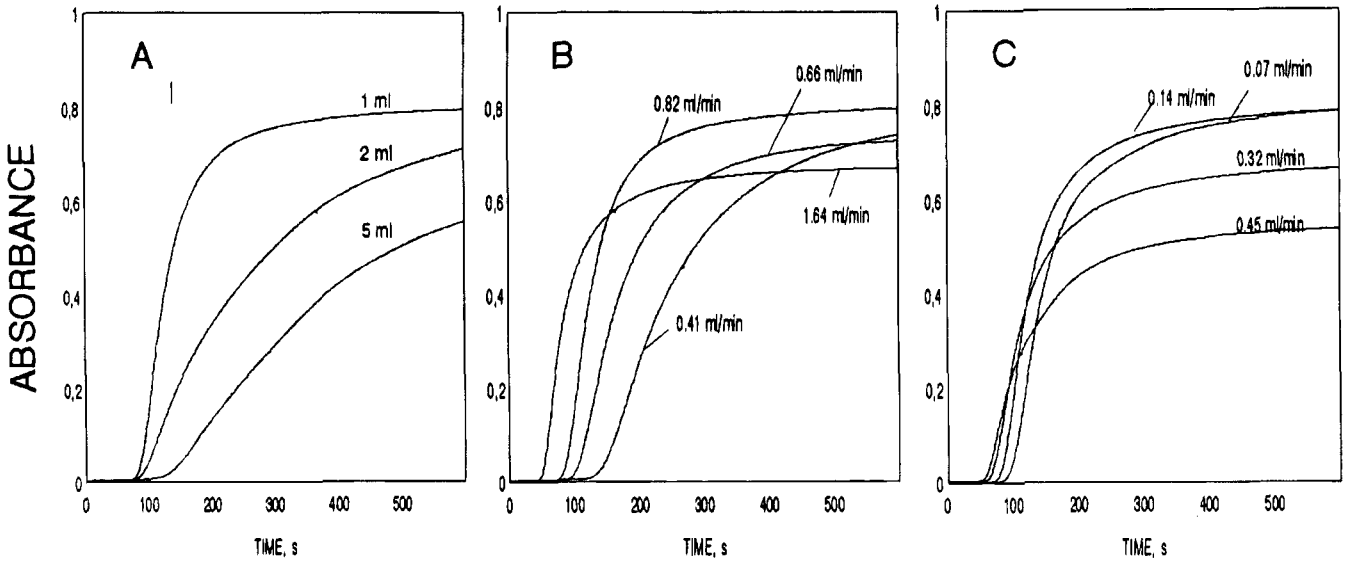

Figure 3. Influence of hydrodynamic variables on the analytical signal. (A) Volume of collecting solution (carrier). (B) Carrier flow rate. (C) Flow rates of the derivatization reagents.

the analytical signal. This is consistent with the variation pattern observed in previous work,[24] as the analyte was more diluted in the present flow system.

(c) Dynamic Variables. Taking into account the gas volume contained in the stream transferred from the collector to the debubbler, a flow rate (q_2) of 1.3 mL/min was set in order to equalize the initial solution level in the collector and debubbler. The flow rate of the eluent stream (q_4) had no effect on the signal, as elution of the monitored product from the support packed in the flow cell was quite efficient.

The dynamic variables clearly influencing the analytical signal were the volume of collecting solution, the carrier flow rate (q_1), and the flow rates of the reagent streams (q_3) (Figure 3). Decreasing the volume of collecting solution (carrier) in the collector increased the absorbance at a fixed time and the slope of the linear portion of the signal and shortened both the residence time (that required for absorbance to start rising) and the time needed for maximum absorbance to be reached (analysis time), as shown in Figure 3A. Increasing carrier flow rates (q_1) up to 0.82 mL/min resulted in increasing maximum absorbances; the opposite held true at greater flow rates. The slope of the linear portion of the rising signal increased and both the residence time and the analysis time decreased with increasing carrier flow rate. The influence of the flow rates of the reagent streams (q_3) on the analytical signal is shown in Figure 3C (increased reagent flow rates increased the slope, to the detriment of the maximum absorbance). Decreasing reagent flow rates down to 0.14 mL/min resulted in increasing maximum absorbances.

In a previous work,[24] the reactions were found to be fast enough at the working pH to make unnecessary the use of long reaction coils.

(d) Influence of Methanol. The presence of methanol was found to affect both the extraction and the retention processes. Using methanol as modifier for the CO_2 increased the extraction efficiency but decreased retention of the monitored product on the C_{18} bonded silica. The overall effect of methanol on the analytical signal is shown in Figure 4. The analytical signal for a standard solution added to the collector was decreased by CO_2 expansion in the collector and saturation of the carrier solution with this gas (signals A and B); however, the effect was more marked when CO_2 containing 10% methanol was used (signal E). The effect of methanol on the extraction efficiency is apparent from the signal obtained after the analyte was extracted with that for a standard added to the collector under identical working conditions (Figure 4): signals C and B, respectively, for pure CO_2 (recovery ~50%) and signals D and E, respectively, for 10% methanol–CO_2 (recovery ~100%).

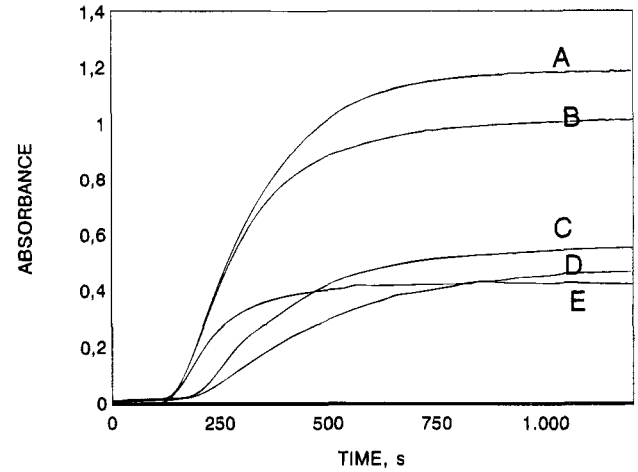

Figure 4. Influence of the methanol concentration on SFE and retention efficiency. Analytical signals obtained for 1 μg of SQX added to the collector solution without (A) and with CO_2 (B), or CO_2–10% methanol (E) bubbling and by on-line SFE of 1 μg of SQX in diatomaceous earth with pure CO_2 (C) and CO_2–10% methanol (D).

Although retention of the reaction product on the support was favored by dilution of the methanol in the stream reaching the detector, merging a water stream with R_3 did not improve on the analytical signal but rather resulted in higher dilution. As the final content of methanol in the solution reaching the flow cell could also be controlled via the reagent flow rates, the influence of this variable was also studied. Increasing the reagent flow rates from 0.06 to 0.32 mL/min resulted in increasing signals that remained constant above this value, which was thus chosen as optimum for further experiments.

Table 2. Features of the Proposed Method

type of measurement	linear range, ng	equation[a]	regression coefficient	RSD,[b] %
maximum absorbance	10–1000	$Y = 2.39 \times 10^{-2} + 7.78 \times 10^{-4}m$	0.999 49	4.1
peak area	10–1000	$Y = 3 \times 57 + 0.15m$	0.998 88	4.5
slope	10–1000	$Y = 1.04 \times 10^{-4} + 4.29 \times 10^{-6}m$	0.995 77	6.2

[a] Y is the maximum absorbance, peak area, or slope; m is the mass (in ng) of SQX. [b] Based on 200 ng of SQX, $n = 11$.

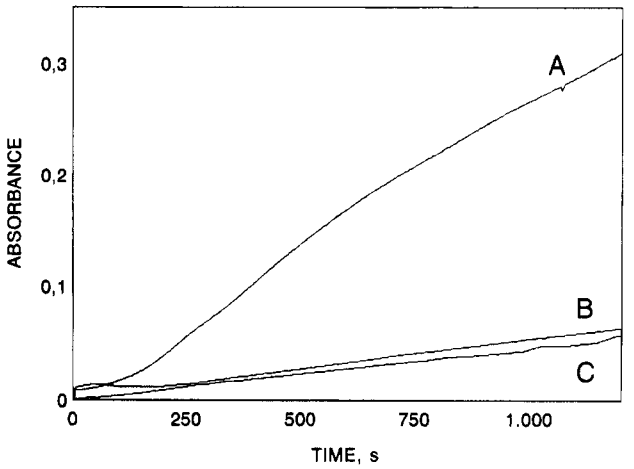

Figure 5. Analytical signals obtained for an SF-extracted blank of a lyophilized milk sample (0 ng of SQX) with (B) and without (A) on-line cleanup. (C) Signal baseline.

(e) On-Line Cleanup. Extraction of insoluble components introduced turbidity in the carrier solution, so it interfered with measurements or even clogged the flow system if a large enough amount of interferents was retained on the flow cell support. The on-line cleanup of the flowing solution was accomplished by passing the solution through a column located after the collector, where undesirable compounds were retained.

Two different materials (C_{18} and silica) and various column dimensions (1 cm × 0.3 cm; 1 cm × 1 cm; and 2 cm × 1 cm (length × i.d.)) were assayed. A 2 cm × 1 cm silica column was found to be the most suitable; in fact, it allowed efficient cleanup of the extract with no alteration of the analytical signal, appearance of which was delayed or even suppressed by C_{18} columns. Figure 5 shows the effect of the column on the blank signal for a milk sample.

Features of the Method. The calibration graph was run by using 100-μL samples of solutions containing different amounts of SQX in methanol, added to the collector solution under the optimum working conditions shown in Table 1. Three calibration curves were obtained by using the maximum absorbance, slope, and peak area as the measurement parameters. All three were linear over the range 10–1000 ng of SQX. The equation for the linear segment obtained, the linear concentration range, the regression coefficient, and the precision (expressed as the relative standard deviation, RSD) are listed in Table 2. The limit of quantitation was 10 ng, and the total analysis time was 35 min. The best results in terms of linearity and precision were obtained by using maximum absorbance measurements. In addition, this was the only type of measurement that allowed quantitation of SQX in the samples since area and slope values were dependent on the kinetics of extraction (see recordings in Figure 2).

Table 3. Recovery from Spiked Samples

sample	spiked concn, μg/g	recovery, %	RSD, %
milk	0.1218	89.6	6.8
	0.0487	82.4	10
feedstuffs	0.5	87.0	9.7
	0.2	102.0	3.0
corn	0.5	91.8	2.3
	0.2	97.7	13
wheat	0.5	100.4	2.9
	0.2	100.9	13
oats	0.5	97.7	1.4
	0.2	103.3	12
mean value		95.3	7.5

The performance of the coupled SFE/sensor system was tested by extracting 500 ng of SQX added to 0.5 g of diatomaceous earth under the optimum working conditions. The recovery thus obtained was 82.8%, with an RSD of 13% for $n = 5$.

Applications. The performance of the proposed method was tested by applying it to the analyses of various samples (lyophilized milk, feedstuff, corn, wheat, and oats) that were spiked with the analyte, as no real samples containing the analyte were available. Unspiked samples provided signals that were identical with the baseline signal. As can be seen from Figure 2, the analyte exhibited rather a different behavior depending on the type of matrix concerned. Analyses were performed in triplicate and provided excellent results. Table 3 shows the added concentration, mean recovery, and RSD ($n = 3$) for each sample. Recoveries ranged from 82.4 to 103.3%, with an average of 95.3% and a mean RSD of 7.5%. The fact that extraction from diatomaceous earth was less efficient than that from grain samples could be due to the affinity of diatomaceous earth for methanol that reduced its proportion in the extracting fluid. Therefore, the supercritical fluid–modifier mixture contained less polar agent and hence extracted less analyte.

CONCLUSIONS

The proposed method is a new approach to automated analyses of solid samples by on-line coupling of a supercritical fluid extractor to a photometric flow-through sensor integrating retention and detection. This combined system allows automation of analyses of solid samples and overcomes the typical slowness, tediousness, and intensive human involvement in conventional methodologies.

Application of the proposed system could be extended from sulfoquinoxaline to any other sulfonamide mixture of members of this compound family. The proposed approach surpasses conventional methodologies (e.g., solid–liquid extraction) in

selectivity because (a) the SFE is performed under optimal conditions for extraction of the analyte, thereby substantially lessening the typical interferences with this procedure, and (b) the cleanup column immediately behind the collector retains extracted interferents (e.g., fat in milk).

This work opens up interesting prospects for SFE in routine sample screening at regulatory laboratories as well as basic studies in this field.

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