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Automated On-Line Renewable Solid-Phase Extraction-Liquid Chromatography Exploiting Multisyringe Flow Injection-Bead Injection Lab-on-Valve Analysis

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In this paper, the third generation of flow injection analysis, also named the lab-on-valve (LOV) approach, is proposed for the first time as a front end to high-performance liquid chromatography (HPLC) for on-line solid-phase extraction (SPE) sample processing by exploiting the bead injection (BI) concept. The proposed microanalytical system based on discontinuous programmable flow features automated packing (and withdrawal after single use) of a small amount of sorbent (<5 mg) into the microconduits of the flow network and quantitative elution of sorbed species into a narrow band (150 μL of 95% MeOH). The hyphenation of multisyringe flow injection analysis (MSFIA) with BI-LOV prior to HPLC analysis is utilized for on-line postextraction treatment to ensure chemical compatibility between the eluate medium and the initial HPLC gradient conditions. This circumvents the band-broadening effect commonly observed in conventional on-line SPE-based sample processors due to the low eluting strength of the mobile phase. The potential of the novel MSFI-BI-LOV hyphenation for on-line handling of complex environmental and biological samples prior to reversed-phase chromatographic separations was assessed for the expeditious determination of five acidic pharmaceutical residues (viz., ketoprofen, naproxen, bezafibrate, diclofenac, and ibuprofen) and one metabolite (viz., salicylic acid) in surface water, urban wastewater, and urine. To this end, the copolymeric divinylbenzene-*co*-n-vinylpyrrolidone beads (Oasis HLB) were utilized as renewable sorptive entities in the micromachined unit. The automated analytical method features relative recovery percentages of >88%, limits of detection within the range 0.02−0.67 ng mL^{-1}, and coefficients of variation <11% for the column renewable mode and gives rise to a drastic reduction in operation costs (~25-fold) as compared to on-line column switching systems.

The determination of organic contaminants in environmental and biological samples using chromatographic techniques often requires appropriate sample processing aimed at removing interfering sample constituents and improving analyte detectability by application of preconcentration schemes. Among the various techniques available for pretreatment of liquid samples, solid-phase extraction (SPE) has become the standard procedure, replacing almost completely liquid−liquid extraction due to its well-known shortcomings.[1] Furthermore, this technique allows straightforward automation and can be coupled on-line to liquid chromatographic (LC) separations.[1,2] This hyphenation leads to an improved precision in chromatographic analyses, as a result of the minimization of manual handling (e.g., transfer, measuring, or evaporation steps), high sample throughput, and minimal generation of organic solvent wastes, thereby decreasing costs for waste disposal.

In practice, the most commonly used approach for on-line coupling of SPE with LC, termed "column switching", involves the implementation of a small precolumn within the injection loop of a six-port rotary valve.[1,2] While the LC separation and analysis of a given sample takes place, the ensuing sample is concurrently loaded on this precolumn by means of a second high-pressure pump, whereupon the valve is switched in order to strip the analytes out of the sorbent by the LC mobile phase and transfer them into the analytical column.[1−7] A second alternative consists of placing the SPE column outside the six-port valve. Under this configuration, sometimes referred to as "heart-cut", the retained analytes are desorbed with a discrete volume of solvent and further

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transferred to the injection loop.[2,8] The major disadvantages of both approaches for on-line SPE-LC coupling are the progressive deterioration of the reusable precolumn material and the risk of sample cross-contamination when highly polluted samples or biological fluids are to be analyzed. As a consequence of the current demands for setting robust and reliable chromatographic methods, cartridge exchangeable modules for on-line SPE are now commercially available. In this context, the various generations of sample processors for single-use sorbent columns from Spark Holland, namely, Prospekt, Prospekt-2, and Symbiosis (Emmen, The Netherlands) and OSP-2 from Merck (Darmstadt, Germany) are particularly worth mentioning.[9−13]

Straightforward and cost-effective alternatives to the aforementioned robotic systems and high-pressure-based SPE modules involve the exploitation of the various generations of flow analysis, i.e., flow injection analysis[14] and sequential injection analysis,[15] that foster the performance of cleaning up and preconcentration schemes in the low-pressure mode with no concern of the ensuing measurement step. Further progress in miniaturization of flowing stream systems for downscaling reagent-based assays and simplification of sample pretreatments launched the development of the third generation of flow injection, the so-called laboratory-on-valve (lab-on-valve, LOV).[16,17] The miniaturized module offers facilities to allow any kind of chemical processes, including among others the microfluidic and accurate handling of sorptive beads into the integrated microchannels. In fact, the use of renewable (i.e., disposable) bead materials in a fully automated fashion, the so-called bead injection (BI) analysis,[18] should be regarded as a promising avenue for overcoming the drawbacks of reusable sorbent precolumns coupled to HPLC as concerns to analyte carryover, column clogging, malfunctions of the active entities, and bounding of nonspecific and irreversible matrix interfering compounds, which deteriorate the lifetime of the extraction column.

The marriage between the microfabricated sample processing LOV unit and modern analytical instrumentation, such as capillary electrophoresis,[19,20] electrothermal atomic absorption spectrometry,[16,21,22] cold-vapor atomic spectrometry,[23] electrospray mass spectrometry,[24,25] and inductively coupled plasma-mass spectrometry,[16] has been recently utilized for downscaling a suite of analytical assays; yet, to the best of our knowledge the integrated LOV manifold has not been exploited so far as a front end to high-performance liquid chromatography. In addition, it should be noted that the BI-LOV system has been exclusively linked to date to sequential injection analysis, which lacks flexibility for on-line manipulation of eluate plugs after sample treatment into the miniaturized unit. As to on-column extraction schemes for organic analytes, postcolumn analyte chemical derivatization or eluate remixing to prevent the broadening of the injection band might be mandatory prior to HPLC separation.[26] This can be efficiently effected by the exploitation of the recently designed multisyringe flow injection analysis (MSFIA) approach[27] that should be viewed as an FI-SI hybrid technique.

The aim of this work is thus to explore the potential capabilities and analytical performance of the new MSFIA-BI-LOV hyphenation for direct on-line coupling of SPE in a renewable fashion with LC for determination of trace level concentrations of organic analytes in environmental (surface and sewage water) and biological (urine) samples. Acidic pharmaceutical residues, including nonsteroidal antiinflammatory drugs (NSAIDs) and lipid regulators, were selected as target analytes because of their current environmental[28,29] and clinical[13,30] relevance.

MATERIALS AND METHODS

Chemicals and Solutions. Pharmaceuticals (naproxen, ketoprofen, bezafibrate, ibuprofen, diclofenac), salicylic acid, and the internal standard (fenoprop, IS) were obtained from Sigma-Aldrich (Milwaukee, WI). Stock solutions of 2 mg mL^{-1} were prepared in methanol, stored in the darkness at 4 °C, and stepwise diluted to the desired concentration with ultrapure water.

Ultrapure water was obtained using a Milli-Q ultrapure water system (Millipore, Bedford, MA). HPLC grade methanol was supplied by Sigma-Aldrich, and formic acid was purchased from Scharlab (Barcelona, Spain).

The sorbent bead material used for preconcentrating the acidic drugs was the *N*-vinylpyrrolidone−divinylbenzene copolymer (Oasis HLB; averaged dry bead size, 30 μm; specific surface area, 800 $m^2\ g^{-1}$; total pore volume, 1.4 $cm^3\ g^{-1}$) purchased from Waters (Mildford, MA). Working bead suspensions were made by moistening 200 mg of the Oasis material with 2 mL of eluent (namely, 95% (v/v) MeOH/water).

Liquid Chromatography. Liquid chromatographic analyses were performed with an analytical setup comprising a Waters 600 high-pressure gradient pump, flow controller, column thermostat, and a Waters 2996 photodiode array detector. LC Eluents were degassed by means of a helium (99.999%) flow at 30 mL min^{-1}. Manual injections were conducted with a Rheodyne high-pressure six-port rotary valve equipped with a 100-μL loop. This valve was also exploited as interface between the flow system and analytical column, as described below.

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Table 1. Target Analytes and Relevant Parameters for LC Separation/Determination

analyte	compound type	retention time (min)	analytical wavelength (nm)
salicylic acid	NSAID metabolite	4.2	300
ketoprofen	NSAID	6.3	256
naproxen	NSAID	7.6	230
bezafibrate	lipid reducing agent	9.1	250
fenoprop	internal standard	12.8	290
ibuprofen	NSAID	13.6	230
diclofenac	NSAID	14.7	275

Reversed-phase chromatographic separation was realized using an Xterra RP-18 (3.9 × 150 mm, 5 μm) analytical column (Waters), preceded by a guard column (3.9 × 10 mm, 5 μm) of the same packing material, at a flow rate of 1 mL min^{-1} and column temperature of 30 °C. Eluent A consisted of MeOH/water (20/80, v/v) and eluent B of MeOH/water (95/5, v/v), both containing 0.1% (v/v) formic acid. The gradient, involving several linear steps, was as follows: 0 min 50% B, 2 min 50% B, 5 min 55% B, 14 min 60% B, and 15 min 50% B. The UV spectra of the various compounds and IS were recorded within the 200−350-nm range and quantitation was done at the analytical wavelengths listed in Table 1 using peak area measurements. Positive identification of analytes in the samples was done by comparison of both retention times and spectra obtained with pure standards. Running of the LC-gradient sequence, chromatogram recording, and data processing were performed automatically by a PC operated under the Empore software (Waters).

Multisyringe-Flow Injection Lab-on-Valve System. A multisyringe piston pump with programmable speed (MicroBu 2030, Crison Instruments, Alella, Barcelona, Spain) was used as a liquid driver for the various on-line SPE operations. It was equipped with three high-precision bidirectional syringes (Hamilton), labeled as S1, S2, and S3, and connected in block to the same stepper motor. S2 with a capacity of 5.0 mL contained the carrier (10^{-2} M HCl), and S3 and S1 with a capacity of 2.5 mL were used for rinsing the sampling tube between samples and postcolumn remixing of the organic eluate with Milli-Q water prior to the LC separation, respectively. A three-way solenoid valve (SV1−SV3, N-Research, Caldwell, NJ) was placed at the head of each syringe, enabling automatic connection with the liquid reservoir (OFF) or flowing system (ON). The multisyringe module was coupled to the integrated LOV sample-processing unit mounted atop a six-port multiposition selection valve (MPV, Valco Instruments, Houston, TX). A schematic illustration of the analytical flow setup is depicted in Figure 1. The LOV microbore assembly encompasses a central port that can communicate with the other six microchannels through the central communication conduit (CC) in the MPV, as reported elsewhere.[22] This CC is connected to S2 for sequential aspiration of the various components of the SPE process (fluids, such as sample and eluent, slurries, i.e., bead suspension, and gases, such as air) from individual ports and placing them into the holding coil (HC). The microchannels connecting S2 with CC and that of port 3 serve as containers for bead microcolumns C_1 and C_2 in which $^1/_{16}$-in. PEEK stoppers (Upchurch Scientific, Oak Harbor, WA) are loosely inserted.[22] The bead container and eluent reservoir were attached to other peripheral ports of the LOV (ports 5 and 6, respectively), while the remaining two single ports were used for bead disposal (port 2) and discrete aspiration of air plugs (port 4), respectively. The specially designed dual channel (port 1) is utilized for sample introduction into the flow network, the outgoing channel being connected to S3, thereby permitting thorough washing of the conduits between samples of different concentration with no risks of analyte carryover. The outlet of microcolumn C_2 and the delivery line of S1 are joined via a poly(methyl methacrylate) T-piece. The flow assembly was built from PTFE tubing of 0.8-mm i.d., except for the 283-cm-long HC, which was made from PTFE tubing of 1.50-mm i.d., corresponding to a volume of 5.0 mL, and the manifold line interfacing the LOV with the injection valve of the LC, which consists of a 0.5-mm-i.d. tube with a total length of 46 cm. The HPLC injection valve was furnished with a 300-μL loop made from PEEK tubing of 0.75-mm i.d. (VICI, Valco Instruments). One-piece hexagonal-headed PEEK fittings (VICI) were used for connecting the injection loop and delivery line to the MSFI−LC interface.

The operational procedures of the MSFIA-BI-LOV system were computer controlled by the software package AutoAnalysis 5.0 (Sciware, Palma de Mallorca, Spain) based on dynamic link libraries (DLLs). The major asset of the dedicated software is the implementation of specific and individual DLLs attending the configuration of the assembled flow analyzer. In our particular arrangement, the principal protocol was loaded with the DLLs designed for the automatic control of the multisyringe device and multiposition valve.

Samples. Influent and tertiary effluent grab samples of a municipal wastewater treatment plant (WWTP) were collected in September 2005. This WWTP is located in the main city of Mallorca and receives urban sewage from 400 000 equivalent inhabitants, also being fed by wastewaters from several hospitals. Surface water samples were collected from a creek near the coast, thus containing an important contribution from seawater. Urine samples were collected from healthy volunteers and analyzed without delay. All samples were filtered through 0.22-μm cellulose esters membrane filters (MF-Millipore) after collection and adjusted to pH 2 with 0.2 M HCl prior to analysis.

Analytical Procedure. The complete operational sequence for on-column SPE treatment of environmental and biological samples exploiting the MSFI-BI-LOV and further LC separation of the target acidic drugs is summarized in Table 2, where details of the multisyringe buret and LOV positions are given, along with the corresponding consumption of sample and reagents. The overall sample processing cycle runs through four steps, namely, system preconditioning and column packing, sample loading, analyte elution and transportation into the MSFI−LC interface, and bead withdrawal. Prior to analysis, the sorbent suspension is aspirated into a 1.0-mL syringe, which is mounted vertically on port 5 of the LOV structure. The tip of the syringe is furnished with a short PEEK tubing piece of 0.76-mm i.d. (Upchurch), which via a gasket fits into the LOV channel. Operation is not initiated until the Oasis beads have settled on the bottom of the container.

The operational details of each single step are detailed in the following:

(1) System Preconditioning and LOV Microcolumn Packing. First, S2 is set to aspirate consecutively 1500 μL of carrier from the external reservoir and a discrete eluent segment (300 μL) from port 6. Thereafter, the solvent plug and 400 μL of carrier

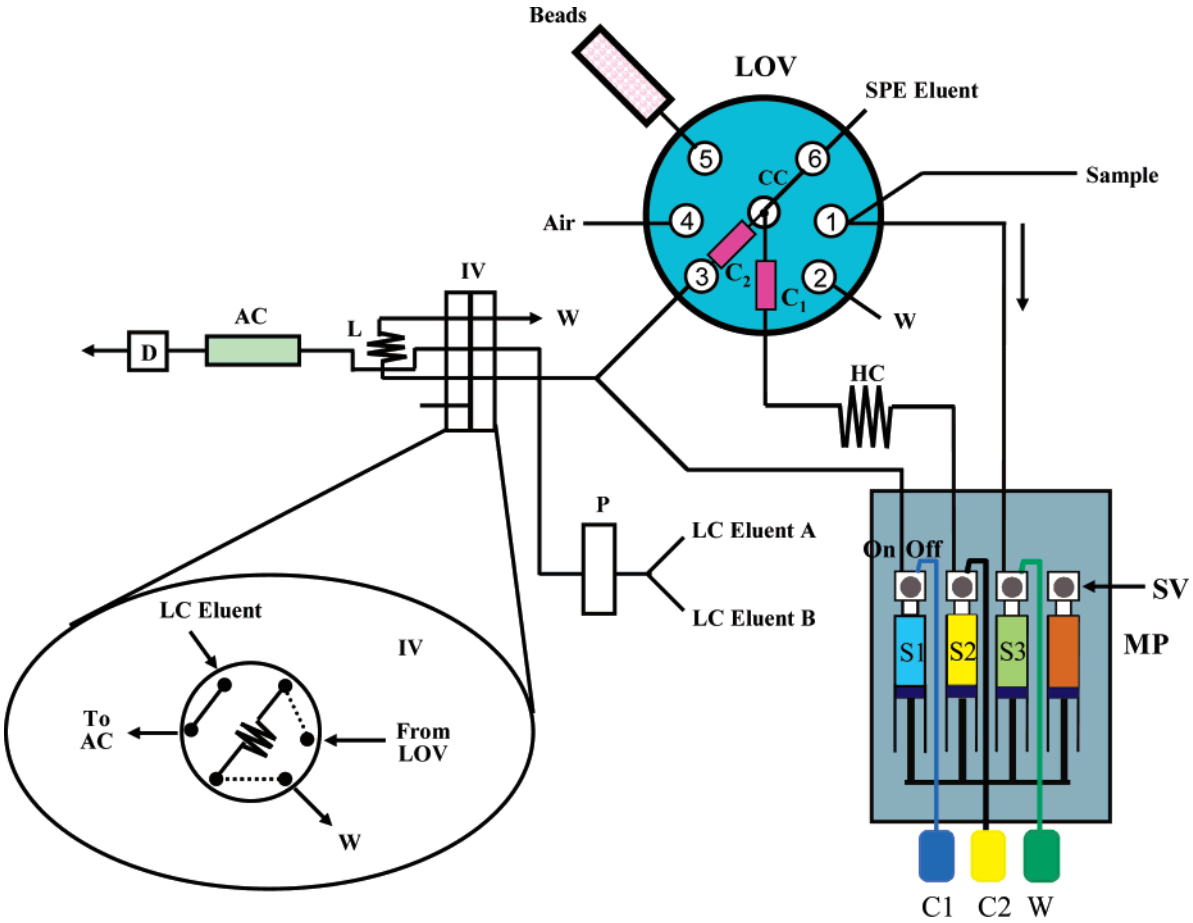

Figure 1. Schematic diagram of the MSFI-BI-LOV manifold hyphenated with liquid chromatography for the determination of trace level concentrations of acidic drugs. LOV, lab-on-valve; MP, multisyringe pump; S, syringe pump; SV, solenoid valve; HC, holding coil; CC, communication channel; C1 and C2, cavities for beads; P, HPLC pump; IV, injection valve; L, loop; AC, analytical column; D, detector; W, waste; C1, carrier (Ultrapure Milli-Q water) C2, carrier (10^{-2} M HCl); LC-eluent A, 80/20 (v/v) MeOH/H_2O + 0.1% (v/v) formic acid; LC-eluent B, 95/5 (v/v) MeOH/H_2O + 0.1% (v/v) formic acid. (The injection valve is illustrated in the loading position).

are delivered, via reversal motion of the multisyringe pump, to port 3 for rinsing the C_2 microcolumn position and the connecting line to the LC. A metered volume of copolymeric beads is next aspirated slowly (viz., 0.5 mL min^{-1}) into C_1 and transferred to C_2 cavity by 150 μL of eluent plus 150 μL of carrier as described in Table 2.

(2) Sample Loading. On changing the sample, S3 is set to aspirate 250 μL of sample solution past the flow-through port 1. Then, S2 is programmed to aspirate consecutively 100 μL of air and either 1 (urine) or 4.5 mL (water) of sample into the HC. The CC is then directed to port 3 to effect the preconcentration of the target analytes on the LOV Oasis microcolumn. As to environmental assays, the latter operational steps are repeated three more times for attaining suitable enrichment factors. The removal of nonretained matrix species from the sorbent material is carried out by rinsing the microcolumn with 1000 μL of carrier solution.

(3) Elution Step. To prevent dispersion of the minute, well-defined organic eluent plug into the carrier solution, which, in turn, would decrease its eluting strength, an air segment is loaded into HC prior to the aspiration of the 150-μL eluent zone. The eluent zone is divided in three parts, each being halted into C2 for effective stripping out of the acidic drugs. S1 is simultaneously activated aimed at mixing the eluate with water before entering the analytical column. The resulting analyte-containing plug is delivered downstream with carrier solution and collected into the injection loop of the rotary valve, whereupon the valve is switched to the injection position and the LC gradient protocol is initiated. Therefore, the LC separation is synchronized with the MSFI-BI-LOV procedure, implying that a sample is analyzed while the ensuing one is being processed in the flow system. Total sample preparation time was ~20 min, thus matching the time frame for both the chromatographic run and column reequilibration to the initial conditions, which amounted to 19 min.

(4) Bead Discarding. The packed column with Oasis beads is facilely removed from LOV microconduits after being moistened with eluent and delivered to waste (port 2) with carrier solution. Hence, the system is ready to initiate a new analysis cycle with a fresh portion of beads, thus eliminating any possibility of carryover between consecutive runs.

RESULTS AND DISCUSSION

Features of MSFIA-BI-LOV as a Front End to HPLC for Organic Analytes. The use of suspended beads as sorbents or microcarriers of immobilized reagents into the LOV microconduits has been restricted so far to inorganic trace element analysis, bioligand interactions, or enzymatic assays. Hence, hydrophilic sorbent materials of Sephadex or Sepharose type with ion-

Table 2. Operating Procedure of the MSFI-BI-LOV System Hyphenated with LC for On-Line Determination of Acidic Drugs in Environmental Waters

sequence and description	S1	S2	S3	HPLC rotary valve (position)	LOV (position)	flow rate (mL min^{-1})	vol (μL)
1. system preconditioning and bead loading							
(a) filling of syringe with carrier	off/aspirate	off/aspirate	off/aspirate	load	–[a]	5 (S1 and, S3) 10 (S2)	750 (S1 and S3) 1500 (S2)
(b) introduction of eluent into HC	–	on/aspirate	–	–	6	0.7	300
(c) rinsing of manifold lines and C2 cavity	–	on/dispense	–	–	3	1.0	700
(d) aspiration of eluent into HC	–	on/aspirate	–	–	6	0.7	150
(e) collection of beads into C_1	–	on/aspirate	–	–	5	0.5	30
(f) transferring beads into C_2, cleansing with eluent and Milli-Q water	–	on/dispense	–	–	3	1.2	330
2. sample loading							
(a) washing of sampling tubing	–	off/aspirate	on/aspirate	–	–	1.5 (S3)	250 (S3)
(b) aspiration of air for preventing dispersion of the sample into the carrier solution	–	on/aspirate	off/aspirate	–	4	0.8	100
start loop							
(c) introduction of sample into HC	–	on/aspirate	–	–	1	2.5	4500
(d) sample loading/analyte preconcn	–	on/dispense	–	–	3	3.0	4500
end loop: repeat 4 times							
(e) sample cleanup with 10^{-2} M HCl	–	on/dispense	–	–	3	3.0	1100
3. elution and eluate transportation to LC							
(a) introduction of air into HC	–	on/aspirate	–	–	4	0.8	100
(b) aspiration of eluent	–	on/aspirate	–	–	6	0.6	150
start loop							
(c) elution of analyte loaded beads and postcolumn mixing of eluate with Milli-Q water	on/dispense	on/dispense	–	–	3	0.5 (S2) 0.25 (S1)	50 (S2) 25 (S1)
(d) stopped flow (10 s)							
end loop: repeat 3 times							
(e) pumping of air segment to waste	off/dispense	on/dispense	–	–	2	0.8	100
(f) transportation of eluate plus water segment into the injection loop	–	on/dispense	–	–	3	0.5	175
switching of rotary valve and activation of LC	–	–	–	inject	–		
4. bead discarding							
(a) aspiration of eluent	–	on/aspirate	–	–	6	0.6	200
(b) wait 20 s							
(c) dispensing of eluent to C_2	–	on/dispense	–	load	3	0.6	200
(d) bead transportation from C_2 to C_1 positions	–	on/aspirate	–	–	3	1.0	200
(e) withdrawal of used beads	–	on/dispense	–	–	2	1.5	300

[a] The symbol (–) means that the position of the valves and/or syringes remains unchanged.

exchange[16,31] or chelating moieties[21,32] or covalently immobilized proteins,[24,25] gel permeation materials,[33] cellulose carriers for cell culture,[34] or hydrophobic sorbents (e.g., C_{18}-poly(styrene–divinylbenzene))[35–37] are the common choice for on-line LOV-SPE or LOV-solid supported (bio)chemical reactions. No research, however, has been conducted so far aiming at expanding the scope of LOV-BI to monitoring trace level concentrations of relevant organic species in biological fluids or environmental samples. Thus, one of the goals of this work was to find a suitable reversed-phase material with excellent capability for retention of a wide spectrum of organic analytes, such as organic pollutants or pharmaceuticals, but at the same time, with potential use as a renewable sorbent. It should be stressed that there are some stringent requirements to be fulfilled by the solid-phase materials for straightforward handling in the LOV structure.[38] They must be (i) perfectly spherical (i.e., in the form of globe-shaped particles), (ii) uniform in size distribution, and (iii) water-wettable to prevent a prompt settlement into the LOV cavities. To this end, a commercially available divinylbenzene-*co*-*N*-vinylpyrrolidone reversed-phase copolymeric sorbent (Oasis HLB) that accom-

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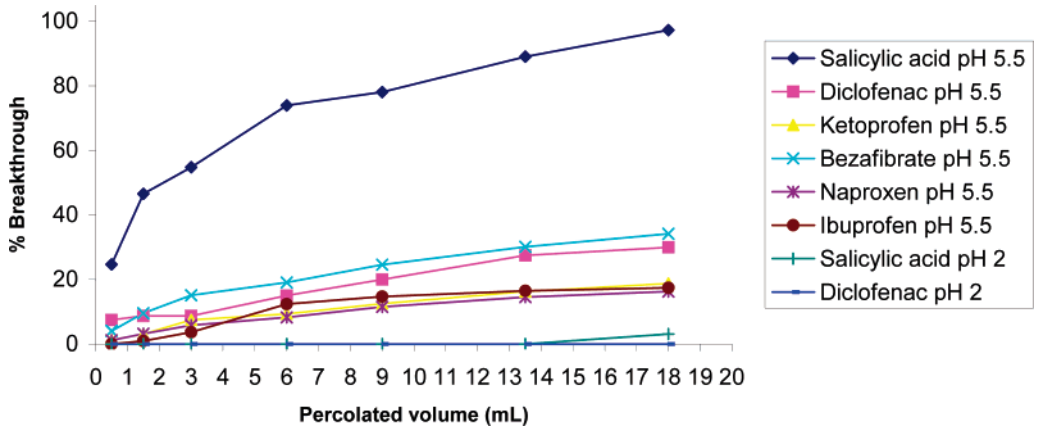

Figure 2. Breakthrough profiles of target analytes (1.7 μg mL^{-1}) obtained by frontal analysis of a 18-mL Milli-Q sample at both pH 5.5 and 2.0. The patterns for the remaining analytes at pH 2.0 overlapped with that of diclofenac.

plishes the three demands above has been for the first time selected as a disposable precolumn packing for the LOV approach. This material is regarded as a universal hydrophilic−lipophilic sorbent for both polar and nonpolar organic compounds, and it has proven excellent for retention of the target polar analytes.[39−43]

Yet, direct hyphenation of SPE using the copolymeric beads with reversed-phase HPLC might be troublesome as a consequence of the incompatibility of the eluent medium with the initial conditions for the HPLC gradient.[26,44] Actually, in the present application, quantitative elution of the target acidic drugs is proven to be merely accomplished with high methanol content solutions (>90% (v/v) MeOH/water), while the maximum tolerated content of organic solvent for appropriate HPLC separation of the most polar compounds (viz. salycilic acid and ketoprofen) without peak-broadening effects is <70% (v/v) MeOH/water. It should be noted that classical on-line column-switching methods[1] or low-pressure FI and SI-SPE assemblies,[2,45] based on eluting the sorbed species with the mobile phase itself, are therefore inappropriate because of the incomplete elution or undue broadening of the elution band.

On the other hand, the marriage of MSFI with LOV facilitates on-line hyphenation of BI with reversed-phase HPLC because both the optimum conditions for SPE and efficient HPLC band focusing are ensured. In our particular application, this is effected by post-SPE merging of the methanolic eluent with a water stream provided concurrently by one of the liquid drivers of the multisyringe device in a 1:2 water/eluent ratio (see Figure 1), thereby rendering a final eluate plug composition of 63% (v/v) MeOH/water, which satisfies the requirements for the LC separation.

Optimization of LOV-SPE Parameters. Once the sorbent material had been selected and its handling into the LOV microsystem tested, as discussed above, the different parameters of the microcolumn SPE method were optimized.

The influence of sample pH on the breakthrough volume for the LOV packed microcolumn was initially assessed by frontal analysis using UV−visible spectrophotometry. To this end, the sorbent was steadily loaded at 3 mL min^{-1} with a standard solution containing one of the analytes at a time at a high concentration level, namely, 1.7 μg mL^{-1}, and the nonretained analyte fraction in the effluent was continuously monitored.[1,46]

Accordingly to literature,[41,42] the copolymeric Oasis HLB material should be able to retain quantitatively acidic pharmaceuticals even at a neutral pH at the concentrations assayed. However, it was found experimentally that analyte breakthrough occurred for all targeted drugs whenever a nonacidified standard was applied (pH 5.5). Thus, breakthrough values between 16 (for naproxen) and 34% (for bezafibrate) were obtained for an 18-mL standard volume as shown in Figure 2, while for the most polar analyte, salicylic acid, breakthrough was almost complete (>97%). On the other hand, no appreciable breakthrough was attained for any of the analytes for acidified standards at pH 2, except for salicylic acid at the 3% level for a percolating volume of 18 mL (see Figure 2). This disagreement with literature data is attributed to the relatively large sorbent content of SPE cartridges used in off-line protocols, which amounts to 60−200 mg,[41,42] as compared to the 4.5 mg accommodated in the LOV cavities. Furthermore, the bead packing efficiency of the automated LOV system is expected to be slightly inferior to that of commercially available prepacked columns. Yet, negligible breakthrough is ensured by appropriate pH sample adjustment before on-column extraction.

One of the outstanding features of the MSFI approach is the inherent capability for automated microfluidic control of the entire SPE process in regard to the dimensions of the injected sample/reagent plugs and flow rates. Percolating flow rates ranging from 1.0 to 4.0 mL min^{-1} were assessed; yet flow rates above 3.0 mL min^{-1} were not applicable in the present LOV arrangement because of the progressive buildup of back pressure. This is attributed to the large surface-to-volume ratio of the copolymeric particles, which causes a compact settlement of the packed LOV microcolumn. Nonetheless, flow resistance can be alleviated to a large extent by application of the renewable sorbent approach. The preconcentrated sample volumes can be fixed according to the sensitivity demands of the assays, which are strongly depend-

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Table 3. Performance of the MSFI-BI-LOV-LC Analytical Method and Comparison with Off-Line SPE Procedures Reported in the Literature[a]

compound	linearity (r)	recovery (%)	repeatability (% RSD, $n = 6$)	reproducibility (% RSD, $n = 6$)	off-line SPE using 60-mg Oasis HLB cartridges (% RSD)		
				this work	LC−UV[43]	LC−MS/MS[40]	GC/MS[39]
salicylic acid	0.9985	101	5.8	7.7		12.5	
ketoprofen	0.9991	91	3.2	6.0	7.8	13.5	9.1
naproxen	0.9989	88	3.2	6.5	8.8	9.3	7.2
bezafibrate	0.9994	95	8.6	10.6		10.9	
diclofenac	0.9971	91	5.2	9.8	7.7	12.1	6.0
ibuprofen	0.9951	109	2.7	10.9	7.9	11.0	14.0

[a] Analytical figures are calculated for acidified standard mixtures (pH 2). Preconcentration volume, 18 mL.

ent on the sample nature. Regarding our application, sample volumes of a few milliliters suffice for appropriate monitoring of acidic drugs in biological fluids (e.g., urine) while loading volumes of >10 mL are typically needed for environmental applications. For surface and sewage water analysis, the sample volume was fixed to 18 mL to guarantee determinations at the subnanogram per milliliter level, while matching the time frame of the current LC separation with that of the on-line BI sample treatment.

As a consequence of the minute dimensions of the LOV sorbent column, effective stripping out of the acidic pharmaceuticals was readily effected with no need for the application of the classical back-flushing[4,6,7] or heart-cut[2,8] eluting schemes for injection of a narrow analyte-containing band into the analytical column. In fact, a multiple-step stopped-flow elution protocol based on the exploitation of the programmable flow of MSFIA was utilized. It involves the splitting of the eluent plug into three portions (50 μL each), which remained in intimate contact with the Oasis precolumn for 10 s. This permitted a more efficient desorption of the analytes as compared to forward-flow elution, where the total eluent volume should be increased to >250 μL for attaining identical peak areas.

Analytical Performance. The performance of the MSFI-BI-LOV method hyphenated to LC was evaluated in terms of recoveries, dynamic linear range, repeatability, reproducibility, enrichment factors, and analysis costs. Analytical figures of merit are compiled in Table 3.

The recovery percentages for the target pharmaceutical residues were calculated as the ratio between the peak areas obtained for each analyte in on-line BI-LOV analysis of spiked water samples at the 10 ng mL^{-1} level (pH 2, 18 mL) and those obtained from direct chromatographic injection of an equivalent mass (100 μL at the 1.8 μg mL^{-1} level) of a standard mixture prepared in the eluent medium. The MSFI-LOV method features excellent extraction efficiencies, ranging from 88 to 109%, as compared with previous off-line Oasis SPE procedures,[39,40,43] which yielded extraction recoveries between 70 and 80% for various NSAIDs, namely, ibuprofen, diclofenac, and ketoprofen. Despite the fact that fully automated SPE approaches do not demand quantitative recoveries as both the samples and standards are processed in exactly the same way through the whole analytical protocol, the higher the recovery the better the sensitivity and enrichment factors, which amounted to more than 100 for the various targeted drugs.

For quantification of the various analytes in real-world environmental samples, seven-level calibration plots based on least-squares linear regression algorithms were established by preconcentrating 18 mL of standard mixtures with concentrations ranging from 0.4 to 40 ng mL^{-1}. The entire set of calibration curves were linear within 2 orders of magnitude with determination coefficients higher than 0.9951.

The overall method repeatability and reproducibility were expressed as the relative standard deviation (RSD) of replicate analysis ($n = 6$) of an acidified standard mixture at the 10 ng mL^{-1} level using either a permanent LOV sorbent or six renewable columns, respectively (see Table 3). Coefficients of variation for the reusable-packed microcolumns ranged from 2.7 to 8.6%, while RSD values for the bead injection scheme increased slightly to 6.0−10.9%. This is likely due to the variations in bead packing and the slight differences in sorbent amount aspirated from the external reservoir whenever the renewable scheme is utilized. Yet, the RSD values are comparable to those obtained for robotic sample processors (e.g., Prospekt-2 system from Spark Holland)[11] capitalized on the use of disposable prepacked cartridges. According to the comparative data compiled in Table 3, the MSFI-LOV-LC approach features better precision than that reported for off-line methods using commercially available Oasis HLB cartridges, as a consequence of the avoidance of common intermediate steps of manual SPE applications.

One of the outstanding advantages of the integrated LOV unit coupled to the MSFI analyzer is the minimal running costs. In fact, as opposed to continuous-flow techniques (e.g., column switching schemes), the programmable flow inherent to the discontinuous-flowing MSFI approach facilitates accurate metering of microliters volumes of solutions, which are injected into the flow network at the precise instant to perform the analytical process. Cost-effectiveness in on-line SPE-LOV systems is not only realized as a result of the minimization of solvent consumption but, most importantly, the expenses of the costly sorptive materials are greatly decreased. Thus, as a result of the miniaturization of the SPE in the LOV microconduits, the sorbent cost per extraction is merely 0.15 euro versus ~3 euros for off-line applications using conventional Oasis HLB cartridges containing 60 mg of sorbent and 4 euros for robotic systems employing Prospekt-2 cartridges.

The system's capability of performing the preconcentration and cleanup of one sample in parallel with the chromatographic analysis of the previously extracted one ensures high sampling throughput, the analysis cycles being hence reduced to 20 min.

Method Validation. To assess the reliability and ruggedness of the hyphenated analytical method, a number of real-life samples

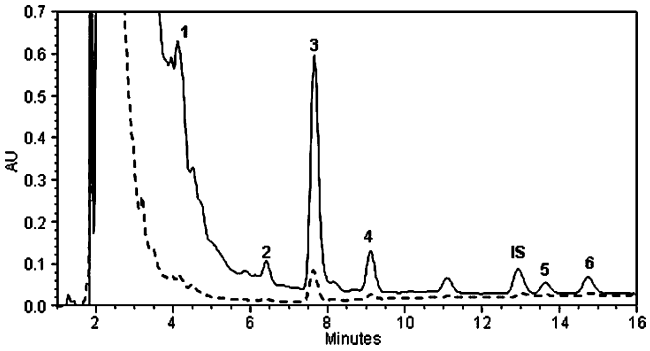

Figure 3. Illustration of the chromatograms recorded for a spiked urine sample (1 μg mL^{-1}) at 230 nm after direct injection of 100-μL standard (dotted line) and on-line MSFI-BI-LOV treatment of 1-mL sample (solid line). Compound identification: (1) salicylic acid, (2) ketoprofen, (3) naproxen, (4) bezafibrate, (5) diclofenac, (6) ibuprofen, and (IS) fenoprop (internal standard). (NB, salicylic acid and ketoprofen were quantified at 300 and 256 nm, respectively, in lieu of 230 nm as represented in the chromatogram).

Table 4. Concentrations, LODs (S/N ≥ 3), and LOQs (S/N ≥ 10) of Acidic Pharmaceuticals Found in Spiked Urine Samples

	concn found ± SD[a]			
compound	1.00 μg mL^{-1} spiked	2.00 μg mL^{-1} spiked	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
salicylic acid	1.09 ± 0.09	2.0 ± 0.2	0.11	0.30
ketoprofen	0.91 ± 0.03	1.98 ± 0.05	0.07	0.17
naproxen	1.16 ± 0.04	2.2 ± 0.1	0.05	0.13
bezafibrate	0.89 ± 0.01	2.0 ± 0.2	0.15	0.37
diclofenac	1.0 ± 0.1	2.0 ± 0.1	0.06	0.17
ibuprofen	1.02 ± 0.01	2.09 ± 0.09	0.06	0.14

[a] The results are expressed as the mean of five replicates ± standard deviation. Loading sample volume, 1 mL.

of variable matrix complexity and nature, namely, surface waters, urban wastewaters, and urine, were processed in the MSFI setup.

Urine Samples. According to the expected concentration levels of NSAIDs in biological samples,[47] the MSFI-BI-LOV protocol is mainly utilized for removal of potentially interfering matrix compounds. In addition, the user-friendly software used for automated control of the multisyringe piston pump and multiposition valve facilitates straightforward selection of the volume to be preconcentrated, which was fixed to 1 mL for urine samples. In Figure 3, the chromatogram of a urine aliquot spiked at the 1 μg mL^{-1} level and recorded following the sample processing step is overlapped with that obtained by direct sample injection. As can be seen, the on-column treatment permits the sensitive and selective determination of the target compounds with negligible matrix interfering effects. Actually, the limits of detection (LOD) and quantification (LOQ) estimated from the on-line analysis of spiked urine samples as the minimum concentration of a given species giving signal-to-noise ratios of 3 and 10, respectively, were found to range from 0.05 to 0.15 μg mL^{-1} and 0.13 to 0.37 μg mL^{-1}, respectively (Table 4), which can be regarded as satisfactory for this kind of biological samples. The analyte concentrations found in the fortified urine samples were in good agreement with the spiked level, with RSD values better than 10% (see Table 5).

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Table 5. Concentrations, LODs (S/N ≥ 3) and LOQs (S/N ≥ 10) of Acidic Pharmaceuticals Found in Spiked Surface Water Samples

	concn found ± SD[a]			
compound	0.40 ng mL^{-1} spiked	1.00 ng mL^{-1} spiked	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
salicylic acid	0.45 ± 0.03	0.95 ± 0.09	0.15	0.38
ketoprofen	0.39 ± 0.01	0.91 ± 0.06	0.10	0.26
naproxen	0.44 ± 0.03	1.0 ± 0.1	0.02	0.05
bezafibrate	0.43 ± 0.03	1.10 ± 0.06	0.10	0.25
diclofenac	0.35 ± 0.05	1.1 ± 0.1	0.12	0.28
ibuprofen	<LOQ	1.1 ± 0.2	0.36	0.85

[a] The results are expressed as the mean of five replicates ± standard deviation. Loading sample volume, 18 mL. The concentration of target compounds in the raw (nonspiked) sample was <LOQ.

The LOV-SPE methodology provided relative recoveries with an average of 102% for both the 1 and 2 μg mL^{-1} level spikes. The application of a statistical *t*-test[48] for assessment of the method's accuracy rendered calculated *t*-values of 0.47 and 0.89, respectively, which are below the critical value (viz., 2.57) at the 0.05 significance level. These results imply that the mean relative recovery ratios for both spike levels are not significantly different from 100%, and thus, there are no significant differences between spiked and measured concentrations.

The RSD values obtained by MSFI-LOV are slightly higher than those previously reported by Mardones et al.[47] (2.2−7.7%) by exploiting an on-line FI-SPE-CE setup for the determination of the same compounds in biological fluids. Yet, the SPE-CE system lacked accuracy because maximum deviations of 43% were found for urine samples at the same concentration level, i.e., 2 μg mL^{-1},[47] than that used for validation of the proposed LOV method.

Environmental Samples. The developed flow method was also applied to the determination of trace level concentrations of the acidic drugs in various environmental samples of variable complexity, namely, surface water samples and both raw and treated wastewaters. The surface water was collected from the mouth of a natural creek, having thus an important seawater contribution. This sample was chosen to assess method's performance for high salt content samples. Tables 5 and 6 summarize the results of the analyses of the various environmental samples and compile the detection and quantification limits obtained. Figure 4 depicts the chromatogram of a spiked (10 ng mL$^{-1)}$ untreated wastewater sample. The spiking levels were selected as to represent low and medium concentration levels according to the LOQ values and the original concentration of the compounds in each sample.

Satisfactory relative recoveries were obtained for both spiked surface and wastewater samples, with an estimated deviation below 13% in all cases. The statistical *t*-tests confirmed the nonexistence of significant differences between the mean recovery ratios for the various spikes and the expected value at the 0.05 significance level. Moreover, the automated method gave rise to averaged RSD values below 10%. Detection limits were found to range between 0.02 and 0.36 ng mL^{-1} for surface water samples (Table 5) and between 0.11 and 0.67 ng mL^{-1} for wastewaters (Table 6). The slightly higher LODs for wastewaters are the consequence of the

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Table 6. Concentrations, LODs (S/N ≥ 3) and LOQs (S/N ≥ 10) of Acidic Pharmaceuticals Found in Raw and Spiked Urban Wastewaters

	concentration found ± SD[a]							
	raw wastewater (influent)			treated wastewater (effluent)				
compound	nonspiked	10.0 ng mL^{-1} spiked	40.0 ng mL^{-1} spiked	nonspiked	4.00 ng mL^{-1} spiked	8.00 ng mL^{-1} spiked	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
salicylic acid	118 ± 7	140 ± 11	155 ± 15	nd	4.3 ± 0.1	7.0 ± 0.4	0.67	1.61
ketoprofen	nd	12 ± 1	46 ± 5	nd	3.8 ± 0.3	7.6 ± 0.5	0.39	1.02
naproxen	13 ± 1	23 ± 2	54 ± 4	1.2 ± 0.2	5.0 ± 0.3	9.1 ± 0.8	0.11	0.30
bezafibrate	nq	11 ± 1	41 ± 5	nd	4.1 ± 0.4	8.6 ± 0.7	0.33	0.93
diclofenac	nd	11 ± 1	41 ± 4	nd	4.2 ± 0.4	7.8 ± 0.4	0.46	1.08
ibuprofen	18 ± 2	27 ± 2	60 ± 3	nd	4.4 ± 0.3	7.2 ± 0.6	0.62	1.38

[a] The results are expressed as the mean of five replicates ± standard deviation. Loading sample volume, 18 mL. nd, below LOD. nq, below LOQ.

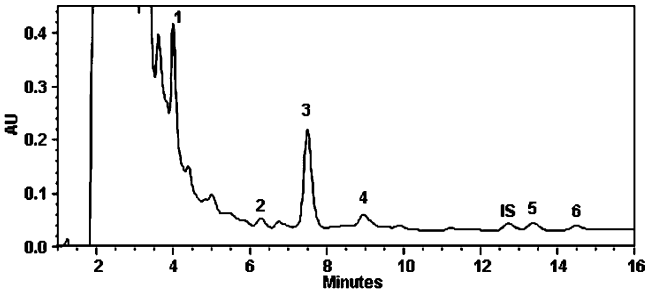

Figure 4. LC chromatogram of a spiked raw wastewater sample (10 ng mL^{-1}) at 230 nm following automated SPE with renewable sorbent. Compound identification as in Figure 3.

increased baseline noise. Yet, the resulting LODs are appropriate for monitoring most of the target analytes in the wastewaters analyzed, where salicylic acid exhibited the maximum concentration (118 μg L^{-1}) in the untreated wastewater sample. Although this concentration level is relatively high, concentrations of ibuprofen of >100 ng mL^{-1} have already been reported in untreated municipal wastewater.[43] Moreover, salicylic acid is known to have many other sources besides acetylsalicylic acid. Experimental results also confirmed that both salicylic acid and ibuprofen were completely removed during the water treatment process, while naproxen was reduced to a large extent, which is in good agreement with earlier observations.[49−51]

On the other hand, the concentration of none of the analytes in surface waters was above the LODs. Therefore, a further reduction of LODs would be desirable. This is feasible by straightforward coupling of the characterized MSFI-LOV-SPE approach with renewable surfaces with liquid chromatography−electrospray tandem mass spectrometry, which has been proven excellent for quantitation of acidic drugs and other environmentally relevant persistent organic pollutants (e.g., endocrine disruptors, polycyclic aromatic hydrocarbons, pesticides, and brominated flame retardants) in environmental samples at concentrations below the levels of concern according to current regulatory authorities.[7,10] Further research is to be focused in further miniaturization of the entire analytical setup by hyphenation of the LOV-BI concept with micro-LC as well the synthesis of novel selective materials fulfilling the stringent demands for their straightforward handling within the LOV conduits.

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