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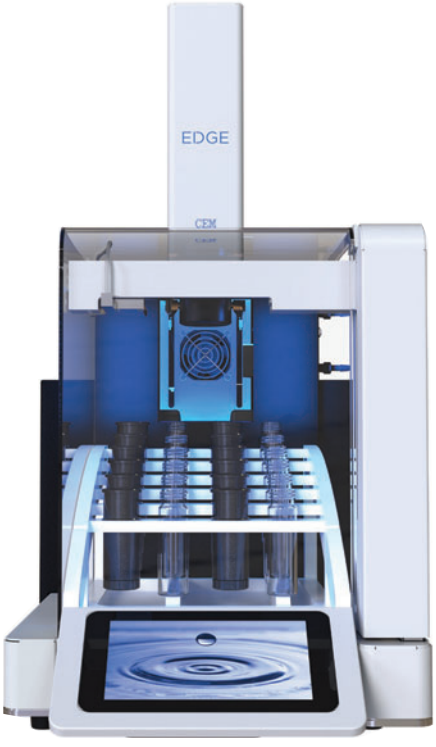

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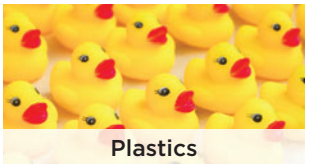
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- Extraction of Fat From Egg Powder
- Extraction of Fat From Pumpkin Seeds
- Extraction of Total Fat From Pepperoni
- Extraction of Total Fat From Feed/Food
- Extraction of Total Fat From Hot Dogs
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- Extraction of Total Fat From Peanuts
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- Extraction of Pesticides From Avocados
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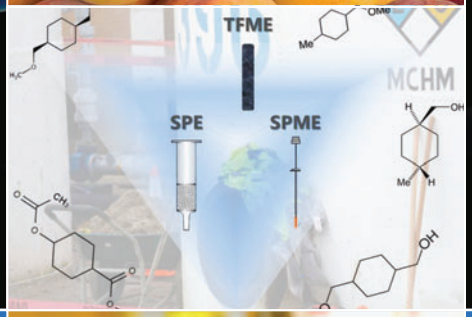
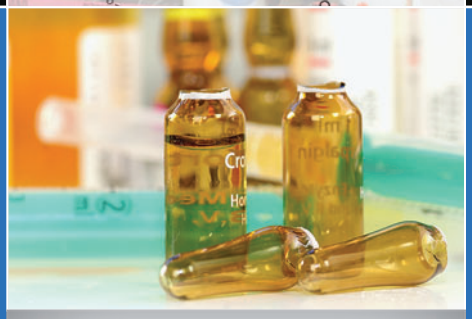


RECENT DEVELOPMENTS IN SAMPLE PREPARATION



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Recent Developments in Sample Preparation

A supplement to LCGC North America

April 2019

Articles

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FROM the GUEST EDITOR

Advancing Chromatographic Analysis Using Selective and Robust Sample Preparation

Jared L. Anderson
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Sample preparation plays a very important role in chemical analysis, and has become an integral step prior to chromatographic separations. Depending on the sample matrix and the overall goal of analysis, the employed sample preparation approach can range from "dilute and shoot" to more laborious solid-phase extraction methods. With ongoing advances accelerating chromatographic separations, sample preparation methods must exhibit most, if not all, of the following characteristics: They must be capable of achieving rapid and high enrichment or preconcentration of target analytes; they must be tolerant of matrix effects; they must exhibit robustness under conditions commonly encountered during sampling and analysis steps; and they must demonstrate compatibility with downstream chromatographic and mass spectrometry methods. The field of sample preparation is undergoing revolutionary change, largely governed by the need for miniaturization while maintaining high selectivity and high analyte enrichment. This change has necessitated the development of new sorbent materials, as well as the design of creative geometries that possess high surface areas and facilitate rapid diffusion of analytes from a variety of samples. This supplement was assembled to showcase advances in various sample preparation approaches from leaders in the field. They have applied their methods towards environmental, food, and pharmaceutically relevant samples to demonstrate the marriage of these techniques with chromatographic separations for solving important challenges in chemical analysis.

Emanuela Gionfriddo and co-workers at The University of Toledo compare the extraction performance of solid-phase extraction (SPE), traditional solid-phase microextraction (SPME), and thin-film SPME (TF-SPME) in the determination of crude 4-ethylcyclohexylmethanol (MCHM) from environmental samples by gas chromatography with mass spectrometry detection (GC–MS). They exploit the rapid preconcentration features of TF-SPME, and report limits of quantitation for MCHM lower than any other reported method in the literature. The versatility of the approach is also demonstrated by determining a metabolite of MCHM through slight modifications to their procedure.

In a demonstration of the growing importance that metal-organic frameworks (MOFs) play in sample preparation, Veronica Pino and co-workers at the University of La Laguna in Spain describe the use of MOFs in dispersive SPE for the determination of personal care products by GC–MS. Many of the previously published approaches using MOF-based materials have been coupled to liquid chromatography. This study showcases the growing importance that MOFs can play as selective sorbent materials when coupled to GC applications.

Amid growing concerns about the release of extractables and leachables during the storage of pharmaceutical drugs, Leandro Hantao and co-workers at the State University of Campinas (Unicamp) in Brazil report a method using SPME coupled to comprehensive two-dimensional gas chromatography mass spectrometry (GC×GC–MS). Their approach consists of direct immersion sampling from a nasal drug solution, and reveals four leachables identified by principal component analysis. Flow modulated GC×GC was employed as a more economical and user friendly alternative to thermal modulation for pharmaceutical analysis.

In an effort to monitor the chiral composition and enantiomeric excess of peach aroma, Cecilia Cagliero and co-workers at the University of Torino in Italy demonstrate a fully automated method based on stir-bar sorptive extraction (SBSE) followed by chiral GC–MS. The components γ- and ∂-lactone were quantified in natural and artificial peach flavored juices. The consolidated approach demonstrates the coupling of a highly sensitive sampling approach with chiral chromatography to achieve rapid quality control metrics of food products.

Last but not least, Javier Hernández-Borges and co-workers, also at the University of La Laguna, discuss the role of nanomaterials in sample preparation. They provide a comprehensive review of sorbent nanomaterials based on carbon, MOFs, covalent organic frameworks, nanoparticles, quantum dots, nanofibers, and dendrimers. Their article highlights some of the challenges and opportunities associated with the preparation, characterization, and toxicity of nanomaterials used in sample preparation.

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Dispersive Miniaturized Solid-Phase Extraction Using the CIM-81 Metal-Organic Framework and Gas Chromatography–Mass Spectrometry to Determine Personal Care Products in Waters

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The use of metal-organic frameworks (MOFs) in miniaturized extraction approaches is already successful in analytical sample preparation, particularly when combined with liquid chromatography. This study shows the determination of several semivolatile personal care products with the CIM-81 MOF, but in combination with gas chromatography and mass spectrometry detection, to expand the applications while still meeting the requirements of green analytical chemistry.

Providencia González-Hernández, Ana B. Lago, Jorge Pasán, Juan H. Ayala, Ana M. Afonso, and Verónica Pino

Personal care products (PCPs) are organic compounds belonging to the group of emerging contaminants, which have shown toxic effects in humans and other living organisms (1,2). There are six groups of PCPs: ultraviolet (UV) filters, preservatives, disinfectants, musk, insect repellents, and siloxanes, with the classification depending on their use, except for siloxanes, which are classified based on their structure. PCPs are present in a wide number of everyday products, such as gels, lotions, and cosmetics. Given their extensive use, it is clear that high amounts of PCPs are released continuously into the environment, mainly into river, lake, and seawaters, where they are diluted. Accurate monitoring of PCPs in environmental samples is undoubtedly important, requiring efficient methodologies of preconcentration and detection.

The determination of nonvolatile PCPs is normally accomplished using high performance liquid chromatography (HPLC) (3–7), whereas gas chromatography (GC) is utilized for volatile or semivolatile PCPs (8–11). Among microextraction and preconcentration techniques, it is important to highlight the increased utilization of miniaturized solid-phase extraction (µSPE) methods because of their advantages: simplicity, low cost, low consumption of solvents, low time requirements, and low amounts of sorbent needed (<500 mg). The dispersive mode (D-µSPE) is probably the simplest approach, with the sorbent added directly to the aqueous sample under gentle stirring, and it is usually accompanied by high enrichment factors (12). D-µSPE, like many other analytical extraction methods, has benefited from recent incorporation of novel materials as sorbents. Among materials tested successfully, molecularly-imprinted polymers (13), graphene (14), multiwalled carbon nanotubes (15), metallic nanoparticles (16), and metal-organic frameworks (17) can be highlighted.

Metal-organic frameworks (MOFs) are crystalline polymers with a three-dimensional structure (18). MOFs result from the autoassembly of metal ions (or metal clusters) and organic linkers through coordination bonds. These materials are characterized by their high porosity and tuneability, quite low crystal densities, and adequate thermal and mechanical stabilities, combined with high surface area (19). These properties fulfil many of the requirements for sorbents in efficient extractions, and thus MOFs have been successfully incorporated into a number of microextraction strategies (20,21), with the majority of applications linked to D-µSPE (12). Thus, different bare MOFs, such as HKUST-1(Cu) (22), MIL-101(Fe) (23), MIL-101(Cr) (24), MIL-53(Al) (25), TMU-5 (26), and UiO-66 (27), among others, have been used as sorbents in D-µSPE for the extraction of different pollutants.

Most applications of bare-MOFs or MOF-based composites as sorbents in D-µSPE are carried out in combination with

chromatographic techniques, and among them, around 80% of the publications use high performance liquid chromatography (HPLC) or ultrahigh-pressure LC (UHPLC) (28,29). The reported applications normally focus on pollutants (such as polycyclic aromatic hydrocarbons, pyrethroids, polychlorinated diphenyl ethers, and hormones) in environmental samples (30–33). In contrast, applications in combination with GC are scarce (34,35). In fact, to date there has not been any study using MOFs as sorbents in D-µSPE-GC for PCPs.

The $[Zn_2(tz)_2(bdc)]$ MOF, termed *CIM-81* in a previous article of our research group (36), is a pillared-layer network constructed from a terephthalic carboxylate ligand (H_2bdc) bridging the 2D layers formed by the Zn(II) metal ions and the 1,2,4-triazole (Htz) motifs. The main advantage of using MOFs with mixed ligands (like CIM-81) in D-µSPE, versus MOFs presenting a single type of ligand, is the increasing number of analyte–MOF interactions that can occur. The extraction efficiency of CIM-81 in D-µSPE for several endocrine disrupting chemicals followed by UHPLC with ultraviolet detection (UHPLC-UV) was demonstrated in a previous study (36). Thus, the main objective of the present study was to develop an analytical application for semivolatile PCPs utilizing this successful CIM-81 MOF in D-µSPE, and in combination with GC combined with mass spectrometry (GC–MS). As a parallel goal, this study aims to expand the analytical applications of MOFs for volatile or semivolatile analytes, while encouraging an increased use of GC when utilizing this microextraction method (D-µSPE) based on MOFs.

Experimental

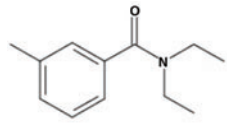
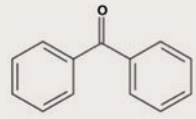
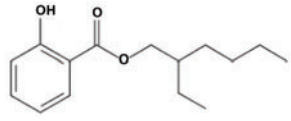
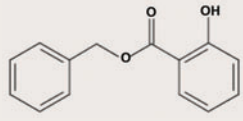
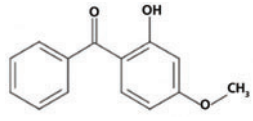
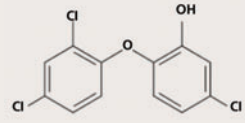
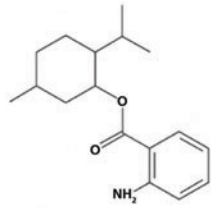
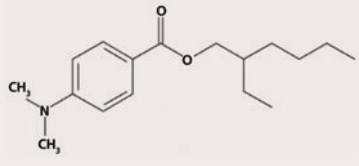
Standards, Reagents and Materials

Six UV-filters: benzophenone (BP), 2-ethylhexyl salicylate (EHS), benzyl salicylate (BS), benzophenone-3 (BP3), menthyl anthranilate (MA), and 2-ethylhexyl 4-(dimethylamino)benzoate (OD-PABA); one disinfectant: triclosan (TCS); and one insect repellent: *N,N*-diethyl-m-toluamide (DEET), were studied. All standards presented purity higher than 98%, being supplied by Sigma-Aldrich (Steinheim, Germany). Individual stock solutions were prepared in cyclohexane (2040–2590 mg/L), or in acetonitrile (1040–1580 mg/L), depending on the experiment. A mix of PCPs as an intermediate standard solution was prepared daily by dilution of the original individual stock solutions, using cyclohexane or acetonitrile as solvents (also depending on the specific experiment). Working aqueous standard solutions were prepared daily by dilution of intermediate solutions in acetonitrile. All solutions were stored at 4 °C, and protected from light.

Ultrapure deionized water was obtained using the Milli-Q gradient A10 water purification system from Millipore (Watford, UK). Methanol and cyclohexane were purchased from Honeywell (Seelze, Germany), ethyl acetate (EA) from Panreac (Barcelona, Spain), and acetonitrile from VWR International (Barcelona, Spain). Glacial acetic acid was acquired from Millipore (Darmstadt, Germany), and anhydrous sodium acetate (>99%) from Sigma-Aldrich. Both were used in the preparation of an acetic–acetate buffer solution.

The following reagents were used in the synthesis of the CIM-81 MOF: 1,2,4-triazol (98%) and benzene-1,4-dicaboxilic acid (98%), from Sigma-Aldrich; zinc nitrate

Table I: Several characteristics of the PCPs studied, together with the ions used in their MS monitoring

PCP	Chemical Structure	Molecular weight ($g \cdot mol^{-1}$)	$logK_{ow}$ [a]	pk_a [a]	Vapor pressure [a] $\times 10^{-8}$ (atm)	Monitoring ions [b] (m/z)
DEET		191.3	2.42	-1.37	177.6	**119,** 91, 190
BP		182.2	3.21	-	108.3	**105,** 182, 77
EHS		250.3	5.93	8.13	10.6	**120,** 138, 121
BS		228.2	4.21	8.11	23.0	**91,** 65, 39
BP3		228.2	3.99	7.56	0.7	**151,** 227, 228
TCS		289.5	5.34	7.80	4.3	**290,** 288, 218
MA		275.4	6.08	2.17	0.6	**137,** 119, 275
OD-PABA		277.4	5.41	2.39	0.6	**165,** 148, 164

a SciFinder 2018 database
b The quantifying ion has been highlighted in bold

hexahydrate (98%), from Honeywell; and *N,N*-dimethylacetamide (DMA), from Merck. The synthesis of the CIM-81 MOF was carried out using 45 mL teflon solvothermal reactors and stainless steel autoclaves, supplied by Parr Instrument Company (Moline, IL, USA).

Pyrex centrifuge tubes (Staffordshire, UK), a 2-mL glass syringe Fortuna Optima (Sigma-Aldrich), and 0.2-µm PVDF (polyvinylidene fluoride) Whatman syringe filters (GE Healthcare, Buckinghamshire, UK), were used in the microextraction procedure.

Sample Collection

Two bottled waters (acquired in local shops) and tap water (collected in the laboratory, and analyzed the same day of the sampling) were used in the analyses.

Instrumentation

The chromatographic analysis was performed on a 7820A gas chromatograph from Agilent Technologies (Waldbronn, Germany), equipped with a HP-5ms ultra inert capillary column (30 m × 0.25 mm × 0.25 µm), and coupled to an Agilent 5977B mass spectrometer equipped electronic impact ionizer and a simple quadrupole as mass analyzer.

A Reax Top vortex of Heidolph (Schwabach, Germany), a centrifuge from Eppendorf model 5720 (Hamburg, Germany), and an IKA RV10 rotary evaporator with a CVR

3000 vacuum controller supplied by VWR, were used in the extraction procedure.

The oven used in the synthesis of the CIM-81 MOF was the Universal UF30 model, supplied by Memmert (Schwabach, Germany).

The phase identification of MOF crystals was performed with an X'Pert diffractometer, supplied by PANalytical (Eindhoven, Netherlands), operating with Bragg-Brentano geometry. Data collection was carried out using Cu K1 radiation (λ = 1.5418 Å) over the angular range from 5.01° to 80.00° (0.02° steps), with a total exposure time of 30 min. IR spectra (450–4000 cm^{-1}) were recorded for the powdered crystals using an IRAffinity1 spectrophotometer from Shimadzu (Kyoto, Japan), equipped with a Pike technologies GladiATR. A Gemini V 2365 model analyzer, supplied by Micromeritics (Georgia, USA), was used to measure the nitrogen adsorption isotherms, in the range $0.02 \leq P/P_0 \leq 1.00$ at 77 K. The Brunauer, Emmet, and Teller (BET) method was used to calculate the surface area. Thermogravimetric analysis on freshly crushed crystals of CIM-81 was carried out in a PerkinElmer Pyris Diamond TG/DTA thermal analyzer (Billerica, MA, USA), requiring a few milligrams under a nitrogen atmosphere, and at a flow rate of 20 mL/min. The temperature was ramped from 25 to 250 °C at a heating rate of 5 °C/min.

Procedures

Synthesis of the MOF

The MOF $[Zn_2(tz)_2(bdc)]\cdot 2DMA$, termed *CIM-81*, was synthesized according to a previous method developed by our group (36). Briefly, $Zn(NO_3)_2\cdot 6H_2O$ (592 mg, 2 mmol), 1,2,4-triazole (140 mg, 2 mmol), and benzene-1,4-dicarboxylic acid (170 mg, 1 mmol), were dissolved in 15 mL of DMA, and then placed in a Parr Teflon-lined stainless steel vessel (25 mL) under autogenous pressure. The synthesis was carried out at 120 °C for 72 h. The obtained colorless crystals were filtered, washed with DMA and acetone, and dried at 50 °C. The obtained MOF was activated by immersion in acetone for 24 h, to ensure removal of the DMA solvent. The obtained yield was 83%. The crystalline structure was ensured by comparing the experimental X-ray diffraction pattern obtained with the theoretical pattern, thus confirming the nature of CIM-81 MOF already reported by our research group (36).

GC–MS Method

The optimal oven program for the GC separation of the PCPs starts at 70 °C, and increases at a rate of 15 °C/min up to 300 °C, being then held for 5 min. Helium was used as carrier gas at 1 mL/min. The temperature of the transfer line was set to 290 °C, 230 °C for the ionization source, and 150 °C for the quadrupole. The ionization by electronic impact (EI) was carried out at 70 eV. The MS ions used for the monitoring of PCPs are summarized in Table I. The volume of the extract (or standard) injected in the GC–MS was 2 µL in splitless mode at 280 °C, and with a solvent delay of 7.5 min.

D-µSPE Method

The optimal microextraction method requires the addition of 10 mg of the CIM-81 MOF to 10 mL of the aqueous sample (containing or not containing PCPs) or to the aqueous standard, at pH 5 adjusted with an acetic–acetate buffer solution. The tube is then subjected to vortex agitation for 1 min, and then centrifuged (2504 *g*) for 3 min. Afterwards, the aqueous supernatant is removed, and 1.2 mL of methanol is added to perform the desorption of the PCPs trapped by the MOF. Desorption is facilitated by the application of vortex agitation for 4 min. Then, the desorption solvent containing the target analytes is filtered (0.2 µm), and evaporated under vacuum, followed by reconstitution in 100 µL of a mixture of cychlohexane:ethyl acetate with a ratio of 9:1 (v/v).

Results and Discussion

GC–MS Method

The optimal chromatographic separation of the eight PCPs took less than 14 min under the conditions described above. Several quality analytical parameters of the GC–MS method are summarized in Table II, obtained with seven calibration levels. For all PCPs, the relative standard deviation (RSD, in %) for the retention times was < 0.07% (n = 40). For all calibrations, the determination coefficients were >0.995.

Limits of detection (LOD) and limits of quantification (LOQ) were calculated as the concentration providing 3 and 10 times, respectively, the standard deviation of the signal generated by a low concentration standard. Thus, the obtained LOQs ranged from 0.4 µg/L for menthyl anthranilate to 4.6 µg/L for triclosan, as can be seen in Table II.

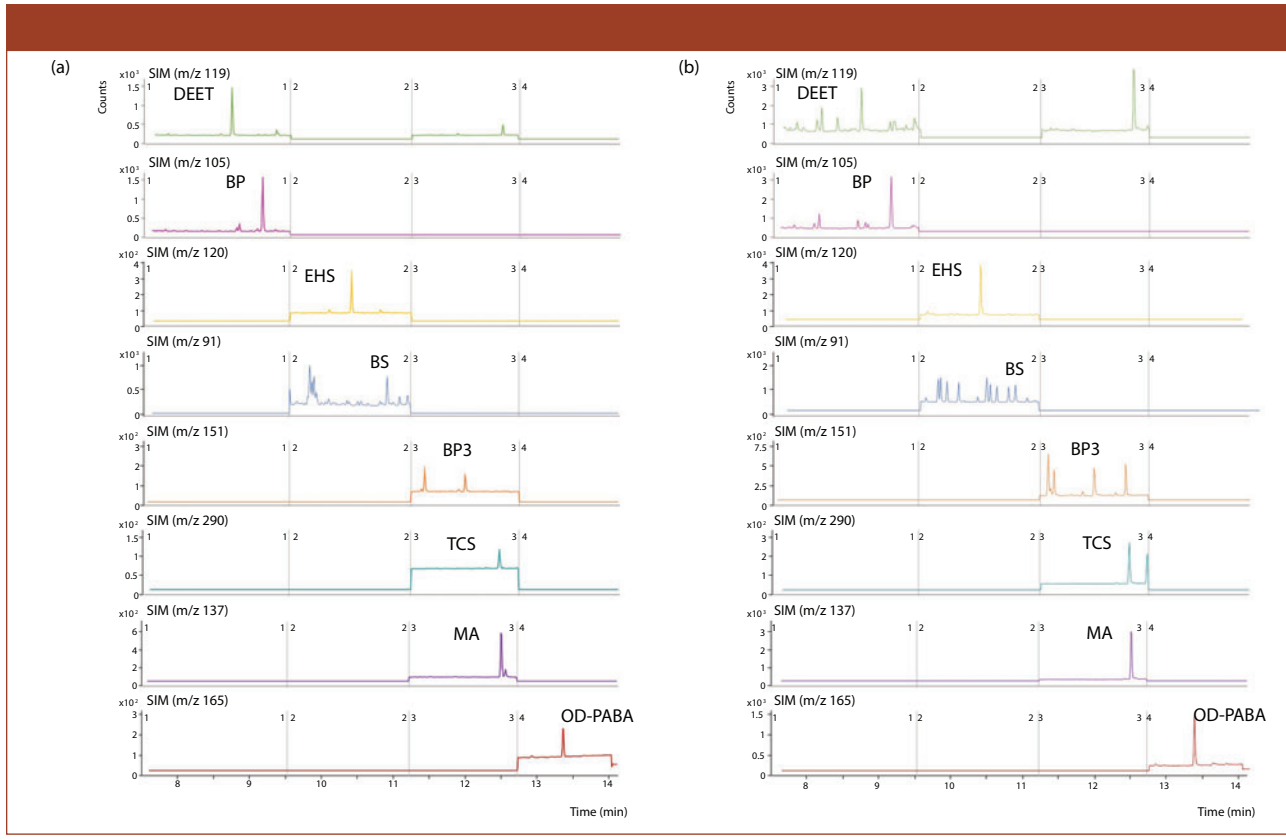


Figure 1: Representative chromatogram of (a) standard solutions of personal care products (PCPs) at a concentration of 10 µg/L, and (b) sample 2 spiked with the PCPs at 4 µg/L concentration and subjected to the entire dispersive mode miniaturized solid-phase extraction-gas chromatography–mass spectrometry (D-µSPE-GC–MS) method. Key to abbreviations: DEET = *N,N*-diethyl-m-toluamide, BP = benzophenone, EHS = 2-ethylhexyl salicylate, BS = benzyl salicylate, BP3 = benzophenone-3, TCS = triclosan, MA = menthyl anthranilate, OD-PABA = 2-ethylhexyl 4-(dimethylamino)benzoate.

The precision of the GC–MS method was evaluated at two levels of concentration: low (15 µg/L) and intermediate (45 µg/L), and in intraday (n = 4) and interday with three different non-consecutive days (n = 12). For the lower concentration level, intraday RSD values ranged between 2.6 and 9.7% for benzyl salicylate and benzophenone-3 respectively, while interday values <15% were obtained in all cases. For the intermediate concentration level, values below 4.8 and 9.4% were obtained for the intraday and the interday precision, respectively. Figure 1(a) shows a representative chromatogram of a standard at a concentration of 10 µg/L.

Optimization of the D-µSPE Procedure Combined with GC–MS

The CIM-81 MOF has been used successfully in our research group as sorbent in a D-µSPE method for a group of PCPs (with only 3 PCPs in common with the current study), when the extraction procedure was combined with UHPLC-UV (36). In fact, the majority of publications for MOFs in D-µSPE are in combination with LC (12,28,29). Clearly, the adaptation of methods using MOFs in D-µSPE with GC would make it possible to monitor semivolatile PCPs. In this particularly case, CIM-81 in D-µSPE, the group of PCPs was extended to incorporate DEET, 2-ethylhexyl salicylate, benzyl salicylate, menthyl anthranilate , and 2-ethylhexyl 4-(dimethylamino)benzoate, which were not previously studied (36).

For the purpose of simplifying the adaptation of LC methods with MOFs to GC methods, the main variables of the microextraction procedure were kept constant. Solvent compatibility is certainly crucial for ensuring proper adaptation of the D-µSPE method to GC–MS. Therefore, the first study evaluated the effects caused by changing the desorption solvent of the D-µSPE method (methanol) to a solvent (such as cychlohexane) compatible with the GC column used. The experiments required the addition of 10 mg of the CIM-81 MOF to 10 mL of the aqueous standard containing PCPs at 5 µg/L (and at pH 5 adjusted with an acetic–acetate buffer solution). After vortex agitation (1 min) and centrifugation (2504 *g* for 3 min) of the tube, the aqueous supernatant was removed, and 1.2 mL of desorption solvent (cychlohexane) is added to perform the desorption step of the PCPs. Desorption was facilitated by the application of vortex agitation for 4 min. Then, cychlohexane, as the desorption solvent, was filtered (0.2 µm), and injected directly in the GC system. Experiments were performed in triplicate.

It was observed that the extraction efficiencies (E_R %) obtained for PCPs were not higher than 7%, except for menthyl anthranilate (E_R ~20%) and 2-ethylhexyl 4-(dimethylamino)benzoate (E_R ~33%). The selection of cychlohexane as the desorption solvent, while adequate for the GC column, was not beneficial in the desorption of PCPs already trapped by the CIM-81 after D-µSPE.

Considering these results, methanol was kept as the desorption solvent in D-µSPE with CIM-81, and therefore a solvent-exchange step was considered. The experiments were

Table II: Several quality analytical parameters of the gas chromatography–mass spectrometry (GC–MS) method

PCP	t_R ± SD (n = 40)	Slope ± SD*	R^2 †	$S_{y/x}$ ‡	LOD/LOQ§ (µg/L)	RSD# (%) intraday/ interday (15 µg/L)	RSD# (%) intraday/ interday (45 µg/L)
DEET	8.688 ± 0.006	142 ± 3	0.998	273	0.4 / 0.8	3.3 / 7.3	2.1 / 9.4
BP	9.130 ± 0.002	174 ± 4	0.997	1	0.3 / 1.4	8.5 / 15	2.0 / 4.8
EHS	10.381 ± 0.001	35 ± 1	0.998	57	0.1 / 1.1	3.3 / 14	2.7 / 4.6
BS	10.888 ± 0.005	64 ± 2	0.997	145	0.2 / 1.4	2.6 / 13	4.0 / 7.6
BP3	11.969 ± 0.005	12 ± 1	0.998	23	1.0 / 3.8	9.7 / 11	4.8 / 8.7
TCS	12.451 ± 0.007	3 ± 1	0.997	7	0.2 / 4.6	3.6 / 7.3	3.3 / 7.9
MA	12.498 ± 0.002	85 ± 2	0.995	232	0.3 / 0.4	2.7 / 8.3	4.7 / 5.1
OD-PABA	13.160 ± 0.009	26 ± 1	0.997	58	1.0 / 2.0	7.7 / 13	4.5 / 9.3

* Standard deviation (SD) associated to the slope
† Determination coefficient (R^2) for 7 calibration levels (calibration range: 5 to 100 µg/L)
‡ Standard deviation (S) of the residuals
§ Limit of detection (LOD) and limit of quantification (LOQ)
# Relative standard deviation (RSD): intraday (n = 4) and interday (n = 12 in three non-consecutive days)
DEET = *N,N*-diethyl-m-toluamide, BP = benzophenone, EHS = 2-ethylhexyl salicylate, BS = benzyl salicylate, BP3 = benzophenone-3, TCS = triclosan, MA = menthyl anthranilate, OD-PABA = 2-ethylhexyl 4-(dimethylamino)benzoate.

Table III: Several quality analytical parameters of the optimum dispersive mode miniaturized solid-phase extraction-gas chromatography–mass spectrometry (D-µSPE-GC–MS) method

PCP	Slope ± SD*	R^2 †	$S_{y/x}$ ‡	LOD/LOQ§ (µg/L)	E_F ‖	Conc. level: 0.8 µg/L		Conc. level: 4 µg/L	
						RSD# (%) intra-day/interday	RR** (%)	RSD# (%) intra-day/interday	RR** (%)
DEET	463 ± 8	0.999	62.6	0.03 / 0.06	3.1	6.6 / 6.1	105	7.7 / 6.9	100
BP	363 ± 2	0.999	12.3	0.03 / 0.15	5.1	11 / 11	112	7.6 / 7.0	95.8
EHS	62 ± 2	0.998	12.3	0.05 / 0.50	4.0	13 / 11	116	6.4 / 8.8	96.1
BS	291 ± 8	0.998	60.9	0.04 / 0.40	8.2	7.7 / 7.4	92.0	5.5 / 7.5	101
BP3	162 ± 1	0.999	7.50	0.04 / 0.16	26	8.6 / 6.2	94.4	5.6 / 11	96.4
TCS	95 ± 4	0.996	27.6	0.05 / 0.50	23	5.7 / 7.7	86.7	6.2 / 6.6	102
MA	1992 ± 48	0.998	369	0.01 / 0.02	21	2.9 / 3.5	96.8	3.7 / 4.2	96.0
OD-PABA	1800 ± 51	0.998	351	0.01 / 0.03	46	1.7 / 1.7	91.3	6.1 / 4.1	102

* Standard deviation (SD) associated to the slope
† Determination coefficient (R^2) for 6 calibration levels (calibration range: 0.2 to 15 µg/L)
‡ Standard deviation (S) of the residuals
§ Limit of detection (LOD) and limit of quantification (LOQ)
‖ Enrichment factor (E_F)
Relative standard deviation (RSD): intra-day (n = 3) and inter-day (n = 9, in three non-consecutive days)
** Relative recovery (RR)
DEET = *N,N*-diethyl-m-toluamide, BP = benzophenone, EHS = 2-ethylhexyl salicylate, BS = benzyl salicylate, BP3 = benzophenone-3, TCS = triclosan, MA = menthyl anthranilate, OD-PABA = 2-ethylhexyl 4-(dimethylamino)benzoate.

carried out using 1.2 mL of methanol as desorption solvent, but followed by an additional step of evaporation and reconstitution. Thus, the methanol was filtered (0.2 µm), and evaporated under vacuum (~8 min), followed by reconstitution in 100 µL of cychlohexane. This volume was the minimum required to ensure proper mixing of PCPs in cychlohexane within the round-bottom flask of the rotary evaporator. As shown in Figure 2, the E_R values obtained for most PCPs improved significantly, reaching values up to 40%, thus supporting the validity of this simple approach. It must be considered that E_R values close to 100% are rarely obtained in microextraction methods (37), and lower values are acceptable, as long as the sensitivity and precision meet the desired requirements.

Once the adequacy of the solvent-exchange step was shown, while keeping the optimum values of performance for the D-µSPE method, the next step was to test whether higher volumes of cychlohexane were required in the reconstitution, to ensure proper solvation of PCPs in the rotary evaporator glass ball. Thus, 200 µL of cychlohexane was tried, but without clear improvements. Therefore, 100 µL was taken as adequate. Lower values were not tried, because they were clearly insufficient with the 2 mL capacity of the rotary evaporator glass ball.

Ultimately, a slight increase in the polarity

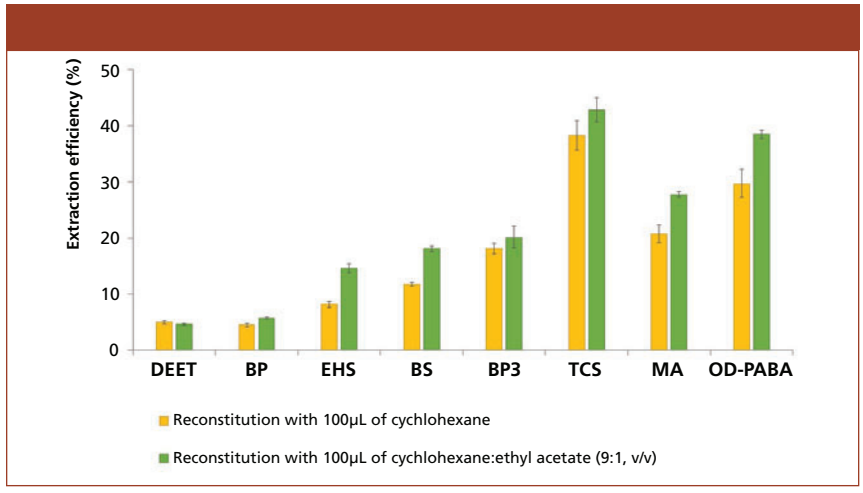


Figure 2: Evaluation of the influence of the volume of the reconstitution solvent: 100 µL (yellow bars), together with the evaluation of the influence of the solvent polarity in the final reconstitution step (green bars). Experiments were carried out in triplicate. Key to abbreviations: DEET = *N,N*-diethyl-m-toluamide, BP = benzophenone, EHS = 2-ethylhexyl salicylate, BS = benzyl salicylate, BP3 = benzophenone-3, TCS = triclosan, MA = menthyl anthranilate, OD-PABA = 2-ethylhexyl 4-(dimethylamino)benzoate.

Table IV: Analysis of samples with the entire dispersive mode miniaturized solid-phase extraction-gas chromatography–mass spectrometry (D-µSPE-GC–MS) method for the monitoring of PCPs, and their recovery study using an intermediate spiked level (4 µg/L)

PCP	Sample 1	Sample 2			Sample 2		
			RR* (%)	E_F†		RR* (%)	E_F†
DEET	n.d.	n.d.	87.1	3.2	n.d.	89.9	3.2
BP	n.d.	n.d.	104	4.9	n.q.	103	4.9
EHS	n.d.	n.d.	108	3.7	5.5 ± 0.9 µg/L	112	3.7
BS	n.d.	n.d.	87.6	8.2	n.d.	109	7.8
BP3	n.d.	n.d.	91.3	26	n.d.	96.4	25
TCS	n.d.	n.d.	112	23	3.0 ± 0.6 µg/L	109	23
MA	n.d.	n.d.	102	21	n.d.	93.1	21
OD-PABA	n.d.	n.d.	91.8	45	n.d.	80.7	43

n.d. = not detected
n.q. = detected, not quantified
* Relative recovery (RR, percent)
† Enrichment factor (E_F)
DEET = *N,N*-diethyl-m-toluamide, BP = benzophenone, EHS = 2-ethylhexyl salicylate, BS = benzyl salicylate, BP3 = benzophenone-3, TCS = triclosan, MA = menthyl anthranilate, OD-PABA = 2-ethylhexyl 4-(dimethylamino)benzoate.

of the reconstitution solvent was considered to aid in the solvation of PCPs in cychlohexane. Therefore, a test was done using a miscible solvent (ethyl acetate) along with cychlohexane, which was compatible with the GC column. Specifically, 100 µL of a mixture of cychlohexane and ethyl acetate with the ratio 9:1 (v/v), was evaluated as the reconstitution solvent. Clearly, the mixture provided better E_R values than those obtained when reconstituting with 100 µL of cychlohexane, as shown in Figure 2, reaching values up to 43%.

In summary, the selected conditions for the D-µSPE-GC–MS method for the group of semivolatile PCPs were the same as the conditions used in the D-µSPE-UHPLC-UV method (36), but subjecting the methanol desorption solvent (containing trapped PCPs) to filtration, evaporation, and further reconstitution in 100 µL of a mixture of cychlohexane and ethyl acetate with a ratio of 9:1 (v/v).

Analytical Performance of the Optimized D-µSPE-GC–MS Method

Several quality analytical parameters of the proposed D-µSPE-GC–MS method, obtained by subjecting aqueous standards to the entire method, are summarized in Table III. The working range of the calibration curve for all PCPs ranged from 0.2 to 15 µg/L. Calibration parameters were obtained with six concentration levels, and the precision study was carried out at two calibration levels: low (0.8 µg/L), and intermediate (4 µg/L) within the calibration range.

Determination coefficients higher than 0.996 were obtained in all cases. LOD and LOQ were estimated in the same manner as in GC–MS, as the concentration that provided 3 and 10 times, respectively, the standard deviation of the signal given for the final extract of an aqueous standard of low concentration, which was subjected to the entire D-µSPE-GC–MS method. LOD values ranged from 0.01 (menthyl anthranilate and OD-PABA) to 0.05 (2-ethylhexyl salicylate and triclosan) µg/L, and LOQ values ranged between 0.02 (menthyl anthranilate) and 0.50 (2-ethylhexyl salicylate and triclosan) µg/L. These values are around 5 and 30 times lower (depending on the PCP) than those obtained with the GC–MS method, and lower than those described in the UHPLC-UV method for the PCPs in common (benzophenone, benzophenone-3, and triclosan) (36).

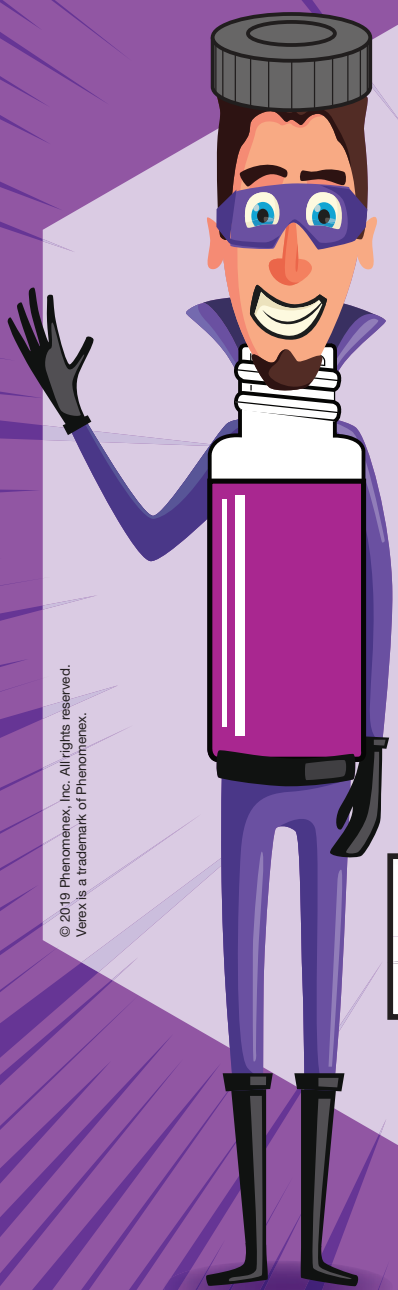
The enrichment factor (E_F) of the developed method was calculated using an intermediate aqueous standard subjected to the entire method in triplicate, ranging from 3.1 for DEET to 46 for OD-PABA, as shown in Table III, being the maximum (theoretical) E_F of 100.

The intermediate precision of the method was evaluated in terms of RSD (in %) for experiments carried out in the same day (intraday, n = 3) and in three different and nonconsecutive days (interday, n = 9, 3 each day), using two concentration levels. For the low concentration level (0.8 µg/L), intraday RSD values were lower than 13%, while interday RSD values were lower than 11%. The relative recoveries (RR) were calculated at both concentration levels, ranging between 86.7% for triclosan and 116% for 2-ethylhexyl salicylate at the low concentration level for triclosan and OD-PABA.

Analysis of samples

The proposed D-µSPE-GC–MS method was applied in the analysis of three samples: two samples of bottled water and one of tap

EVERY LAB SUPER HERO NEEDS A SIDEKICK



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TRAIT:
100% defect-free glass

LIKES:
Reproducible results and cleanroom packaging

DISLIKES:
Extraneous peaks

SECRET POWER:
MEGA TITAN has the ability to transform into 3 levels of certification to guarantee pinpoint accuracy:

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water. Table IV summarizes the results obtained. PCPs were not detected in the bottled water (samples 1 and 2). However, three PCPs were detected in tap water (sample 3). Thus, benzophenone was detected but not quantified, and the quantified PCPs included 5.5±0.9 µg/L of 2-ethylhexyl salicylate and 3.3±0.6 µg/L of triclosan.

Furthermore, two of the studied samples were spiked to carry out a recovery study to evaluate possible matrix effects. The results obtained for samples 2 and 3 using an intermediate concentration level (4 µg/L) are shown in Table IV. RR values ranged from 87.1% for DEET to 112% for triclosan in sample 2, and from 80.7% for OD-PABA to 112% in sample 3, thus indicating that matrix effects were negligible. Moreover, the obtained E_F values for the spiked samples are in agreement with those obtained with the aqueous standards (see Table III), except for OD-PABA.

Figure 1(b) shows a representative chromatogram obtained after the analysis of sample 2 spiked at 4 µg/L and subjected to the entire D-µSPE-GC–MS method.

Conclusions

The promising CIM-81 MOF has been used successfully in a dispersive miniaturized solid-phase extraction method combined with GC–MS to determine a group of personal care products in waters. The resulting method is characterized by being fast (~8 min overall for the entire extraction-desorption step), for requiring low amounts of sorbent (10 mg of CIM-81), for its low sample volume requirements (10 mL of water) and low desorption solvent volumes (1.2 mL of methanol), for ensuring simple compatibility with GC (solvent-exchange step reconstituting with 100 µL of cyclohexane:ethyl acetate, 9:1 ratio), while achieving adequate precision (<13% as interday RSD) and sufficient sensitivity (LODs down to 0.01 µg/L). The reported method ensures a simple adaptation of the D-µSPE approach previously developed for LC, to this method reporting for first time the determination of semivolatile PCPs with GC–MS using D-µSPE with MOFs.

Acknowledgments

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Solid-Phase Microextraction Combined with Flow-Modulated Comprehensive Two-Dimensional Gas Chromatography for Screening Leachables in the Pharmaceutical Industry

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Extractables and leachables (E&Ls) are compounds that may be released from materials that contact drugs during drug storage, which may affect product quality and safety. Such compounds typically are found at trace levels, making their evaluation a difficult task. In this article, we propose a simple method for the analysis of leachables employing solid-phase microextraction (SPME) and comprehensive two-dimensional gas chromatography, coupled to mass spectrometry (GC×GC–MS). This fully automated method bypasses the use of solvents and excessive sample handling. Four potential leachables were tentatively identified.

The United States Food and Drug Administration (US FDA) has recently given increased attention to non-intentionally added substances from contact materials, which may contaminate biopharmaceutical products during the manufacturing process or product storage. Extractables and leachables (E&Ls) are chemical compounds that may have both an organic or inorganic nature (1). Extractables are compounds that can migrate from a contact material under laboratory conditions, like exaggerated temperature, solvent, and time exposure (2). Leachables are contaminants that migrate spontaneously from a contact material (container closure system, packaging, or processing equipment) under normal conditions of product use or storage (2). E&Ls have potential effects on product quality and safety, as they may interact with the drugs reducing its efficacy, and may be toxic to patients, as shown in Figure 1 (3) E&Ls result from product interaction (contact) with filters, gaskets, stoppers, storage bags, cartridges, prefilled syringes, septa, caps, and even from the surface of components used in the manufacturing line (pistons and grinders) (2,3). These impurities are of concern for patients, because of potential effects on product quality and safety. For instance, leachables could interact with a protein drug and form protein adducts, and thus affect the quality of the biopharmaceutical product (4). Moreover, drug efficacy may be dampened by chemical reactions between drug and leachables (cross-reaction), as shown in Figure 1, or by impacting a property of the drug product (pH, appearance, particulate matter) (3). Also, E&L testing should be conducted during manufacturing scale-up after drug regulatory approval.

The degree of concern about E&Ls for any given biopharmaceutical product must be evaluated through a risk-assessment process that accounts for the route of administration and the likelihood of interactions between the dosage form and any leachables. Hence, inhalation aerosols and solutions, injections, and injectable suspensions are of the highest concern; ophthalmic solutions and suspensions, transdermal ointments and patches, and nasal aerosols and sprays are of high concern, as shown in Table I (5). The Product Quality Institute has established a Safety Concern Threshold (SCT) for some contaminants; when above the SCT value, a toxicological evaluation must be performed for leachables (6). Although some contaminants have SCT values, there is still no specific standard or guidance for E&L assessment (5). This is due in part to the difficulty of detecting and identifying such compounds, normally present in trace concentrations. Accordingly, there is

Juliana Crucello, Andre Cunha Paiva, and Leandro W. Hantao

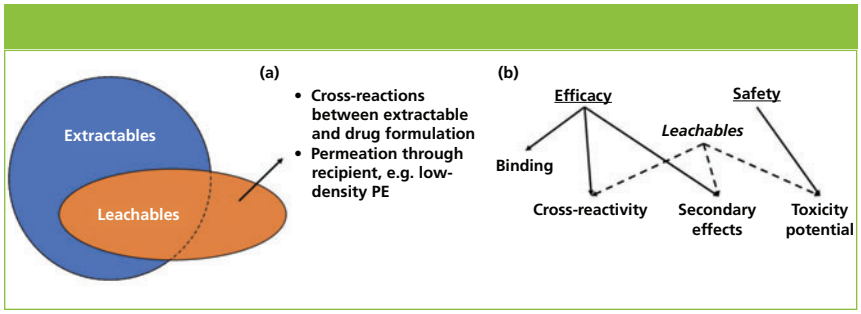

Figure 1: (a) Scope of extractables and leachables (E&L) investigation and (b) dimensions of compatibility assessment.

an urgent need to develop highly efficient and sensitive analytical methods for quality assurance (QA) and quality control (QC).

For instance, some drug impurities are genotoxic and may induce genetic mutations, chromosomal breaks, or rearrangements in humans (7). For example, a tolerable daily intake may be as low as 1.5 µg per day (8). Considering a typical contaminant, such value may translate to low parts per million of impurities in drug substances. However, molecular investigations using conventional techniques such as vibrational spectroscopy, nuclear magnetic resonance, and elemental analysis are somewhat insensitive to trace-level determination of pharmaceutical impurities. The main problem associated with such investigations is the absence of standard protocols. Consequently, E&L research is highly dynamic and generates an aggressive demand for novel and modern analytical methods because E&L studies are product related, which requires extensive and continuous research and development (R&D).

The global pharmaceutical industry has reached unprecedented revenues of $1.11 trillion dollars in 2017 (1). For 2020, the growth is estimated to reach $1.43 trillion dollars. This is a research-driven industry that just in 2015 invested nearly $149.8 billion in R&D projects, being the leading segment in R&D investments. The largest market today is the United States, which accounts for more than 45% of the pharmaceutical market share. In the coming years, it is expected that South American markets will begin to emerge as well. Most of the research efforts are dedicated to drug research, development, and approval. E&L research is an emerging demand of the pharmaceutical industry, and should require considerable investment in R&D projects for quality assurance of E&L from contact materials used in the manufacturing process of the medicines and during product storage. In this context, analytical chemistry is fundamental to tackle the challenges of E&Ls in the biopharmaceutical industry by using high-resolution and sensitive instrumental analysis, such as separations using chromatography and mass spectrometry.

Sample preparation is a mandatory step in almost every analytical method, allowing analyte extraction, cleanup, and preconcentration. Such methods have a high importance for the analysis of trace-level compounds. Contemporary sample preparation techniques have been used for E&L analysis, including Soxhlet extraction, and liquid–liquid extraction (9). Such methods use exhaustive techniques that are both time- and solvent-consuming (10). Besides, exhaustive techniques require multistep procedures that may lead to inaccurate measurements (11). Furthermore, excessive sample handling and multistep methods are more susceptible to sample contamination, compared to single-step procedures, and thus may jeopardize accurate leachable assignment (12,13).

Solid-phase microextraction (SPME) (14) is a solventless and miniaturized technique that enables analyte extraction and preconcentration in a single step (15). This equilibrium-based technique was introduced by Pawliszyn in the early 1990s, and has been applied successfully in many fields of analytical chemistry. SPME may be used to isolate analytes from the headspace (HS), or by direct immersion (DI) into the sample solution, depending on the Henry's law constant of the target compound (16). The latter, DI mode, is recommended for analysis of compounds in low levels in clean matrices, such as drug products (17), being a promising technique for E&L screening.

Comprehensive two-dimensional gas chromatography coupled to mass spectrometry (GC×GC–MS) is a powerful technique for the analysis of volatile and semivolatile complex mixtures, offering unprecedented peak capacity and detectability (18). The principle of GC×GC resides in the hyphenation of two sequential and complementary GC separations in a single run (18). Improved resolving power is attained by a two-factor combination, namely, coupling of two GC columns with distinct selectivity, and highly efficient transfer of the effluent of the primary (^{1}D) to the secondary column (^{2}D) as sharp solute bands. Traditional GC×GC applications relied mostly on thermal and cryogenic interfaces for solute reinjection (19). As a consequence, the costs of acquisition, operation, and upkeep have prevented GC×GC from being considered for routine analysis. Conversely, flow modulation (FM) is an inexpensive and robust technique for GC×GC interfacing, bypassing the need for expert users. For instance, we estimated that a flow modulator enabled a twentyfold reduction in the operating costs compared with a liquid nitrogen (LN_2) based cryogenic interface based on our laboratory expenses in the years 2017 and 2018. Furthermore, an 86% savings may be attained with FM when compared to a consumable-free N_2 based thermal modulator. In the most efficient setup (20), the modulator uses a microfluidic interface with a small footprint and an auxiliary gas, which operates at typical flow rates up to 20 mL/min. Flow modulation comprises two steps, namely, sampling and reinjection. For solute sampling, the auxiliary gas directs the ^{1}D effluent to an 0.53-mm-id sampling loop (up to 50 µL). Next, the auxiliary gas is actuated and a countercurrent surge of gas sweeps the bands into the ^{2}D. Under typical chromatographic conditions, comprehensive modulation is ensured for sensitive analysis. Flow modulators have been successfully used in GC×GC for the analysis of fragrances and allergens, aroma-related compounds in beverages, and analysis of fuels (21–23). Hence, FM-GC×GC analysis may be an interesting alternative for reliable E&L screening, being particularly suited for trace level investigations.

In this article, we discuss the potential of direct immersion solid-phase microextraction and flow-modulated GC×GC–MS for screening leachables in biopharmaceutical drugs. This case study evaluated potential extractables from a nasal drug solution by DI-SPME and FM-GC×GC–MS. Data mining was performed by using a pixel-based approach, namely, multilinear prin-

Table I: Modified FDA/CDER/CBER risk-based approach to consideration of leachables. Reproduced from *USP* 39 (2016). Note: CDER is the FDA Center for Drug Evaluation and Research, and CBER is FDA Center for Biologics Evaluation and Research.

Examples of packaging concerns for common classes of drug products			
Degree of concern associated with the route of administration	Likelihood of packaging component–dosage form interaction		
	High	Medium	Low
Highest	Inhalation aerosols and sprays	Injections and injectable solutions	Sterile powders and powders for injection; inhalation powders
High	Transdermal ointments and patches	Ophthalmic solutions and suspensions; nasal aerosols and sprays	–
Low	Topical solutions and suspensions; topical and lingual aerosols; oral solutions and suspensions	–	Oral tablets and oral (hard and soft gelatin) capsules; topical powders; oral powders

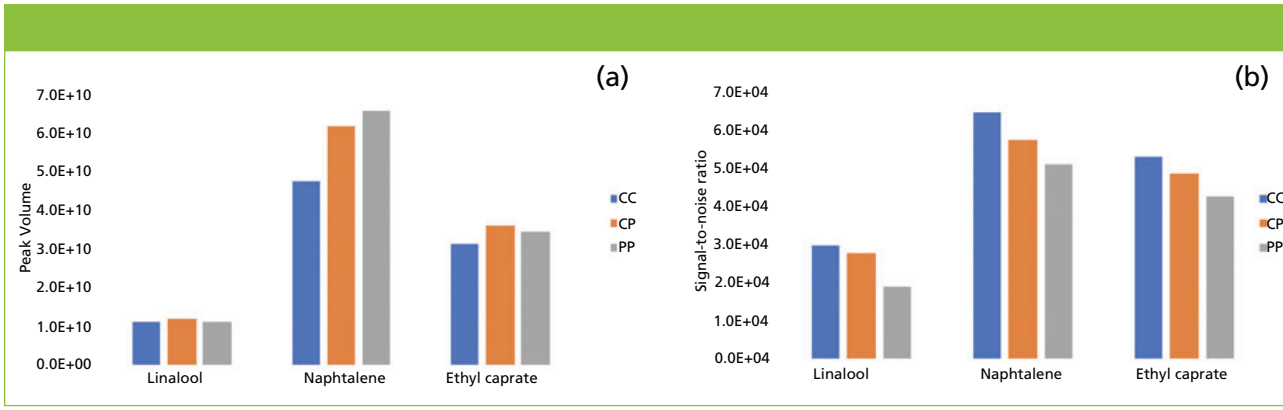

Figure 2: Bar graph comparing GC×GC methods considering (a) peak volume, and (b) signal-to-noise ratio. Carrier and auxiliary gases were operated at constant–constant (CC) flow; constant–programmed flow (CP), and programmed–programmed (PP) flow.

cipal component analysis (MPCA). The proposed method eliminated the use of organic solvents and greatly reduced sample manipulation, being an ideal solution for fully automated and high sample throughput QA and QC methods.

Materials and Methods

Samples and Chemicals

The nasal solutions studied (Batch ABCD-2044) were purchased from a local drug store. Ethanol and methanol were purchased from Honeywell (Morris Plains, NJ, USA). The ultrapure water (18.2 MΩ-cm) used was supplied by a Direct-Q water purification system (Merck KGaA, Darmstadt, Germany). Linalool, ethyl caprate, and naphthalene standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). A 50:30 mm polydimethylsiloxane:divinylbenzene (PDMS–DVB) SPME fiber (Sigma Aldrich) was used for analyte isolation. A set of 20 mL clear glass vials, magnetic screw caps, and PTFE–PDMS septa were used throughout the SPME extractions (Sigma-Aldrich).

Evaluation of SPME Desorption Conditions

The PDMS–DVB SPME fiber was conditioned as recommended by the manufacturer. An aqueous working solution composed of 50 mg/mL of linalool, ethyl caprate, and naphthalene was used to evaluate SPME desorption conditions.

In the SPME experiments, 20 mL of working solution was placed into a 20 mL glass vial. Sample was pre-equilibrated at 40 °C for 5 min. DI-SPME was performed by exposing the fiber to the working solution at 40 °C for 15 min. Afterwards, the fiber was desorbed in the GC inlet at 260 °C for 1 min. Each sample was analyzed in quadruplicate for each desorption method, as detailed in Table II.

Profiling Leachables in Nasal Solution

Assignment of leachables used a side-by-side comparison between a control group and a stressed group of nasal solution samples. A total of eight commercial samples were purchased from the same batch (undisclosed manufacturer). In all experiments, the samples were used as received. An accelerated stability test was performed to produce the stressed group of commercial samples, which were kept at 50 °C for 30 d (Jeio Tech, Seoul, South Korea). In the stability experiments, the positioning of the sample boxes was standardized and kept horizontal to ensure solution contact with all components of the low-density polyethylene (PE) flask.

Sample unboxing was only performed prior to chromatographic analysis. For the SPME experiments, aliquots of 20 mL of nasal solution were transferred to 20 mL glass vials. Samples were pre-equilibrated at 45 °C for 5 min. The PDMS–DVB fiber was exposed to the sample solution for equilibration at 45 °C for 30 min. For GC×GC analysis, the fiber was desorbed at the GC inlet for 1 min at 260 °C. The SPME fiber was rinsed with 1:1 (*v/v*) water:methanol solution between extractions to prevent salt deposit.

GC×GC–MS Method

All analyses were performed on a GC×GC system, comprised of a Trace 1310 GC

Table II: Carrier gas and auxiliary gas conditions evaluated for the SPME fiber desorption optimization

Condition	Carrier gas flow	Auxiliary gas flow
Constant carrier gas and constant auxiliary gas (CC)	1.00 mL/min	12.80 mL/min
Constant carrier gas and programmed auxiliary gas (CP)	1.00 mL/min	5.00 mL/min for 1 min and then 12.80 mL/min at 8 mL/min^2
Programmed carrier gas constant and programmed auxiliary gas (PP)	1.50 mL/min for 1 min and then 1 mL/min at 1 mL/min^2	5.00 mL/min for 1 min and then 12.80 mL/min at 8 mL/min^2

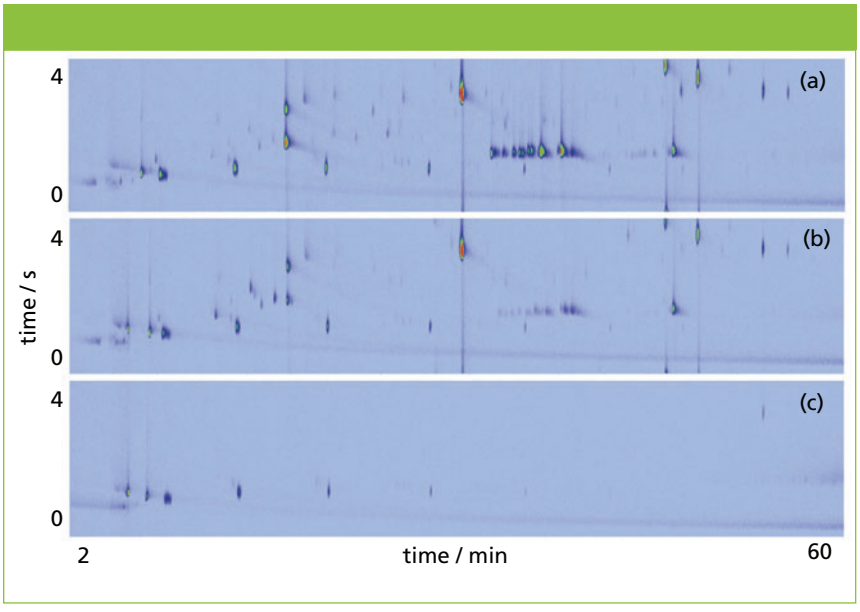

Figure 3: Total ion chromatograms obtained by GC×GC–MS of (a) the control sample, (b) the stressed sample, and (c) the blank using DI-SPME sampling with PDMS–DVB fiber.

intrument, ISQ single quadrupole mass spectrometer, and Triplus RSH Autosampler (Thermo Scientific, Waltham, MA, USA). Differential flow modulation using the reverse fill-flush configuration was attained using Insight modulator (SepSolve Analytical, Frankfurt, Germany).

The final method employed helium as the carrier gas and auxiliary gas at constant flow rates of 1.00 mL/min and 12.80 mL/min. Sample introduction was performed using splitless injection and 1.00 min sampling time.

All separations were carried out using a 30 m x 0.25 mm (0.20 µm) Mega-5 primary column and a 5 m x 0.25 mm (0.20 µm) Mega-17 secondary column (Mega, Legnano, MI, Italy). A restriction capillary of 5 m x 0.10 mm and 21 cm x 0.53 mm (50 µL) sampling loop were used for flow modulation. A scanning range of 50 to 450 *m/z* units was used for signal acquisition (19 scans/s). Transfer line and the ion source were operated at 300 and 275 °C, respectively.

Data Analysis

XCalibur software (Thermo Scientific) and ChromSpace (SepSolve Analytical) were used for instrument control and data acquisition. GC Image (Zoex, Houston, TX, USA) was employed for tentative identification of analytes by combining mass spectrum similarity and linear temperature programmed retention index (LTPRI) filtering. A minimum similarity match of 80% was considered. In addition, the chromatograms were exported to ANDI/netCDF. MATLAB R2014b (MathWorks, Natick, MA, USA) was used to perform pattern recognition. PLS Toolbox 7.5 (Eigenvector Research Inc., Wenatchee, WA, USA) was used to perform multilinear principal component analysis (MPCA).

Results and Discussion

General Considerations for Method Optimization

Flow modulation comprises two stages, namely, sampling (fill) and re-injection (flush) (20). The analyst must select the modulation period within the maximum sampling time allowed by the chromatographic conditions. A general rule is to avoid modulation conditions that exceed 75% of the capacity of the sampling loop to ensure sensitive analyses.

A general guideline may be attained by considering the maximum sampling duration and minimum reinjection duration. Such parameters may be estimated by the equations listed below.

Fill = (0.75 × volume of sampling loop) / ^{1}D flow rate

Flush = (1.5 × sampling loop) / ^{2}D flow rate

Analyte desorption must also be considered when coupling SPME and GC×GC. High linear velocities of carrier gas around the coating during analyte desorption is required to improve mass transfer and peak efficiency (17). Flow modulators, unlike thermal interfaces, typically operate at lower flow rates in the primary column. Therefore, we have designed three representative methods, as detailed in Table II, that may be used to improve mass transfer in the injection port, while retaining the flows required for proper band modulation. The impact of such conditions was evaluated using a stock solution of linalool, ethyl caprate, and naphthalene.

The flow rate in the primary column increased in the following order: method CC < method CP < method PP, where CC is constant–constant flow, CP is constant–programmed flow, and PP is programmed–programmed flow. Clearly, there is a significant improvement in peak volume for all analytes when increasing ^{1}D flow rate, as shown in Figure 2. Although there was an improvement in mass transfer under such conditions, the signal-to-noise ratio was compromised by increasing the injection pulse. Considering that signal-to-noise ratio plays a critical role in peak detection, method CC that uses constant carrier gas flow and constant auxiliary gas flow rates was selected for the analysis of leachables.

Table III: Tentative identification of leachables in nasal spray after accelerated stability experiment

Compound name	Formula	CAS	LTPRI	Possible source
Methoxy-phenyl-oxime (MPO)	$C_8H_9NO_2$	241-114-2	922	Used in polymer synthesis, crosslinker
4-Methyl-2-propan-2-yl-1,3-dioxolane (MPD)	$C_7H_{14}O_2$	67879-60-1	985	Used in polymer synthesis, solvent or reactant
Butyl isocyanato-acetate (BIC)	$C_7H_{11}NO_3$	17046-22-9	704	Used in polymer synthesis, Passerini reaction
Diethyltoluamide (DEET)	$C_{12}H_{17}NO$	134-62-3	1622	Active ingredient in insect repellent

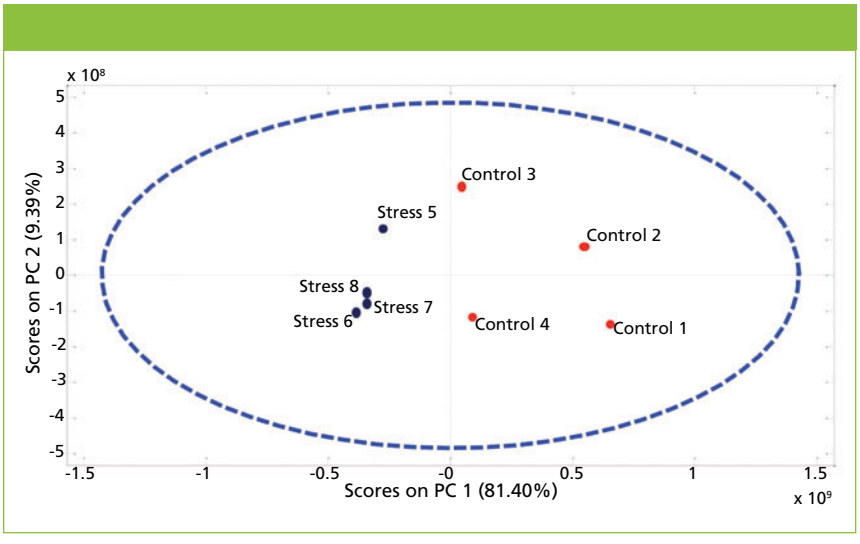

Figure 4: Pixel-based, two-component MPCA scores plot of evaluated samples, namely, nasal solutions after accelerated stability experiment (Stress) and control group (Control).

Screening of Leachables from a Nasal Drug Solution

Nasal solutions are of high concern in risk assessment for E&Ls. Since leachables may exhibit high toxicity, there is an urgent need to detect and assign such contaminants. An accelerated stability assay uses exaggerated exposure conditions to evaluate leachables. Thus, comparative analysis of samples from the control and stressed groups readily enabled assignment of these contaminants.

The selected nasal spray is sold in a low density polyethylene (PE) bottle. This polymer is readily susceptible to contamination by diffusion of contaminants through the wall into the drug solution. Consequently, components from the cardboard box, label, ink, and adhesive may leach into the solution during the accelerated stability experiment. To screen for leachables, control and stressed samples were analyzed by DI-SPME and GC×GC–MS.

GC×GC–MS generates a large amount of structurally complex data, wherein important information may be lost or overlooked during conventional data analysis. For instance, visual inspection of the chromatograms in Figure 3 indicated variations in profile of the control and stressed samples. For proper assignment of leachables, multiway principal component analysis (PCA) was applied for pattern recognition. A two-component scores plot revealed significant differences between the GC×GC profiles of stressed and control samples along the first principal component (PC), as shown in Figure 4.

It can be readily seen from Figure 4 that stress-related peaks can be found in the negative scores and loadings. Therefore, a leachable assignment was performed by template matching using GC Image software. First, a virtual chromatogram was reconstructed using a vector that comprised the negative loadings of PC-1. This image was composed of pixels with proven variance between stressed and control samples and exhibited the shapes of chromatographic peaks (Figure 5). Second, the virtual chromatogram was used to create a template. This template was loaded and applied to the sample chromatograms for leachable identification. The reader is directed elsewhere for a detailed description of this procedure (22,24).

Tentative peak identification of leachables is shown in Table III. Four analytes were found exclusively in stressed samples and assigned as potential leachables, namely methoxy-phenyl-oxime (MPO), 4-methyl-2-propan-2-yl-1,3-dioxolane (MPD), butyl isocyanatoacetate (BIC) and N,N-diethyl-m-toluamide (DEET). MPO is a common non-intentionally added substance that was reported in PE-based milk packaging (25–27). BIC is commonly used in polymer synthesis as part of Passerini reaction (28), while 1,3-dioxolane is used as an aprotic solvent for use in formulation, production processes, and as a reactant in polymer synthesis, which may explain the presence of MPD. Noteworthy, DEET is an active ingredient of commercial insect repellents. This compound is extremely mobile and persistent (29). DEET may have originated from the cardboard contamination or shipping pellets.

Conclusions

Advances in research in the biopharmaceutical industry have contributed to the decline of diseases and to the industry's economic expansion. However, there are still high-risk areas that need to be studied, such as those involving drug storage materials. These materials may represent a potential source of contamination from the migration of extractables and leachables. In this article, we discussed the application of solid-phase microextraction and comprehensive two-dimensional gas chromatography coupled to mass spectrometry for screening leachables in a nasal spray solution. SPME is an interesting alternative to solvent-based techniques for E&L experiments, bypassing the need for solvents and cumbersome liquid handling. Furthermore, flow

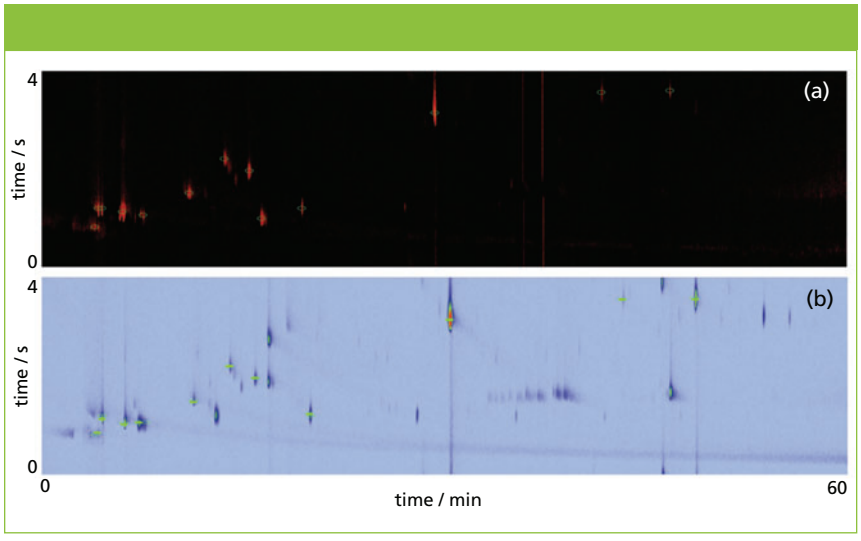

Figure 5: Creation of peak template using the (a) loadings vector, reconstructed chromatogram. (b) showing template matching process using GC Image highlighting potential leachables in the GC×GC–MS total ion chromatogram of a stressed sample of nasal solution.

modulated GC×GC–MS is an inexpensive technique that may be adopted by the pharmaceutical industry for routine analysis, given that FM is more robust and user-friendly than thermal–cryogenic modulation. This method is fully automated, making it ideal for routine analysis in QA and QC laboratories. The results from this study may assist both industry and regulatory agencies to ensure quality and safety for drugs. We hope this proof-of-concept application may also guide regulatory agencies to develop standardized protocols extractable and leachable studies.

Acknowledgments

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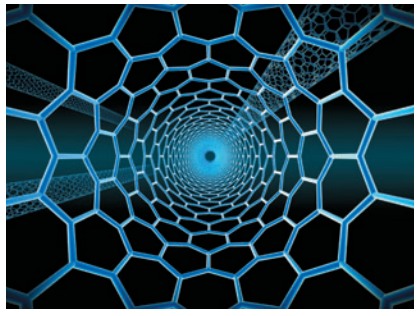
Nanomaterials Have Come to Stay: An Overview of their Use as Sorbents in Sample Preparation



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Nanomaterials occupy an important place in current research and applications in analytical chemistry and, in particular, as extraction sorbents for sample preparation. Nanomaterials are useful as sample preparation sorbents as a result of their varied morphologies, high surface area, surface-to-volume ratio, and porosity, as well as their ability to interact in a variety of ways. The sorbent-based extraction procedure in which nanomaterials have been mostly commonly applied is dispersive solid-phase extraction (dSPE), but these materials also have been applied in other sample preparation techniques, such as solid-phase microextraction, stir-bar sorptive extraction, and matrix solid-phase dispersion. Carbon-based nanomaterials are the most commonly used in sample preparation, but other types of materials used include metal–organic frameworks, covalent organic frameworks, nanoparticles combined or modified with other materials or nanomaterials, quantum dots, electrospun nanofibers, and dendrimers.

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Judging from the high number of advances and applications of nanoscience and nanotechnology in recent years, it is clear that nanomaterials (structures with at least one dimension in the 1–100 nm range) have come to stay in our daily life, and, in particular, in the analytical chemistry laboratory. Some of these are nanomaterials themselves, whereas others can be suitably modified or combined to create nanomaterial units.

Sample preparation, the workhorse of analytical chemists, has found in nanomaterials an extraordinary ally, as a result of their varied morphologies, high surface area, surface-to-volume ratio, and porosity, as well as their ability to interact in a variety of ways. At the nanometer level, surface physics and chemistry start to dominate a material's properties, which also depend on the material's three-dimensional morphology, the surrounding media, and the material's arrangement in space (1). Properly considered and managed, these properties can be of high utility. In particular, such properties are highly beneficial in sorbent-based extraction procedures like solid-phase extraction (SPE), solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE), membrane stir extraction (SME), stir fabric phase sorptive extraction (SFPSE), and matrix solid-phase dispersion (MSPD) (2,3), and, therefore, they have been applied mainly in such procedures for analyte enrichment or storage, sample fractioning, or cleanup, as well as support for derivatization reactions in nearly any type of sample matrix. Moreover, nanomaterial applications in sample preparation have also facilitated the development of miniaturized and more environmentally friendly procedures, which are key challenges and trends in analytical chemistry (4).

To date, the main use of nanomaterials in sorbent-based extraction methods has been in dispersive SPE (dSPE), in various operational modes, assisted by stirring, vortexing, or ultrasound procedures (Figure 1). With dSPE, the fact that bulk materials that quickly enter in contact with the sample or extract can be used, along with

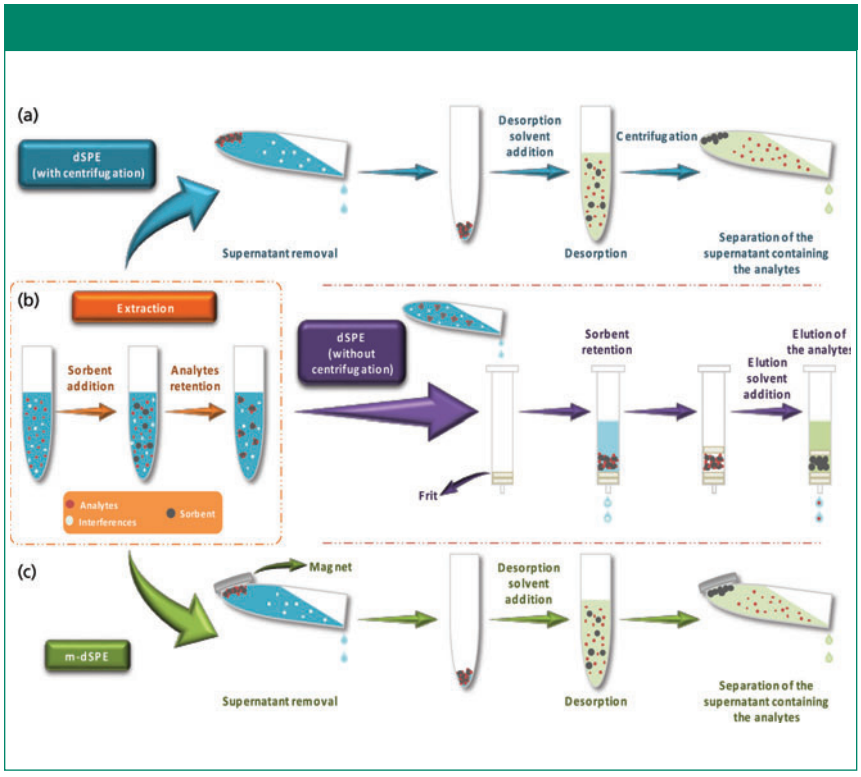

Figure 1: The most common dispersive solid-phase extraction (dSPE) modes in which nanomaterials are applied: (a) dSPE with centrifugation, (b) dSPE without centrifugation, and (c) magnetic dSPE (m-dSPE) procedures. Adapted with permission from (5).

its simplicity and rapidity, has made dSPE an excellent choice for many applications. Of particular interest has been the application of magnetic sorbents, which were introduced in 1996 by Towler and colleagues (6), because the application of an external magnetic field considerably simplifies and facilities the procedures, saving time and effort. With SPE, in some cases nanomaterials have even been used in pipette-tip SPE (7), confined in small devices, bags, or disks that are inserted into the sample or extract, or in multiwell plates (8). As for SPME or SBSE coatings, when using nanomaterials, suitable, repeatable, and durable coating procedures must be achieved, which is not an easy task. For that purpose, physical adhesion, in-situ growth, or chemical bonding methods are used (9).

In general, the nanoscale materials that scientists are able to produce have some uncertainties and irregularities in their shape, size, and chemical composition. That fact has not limited their application as sorbents, however, because a certain degree of control over them can be ultimately achieved.

Table I: Examples of the application of nanomaterials in sorbent-based extraction procedures

Analytes	Matrix	Nanomaterial	Extraction technique	Determination technique	Recovery (%)	Reference
6 PCBs	Snakehead fish, crucian carp, grass carp	Fe_3O_4@MOF-5(Fe)	SBSE	GC–MS	93–97	(12)
6 PAHs	Grilled eggplant, soil	Graphene-polydopamine-stainless steel	SBSE	HPLC-FD	89-114	(13)
5 pesticides	*Annona muricata*	$[(Nd_{0.9}Eu_{0.1})_2(DPA)_3(H_2O)_3]$	MSPD	HPLC-DAD	78–88	(14)
Acrylamide	Potato, flour	Chitosan-MWCNT-o	MSPD	HPLC-UV	85–95	(15)
Chloramphenicol	Pork and honey	Fe_3O_4@MIP	m-dSPE	HPLC-UV	95-107	(16)
15 PAHs	Pork, fish, grilled fish, smoked bacon, coffee	F_3O_4@COF-(TpBD)	m-dSPE	HPLC-DAD	84–107	(17)
6 adulterants	Tea	MWCNTs	dSPE	CE-PDA	71–105	(18)
7 herbicides	Peanuts	MIL-101	dSPE	HPLC-DAD	90–103	(19)
6 PAHs and 3 PAEs	Potatoes	GO@IL	SPME	GC-FID	78–102	(20)
6 penicillins	Milk	MIL-53@MIP	SPME	NanoUHPLC-UV	81–101	(21)
4 pesticides	Pear, grape, and eggplant	MWCNTs@SiO_2	SPME	GC-IMS	79–99	(22)

Key to Abbreviations: BD, benzidine; CE, capillary electrophoresis; COF, covalent organic framework; DAD, diode array detector; DPA, pyridine-2,6-dicarboxylic acid; dSPE, dispersive solid-phase extraction; FD, fluorescence detection; FID, flame ionization detection; GC, gas chromatography; GO, graphene oxide; HPLC, high performance liquid chromatography; IL, ionic liquid; IMS, ion mobility spectrometry; m-dSPE, magnetic-dispersive solid-phase extraction; MIL, Material of the Institute Lavoisier; MIP, molecularly imprinted polymer; MOF, metal–organic framework; MS, mass spectrometry; MSPD, matrix solid-phase dispersion; MWCNT, multiwalled carbon nanotube; PAE, phthalic acid ester; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; PDA, photodiode array; SBSE, stir-bar sorptive extraction; SPME, solid-phase microextraction; Tp, 1,3,5-triformylphloroglucinol; UHPLC, ultrahigh-pressure liquid chromatography; UV, ultraviolet.

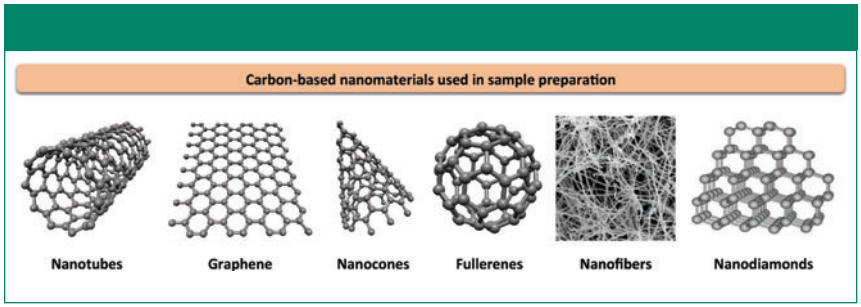

Figure 2: Structures of carbon-based nanomaterials used in sample preparation.

Carbon-Based Nanomaterials

Carbon-based nanomaterials are one of the most extensively used types of nanomaterials applied to sample preparation. The reason for their extensive application probably lies in their ability to establish strong π-π interactions, because carbon-based structures are composed of large conjugated π-systems, as well as Van der Waals interactions (as a result of fluctuating dipole moments caused by the distortion of the planar graphene sheet), in combination with their high stability, tensile strength, and so on, and the abovementioned typical features of nanomaterials. In this sense, pristine carbon-based nanomaterials have provided excellent results for the extraction of a wide variety of nonpolar organic compounds (10). The extraction of metals can also be achieved with pristine materials, though in that case, a prior complexation step is often necessary. On the other hand, polar compounds normally can be extracted using these nanomaterials, following a suitable functionalization (covalent or noncovalent) of the nanomaterial surface (11). Table I lists some research in which different nanomaterials, including carbon-based nanomaterials, have been applied as sorbents.

Among carbon-based nanomaterials, multiwalled carbon nanotubes (MWCNTs) and graphene are the two carbon allotropic forms most commonly used in sample preparation; in the first case, probably because they were the first to be synthesized and also the most commercialized. Others, such as single-walled carbon nanotubes (SWCNTs), fullerenes, carbon nanohorns, carbon nanofibers, and nanodiamonds (Figure 2) have also been used in this sense, though less frequently (23).

Although the application of pristine MWCNTs or graphene continues to have interest, current research is mainly focused on modifying their surface to increase their extraction selectivity and capacity, as well as to improve their solubility in many solvents, which is one of the main drawbacks for their application in extraction procedures. Thus, the particularities of their structures make it easy to modify them with molecules with aromatic systems (such as surfactants and polymers) by means of noncovalent interactions (11). However, covalently functionalized MWCNTs are now used more extensively, because of their simplicity and because a wide variety of molecules can be bound to the surface of these nanomaterials, generally starting by the simple oxidation of their sidewalls, which mainly introduces –COOH, –OH and –CO– groups into their structures (11,23).

Although graphene can be modified with molecules containing benzene rings, graphene oxide (GO) has become the most widely used way to functionalize this material; it is the most common method to obtain graphene based on the oxidation and exfoliation of graphite, which is also really cheap. In addition, carboxyl and hydroxyl groups present on the surface of graphene enable the binding of a wide variety of molecules, resulting in an important expansion of graphene's applicability (22,24), though one of its main drawbacks is the fact that it is an ultralight material that is difficult to retrieve from a suspension, even by high-speed centrifugation.

In general, the great surface versatility of carbon-based nanomaterials is the main reason for their success in sample preparation, because it has allowed not only their use in all SPE modes, but also in the extraction of a broad spectrum of compounds, having been principally applied to the analysis of pesticides (10).

Lastly, it is important to mention that, despite the good features that carbon-based nanomaterials have proven to have as sorbents, these materials also have been combined with other materials to provide them with other properties or to improve their selectivity. In this sense, as it will be later shown, their combination with magnetic nanoparticles (mNPs) has been the most common. However, they also have been combined with polymers, metal-organic frameworks (MOFs), or even other carbon-based nanomaterials, among others (2,3,9). At this point, it is important to highlight that such combinations should be properly justified, demonstrating that the extraction performance of the composite is better than that of the materials separately, which has not always been proven in the literature.

Metal–Organic Frameworks

MOFs are microporous inorganic–organic crystalline structures presenting a three-dimensional and highly ordered structure (8) that when produced at extremely small sizes, can be considered and used as nanomaterials. Among current nanomaterials, MOFs have probably been the least explored in sample preparation, perhaps as a consequence of the high number of crystalline structures that can be created. However, it is precisely this fact that makes them so interesting as sorbents, because it makes it possible to control the features of the material. In addition, MOFs can boast being the nanomaterials with the largest surface area (from ~200 to ~7000 m^2/g), as a result of their extremely high porosity. Finally, their capacity to be modified makes them almost the ideal sorbent to be used in SPE and dSPE procedures (8). In recent years, there also has been a noticeable increase in the number of studies in which MOFs have been used as SPME coatings (25), even more than other nanomaterials.

When working with MOFs, it is important to consider that these structures are not normally stable in solution, because their open metal sites are easily occupied by water, resulting in damage to the structure and a loss of extraction capacity (8,26). Thus, although bare MOFs have demonstrated a high extraction capacity, their post-synthesis modification constitutes one of the main approaches to improve their hydrostability. Another common practice consists of the carbonization of the MOF structure to obtain an extremely porous carbon material that preserves the structure of the MOF used (though it is not a MOF itself). In some cases, it has been suggested that MOFs should be thermally activated before their use as sorbents to prevent solvent molecules from getting trapped in the

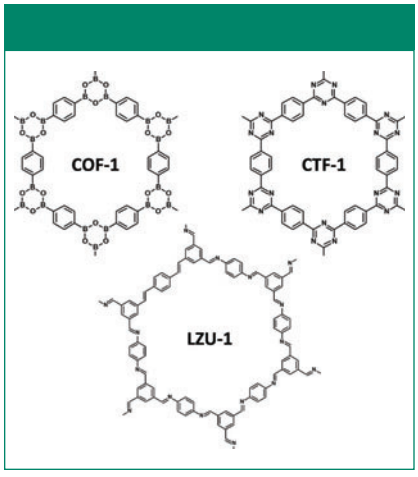

Figure 3: Structures of typical covalent organic frameworks (COFs) used in separation science.

MOF's pores during synthesis (27). Combining MOFs with water-repellent materials constitutes another alternative, though less frequently used (26). However, water-stable MOFs sometimes need to be functionalized, principally for three reasons: to reduce their hydrophobicity (to improve their contact with aqueous samples and to increase the adsorption of hydrophilic compounds); to enhance the selectivity of the nanomaterial; and to meet the demands of the extraction mode in which they are to be applied (26).

Like carbon-based nanomaterials, MOFs also have been combined with other materials to modify their extraction performance or to facilitate their separation from the sample. Once more, mNPs have been the material selected in most applications because of the advantages derived from their use. However, many combinations with carbon-based nanomaterials, polymers, or ionic liquids have also been developed (2,9).

Covalent Organic Frameworks

Although MOFs have been demonstrated to be excellent sorbents for the extraction of a wide variety of molecules of different natures and sizes from a broad variety of matrices, during the last decade, covalent organic frameworks (COFs) have emerged as an alternative. COFs contain only light elements like H, B, C, N, O, and Si, providing an ordered crystalline organic polymeric structure via covalent bonds, but without metallic ions (28). They have good chemical and thermal stability, a tunable porous structure (though their porosity is not as high as that of MOFs), and low density (29). Their application as sorbents has been mainly carried out in SPME, though much less frequently than the rest of the materials discussed here. Figure 3 shows a schematic representation of typical COFs used in separation science.

Nanoparticles Combined or Modified with Other Materials or Nanomaterials

As previously indicated, since the introduction of magnetic SPE (mSPE) (6), mNPs have gained importance as sorbents in sample preparation procedures, because they provide clear advantages, such as a significant reduction of analysis time and an important simplification of the extraction procedure. The iron oxides magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3), especially the former, have been the mNPs most often used, principally because of their low toxicity, ease of manipulation and synthesis, and their ability to be modified, compared to other mNPs (30,31). Others composed of metals like iron, nickel, and cobalt, or their oxides, and spinel-type ferromagnets such as $MnFe_2O_4$, $MgFe_2O_4$, and $CoFe_2O_4$, have also been used.

It should be noted that, although in certain applications this kind of mNP has been used as synthesized, without further modifications, they are only rarely used in analytical extraction methods, because of their high tendency to aggregation and their chemical reactivity, which results in a loss of dispensability (dispersibility) and magnetism (30). Trying to solve these problems, as well as to improve their selectivity and extraction capacity, the most common strategy consists of coating mNPs using different organic and inorganic materials, resulting in a core–shell structure (30,31) as shown in Figure 4. However, achieving a perfect core–shell structure is extremely difficult and rare.

Among the various inorganic materials that have been selected as coatings, silica is one of the most widely used, probably because of its stability and low price. However, an important factor that has favored the extensive use of silica has been its numerous silanol groups, which can be easily modified with other functional groups, with octadecylsilane being the most common, though the most extended practice involves the use of the silica shell only as a support to attach other materials (30,31). On the other hand, the variety of organic coatings that has been used with this aim is much more extensive, including a good variety of surfactants, ionic liquids, antibodies, and aptamers, as some of the most common. Polymeric coatings (polymer-based nanosorbents) have been the most widely used by far, because they have demonstrated good stability and some of them can even bind directly to the surface of mNPs. Among them, molecularly imprinted polymers have become one of the preferred alternatives, as a consequence of their high selectivity and specificity (30,31).

The mNPs previously mentioned have been demonstrated to have a good surface-to-volume ratio, and provide a high extraction capacity in a wide number of applications. However, the trend in recent years has been to focus on combining mNPs with new sorbent materials, and especially nanomaterials, with the goal of combining the high adsorption capacity of nanomaterials and the separation convenience of magnetic materials (30). In this sense, the combination of mNPs with carbon-based materials, especially CNTs and graphene, has been one of the most extensive, as a result of their abovementioned excellent properties (2,3). Such combinations normally consist of the deposition of mNPs onto the surface of the carbonaceous materials, taking advantage of electrostatic forces. However, in certain applications using CNTs, the mNPs have been inserted inside the tubular structure (9). In addition, in recent years mNPs have been combined with several MOFs (Figure 5) (8) or COFs (29), providing excellent results. The synthesis procedures for these combinations are very diverse. Thus, carbon-based materials need to be first functionalized, while MOFs and COFs can be synthesized around the surface of the mNPs, though in some cases these materials can be combined using an ultrasound process (9).

As previously mentioned, all these magnetic materials are isolated from the sample matrix using an external magnetic field, which is highly convenient. However, it is important to highlight that, when working with such strong magnets in the laboratory, suitable precaution should be taken, because they can stick to any metallic furniture of the laboratory or even to other magnets.

Although mNPs are the type of NP most extensively used in sample preparation, nonmagnetic NPs have also been used as sorbents in various extraction techniques, although in fewer applications. In this sense, Al_2O_3 and especially SiO_2 NPs, have been the most commonly used, probably as a result of their stability and the fact that

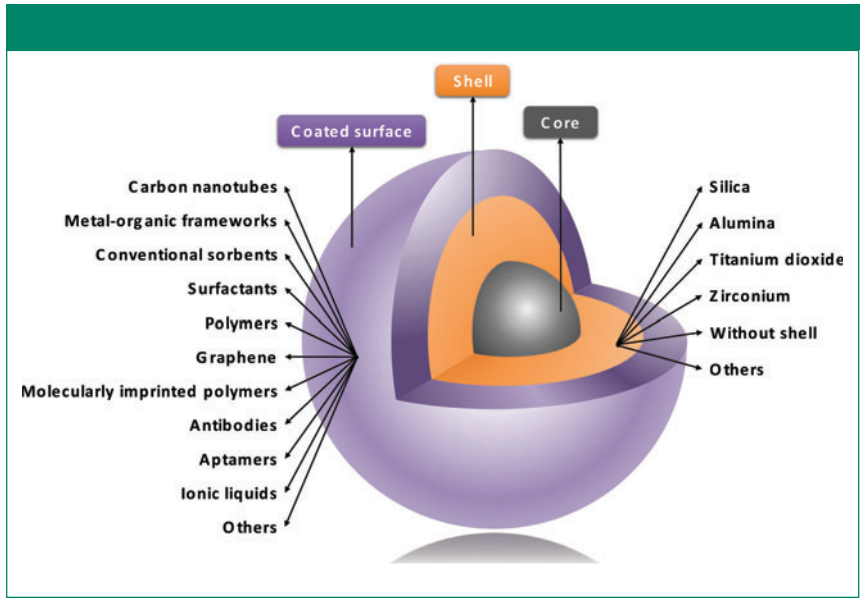

Figure 4: Scheme of the core–shell structure and materials most commonly used as shells and coatings for magnetic nanoparticles used for extraction purposes. Adapted from (35).

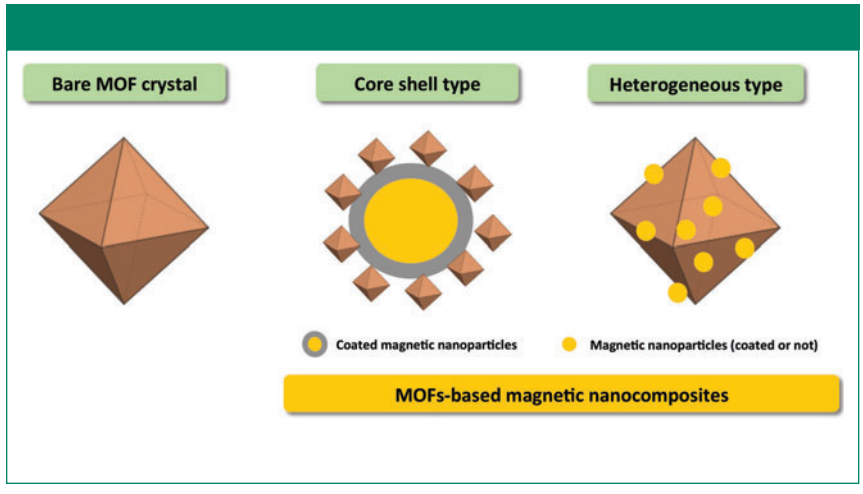

Figure 5: Schematic representation of a bare MOF, a core-shell type magnetic composite and an heterogenous magnetic composite based on a MOF.

they can be functionalized or coated with other materials, particularly polymers (2,3).

Quantum Dots

Quantum dots (QDs) are defined as "small crystals containing a variable number of electrons that occupy well-defined, discrete quantum states and have electronic properties intermediate between bulk and a discrete fundamental particle" (32). QDs usually have diameters ~2–10 nm, and consist of a semiconductor core overcoated by a shell, and a cap that improves solubility in aqueous buffers (33). During synthesis, different compounds are added so that they ultimately form ligands on the surface of the core. Such ligands are key parameters in determining their applicability.

In general, QDs are characterized by poor extraction selectivity, so they are normally used as sorbents after a modification of their surface or their combination with other materials (3). Carbon-based QDs have been some of the most widely used for extraction purposes. They are a new class of environmentally friendly carbon materials with high water solubility, negligible toxicity, good stability, and small size (34). Graphene QDs, which were first fabricated 10 years ago, possess a graphene lattice inside the dots. They can be modified on their surface, like graphene. In general, QDs are in their infancy in extraction procedures, though they have found an important role in other disciplines.

Electrospun Nanofibers

Another current trend is the production and use of polymers with nanoscale fibrous structures, which can be achieved mainly by electrospinning (35), which is why they are frequently called *electrospun nanofibers*. Electrospinning relies on repulsive electrostatic forces to produce a solution of optimal viscoelasticity to form nanofibers. The analyte–sorbent interaction mechanisms are similar to those of conventional SPE polymers. The nanofibers most frequently used as sorbents have been those based on polystyrene and nylon 6 polymers (35). Nowadays, it is possible to modify the nanofibers pre- and post-electrospinning, so that a broad spectrum of materials is obtained, to be used mainly in SPE and SPME (36). Like other nanomaterials, electrospun nanofibers have also been combined with other materials to improve their performance (36).

Dendrimers

Dendrimers are polymer-based macromolecules with highly repetitive branched three-dimensional structures of several nanometers in size. Their properties mainly depend on the functional groups on their molecular surfaces, although sometimes they also depend on their internal cores. These structures can be built in a controllable manner, in which the end groups can be tailored and modified with desired functionalities (35,37).

With proper design of a dendrimer structure, good selectivity for various target molecules can be achieved (37). In fact, dendrimers can also be used to provide greater selectivity to other sorbents whose high extraction capacity has been demonstrated in order to improve their selectivity towards certain analytes. In this sense, dendrimers have been used to coat magnetic and nonmagnetic NPs, to modify other nanomaterials (such as the surface of CNTs), or even to form composites (37). Dendrimers act by encapsulating the target molecules, which have generally included dyes, polycyclic aromatic hydrocarbons (PAHs), or heavy metals.

Conclusions

Today, nanomaterials occupy an important place in current research and applications in analytical chemistry and, in particular, as extraction sorbents. A clear indicator of the exceptionally high interest in these materials is the extremely high number of applications that are being published every year.

The current variety of nanomaterials available, as well as their combinations, is quite high, providing an arsenal of sorbents ready for use, a good number of which have even been commercialized, though not always at an affordable cost.

The sorbent-based extraction procedure in which nanomaterials have been mostly commonly applied is dSPE, especially for nanomaterials with magnetic properties. Indeed, mNPs constitute one of the most widespread classes of nanomaterials used, thanks to their extraordinary high surface area, extraction capacity, chemical stability, and possibility and ease of functionalization and coating.

In general, when a new nanomaterial or combination of nanomaterials is used, suitable characterization to elucidate its morphology, composition, size, and so on, must be carried out, using complementary surface characterization techniques such as X-ray diffraction (XRD), thermogravimetric analysis/differential thermal analysis (TGA/DTA), gas adsorption isotherms, transmission electron microscopy (TEM), scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS) or infrared (IR), and UV–vis spectroscopy.

Given the excellent results provided by nanomaterials and the growing research into their synthesis, it is expected that the number of methodologies using such materials will continue to increase, though further research is needed to fully evaluate the convenience of their routine use in the laboratory. Furthermore, given that their synthesis is often complex and not so environmentally friendly, suitable efforts must be made in that direction. Finally, it should be highlighted that there is particular concern about the toxicity and environmental fate of many of these materials, which is associated with their extremely low size and sorption and binding properties, and which are still not fully described or understood.

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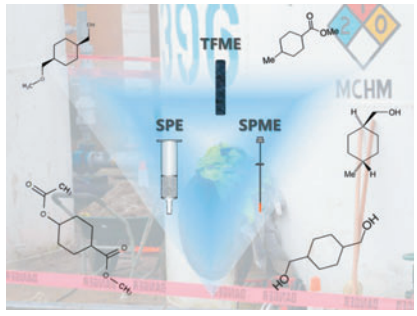
Exploring the Efficiency of Various Extraction Approaches for Determination of Crude (4-methylcyclohexyl)methanol (MCHM) Constituents in Environmental Samples



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Crude (4-methylcyclohexyl)methanol (MCHM) is a chemical blend, mainly used in the coal industry for the separation of usable coal from rocks, debris, and coal dust by froth flotation. Following a 2014 MCHM spill in the Elk River in West Virginia, studies demonstrated that MCHM sorbed into water pipes and linings readily desorbed from polyethylene into water at levels above the odor threshold, confirming the risk of its long-term exposure from contaminated tap water pipelines. In light of this, it is imperative to develop analytical methods able to detect crude MCHM components in environmental water samples. In this work, two microextractive methods based on solid-phase microextraction (SPME) in fiber format and thin film microextraction (TFME) were developed and validated. Their performance was compared with a modified solid-phase extraction (SPE) method based on U.S. Environmental Protection Agency (EPA) Method 522 for analysis of volatiles in water. SPME and TFME methods both showed enhanced performance in terms of achievable limit of quantitation (LOQ) compared to the SPE protocol. Moreover, the sensitivity of the TFME method, coupled with its higher analytical throughput, established TFME as the optimal extraction approach for 4-MCHM and other constituents of crude MCHM, with limits of quantitation below the odor threshold for aqueous crude MCHM in 19–21 °C deionized water (0.55 µg/L).

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On January 9, 2014, an estimated 37,800 liters (9,986 gallons) of a chemical mixture was spilled into the Elk River upriver from Charleston, West Virginia. This mixture, used in a purification process for coal, contained crude (4-methylcyclohexyl) methanol (MCHM; 88.5%), a proprietary blend of stripped polyglycol ethers (PPh; 7.3%) and water (4.2%). Crude MCHM is composed of (4-methylcyclohexyl)methanol (4-MCHM; 68–89%), 4-(methoxymethyl)cyclohexanemethanol (4MMCH; 4–22%), methyl 4-methylcyclohexanecarboxylate (MMCHC; 5%), dimethyl-1,4-cyclohexanedicarboxylate (DM-1-4-CHC; 1%), 1,4-cyclohexanedimethanol (1-4CHDM; 1-2%), water (4–10%) and methanol (1%) (1). The drinking water treatment plant located in Charleston, operated by West Virginia America Water (WVAW), was contaminated along with the water supply of approximately 300,000 residences. This may be an ongoing problem, given that it has been demonstrated that 4-MCHM readily adsorbs and desorbs from pipes made from polyethylene materials (2), creating a risk of chronic exposure for contaminated households. Analysis was not performed on the day of the spill, but the next day contamination levels as high as 2400 µg/L 4-MCHM were detected exiting the WVAW treatment plant (3). Concentrations of 4-MCHM in the range of 2–5 µg/L were found persisting from January 20 to February 2, 2014 (4). Analysis of tap water from households indicated that contamination was not limited to the WVAW facility, but also affected the entire water distribution system, evidenced by concentrations as high as 420 µg/L being found in drink-

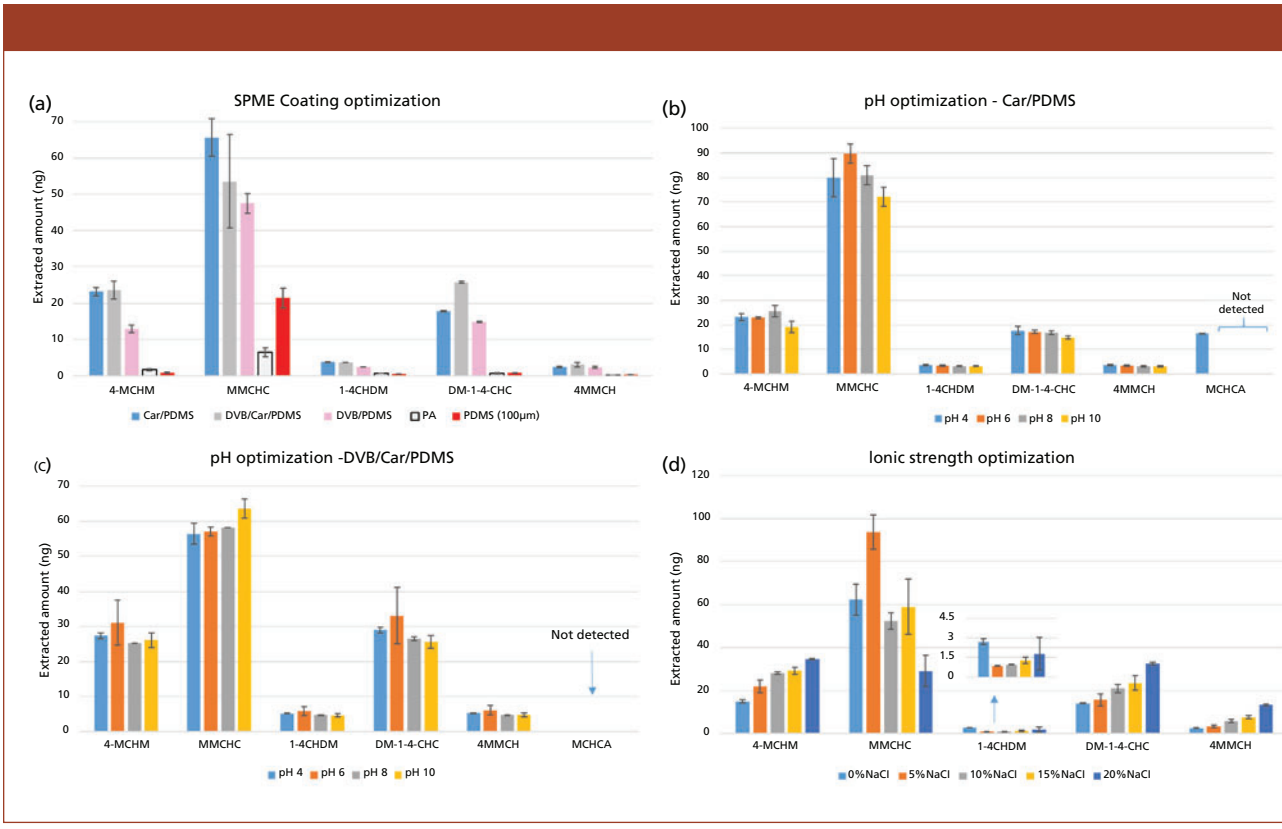

Figure 1: Optimization of SPME parameters including: (a) coating evaluation of five commercially available fibers; pH optimization using (b) Car/PDMS fiber and (c) DVB/Car/PDMS fiber; and (d) ionic strength optimization with Car/PDMS fiber.

ing water two weeks after the spill (5). Tap water from residences with different water suppliers were demonstrated to be contaminated by crude MCHM, with 4-MCHM being identified over 600 km from the spill site in Louisville, Kentucky (6). Of the approximate 300,000 households directly affected by the spill, an estimated of 25,623 families exhibited health problems, such as rash, skin irritation, respiratory problems, nausea, diarrhea, and other symptoms linked to crude MCHM, according to the Centers for Disease Control and Prevention (7). It has been established that 4-MCHM damages DNA, with its metabolites being more toxic as a result of their potential to cause greater oxidative stress (8). It has also been shown that low levels of 4-MCHM can be cytotoxic when paired with PPh, with concentrations as low as 1.28 µg/L 4-MCHM and 1.52 µg/L PPh (9). It is thus important to develop analytical methods able to quantitate the components of crude MCHM, as well as the associated metabolites, so as to monitor long term accumulation and release of these contaminants in environmental waters. Previous studies employed liquid–liquid extraction (LLE), coupled with gas chromatography with flame ionization detection (GC–FID) (10), or mass spectrometry (GC–MS) (11). Also, headspace (HS) sampling coupled with GC–FID (2,12), purge and trap (P&T) with GC–MS (6), and headspace solid-phase microextraction (HS-SPME) with GC–MS (13) have also been used to detect 4-MCHM only, given that other components of crude MCHM contribute less in the leaked mixture. In light of new toxicological data (8,9), accompanied by a lack of adequate research on the toxicology and sorption behavior of the minor constituents of crude MCHM, we herein propose quantitative methods for analysis of 4-MCHM and all associated compounds, including a primary metabolite of 4-MCHM, trans-4-methyl-1-cyclohexanecarboxylic acid (MCHCA) (14). The goal of this study is to quantitate, for the first time, all known components of crude MCHM, comparing the efficiency of various extraction approaches across different methods. The methods developed in this study utilized solid-phase extraction (SPE), direct immersion solid-phase microextraction (DI-SPME), and direct immersion thin film microextraction (DI-TFME). Each method employed GC–MS for separation and detection. SPE is a sample preparation technique commonly used in the analysis of environmental matrices, utilizing a solid-phase sorbent to exhaustively extract and preconcentrate target analytes in the same procedure (15). SPME is a non-exhaustive sample extraction method accomplished by a supported solid-phase coating, sharing the same advantages as SPE, but with a geometry that allows ease of automation and direct desorption of the SPME device into analytical instrumentation (16). TFME differs in geometry compared to SPME, with the extraction phase being coated on a carbon-mesh film in contrast to the fiber geometry of SPME. The increased surface area of a TFME device compared to a SPME fiber allows it to achieve greater sensitivity, thus making it more suitable for multiresidue trace analysis in environmental matrices (17). Among the methods developed, TFME provided the best results in terms of throughput and limits of quantitation (LOQs) achievable. The TFME based

Table I: Figures of merit obtained for SPE, SPME, and TFME protocols, comparing linear dynamic range (LDR), correlation coefficient (R^2), and limits of quantitation (LOQ)

	SPE			SPME			TFME		
	LDR (mg/L)	R^2	LOQ (mg/L)	LDR (µg/L)	R^2	LOQ (µg/L)	LDR (µg/L)	R^2	LOQ (µg/L)
MCHM	0.5–25	0.983	0.5	1–100	0.992	1	0.1–75	0.994	0.1
MMCHC	0.25–25	0.987	0.25	2.5–200	0.992	2.5	0.1–100	0.992	0.1
4MMCH	0.5–25	0.984	0.5	2.5–200	0.991	2.5	1–100	0.995	1
1-4CHDM	5–25	0.987	5	0.25–100	0.985	0.25	0.5–75	0.995	0.5
DM-1-4CHC	0.5–25	0.987	0.5	0.5–100	0.992	0.5	0.1–75	0.988	0.1
MCHCA	25–50	0.983	25	2.5–200	0.992	2.5	2–70	0.995	2

Table II: Accuracy values of the validated SPE, SPME, and TFME protocols.

	Accuracy (%) and Relative Standard Deviation (%)							
	SPE		SPME			TFME		
	7.5 (mg/L)	30 (mg/L)	3.5 (µg/L)	35 (µg/L)	75 (µg/L)	3.5 (µg/L)	35 (µg/L)	50 (µg/L)
MCHM	95.2 (7.5)	95.3* (28.1)	92.0 (5.2)	97.7 (6.6)	90.4 (7.8)	137.5 (10.7)	93.2 (19.6)	90.3 (29.9)
MMCHC	100.0 (9.2)	93.3* (20.5)	95.6 (11.3)	94.3 (21.4)	97.8 (16.5)	134.6 (11.1)	86.3 (3.8)	95.0 (23.2)
4MMCH	97.1 (6.3)	102.6* (24.7)	99.7 (7.9)	104.1 (13.0)	105.7 (8.9)	181.3 (14.6)	93.7 (2.3)	87.7 (18.9)
1-4CHDM	95.3 (6.3)	98.3* (18.7)	73.2 (22.2)	86.0 (29.7)	80.7 (9.2)	151.2 (5.7)	107.2 (16.2)	82.0 (37.9)
DM-1-4CHC	106.8 (7.6)	90.8* (28.7)	95.4 (15.6)	89.9 (28.5)	85.6 (16.9)	250.8 (15.9)	108.8 (9.0)	95.5 (19.8)
MCHCA†	- -	58.2 (5.5)	104.8 (23.9)	98.8 (11.8)	97.9 (6.2)	92.1[a] (18.5)	104.1[b] (12.2)	90.6[c] (7.4)

* Concentration value above upper limit of quantitation
† Accuracy values for MCHCA were calculated for TFME at
a: 7 (µg/L)
b: 70 (µg/L)
c: 150 (µg/L)

method was thus used for analysis of tap, lake, and river water.

Experimental

Method and Apparatus

Reference standards of 4-MCHM, MMCHC, DM1-4CHC, and MCHCA were purchased from Sigma Aldrich (St. Louis, MO, USA), along with the internal standards toluene-D8, and methyl benzoate-D8. A standard solution of 4MMCH was obtained from Toronto Research Chemicals Inc (Toronto, ON, Canada) and 1,4-CHDM was purchased from Tokyo Chemical Industries Co., Ltd. (Tokyo, Japan). Buffer solutions with pH 4, 6, 8, 10 were purchased from Honeywell Specialty Chemicals (Seelze, Germany). Materials for SPE included Sep-Pak AC2 Plus short Cartridge (400 mg) from Waters (Milford, MA, USA), and Cole-Parmer 2 mL wide neck volumetric vial with PTFE/silicone lined cap (Vernon Hills, IL, USA). Disposable Falcon tubes (3, 10, and 50 mL) were obtained from Becton Dickinson and Company (Franklin Lakes, NJ, USA). HPLC grade water, methanol, and dichloromethane were bought from ThermoFisher Scientific (Fair Lawn, NJ, USA). SPME fibers were purchased from Supelco (Bellefonte, PA). Car/PDMS thin films were obtained from Gerstel (Linthicum, MD, USA). River and tap water samples were collected in glass containers, being completely filled to minimize headspace. The river sample was collected ~0.5 m below the water surface along the bank of the Ohio River located at Portsmouth, Ohio. The lake water was obtained from the west fork of Keuka Lake near Pulteney, New York, in Steuben County, approximately 10 m off shore. The lake water was collected in a plastic container ~1.5 m below the surface. Tap (drinking) water was obtained at a residence in Louisville, Kentucky. Samples were stored in their original containers at 4 °C until analysis. An Agilent 7890 B GC instrument hyphenated to a 5977 B single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) and equipped with a DB-5ms column (30 m x 250 µm x 0.25 µm, Agilent Technologies), MultiPurpose Sampler, MPS, (Gerstel, Inc., Linthicum, MD, USA), a cooled injection system, CIS 4, (Gerstel, Inc., Linthicum, MD, USA) and

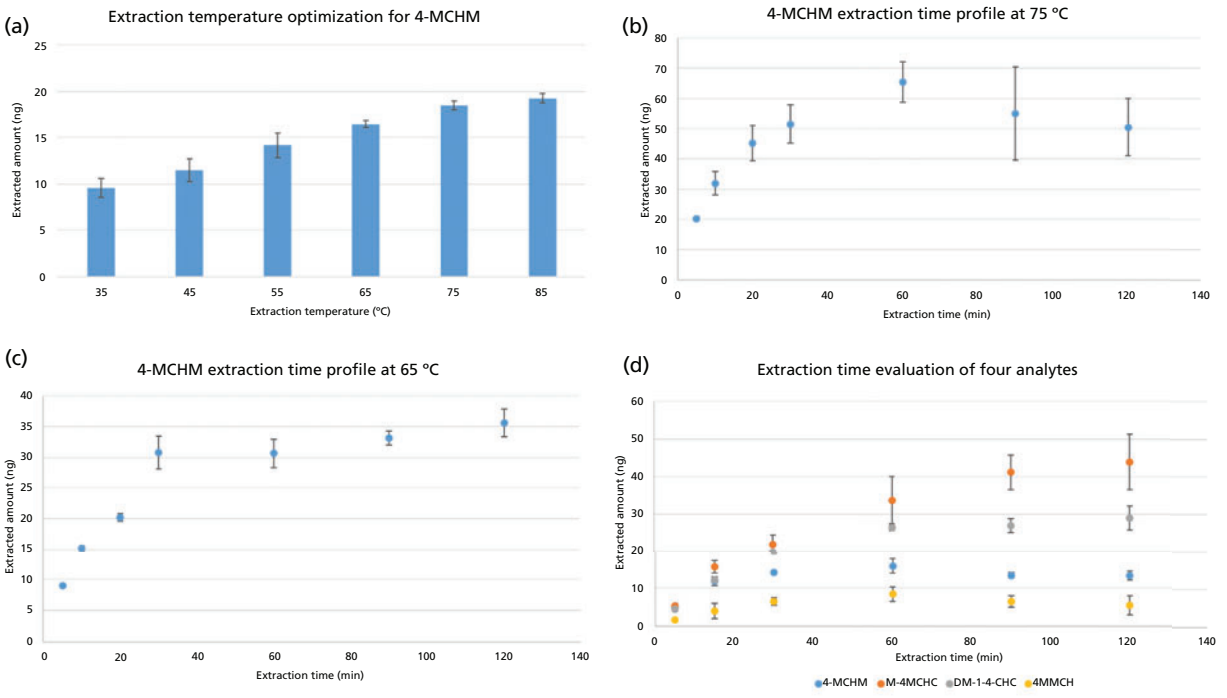

Figure 2: (a) Extraction temperature optimization for 4-MCHM. Extraction time profile of 4-MCHM at (b) 75 °C and (c) 65 °C. (d) Extraction time profile at 65 °C of four crude MCHM constituents.

a thermal desorption unit, TDU, (Gerstel, Inc., Linthicum, MD, USA) was used for analysis of the crude MCHM constituents. Ultrapure helium (99.999%) was used as the carrier gas at a flow of 1.5 mL/min for each method. For SPE and SPME analysis, the GC oven temperature was held at 50 °C for 2 min, then raised to 220 °C at a rate of 20 °C/min, then held at held 220 °C for 2 min. During TFME analysis, the GC oven was programmed at an initial temperature of 50 °C (held for 2 min), then raised to 290 °C at a rate of 20 °C/min, then held at 290 °C for 2 min. The mass spectrometer was used in electronic ionization (EI) mode at 70 eV with the MS source being set at 230 °C and the quadrupole at 150 °C, collecting full scan mass spectra. All data analysis was performed with Agilent Masshunter Workstation Quantitative Analysis software (Agilent Technologies, Santa Clara, CA, USA) for GC–MS. The response for each analyte was obtained as the sum of individual peak areas for trans and cis isomers, with exception of DM1-4-CHC and MCHCA.

SPE Protocol

The SPE extractions were conducted according to a modified-protocol based on EPA method 522 (18) before GC–MS analysis. SPE cartridges were first conditioned with 2 mL of dichloromethane. The cartridges were then equilibrated by passing 2 mL of HPLC grade methanol, followed by 10 mL of HPLC grade water through the cartridge. Throughout the equilibration step and onward, care was taken not to allow the cartridges to come to dryness during solvent exchange and sample loading. For extraction, 18 mL of each aqueous solution spiked with the targeted analytes was loaded onto the SPE cartridge. Any water residues was removed from the cartridge by purging it with high purity argon gas for 3 min until dryness. A 2 mL volumetric vial was used for collection of the eluate. Elution of the analytes was performed with the addition of dichloromethane to the cartridge. During this step, dichloromethane was initially used to soak the SPE cartridge for 1 min. Elution then proceeded in a dropwise fashion into a volumetric vial. Additional dichloromethane was added to the cartridge until the eluted volume in the vial was 1.5 mL.

To eliminate residual water in the eluate, 0.4 g of anhydrous sodium sulfate was added to each vial. The samples were sealed, and then stored in the refrigerator at 4 °C until GC–MS analysis.

SPME Protocol

The extraction efficiency of five commercially available SPME fibers, namely Carboxen/polydimethylsiloxane (Car/PDMS), divinylbenzene/Carboxen/polydimethylsiloxane (DVB/Car/PDMS), divinylbenzene/polydimethylsiloxane (DVB/PDMS), polyacrylate (PA), and polydimethylsiloxane 100 µm (PDMS), was assessed using a 100 µg/L aqueous solution containing the targeted analytes. Optimization of pH was performed using both Car/PDMS and DVB/Car/PDMS by adjusting the pH of the 100 µg/L aqueous solution containing the targeted analytes to pH 4, 6, 8, 10 using disodium hydrogen citrate plus sodium dihydrogen citrate plus sodium chloride buffer solution at pH 4, trisodium citrate-2-hydrate plus disodium hydrogen citrate buffer solution at pH 6, sodium chloride plus disodium tetraborate buffer solution at pH 8, and disodium tetraborate plus sodium hydroxide buffer solution at pH 10. The optimized SPME protocol consisted of 1 min incubation, followed by 30 min extraction, both performed at 65 °C and 300 rpm agitation

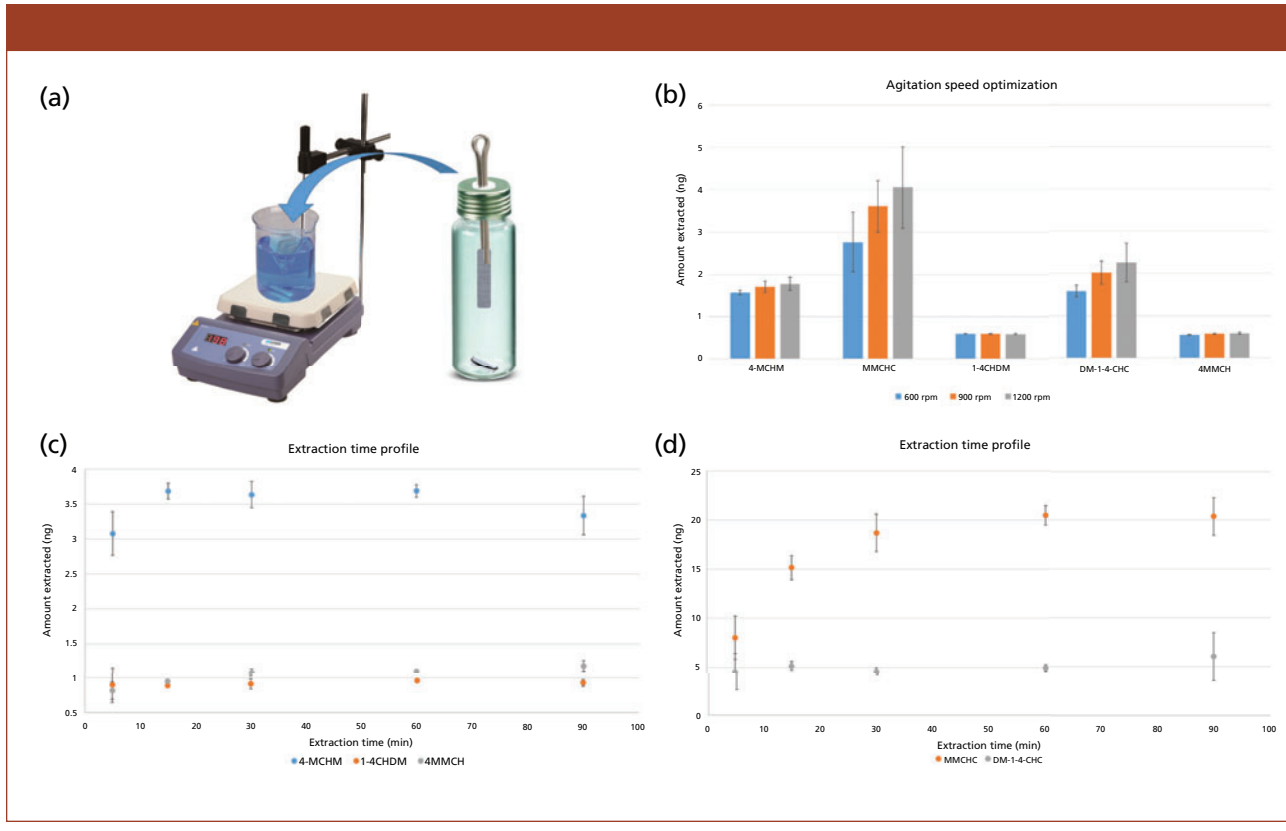

Figure 3: (a) Diagram of apparatus used for extraction procedures by TFME; (b) Agitation rate optimization; (c) Extraction time profile by TFME for 4-MCHM, 1-4 CHDM, and 4 MMCH; (d) Extraction time profile by TFME for MMCHC and DM-1-4-CHC.

speed. Desorption was performed for 10 min in splitless mode at 300 °C for Car/PDMS, 270 °C for DVB/Car/PDMS, 300 °C for PA, 270 °C for DVB/PDMS and 280 °C for PDMS. The conditions used for desorption prevented the occurrence of carryover of the analytes on the SPME coatings. Carryover was tested by re-desorbing the fibers immediately after the first extraction and desorption cycle and verifying the absence of peaks attributable to the targeted analytes in the obtained chromatograms.

TFME Protocol

Samples for TFME method optimization were prepared following the same procedures described for SPE and SPME to ensure direct comparison between extraction methods. During extraction, TFME devices were held in place by stainless steel pins penetrating the vial septum, with the thin films fully submerged in the sample solution. Samples were placed in a water bath at 65 °C for 5 min without agitation, and subsequently stirred at 900 rpm for 15 min by a magnetic stir bar placed in the vial. After agitation, the TFME device was quickly wiped, and then placed into a baffled glass desorption liner prior to insertion into the TDU. TDU parameters were first set at 30 °C, held at 30 °C for 0.5 min, raised to 270 °C at a rate of 700 °C/min, then held at 270 °C for 8 min, all performed in splitless mode at a 280 °C transfer temperature. Coupled to the TDU was a CIS 4, with an initial temperature of -50 °C, raised to a final temperature of 280 °C at 10 °C/s, then held at 280 °C for 3 min. Under the desorption conditions used for TFME devices, minimum carry over (<1%) was achieved for the targeted analytes.

Method Validation

The methods developed were validated with respect to linearity, precision, accuracy, and limits of quantitation (LOQs). Calibration curves were obtained for each target by plotting the signal ratio of the analyte and the isotopically labeled internal standards (A/Is) for various concentration levels (8 for the SPE protocol and 10 for the SPME and TFME protocols) in three independent replicates. Toluene-D8 was used as internal standard for the SPE method, while methylbenzoate-D8 was used for the SPME and TFME methods. Furthermore, three validation points selected within the linear range of each method were analyzed to assess precision and accuracy. LOQs were calculated as the lowest calibration point, with precision values lower than 20% and accuracy within 70–120%.

Results and Discussion

SPME Optimization

Various parameters affecting SPME were optimized to achieve the most suitable conditions for extraction of the major components of crude MCHM and a metabolite of 4-MCHM, namely MCHCA. At first, we tested the extraction performance of 5 commercially available SPME coatings, PDMS 100 µm, PA, PDMS/DVB, Car/PDMS, and DVB/Car/PDMS in order to select the most suitable extraction phase. Results shown in Figure 1(a) reveal that the best performance was achieved with Car/PDMS and DVB/Car/PDMS. Considering that SPME coating optimization was performed before optimization of other parameters such as pH and ionic strength of the solution, it was not possible to achieve extraction of MCHCA (a carboxylic acid with pKa = 4.89). Con-

Table III: Comparison of extraction efficiency between the methods developed in this work and other methods found in the literature

	Limits of quantitation (µg/L)			
	Total 4-MCHM	*trans*-4-MCHM	*cis*-4-MCHM	Reference
SPME-GC–MS	1	–	–	This work
TFME-GC–MS	0.1	–	–	This work
LLE-GC-FID	–	[100]	[100]	(10)
LLE-GC–MS	–	[30]	[30]	(11)
HS-GC-FID	5380	–	–	(12)
H-P&T-GC-MS	[0.4]	[0.16]	[0.28]	(6)
HS-SPME-GC-MS	–	[23]	[10]	(13)

[ ] Indicates the value to be reported as a limit of detection

Table IV: Analysis of crude MCHM constituents spiked at 45 µg/L in different environmental matrices

	Tap Water		Lake Water		River Water	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
4-MCHM	127.2	13.1	123.6	4.5	141.1	6.3
MMCHC	69.6	5.9	87.4	6.2	78.5	21.4
4MMCH	63.3	6.2	63.9	1.9	63.3	8.0
1-4CHDM	117.5	12.6	115.0	6.0	134.8	13.1
DM-1-4CHC	77.1	13.9	94.6	14.0	88.7	12.2
MCHCA	61.6	20.6	65.8	12.7	88.6	10.0

sequently, Car/PDMS and DVB/Car/PDMS were both used to perform the optimization of matrix pH with the purpose of determining which of the coatings could best extract MCHCA. Figures 1(b) and 1(c) compare results obtained adjusting the pH of the aqueous solution at 4, 6, 8, and 10 pH units. The results demonstrate that MCHCA is most efficiently extracted by the Car/PDMS coating, which was selected for further optimization and method validation. Moreover, it was also noticed that adjusting the pH of the aqueous solution to 4 guaranteed simultaneous and best extraction of all the analytes targeted in this study. The next step was the optimization of the sample ionic strength. The adjustment of ionic strength was performed by adding opportune concentrations of NaCl to aqueous samples adjusted to pH 4. However, changes in ionic strength also resulted in variations of the final pH of the sample originally adjusted at 4. A similar effect was noticed by changing the type of salt (potassium nitrate and magnesium nitrate) used for ionic strengths adjustments. Therefore, we decided to perform two separate sample preparations: to extract MCHCA. The sample was adjusted to pH 4 with no adjustment of ionic strength; for the remaining analytes, the sample was kept at pH 7, adjusting the ionic strength to 20% with NaCl, per the results obtained in Figure 1(d).

Extraction temperature was also evaluated from 35 to 85 °C (Figure 2(a), representative results for MCHM). From the trend obtained, the best extraction performance was achieved at 85 °C. However, due to pressure built up in the vial with consequent partial deformation of the vial cap septum, 75 °C was selected as the optimum, since no pressure buildup occurred in the vial. While performing the extraction time profile for MCHM at optimized extraction conditions (pH 7, 20% NaCl content, 75 °C extraction), high variability of the measurements and a decline in the MCHM response over time were observed (Figure 2(b)). This indicates that a probable degradation of the analyte occurred under the used experimental conditions. To investigate if the extraction temperature were the main contributing factor to the trend observed, the extraction time profile was repeated at 65 °C, while keeping all the other parameters constant. As can be seen in Figures 2(c) and 2(d), good reproducibility was obtained with a reasonable trend for the equilibration process. Therefore, 65 °C was considered the optimum extraction temperature for further testing and validation. Moreover, 30 min extraction time was selected as the best compromise between analyte response and analysis throughput. In summary, the extraction conditions used for 4-MCHM, 4MMCH, MMCHC, 1-4CHDM, and DM-1-4-CHC were Car/PDMS SPME fiber, 30 min extraction time, 65 °C extraction temperature with the sample adjusted at pH 7 with 20% (w:w) of NaCl. For analysis of the MCHM metabolite MCHCA, the optimized extraction conditions were: Car/PDMS SPME fiber, 30 min extraction time, 65 °C extraction temperature, with the sample adjusted at pH 4 with no adjustment of the ionic strength of the solution.

TFME Optimization

During SPME optimization, it was determined that the extraction phase Car/PDMS was best suited for analysis of crude MCHM components. Consequently, we used a Car/PDMS TFME device for further method optimization and validation. Some of the optimized operating conditions, such as sample ionic strength, pH, and extraction temperature, are unaffected by the geometry of the microextraction device, and thus further optimization of these parameters for the TFME method was unnecessary. However, parameters such as extraction speed and extraction time required further optimization as extraction by TFME devices was not automated (Figure 3[a]). Moreover, the microextraction device geometry, as well the agitation method used, directly influence the kinetics of the extraction process. Figure 3(b) compares the extraction performance achieved at agitation speeds of 600, 900, and 1200 rpm during extraction, each experiment being performed at optimized conditions. Results show that at 1200 rpm extraction efficiency is the greatest, however, with greater variability compared to the comparably efficient 900 rpm; thus, 900 rpm was chosen as the optimal agitation speed. Using this new optimal agitation speed, figures 3(c) and 3(d) show the extraction time profile for the analysis of crude

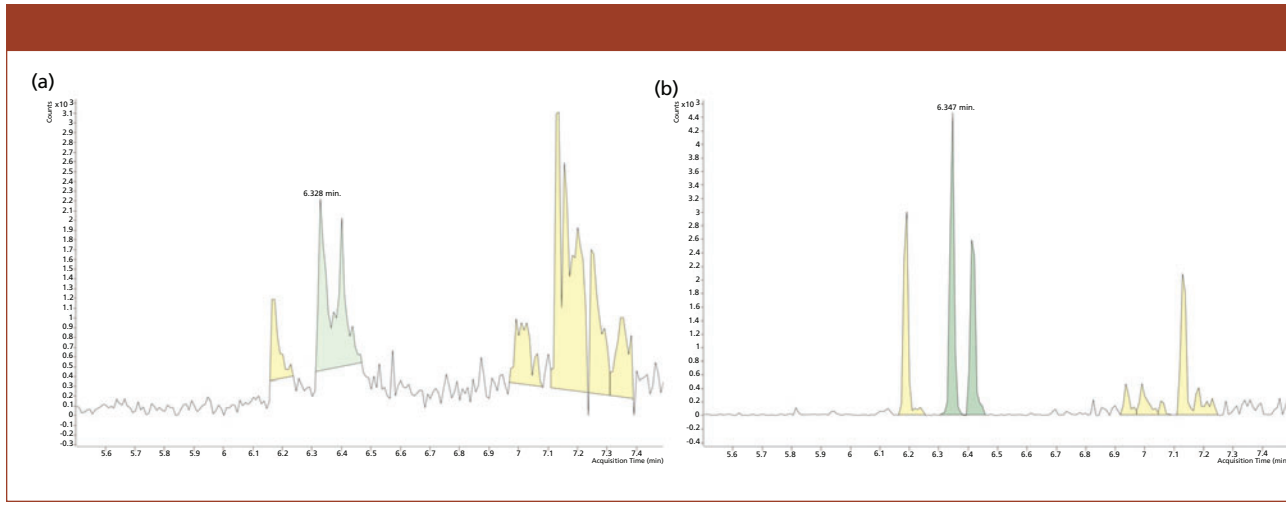

Figure 4: Comparison of signal obtained for 4-MCHC spiked at 0.25 µg/L for (a) SPME and (b) TFME methods.

MCHM constituents by TFME using all optimized parameters. Obtained results clearly demonstrate that all analytes, with the exception of MMCHC, equilibrate at the optimal 15 min extraction time. MMCHC equilibrated soon after 15 min, however, considering its high affinity for the extraction phase, extraction was carried conveniently at 15 min for all the target analytes.

Method Validation and Analysis of Real Samples

A summary of the results obtained from the method validation of the SPE, DI-SPME, and DI-TFME methods are presented in Table I. LOQs obtained with the SPE protocol were several orders of magnitude higher than the results of the other two extraction techniques tested, indicating that this technique is not suitable for extraction of crude MCHM constituents at trace level. Linear ranges of most compounds were very narrow, notably for MCHCA; some ranges spanned only one order of magnitude or less. SPME and TFME both circumvent the caveats of using SPE, with the two microextraction protocols exhibiting similar linear ranges and coefficients of determination. TFME recorded lower limits of quantitation than both SPE and SPME for most compounds (Table I) with comparable accuracy, except for the accuracy level at 3.5 ppb (Table II).

Further comparison with previously published work demonstrates that the microextraction methods developed in this study, in addition to being the first to simultaneously detect and quantitate all known components of crude MCHM, also quantitate 4-MCHM at concentrations at levels lower than any previous published method. These results are shown in Table III.

Compared to other extraction techniques evaluated in this study, TFME recorded lower limits of quantitation and better throughput; extraction times are shorter than SPME (15 min for TFME versus 30 min for SPME). Consequently, TFME was chosen as the best extraction protocol to follow for analyzing real matrices. Tap, river, and lake water samples were chosen to compare different possible matrices where 4-MCHM and its constituents might be detected. No detectable amount of the target analytes was discovered in the samples. Subsequently, each sample was fortified with the targeted analytes and tested for accuracy using the TFME method developed and validated as mentioned above. Results shown in Table IV reasonably validate the TFME protocol for analysis of real matrices for the contributing compounds of crude MCHM and one 4-MCHM metabolite, MCHCA.

Conclusions

For the first time, methods utilizing SPME and TFME were developed and optimized for simultaneous analysis of 4-MCHM and all other known components of crude MCHM. MCHCA, a primary metabolite of 4-MCHM, was also determined independently with only minor modifications to the sample preparation protocol. The performance of these microextraction-based analytical methodologies was compared to an SPE method that was also tested in this study. Our results show that lower limits of quantitation can be achieved with microextraction methods. Relating these results to previously developed methods, both SPME and TFME are competitive in detecting and quantitating 4-MCHM. The TFME-GC–MS method recorded a limit of quantitation for 4-MCHM lower than any currently known method, with only 15 min of extraction time needed. To validate this, water samples from various sources were analyzed using TFME and the accuracy of the method was determined. The sensitivity of the TFME method, coupled with its increase in analytical throughput, made it clear that TFME is the optimal extraction approach for 4-MCHM and its constituents found in crude MCHM.

Acknowledgments

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(Continued on page 42)

"Truly Natural": Fully Automated Stir-Bar Sorptive Extraction with Enantioselective GC–MS Quantitation of Chiral Markers of Peach Aroma

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The volatile fraction of a food plays a fundamental role in its characterization and appreciation by consumers, and thus can be used to authenticate and assess the quality of food products. Key odorants in foods are very often chiral molecules with an enantiomeric excess. Reliable quality control therefore entails fast, fully automated methods that can quantify key odorants, and determine their enantiomeric compositions. This study reports the development of a simple, fast, simultaneous, and fully automated total analysis system to quantify and measure the enantiomeric excess of γ- and δ- lactones, in natural and artificial peach flavored juices. Stir-bar sorptive extraction (SBSE) is combined with fast enantioselective GC–MS analysis and online statistical processing to quantify target quality components, including at trace levels, and effectively discriminate between samples.

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The volatile fraction of a food plays a fundamental role not only in its characterization, acceptance, and appreciation by consumers, by originating its aroma, but also in its authentication and quality assessment. Volatiles directly affect the sensorial quality of fruit products, and are responsible for specific sensory characters (1,2). The concentration of volatiles is generally low; it can be affected by a number of agronomic and technological factors, and also reflects the contribution of those factors to a food's aroma. The aroma of a food product is often a complex mixture of chemicals belonging to different chemical classes, but only those (few) of them present in a concentration above their odor threshold are responsible for the sensory perception (3). The contribution of each analyte to smell can be expressed numerically in various ways, including through an odor activity value (OAV) or odor unit (3); this value is the concentration of the analyte in the food divided by its odor threshold.

Enantiomer recognition in the food field is very important, not least because biological interactions and biosynthetic processes are mostly stereospecific; this means that chiral components in natural products often show a specific enantiomeric composition (4). Conversely, many commercially relevant fruit aromas are expensive and, therefore, quite often replaced by cheaper synthetic products containing racemic compounds, or natural ingredients from different cheaper sources or origins that may contain chiral components with a different enantiomeric composition (5,6).

The flavoring of food and the discrimination between "natural" and "artificial" flavored food is also regulated by the current European legislation, in Regulations (EU) 1334/2008 and 1169/2011. These regulations state that flavorings must be labeled in the list of ingredients of a food product, and limit the use of the term *natural* to flavored preparations containing exclusively "natural flavoring substances," meaning those obtainable enzymatically, microbiologically, or by an appropriate physical process, the latter being, ". . . a process which does not intentionally modify the chemical nature of the components of the flavoring . . . ". Chiral recognition thus becomes a decisive tool for a quality control laboratory, to monitor conformity of a food to legal requirements, and at the

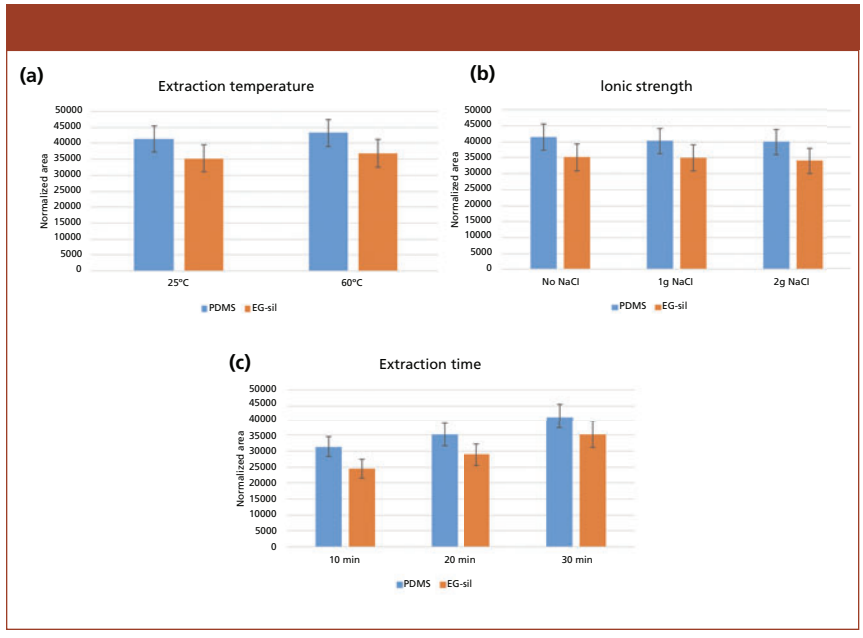

Figure 1: Extraction efficiency for (R)-γ-decalactone under different sampling conditions for stir-bars coated with PDMS (blue) and EG-silicone (red). Normalized peak areas obtained at (a) different extraction temperatures, (b) ionic strengths, and (c) and extraction times are reported.

Table I: Examples of odor features of the enantiomers of some lactones (19)

Compound	Enantiomer	Features
γ-Decalactone	*(R)*-(+) *(S)*-(-)	Sweet, fruity, milk note Sweet, fruity, peach note, fatty, butter-like
δ-Pentalactone	*(R)* *(S)*	Faint, sweet Nearly odorless
δ-Hexalactone	*(R)* *(S)*	Faint, sweet coconut with a fatty-herbaceous hay note Sweet, creamy coconut with some woody aspects
δ-Heptalactone	*(R)* *(S)*	Sweet, spicy, herbaceous hay note, reminiscent of coumarin Fatty, coconut note with fruity-sweet aspects, less intense than the opposite enantiomer
δ-Octalactone	*(R)* *(S)*	Spicy-green, coconut note, with almond notes Fatty, coconut note, less intense than the opposite enantiomer
δ-Nonalactone	*(R)* *(S)*	Strong, sweet, soft coconut with fatty-milky aspects Fatty, moldy, weak coconut less intense than the opposite enantiomer
δ-Decalactone	*(R)* *(S)*	Strong, fatty-sweet fruity note, some reminiscence to coconut, caramel Soft, sweet coconut note with fruity-fatty aspects
δ-Undecalactone	*(R)* *(S)*	Strong, fatty-sweet, reminiscent of peach, with some bloomy aspects Fatty-sweet aldehyde note, less intense than the opposite enantiomer
δ-Dodecalactone	*(R)* *(S)*	Strong, fatty-sweet, bloomy note with aldehyde and woody aspects Fatty-fruity, milky notes, less intense than the opposite enantiomer

same time to check authenticity and reveal any adulteration or fraud. In addition, without specifying the use of flavoring in the list of ingredients or technological treatment, the concentrations of the key odorants should be compatible with those of the original ingredients in nature.

An optimal method for quality control of fruit products should, therefore, be able to quantify their key odorants, and simultaneously determine the enantiomeric composition of their chiral components.

For a routine quality control laboratory working in the food field, processing a large number of relatively similar samples daily, the primary requirement is to develop automated systems to satisfy the ever-increasing number of analyses required. At the same time, automated systems are also important in food research; for instance, to characterize a biological property (OAV) requiring a statistically significant number of reliable data to be described. It is ,therefore, important to develop inclusive platforms, the so-called *Total Analysis System* (TAS), in which the sample preparation, analysis, and data processing steps are merged online into a single workflow (7). In a TAS, the sample preparation step should be considered the "zeroth" dimension of the analytical platform, because it discriminates the analytes as a function of their characteristics (such as polarity and volatility) (8); it should, therefore, be chosen to recover the fraction of interest selectively.

Stir-bar sorptive extraction (SBSE) is a solventless sampling technique introduced by Baltussen and colleagues in 1999 (9); its main features were recently reviewed by David and associates (10). This technique is based on the recovery of the investigated fraction or analytes in a liquid sample by sorption, with thick-film polydimethylsiloxane (PDMS) coated stir bars. As well as PDMS, several other materials have been proposed, mainly to improve the recovery of more polar compounds; to the best of the authors' knowledge, only a PDMS-ethylene glycol (EG-silicone) copolymer is commercially available at present (11). SBSE is characterized by high extraction efficiencies and sensitivities, and good repeatability and reproducibility, especially when combined online with thermal desorption (TD)-GC–MS and multiple use. Furthermore, the high volume of the stir-bar coating provides an extraction yield at least tenfold that of the recently introduced solid-phase microextraction (SPME)

Table II: Figures of merit of the quantitative method for the investigated stir-bars

	PDMS						EG-silicone					
	Slope	Intercept	Linearity (R^2)	LOD (µg/L)	LOQ (µg/L)	Repeatability RSD% 5 µg/L (n = 5)	Slope	Intercept	Linearity (R^2)	LOD (µg/L)	LOQ (µg/L)	Repeatability RSD% 25 µg/L (n = 5)
(R)-γC6	4.7	210.7	0.985	4.5	6.1	9.8	2.9	108.5	0.999	21	49	18
(S)-γC6	6.3	225.7	0.961	3.4	4.6	10	3.2	139.0	0.998	17	32	21
(R)-γC7	24.6	446.2	0.963	0.94	1.2	1.0	13.3	330.5	0.999	4.5	8.5	13
(S)-γC7	22.7	480.2	0.953	1.4	1.9	1.6	11.6	350.8	0.999	6.0	11	8.8
(R)-γC8	72.9	914.7	0.982	0.60	0.80	8.9	45.8	493.4	0.978	2.6	4.9	19
(S)-γC8	73.8	919.6	0.981	0.49	0.66	12	44.7	532.4	0.976	2.2	4.1	18
(R)-γC9	252.7	1681.5	0.990	0.22	0.29	13	138.6	1067.0	0.989	0.82	1.5	19
(S)-γC9	256.7	1713.0	0.991	0.21	0.28	9.7	141.2	1030.1	0.990	0.73	14	20
(R)-γC10	665.8	3988.2	0.992	0.088	0.12	7.3	361.1	4837.4	0.966	0.17	0.32	12
(S)-γC10	669.9	3932.9	0.992	0.10	0.14	6.8	357.8	4878.8	0.965	0.16	0.31	13
(R)-γC11	1279.4	5377.7	0.994	0.061	0.081	0.7	733.3	4476.2	0.983	0.11	0.20	13
(S)-γC11	1341.0	5376.6	0.995	0.056	0.076	1.9	767.5	4416.3	0.981	0.10	0.20	12
(R)-γC12	1447.3	7429.7	0.995	0.053	0.071	9.7	1013.4	4657.3	0.977	0.090	0.17	11
(S)-γC12	1475.4	6132.5	0.995	0.060	0.080	9.2	959.5	4959.5	0.962	0.087	0.16	12
(S)-δC8	10.5	7.9	0.999	4.1	5.51	13	5.9	–29.1	0.988	20	39	18
(R)-δC8	11.2	24.2	0.994	3.5	4.75	11	7.7	–61.0	0.982	22	41	19
(S)-δC9	19.3	13.7	0.995	2.5	3.32	5.2	9.9	–17.4	0.997	22	41	19
(R)-δC9	18.7	21.7	0.992	1.9	2.56	5.9	9.6	–21.0	0.999	20	37	18
(S)-δC10	191.9	-26.9	0.996	0.32	0.43	9.8	76.2	–161.2	0.997	1.8	3.5	17
(R)-δC10	195.9	-23.6	0.995	0.34	0.45	8.7	74.3	–171.0	0.996	2.8	5.2	19
(S)-δC11	497.8	-614.2	0.997	0.18	0.24	6.5	162.1	–433.8	0.997	1.9	3.6	14
(R)-δC11	505.6	-548.2	0.997	0.17	0.23	7.1	159.3	–400.6	0.997	1.6	3.0	16
(S)-δC12	1023.7	-2511.5	0.997	0.10	0.14	5.8	368.2	–1153.4	0.996	0.98	1.8	11
(R)-δC12	1022.8	-2447.2	0.998	0.10	0.14	2.8	359.3	–1151.9	0.997	0.98	1.8	8.5

Arrow devices (12), and hundredfold that of conventional SPME fibers at equilibrium, in particular for solutes with low log octanol-water partition coefficient (K_{ow}). PDMS stir bars are currently widely applied in different fields (such as environment, food, biofluids, and drugs) for trace analysis of solutes with log P above 3 (13). The high concentration capacity of SBSE also permits working under nonequilibrium conditions, making this a fast sampling approach.

This study aims to develop a fast, fully-automated method for the simultaneous quantification and enantiomeric recognition of chiral key odorants in fruit products. The main focus is on the suitability of SBSE as a sampling technique to detect and quantify chiral compounds, even at trace levels. The simultaneous quantification and enantiomeric recognition of chiral analytes by headspace solid-phase microextraction (HS-SPME), combined with enantioselective gas chromatography–mass spectrometry (GC–MS), has already been applied to characterizing chiral terpenoids in tea leaves (14) and for authenticity control in wine (15); however, both methods were multistep and time-consuming. Peach (*Prunus persica* [L.] Batsch) was taken as model fruit, because it is an economically important crop, the production of which is continually rising. The peach volatile fraction has been investigated intensively, and more than 100 compounds have been identified (16). Among them, γ- and δ-lactones, in particular γ-decalactone (odor threshold is 7 ppb), have been named as character impact compounds in peach aroma (17,18), in association with C6 compounds, alcohols, esters, terpenoids, and phenolic compounds. A particular feature of lactones, very often neglected during aroma investigations, is their chirality (Table I) (19). Determination of the natural enantiomeric abundance of the two enantiomers of γ- and δ-lactones in peaches has revealed a strong enantiomeric excess of the *(R)*-enantiomers of both lactones (20), whereas racemic lactones are in general used for artificial flavoring of peach products.

In a previous study (4), the present group developed a rapid total analysis system to detect the authenticity of fruit-flavored foods and beverages, combining headspace (HS)-SPME with enantioselective GC–MS and statistical multivariate methods, with the goal of speeding up the entire method, without tackling quantification.

In this study, a TAS to quantify a homologous series of γ- and δ- lactones was developed. In particular, two steps were first investigated: first, selection among stir bars with different coatings (PDMS and EG-silicone) and optimization of sampling conditions, and second, tuning the optimal enantioselective GC–MS conditions to combine a fast analysis method with

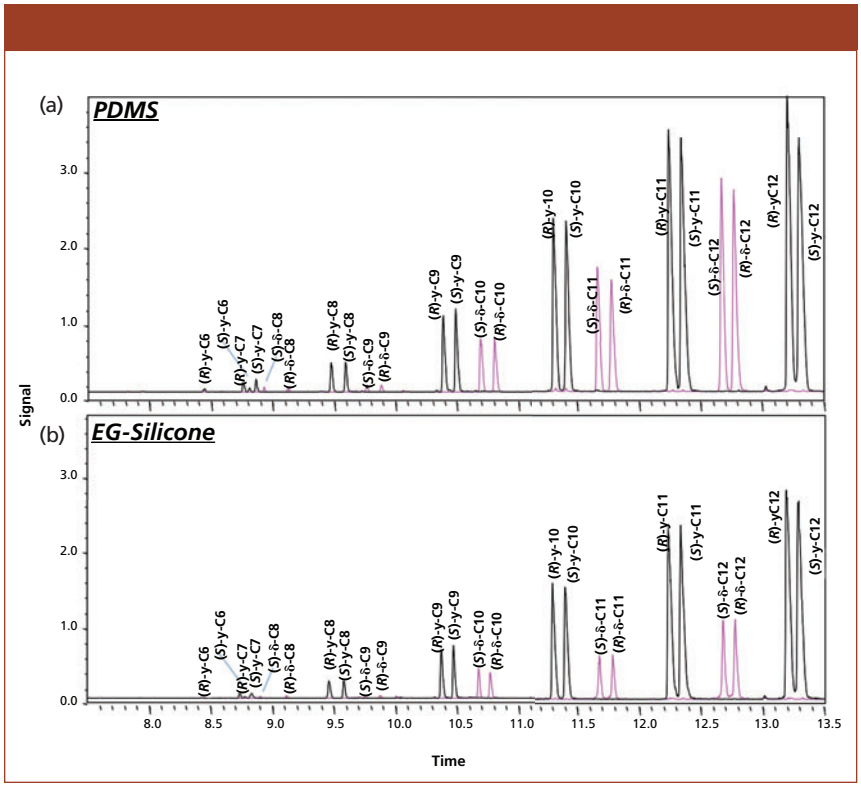

Figure 2: Enantioselective GC–MS profiles of the of 100 ppb mixture of γ-lactones (black) and δ-lactones (pink) sampled with stir-bars coated with (a) PDMS and (b) EG-silicone.

baseline separation of all enantiomers in a single run. The performance of the analytical method was then evaluated in terms of linearity, limits of detection, quantification, and repeatability.

Lastly, the method was applied to a set of peach-flavored beverages, and the quantitative results processed by applying statistical multivariate methods (principal component analysis) for sample characterization and authentication.

Experimental

Materials and Reagents

Racemic γ- and δ-lactones (γ-hexalactone, γ-heptalactone, γ-octalactone, γ-nonalactone, γ-decalactone, γ-undecalactone, γ-dodecalactone and δ-octalactone, δ-nonalactone, δ-decalactone, δ-undecalactone, and δ-dodecalactone) and methyl octanoate were from the authors' standards collection. Ethanol and cyclohexane were purchased from Millipore (Milan, Italy), with purities equal to or above 99%. Deionized water (18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

A model juice solution was also prepared, by mixing 100 g of saccharose and 4 g of citric acid in 1 L of distilled water, as reported elsewhere (2).

A set of one homemade and 19 commercial naturally and artificially flavored peach juices (*P. persica* [L.] Batsch) were also analyzed. The commercial juices were purchased in a local supermarket.

Stir Bars

Stir bars (1 cm long), coated with 25 µL of polydimethylsiloxane (PDMS) and 32 µL of polyethyleneglycol-modified silicone copolymer (EG-silicone), were used. All stir bars were supplied by Gerstel GmbH & Co. (Mülheim a/d Ruhr, Germany) and conditioned 3 d under inert gas flow at 220 °C.

Standard and Sample Preparation

Individual stock solutions of γ-lactones, δ-lactones, and methyl octanoate (used as an internal standard) were prepared in a 10 mL sealed vial by dissolving 1 mg of pure standard in a mixture of 50:50 ethanol:ultrapure water to obtain a concentration of 100 ppm. Working standards of γ- and δ-lactones were prepared by adding appropriate amounts of stock standard solutions and 2 µL of methyl octanoate internal standard (IS) stock solution (final concentration was 10 ppb) to a 20 mL sealed vial, and further diluting with model juice solution to obtain a final volume of 20 mL. The natural peach juices were prepared by adding 2 µL of the IS to 20 mL of pure juice in a 20 mL sealed vial. The artificial flavored juices were first diluted 1:5 with model juice solution, because of the great abundance of lactones in these samples.

The optimized sampling conditions for SBSE sampling of the standard and sample solutions with each stir bar (PDMS and EG-silicone) were 15 min at room temperature (25 °C) under stirring at 1500 rpm.

After extraction, stir bars were removed from the sample, dried with filter paper, and placed in a glass tube, and in turn installed in a thermal desorption unit (TDU, Gerstel GmbH & Co., Mülheim a/d Ruhr, Germany). The thermally desorbed analytes were online injected into a GC–MS system for further analysis (see below). Blank runs were completed with each stir bar before and after each set of analyses, and no carryover effects were observed. Each experiment was repeated three times.

SBSE with Thermal Desorption–Enantioselective GC–MS Analysis

The thermal desorption unit (TDU) from Gerstel GmbH & Co. (Mülheim a/d Ruhr, Germany) was installed on an Agilent 6890 GC unit coupled to an Agilent 5973N MSD (Little Falls, DE, USA). Thermal desorption conditions: desorption temperature program: 30–230 °C (5 min) at 60 °C/min; flow mode: splitless, transfer line: 250 °C. A Gerstel CIS-4 PTV injector was used to cryogenically focus the analytes thermally desorbed from the stir bars. The PTV was cooled to –40 °C with liquid CO_2; injector: PTV; injection temperature: from –40 to 250 °C (5 min) at 12 °C/s. The inlet was operated in split mode: split ratio 1:5.

Chromatographic conditions: column: 30% $6^{I–VII}$-O-TBDMS-$2^{I–VII}$-$3^{I–VII}$-O-acetyl-β-cyclodextrin diluted in PS-086 (10 m x 0.10 mm d_c, 0.10 µm d_f) from Mega Srl (Legnano, Italy). Temperature program: 0 °C for 0.5 min, then 0–90 °C at 80 °C/min, 90–195 °C at 8.1 °C/min, 195–230 °C at 15 °C/min, then hold at 230 °C for 2 min. For all experiments, carrier gas was helium, flow-rate: 0.4 mL/min, in constant flow mode. MS operated in EI mode at 70 eV. Ion source temperature: 230 °C; quadrupole temperature: 150 °C.

Table III: Concentration of the main lactones in the samples investigated, together with the experimental odor activity values (OAV). Legend: HMJ, reference juice; NJ, natural juice, SJ, artificial flavored juice; OAVs in bold when above 1.0

	γC8 (odor threshold 7 µg/L)		γC10 (odor threshold 11 µg/L)		γC12 (odor threshold 7 µg/L)		δC10 (odor threshold 100 µg/L)	
	Concentration (µg/L)	OAV	Concentration (µg/L)	OAV	Concentration (µg/L)	OAV	Concentration (µg/L)	OAV
HMJ	4.2	0.6	55	**5.0**	11	**1.5**	20	0.2
NJ_1	15	**2.1**	75	**6.8**	2.5	0.4	24	0.2
NJ_2	13	**1.9**	102	**9.3**	4.1	0.6	27	0.3
NJ_3	3.3	0.5	19	**1.7**	0.80	0.1	9.5	0.1
NJ_4	7.1	1.0	53	**4.8**	1.2	0.2	7.1	0.1
NJ_5	35	**5.0**	370	**33.6**	14	**2.0**	92	0.9
NJ_6	13	1.8	100	**9.1**	3.9	0.6	34	0.3
NJ_7	5.6	0.8	28	**2.6**	0.71	0.1	12	0.1
NJ_8	7.0	1.0	53	**4.9**	2.2	0.3	17	0.2
NJ_9	6.7	0.9	36	**3.3**	1.1	0.2	13	0.1
NJ_10	17	**2.4**	105	**9.6**	2.5	0.4	28	0.3
NJ_11	5.7	0.8	52	**4.7**	1.8	0.3	15	0.1
NJ_12	5.6	0.8	56	**5.1**	5.2	0.7	25	0.2
NJ_13	4.9	0.7	33	**3.0**	2.6	0.4	16	0.2
SJ_1	12	**1.7**	181	**16.4**	4.7	0.7	19	0.2
SJ_2	55	**7.9**	133	**12.1**	6.2	0.9	23	0.2
SJ_3	37	**5.3**	577	**52.5**	73	**10.5**	29	0.3
SJ_4	not detected	/	2862	**260.1**	115	**16.4**	241	**2.4**
SJ_5	not detected	/	16	**1.4**	573	**81.9**	206	**2.1**
SJ_6	14	**2.1**	188	**17**	4.6	0.7	10	0.1

Mass range: from 35 to 350 amu (*m/z*).

The elution order of the enantiomers of the chiral lactones was assigned according to an in-house library based on mass spectra and linear retention indices, built up with reference compounds (21).

Quantification Method and Analytical Performance

Quantification was carried out by the external calibration method on 85 *m/z* and on 99 *m/z* target ions, for γ- and δ-lactones, respectively. The absolute amount of each enantiomer in the racemate was determined by analyzing each standard diluted in cyclohexane at 100 ppm by GC–FID, and determining the area ratio between the two enantiomers. The calibration curves of γ- and δ-lactones were prepared with five different concentrations, over ranges of 1–100 ppb and 1-50 ppb, respectively.

Repeatability and intermediate precision were determined by analyzing the standard mixtures under the conditions reported in the sections above, three times every two days over one week in the same laboratory, with the same instrument and operator. Five stir bars for each coating were used at random. Repeatability is expressed as relative standard deviation % (RSD%). Stir-bar repeatability was rigorously controlled by sampling the standard mixtures with two stir bars for each PDMS and EG-silicone coating from three different lots, for a total of six stir bars.

Limits of detection (LOD) and quantification (LOQ) were calculated following IUPAC recommendation:

$$x_L = \bar{x}_{bi} + k * s_{bi} \quad [1]$$

where $\bar{x}_{bi}$ is the mean of the blank measures, s_{bi} is the standard deviation of the blank measures, and k is a numerical factor chosen depending on the confidence level desired (3 for LOD and 10 for LOQ). LOD and LOQ, expressed in terms of signal in equation 1, can be transformed into concentration by applying the following equation:

$$\text{LOD/LOQ conc} = x_L * conc / h \quad [2]$$

where *conc* is the analyte concentration and *h* is the peak height.

Data Analysis

All data analyses were conducted with Shimadzu GC–MS Solution 2.51 software (Shimadzu, Milan, Italy). All statistical processing was carried out using Statistica 10 (StatSoft. Inc., Tulsa, OK, USA) software.

Results and Discussion

This section reports the optimization of the enantioselective GC–MS and sampling methods to quantify lactones in peach juices; the method is then applied to a set of real-world samples, to illustrate its reliability in authenticating fruit-flavored beverages.

Optimization of the Sampling Method

The SBSE sampling procedure was optimized on two standard mixtures of racemic γ-lactones and δ-lactones (from γ-hexalactone to γ-dodecalactone, and from δ-octalactone to δ-dodecalactone), both diluted at 100 ppb (each enantiomer at 50 ppb) in a mixture of ethanol:water

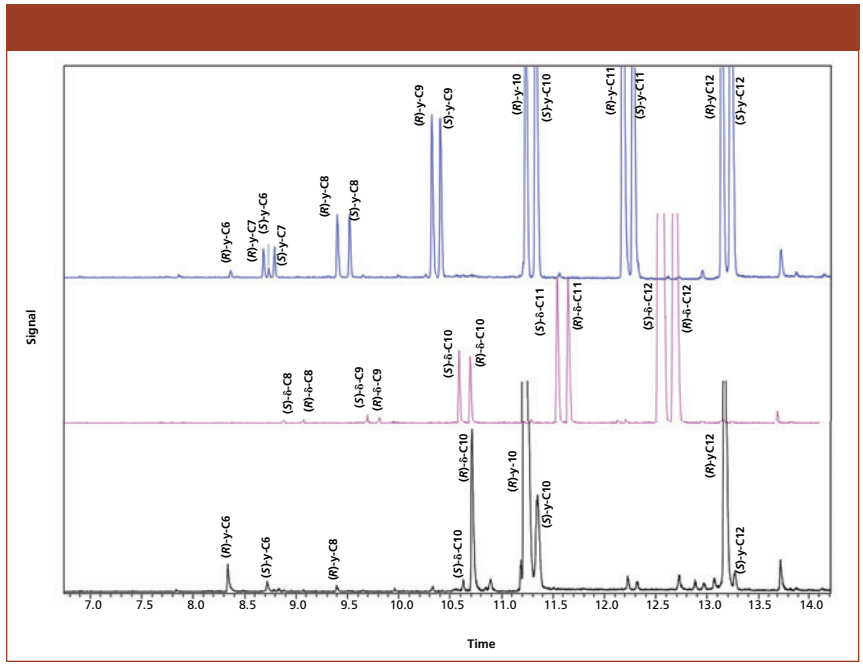

Figure 3: Optimized enantioselective GC–MS profiles of the homologous series of γ-lactones (blue), δ-lactones (pink) and peach juice (black) sampled with a PDMS stir-bar.

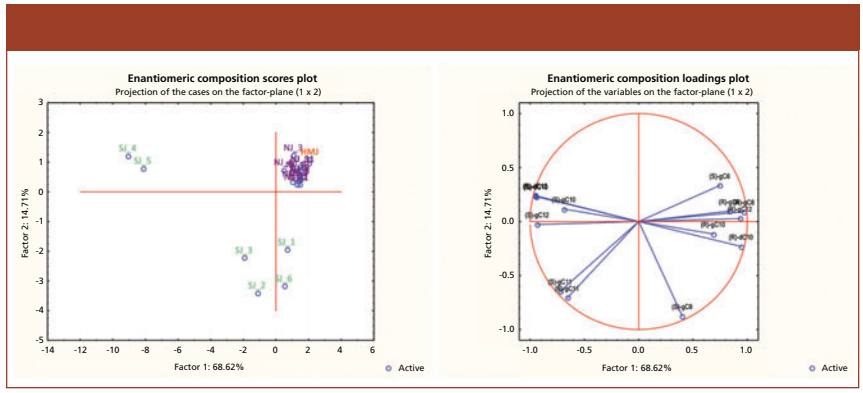

Figure 4: PCA scores and loading plots of the enantiomeric composition for the samples investigated. Legend: HMJ (red), reference juice; NJ (black), natural juice, SJ (green), artificially flavored juice.

50:50 under the chromatographic conditions reported in the section describing the optimization of the enantioselective GC–MS method. The following parameters were evaluated: extraction time, extraction temperature, and ionic strength. Furthermore, the performances of two stir-bar coatings (PDMS and EG-silicone copolymer) were tested and compared under the following conditions: extraction time: 15 min, extraction temperature: 25 °C, without salting out.

These conditions were derived from a series of experiments to optimize extraction time and temperature, and ionic strength. Figure 1 shows the results obtained for *(R)*-γ-decalactone, which was taken as reference as all the investigated lactones behaved similarly. Two sampling temperatures (25 °C and 60 °C) were tested, applying an extraction time of 30 min and without salting out. The extraction efficiency was comparable at both temperatures (Figure 1a); room temperature (25 °C) was therefore chosen. The influence of the ionic strength was tested by adding 1 or 2 g of NaCl to the standard mixtures. The results (Figure 1b) were again comparable, making salting out unnecessary. Lastly, different extraction times (10, 20, and 30 min) were applied: as expected, the results (Figure 1c) showed that analyte recoveries increased over time. However, given that even the shorter extraction times produced high extraction yield, good repeatability and intermediate precision, a nonequilibrium sampling approach under rigorously standardized conditions (15 min) was chosen to reduce the total analysis time.

The results showed that PDMS-coated stir-bars provided higher recoveries, probably because of the apolar nature of the lactones, with $\log K_{ow}$ ranging from 0.6 for γ-hexalactone to 3.6 for γ-dodecalactone. In particular, the normalized area of the low molecular weight lactones was almost double that obtained with the EG-silicone polymer. Figure 2 reports the comparison between the patterns of the 100 ppb lactone standard mixtures obtained with the two investigated stir-bars. The intermediate precision of the recoveries of the stir-bars coated with the two polymers (n = 5) was satisfactory, RSD% being below 12% for PDMS and only slightly higher (15%) for EG-silicone.

Optimization of the Enantioselective GC–MS Analysis Method

Enantioselective GC–MS with cyclodextrin (CD) as chiral selector can successfully discriminate between the enantiomers of the key odorants of peach, γ- and δ- lactones. This analysis requires the appropriate cyclodextrin derivative to be used, which can separate the enantiomers of all target compounds in a single run, and chromatographic conditions must be optimized (22,23). In agreement with this group's previous studies (4, 21), the $6^{I–VII}$-O-TBDMS-$2^{I–VII}$-$3^{I–VII}$-O-acetyl-β-CD was adopted as chiral selector, because of its ability to separate enantiomers of γ- and δ- lactones simultaneously. In chiral analyses, the chromatographic conditions are conditioned by the host–guest interaction mechanisms between each enantiomer and the cyclodextrin chiral selector, and are thermodynamically driven, thus limiting the heating rates and resulting in long analysis times. A short narrow-bore column (10 m x 0.10 mm d_c, 0.10 µm d_f) had therefore to be chosen to achieve an analysis time compatible with the requirements of quality control. The chromatographic method was optimized to obtain a fast analysis, while maintaining baseline separation of all enantiomers in a single run (24). Moreover, thermodesorption of the analytes from the stir-bar required them to be cryofocused at the head of the column. The resulting optimized enantioselective GC temperature program was as follows: 0 °C for 0.5 min, then 0–90 °C at 80 °C/min, 90–195 °C at 8.1 °C/min, 195–230 °C at 15 °C/min, and hold at 230 °C for 2 min, with a total analysis time of 19 min.

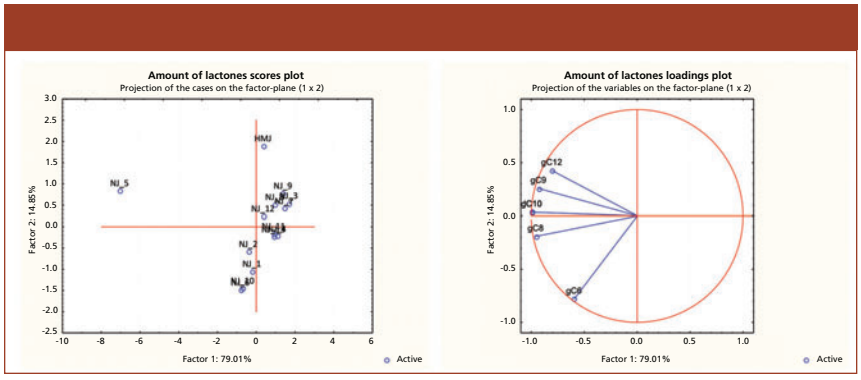

Figure 5: PCA scores and loading plots of the absolute amount of lactones for the natural juices. Legend: HMJ, reference juice; NJ, natural juice.

Figure 3 reports the patterns of the homologous series of γ- and δ- lactones and of a peach juice sampled with a PDMS stir-bar.

Development of the Quantification Method and Evaluation of its Performance

The key odorants of the peach juices were quantified by the external calibration method. The calibration curves were built by analyzing different concentrations of the racemate standards of γ- and δ-lactones, diluted in a model juice prepared by mixing 100 g of saccharose and 4 g of citric acid in 1 L of distilled water (2). The investigated ranges were 1–100 ppb for γ-lactones and 1–50 ppb for δ-lactones.

The performance of the method was evaluated in terms of linearity, limits of detection and quantification, and repeatability, for both PDMS and EG-silicone stir-bars. Table II reports the results and shows the method's excellent linearity over the ranges investigated for all analytes with both coatings.

PDMS was chosen as stir-bar coating material because its limits of detection and quantification were considerably lower than those obtained with EG-silicone coatings (see above). LODs ranged from 0.053 ppb for *(R)*-γ-dodecalactone to 4.5 ppb for *(R)*-γ-hexalactone for PDMS, and from 0.087 ppb for *(S)*-γ-dodecalactone to 21 ppb for *(R)*-γ-hexalactone for EG-silicone. LOQs ranged from 0.071 ppb for *(R)*-γ-dodecalactone to 6.1 ppb for *(R)*-γ-hexalactone for PDMS, and from 0.16 ppb for *(S)*-γ-dodecalactone to 49 ppb for *(R)*-γ-hexalactone for EG-silicone. The repeatability performance was similar, with RSD% below 15% at 5 ppb with the PDMS coating and below 20% at 25 ppb for EG-silicone. Lastly, the performances of SBSE sampling were compared to those of the method developed previously with HS-SPME-enantioselective-GC–MS (4). The LODs with PDMS stir-bars for both γ-decalactone and δ-decalactone were about two orders of magnitude lower than those with HS-SPME (4), enabling the key odorants in peach juice to be analyzed at the ng/L (ppt) level for γ-decalactone (<100 ppt (ng/L) and few µg/L (ppb) for δ-decalactone (<5 µg/L (ppb)).

Analysis of Real-World Samples and Data Processing

The method was then applied to a set of real-world samples (19 commercial natural and artificial flavored peach juices), and the results compared to those of the reference standard.

The results were evaluated in terms of enantiomeric composition and absolute amount of the target analytes, and were processed with multivariate statistical analysis using principal component analysis (PCA) for rapid sample discrimination.

Figure 4 reports the PCA scores and loading plots of the enantiomeric composition of the investigated samples. The results show clear discrimination between natural and artificial flavored juices, based on the presence of non-natural lactones (lactones with an odd number of carbon atoms, or *(S)*-enantiomers for the even-carbon-atom lactones).

Figure 5 reports the PCA scores and loading plots obtained by processing the absolute amount of lactones for the natural peach juices. The results showed clear discrimination of sample 5, both from the other commercial juices and from the reference juice, not detectable from the enantiomeric composition. This is because, although the enantiomeric composition of sample 5 is in accordance with that occurring naturally in peach, the absolute amount of all compounds is considerably higher than that of all other commercial and reference juices, indicating the possible addition of a natural aroma not mentioned in the label. This result clearly emphasizes the need to determine both enantiomeric composition and absolute amount of the chiral key odorants, to authenticate fruit flavored foods.

Finally, the absolute amount of the sum of the enantiomers was used to determine the odor activity value (OAV) of those lactones identified by Horvat and associates (18) as major contributors to peach aroma. The results, reported in Table III, show that only for γ-decalactone is the OAV above 1 in all the samples investigated, confirming that it may be considered the peach juice key odorant. The OAVs of the other lactones investigated are more variable, partly because flavoring ingredients are almost always added at concentrations above their odor threshold.

Conclusions

The study describes the development of a total analysis system to authenticate and assess the quality of fruit flavored food, based on quantification and enantiomer recognition of their chiral key odorants, in a single run. Peach was used as model fruit, and the enantiomers of its key aroma components (γ- and δ- lactones) were quantified in a set of natural and artificial peach-flavored juices.

Non-equilibrium fast SBSE with PDMS coating was combined with fast enantioselective GC-MS analysis, producing excellent results in terms of sensitivity (limits of detection and quantification in the ppt range), linearity, and repeatability. The quantitative data and enantiomeric composition were processed through online statistical elaboration (PCA) providing an effective and complementary discrimination among samples.

The total analytical method takes only 30 min, and does not require any sample pretreatment. It is simple, fast, and fully automated, and compatible with the processing of a large number of samples, as required in a routine quality control laboratory.

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(Continued from page 34)

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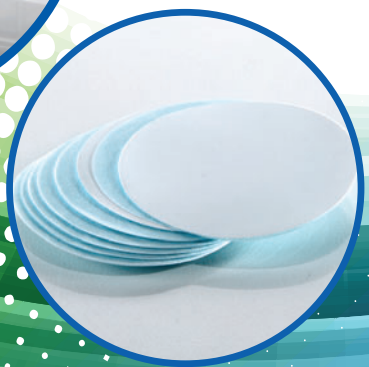

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