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Evaluation of Commercial Solid-Phase Extraction (SPE) Carrier Materials for the Selective Automated Enrichment of Monoterpenoides and their Analysis in Cough Drops, Mouthwashes and Bath Additives by Gas-Chromatography Mass Spectrometry (GC-MS)

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Abstract: For sample preparation solid-phase extraction (SPE) became very popular during the 1980s and 1990s. Because method development and its optimization is very time consuming, in most cases C_{18} is used as standard carrier material without knowing if it possesses the highest efficiency. Therefore detailed studies with different stationary phases are necessary for optimal recovery rates. Due to the resulting high number of samples, automation is of crucial importance. In the study described, the extraction of five monoterpenoids (myrcene, citronellal, p-cymene, menthol and thymol) out of an ethanolic solution was optimized using automated SPE. An automated robotic system, the MEA Personal Purification System[®] from PhyNexus™ Inc. (http://www.phynexus.com/), was adopted for high-throughput SPE. It allows the simultaneous automatic extraction of 12 samples in parallel employing different carrier materials. Additionally, critical parameters of automation (speed, volume uptake, skipping of drying step) were variegated to obtain optimal recovery rates. Furthermore, extraction efficiency was compared with manual handling to evaluate its performance. The application of 18 different SPE carrier materials, C_{18} Hydra, C_{18} ec, C_8, C_4, C_2, C_6H_5, C_6H_{11} ec, NO_2, CN, NH_2/C_{18}, Diol, HR-P, EASY, PA, Florisil[®], Davisil[®], Strata-X[®] and cellulose pointed out huge differences in extraction efficiency. Finally, the optimized automated procedure was applied for the enrichment of monoterpenoides and their analysis in selected real samples including cough drops, mouthwash and bath additives by gas-chromatography mass spectrometry (GC-MS).

Keywords: Solid-Phase extraction, monoterpenoides, automation, gas-chromatography mass spectrometry, real sample.

1. INTRODUCTION

Solid-phase extraction (SPE) is one of the most important sample preparation techniques [1]. Its popularity increased with the need to decrease organic solvent usage [2]. It can be used for different tasks, also in combination: trace enrichment (concentration), matrix simplification (sample clean-up) and medium exchange (transfer from the sample matrix to a different solvent or to the gas phase) [3]. One can differentiate between cartridge based SPE and dispersive SPE. In dispersive SPE, the stationary phase is added to the liquid sample, mixed and finally filtered to remove the liquid phase or parts of the liquid phase are used for further analysis. This technique is very popular for the cleanup of e.g. pesticide samples [4]. There it is part of the so called QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe) [5].

In the beginnings of SPE C_{18}-silicas differing only in particle size from those in high-performance liquid chromatography (HPLC) were used [2]. Nowadays sorbents especially designed for SPE are available, based on silica or on polymeric beads with a broad variety of functionalities [6]. The most common SPE materials are C_{18}-silicas, but there are a wide range of silica sorbents with varying alkyl chain length available, e.g. C_2, C_4, C_8. These materials show in most cases lower retention, but have the advantage to retain less interference. There are also phenyl and cyclohexyl phases available providing higher retention for some aromatic compounds [2]. To achieve high recovery rates, a large surface area is of great importance as the extraction efficiency depends on the equilibrium between the two phases. Typically C_{18}-silica materials are made from silica with specific surface areas in the range of 500 to 600 $m^2\,g^{-1}$, while certain polymeric resins designed for SPE reach high specific surface areas in the range of 700 to 1200 $m^2\,g^{-1}$ (e.g. Chromabond[®] HR-P 1200 $m^2\,g^{-1}$, Oasis[®] HLB 800 $m^2\,g^{-1}$) and thus higher capacities [2,3,7].

Another advantage of polymeric stationary phases is the better pH stability, while silica-based sorbents are only stable in a pH range of 2 to 8 [3,8]. Sun and Fritz found that extraction with polymeric resins resulted in higher recovery for many types of analytes [9,6]. Both silica and polymeric stationary phases have some disadvantages [10]. Due to its polar silanolic groups, silica is hydrophilic but is getting hydrophobic by the immobilized carbon chains. Polystyrene based materials have a hydrophobic character, too, resulting in poor surface contact under aqueous conditions [1,6,11].

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To overcome this disadvantage, the material has to be exposed to an activating solvent, in most cases methanol. Low recovery rates can also be caused in case the column runs dry and air is introduced [2, 12]. The new hydrophilic lipophilic based materials, like Oasis® HLB, a macroporous poly(divinylbenzene-co-N-vinylpyrrolidone) polymer (Waters, Eschborn, Germany), or Strata-X® (Phenomenex, Aschaffenburg, Germany), are water-wettable and presented as "universal" sorbent for acidic, basic and neutral analytes whether polar or unpolar. In addition to the hydrophobic interaction and the π-π-interactions of the divinylbenzene, the pyrrolidone groups acts as a hydrogen acceptor [2, 3].

Manual SPE procedure is time consuming and a lot of mistakes can be made. The basic equipment necessary for manual SPE is the SPE cartridge, a syringe and a special adapter. This way the necessary pressure is applied. Using this equipment only one sample can be processed at a time. To increase the amount of sample processed at a time, vacuum manifolds for 6, 12 or 24 samples are available. For high sample throughput especially in routine analysis there are automated systems on the market. Extraction plate systems are able to handle 96 samples at a time. The SPE material is embedded in every well. The waste well plate is replaced after running the sample through the system by a collection well plate and the analytes are eluted. This system has a big disadvantage, the restriction in choice of SPE materials as the material is the same in each well and cannot be chosen separately [13]. PhyNexus Inc. developed a Personal Purification and Enrichment System, MEA™. The MEA™ robot offers automated SPE for the simultaneous handling of up to 12 samples, while the sorbent can be chosen separately for each sample. Therefore the main application of the system lies in method development, optimization and medium throughput screening [13]. In case of really high sample numbers, Tecan® and Hamilton® offer fully automated SPE systems using 96 deep well plates for industrial and clinical applications.

The aim of the present study was to evaluate commercial available SPE materials for the selective automated enrichment of monoterpenoids and their analysis in cough drops, mouthwashes and bath additives by gas-chromatography mass spectrometry (GC-MS)

2. EXPERIMENTAL

2.1. Chemicals

Methanol (gradient grade) was purchased from Sigma-Aldrich (Steinheim, Germany) and water was provided by the in-house water treatment plant (Barnstead Nanopure Infinity, Thermo Fisher Scientific, Dreieich, Germany).

2.2. Samples

Standards were purchased from Sigma-Aldrich (Steinheim, Germany). The mixed standard solutions contained alpha-pinene (98%), beta-pinene (99%), myrcene (> 90%), citronellal (purum), L-menthol (purum), p-cymene (99%), borneol (puriss, p.a.) and thymol (99.5%). High concentrated standard solution contained 500 mg L^{-1} alpha-pinene, 570 mg L^{-1} beta-pinene, 591 mg L^{-1} myrcene, 502 mg L^{-1} p-cymene, 543 mg L^{-1} citronellal, 740 mg L^{-1} L-menthol, 730 mg L^{-1} borneol and 485 mg L^{-1} thymol in methanol. Low concentrated standard solution contained 123 mg L^{-1} alpha-pinene, 157 mg L^{-1} beta-pinene, 118 mg L^{-1} myrcene, 132 mg L^{-1} p-cymene, 159 mg L^{-1} citronellal, 182 mg L^{-1} L-menthol, 185 mg L^{-1} borneol and 128 mg L^{-1} thymol in methanol.

For SPE 500 µL of the mixed standard were diluted with 9.5 mL water to reduce the methanol concentration to 5%. The resulting 10 mL standard solution was used for one extraction.

Furthermore a mouthwash, two bath additives and cough drops were purchased in the local supermarket.

Mouthwash: 15 mL of the mouthwash were filled up to 250.0 mL with ultrapure water instead of 5% methanolic solution, as the mouthwash contains about 20% ethanol. For each extraction procedure 10.0 mL of this solution were used; thymol and menthol were quantified.

Bath additive 1: 5 g of the bath additive were filled up to 250 mL with 5% methanolic solution and was allowed to stand overnight to achieve phase separation. For each extraction procedure 10.0 mL of the aqueous phase were used; thymol and p-cymene were quantified.

Bath additive 2: 5 g of the bath additive were filled up to 250 mL with 5% methanolic solution. For each extraction procedure 10.0 mL of this solution were used; menthol and p-cymene were quantified.

Cough drops: 5 cough drops (3.6 g each) were dissolved in 200 mL of ultrapure water. 25 mL methanol was added and the flask was filled up to 500.0 mL with ultrapure water. For each extraction procedure 10.0 mL of this solution were used; menthol was quantified.

2.3. Solid-Phase Extraction (SPE)

Automated SPE: Solid-phase extraction was done employing the MEA Personal Purification System® (PhyNexus™ Inc., San Jose, CA, USA) [14] using tips containing 160 µL SPE-material (Fig. **1**). 10 mL prepared sample solution were pipetted into a 2.2 mL storage plate (Thermo Fisher Scientific, Dreieich, Germany) as depicted in Fig. (**2**) (1 mL per well). SPE tips were manually installed on the pipette head of the Personal Purification System® - two materials, six tips of each material - and the extraction method was started. Exact method executed with the Personal Purification System® is provided in Table **1**. The loading step took up 1 mL with a speed of 0.5 mL min^{-1} and a delay of 20 sec. Expelling step blew out 1 mL with a speed of 0.4 mL min^{-1} and a delay of 180 sec. The drying step took up and blew out 1 mL with a speed of 30 mL min^{-1} and a delay of 1 sec.

Finally the analytes were manually eluted from the tips using 700 µL methanol into a 1 mL volumetric flask. The volumetric flask was filled with methanol to the calibrating mark and the resulting sample analyzed by GC-MS. Total extraction procedure of the Personal Purification System® took 5 h 15 min. Extraction was done 6 times with each SPE material. Tips were purchased from PhyNexus™ Inc. (San Jose, CA, USA) except for Strata-X®, Cellulose, Florisil and Davisil which were manually packed into empty tips from PhyNexus™ Inc. Strata-X® was gained out of SPE cartridges (Phenomenex, Aschaffenburg, Germany). Cellulose (Avicel®, PH 101, Fluka, Steinheim, Germany), Florisil

Fig. (1). MEA Personal Purification System® (PhyNexus Inc., San Jose, CA, USA).

(Carl Roth GmbH + Co.KG, Karlsruhe, Germany), Davisil (Grace Davison, Worms, Germany) were purchased as bulk material.

2.4. Variations of Solid-Phase Extraction Procedure

To study the effects of extraction parameters, method and protocol were changed in different points: Each well extracted once and twice, uptake of only 0.8 mL sample, skipping of drying step and changes in loading and expelling speed.

Each well extracted once: Extraction procedure was mainly the same as described before (Table **1**) with the difference that each well was only extracted once. This way the extraction time was reduced to 2 h 07 min. Extraction was done with C_{18} Hydra and C_{18} ec.

Each well extracted twice: Extraction procedure was mainly the same as with normal extraction (Table **1**) with the
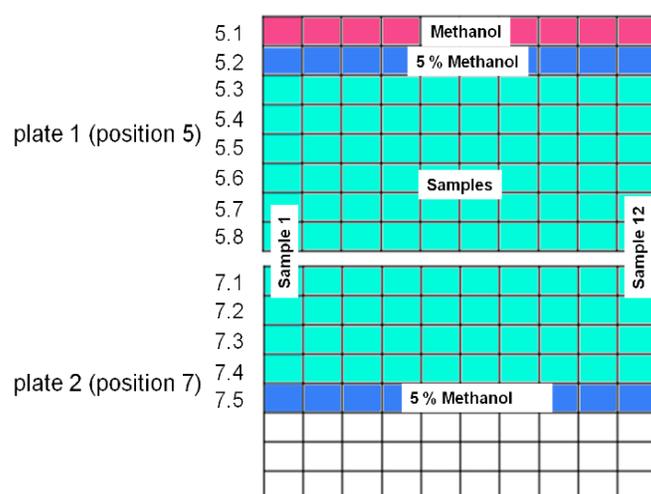
Fig. (2). Schematic view of storage plate.

Table 1. Method Used for Automated SPE

Row	Filling	Repetitions	Step
5.1	1 mL methanol	2	conditioning
5.2	1 mL water with 5% methanol	2	conditioning
5.3	1 mL sample	3	extraction (loading + expelling)
5.4	1 mL sample	3	extraction
5.5	1 mL sample	3	extraction
5.6	1 mL sample	3	extraction
5.7	1 mL sample	3	extraction
5.8	1 mL sample	3	extraction
7.1	1 mL sample	3	extraction
7.2	1 mL sample	3	extraction
7.3	1 mL sample	3	extraction
7.4	1 mL sample	3	extraction
7.5	1 mL water with 5% methanol	3	washing
7.6	air	30	drying

difference that each well was only extracted twice. This led to a reduced extraction time of 3 h 41 min. Extraction was done with C_{18} Hydra and C_{18} ec.

Uptake of 0.8 mL sample: Extraction procedure was mainly the same as with normal extraction with the difference that only 0.8 mL sample was taken up. This resulted in a reduced extraction time of 4 h 39 min. The extraction was done with C_{18} Hydra and C_{18} ec.

Skipping of drying step: Extraction procedure was mainly the same as with normal extraction with the difference that the drying step was skipped. The extraction was done with C_{18} Hydra and C_{18} ec and took 5 h 13 min.

Changes in loading and expelling speed: Extraction procedure was mainly the same as with normal extraction with the difference that the loading speed was changed to 0.5 mL min^{-1} and the expelling speed to 0.4 mL min^{-1}. This led to a reduced extraction time of 2 h 38 min. The extraction was done with C_{18} Hydra and C_{18} ec.

2.5. Manual Solid-Phase Extraction

To compare recovery rates all extractions were done manually, too, employing classic cartridges and tips. In principal extraction was done the same way as with the MEA™-system. For each material three extractions were performed. Cartridges including the stationary phase were purchased from Macherey-Nagel except Strata-X® which was purchased from Phenomenex. Manual extraction with cartridges was done with a syringe and adapter. For the manual extraction of tips, liquid was pipetted on the column bed of the tip and pushed through with a fitting Rainin® pipette (Mettler-Toledo, Gießen, Germany). SPE cartridges or tips were conditioned with 1 mL of methanol and 1 mL water containing 5% methanol. After that 1 mL of diluted standard solution was pipetted on top of the column bed and pushed through. The same was done with the remaining 9 mL. In the next step the cartridges or tips were washed with 1 mL of water containing 5% methanol. Finally the analytes were eluted from the cartridges or tips using 700 µL methanol into a 1 mL volumetric flask. The volumetric flask was filled with methanol to the calibrating mark and the resulting sample analyzed by gas chromatography mass spectrometry (GC-MS).

2.6. Gas Chromatography – Mass Spectrometry (GC-MS) Measurements

The GC-MS method used for recovery experiments was developed by the authors. Measurements were carried out on an Agilent 6890N gas chromatograph coupled to a 5973 inert mass selective detector (Agilent Technologies, Waldbronn, Germany) equipped with a Combi PAL autosampler (CTC, Zwingen, Switzerland). For data acquisition MSD ChemStation E.02.01.1177 was used (Agilent Technologies, Waldbronn, Germany). Separation was done on a HP-20M (Carbowax 20M) column; 25 m x 0.20 mm x 0.2 µm (Hewlett-Packard, Palo Alto, CA, USA). The temperature program used was 45 °C – 0.5 min – 10°C min^{-1} – 180°C – 20°C/ min^{-1} – 200°C – 3.0 min; the carrier gas flow (helium) was 0.5 mL min^{-1}. Injected sample volume: 1 µL; split ratio: 10:1; inlet temperature 280 °C. The sample was analyzed in the full scan mode using a mass range of 100–250 Th after

electron impact ionization (70 eV). The interface and ion source temperatures were 250 °C and 230 °C, respectively, the quad temperature 150 °C. 100 µL of sample were mixed with 10 µL of an internal standard solution containing 25.05 mg mL^{-1} o-cresol. For the quantification of thymol the ion with 135 Th, for borneol 95 Th, for menthol 71 Th, for citronellal 69 Th, for p-cymene 119 Th, for myrcene 93 Th and for the external standard o-cresol 108 Th were used.

The method was validated regarding selectivity, linearity, limit of quantification and precision. Method linearity was given between 2.8 mg L^{-1} and 136 mg L^{-1} for thymol, between 8 mg L^{-1} and 200 mg L^{-1} for borneol, between 3.8 mg L^{-1} and 180 mg L^{-1} for menthol, between 3.3 mg L^{-1} and 157 mg L^{-1} for citronellal, between 2.9 mg L^{-1} and 138 mg L^{-1} for p-cymene and between 2.6 mg L^{-1} and 127 mg L^{-1} for myrcene with $R^2 \geq 0.99$. Limit of quantification was set to 2.8 mg L^{-1} for thymol, 8 mg L^{-1} for borneol, 3.8 mg L^{-1} for menthol, 3.3 mg L^{-1} for citronellal, 2.9 mg L^{-1} for p-cymene and 2.6 mg L^{-1} for myrcene. From a sixfold injection a coefficient of variation of 3.2% resulted for myrcene, 4.7% for p-cymene, 3.6% for citronellal, 3.5% for menthol, 3.4% for borneol and 4.4% for thymol. Accuracy data is not given, as the complete work consists of recovery experiments.

3. RESULTS AND DISCUSSION

In the first step the system was adopted, evaluated and validated applying standards and mixtures thereof. In the following experiments the optimized protocol was extrapolated to the analysis of real samples.

3.1. Enrichment and Analysis of Standards

The gas chromatogram of mouthwash after extraction with Strata-X® is depicted in Fig. (**3**). In principal separation is achieved in order of the analytes' polarity: myrcene, p-cymene, citronellal, menthol, borneol, o-cresol (internal standard) and thymol. Recovery rates for high concentration are given in Table **2**, while those for low concentration can be deduced from Table **3**.

At high concentration thymol, borneol, menthol and citronellal showed highest recovery rates for Strata-X®. For thymol second best material was C_{18} ec closely followed by C_8, for borneol C_{18} Hydra followed by C_4. For menthol second best material was C_{18} ec closely followed by C_8 and C_{18} Hydra, for citronellal C_8 and C_{18} ec closely followed by C_{18} Hydra. For p-cymene only C_{18} Hydra and for myrcene C_{18} Hydra and Davisil showed recovery $\geq$ 10%. alpha-Pinene and beta-pinene were not interpreted because of improper peak shape.

Low concentration resulted for thymol, borneol, menthol and citronellal in highest recovery rates with Strata-X®. For thymol second best material was C_{18} ec closely followed by C_{18} Hydra and C_6H_{11} ec, for borneol by C_{18} Hydra and C_{18} ec. For menthol second best material was C_{18} Hydra closely followed by C_{18} ec and C_6H_{11} ec. Citronellal showed second best result with C_8 and C_{18} ec closely followed by C_{18} Hydra and C_6H_{11} ec. For p-cymene and myrcene achieved recovery rates were very low with all materials (< 10%).
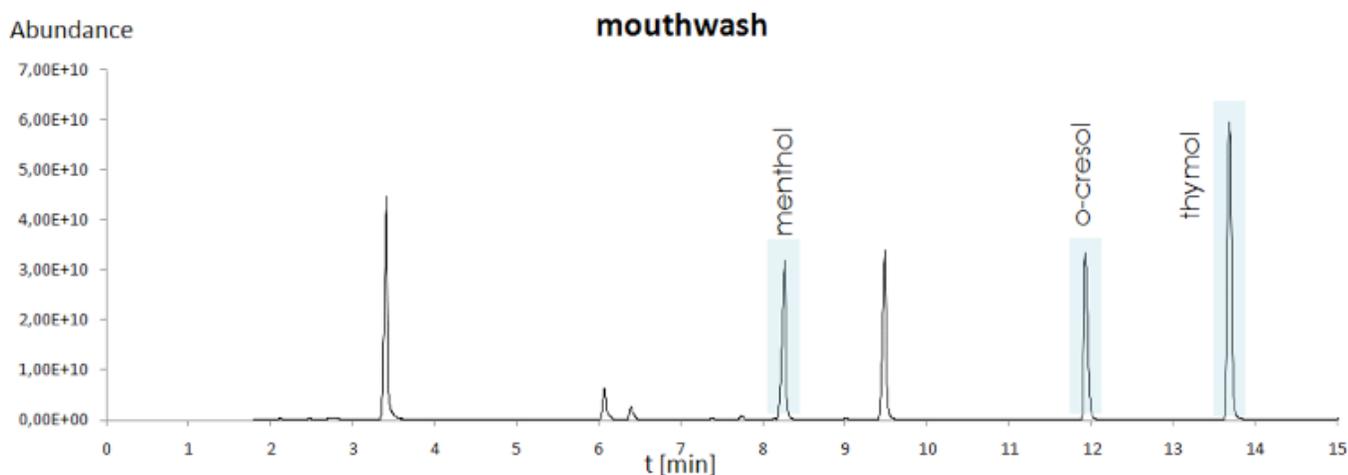
Fig. (3). Chromatogram of mouthwash extracted with Strata-X.

Table 2.   **Recovery Rates of High Concentrated Monoterpene Standards in % Including Standard Deviation and Coefficient of Variation in Italics**

	Myrcene	p-Cymene	Citronellal	Menthol	Borneol	Thymol
C_2	< 10	< 10	< 10	< 10	20 ± 4 (*19*)	< 10
C_4	< 10	< 10	15 ± 5 (*33*)	26 ± 7 (*27*)	55 ± 10 (*18*)	20 ± 5 (*24*)
C_8	< 10	< 10	34 ± 2 (*7*)	64 ± 9 (*14*)	50 ± 8 (*16*)	62 ± 8 (*13*)
C_{18} Hydra	12 ± 1 (*7*)	12 ± 1 (*6*)	24 ± 2 (*7*)	62 ± 6 (*10*)	58 ± 10 (*17*)	49 ± 8 (*17*)
C_{18} ec	< 10	< 10	28 ± 3 (*9*)	69 ± 6 (*9*)	37 ± 3 (*7*)	64 ± 9 (*14*)
C_6H_{11} ec	< 10	< 10	25 ± 3 (*12*)	48 ± 5 (*10*)	15 ± 3 (*22*)	45 ± 4 (*9*)
C_6H_5	< 10	< 10	15 ± 5 (*31*)	21 ± 5 (*25*)	< 10	16 ± 4 (*25*)
CN	< 10	< 10	< 10	< 10	27 ± 1 (*4*)	< 10
NH_2/C_{18}	< 10	< 10	19 ± 2 (*10*)	39 ± 3 (*7*)	< 10	27 ± 2 (*6*)
OH	< 10	< 10	< 10	< 10	< 10	< 10
NO_2	< 10	< 10	< 10	< 10	36 ± 3 (*7*)	< 10
Easy	< 10	< 10	21 ± 2 (*7*)	40 ± 3 (*7*)	35 ± 5 (*16*)	43 ± 3 (*7*)
HR-P	< 10	< 10	19 ± 3 (*14*)	37 ± 5 (*14*)	< 10	39 ± 6 (*15*)
PA	< 10	< 10	< 10	< 10	< 10	16 ± 1 (*6*)
Strata-X®	< 10	< 10	58 ± 5 (*9*)	88 ± 6 (*7*)	87 ± 6 (*7*)	92 ± 7 (*7*)
Cellulose	< 10	< 10	< 10	< 10	< 10	< 10
Davisil	15 ± 2 (*13*)	< 10	< 10	< 10	< 10	< 10
Florisil	< 10	< 10	< 10	< 10	20 ± 4 (*19*)	< 10

From those results one can see that in general especially polymeric materials like Strata-X®, Easy, HR-P and PA have low standard deviations and coefficients of variation in comparison to silica based materials. Furthermore results show that taking standard deviation into account, recovery rates were in the same range for low and high concentration. Thus breakthrough of analytes resulting from exceeding capacity of column bed can be excluded. Only in case of borneol extraction with Strata-X® standard deviation cannot compensate the difference in recovery rates, but most probably is a statistic variation.

The results clearly show that C_{18} is not the most effective SPE material for this task. Furthermore it can be seen that recovery rates for thymol, borneol and menthol are much higher than for myrcene, p-cymene and citronellal. The reason may be the higher volatility of the more nonpolar compounds. As the extraction took quite a long time (5 h 15 min) analytes had the opportunity to vaporize. Another explanation for lower recovery of more nonpolar compounds is the low solubility of the analytes in the sample solution, which consisted of water with 5% methanol. Analytes can have precipitated before or during SPE

Table 3.   Recovery Rates of Low Concentrated Monoterpene Standards in % Including Standard Deviation and Coefficient of Variation in Italics (*Myrcene and p-Cymene in All Cases < 10%*)

	Citronellal	Menthol	Borneol	Thymol
C_2	< 10	< 10	< 10	< 10
C_4	12 ± 4 (*30*)	27 ± 6 (*22*)	21 ± 3 (*13*)	21 ± 4 (*17*)
C_8	32 ± 5 (*16*)	53 ± 10 (*19*)	48 ± 10 (*22*)	53 ± 11 (*21*)
C_{18} Hydra	27 ± 5 (*19*)	69 ± 9 (*13*)	58 ± 11 (*19*)	58 ± 11 (*19*)
C_{18} ec	31 ± 5 (*15*)	64 ± 9 (*13*)	57 ±12 (*21*)	61 ± 12 (*19*)
C_6H_{11} ec	26 ± 5 (*18*)	62 ± 12 (*20*)	48 ± 8 (*16*)	56 ± 11 (*19*)
C_6H_5	13 ± 3 (*24*)	20 ± 2 (*11*)	17 ± 2 (*9*)	17 ± 1 (*8*)
CN	< 10	< 10	< 10	< 10
NH_2/C_{18}	23 ± 6 (*27*)	47 ± 14 (*30*)	38 ± 16 (*43*)	39 ± 16 (*42*)
OH	< 10	< 10	< 10	< 10
NO_2	< 10	< 10	< 10	< 10
Easy	21 ± 2 (*9*)	37 ± 3 (*8*)	36 ± 3 (*7*)	42 ± 3 (*6*)
HR-P	19 ± 2 (*9*)	36 ± 3 (*8*)	35 ± 3 (*7*)	39 ± 3 (*7*)
PA	< 10	< 10	< 10	16 ± 2 (*10*)
Strata-X®	50 ± 3 (*5*)	76 ± 4 (*5*)	94.8 ± 4 (*4*)	94 ± 3 (*3*)
Cellulose	< 10	< 10	< 10	< 10
Davisil	< 10	< 10	< 10	< 10
Florisil	< 10	< 10	< 10	< 10

cleanup. Strata-X® shows highest recovery of all tested sorbents for each standard analyte. This might be based on the special chemistry combined the high specific surface [15]. Wissiack *et al.* obtained similar results, when they compared different sorbents for online SPE coupled with liquid chromatography – mass spectrometry of phenols. They obtained high recoveries with good relative standard deviations with Oasis® HLB compared to silica based sorbents or conventional polymer based sorbents [16].

Strata-X® offers π- π bonding due to the phenyl ring in the ligand. This can increase recovery of aromatic analytes, e.g. p-cymene and thymol. Hydrophobic interactions of the styrene-divinylbenzene backbone leads to high recovery of neutral hydrophobic analytes, e.g. monoterpenes. The pyrrolidone ligand is able to form hydrogen bonds with analytes containing hydroxyl groups, e.g. thymol, borneol or menthol. Furthermore it can interact with acidic compounds. Additionally the pyrrolidone ligand can participate in dipole-dipole interactions, e.g. with aldehydes like citronellal. Highest recovery rates were observed with thymol and borneol. Thymol is able to use four interactions: π- π bonding, hydrophobic interactions, hydrogen bonds and interaction with acidic compounds. As thymol is a derivate of phenol having the character of a weak acid it can interact with the basic pyrrolidone ligand. Borneol can interact by hydrogen bonding and hydrophobic interaction.

C_2 and C_4 are traditionally used for the extraction of analytes, which are too strongly retained with C_{18}-silica and show, as expected, low recovery rates for all analytes. C_8 is used for the same analytes as C_{18}, but as the alkyl chain is

shorter, it offers the possibility of polar interactions with the free silanols on the silica surface. But in this experiment, this does not have a great influence as the recovery rates for C_8, C_{18} ec and C_{18} Hydra do not differ, especially when taking standard deviation into account. C_6H_{11} ec showed recovery rates in the same range as C_8, C_{18} ec and C_{18} Hydra, even for thymol, although it is recommended for the extraction of phenols from water. Surprisingly C_6H_5 showed lower recovery than C_8, C_{18} ec and C_{18} Hydra, so π- π bonding seems to have lower influence on recovery than hydrophobic interaction. CN, OH, NO_2, PA, Cellulose, Davisil and Florisil are normal phase sorbents, which were used in reversed-phase (RP) mode and thus it is no surprise that they show quite low recovery. According to Machery-Nagel®'s product information, HR-P is a poly(styrene-*co*-divinylbenzene) polymer with a high specific surface of 1200 m²/g recommended for the extraction of aromatic compounds and phenols from water. Easy is a polystyrene-divinylbenzene polymer (650 – 700 m²/g) functionalized with a weak ion exchanger to be more hydrophilic and is recommended for the extraction of polar phenols from water. In comparison to HR-P, Easy did not show better recovery due to its ion exchange capacity.

3.2. Variation of Extraction Procedure

Tables **4** and **5** show the recovery rates obtained with C_{18} Hydra and C_{18} ec using the different SPE methods as described in the experimental section 2.4.

For C_{18} Hydra in case of thymol, borneol and menthol extracting each well only one time and extraction of 0.8 mL led to lower recovery rates in comparison to the "normal"

Table 4.   Recovery Rates of Monoterpenes Achieved for Different Extraction Variations with C$_{18}$ Hydra in % Including Standard Deviation and Coefficient of Variation in Italics (Myrcene and p-Cymene in all Cases < 10%)

	Citronellal	Menthol	Borneol	Thymol
normal	27 ± 5 (*19*)	69 ± 9 (*13*)	58 ± 11 (*19*)	58 ± 11 (*19*)
2 x extraction	31 ± 4 (*13*)	76 ± 10 (*13*)	64 ± 13 (*21*)	65 ± 15 (*23*)
1 x extraction	44 ± 3 (*6*)	54 ± 6 (*11*)	39 ± 8 (*19*)	40 ± 8 (*19*)
0.8 mL extraction	32 ± 6 (*19*)	49 ± 10 (*21*)	40 ± 12 (*31*)	41 ± 13 (*32*)
fast extraction	31 ± 2 (*6*)	77 ± 2 (*2*)	63 ± 2 (*4*)	65 ± 3 (*4*)
without drying	16 ± 1 (*5*)	65 ± 2 (*3*)	52 ± 4 (*7*)	53 ± 5 (*9*)

Table 5.   Recovery Rates of Monoterpenes Achieved with Different Extraction Variations for C$_{18}$ ec in % Including Standard Deviation and Coefficient of Variation in Italics (*Myrcene in All Cases < 10%*)

	p-Cymene	Citronellal	Menthol	Borneol	Thymol
normal	< 10	31 ± 5 (*16*)	64 ± 9 (*14*)	57 ± 12 (*21*)	61 ± 12 (*20*)
2 x extraction	< 10	35 ± 2 (*5*)	82 ± 3 (*3*)	73 ± 10 (*14*)	81 ± 9 (*11*)
1 x extraction	< 10	38 ± 6 (*15*)	47 ± 8 (*17*)	40 ± 10 (*26*)	44 ± 10 (*23*)
0.8 mL extraction	< 10	39 ± 7 (*18*)	59 ± 10 (*17*)	51 ± 9 (*17*)	54 ± 9 (*16*)
fast extraction	11 ± 1 (*10*)	37 ± 1 (*3*)	88 ± 2 (*2*)	83 ± 4 (*4*)	90 ± 3 (*3*)
without drying	< 10	16 ± 4 (*23*)	73 ± 8 (*12*)	69 ± 11 (*16*)	77 ± 11 (*15*)

procedure. Taking standard deviation into account, differences are compensated for the most part. Regarding standard deviation no significant difference was obtained in case of citronellal. Skipping of drying step and fast extraction resulted in most cases in lower coefficients of variation. Best results were achieved with fast extraction as it resulted in high recovery and low coefficient of variation. For C$_{18}$ ec in case of thymol, borneol and menthol fast extraction led to higher recovery rates in comparison to the "normal" procedure. The same was obtained for two times extraction, but standard deviations are compensating the differences. Extracting only once or extracting 0.8 mL resulted in reduced recovery rates. Regarding standard deviation no significant difference was obtained in case of citronellal. Fast extraction additionally led to lower coefficient of variation.

3.3. Manual Solid-Phase Extraction

In the first step cartridges and in the following tips were employed for SPE.

3.3.1 Cartridges

Table **6** gives the results obtained by manual extraction with classical SPE cartridges. High recovery rates with nearly equal results were obtained with C$_4$, C$_8$, C$_{18}$, C$_{18}$ Hydra, C$_{18}$ ec, C$_6$H$_{11}$ ec and C$_6$H$_5$ and Strata-X$^®$. HR-X did not deliver as high results as Strata-X$^®$ with only 76% recovery for thymol, 81% for menthol and 77% for borneol. Even in the manual operation mode, overall recovery rates for myrcene and p-cymene were very low. The difference in standard deviation and coefficient of variation obtained with automation was not found in the same dimensions, especially in case of menthol, borneol and thymol.

Manual extraction with cartridges led to high recoveries with a wide range of sorbents (C$_4$, C$_8$, C$_{18}$, C$_{18}$ Hydra, C$_{18}$ ec, C$_6$H$_{11}$ ec, C$_6$H$_5$ and Strata-X$^®$), especially for thymol, borneol and menthol. So here the hydrophobic interactions of C$_4$, C$_8$, C$_{18}$, C$_{18}$ Hydra, C$_{18}$ ec, C$_6$H$_{11}$ ec work as well as the additional π- π interactions of C$_6$H$_5$ and the many possible interactions of Strata-X$^®$ already discussed in the previous chapters. 3 Endcapping does not seem to have significant influence on recovery of all of the analytes as recovery rates achieved with C$_{18}$ Hydra, C$_{18}$ and C$_{18}$ ec show similar resuts. As expected, the extraction with the normal-phase(NP) sorbents OH, NO$_2$, Florisil, SiOH and NH$_2$ did not reach high recoveries. CN is a normal-phase sorbent that can also be used in reversed-phase mode. Due to its high electron density it can show selective interaction, but is less hydrophobic than C$_{18}$ or C$_8$ and is recommended for the extraction of lipophilic compounds from aqueous solutions, which are too strongly retained with C$_8$ or C$_{18}$. As the extraction of the applied analytes works well with C$_{18}$ and C$_8$, the hydrophobic interaction of CN seems to be too low to reach satisfying recovery. HR-X, a hydrophobic poly(styrene-*co*-divinylbenzene) polymer, which is recommended for the extraction of phenols from water, achieved moderate results in case of thymol, borneol and menthol. But as the exact composition and modification of this material is not available, further discussion would be wild guess. HR-P, a poly(styrene-*co*-divinylbenzene) copolymer, did only reach moderate recovery.

3.3.2. Tips

Table **7** gives the results obtained by manual extraction with SPE tips. For thymol, borneol and menthol very high recovery rates of about 100% were found with C$_4$, C$_8$,

Table 6.   Recovery Rates of Monoterpene Standards in % Including Standard Deviation and Coefficient of Variation in Italics (Myrcene and p-Cymene in All Cases < 10%) for Manual Extraction with Cartridges

	Myrcene	p-Cymene	Citronellal	Menthol	Borneol	Thymol
C_2	< 10	< 10	< 10	< 10	< 10	< 10
C_4	45 ± 9 (*19*)	35 ± 6 (*16*)	92 ± 4 (*4*)	102 ±4 (*4*)	102 ± 4 (*4*)	99 ± 2 (*2*)
C_8	39 ± 3 (*8*)	48 ± 5 (*11*)	93 ± 6 (*6*)	105 ± 3 (*3*)	105 ± 3 (*3*)	103 ± 3 (*3*)
C_{18}	20 ± 14 (*66*)	26 ± 10 (*39*)	79 ± 8 (*11*)	99 ± 5 (*5*)	98 ± 5 (*5*)	99 ± 5 (*5*)
C_{18} Hydra	32 ± 2 (*5*)	42 ± 2 (*5*)	90 ± 4 (*5*)	103 ± 3 (*3*)	103 ± 3 (*3*)	104 ± 4 (*4*)
C_{18} ec	31 ± 1 (*2*)	38 ± 1 (*4*)	89 ± 4 (*4*)	104 ± 5 (*5*)	101 ± 4 (*4*)	100 ± 5 (*4*)
C_6H_{11} ec	23 ± 2 (*7*)	37 ± 2 (*5*)	83 ± 1 (*2*)	97 ± 2 (*2*)	98 ± 2 (*2*)	94 ± 2 (*2*)
C_6H_5	20 ± 2 (*9*)	35 ± 4 (*10*)	82 ± 6 (*7*)	104 ± 6 (*6*)	94 ± 6 (*6*)	99 ± 7 (*7*)
CN	17 ± 2 (12)	22 ± 1 (*7*)	37 ± 2 (*5*)	21 ± 1 (*4*)	< 10	43 ± 1 (*3*)
OH	< 10	< 10	< 10	< 10	< 10	< 10
NO_2	< 10	< 10	< 10	< 10	< 10	< 10
HR-P	17 ± 3 (*17*)	17 ± 2 (*12*)	48 ± 7 (*14*)	65 ± 9 (*14*)	65 ± 9 (*13*)	57 ± 6 (*11*)
Strata-X®	30 ± 4 (*13*)	44 ± 1 (*3*)	82 ± 1 (*2*)	93 ± 2 (*2*)	93 ± 2 (*2*)	94 ± 2 (*2*)
Florisil	< 10	< 10	21 ± 1 (*3*)	13 ± 1 (*6*)	< 10	< 10
HR-X	< 10	< 10	32 ± 3 (*10*)	77 ± 5 (*7*)	81 ± 5 (*6*)	76 ± 5 (*6*)
SiOH	< 10	< 10	< 10	< 10	< 10	< 10
NH_2	< 10	< 10	< 10	< 10	< 10	< 10

C_{18} Hydra, C_{18} ec, C_6H_{11} ec, C_6H_5, NH_2/C_{18} and Strata-X®. Second best results around 70% gave Easy and HR-P. For citronellal recovery rates of about 100% (between 90 and 116%) were achieved with C_4, C_8, C_{18} Hydra, C_{18} ec, C_6H_{11} ec and C_6H_5. Second best was Strata-X® with a recovery rate of about 80%. Overall recovery rates for myrcene and p-cymene were very low also here in manual mode with SPE tips.

Table 7.   Recovery Rates of Monoterpene Standards in % Including Standard Deviation and Coefficient of Variation in Italics (*Myrcene and p-Cymene in All Cases < 10%*) for Manual Extraction with Tips

	Myrcene	p-Cymene	Citronellal	Menthol	Borneol	Thymol
C_2	< 10	< 10	< 10	< 10	< 10	< 10
C_4	44 ± 1 (*3*)	62 ± 5 (*8*)	92 ± 11 (*12*)	103 ± 4 (*4*)	102 ± 2 (*2*)	101 ± 1 (*1*)
C_8	68 ± 8 (*11*)	68 ± 12 (*18*)	107 ± 18 (*17*)	108 ± 15 (*14*)	109 ± 15 (*14*)	110 ± 14 (*13*)
C_{18} Hydra	68 ± 6 (*9*)	67 ± 3 (*4*)	99 ± 6 (*6*)	101 ± 7 (*7*)	102 ± 7 (*7*)	101 ± 7 (*7*)
C_{18} ec	78 ± 4 (*5*)	73 ± 2 (*3*)	97 ± 0 (*0*)	99 ± 2 (*2*)	100 ± 2 (*2*)	100 ± 2 (*2*)
C_6H_{11} ec	46 ± 5 (*12*)	62 ± 7 (*11*)	116 ± 3 (*3*)	101 ± 1 (*1*)	102 ± 2 (*2*)	103 ± 1 (*1*)
C_6H_5	53 ± 0 (*0*)	23 ± 6 (*25*)	92 ± 10 (*11*)	107 ± 5 (*5*)	95 ± 4 (*4*)	107 ± 3 (*3*)
CN	12 ± 1 (*7*)	12 ± 1 (*6*)	14 ± 2 (*13*)	< 10	< 10	13 ± 2 (*17*)
NH_2/C_{18}	56 ± 2 (*3*)	65 ± 3 (*4*)	35 ± 6 (*16*)	98 ± 3 (*3*)	99 ± 2 (*2*)	99 ± 1 (*1*)
OH	< 10	< 10	< 10	< 10	< 10	< 10
NO_2	18 ± 1 (*5*)	< 10	13 ± 1 (*8*)	< 10	< 10	< 10
Easy	41 ± 5 (*13*)	39 ± 3 (*7*)	61 ± 3 (*5*)	75 ± 6 (*7*)	77 ± 5 (*7*)	70 ± 5 (*8*)
HR-P	47 ± 1 (*3*)	43 ± 8 (*19*)	60 ± 5 (*8*)	75 ± 5 (*7*)	75 ± 6 (*8*)	70 ± 6 (*8*)
PA	18 ± 2 (*12*)	< 10	<10	<10	< 10	19 ± 2 (*11*)
Strata-X®	47 ± 4 (*10*)	51 ± 5 (*9*)	84 ± 4 (*5*)	100 ± 2 (*2*)	102 ± 2 (*2*)	102 ± 1 (*1*)
Cellulose	< 10	< 10	< 10	< 10	< 10	< 10
Davisil	< 10	< 10	< 10	< 10	< 10	< 10
Florisil	< 10	< 10	14 ± 2 (*15*)	< 10	< 10	< 10

Comparable to manual extraction with cartridges for thymol, borneol and menthol a wide range of sorbents achieved recovery around 100%. The mainly on hydrophobic interaction based C_4, C_8, C_{18} Hydra, C_{18} ec, C_6H_{11} ec and HR-P as well as the mixed NH_2/C_{18} sorbent and the "universal" Strata-X®. Easy and HR-P showed nearly equal recovery for all analytes, although HR-P is completely nonpolar, while Easy is modified to show hydrophilic character. For citronellal the hydrophobic interaction capacity of the silica based sorbents seems to work more efficiently than the many interactions of Strata-X®. Surprisingly recovery with C_6H_5 was quite low for p-cymene, although π- π interactions due to the aromatic ring systems should be expected.

3.4. Real Sample Analysis

In the following the above optimized protocols were applied for the analysis of real samples including mouthwash, bath additives and cough drops.

3.4.1. Mouthwash

Menthol and thymol were determined in mouthwash. Strata-X showed best results for thymol as well as menthol. Second best results were achieved with C_8 followed by C_{18} ec, C_6H_{11} ec and C_{18} Hydra (Table **8**). In general, the SPE materials showed similar results as with standard solution.

Table 8.   **Thymol and Menthol Concentration in mg mL^{-1} Found After Automated SPE of Mouthwash. Also Given are Standard Deviation and Coefficient of Variation in Italics**

	Menthol	Thymol
C_2	0.10 ± 0.00 (*12.2*)	0.06 ± 0.01 (*10.3*)
C_4	0.29 ± 0.04 (*39.3*)	0.18 ± 0.06 (*34.9*)
C_8	0.68 ± 0.06 (*26.0*)	0.41 ± 0.10 (*24.0*)
C_{18} Hydra	0.54 ± 0.05 (*21.9*)	0.33 ± 0.05 (*16.7*)
C_{18} ec	0.64 ± 0.05 (*22.4*)	0.38 ± 0.05 (*13.8*)
C_6H_{11} ec	0.60 ± 0.04 (*21.6*)	0.36 ± 0.05 (*14.7*)
C_6H_5	0.34 ± 0.02 (*19.5*)	0.20 ± 0.04 (*18.6*)
CN	0.19 ± 0.01 (*26.1*)	0.11 ± 0.02 (*17.2*)
NH_2/C_{18}	0.40 ± 0.04 (*31.4*)	0.24 ± 0.05 (*20.0*)
OH	< LOQ	0.01 ± 0.00 (*20.9*)
NO_2	0.10 ± 0.00 (*29.3*)	0.06 ± 0.02 (*28.2*)
Easy	0.39 ± 0.02 (*14.2*)	0.23 ± 0.03 (*14.7*)
HR-P	0.43 ± 0.02 (*10.4*)	0.26 ± 0.02 (*8.8*)
PA	0.24 ± 0.00 (*1.9*)	0.14 ± 0.01 (*5.8*)
Strata-X	0.97 ± 0.01 (*4.0*)	0.58 ± 0.02 (*3.1*)
Cellulose	< LOQ	< LOQ
Davisil	< LOQ	0.01 ± 0.01 (*58.2*)
Florisil	0.05 ± 0.01 (*20.2*)	0.03 ± 0.00 (*16.6*)

3.4.2. Bath Additive

In bath additive 1, thymol and p-cymene were determined (Table **9**). For thymol Strata-X showed best results followed by C_8 and C_6H_5. Similar results were achieved for p-cymene but the differences to other materials were quite small. In comparison to the experiment using pure standards, CN and NO_2 had high recovery rates.

Table 9.   **Concentration of p-Cymene and Thymol in mg g^{-1} Found After Automated SPE of Bath Additive 1. Also Given are Standard Deviation and Coefficient of Variation in Italics**

	p-Cymene	Thymol
C_2	0.146 ± 0.014 (*9.9*)	0.567 ± 0.014 (*2.4*)
C_4	0.139 ± 0.035 (*24.9*)	0.645 ± 0.138 (*21.5*)
C_8	0.261 ± 0.042 (*16.2*)	0.611 ± 0.041 (*6.8*)
C_{18} Hydra	0.204 ± 0.028 (*13.6*)	0.673 ± 0.072 (*10.7*)
C_{18} ec	0.209 ± 0.048 (*22.9*)	0.576 ± 0.107 (*18.6*)
C_6H_{11} ec	0.160 ± 0.013 (*8.0*)	0.649 ± 0.043 (*6.6*)
C_6H_5	0.255 ± 0.083 (*32.5*)	0.572 ± 0.054 (*9.5*)
CN	0.134 ± 0.007 (*5.2*)	0.554 ± 0.023 (*4.1*)
NH_2/C_{18}	0.223 ± 0.030 (*13.4*)	0.614 ± 0.065 (*10.6*)
OH	< LOQ	0.067 ± 0.006 (*8.8*)
NO_2	0.133 ± 0.007 (*5.4*)	0.557 ± 0.025 (*4.5*)
Easy	0.199 ± 0.028 (*14.3*)	0.553 ± 0.068 (*12.3*)
HR-P	0.184 ± 0.022 (*11.7*)	0.520 ± 0.046 (*8.8*)
PA	< LOQ	0.075 ± 0.006 (*7.9*)
Strata-X	0.284 ± 0.042 (*14.9*)	0.963 ± 0.034 (*3.5*)
Cellulose	< LOQ	< LOQ
Davisil	< LOQ	< LOQ
Florisil	< LOQ	0.046 ± 0.011 (23.9)

In bath additive 2, menthol and p-cymene were determined. For menthol Strata-X followed by C_{18} Hydra gained highest amounts. Strata-X, C_8 and C_4 had comparable results in case of p-cymene. Surprisingly Normal Phases like Davisil, CN, and NO_2 showed good results, probably caused by tensides in the matrix.

3.4.3. Cough Drops

In the cough drops, menthol was quantified (Table **10**). Highest amounts were found with Strata-X, C_{18} Hydra and C_{18} ec.

4. CONCLUSION

These experiments clearly show the necessity to test a broad range of available SPE materials for method development, as the matrix has great influence on recovery rates of different analytes. Automation is a useful tool to

Table 10. **Concentration of Menthol in mg drop^{-1} After Automated Extraction of Cough Drops. Also Given are Standard Deviation and Coefficient of Variation**

	Menthol
C_2	0.082 ± 0.016 (*19.0*)
C_4	0.529 ± 0.087 (*16.4*)
C_8	1.724 ± 0.312 (*18.1*)
C_{18} Hydra	2.241 ± 0.263 (*11.7*)
C_{18} ec	2.375 ± 0.261 (*11.0*)
C_6H_{11} ec	0.585 ± 0.162 (*27.7*)
C_6H_5	1.399 ± 0.315 (*22.5*)
CN	1.807 ± 0.741 (*41.0*)
NH_2/C_{18}	<LOQ
OH	0.056 ± 0.024 (*43.8*)
NO_2	0.208 ± 0.030 (*14.2*)
Easy	0.844 ± 0.100 (*11.9*)
HR-P	0.798 ± 0.039 (*4.9*)
PA	0.090 ± 0.009 (*9.6*)
Strata-X	2.404 ± 0.190 (*7.9*)
Cellulose	< LOQ
Davisil	< LOQ
Florisil	0.075 ± 0.007 (*8.8*)

accomplish the emerging high number of samples. Fully automated extraction procedure with the MEA Personal Purification System® took 5 h 15 min for 12 samples. Preparing the system takes about 10 min, extraction of analytes about 15 min. So laboratory staff is occupied for 25 min, while the rest is done by the robot. To do the same extraction manually one would have to invest about 4 hours using a syringe and adapter and about 2 hours using a vacuum manifold. Apart from time, another advantage of automation is the fact, that mistakes made by laboratory staff are minimized. They will not be exhausted due to the long extraction procedure and there will not be deviations due to the fact that different laboratory workers are doing the extractions.

In many cases, the most commonly used SPE material C_{18} showed lower recovery rates compared to Strata-X or C_8. Strata-X was in 12 cases the best material. Only for the extraction of menthol out of cough drops, C_{18} ec and C_{18} Hydra obtained equal results. In case of the extraction of p-cymene out of bath additive 2 nearly equal results were obtained with C_8 and C_4.

Furthermore results show, that modern SPE materials like Strata-X can be better used in automated extraction procedures than the "old schooled" ones like silica based materials.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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