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Review

Solid-phase microextraction in biomedical analysis

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Abstract

Chromatographic methods are preferred in the analysis of organic molecules with lower molecular mass (<500 g/mol) in body fluids, i.e., the assay of drugs, metabolites, endogenous substances and poisons as well as of environmental exposure by gas chromatography (GC) and liquid chromatography (LC), for example. Sample preparation in biomedical analysis is mainly performed by liquid–liquid extraction and solid-phase extraction. However, new methods are investigated with the aim to increase the sample throughput and to improve the quality of analytical methods. Solid-phase microextraction (SPME) was introduced about a decade ago and it was mainly applied to environmental and food analysis. All steps of sample preparation, i.e., extraction, concentration, derivatization and transfer to the chromatograph, are integrated in one step and in one device. This is accomplished by the intelligent combination of an immobilized extraction solvent (a polymer) with a special geometry (a fiber within a syringe). It was a challenge to test this novel principle in biomedical analysis. Thus, an introduction is provided to the theory of SPME in the present paper. A critical review of the first applications to biomedical analyses is presented in the main paragraph. The optimization of SPME as well as advantages and disadvantages are discussed. It is concluded that, because of some unique characteristics, SPME can be introduced with benefit into several areas of biomedical analysis. In particular, the application of headspace SPME–GC–MS in forensic toxicology and environmental medicine appears to be promising. However, it seems that SPME will not become a universal method. Thus, on-line SPE–LC coupling with column-switching technique may be a good alternative if an analytical problem cannot be sufficiently dealt with by SPME.

Keywords: Reviews; Solid-phase microextraction; Forensic analysis; Pharmaceutical analysis; Antidepressants; Beta-blockers; Benzodiazepines; Amphetamine; Phencyclidine; Polynuclear aromatic hydrocarbons; Pesticides; Volatile organic compounds; Polychlorinated biphenyls; Antihistamines

Contents

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1. Introduction

Biomedical analysis of lower-molecular-mass organic molecules (<500 g/mol) comprises, for the main part, the analysis of drugs, metabolites, poisons, chemicals of environmental exposure and endogenous substances in body fluids and tissues. The quantitative and qualitative analysis of drugs and metabolites is extensively applied to pharmacokinetic studies. Variables such as time to maximal concentration in plasma, clearance and bioavailability have to be known for the approval of a new drug [1,2]. Pharmacokinetic interactions, the pharmacokinetics in special populations and relationships between the concentration of drug and pharmacological effect, for example, are investigated in post-marketing surveillance. Therapeutic drug monitoring (TDM) may be used as a tool for the improvement of drug therapy [3–6]. Drugs of abuse, illicit drugs and intoxications by drugs and poisons are analyzed in clinical and forensic toxicology [7–10]. As part of environmental chemistry and environmental medicine, a wide variety of chemicals such as pesticides, herbicides, volatile organic compounds (VOCs), dioxins and polynuclear aromatic hydrocarbons (PAHs), for example, are analyzed in human body fluids for the investigation of environmental and occupational exposure [11,12]. Endogenous substances such as neurotransmitters, arachidonic acid metabolites and fatty acids, for example, are analyzed in biological and medical research and in clinical diagnostics [13–15].

Capillary gas chromatography (GC) and column liquid chromatography (LC) are mainly applied. High sensitivity and high selectivity are the most prominent advantages of chromatographic methods compared with, for example, enzyme-linked immunoassays (ELISAs) and fluorescence polarization immunoassays (FPIAs) [16–20]. However, the main disadvantage of chromatographic methods is the need for sample preparation. The sample cannot be applied to the chromatograph in its original form. Therefore, the task of sample preparation is to transfer the analyte into a form that is (1) pre-purified, (2) concentrated and (3) with the chromatographic system fitting solvent. Prior to sample preparation the analyte is found in a low concentration and in a great volume of an aqueous matrix which

consists of a huge number of highly concentrated proteins, lipoproteins, lipids and salts as well as other lower concentrated endogenous and exogenous organic substances. Because of this complex matrix the trace analysis in body fluids is more complicated than trace analysis in surface water in environmental chemistry. However, it is comparable with the trace analysis in water enriched with dissolved polymer organic material (DOM). The sample as prepared for the chromatograph should be concentrated and prepurified in an organic solvent. This is accomplished mainly by liquid–liquid extraction (LLE) and solid-phase extraction (SPE). Nearly all analytical problems can be solved by LLE and SPE. Therefore, these methods can be characterized as universal from a scientific and technical view.

However, the disadvantage of LLE and SPE is the considerable expense of time and manual operations. Sample throughput is low and the economic expense is high. In other words, sample preparation is the bottleneck of the entire analytical method. Furthermore, some advantages claimed for SPE over LLE must be regarded critically. For example, laborious operations such as conditioning, washing, elution and solvent evaporation are needed, too. The volume of organic solvents needed in SPE cannot be neglected with regard to environmental pollution. It can be even higher than in a simple one-step LLE or even in a three-step LLE [21–23]. Evaporation of the eluate is more time-consuming than in LLE because protic solvents are mainly used, aqueous methanol for example, which have a lower vapor pressure than chloroform and hexane in LLE. In addition, clotting, channeling and percolation are typical problems of SPE encountered in every-day laboratory work. Off-line automation of LLE and SPE is complicated. Although some systems were presented they did not lead to a break-through in the economics of sample preparation. Despite automation of SPE being easier than automation of LLE, it is also beset with technical problems. However, the comparison of LLE and SPE is discussed controversially [9,12,24,25]. Some promising approaches in SPE are based on special packings such as restricted access materials (RAMs), and molecular imprinting materials (MIPs), for example [26].

A alternative simple approach in LC is protein precipitation of plasma and injection of plasma water. Even automated systems were described [27]. However, the analyte enrichment and sample purification is poor. Another relatively simple approach is the headspace (HS) technique in GC. However, HS can only be applied to analytes with high vapor pressure [28]. Other methods of sample preparation are supercritical fluid extraction (SFE) [29], on-column sample preparation with column-switching techniques and on-line SPE in LC using RAMs [26,30–32], LC–GC coupling [33,34] and membrane-based sample preparation (dialysis, electrodialysis, ultrafiltration) [35]. Although these methods have their own merits, most of them are only found in isolated applications, oftentimes, they do not achieve sensitivity and selectivity of LLE and SPE and, finally, some methods need expensive equipment. Other problems are fouling of membranes in membrane-based sample preparation and irreversible binding of some high-molecular-mass material in on-line SPE, for example. However, on-line SPE–LC using RAMs appears to be promising [26].

This situation is the reason for the permanent search for new sample preparation methods. One approach is solid-phase microextraction (SPME). The present review provides a survey and discussion of the application of SPME in biomedical analysis.

2. Solid-phase microextraction – a new principle in sample preparation

SPME is based on a modified syringe which contains a stainless steel microtubing within its syringe needle. This microtubing has an about 1-cm fused-silica fiber tip which is coated with an organic polymer. The coated silica fiber can be moved between two positions, inside and outside the needle, with a plunger as in the case of a normal syringe. The diameter of the syringe needle housing the microtubing and coated silica fiber is not much increased in comparison with a normal GC syringe. Thus, by means of this simple equipment several steps of sample preparation are combined in one device. Extraction and enrichment of the analyte is completed by the coating in the position outside the syringe needle. Penetration of the septum of a GC injection port is possible if the fiber was withdrawn

into the syringe needle. Desorption of the analyte and transfer to the capillary is performed after again moving the fiber to the position outside the syringe. This procedure can be repeated with one device several times (Fig. 1).

It should be emphasized that the term ''solid-phase microextraction'' may undervalue the advantages of SPME. Advantages of this principle should be greater than those of other extraction methods with only a very low quantity (''*micro*'') of the extraction agent, for example, SPE with disc technology. The outstanding and crucial idea of this principle named SPME is the intelligent geometry of the extraction agent and extraction device. In contrast to conventional SPE with packed-bed columns, micro or non-micro columns, this arrangement allows the combination of all steps of sample preparation in one step as described above. For this reason, the main advantage of SPME is its simplicity and automation is anticipated to be considerably easier than with other sample preparation methods.

SPME was invented and first described by Pawliszyn and co-workers in 1990 [36,37]. The invention of SPME appears to be a logical development based on open-tubular capillary columns used in GC. These capillaries had their break-through in analytical laboratories in the mid-1980s. The conception of SPME may have been derived from the idea of an inversed GC capillary. Thus, a SPME device constituting a tubing with a coated inner surface was described, too [38]. During the initial years SPME was mainly described for applications in environmental analysis [39–41]. About 110 applications to environmental analysis were published up until 1996 [42]. By nature, SPME is used mainly for GC. However, an adaptation for LC is possible with a special interface [43]. Two main variants of SMPE can be chosen: direct SPME with dipping the fiber

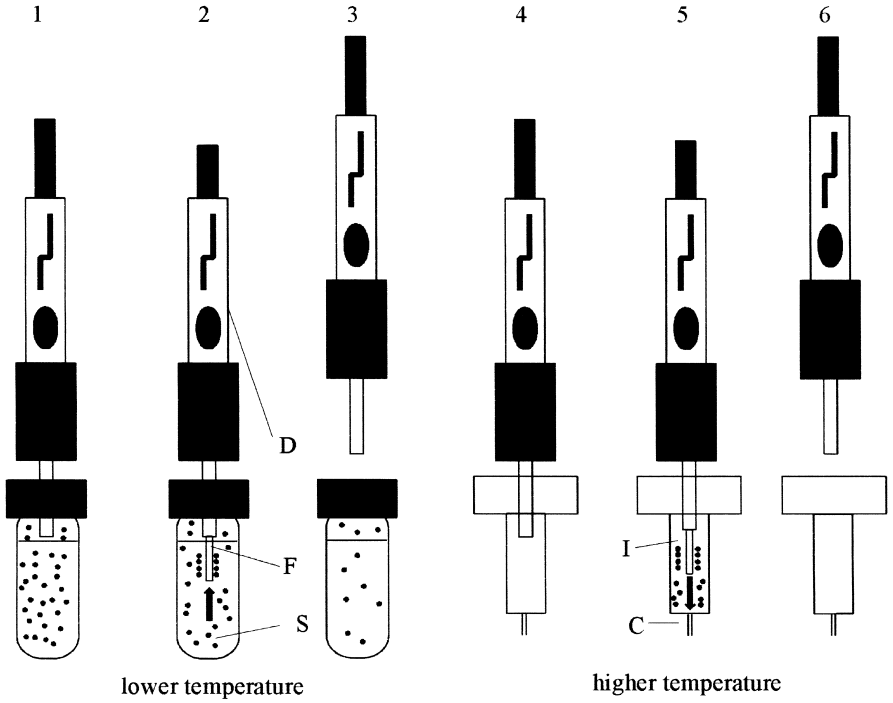

Fig. 1. The principle of SPME: 1=introduction of syringe needle of the SPME device (D) into the sample vial and close to the sample (S), 2=moving the fiber (F) into the position outside the syringe and into the sample (**extraction**), 3=moving the fiber back into the syringe needle and subsequent **transfer** of the device to the GC injector port (I) and capillary head (C), 4=penetration of the septum with syringe needle, 5=moving the fiber into the position outside the syringe (**desorption**), 6=moving the fiber back into the syringe needle and withdrawing the syringe needle.

directly into the aqueous sample and HS-SPME with extraction of the analyte from the HS of the sample. Minor variants are derived from whether or not derivatization is applied and in which phase, the type of sample agitation as well as the option of cooling of the fiber, for example.

3. Theory of solid-phase microextraction

3.1. Thermodynamics

Because of the physicochemical properties of, for example, polydimethylsiloxane (PDMS, melting point: −50°C, glass transition temperature: −126°C), which is most often applied in SPME, the extraction obeys the rules of liquid–liquid equilibrium:

$$\text{analyte}_{\text{w}} \overset{K^{\text{fw}}}{\rightleftharpoons} \text{analyte}_{\text{fiber}}$$

$$K^{\text{fw}} = \frac{c_{\text{f}}}{c_{\text{w}}} \tag{1}$$

where K^{fw} is the equilibrium constant of liquid–liquid equilibrium, c_{f} is the equilibrium concentration of the analyte in the coating and c_{w} is the equilibrium concentration of the analyte in the aqueous matrix. Eq. (1) can also be written as:

$$K^{\text{fw}} = \frac{n_{\text{f}} V_{\text{w}}}{n_{\text{w}} V_{\text{f}}} \tag{2}$$

and because $n_0 = n_{\text{f}} + n_{\text{w}}$ a rearrangement is possible to:

$$n_{\text{f}} = \frac{K^{\text{fw}} V_{\text{f}} n_0}{(K^{\text{fw}} V_{\text{f}} + V_{\text{w}})} \tag{3}$$

where n_{f} is the number of molecules in the fiber in equilibrium, n_{w} is the number of molecules in the aqueous phase in equilibrium, n_0 is the number of molecules in the aqueous phase prior to SPME, V_{w} is the volume of aqueous phase and V_{f} is the volume of the coating. It is evident from Eq. (3) that the basis for a quantitative method is given because of the linear relationship between n_{f} and n_0. However, SPME is an equilibrium extraction but not an exhaustive extraction. A simple rearrangement of Eq. (3) gives an expression for the recovery of SPME in equilibrium which is also the maximum recovery

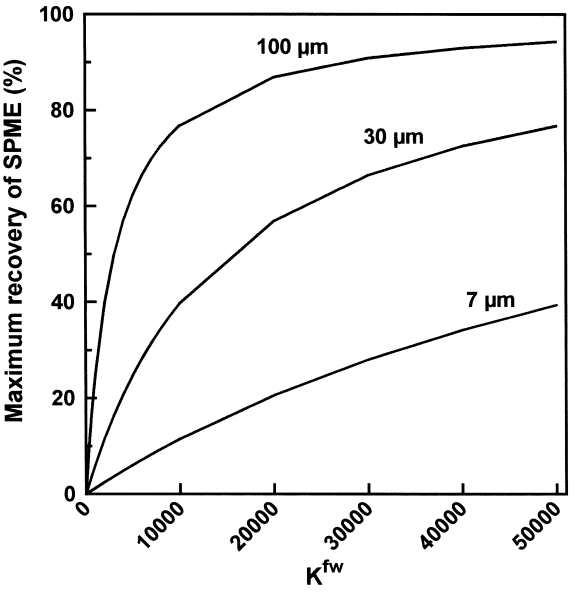

Fig. 2. Dependence of maximum recovery by SPME on K^{fw} according to Eq. (4) for three fibers with a length of 1 cm and coatings of 7 µm ($V_{\text{f}} = 2.6 \cdot 10^{-5}$ ml), 30 µm ($V_{\text{f}} = 1.3 \cdot 10^{-4}$ ml) and 100 µm ($V_{\text{f}} = 6.6 \cdot 10^{-4}$ ml) with $V_{\text{w}} = 2$ ml.

(Eq. (4)). Thus, it is also evident that SPME will mainly have a low or very low recovery (Fig. 2) because K^{fw} is in the range of 100 to 10 000 for many analytes, e.g., K^{fw} (benzene) = 125, K^{fw} (*p*-xylene) = 831 [44], K^{fw} (clozapine) = 226 and K^{fw} (loxapine) = 2671 [45]. The values K^{fw} of polychlorinated biphenyls (PCBs) were found between 250 and 11 000 [46]. Octanol–water partitioning coefficients (K^{ow}) can be a good estimate of K^{fw}, however, this has to be confirmed for a special group of substances. The K^{fw} of PCBs did not correlate with K^{ow} [46].

$$\text{Maximum recovery} = \frac{n_{\text{f}}}{n_0} = \frac{K^{\text{fw}} V_{\text{f}}}{(K^{\text{fw}} V_{\text{f}} + V_{\text{w}})} \tag{4}$$

The values of K^{fw} are influenced by temperature, salt, pH and organic solvents. The dependence of K^{fw} on temperature is expressed by Eq. (5) where K_0^{fw} is the equilibrium constant at T_0 and ΔG^{fw} is the free enthalpy of the transfer of analyte between the two phases:

$$K^{fw} = K_0^{fw} \exp\frac{-\Delta G^{fw}}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_0}\right) \quad (5)$$

$$\Delta G^{fw} = G^{f} - G^{w} \quad (6)$$

$$\ln \frac{K^{fw}}{K_0^{fw}} = \frac{-\Delta G^{fw}}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_0}\right) \quad (7)$$

Because of the interference of organic molecules with the intermolecular interactions of water the free enthalpy in water (G^{w}) is always higher than in PDMS (G^{f}). Thus, according to Eq. (6) ΔG^{fw} should be negative except for, perhaps, rare cases with a high entropy term. It can be concluded that K^{fw} decreases with increased temperature and, therefore, also the amount of analyte extracted and the recovery of SPME decrease. This is shown for the antipsychotic drug clozapine in Fig. 3. According to Eq. (7) which can be received after rearrangement of Eq. (5) a linear relationship was found (Fig. 4) and $\Delta G^{fw} = -25.9$ kJ/mol was calculated [47]. The relationship between K^{fw} and concentration of salt (c_s) can be expressed with Eq. (8) where K_0^{fw} is K^{fw} at $c_s = 0$ and k_s is a specific constant [48]. The higher the concentration of salt the higher is K^{fw} and the amount of analyte extracted [49]. However, this was not always confirmed in real samples [50,51]. The relationship between K^{fw} and pH can be described with Eq. (9) if only the acid is extracted where K_0^{fw} is K^{fw} of the undissociated form. This was confirmed for short-chain fatty acids [52]. The analyte is better extracted at low pH. Eq. (10) can be used if only the basic form is extracted. The analyte can be better extracted at high pH. Finally, the presence of an organic solvent in the aqueous sample usually decreases K^{fw} [53]:

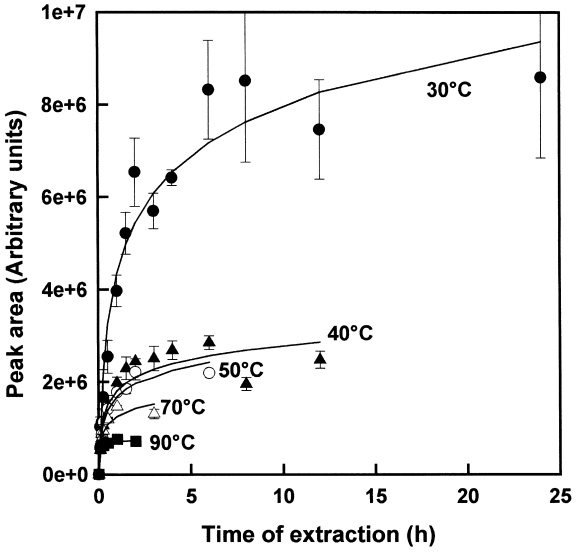

Fig. 3. SPME of clozapine in aqueous solution at various temperatures (filled circles 30°C, filled triangles 40°C, circles 50°C, triangles 70°C, filled squares 90°C), $c_0 = 500$ ng/ml, $V_w = 1.5$ ml, 100-μm PDMS fiber, pH 12.

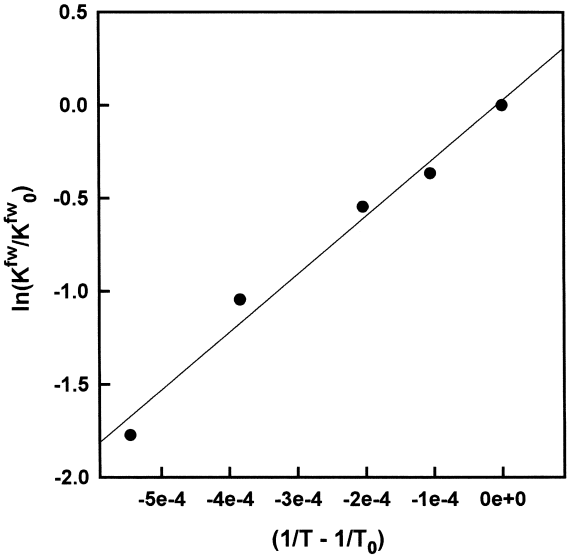

Fig. 4. Linear relationship of K^{fw} and temperature according to Eq. (7) for the SPME of clozapine by a 100-μm PDMS fiber, $c_0 = 500$ ng/ml, $V_w = 1.5$ ml, 100-μm PDMS fiber, pH 12.

$$\ln \frac{K^{fw}}{K_0^{fw}} = k_s c_s \quad (8)$$

$$\log\left(\frac{K_0^{fw}}{K^{fw}} - 1\right) = \mathrm{pH} - \mathrm{p}K_a \quad (9)$$

$$\log\left(\frac{K_0^{fw}}{K^{fw}} - 1\right) = \mathrm{pH} + \mathrm{p}K_a - 14 \quad (10)$$

In HS-SPME Eq. (3) is extended to Eq. (13) where K^{hw} is the equilibrium constant of HS and aqueous sample (Eq. (11)), K^{fh} is the equilibrium constant of fiber and HS (Eq. (12)), c_h is the equilibrium concentration of the analyte in HS and V_h is the volume of HS:

$$K^{hw} = \frac{c_h}{c_w} \quad (11)$$

$$K^{fh} = \frac{c_f}{c_h} \quad (12)$$

$$n_f = \frac{K^{fh}K^{hw}V_f n_0}{(K^{fh}K^{hw}V_f + K^{hw}V_h + V_w)} \quad (13)$$

K^{hw} and K^{fh} can be calculated with the Henry's Law constants of the analyte in water (H_w) and in the coating (H_f), respectively (Eqs. (14a) and (14b)). The vapor pressures in aqueous sample (p_w) and coating (p_f) are given in Eqs. (15a) and (15b):

$$K^{hw} = \frac{H_w}{RT} \quad (14a)$$

$$K^{fh} = \frac{RT}{H_f} \quad (14b)$$

$$p_w = H_w c_w \quad (15a)$$

$$p_f = H_f c_f \quad (15b)$$

Eq. (16) and an alternative expression for the amount extracted (Eq. (17)) can be derived from Eqs. (1), (14a), (14b), (15a) and (15b) because the equation $p_w = p_f$ is valid in equilibrium. A similar rearrangement as shown in Eqs. (3) and (4) provides the recovery of HS-SPME (Eq. (17a)). Accordingly, the recovery of HS-SPME should be lower than that of direct SMPE (Eq. (17b)):

$$K^{fw} = \frac{H_w}{H_f} = K^{hw}K^{fh} \quad (16)$$

$$n_f = \frac{K^{fw}V_f n_0}{(K^{fw}V_f + K^{hw}V_h + V_w)} \quad (17)$$

$$\text{Maximum recovery (HS-SPME)} = \frac{n_f}{n_0} = \frac{K^{fw}V_f}{(K^{fw}V_f + K^{hw}V_h + V_w)} \quad (17a)$$

$$\frac{\text{Maximum recovery (HS-SPME)}}{\text{Maximum recovery (direct SPME)}} = \frac{1}{1 + \frac{K^{hw}V_h}{K^{fw}V_f + V_w}} \quad (17b)$$

3.2. Kinetics

The relationship of the SPME with time as shown in Fig. 3, for example, was mathematically described in a model which used several prerequisites with regard to geometry, size of sample and access of analyte molecules to the fiber [42,44]. If all analyte molecules have access to the coating, i.e., the perfectly agitated model, the time to equilibrium (t_e) is given by Eq. (18) with r_o the outer radius of the coating, r_i the inner radius of the coating and D_f the diffusion coefficient of the analyte in the coating. Taking into account the experimental error it can be assumed that t_e is reached when 95% ($t_{95\%}$) of the maximal amount was extracted. Otherwise, the theoretical t_e is infinitely long according to the model used:

$$t_e = t_{95\%} = \frac{(r_o - r_i)^2}{2D_f} \quad (18)$$

Not all analyte molecules have simultaneous access to the coating in a more real approach. This is described in a model using a hypothetical boundary layer of radius δ with no agitation. Perfect agitation occurs only in the sample outside the boundary layer. The radius δ of this static layer depends on the rate of agitation. The higher the rate of agitation the lower is δ and vice versa. The time to maximal extraction can be calculated with Eq. (19) where D_w is the diffusion coefficient of the analyte in water:

$$t_e = t_{95\%} = 3 \cdot \frac{\delta K^{fw}(r_o - r_i)}{D_w} \quad (19)$$

It is concluded that the time of extraction is increased with increased K^{fw}, a higher fiber thickness ($r_o - r_i$) and lower diffusion coefficients of the analyte molecule in the sample (D_w). The time of extraction may be decreased with an improved agitation method, thus by decreasing δ. In the case of perfect agitation the minimal time of extraction is reached and t_e only depends on the geometry of the fiber and the analyte's diffusion coefficient in the fiber (Eq. (18)). However, it is emphasized that equilibrium is not a prerequisite for a quantitative method. The time of extraction t_e is independent of the concentration of analyte in the sample. The relative number of molecules extracted at a distinct

time (n_f^t/n_f) is also independent of the concentration of analyte. Finally, the absolute number of molecules extracted at a distinct time (n_f^t) is linearly proportional to the concentration of analyte [44].

In HS-SPME Eq. (18) is also valid for the estimation of t_e if the aqueous phase and the HS are perfectly agitated. Several variables have to be taken into account for the estimation of t_e in the case of practical agitation (Eq. (20)): thickness of coating, HS and aqueous phase (L_f, L_h and L_w, respectively), revolution rate of the stir bar (N), radius of the stir bar (R), D_w and diffusion coefficients of analyte in HS (D_h) as well as K^{hw} and K^{fw}. A simple model was applied with the assumptions of only one-dimensional diffusion and R only slightly smaller than the radius of the vial [42]:

$$t_e = t_{95\%} = 1.8 \cdot \left(\frac{L_h}{K^{hw} \cdot (D_h + 2 \cdot 10^{-5} NR^2)} + \frac{L_w}{1.6 \cdot (D_w + 0.03NR^2)} \right) \cdot K^{fw} L_f \quad (20)$$

3.3. Solid-phase microextraction in biological fluids

The analysis of biological fluids is hampered by the presence of dissolved biopolymers. For example, human plasma consists of about 7 to 8% of proteins. The main portion is albumin (about 55%). Immunoglobulins account for about 20% and lipoproteins for about 11% of proteins. Serum is formed from nonstabilized plasma after coagulation. Thus, fibrinogen (about 3.5% of plasma proteins) is not present in serum. Other components are triglycerides and electrolytes as well as a huge number of trace components such as hormones, transmitters and metabolites. The composition of plasma can be subject to considerable differences due to pathological and nonpathological influences. For example, plasma albumin can be decreased to about 50% of normal in hepatic diseases and the concentration of triglycerides depends on dietary status. However, in a more general view plasma can also be considered as a relatively fixed and well-described matrix in comparison to real samples in some areas of environmental analysis, for example. Problems of the qualitative and quantitative analysis in plasma arise from (1) problems of selectivity because of interferences of endogenous substances and (2) problems of quantitation because of binding of the analyte with biopolymers. A short discussion of the impairment of quantitation by protein binding of the analyte is presented.

The binding of the target analyte, a drug for example, to proteins can be described by a chemical equilibrium reaction as shown in Eqs. (21) and (22), where c_w is the free concentration of drug in plasma water in equilibrium, c_{pr} is the concentration of binding sites of protein in equilibrium, c_b is the concentration of bound drug in equilibrium and K^{pr} is the equilibrium constant:

$$c_w + c_{pr} \overset{K^{pr}}{\rightleftharpoons} c_b \quad (21)$$

$$K^{pr} = \frac{c_b}{c_w c_{pr}} \quad (22)$$

Eq. (23) can be derived with c_{pr}^0 the concentration of binding sites prior to equilibrium. Eq. (24) is obtained with the assumption $c_b << c_{pr}^0$, which should be valid for trace analysis. Substitution of concentrations yields Eq. (25) where n_b is the amount of bound drug, n_w is the amount of free drug and n_{pr}^0 is the amount of binding sites of protein (in moles):

$$K^{pr} = \frac{c_b}{c_w \cdot (c_{pr}^0 - c_b)} \quad (23)$$

$$K^{pr} = \frac{c_b}{c_w c_{pr}^0} \quad (24)$$

$$K^{pr} = \frac{n_b V_w}{n_w n_{pr}^0} \quad (25)$$

With Eq. (26) and introduction of Eq. (2) an expression is obtained for the amount of analyte extracted by SPME (n_f) in the ternary system fiber–plasma water–protein (Eq. (27)). A considerably more complex result was described without the assumption made in Eqs. (23) and (24) [54]. The main problem of analysis by SPME in matrices containing protein can be concluded from Eq. (27), i.e., a decrease of sensitivity. The factor of decrease of sensitivity (f_{sens}) can by calculated by the combination of Eqs. (3) and (27), where n_f is the amount

extracted in the absence of proteins and n_f' is in presence of proteins (Eq. (28)). Accordingly, the sensitivity of SPME is decreased for a high capacity of protein binding, i.e., high $K^{pr}n_{pr}^0$. In addition, the sensitivity of SPME may be decreased in the presence of proteins if the coating is changed by the irreversible adsorption of proteins, i.e., lower K^{fw} due to protein fouling:

$$n_0 = n_f + n_w + n_b \tag{26}$$

$$n_f = \frac{K^{fw}V_f n_0}{K^{fw}V_f + K^{pr}n_{pr}^0 + V_w} \tag{27}$$

$$f_{sens} = \frac{n_f}{n_f'} = 1 + \frac{K^{pr}n_{pr}^0}{K^{fw}V_f + V_w} \tag{28}$$

The amount of analyte in plasma water is given by Eq. (29) (n_w', with SPME) and by Eq. (30) (n_w, without SPME):

$$n_w' = \frac{n_0 V_w}{K^{fw}V_f + K^{pr}n_{pr}^0 + V_w} \tag{29}$$

$$n_w = \frac{n_0 V_w}{K^{pr}n_{pr}^0 + V_w} \tag{30}$$

The ratio of n_w and n_w' provides a criterion for the interference of SPME with the equilibrium between bound and free analyte (Eq. (31)). If the experimental conditions are chosen according to Eq. (32) the interference of SPME with the equilibrium between bound and free analyte can be neglected because the amount in plasma water is changed by less than 10%. Thus, the free concentration of analyte can be measured:

$$\frac{n_w}{n_w'} = 1 + \frac{K^{fw}V_f}{K^{pr}n_{pr}^0 + V_w} \tag{31}$$

$$\frac{K^{fw}V_f}{K^{pr}n_{pr}^0 + V_w} < 0.111 \tag{32}$$

This is a unique advantage of SPME over other sample preparation methods. A direct assay of free concentration can be performed without the separation of phases. This is possible because the binding of analyte to proteins is not impaired by the SPME, i.e., no loosening occurs of the protein–analyte bonding as in LLE, and because SPME is an equilibrium extraction but not an exhaustive extraction. However, the experimental conditions needed imply a very low recovery and sensitivity of SPME and, therefore, this approach may be limited to selected problems. This was first shown and experimentally confirmed for the SPME of organic pollutants in waste water which was enriched with DOM [55,56]. The free concentration of analytes (c_w) was analyzed directly by external calibration as discussed above. The total concentration ($c_w + c_b$) was analyzed by internal calibration with isotopically labeled spikes. The total concentration can also be assessed by LLE, for example. Thus, the portion of freely dissolved analyte $x_w = c_w/(c_w + c_b)$ and the product $K^{pr}n_{pr}^0$ are available. The knowledge of x_w of drugs is important in pharmacology, for example, because only the free concentration is the pharmacologically active portion in plasma.

If the matrix is diluted by a dilution factor $D = n_{pr,D}^0/n_{pr}^0$ ($D = 0$ to 1) and with $x_w = c_w/(c_w + c_b)$ Eq. (33) can be derived where $n_{f,D}$ and $n_{pr,D}^0$ are the amount of analyte extracted and the amount of binding sites after dilution, respectively:

$$\frac{n_0}{n_{f,D}} = 1 + \frac{V_w}{K^{fw}V_w} + D\frac{V_w}{K^{fw}V_f} \cdot \left(\frac{1}{x_w} - 1\right) \tag{33}$$

Eq. (34) is obtained after rearrangement of Eq. (3) with an expression for the ratio $V_w/K^{fw}V_f$ in buffer solution and introduction in Eq. (33). Thus, x_w can be calculated from a linear plot according to Eq. (34). If no linear relationship is found the assumptions made in the model are not valid, i.e., $c_b << c_{pr}^0$, preformed binding sites, linear dependence of number of binding sites on the concentration of proteins, only one type of binding sites of proteins:

$$\frac{\left(\frac{n_0}{n_{f,D}} - 1\right)^{Plasma}}{\left(\frac{n_0}{n_f} - 1\right)^{Buffer}} = 1 + \left(\frac{1}{x_w} - 1\right) \cdot D \tag{34}$$

In contrast to Eq. (21) an alternative approach is possible. The protein is regarded as a third phase and binding of analyte is regarded as an extraction but not a chemical reaction. Thus, Eqs. (35) and (36) are used for further calculations where $K^{pr'}$ is the equilibrium constant of extraction between the aqueous phase and the protein phase and $c_{b'}$ is the

concentration of analyte in the protein phase. The results of this approach are similar to the equations presented above:

$$c_w \overset{K^{pr'}}{\rightleftharpoons} c_{b'} \quad (35)$$

$$K^{pr'} = \frac{c_{b'}}{c_w} \quad (36)$$

Apart from the changes of equilibrium, the extraction profile of SPME is influenced by proteins or other DOM in the sample, too. This can be explained if the equilibrium between free and bound analyte of Eq. (21) is written kinetically with k_b the rate constant of association with protein and k_{-b} the rate constant of the dissociation of the protein–analyte binding (Eqs. (37) and (38)). The rapidity of extraction is determined by the rate of dissociation ($r_{-b} = k_{-b} c_b$) if the rate of dissociation is slower than the diffusion of analyte from the aqueous phase to the coating. This may occur for some analytes. Furthermore, the viscosity (η) of plasma and blood in vitro is about 2- and 4.5-times higher, respectively, than the viscosity of water. Because the diffusion coefficients are inversely related to η [$D = f(1/\eta)$] diffusion coefficients of the analyte in the aqueous phase are about 2- and 4.5-times decreased in plasma and blood, respectively. Thus, t_e is increased according to Eq. (19) by factors of about 2 and 4.5 in plasma and blood, respectively, in comparison to water. Finally, the formation of a diffusion barrier by polymer molecules is supposed close to the surface of the coating which diminishes the transfer of analyte into the coating. However, this mechanism is only little understood. In conclusion, the SPME in biomedical samples may be substantially impaired with respect to sensitivity and rapidity:

$$c_w + c_{pr} \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} c_b \quad (37)$$

$$K^{pr} = \frac{k_b}{k_{-b}} \quad (38)$$

4. Application of solid-phase microextraction in biomedical analysis

The number of published data of the application of SPME in biomedical analysis has increased since 1998. A survey of these methods and approaches is presented. Some methods are presented in more detail to provide the reader with deeper insight into the practice of SPME and to compare different approaches.

4.1. Direct solid-phase microextraction

The vapor pressure of many important analytes is low because of a molecular mass between 150 to 450 g/mol and the presence of hydrophilic groups in their molecule. Thus, according to Eqs. (13), (14a), (15a) and (20) the concentration of the analyte in the HS is low and the transfer to the fiber is slow at ambient temperature. The application of increased temperatures appears to be problematic because of denaturation of proteins and decomposition of analytes. An advantage of low vapor pressure is the option of storage of fibers after extraction and prior to desorption and GC analysis. Thus, field analysis is possible without the need of transport of the sample. Furthermore, several fibers can be processed simultaneously in the extraction and analyzed subsequently by GC thereafter as it is well-known in LLE. Direct SPME was studied in several methods for the assay of drugs and other analytes in plasma and urine. Methods without derivatization and methods with derivatization were described.

4.1.1. Without derivatization

A method for the assay of eight barbiturates in urine was described [57]. A 65-μm Carbowax–divinylbenzene (DVB) fiber was found to have the highest extraction efficiency in comparison with 100-μm PDMS and 85-μm polyacrylate (PA) fibers. The time of extraction as indicated by t_e was about 5 to 15 min with agitation by a stir-bar. The Carbowax–DVB coating stripped off the fused-silica at temperatures $>$ 265°C during desorption, therefore, a desorption temperature of 250°C was used. However, a considerable carryover effect was found and a special clean-up procedure of the coating was necessary after the 12-min desorption. For this purpose, after each analytical run the fiber was cooled for 3 min, exposed to methanol–water (20:80) solution for 3 min and again desorbed in the hot injector for a period of 4 min. The carryover was decreased to 2%. The GC separation was performed with a PTE-5

column (30 m×0.25 mm I.D., 0.25 μm film thickness) with helium as the carrier gas and a temperature program starting at 60°C, a 40°C/min ramp to 110°C and a 10°C/min ramp to the final temperature of 250°C. Ion-trap mass spectrometric detection (IT-MS) in the electron ionization mode (EI) was applied and selected ions were used for quantitation. Calibration was linear (correlation coefficient $r^2 =$ 0.990), precision was between 1.4 and 12.0% (relative standard deviation, RSD) and limit of detection (LOD) was 1 to 5 ng/ml. The recovery as calculated by Eq. (4) was considerably lower than the value given by the authors (93–104%). It is emphasized that some authors currently reporting on SPME ''recovery'' seem to be using the term interchangeably to mean both: absolute recovery as given in Eq. (4) and relative recoveries, for example, the recovery in a complex matrix relative to that from water. It is strongly recommended to provide absolute recoveries for a comparison with other methods. Fibers were used for at least 100 extractions.

Chlorophenols were analyzed in urine with a 85-μm PA fiber [58]. A time $t_e = 50$ min was found and used for the extraction at $N = 1000$ of a magnetic stir-bar. Desorption in the injector port was at 290°C for 2 min. The GC separation was performed with a DB-5.625 capillary (30 m×0.25 mm I.D., 0.5 μm film thickness, J&W Scientific, Folsom, CA, USA), with helium as the carrier gas and a temperature program starting at 60°C, with a ramp of 30°C/min to 190°C, a second ramp of 10°C/min and a final temperature of 310°C. EI as well as negative chemical ionization (NCI) with selected ion monitoring (SIM) MS was used for detection. The LODs were between 1 and 41 pg/ml. They were lower than with sample preparation by LLE, however, with full-scan MS detection. The values for K^{fw} were between 8 and 212 at pH 6.2. Low pH (pH 1) increased K^{fw} by factors of 1.2 to 9.2. Addition of salt (NaCl, KCl) increased K^{fw}, too, but the combined effect of salt and pH was not better, even poorer than the single effects. The method was linear over a range of three orders of amounts with $r = 0.999$ and the precision was 5 to 10% (RSD is always used for precision) at a concentration of 25 ng/ml. The authors estimated the SPME method was better than SPE and LLE for the assay of chlorophenols in urine.

The stable nitroxide radical 2,2,6,6-tetramethylpiperidine-1-oxide (TEMPO) and the metabolite 2,2,6,6-tetramethylpiperidine were measured in human cell cultures with thymol as the internal standard [59]. A 100-μm PDMS fiber was used and a time of 5 min was sufficient for extraction ($t_e \approx 5$ min). The recovery from the cell culture was about 10 to 20% for TEMPO and about 40% for the amine. Clotting of proteins on the surface of the fiber and formation of a diffusion barrier was discussed. Desorption at 250°C was for 1 min and a HP-5 capillary (30 m×0.25 mm I.D., 0.25 μm film thickness) was used for the GC separation with helium as the carrier gas and flame ionization detection (FID). Peak areas increased with increasing temperatures from about 5 to 25°C but decreased at higher temperatures.

The analysis of eight antidepressant drugs in human plasma and serum was described by direct SPME with a 100-μm PDMS fiber [54]. Aqueous NaOH was added to the plasma and an internal standard was used as usual also in the LLE of antidepressants. After 10 min of SPME the fiber was successively washed for about 20 s in a 50% aqueous methanol solution and in water. This step was found to be important to prevent burning-in of proteins adsorbed on the surface of the fiber during desorption. After 1 min of desorption at 260°C the GC separation was performed with a DB-17 capillary (30 m×0.25 mm I.D., 0.25 μm film thickness) and nitrogen at 0.7 ml/min as the carrier gas. The temperature program started at 140°C with a steep ramp of 20°C/min to 220°C and a second ramp of only 2°C/min to 270°C. Nitrogen–phosphorus selective detection (NPD) and MS detection (SIM) were used. Calibration was linear between 125 and 1000 ng/ml with r from 0.989 to 0.999. Precision was 6.1 to 39.6% at 125 ng/ml and 1.9 to 11.8% at 250 ng/ml, for example. The limit of quantitation (LOQ) was 90 to 200 ng/ml. The assay provided good agreement with a standard method which was based on LLE. The time of SPME applied was chosen because of an attempt to optimize the method with respect to a minimum time. In fact, equilibrium was not reached even after 300 min of extraction. The method was not sensitive for the assay of antidepressant drugs in patients taking therapeutic doses, i.e., for TDM. The LOQ required for example for amitriptyline and its active metabolite nortriptyline

should be at least 10 to 40 ng/ml because the therapeutic window is at about 80 to 250 ng/ml for the sum of both substances and the ratio of nortriptyline and amitriptyline concentrations is about 0.5 to 1.5. Thus, only the assay of increased concentrations is possible with this method as usually encountered in intoxications. A case of a suicidal intoxication was presented. It was discussed that the sensitivity could be considerably improved by increasing the time of extraction, however, then the goal of SPME to present a fast method is abandoned. It was emphasized that the selection of a well-suited internal standard is crucial for the direct SPME in plasma. Of course, internal standard calibration with isotopically labeled spikes would be ideally. The chemical structure of the internal standard should be very similar to the analyte. The internal standard used (chloramitriptyline) may not have met this aim for the secondary amine antidepressants and, therefore, the poor precision of some antidepressants can be explained. Finally, it was shown that the peak area increased with decreased concentration of proteins if the concentration of analytes was held constant. This was explained by the considerable binding of antidepressant drugs (90 to 99%) to proteins (Fig. 5).

A method for the assay of the antipsychotic drug clozapine was described by the same authors [45,60]. In contrast to the method for antidepressants the plasma was diluted with water 1:7 (v/v) and the time of extraction was increased to 30 min. Loxapine which has a similar chemical structure was chosen as the internal standard. Desorption was carried out at 260°C for 1 min. The time of desorption was shown to be sufficient in a desorption–postdesorption graph, i.e., no carryover effect was found. A BPX-5 megabore capillary (SGE, Weiterstadt, Germany) with the dimensions 30 m×0.53 mm I.D. and 1.0 μm film thickness was used for the separation with nitrogen as the carrier gas (20 ml/min) and a temperature programme (T_1=160°C, T_2=260°C, T_3=288°C, $ramp_1$=40°C/min, $ramp_2$=4°C/min). A linear calibration curve was found for the peak-area ratio of clozapine and loxapine from 100 to 1000 ng/ml of clozapine (r=0.987). The within-day precision was between 7.9 and 14.5% at concentrations of 100 to 1000 ng/ml. The between-day precision was 7.9 to 12.7% at 200 to 1000 ng/ml. The between-day

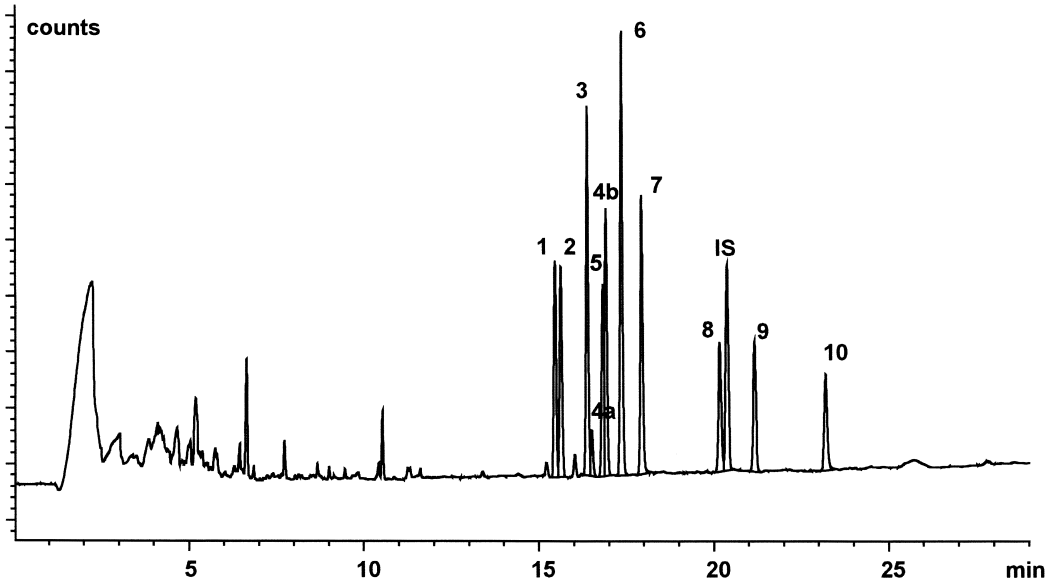

Fig. 5. Typical SPME–GC–NPD chromatogram of antidepressant drugs and metabolites in human plasma [1=amitriptyline, 2=trimipramine, 3=imipramine, 4a=*cis*-doxepin, 4b=*trans*-doxepin, 5=nortriptylin, 6=mianserine, 7=desipramine, 8=maprotiline, IS=internal standard (chloramitriptyline), 9=clomipramine, 10=desmethylclomipramine, 375 ng/ml each, 30 min time of extraction].

precision of 22% at 100 ng/ml was an indication of the LOQ. The LOD was 30 ng/ml. The method was compared with two standard methods [three-step LLE–GC–NPD and on-line-SPE–LC–ultraviolet detection (UV)] and good agreement was demonstrated. Thus, the method may be applied in TDM because the therapeutic window of clozapine is 350 to 600 ng/ml. As in the case of antidepressants the LOD may be improved by increasing the extraction time. Maximal peak areas were obtained after about 12 h in plasma and 2 h in water only. Increased concentration of triglycerides decreased the peak areas of clozapine and loxapine, however, the effect was negligible for the peak-area ratio. This also applied for the effect of salt, however, in contrast to theoretical expectation the peak areas remained constant over a wide range of salt concentration and even decreased at high concentrations of salt.

Methadone and amphetamines were analyzed in urine with a 100-μm PDMS fiber and extraction for 20 min at a temperature of 40°C. The desorption time was also 20 min at a temperature of 250°C. A three-ramp temperature program with an initial temperature of 70°C and final temperature of 300°C was used. Helium was the carrier gas, a HP-5 capillary (30 m×0.32 mm I.D., 0.33 μm film thickness) was used for separation and MS (SIM) for detection. Calibration of methadone was linear between 10 and 100 ng/ml but nonlinear at higher concentrations. Precision was between 3 and 6%. It was claimed that the recovery of SPME of methadone was higher than of LLE with dichloromethane–isopropanol (4:1, v/v) [61].

Pethidine and methadone were analyzed in human urine by SPME–GC–NPD with LODs below 1 ng/ml [62]. A deuterated internal standard was used for the assay of methadone and the main metabolite 2-ethylene-1,5-dimethyl-3,3-diphenylpyrrolidine (E-DDP) in saliva [63]. Methadone and EDDP in hair and in plasma were assayed by SPME–GC–MS [64,65]. An interesting approach is the degradation of proteins by hydrolases prior to SPME [64]. PCBs in human blood were analyzed by SPME–GC–electron-capture detection (ECD). Precision was considerably improved by enzymatic proteolysis [66]. Cannabinoids in hair were analyzed by SPME–GC–MS and enzymatic proteolysis was also tested [67]. A similar approach as shown in Eqs. (33) and (34) was used to estimate the protein binding of the local anesthetic drug lidocaine in plasma. However, the pH 9.5 indicates nonphysiological conditions [68].

4.1.2. With derivatization

As in the case of LLE and SPE derivatization can be used also in SPME for the chemical transformation of the analyte into a more suitable form for GC, i.e., polar groups should be eliminated or masked. Derivatization can be performed in situ or after transfer of the analyte into the coating. The second approach, however, is more time-consuming than simply adding the derivatization agent to the sample because, in fact, a second extraction is needed. Therefore, in situ derivatization may be preferred in SPME. For this purpose, only a limited number of agents can be used because many derivatization agents are unstable in aqueous matrix.

Benzodiazepines in urine were analyzed after acid hydrolysis of glucuronides for 30 min at a temperature of 100°C. An 85-μm PA fiber was found superior to a PDMS fiber for some benzodiazepines. The conditions of GC separation were as described above. However, no more data were presented [61].

Amphetamine and methamphetamine were analyzed in urine by direct SPME with a 100-μm PDMS fiber after in situ derivatization with methyl-, propyl- and butylchloroformate at pH 10.8 for 1 min (Fig. 6). Methoxyphenamine was used as internal standard. Water–stable carbamates were formed during the reaction. The SPME of carbamates was found to be complete after 14 min. The desorption needed 1 min at 300°C. GC separation and detection were performed with a SPB-1 capillary (30 m×0.25 mm I.D., 0.25 μm film thickness), with helium (1 ml/min) as carrier gas and NPD. The temperature program was 180°C for the initial temperature and a ramp of 20°C/min to a temperature of 300°C. A

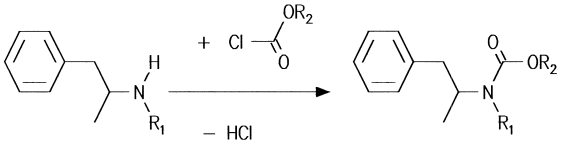

Fig. 6. In situ derivatization of amphetamine (R_1=H) and methamphetamine (R_1=methyl) with alkylchlorformates (R_2=methyl, propyl, butyl) for the assay in urine by SPME.

SPME autosampler (Varian 8200 CX; Varian, Walnut Creek, CA, USA) was used and one sample needed 15 min for analysis. Alternatively, GC–MS analysis with a HP-1 capillary (12 m×0.2 mm I.D., 0.33 μm film thickness) was applied. The calibration was linear ($r=0.999$) with an LOD of 50 ng/ml. Precision was 2.1 to 20.3%. The recovery was 2 to 7%. The PDMS fiber was found to be more efficient and robust than PA, PDMS–DVB and Carbowax–DVB. The fiber had to be replaced by a new fiber after 100 analyses. The authors concluded that the method was sufficient for bioanalysis [69].

Derivatization with trimethyloxonium tetrafluoroborate and SPME with an 85-μm PA fiber for 20 min of the resulting methyl esters (Fig. 7) was described for the analysis of 29 organic acids in urine [70]. The fiber was conditioned for 2 h at 300°C in the injection port of the gas chromatograph to get no peaks in the blank analysis. This procedure was repeated for 5 min after every analysis to avoid carryover effects. Desorption was performed at 280°C for 4 min. The GC separation was completed with a capillary (25 m×0.25 mm I.D., film thickness not given) and an OV-1701 coating. FID and MS were used alternatively for detection. No validation data of the method were presented and, indeed, the derivatization was rather complicated because five steps of successively adding the derivatization agent and sodium hydrogencarbonate for neutralization were needed at a temperature of 100°C.

A well-known derivatization method for benzodiazepines was adapted to SPME (Fig. 8). Thus, a method was described for the assay of 10 benzodiazepines in urine by acid hydrolysis to the corresponding benzophenones with 8 *M* HCl at a temperature of 100°C and with direct SPME of the benzophenone derivatives [71]. A 100-μm PDMS fiber and an 85-μm PA fiber were tested. Both coatings gave nearly the same recovery for benzodiazepines,

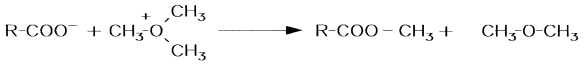

Fig. 7. In situ derivatization of organic acids with trimethyloxonium tetrafluoroborate for the assay in urine by SPME.

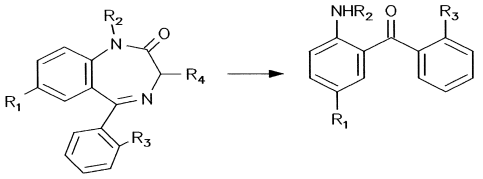

Fig. 8. In situ derivatization of benzodiazepines with formation of benzophenones for the assay of benzodiazepines in urine.

however, the PDMS fiber was selected for further method evaluation because of a lower extraction of interfering substances. This was explained by the lower affinity of PDMS to polar endogenous substances in urine. The time of derivatization was 40 min and after neutralization and cooling to ambient temperature the SPME was conducted for 30 min. Maximum peak areas were found after 20 to 40 min of SPME. The recovery of each benzophenone was not affected by the pH of SPME in a range of pH 7.7–10.4. Desorption was performed at a temperature of 270°C for 1 min. A DB-17 capillary (30 m×0.32 mm I.D., 0.25 mm film thickness) was used with helium as the carrier gas. The temperature program was similar to other methods presented above and ECD was used. Calibrations were linear in two separate ranges of 10 to 100 ng/ml and 50 to 500 ng/ml with values of r from 0.981 to 0.998. LODs were between 2 and 80 ng/ml. The within-day precision was from 2.1 to 14% and the between-day precision was from 4.2 to 17%. The recoveries ranged from 1 to 25%. This was lower than the recoveries of a LLE standard method. However, the authors discussed that the amount of analyte on column is higher in SPME than in LLE because only 1 μl of 100 μl of the LLE extract was injected to the chromatograph. The transfer ratio is 100% in SPME. Finally, it should be taken into account that the derivatization method via benzophenones is not selective for all benzodiazepines, i.e., some benzodiazepines form the same benzophenone.

SPME–GC–IT-MS after derivatization with hexylchloroformate was used for the assay of benzoylecgonine in urine with an LOD of 30 ng/ml, linear calibration between 100 ng/ml and 20 μg/ml ($r^2=0.999$) as well as precision below 9% [72].

Thioglycol methylate derivatization with SPME–GC–IT-MS was used for the assay of arsenic species in human urine [73].

4.1.3. Other methods

A solvent-modified SPME for the assay of diazepam in plasma was described [74]. Thus, a 100-μm PDMS fiber and an 85-μm PA fiber were soaked in 1-octanol and 2-octanone for 2 min and these modified coatings were used for SPME instead of the original coatings. Plasma was pretreated by adding methanol and precipitation of proteins with trichloroacetic acid. The t_e was lower than 10 min in buffer solution and in the pretreated plasma. The enrichment with the solvent modified fibers was about two- to three-times improved in comparison with the original coatings. PA was superior to PDMS. Desorption was at a temperature of 300°C for 1 min. A DB-1 capillary (30 m×0.2 mm I.D., 0.25 μm film thickness) was used for GC with either FID or NPD. The LOD was 30 ng/ml and the precision was between 3.2 and 6.5%. The method was extended to other benzodiazepines, however, it was recognized that the sensitivity was insufficient for low-dose benzodiazepines such as flunitrazepam [75].

Automated equilibrium dialysis was applied as a sample pretreatment for the assay of the free concentration of valproic acid in plasma with caprylic acid as the internal standard. A 100-μm PDMS fiber was used and a time of extraction of 3 min was sufficient although equilibrium was not reached. No extraction occurred at pH 7.4, a partial extraction was found at pH 5 and optimum extraction was at pH 2.5, i.e., below the pK_a of valproic acid of 5.0. Thus, the results for other organic acids and the theoretical description of the influence of pH (Eq. (9)) were confirmed [52]. The recovery of SPME was about 4%. The analyte and internal standard were desorbed at a temperature of 210°C for 1 min. Capillary GC–FID was used with a Nukol column (30 m×0.2 mm I.D., 0.25 μm film thickness, Supelco) with a temperature program beginning with 60°C, a first ramp of 30°C/min to 150°C and a second ramp of 10°C/min to 190°C. The calibration of peak-area ratios of analyte and internal standard was linear between 2 and 20 μg/ml with $r=0.999$. The LOD was 1 μg/ml and precision was 1.3 to 5% [76].

Lidocaine was analyzed in human plasma after protein precipitation with trichloroacetic acid. The calibration was linear in a range of 25 to 2000 ng/ml ($r=0.998$) with an LOD of 5 ng/ml [68].

4.2. Headspace solid-phase microextraction

The outstanding advantage of HS-SPME in biomedical analysis is the prevention of direct contact of the fiber with the sample and, therefore, prevention of contamination of the surface of the fiber with organic polymers. No diffusion barrier of clotted proteins is formed, no burning-in of adsorbed organic material is possible during desorption in the hot injector, the risk of decreased K^{fw} due to changes of the coating is decreased and the life-time of fibers is considerably increased. The advantages of SPME can be completely and easily exploited in HS-SPME. The enrichment of analyte from the HS by SPME is unique in comparison to other HS sample preparation methods. It is considerably simpler than purge-and-trap techniques with cryofocusing of HS, for example. It should be kept in mind that no enrichment takes place in the sampling from the HS by gas-tight syringes. On the other hand, HS-SPME is limited to special analytes because of the requirement of a high vapor pressure of the analyte. Furthermore, the transfer of fibers to the gas chromatograph and desorption should be performed immediately after extraction because of the high vapor pressure of analytes also in the coating and the risk of loss of analytes during storage of the loaded fiber.

4.2.1. Without derivatization

A method for the assay of inhalation anesthetics, i.e., nitrous oxide, isoflurane and halothane, in human urine was developed for the investigation of occupational exposure of operating room personnel [77]. A 75-μm Carboxen–PDMS fiber and a 50/30-μm DVB–Carboxen–PDMS fiber were applied for 15 to 20 min at a distance of 2 cm above the solution. Equilibrium was reached within this time. The recoveries were 0.3% for nitrous oxide, 20 to 60% for isoflurane and 30 to 80% for halothane. Desorption was carried out at a temperature of 240°C

for 16 min. An RT-QPLOT capillary (30 m×0.32 mm I.D., Restek, Bellafonte, PA, USA), i.e., a capillary with a DVB porous homopolymer as the stationary phase, was used for GC analysis with MS detection (SIM). The temperature program was: 40°C initial temperature, a first ramp of 30°C/min to 130°C and a second ramp of 10°C/min to 180°C. Calibrations were linear with r from 0.994 (nitrous oxide) to 0.999 (halothane). LODs were 75 pg/ml (nitrous oxide), 15 pg/ml (isoflurane) and 20 pg/ml (halothane) with the Carboxen–PDMS fiber. Within-day precision was 3.0 to 7.2% and between-day precision was 6.5 to 12.9% (Carboxen–PDMS fiber). Addition of 10% of salt (NaCl) increased the peak areas by about 30%, however, no further increase of peak areas was found at higher salt concentrations. The influence of temperature was investigated and analyzed according to Eq. (7). Linear relationships emerged for isoflurane and halothane with decreased K^{fw} at increased temperatures. No linear relationship was found for nitrous oxide. Values of $\Delta G^{fw} \approx -20$ kJ/mol were calculated for isoflurane and halothane.

The method described above for the assay of TEMPO and the metabolite 2,2,6,6-tetramethylpiperidine by direct SPME was extended to HS-SPME with a 7-μm PDMS fiber at a temperature of 90°C [59]. Equilibrium was reached earlier and the recovery was higher than in direct SPME, i.e., about 90 to 100%. The lower recovery of direct SPME was explained by the adverse effects of proteins which are more pronounced for the direct contact of coating and proteins. The peak areas increased with increased temperature. This is unexpected with regard to theory and other experimental results, however, the authors did not study and discuss the effect in more detail. The calibration was linear between 5 and 500 μg/ml (LOQ 35 μg/ml) and the precision was between 5 and 9%. The authors claimed an improved recovery and sensitivity of HS-SPME in a comparison with SPE and LLE.

Amphetamine and methamphetamine were measured in hair by HS-SPME–GC–NPD after a pretreatment of the sample with 5 M aqueous NaOH for 5 min at 75°C [78]. The HS-SPME was performed with a 100-μm PDMS fiber at a temperature of 55°C for 20 min. A temperature of 220°C was chosen for the desorption and a time of 30 s was shown to be sufficient. GC separation was performed with a CBJ-17 capillary (30 m×0.53 mm I.D., 1 μm film thickness) and with helium as the carrier gas with a flow of only 4 ml/min. The temperature program was similar to other methods with an initial lower temperature of 100°C and a ramp of 10°C/min to 220°C. The final temperature was held for 3 min. The recovery was 48 to 62%. The calibration according to an internal standard method was linear between 0.4 and 15 ng/mg (r=0.998, LOD 0.1 ng/mg) for amphetamine and between 4 and 160 ng/mg (r=0.999, LOD 0.4 ng/mg) for methamphetamine. Precision was below 5%. The peak-area ratios of analyte and internal standard were not influenced by the extraction time.

Dinitroaniline herbicides were analyzed by HS-SPME in water, urine and blood. A 100-μm PDMS fiber was superior to a 85-μm PA fiber. A time of about 40 min was needed to reach equilibrium, thus, 30 min was chosen as the exposure time. Addition of salt increased peak areas in water and urine, however, salt decreased peak areas in the analysis of blood. No linear relationships emerged for the dependence of peak areas on temperature. Maximum peak areas in water and urine occurred at 70°C. The maximum peak areas in blood were found for 90°C, however, coagulation and decreased peak areas were a problem in nondiluted blood at increased temperatures. Dilution of blood with water also exhibited a nonlinear relationship with peak areas. The maximum peak area was at a dilution of 0.5 ml of blood with 0.5 ml of water. The recovery was 35 to 64% from water and urine. A low recovery of only 3.2 to 7.2% was found for blood. A time of 1 min was sufficient for complete desorption at a temperature of 270°C. A good GC separation was obtained with a DB-1 capillary (30 m×0.32 mm I.D., 0.25 μm film thickness), helium as the carrier gas and a temperature program: T=100°C (hold for 1 min), $ramp_1$=20°C/min to 170°C (7 min), $ramp_2$=20°C/min to 190°C (3 min) and $ramp_3$=20°C/min to 300°C (5 min). ECD was used for detection. Calibrations were linear according to an internal standard method with values of r from 0.994 to 0.999 in blood, for example. The LODs were about 0.1 ng/ml in water and urine and 1 ng/ml in blood. Precision was below 14%. The authors valued the HS-SPME method as being superior to a SPE standard method [79].

Five local anesthetics were analyzed in blood

using HS-SPME–GC–EI-MS-SIM [80]. After addition of 5 *M* aqueous NaOH a 100-μm PDMS fiber was exposed to the HS of a sample at a temperature of 120°C for a time of 45 min. Two compounds exhibited an unusual extraction profile with time. The amount extracted reached a maximum after 60 min and decreased thereafter. This was explained by a retarded heating of the fiber in comparison to the sample, i.e., the fiber had an increased K^{fw} during the first period of the experiment. Nonlinear relationships were found between peak area and temperature. The recovery was low, i.e., only 0.6 to 8.5%. GC separation was performed with a DB-1 capillary (30 m×0.32 mm I.D., 0.25 μm film thickness) with helium as the carrier gas (flow-rate 1.8 ml/min) and a temperature program beginning with a temperature of 100°C (for 5 min) and a ramp of 20°C/min to 280°C. Desorption was performed at a temperature of 250°C for 5 min. Calibrations were linear between 0.1 and 20 μg/ml for lidocaine (LOD 0.05 μg/ml) and mepivacaine (LOD 0.05 μg/ml), between 0.5 and 20 μg/ml for bupivacaine (LOD 0.01 μg/ml) and between 1 and 20 μg/ml for prilocaine (LOD 0.25 μg/ml) with values of $r>0.999$ in each case. This is sufficient when taking into account the therapeutic concentrations of the drugs [81]. The within-day precision was between 1.3 and 6.7%. The between-day precision was between 1.4 and 8.3%. In contrast, the validation appeared insufficient for dibucaine. It was discussed that ester-type local anesthetics such as procaine, tetracaine, benoxinate and T-cain cannot by analyzed with this method because of hydrolysis in the strong alkalic solution and at increased temperatures. Thus, HS-SPME methods with drastic conditions as in the present case have the disadvantage of a limitation to only very stable analytes.

The sedative drug chlormethiazole was analyzed in plasma by HS-SPME–GC–NPD. SPME was carried out with a 100-μm PDMS fiber at ambient temperature [82]. After a 30-min extraction time the recovery was only 0.5%. However, calibration was linear between 0.5 and 5 μg/ml with an LOD of 0.15 μg/ml and precision<10%. A HP-5 megabore capillary (30 m×0.53 mm I.D., 0.88 μm film thickness) was used for GC separation with nitrogen as the carrier gas (Fig. 9).

A less detailed survey of other HS-SPME methods without derivatization should be added: trimethylamine was analyzed in urine by GC–MS to detect

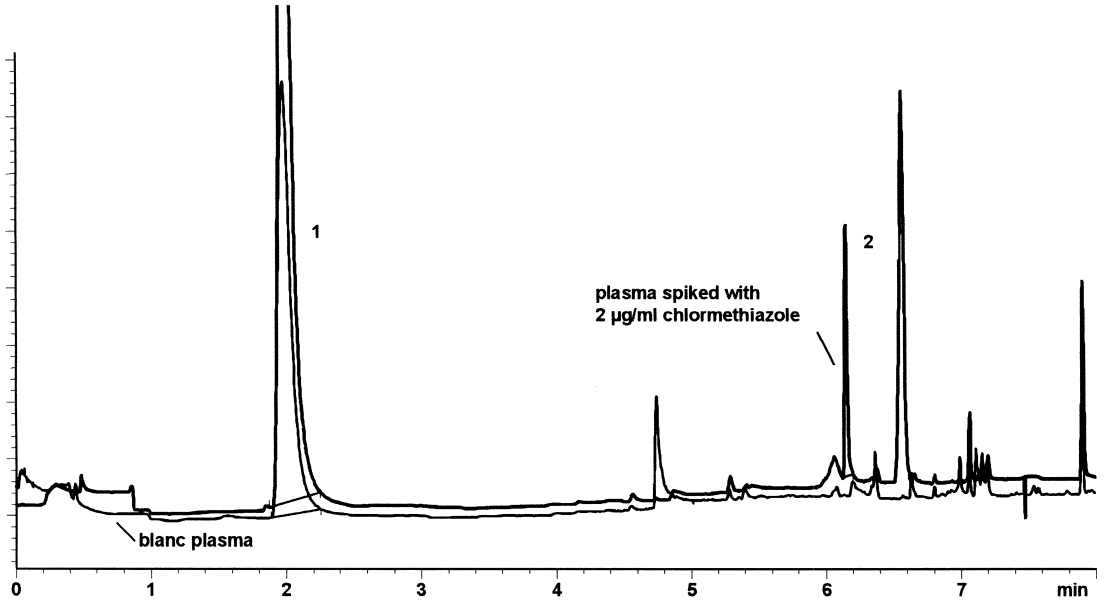

Fig. 9. HS-SPME–GC–NPD chromatogram of chlormethiazole in plasma [1=5-methylthiazole, $t_R=2.01$ min (internal standard), 2=chlormethiazole, $t_R=6.16$ min, $c_0=2.0$ μg/ml].

trimethylaminuria [83]. Nereitoxin and metabolites in human serum were analyzed with GC–MS and benzylacetone as internal standard. Ingestion of herbicides was confirmed [84]. Parathion, dichlorobenzene isomers and VOCs in blood were assayed by HS-SPME in combination with GC–MS, too [85–87]. The GC–FID combination with HS-SPME was sufficient for the assay of chloroform and methylene chloride in human blood and urine [88]. Approaches were described for the assay of nicotine, amphetamine derivatives, local anesthetics, phencyclidine, ketamine, methadone, diphenhydramine, tramadol, tricyclic antidepressants, phenothiazines and chlormethiazole by HS-SPME–GC–MS in hair [89]. HS-SPME–GC–ECD was used for the assay of tetrachloroethylene and trichloroethylene in tissues [90]. Finally, an interesting approach was the assay of residues of ignitable liquids in the HS of human skin [91].

4.2.2. With derivatization

The assay of formic acid in urine and blood was performed by in situ derivatization with methanol and sulfuric acid to methyl formate at a temperature of 35°C for a reaction time of 5 min. A 75-μm Carboxen–PDMS fiber was exposed to the HS at 35°C for 10 min. Equilibrium was reached within this time. It was found that several alternative fibers displayed a lower performance for the SPME of methyl formate. Various salts increased the peak areas by a factor of about 2 to 4. Desorption was carried out at a temperature of 280°C for 1.5 min. A Supelcowax capillary (30 m×0.25 mm I.D., 0.25 μm film thickness) was used for separation with helium as the carrier gas (0.7 ml/min flow-rate) and with a temperature program as follows: 3 min hold at 30°C, a ramp of 25°C/min to 105°C and a second ramp of 10°C/min to 145°C. In spite of a low recovery of only 0.4 to 0.8% the calibration was linear between 3 and 1000 μg/ml ($r=0.999$) and the within-day precision was 1.3 to 3.3% according to an internal standard method. The LOD was 1.2 μg/ml (FID) and the method was, therefore, described as excellently for the detection of intoxications, for example [92].

The method described above for the assay of benzodiazepines by in situ hydrolysis to benzophenones was extended to HS-SPME [71]. Fibers were exposed to the HS of the reaction mixture at a temperature of 100°C for 30 min. However, in contrast to direct SPME only five of the 11 drugs were extracted and the HS method was not investigated in more detail.

On-fiber derivatization with 1-pyrenyldiazomethane was developed for the assay of 12 short-chain fatty acids in feces (Fig. 10) [93]. The fibers were loaded with the reagent by placing them into a solution of 1-pyrenyldiazomethane in *n*-hexane (5 mg/ml) for 15 min. The HS-SPME was performed with a 85-μm PA fiber at a temperature of 50°C for 30 min. A desorption time of 4 min at a temperature of 260°C was needed with no carryover between samples. GC separation was obtained with a BPX-5 capillary (30 m×0.22 mm I.D., 0.25 μm film thickness) and helium as the carrier gas (flow-rate 1 ml/min). The temperature program was as follows: 100°C for 2 min, a first ramp of 20°C/min to 280°C (1 min) and a second ramp of 2°C/min to 310°C (10 min). An internal standard method with deuterated standards and MS detection was used for quantitation. The effect of temperature on the extraction was dependent on the chain length of acids. A plateau of peak areas was reached at a temperature of 40°C for C_1 to C_3 acids. Peak areas of acids $>C_4$ continued to increase at higher temperatures. No equilibrium of extraction was reached within 60 min of extraction. The addition of lithium salts to the sample and low pH caused damage of the PA coating. Thus, sodium chloride was added and acidification of the sample was not applied. It was demonstrated that silanization of glassware increased peak areas. Recoveries were between about 60 and 100%. Calibrations of C_2 to C_6 acids were linear between 1.9 to 32.4 μmol (acetic acid) and 0.004 to 0.07 μmol (*n*-hexanoic acid), for example, with values of r of 0.987 to 0.993. In contrast, linearity of the calibration of formic acid was poor with a value of $r=0.939$

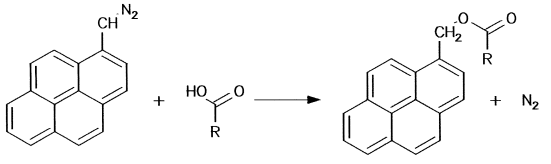

Fig. 10. SPME on-fiber derivatization of fatty acids in feces with 1-pyrenyldiazomethane.

between 2.2 and 7.2 μmol. Within-day precision was estimated to 2.4 to 12.7%, however, the value of formic acid was 15.3%. The authors concluded that HS-SPME–GC–MS is an exciting new approach to the analysis of fatty acids in feces. They further concluded that the method is superior to previous methods because it is easier to perform, sensitive and capable of accurate quantitation.

Heptafluorobutyric anhydride as an derivatization agent was injected to the injector port immediately prior to desorption of the fiber for the assay of amphetamine and fenfluramine in blood by HS-SPME–GC–MS. Good linearity between 10 and 1000 ng/g with LODs of 5 ng/g for fenfluramine and 10 ng/g for amphetamine were obtained using a calibration method with a deuterated internal standard [94]. Another principle of derivatization was applied for the assay of four amphetamine derivatives in urine by HS-SPME–GC–MS. After HS-SPME of the analytes with PDMS for 10 min the derivatization agent trifluoroacetic anhydride was applied to the analyte in the HS of a separate vial for 20 min. Calibration was linear between 50 and 1000 ng/ml ($r=0.995$ to 0.999) with LOQs of 10 to 20 ng/ml [95]. Finally, lead was assayed in blood and urine after derivatization with sodium tetraethylborate [96].

4.3. Miscellaneous

An alternative geometry of SPME is described as in-tube SPME. Thus, the SPME coating is applied to an open tubular capillary column which is well-known from GC separation. Agitation is performed by repeated aspiration and dispension of the sample in the capillary. An aqueous solvent is used for desorption and, therefore, on-line coupling with LC can be used. The H_2-antihistaminic drug ranitidine and nine beta-blockers were analyzed in urine and serum by in-tube SPME–LC–electrospray ionization MS. Ten to 15 aspiration–dispension cycles of a sample volume of 30 μl were used. The calibration of ranitidine was linear in a range of 5 to 1000 ng/ml ($r=0.999$) and LOD of 1.4 ng/ml. Within-day and between-day precisions were 2.5% and 6.2% ($n=5$), respectively. Calibrations of beta-blockers were linear in a range of 2 to 100 ng/ml ($r>0.998$) with LODs of 0.1 to 1.2 ng/ml. Recoveries in urine and serum were above 84% and 71%, respectively [97,98]. The binding constants K^{pr} between VOCs and bovine serum albumin were studied by SPME. It was demonstrated that SPME is a tool to measure K^{pr} and the freely dissolved analyte concentration in biomedical samples [99].

5. Optimization of solid-phase microextraction

A discussion on the theory of SPME and a survey of SPME methods for biomedical analysis were presented in the previous paragraphs. Some theoretical insight and practical experiences are available. Thus, a discussion should be possible of how to establish a new SPME method for biomedical analysis and how to perform an optimization of a method. Several variables have to be taken into account for this purpose, for example temperature, agitation, pH, addition of salt. These variables are discussed successively and in detail below.

5.1. Coating

Several types of coatings are commercially available now. They consist of one or two polymers: PDMS, PA, Carboxen–PDMS, PDMS–polydivinylbenzene and Carbowax–DVB, for example. The coatings with a phase of DVB consist of porous particles of DVB which are held together either by PDMS or Carbowax as a glue. Alternatively, the DVB phase is a template resin in another coating. Recently, coatings prepared with three polymers have also become available, e.g., DVB–Carboxen–PDMS. The thicknesses of the usual coatings are 7 μm, 30 μm and 100 μm for PDMS, 85 μm for PA, 75 μm for Carboxen–PDMS, 65 μm for PDMS–DVB and Carbowax–DVB. The 7-μm PDMS coating is a bonded phase and the 30-μm and 100-μm PDMS coatings are nonbonded phases. It should be recognized that some coatings cannot be regarded as liquids as presumed in Section 3.1, i.e., Carbowax–DVB and PDMS–DVB are solids and the mechanism of analyte enrichment is sorption instead of extraction.

Two simple rules should be applied for the selection of the coating in a first attempt for a new SPME method. The polarity of the coating should

match the polarity of the analyte, i.e., according to ''similar attracts similar'' the value of K^{fw} is expected to be high for a nonpolar coating and a nonpolar analyte. The number of groups forming hydrogen bonds is a special property of analytes, i.e., the number of NRH groups, NH_2 groups and OH groups. These groups mainly determine the hydrophilicity of an analyte and, therefore, also the affinity to the coating. As a second rule the coating should be resistant to extreme chemical (pH, salts, additives) and physical (high temperature) conditions. For the main part, these requirements are met by PDMS coatings. Many organic analytes investigated in bioanalysis are nonpolar molecules and they have, therefore, a good affinity to the nonpolar PDMS. PDMS is the most resistant coating in SPME and the performance of fibers is high also after many repeated extractions and desorptions. PDMS should be first tried for a new method. The PA coating can be superior in the case of slightly more polar analytes such as chlorophenols [58] and benzodiazepines [61]. The affinity was equal for PDMS and PA if the benzodiazepines were derivatized to the less polar benzophenones. However, PA was worse because of the co-extraction of interfering substances. This is important for the analysis of complex matrices such as body fluids. Furthermore, the PA coating is damaged more easily than PDMS [93]. The selection of the other coatings is even more empirical. However, PDMS–DVB and Carbowax–DVB are regarded to be suitable for more volatile analytes because of the adsorption to porous particles. The Carbowax–DVB coating stripped off the fiber in direct SPME at various temperatures and at various pH [47]. The Carbowax additive has a special affinity to alcohols. The linear range of Carbowax–DVB and PDMS–DVB is smaller than that of PDMS.

The option of selecting a coating thickness is limited to the PDMS coating. Because it is advantageous to reach equilibrium of extraction and according to Eq. (4) and Eq. (19) the sensitivity is increased with K^{fw} and $r_o - r_i$ but the rapidity of the method is decreased with K^{fw} and $r_o - r_i$. A compromise is necessary with regard to coating thickness between these two criteria of performance of a method. A 100-μm coating can be used if sensitivity should be maximum and the time of extraction is of lower concern. A 7-μm coating should be preferred if the method should be fast and sensitivity is of lower concern because the concentrations of analytes are sufficiently high. It is concluded that the higher is the affinity of an analyte to the coating (high K^{fw}) the lower should be $r_o - r_i$. For biomedical analysis, because of lower diffusion coefficients of the analyte molecule in plasma, for example, the decision for a 7-μm coating may be drawn already at lower K^{fw} than in water. However, if it was decided to stop extraction prior to equilibrium the validity of these simple calculations is limited and the fiber thickness may play a minor role in method optimization. Finally, it should be taken into account that also the time of desorption is increased with fiber thickness.

5.2. Extraction method and sample pretreatment for solid-phase microextraction

A HS method should be applied whenever possible in SPME of body fluids. The burden of the fiber with proteins, for instance, is considerably decreased. The lifetime of the fiber is increased because irreversible damage is delayed. A reversible change of extraction properties of the coating is also avoided and, therefore, the precision of the method is improved. In addition, endogenous trace substances with molecular masses between about 200 and 450 g/mol are better separated from a volatile analyte in HS-SPME, too. As a rule of thumb analytes with a molecular mass below 200 g/mol and (or) without groups forming hydrogen bonds, i.e., NRH groups, NH_2 groups and OH groups, are suitable for HS-SPME because they are likely to have a high vapor pressure. Direct SPME should only be tried if HS-SPME failed to give sufficient peak areas. Derivatization methods should be considered critically because they are often laborious, time-consuming and not easy to automate. Another sample preparation without derivatization may be preferred in many cases if SPME is possible only with derivatization. For example, the assay of benzodiazepines by hydrolysis to benzophenones [71] can be easily performed with a simple one-step LLE. Furthermore, laborious sample pretreatment for SPME should be avoided if an alternative sample preparation method is available without an extended sample pretreatment. For example, precipitation of proteins prior to

SPME is questionable for the assay of diazepam because other methods are known devoid of this additional step [74].

5.3. Agitation

The time to reach equilibrium is determined by the effectiveness of sample agitation. The radius δ of the boundary layer of the practical agitation model is decreased with increased revolution rate N of a magnetic stirrer, for example. Thus, according to Eqs. (19) and (20) the equilibration time t_e is decreased with an improved agitation. Apart from magnetic stirring the following agitation methods can be applied: vortex mixing (moving vial), fiber movement, flow through agitation and sonication. Magnetic stirring was mainly applied for SPME in biomedical analysis. Disadvantages of magnetic stirring are a more complicated automation, problems to maintain a constant N and, perhaps most important, the potential for carryover. Advantages are the good effectiveness of agitation and the availability in analytical laboratories. The moving vial approach is mainly applied in the LLE of drugs in body fluids. One apparatus can be applied to SPME with the simultaneous agitation of up to 24 1.5-ml vials and the options of setting a selected revolution rate of the vials and temperature [45,60]. The fiber movement agitation method, i.e., a vibration of the fiber, is realized in a commercial autosampler (Varian). Both methods provide good agitation similar to the effectiveness of magnetic stirring. As an advantage, no external object is needed in the sample. However, HS-SPME is impossible with the moving vial method and it is less effective with the vibration of fiber. The flow through method was rarely applied because of several disadvantages. Sonication was expected to provide a better agitation than magnetic stirring, for example. However, this was not confirmed for the assay of clozapine in plasma [47]. Thus, it is concluded that magnetic stirring at the highest N should be applied for the sample agitation in direct and HS-SPME and, additionally, fiber movement and moving vial can be applied for direct SPME. An effective agitation is needed in biomedical analysis because of a higher viscosity of samples and lower diffusion coefficients.

5.4. Sample volume and volume of the headspace

The amount of analyte extracted in equilibrium is increased with the sample volume V_w according to Eq. (39), which was derived from Eq. (3), with c_0 the concentration of analyte in the sample prior to SPME. Therefore, V_w should be as large as possible. However, because V_w is present also in the denominator of Eq. (39) no further increase of V_w is needed over a limit of about $V_w = 10K^{fw}V_f$:

$$n_f = \frac{K^{fw}V_f c_0 V_w}{(K^{fw}V_f + V_w)} \quad (39)$$

Analogously, Eq. (40) can be derived from Eq. (17) for HS-SPME. The situation is more complicated than for direct SPME because of three terms in the denominator of Eq. (40). However, V_h should be small for highly volatile analytes, i.e., for analytes with high K^{hw}. The denominator is mainly determined by the term $K^{hw}V_h$. In the case of lower K^{hw} the degree of the influence of V_h is modified by K^{fw} and V_w:

$$n_f = \frac{K^{fw}V_f c_0 V_w}{(K^{fw}V_f + K^{hw}V_h + V_w)} \quad (40)$$

According to Eq. (19) the rapidity of extraction can be regarded as independently of V_w in direct SPME. The linear model used for the description of extraction kinetics in HS-SPME (Eq. (20)) provides no explanation for the influence of sample volume and volume of the HS on the time of extraction. However, it is discussed that the capacity of HS should exceed the capacity of the fiber for about 20 times for a rapid extraction, i.e., $K^{hw}V_h > 20K^{fw}V_f$ [42]. Thus, a compromise between a rapid and a sensitive method must be found in HS-SPME. Finally, it is emphasized that this discussion only applies to methods with equilibrium conditions of extraction. This may not be accomplished in the analysis of drugs in plasma [45], for example, because of increased equilibration times. Finally, in practice, the sample volume is also determined by the available volume of sample and the available vials and equipment of agitation.

5.5. Extraction time

A graph of the relationship of peak areas and time

of extraction is a prerequisite for method optimization. The time t_e can be obtained when no further increase of peak areas is detected with increased time of extraction. Care should be taken because the slope may decrease considerably without reaching t_e. An overnight experiment may be necessary. It has to be decided with the known t_e whether the method should work in equilibrium or in nonequilibrium. Of course, an experimental time exceeding t_e is desirable because experimental errors are decreased and sensitivity is at maximum. However, the values of t_e in biomedical analysis can be very large (Fig. 3) and the chosen times of extraction were considerably shorter than t_e in some methods [45] because of practical reasons. This is possible because SPME is a quantitative method at every time of the extraction time profile as already discussed above and because sensitivity may be sufficient prior to equilibrium. Furthermore, internal standard calibration as usual in drug analysis, for example, can compensate for errors due to variable time of extraction and variable agitation. Thus the use of internal standard methods is a general recommendation for SPME. An internal standard with very similar extraction time profile should be applied. This is best realized by isotopically labeled spikes.

5.6. pH

The pH of the sample is crucial for the SPME of acids and bases. This is explained theoretically by the coupled extraction equilibrium and acid–base equilibrium (Eqs. (9) and (19)) and was confirmed in experiments. Thus, basic drugs such as antidepressants were analyzed in aqueous NaOH [45] and acid analytes such as chlorophenols [58] and valproic acid [76] were shown to be extracted better at low pH.

5.7. Salt and other additives

A salting-out effect is expected according to Eq. (8) and was described in several methods [58,77,79,92]. However, the effect is less clearly than the effect of pH. No increase of peak areas was found in plasma [48,79], the salting-out effect was limited to low salt concentrations ($<10\%$) [76] and the effect of salt interfered with the effect of pH [58]. Lithium salts caused damage of the fiber [93]. Another important goal of the addition of salt in SPME is to compensate for a variable salt concentration of the samples. This may be the only reason of adding salts to plasma samples and for the assay of acid and basic analytes at low or high pH. It is recommended to add no more than about 10% of NaCl, for example.

The use of organic additives was recommended for matrices with polymer components, e.g., plasma. It is suggested that the binding of target analyte to the proteins can be decreased and, therefore, the sensitivity of the method can be substantially increased. About 25 organic chemicals of different classes were tested to improve the sensitivity of direct SPME of antidepressant drugs in plasma. However, no substantial increase of peak areas was found and even peak areas decreased for some additives. This also applied to the addition of drugs with very similar structure or drugs which are known to decrease protein binding [47].

5.8. Temperature

As discussed above K^{fw} is decreased with increased temperatures. The sensitivity of the method is decreased in equilibrium. Therefore, ambient temperature is applied for the direct SPME. Because diffusion coefficients are increased with lower viscosity and, therefore, also with increased temperature the rapidity of the extraction may be improved according to Eq. (19), however, at cost of a loss of sensitivity. It may be tested whether the sensitivity of a method can be improved at a nonequilibrium extraction time and at increased temperatures due to this effect. This may be more important for plasma because of the higher viscosity than water or urine. The extraction profiles at various temperatures should cross one another. However, this was not found in Fig. 3 [45] and no more data were available in the literature. In HS-SPME the values of K^{fh} and K^{hw} are decreased and increased, respectively, with increased temperature. Thus, it is expected that the sensitivity of the method in equilibrium is also decreased at higher temperatures according to Eqs. (13) and (17). On the other hand, the rapidity of extraction is considerably increased in HS-SPME at increased temperatures as can be concluded from Eq. (20). Increased K^{hw} can determine the improvement

of extraction by HS-SPME for instance for analytes with low to moderate volatility. Therefore, increased temperatures were indeed recommended for HS-SPME in many applications [59,71,80,93]. Furthermore, peak areas increased at higher temperatures [59] and nonlinear relationships between peak area and temperature were described [80,93]. In addition, due to the slow transfer of heat in the HS the temperature of the fiber was lower than the temperature of the sample for a considerable time of extraction. Thus, the amount of analyte extracted can be decreased with time because of the delayed heating of the fiber [80]. Coagulation was a problem at increased temperatures for nondiluted blood samples [79]. In conclusion, the optimum temperature for HS-SPME of a distinct analyte is determined by several variables. This optimum temperature can only be found in a trial of various temperatures. Nevertheless, for the main part an increased temperature of about 50 to 100°C was used in the HS-SPME methods described above and may be a guideline for other methods.

5.9. Desorption

The time of desorption should be short as possible and carryover effects must be excluded. Thus, the highest temperature without damage of the selected coating and the smallest diameter of the injector insert should be applied because K^{fh} is decreased with increased temperatures and the linear flow-rate is increased with a smaller diameter of the insert. The maximum temperatures are about 340°C (7 μm) and 280°C (30 and 100 μm) for PDMS, 270°C for PDMS–DVB, 320°C for PA and Carboxen–PDMS, 265°C for Carbowax–DVB and 270°C for DVB–Carboxen–PDMS. Special injector inserts are available for SPME with I.D.s of 0.75 and 1.5 mm. The diameter of the insert is less critical if a megabore capillary is used with flow-rates between 10 and 20 ml/min. However, the optimum time of desorption has to be found experimentally in a desorption–postdesorption graph as described for clozapine [45], for example. Apart from this theoretical consideration, the maximum temperatures as provided by the manufacturer were not applied in practice with the aim to increase the lifetime of fibers. Indeed, the maximum temperatures of coatings may be considerably lower after direct application in an aqueous matrix.

After the needle was introduced into the insert the fiber should be exposed fast as possible because partial desorption already within the needle can result in split peaks. This applies for instance for volatile analytes and the temperature of desorption may be decreased considerably below the maximum temperature. Carryover of interferences of the matrix must also be taken into account for instance in biomedical analysis apart from the carryover of analytes only. Otherwise, accumulation of interfering substances can occur in the coating and, easily unnoticed, provide bias. Thus, the definition of the desorption time to prevent carryover effects of interferences from the matrix is also necessary. Blank samples should be analyzed repeatedly and the accumulation of trace interferences should be observed.

5.10. GC temperature program

The temperature program of the GC oven after SPME sample preparation is determined by the need for refocusing of the analyte on the head of the capillary. Otherwise, large peak widths, or even no peaks, can be found in the chromatogram because of the very slow injection, i.e., the relative long period of desorption. Therefore, the temperature program is usually started at a temperature T_1 which is considerably lower than the desorption temperature and which is low enough for refocusing. T_1 may be maintained for 1 to 3 min. This is followed by a steep ramp of 10 to 30°C/min until temperature T_2 is reached as is usual for the GC separation of the analyte. Then a less steep ramp of 1 to 5°C/min to the final temperature T_3 can be used or, alternatively, the temperature can be maintained isothermally. This principle may be modified and even only 1 ramp was described as being convenient. However, the low T_1 is crucial except in cases of very volatile analytes and a high film thickness of the analytical capillary.

5.11. GC capillary

SPME is a dirty extraction because it is only a one-step extraction and because of the limited selectivity of coatings. Therefore, endogenous trace sub-

stances of plasma, urine or other biological fluids are easily co-extracted by SPME and they have to be separated by the GC thereafter. A three-step LLE provided considerably purer extracts than SPME and the risk of interferences in the chromatogram was low in the analysis of drugs in plasma. The chromatograms after SPME were similar to chromatograms after a one-step LLE and the GC separation was considerably less comfortable than after three-step LLE [45,47,60]. Furthermore, the production of SPME coatings does not exclude an inherent contamination of fibers. Despite the usual conditioning of fibers prior to first use, i.e., desorption for several hours, interferences originating from the fiber were found in the chromatograms. This effect prevented the analysis of some antidepressants in plasma at therapeutic concentrations [47]. Thus, the selection of a GC capillary may be more critical in biomedical analysis for instance with direct SPME. More polar phases can be tried. The I.D. of the capillary, i.e., the linear flow-rate of carrier gas, is a variable which determines the desorption time. A megabore capillary should be tried to decreases the time of desorption. Finally, the use of a retention gap may improve the refocusing because of no temperature gradient in the head of the capillary.

5.12. GC detector

A more selective GC detector may separate interferences and analyte if the selectivity of sample preparation by SPME and subsequent GC separation was insufficient. Thus, GC detection by MS with SIM or even IT-MS should be preferred for the application of SPME in complex matrix. NPD as usual for the analysis of drugs in plasma is prone to problems with interferences because the majority of endogenous substances also contain nitrogen atoms in their molecule, i.e., they are also sensitively detected by NPD. This problem of chemical noise in SPME–GC–NPD is expected to be considerably decreased by the application of ECD. The application of ECD for the analysis of benzodiazepines in urine [71] and dinitroaniline herbicides in water, urine and plasma [79] was described. ECD should be increasingly tried in the SPME–GC analysis of biological fluids.

5.13. Automation

Ease of automation can be regarded as an important advantage of SPME. This is caused by the simple principle of SPME. Some complex processes as known from SPE and LLE are not needed, i.e., transfer, separation and evaporation of liquid phases. The development of an instrumental periphery is expected for the automation of SPME. An autosampler for SPME–GC was developed using agitation by vibration of the fiber. It can successively perform the SPME and desorption of several samples with one fiber [100]. However, the agitation method is less effective in HS-SPME and the sample throughput is low for methods with large extraction time and short GC separation time. Therefore, an equipment is needed which prepares several samples in a batch by SPME and subsequently makes the desorption in a batch of fibers. This principle of automation is known from LLE–GC, although it is not a fully-automated system. It can be easily calculated that the sample throughput of this approach is higher for methods with large time of extraction and short time of GC separation. This applies to the analysis of drugs in plasma by direct SPME, for example.

6. Advantages and disadvantages of solid-phase microextraction

The advantages claimed for SPME were (1) no use of solvents, (2) easy handling, (3) little equipment necessary, (4) fast method, (5) ease of automation as well as (6) good linearity and high sensitivity [44]. However, taking into account the survey of methods as provided above it is obvious that SPME can display these advantages only in some areas of biomedical analysis, i.e., the matrix and the volatility of target analyte have to be taken into account. First of all, the combination of low volatility of analyte and a complex matrix with polymer components, e.g., proteins in plasma or cell cultures, considerably limits the application of SPME. The extraction is very slow in contrast to LLE and SPE with packed bed columns. Extraction times considerably lower than t_e must be used because of practical requirements. Thus, the recovery was very low and the

sensitivity was critical for some analytes within an acceptable time of extraction [45,47]. In general, recoveries reported for SPME were considerably lower in many cases than it is usually known for LLE and SPE. Another disadvantage of SPME is uncovered if the sensitivity is low with regard to the target concentrations of analyte, the therapeutic concentration of a drug in plasma for example: direct SPME is a dirty sample preparation. The occurrence of a huge number of interferences in the chromatogram which come from endogenous trace substances in biological fluids prevents the analysis of target analyte at low concentrations. This is not encountered in three-step LLE or SPE. The values r of internal standard calibration of SPME were not excellent and the precision was about 8 to 10%. Attempts to by-pass this problem by special methods such as solvent-modified SPME [74], complicated derivatization methods [71] or equilibrium dialysis [76] can be regarded as misleading because the advantages of easy handling, little equipment and fast sample preparation are abandoned. A good SPME method should remain simple, otherwise, alternative sample preparation methods may be superior. It was concluded that the application of SPME for the assay of low volatile drugs and metabolites in plasma may be limited to some drugs with high therapeutic concentrations in the range of 1 to 100 μg/ml. Because the analysis of drugs in plasma for pharmacokinetic studies and for TDM is extensively applied an important area of biomedical analysis may be only little accessible for SPME.

The assay of volatile analytes in plasma and similar matrices is more convenient because of the option of HS-SPME. SPME may clearly display some advantages over other sample preparation methods in this area. No solvent or only very little solvent is needed, the handling of samples is easy with little equipment and the available SPME autosampler can be applied efficiently because the extraction time is short. Thus, the SPME of one sample is completed during the GC separation of another. Methods with good linearity and sensitivity were described [77–80]. HS-SPME with derivatization may also display a good performance with regard to effectiveness, precision, accuracy, sensitivity and selectivity [92]. Complicated and long-term derivatization methods should be avoided [71] if alternative sample preparation methods are available. Again, the labels fast, efficient and simple should not be abandoned.

The advantages of SPME can be used for both the assay of low volatile and highly volatile analytes in urine or other body fluids with no or only a low concentration of polymer biomolecules. The problems of sensitivity and delayed t_e are considerably decreased in comparison to plasma. A number of methods with good precision, accuracy, sensitivity and selectivity were demonstrated which were also simple and fast [58,69,77–79,92].

Apart from the problems discussed above SPME has also some principle disadvantages. (1) Because of desorption times of at least 1 min cryofocusing of the analyte is needed. Thus, temperature programs with a very low initial temperature are needed for the GC separation. The time of the GC program is, therefore, longer than the time of an alternative sample preparation. (2) The desorption needs more time than the injection of liquid extracts after LLE and SPE. Desorption times of 12 and 20 min [57,61] waste the advantages of SPME and may be not accepted. (3) By nature, carryover effects occur very easily in SPME methods because of the repeated use of one fiber. Additional efforts are necessary to handle this problem. Extended extra clean-up procedures for the fiber after every extraction may not be accepted for routine methods. In fact, a carryover of 2% is very questionable [57]. (4) The conditioning of fibers prior to first use is not necessary in LLE, for example. (5) By nature, SPME is a relatively dirty extraction if it is compared with multiple step LLE. (6) Because SPME is a nonexhaustive sample preparation the methods cannot equally compensate for changes of the composition of the matrix as in the case of LLE. Quantitation is more prone to errors due to changes of the matrix also if internal standard methods are applied. Thus, matrix effects must be extensively investigated during method validation. (7) SPME is not an universal sample preparation method because of the restrictions and limitations discussed.

7. Conclusions

SPME is an encouraging development for sample

preparation in biomedical analysis. The majority of applications were encountered in environmental chemistry in the early developmental period. However, an increased number of publications are now available on biomedical analysis. The special geometry of the extraction agent is an unique feature of SPME and, therefore, special advantages and disadvantages are involved as discussed above. The most striking attribute of SPME is a low recovery as reported for many methods. This is not unexpected because SPME is an equilibrium extraction but not an exhaustive extraction. However, several SPME methods also presented with high recovery and the performance of the majority of methods was high with regard to sensitivity, linearity, precision and accuracy. It is concluded that SPME can be used as a substitution and improvement of classical sample preparation methods. Thus, it can be applied for the analysis of drugs, metabolites, environmental pollution and endogenous substances for instance in urine and body fluids without or with low concentration of biopolymers. It appears from the survey of published methods that HS-SPME–GC–MS, for instance, becomes increasingly popular in forensic and environmental toxicology. The need for an alternative method in the confirmatory analysis of positive tests by ELISA and FPIA may be a special requirement which promoted the application of SPME in forensic toxicology. The trace analysis in plasma may be limited to highly volatile analytes. The SPME assay of substances with low volatility is more complicated in plasma and may be easily performed, therefore, only in samples with high concentrations. For this reason, a wide application of SPME in TDM cannot be expected for the near future. On-line SPE–LC with column-switching techniques may be more recommended in this area. However, more studies are necessary also by SPME in TDM applications. Apart from this role of SPME as a substitution and improvement of classical methods, SPME may lead to new approaches in biomedical analysis. For example, the potential of SPME to analyze directly the free concentration of drugs in plasma should be more investigated in future. The in-vivo analysis of metabolites in animal experiments should be tried using new geometries. New developments are expected because the important role of the geometry of extraction agent was first recognized in the invention of SPME.

8. Nomenclature

DOM	Dissolved polymer organic material
DVB	Divinylbenzene
ECD	Electron-capture detection
EI	Electron ionization mode
ELISA	Enzyme-linked immunoassay
EDDP	2-Ethylene-1,5-dimethyl-3,3-diphenylpyrrolidine
FID	Flame ionization detection
FPIA	Fluorescence polarization immunoassay
GC	Capillary gas chromatography
HS	Headspace
I.D.	Inner diameter
IT	Ion-trap
LOD	Limit of detection
LOQ	Limit of quantitation
LC	Liquid chromatography
LLE	Liquid–liquid extraction
MS	Mass spectroscopy
MIP	Molecular imprinting material
NCI	Negative chemical ionization
NPD	Nitrogen–phosphorus selective detection
PA	Polyacrylate
PAH	Polynuclear aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PDMS	Polydimethylsiloxane
RSD	Relative standard deviation
RAM	Restricted access material
SFE	Supercritical fluid extraction
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TEMPO	2,2,6,6-Tetramethylpiperidine-1-oxide
TDM	Therapeutic drug monitoring
UV	Ultraviolet detection
VOC	Volatile organic compound

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