Analytical Methods

DOI: 10.1002/anie.201006896

In Vivo Solid-Phase Microextraction in Metabolomics: Opportunities for the Direct Investigation of Biological Systems

*Dajana Vuckovic, Sanja Risticevic, and Janusz Pawliszyn**

analytical methods · gas chromatography · in vivo sampling · liquid chromatography · metabolomics

Sample preparation has a strong impact on the quality of metabolomics studies. The use of solid-phase microextraction (SPME), particularly its in vivo format, enables the capture of a more representative metabolome and presents opportunities to detect low-abundance, short-lived, and/or unstable species not easily captured by traditional methods. The technique is ideally suited for temporal, spatial, and longitudinal studies of the same living system, as well as multicompartmental studies of the same organism. SPME is useful for the investigation of biological systems ranging in complexity from cells to mammalian tissues. Selected examples are highlighted in this Minireview in order to place the technique within the context of conventional methods of sample preparation for metabolomics.

1. Introduction to Solid-Phase Microextraction

SPME is a non-exhaustive environmentally friendly sample preparation procedure which combines sampling, analyte extraction, and sample introduction in a single step while minimizing or completely eliminating the use of solvents.[1] SPME enables solventless extraction by means of a fused-silica or stainless-steel fiber coated with a thin film of polymer, which acts as the sorbent/solvent during the extraction of compounds (Figure 1). The ratio of the extraction-phase volume to the sample volume is very low, so the complete removal of the analyte from the sample is not typically achieved. Instead, the amount of analyte extracted is governed by the distribution coefficient of the analytes between the SPME coating and sample matrix if the equilibrium is reached [Eq. (1)], or by the rate of mass transfer (defined by the diffusion coefficient and the convection/agitation conditions) if a short sampling time is used. In Equation (1) n_e is the amount of analyte extracted at equilibrium, K_{fs} is the distribution constant for the analyte between the fiber coating and the sample, V_s is the sample volume, V_f is the volume of the extraction phase immobilized on the fiber, and C_0 is the initial concentration of the analyte in the sample.

$$n_e = \frac{K_{fs} V_s V_f}{K_{fs} V_f + V_s} C_0 \quad (1)$$

Although SPME was originally developed as a green and simple alternative for the monitoring of organic pollutants in aqueous, gaseous, and solid environmental samples, the utility of this technique has rapidly extended to many other fields including food, pharmaceutical, forensic, toxicological, biological, and clinical analyses. As a result, the number of journal articles featuring SPME as an analytical method for various applications (both qualitative and quantitative) has exceeded 5000 over the past decade, indicative of a mature and well-understood technique. The main focus of this Minireview is to discuss the emerging opportunities for the use of SPME in the field of metabolomics,[2] and to show the capability of this technique to provide valuable information not easily obtained by conventional methodologies.

[*] Dr. D. Vuckovic, S. Risticevic, Prof. J. Pawliszyn
Department of Chemistry, University of Waterloo
200 University Avenue West, Waterloo, ON, N2L 3G1 (Canada)
Fax: (+1) 519-746-0435
E-mail: janusz@uwaterloo.ca

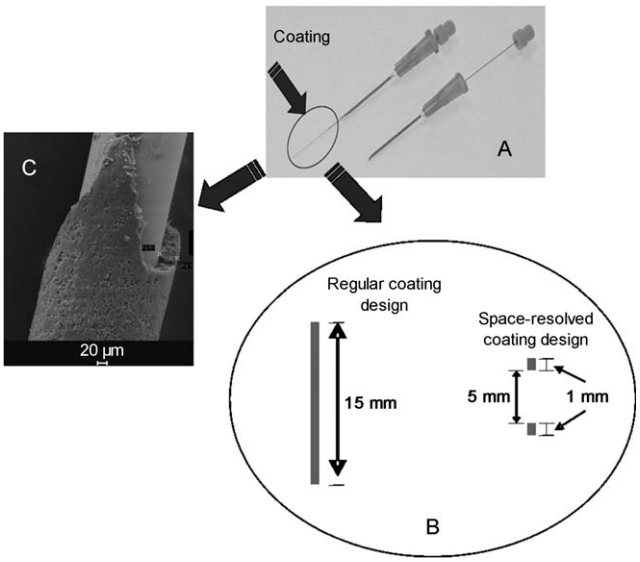

Figure 1. Design of a needle-based SPME device. A) Photograph of a prototype device shown with the coating exposed and retracted. B) Schematic of regular (coating length 15 mm) versus space-resolved coating (two segments each 1 mm in length). C) SEM image of SPME coating showing the coverage of sorbent with a biocompatible layer.

2. In Vivo SPME for Metabolomics

The choice of the sample preparation method plays an extremely important role in metabolomic studies because it affects both the metabolite content and the data quality.[3–5] For instance, Canelas et al. showed that even the relative levels of metabolites (up- or down-regulation in the comparison of two sets of conditions) could be distorted on the basis of the method of sample preparation selected and could lead to erroneous conclusions.[6] An ideal sample preparation method for the metabolomic analysis of biological samples by GC–MS or LC–MS should 1) be simple and fast to prevent metabolite loss and/or degradation during the preparation procedure and enable high-throughput, 2) incorporate a metabolism-quenching step to preserve the chemical identity of the metabolites, 3) be reproducible and allow for adequate metabolite solubilization, and 4) be nonselective. First, we briefly discuss theoretical and experimental approaches to performing in vivo SPME (Sections 2.2 and 2.3) in order to show that this simple and rapid approach combines sampling, metabolism quenching, and sample preparation in a single efficient step, thus addressing the first two requirements of an ideal metabolomics method. Section 3 discusses the metabolite coverage that can be achieved by in vivo SPME and the selection of the appropriate coating which reduces or increases the selectivity of the SPME procedure to meet the demands of a given application. In Section 5, we discuss the reproducibility of the technique, and show it is satisfactory for metabolomics studies.

Dajana Vuckovic completed PhD studies in analytical chemistry at the University of Waterloo, Canada in 2010 under the supervision of Dr. Pawliszyn. Her main research interests include the development of in vivo SPME for metabolomics as well as the development of high-throughput mass-spectrometry-based workflows for biomarker discovery, chemical proteomics, and bioanalysis.

Sanja Risticevic is a PhD student in the Pawliszyn research group at the University of Waterloo. Her expertise is in the area of food and complex sample analysis. Her PhD project is focused on the application of non-invasive sample preparation combined with high-resolution gas chromatography for metabolomic profiling in plant-based food commodities.

Janusz Pawliszyn is a professor of analytical chemistry at the Department of Chemistry, University of Waterloo, Canada. He has written over 400 scientific publications and invented SPME. He presently holds the Canada Research Chair and NSERC Industrial Research Chair in New Analytical Methods and Technologies. The primary focus of his research program is the elimination of organic solvents in sample preparation to facilitate on-site monitoring and in vivo analysis.

2.1. Why Consider In Vivo Sampling during Experimental Design?

Although the majority of research on biological systems is currently performed using in vitro methods, it is important to remember that removing a sample from its natural biological milieu can result in important changes in sample composition owing to processes such as oxidation and enzymatic degradation. For example, the composition of the volatile emissions obtained from detached or damaged plants is significantly different from that of the volatile emissions collected from living, undamaged, and undisturbed specimens; thus, in vitro approaches may not be adequate depending on the goals of a particular study.[7] In the context of collecting representative metabolomes of plant systems, it has been reported that even harvesting (separating the investigated material from the original plant) should be conducted very rapidly and followed by immediate freezing in liquid nitrogen in order to avoid changes in the metabolome resulting from enzymatic reactions triggered by plant handling and wounding and to stabilize labile metabolites.[8] In vivo research with minimal perturbation to the system under study also allows the monitoring of dynamic processes as they occur in the same biological entity, for example scent development in a flower, release of pheromones, or disease onset in an animal. Thus,

the availability of appropriate in vivo methodologies can play an important role during experimental design to ensure the collection of high-quality information for subsequent biological interpretation.

2.2. Theoretical Basis for In Vivo Sampling by SPME

SPME can be employed for in vivo sampling because the amount of analyte extracted is independent of the sample volume, under the conditions of negligible depletion.[1] In the context of Equation (1), the conditions of negligible depletion are met when the sample volume is much larger than the product of the distribution coefficient and the fiber volume ($V_s \gg K_{fs}V_f$). Thus SPME can be used to sample various living systems without the need to isolate a defined sample volume while maintaining the ability to perform quantitative analysis.

2.3. Experimental Approach

The main steps of an in vivo SPME process include 1) the extraction of analytes from the sample into the SPME coating and 2) the removal of analytes from the device by thermal desorption for GC applications or by solvent desorption for LC applications. Figure 2 illustrates the workflow of in vivo

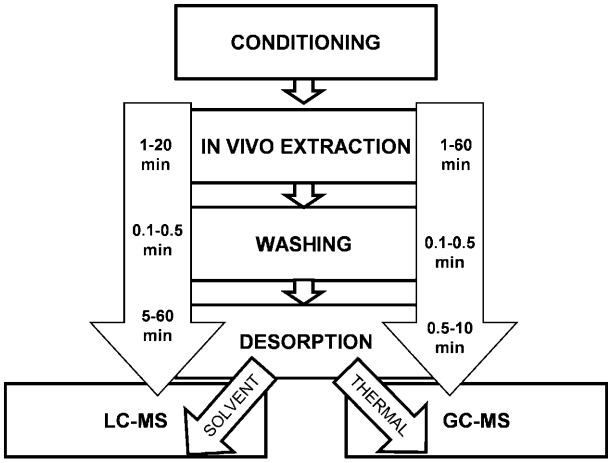

Figure 2. Overall workflow of in vivo SPME in combination with LC–MS and GC–MS.

SPME. The entire procedure is very rapid and requires few steps and minimal sample handling. This can effectively minimize the potential for metabolite losses throughout the sampling/sample preparation procedure and also eliminate the generation of artifacts arising from sample preparation, extraction, and storage. In addition, the desorption step can be coupled directly online to LC–MS or GC–MS analysis by thermally desorbing the sample from the fiber directly in the GC injector port or in the stream of the mobile phase for LC applications. This permits the complete elimination of glassware or plasticware in sample preparation and reduces possible contamination and/or the adsorptive loss of analytes.

The extraction process itself can be performed either in headspace mode or in direct extraction mode depending on the volatility of the analytes to be studied. To date, in vivo SPME has been successfully applied to various biological systems including microorganisms, plants, plant-based foods, animals, insects, and human emissions (Table 1). For example,

Table 1: Sampling strategies in various biological systems using in vivo SPME.

SPME mode	Living system	Biological matrix	Ref.
direct extraction	dogs	blood	[14–17]
direct extraction	rats	blood	[18]
direct extraction	mice	blood	[19]
direct extraction	fish	muscle, adipose	[20–22]
direct extraction	plants	stem, leaf, onion bulb	[23–25]
headspace	plants	volatile emissions	[26, 27]
headspace	cell cultures	volatile emissions	[28–30]
headspace	human	breath	[31]
headspace	insects	volatile emissions	[32–34]

the SPME fiber can be exposed directly in the headspace of a cell culture or plant to study volatile emissions (Figure 3). In addition, metabolite fingerprinting and profiling can be

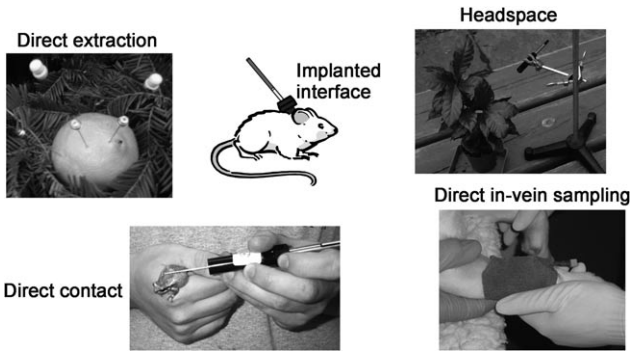

Figure 3. Examples of experimental approaches used for in vivo SPME sampling.

achieved by exposing the SPME coating directly to intact fruits; this approach could be a promising alternative in the rapidly growing area of food metabolomics.[9] For animal studies, SPME can be used not only to sample volatile emissions, but also to sample directly circulating blood (directly though a catheter in large blood vessels or using a specially developed interface for small blood vessels in mice and rats) or even tissue such as muscle, adipose, brain, and liver. A recent review article describes in detail various sampling approaches and appropriate considerations during method development.[10]

For direct extraction applications, the main point to emphasize is that the in vivo SPME device is specially designed to prevent adverse reactions in the living system. This is accomplished by placing a layer of a biocompatible polymer (such as polyethylene glycol or polyacrylonitrile) on the outside of the coating. This biocompatible layer minimizes the adhesion of biomolecules to the surface, which can affect

analyte uptake into the coating, and also minimizes possible toxic/adverse reactions such as clotting on the surface of the coating. Figure 1 A depicts a SPME fiber with a biocompatible coating which is housed a commercial needle. Figure 1 C shows a scanning electron microscopy image of the fiber to illustrate the coverage of the biocompatible polymer. These devices are aimed primarily for LC applications; they are inexpensive, recommended for single use, and are now commercially available. In contrast, commercially available mixed-polymer SPME GC fiber assemblies that can be implemented for in vivo sampling are not biocompatible. This means that multiple direct extraction processes inside a biological system using the same coating can lead to fouling of the extraction phase.[11] The adsorption of interfering high-molecular-weight and nonvolatile macromolecules to the coating surface changes the chemical properties of extraction phase and may lead to a decrease in sample reproducibility, extraction efficiency, and fiber-coating lifetime.[12] One way to eliminate the fouling of the extraction phase for GC metabolomics studies is to introduce a gaseous barrier between the sample matrix and the fiber coating (headspace extraction mode) or alternatively to employ sample dilution.[13] However, as none of these alternatives are compatible with the direct in vivo extraction of metabolites from biological systems, a washing step in water immediately following extraction and prior to thermal desorption is the most practical solution to date. The introduction of a washing step may minimize not only the fouling of the extraction phase but also the potential formation of artifacts in the injector port and the generation of unrepresentative chromatographic profiles. For example, during the analysis of volatile and semivolatile metabolites of intact strawberry fruits, Verhoeven et al. reported that implementation of a rinsing step significantly reduced the production of artifacts (namely Maillard products of carbohydrates and amino acids adsorbed on the surface of extraction phase) during thermal desorption.[9]

From the perspective of metabolomics, the SPME coating plays another crucial role. Small molecules can diffuse through the outer biocompatible layer and enter the sorbent pores, whereas large biomolecules cannot. This means that once a metabolite partitions into the coating, it is protected from further enzymatic reactions thus ensuring the capture of short-lived and labile species.

3. Metabolite Coverage by SPME

Existing sample preparation procedures for untargeted metabolomics studies in various matrices have been evaluated systematically in the recent literature, but the question often not asked is "How well does the metabolome at the time of analysis represent the true metabolome at the time of sampling?" For untargeted metabolomics methods where the aim is to accurately capture as many of metabolites as possible, it becomes impossible to ensure that sampling, sample storage, and preparation conditions are suitable for all metabolites present in the sample. After all, it is impossible to validate methods in a truly quantitative fashion for thousands of endogenous/exogenous metabolites whose identity may not be known and for which authentic standards may not be available. Thus, currently there is no way to objectively evaluate how representative the collected metabolome actually is. Many of the existing procedures aim to incorporate a metabolism-quenching step through the use of low temperatures (addition of cold solvent, freezing in liquid nitrogen), addition of acid, freeze-drying, or fast heating.[35,36] As metabolic processes can be very fast with time scales of < 1 s, the quenching step must be extremely rapid to be fully effective. This can be very difficult to implement for biological samples. Furthermore, the application of a quenching step can cause inadvertent degradation or loss of some metabolites, and no single metabolism-quenching method can be considered optimum.[35,37] For example, in plant metabolomics it was reported that the addition of an acid can reduce the number of captured metabolites, while freeze-drying may lead to the irreversible adsorption of metabolites on cell walls and membranes.[38] During subsequent sample handling, appropriate steps must be undertaken in order to avoid warming/thawing which may give rise to potential enzymatic activity and alteration of the composition of the metabolite pool.[39] Alternatively, saturated salt solutions may be added to plant material to stop enzymatic activity after the material is ruptured.[39,40] A quenching step is routinely included in most studies on microorganisms and plants, but metabolomics studies on biofluids typically do not employ any quenching step at least until the plasma is isolated, thus leaving plenty of opportunity for additional changes in metabolome to take place. The use of in vivo SPME to sample the circulating blood of animals addresses this difficulty as the metabolism-quenching step is incorporated directly in the sampling procedure. In fact, our results show that using this approach 70 (positive-ion ESI reversed-phase LC–MS) and 85 (negative-ion ESI reversed-phase LC–MS) features were unique to in vivo SPME and were not detected when the blood was withdrawn and the subsequent plasma sample subjected to SPME, ultrafiltration, or solvent precipitation.[41] For example, β-NAD is one of the metabolites identified using in vivo SPME but was missed completely by other methodologies (Figure 4). Although we are currently working on characterizing the remaining unique metabolites, preliminary data (accurate mass, polarity, database search, and retention time) indicate that these species may include carotenes, nucleosides and other phosphorylated compounds, thionines, and glucuronide species. We also showed that the methods based on blood withdrawal resulted in extremely elevated levels of oxidized glutathione and incorrect glutathione ratios, while in vivo SPME was able to measure true glutathione concentrations (both reduced and oxidized; Figure 5).

Headspace and/or direct contact in vivo SPME in combination with GC–MS can also help improve metabolite coverage in comparison to that of traditional extraction approaches. Gallagher et al. compared the performance of in vivo SPME versus hexane extraction for the analysis of human skin emissions.[42] They tentatively identified a total of 92 compounds: 58 were found using in vivo SPME while 49 were found in hexane extracts, indicating that the two techniques are complementary in nature. Higher-molecular-

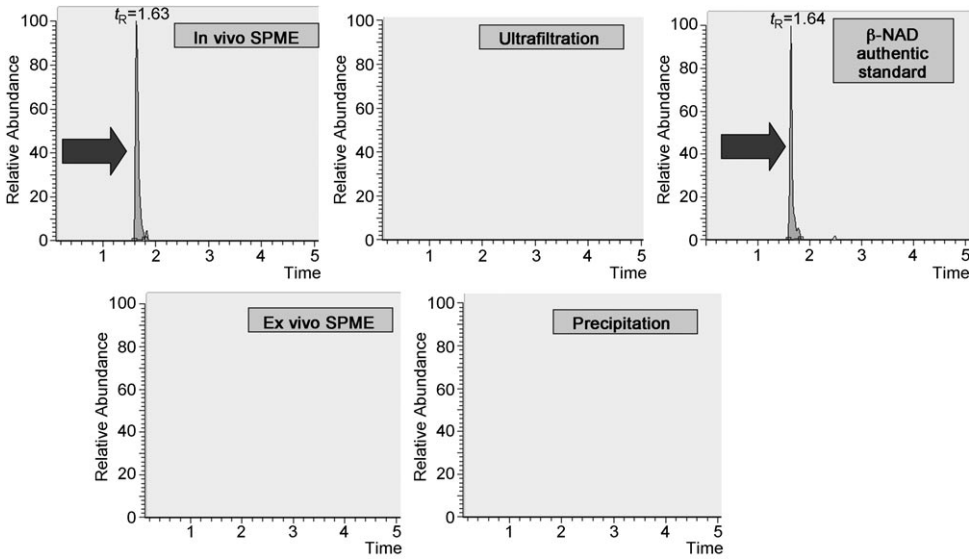

Figure 4. Example of metabolite (β-NAD) identified in mouse blood using in vivo SPME and not detected after blood withdrawal using SPME, solvent precipitation, or ultrafiltration methods.

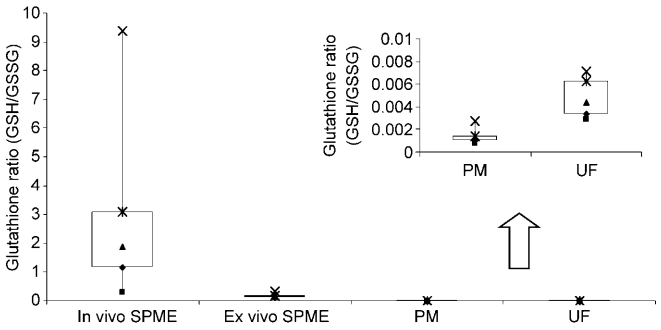

Figure 5. Plots of glutathione ratio (reduced/oxidized forms) obtained using in vivo SPME sampling in circulating mouse blood and after blood withdrawal using SPME, solvent precipitation (PM), and ultrafiltration.

weight compounds predominated in hexane extracts, while SPME was more suitable for collection of lower-molecular-weight aldehydes and ketones. On the basis of SPME results, the authors propose dimethylsulfone, benzothiazole, and nonanal to be biomarkers of aging. In another example, Zimmermann et al. were able to identify for the first time methyl dodecanoate, decan-1-ol, heptan-1-ol, 3-methylbutan-1-ol, pentadecan-2-one, nonan-2-one, and undecan-2-ol in human colon cells; this indicates that SPME can be very useful in global metabolomic studies to identify previously unobserved metabolites.[28] Furthermore, in vivo SPME (both headspace and direct contact) has been extensively applied for the study of insect emissions such as semiochemicals and defense chemicals. For example, undecane was isolated as a recruitment pheromone in ants; it enables long-range intra-species communication when a good supply of food is discovered.[43] In another study, SPME was used to identify for the first time a sex pheromone (blend of tetradecanal and pentadecanal) in praying mantid.[44] Also, Cai et al. used in vivo headspace SPME for the determination of metabolites potentially affecting the quality of fruit as well as yields; these metabolites were emitted by *Harmonia axyridis* beetles, and the researchers were able to attribute the presence of characteristic odors to several identified methoxypyrazines.[34] Djozan et al. compared traditional solvent extraction, in vitro headspace SPME, and in vivo headspace SPME for the isolation of defense chemicals produced in the scent gland of the shield bug *Graphosoma lineatum*, and they noticed a remarkable increase in sensitivity with in vivo mode.[33] Prior to the availability of SPME for this type of application, the insects were killed and extracted with solvent; it was not possible to monitor dynamic changes in the emissions of semiochemicals over time or changes in emissions upon interaction with other individuals or exposure to environmental stimuli. In vivo SPME can also be used in to study insect–plant relationships, a very important topic in pest management, chemical ecology, and entomology.[32] Several systems have previously been designed and implemented to capture volatile and semivolatile components emitted by insects, for example, glass–Teflon chambers with adsorption by means of Tenax traps and wind tunnels.[32] However, several difficulties and disadvantages were encountered: the organisms' stress levels were too high, and since most of the studies focused on insect–plant system as a whole, the contribution of the insect alone could not be isolated. In vivo SPME addresses these difficulties. For example, Fernandes et al. found a significant difference in the metabolites emitted by kale before and after attack by an insect, and also detected an in vivo accumulation of limonene and camphor in the insect, thus adding to the knowledge of the ecological interactions of the two species.[32]

4. Investigation of Biochemical Individuality

The study of interanimal variability or biochemical individuality can provide fascinating insight into the biology of various processes.[45] For example, in a recent study Coen et al. studied the metabolome of various compartments to understand the toxic response of rats to the administration of galactosamine.[46] This study not only provided interesting insight into the mechanism of galactosamine toxicity, but it also effectively illustrates the power of metabolomic studies to examine the differences in individual response since 25% of the rats were found to be nonresponders while 75% of the rats displayed various degrees of hepatotoxicity. We believe that in vivo SPME can play an important role in this type of study as it permits repeated sampling of the same animal over time and multicompartmental sampling (for example, blood

and tissue), and can also be used to measure the concentration of free (unbound) metabolites.

4.1. Concentration of Unbound Metabolites

The amount of analyte extracted by SPME is proportional to the concentration of the free (unbound) metabolite in the biological sample. Only unbound metabolites are biologically active, so using SPME as the method of sample preparation may be helpful in metabolomics studies aiming at understanding biological processes. For example, our results for in vivo SPME sampling of blood show that SPME can be used to study the variability in concentrations of various metabolites (even highly unstable species such as glutathione, retinol, and adenosine), both to examine the variation of metabolite concentration in the same individual over time, as well as the variation in different individuals (Figure 6).[41] Interestingly, the availability of information on unbound metabolites was important for distinguishing between control and test groups when small cohorts of animals were used.[41] In our study, we were able to find potential biomarkers of carbamazepine dosing using both in vivo SPME (sampling of the same four animals before and after dosing) and ultrafiltration (sampling of four animals per control and test group). However, when solvent precipitation was used on the same samples, it was not possible to distinguish between the two groups. In a GC–MS example, Soini et al. developed a very rapid sampling technique (10–12 s sampling time) using a commercially available Twister polydimethylsiloxane stir bar for high-throughput metabolomic studies of human skin emissions to study whether these emissions can serve as fingerprints.[47,48] Long-term reproducibility of 14.3% and 14.7% relative standard deviation (RSD) was achieved for the two internal standards. This data was obtained from the analysis of a total of 960 in vivo samples over a three-month period, thus enabling the successful comparison of individual emission profiles over time.

4.2. Temporal and Spatial Resolution

Temporal resolution is the ability to accurately determine analyte concentrations at an instantaneous time point and to clearly resolve two different concentrations in rapid succession. In vivo SPME sampling is not instantaneous but takes place over a short, well-defined time interval. The temporal resolution achievable by in vivo SPME depends on a variety of factors including instrumental sensitivity of the subsequent analysis method and the amount of analyte extracted by SPME probe, which in turn is dependent on coating dimensions, analyte concentration in the sample, analyte distribution coefficient, the rate of change of analyte concentrations over the time period studied, and sample conditions such as agitation rate. To estimate the minimum sampling times for appropriate temporal resolution in a given dynamic system, Zhang et al. recently developed and validated a set of equations taking into account all of these factors.[49] With the increased sensitivity of modern analytical instrumentation (GC–MS and LC–MS in particular), in vivo SPME sampling times of 0.5–5 min are often employed because they result in sufficient amounts extracted and give reasonable temporal resolution for many processes as confirmed by the developed theoretical model (for example, pharmacokinetic studies of drugs and metabolites).[14,15,18,19] The length of these sampling times implies that in vivo SPME is typically not applicable for quantitative studies of very rapid processes occurring on time scales of seconds and is also not generally applicable for

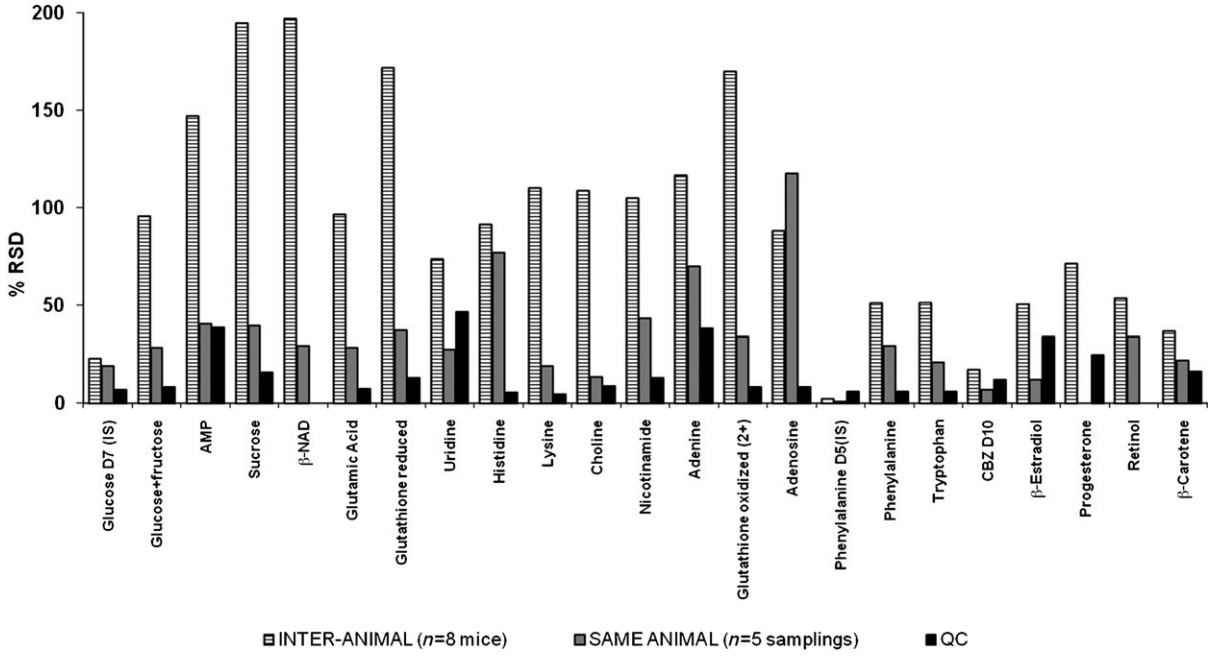

Figure 6. Evaluation of intra-animal ($n=5$ consecutive 2-min samplings) and inter-animal variability ($n=8$ mice) of selected metabolites using in vivo SPME sampling in circulating mouse blood. QC = quality control.

processes with large changes in concentration per unit time. Although even shorter sampling times can be envisioned, issues such as the convection introduced into the system by the introduction of SPME probe and the time needed to establish steady-state diffusion at the interface become important when sampling times of less than one minute are considered.[49] Further investigation of the fundamental theory is needed to evaluate these effects on the accurate determination of true analyte concentration. However, such rapid methodology is still inherently suitable for qualitative analysis applications, for example to check which metabolites are present at a given time or to confirm pathway mechanisms through the capture of a short-lived intermediate.

Temporal analyses were performed to investigate the kinetics of the release of volatile and semivolatile metabolites during the consumption of a complex flavored model cheese.[50] In this particular study, the SPME device was inserted into a Y-junction, which connected the entry of the API-MS capillary, the entry for the SPME fiber, and the nose of the subject, at different times during cheese mastication to sample the expired air for 8 s. The authors were able to explain the pattern in the temporal release profile of heptan-2-ol by actual differences between individuals which are known to be controlled by a number of oral parameters.[50] In another interesting study, in vivo SPME was implemented to measure allelochemical uptake by tomato plants.[23] Allelochemical solutions were exogenously applied to the soil, and the tomato stem was treated by several one-hour-long extraction cycles (up to 72 h after treatment application). Even though a fairly long extraction time was implemented in this particular case, the authors were able to elucidate the time course of the persistence of 1,8-cineole in tomatoes. Thus in vivo SPME should be a promising alternative methodology for measuring allelochemical uptake by target plants and subsequently studying allelopathic phenomena.[23] In vivo SPME was used to study scent production in petunia flowers.[51] The circadian rhythm in the emission of major benzoid compounds is evident in the results shown in Figure 7. In vivo data demonstrated de novo production of volatiles during low emission periods rather than the release of stored metabolites, as confirmed by both targeted metabolomics and genomics approaches.

Spatial resolution refers to the ability of a technique to distinguish between different analyte concentrations in space continuum. Uneven spatial distribution of analytes is particularly common in biological systems and arises from the specialization of tissue function as well as from differential uptake, metabolism, external stimuli, and storage of analytes. For example, in the area of plant metabolomics, most approaches employ homogenization to enhance the release of metabolites during extraction.[52] However, homogenization prevents the acquisition of data associated with metabolite distribution across a sample which may be valuable since metabolite content can vary not only from organ to organ, but also within the same organ.[8,39] For example, Biais et al. reported a method for the spatial localization of metabolites in melon where considerable effort was required to dissect, homogenize, and analyze specific plant parts.[53] In vivo SPME may be particularly attractive for collecting valuable information about the spatial distribution of metabolites in smaller tissues and compartments of interest. The spatial resolution of the technique is determined by the dimensions of the SPME

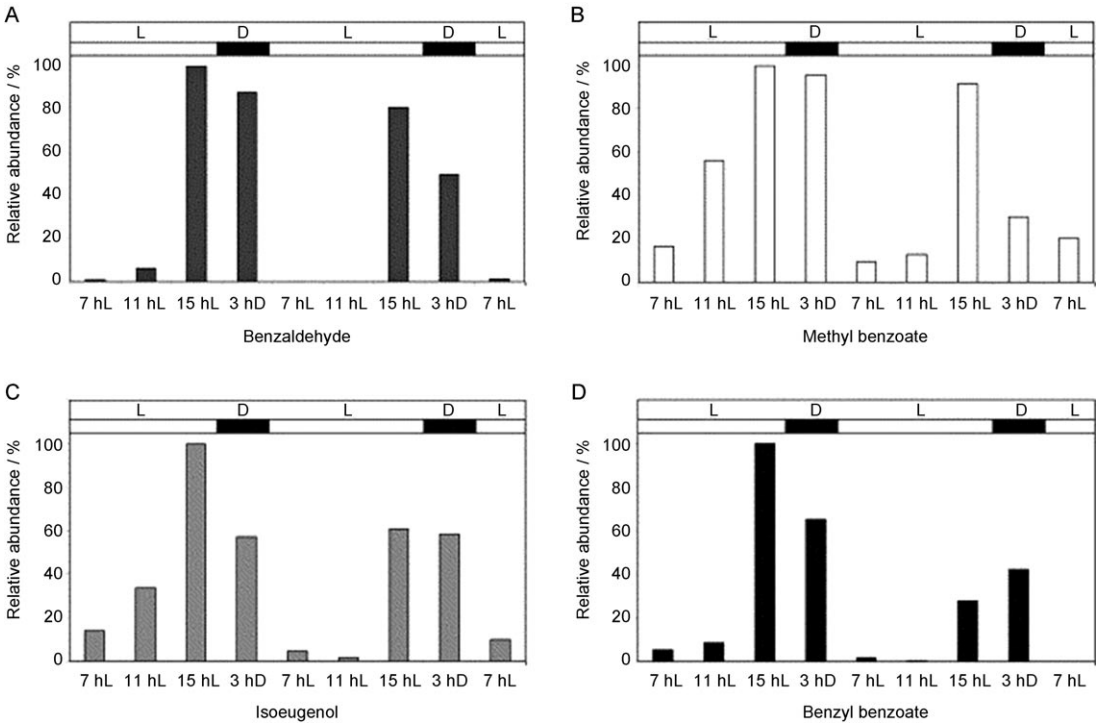

Figure 7. Rhythmic emission of four major volatile benzenoids A) benzaldehyde, B) methylbenzoate, C) isoeugenol, and D) benzyl benzoate. After flower opening, flower volatiles were analyzed four times a day, over a 48 h period, using in vivo headspace SPME. The top white bars indicate light periods (L); top black bars indicate dark period (D). Each component is plotted as a percentage of its maximum value. Figure reproduced from Ref. [51] with permission.

probe as well as sampling time.[20–22] To improve the spatial resolution, SPME coatings with lengths of 1–2 mm have been developed in contrast to commercial coatings with lengths of 10–15 mm.[20–22] For this type of study, a segmented fiber is useful because it can be customized and used to simultaneously sample adjacent tissue (Figure 1 B). For desorption purposes, the lower segment is exposed to solvent first to remove the analytes. Once the desorption of the lower segment is complete, the upper segment is desorbed using a fresh portion of solvent. In an alternative approach Loi et al. reported the use of commercial GC fiber assemblies in an in vivo spatial resolution study in which they determined the concentration of 1,8-cineole as a function of tomato stem sampling height 24 h after the chemical had been applied exogenously to soil.[23] The authors found a linear decrease in the concentration of 1,8-cineole with sampling stem height. Furthermore, the determination of floral scents and their patterns within a single flower can lead to a better understanding of pollination ecology, plant–animal relationships, and plant defense mechanisms.[27] For example, commercial SPME fiber assemblies were implemented for in vivo headspace SPME metabolomic profiling of different plant compartments (such as living flowers, leaves, and bracts) of four different *Lamium* (deadnettle) species as well as the intercompartmental metabolome composition of grapefruit at different developmental stages.[26,27] In one of the *Lamium* species examined, different compartments were distinguished by differing levels of monoterpenes, namely α- and β-pinene. The authors were also able to determine differential metabolites responsible for variability between species.[26]

It is also important to note that acceptable temporal and spatial resolution using in vivo SPME may not be achievable simultaneously, and appropriate experimental design should be chosen depending on the specific goals of a given study. For in vivo SPME with good spatial resolution, miniaturized probes are required; this in turn necessitates longer sampling times to ensure that a sufficient amount of analyte is extracted for detection by the analytical instrument. Thus, sampling times of 20–30 min are usually employed in contrast to sampling times of 0.5–5 min typically employed in temporal studies.[20–22,49] However, extended sampling times can average the effects of concentration change through diffusion, and thus the benefits of improved spatial resolution through fiber miniaturization are lost. Careful experimental design is necessary to ensure a successful space-resolved in vivo SPME experiment.

5. Comparison of SPME to Traditional Methods for Global Metabolomics Studies

5.1. Comparison of SPME to Solvent Precipitation and Ultrafiltration

The performance of SPME as a sample preparation method was compared to that of ultrafiltration and solvent precipitation in both in vitro (human plasma) and in vivo experiments (mouse circulating blood).[41,54] In general, SPME provided lower overall metabolite coverage (Table 2), except when detection by negative ESI in combination with reversed-phase LC–MS was used; in this case, SPME with long extraction times improved coverage by approximately 50%. If one examines the metabolite coverage in more detail, SPME gives significantly better coverage of hydrophobic species in comparison to ultrafiltration as shown by the increased number of metabolites with retention time >10 min (Figure 8). As such, the two techniques can be regarded as complementary in nature where ultrafiltration is ideally coupled to HILIC–MS (HILIC: hydrophilic interaction liquid chromatography) for analysis of polar metabolites, and SPME is ideally coupled to reversed-phase LC–MS for analysis of more hydrophobic analytes. On the other hand, if a balanced extraction of both hydrophilic and hydrophobic species in a single sampling is desired, in vivo SPME presents a better alternative.

Table 2: Summary of the results for the comparison of SPME, ultrafiltration (UF), plasma protein precipitation with acetonitrile (PP), and plasma protein precipitation with methanol/ethanol (PM) for the extraction of pooled human plasma sample.[54]

Method	Number of features detected		Median RSD of peak area (n=7 replicates)	
	positive-ion ESI	negative-ion ESI	positive-ion ESI	negative-ion ESI
PP	2975	2082	19	12
PM	3245	2252	12	8
UF	2686	2093	20	22
SPME (5 min)	1592	2005	16	18
SPME (overnight)	1821	3320	11	17

In terms of method precision, the performance of SPME was found to be comparable to that of both solvent precipitation and ultrafiltration, with a median RSD for SPME of 11–18% (Table 2) reflecting the good quality of the collected data. This is especially important because the sensitivity of the SPME method was considerably lower than that of traditional sample preparation methods owing to the very small amount of analyte extracted, and method precision usually deteriorates as signal strength decreases in LC–MS studies. The results shown in Table 2 are in agreement with other literature reports in which solvent precipitation or ultrafiltration is used in global metabolomics studies.[55–57] It is also interesting to note that solid-phase extraction using C18 sorbent resulted in 1500 features recorded with an ultrahigh-performance liquid chromatography (UHPLC)–MS platform;[56] this is in line with the results we observed for SPME combined with positive ESI reversed-phase liquid chromatography. However, with solid-phase extraction only 48% of the detected peaks had an acceptable RSD of ≤ 30% versus 80–92% of peaks using SPME; this underlines the better overall performance of SPME method.

One of the biggest issues in quantitative analysis by LC–MS is ionization suppression owing to the presence of co-eluting matrix components. For metabolomics studies, this becomes especially problematic because an unselective sample preparation method is desired. Highly abundant signals can cause ionization suppression of co-eluting species due to the competitive nature of the ionization process. This can be detrimental for any type of quantitative analysis,

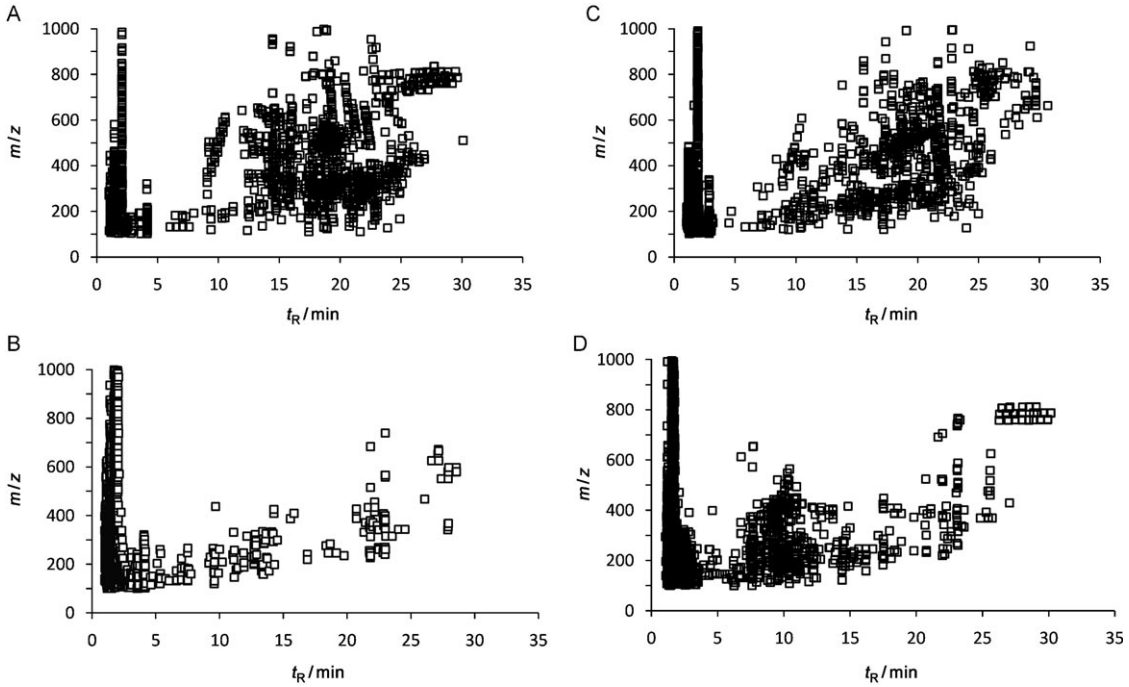

Figure 8. Ion maps (retention time versus *m/z*) comparing the metabolite coverage of in vitro SPME (A) versus that of ultrafiltration (B) of human plasma, and of in vivo SPME in circulating mouse blood (C) versus that of ultrafiltration (D) of mouse plasma.

including relative quantitation, as the observed differences between control and treatment groups may simply be caused by different sample compositions resulting in a different degree of ionization suppression effects, rather than true differences between samples for a given metabolite. SPME successfully minimizes ionization suppression because only a small proportion of metabolite is extracted. In addition, highly abundant polar metabolites usually have very low extraction efficiencies in SPME further minimizing the potential for ionization suppression throughout the entire chromatographic analysis. A detailed investigation of absolute matrix effects was carried out for SPME after extraction of human plasma in order to evaluate the extent of ionization suppression.[54] Briefly, the human plasma extract obtained by SPME was spiked post-extraction with a known concentration of selected metabolites which eluted throughout the entire chromatographic space in both reversed-phase and HILIC–MS methods. The results were compared against a standard containing the metabolites at the same concentration, after subtraction of any endogenous levels of the same metabolites in the plasma samples. Only the region of elution of the anticoagulant sodium citrate was found to be prone to ionization suppression in reversed-phase method, while with the HILIC method $<20\,\%$ of metabolites studied exhibited significant matrix effects.

5.2. Comparison of SPME to Microdialysis

Both in vivo SPME and microdialysis allow for monitoring analytes in awake and/or freely moving animals. For the targeted quantitative analysis of selected pesticides in jade plant, the performance of in vivo SPME was shown to be equivalent to that of microdialysis, although some loss of pesticides to the membrane was observed for microdialysis sampling.[24] Unfortunately, limited data currently exists regarding the use of microdialysis in metabolomics. Recently, Wibom et al. showed the utility of the technique to intracranially sample extracellular fluid from glioblastoma patients.[58] With this stereotactic microdialysis approach, the authors were able to detect 151 metabolites after GC–MS analysis and find distinct metabolic differences between tumor and tumor-adjacent regions of the brain. Similar studies combining microdialysis with LC–MS have not yet been reported, perhaps indicating the difficulties in coupling this technique to LC–MS because of the severe ionization suppression caused by the salt-containing buffers typically used in microdialysis. Furthermore, considering microdialysis is a membrane-based technique, it is likely that its performance is analogous to that of ultrafiltration, in which case a severe loss of hydrophobic species can be anticipated in untargeted metabolomics studies as demonstrated by our results shown in Figure 8[41,54] and supported by microdialysis literature.[59] In addition, dialysis is more damaging to the living system since microdialysis probes are much larger than SPME fiber devices and offer poorer spatial resolution than that obtained with space-resolved SPME.[20,21] For example, a typical microdialysis probe employed in brain studies is 15 mm long with an outer diameter of 200–500 μm;[60] in contrast, the fibers used in space-resolved SPME are 1–2 mm long with typical diameters of less than 200 μm. On the other hand, microdialysis is useful for continuous monitoring of metabolites in almost real time, especially when it is coupled to analytical instrumentation online. Consequently micro-

dialysis is better suited for short-term studies requiring a high degree of temporal resolution, for example to monitor very fast processes directly in vivo.

6. Summary and Outlook

In vivo SPME is a powerful new tool for metabolomics studies because it has a rapid, minimum-step workflow and incorporates a metabolism-quenching step directly during sampling. The precision achievable with this technique is comparable to that of traditional methods, and information on biologically important unbound metabolites is obtained. Recent data demonstrates that in vivo SPME can be extended to the sampling of biological fluids in an untargeted metabolomic workflow where it can play an important role in capturing metabolites with fast turnover rates and/or reactive metabolites. Further work in this area could explore the trapping of known reactive metabolites and intermediates by using glutathione or methoxylamine trapping agents, for example.[61,62] In addition, this metabolomic approach can also be extended to include tissue sampling where in vivo SPME can play an important role as a less-invasive alternative to biopsy sampling. Also, in vivo SPME in either headspace or direct modes has the potential of providing insight into other areas such as understanding of secondary metabolism, assessing the quality of food commodities, understanding the unintended effects of genetically modified foods and crops, understanding the genotype–environment interaction, and deducing factors responsible for variations in the content of nutritionally relevant metabolites. Additional miniaturization of SPME devices can further improve spatial resolution which is exceedingly important for the studies of heterogeneous samples, and also facilitate new areas for exploration such as single-cell studies. In summary, because of the convenient format and versatility, we envision that in vivo SPME can play an important role as a sample preparation tool to facilitate the elucidation of chemical processes in living systems.

Received: November 3, 2010
Published online: May 23, 2011

[1] J. Pawliszyn in *Solid Phase Microextraction, Theory and Practice*, Wiley-VCH, Wiley, **1997**.
[2] W. J. Griffiths, T. Koal, Y. Wang, M. Kohl, D. P. Enot, H.-P. Deigner, *Angew. Chem.* **2010**, *122*, 5554 – 5575; *Angew. Chem. Int. Ed.* **2010**, *49*, 5426 – 5445.
[3] S. J. Bruce, I. Tavazzi, V. Parisod, S. Rezzi, S. Kochhar, P. A. Guy, *Anal. Chem.* **2009**, *81*, 3285 – 3296.
[4] S. Moco, J. Vervoort, S. Moco, R. J. Bino, R. C. H. De Vos, R. Bino, *TrAC Trends Anal. Chem.* **2007**, *26*, 855 – 866.
[5] O. Teahan, S. Gamble, E. Holmes, J. Waxman, J. K. Nicholson, C. Bevan, H. C. Keun, *Anal. Chem.* **2006**, *78*, 4307 – 4318.
[6] A. B. Canelas, A. Ten Pierick, C. Ras, R. M. Seifar, J. C. Van Dam, W. M. Van Gulik, J. J. Heijnen, *Anal. Chem.* **2009**, *81*, 7379 – 7389.
[7] F. Augusto, A. Luiz Pires Valente, *TrAC Trends Anal. Chem.* **2002**, *21*, 428 – 438.
[8] H. K. Kim, R. Verpoorte, *Phytochem. Anal.* **2010**, *21*, 4 – 13.
[9] H. Verhoeven, T. Beuerle, W. Schwab, *Chromatographia* **1997**, *46*, 63 – 66.
[10] D. Vuckovic, X. Zhang, E. Cudjoe, J. Pawliszyn, *J. Chromatogr. A* **2010**, *1217*, 4041 – 4060.
[11] J. Pawliszyn, *Anal. Chem.* **2003**, *75*, 2543 – 2558.
[12] L. S. De Jager, G. A. Perfetti, G. W. Diachenko, *J. Chromatogr. A* **2008**, *1192*, 36 – 40.
[13] S. Risticevic, D. Vuckovic, J. Pawliszyn, in *Biophysico-chemical Processes of Anthropogenic Organic Compounds in Environmental Systems*, (Eds: B. Xing, N. Senesi and P. M. Huang), Wiley, **2010**, in press.
[14] X. Zhang, A. Es-Haghi, F. M. Musteata, G. Ouyang, J. Pawliszyn, *Anal. Chem.* **2007**, *79*, 4507 – 4513.
[15] F. M. Musteata, M. L. Musteata, J. Pawliszyn, *Clin. Chem.* **2006**, *52*, 708 – 715.
[16] H. L. Lord, R. P. Grant, M. Walles, B. Incledon, B. Fahie, J. B. Pawliszyn, *Anal. Chem.* **2003**, *75*, 5103 – 5115.
[17] A. Es-haghi, X. Zhang, F. M. Musteata, H. Bagheri, J. Pawliszyn, *Analyst* **2007**, *132*, 672 – 678.
[18] F. M. Musteata, I. de Lannoy, B. Gien, J. Pawliszyn, *J. Pharm. Biomed. Anal.* **2008**, *47*, 907 – 912.
[19] D. Vuckovic, B. Gien, I. de Lannoy, F. M. Musteata, R. Shirey, L. Sidisky, J. Pawliszyn, *J. Chromatogr. A* **2011**, *1218*, 3367 – 3375.
[20] X. Zhang, K. D. Oakes, S. Cui, L. Bragg, M. R. Servos, J. Pawliszyn, *Environ. Sci. Technol.* **2010**, *44*, 3417 – 3422.
[21] X. Zhang, J. Cai, K. D. Oakes, F. Breton, M. R. Servos, J. Pawliszyn, *Anal. Chem.* **2009**, *81*, 7349 – 7356.
[22] S. N. Zhou, K. D. Oakes, M. R. Servos, J. Pawliszyn, *Environ. Sci. Technol.* **2008**, *42*, 6073 – 6079.
[23] R. X. Loi, M. C. Solar, J. D. Weidenhamer, *J. Chem. Ecol.* **2008**, *34*, 70 – 75.
[24] S. N. Zhou, G. Ouyang, J. Pawliszyn, *J. Chromatogr. A* **2008**, *1196–1197*, 46 – 56.
[25] H. L. Lord, M. Möder, P. Popp, J. B. Pawliszyn, *Analyst* **2004**, *129*, 107 – 108.
[26] G. Flamini, P. L. Cioni, I. Morelli, *Food Chem.* **2005**, *91*, 63 – 68.
[27] G. Flamini, P. L. Cioni, *Food Chem.* **2010**, *120*, 984 – 992.
[28] D. Zimmermann, M. Hartmann, M. P. Moyer, J. Nolte, J. I. Baumbach, *Metabolomics* **2007**, *3*, 13 – 17.
[29] B. Buszewski, A. Ulanowska, T. Ligor, M. Jackowski, E. Kłodzińska, J. Szeliga, *J. Chromatogr. B* **2008**, *868*, 88 – 94.
[30] X. Chen, F. Xu, Y. Wang, Y. Pan, D. Lu, P. Wang, K. Ying, E. Chen, W. Zhang, *Cancer* **2007**, *110*, 835 – 844.
[31] W. Miekisch, P. Fuchs, S. Kamysek, C. Neumann, J. K. Schubert, *Clin. Chim. Acta* **2008**, *395*, 32 – 37.
[32] F. Fernandes, D. M. Pereira, P. Guedes de Pinho, P. Valentão, J. A. Pereira, A. Bento, P. B. Andrade, *Food Chem.* **2010**, *119*, 1681 – 1693.
[33] D. Djozan, T. Baheri, R. Farshbaf, S. Azhari, *Anal. Chim. Acta* **2005**, *554*, 197 – 201.
[34] L. Cai, J. A. Koziel, M. E. O'Neal, *J. Chromatogr. A* **2007**, *1147*, 66 – 78.
[35] W. B. Dunn, D. I. Ellis, *TrAC Trends Anal. Chem.* **2005**, *24*, 285 – 294.
[36] C. J. Bolten, P. Kiefer, F. Letisse, J. -. Portais, C. Wittmann, *Anal. Chem.* **2007**, *79*, 3843 – 3849.
[37] J. L. Griffin, R. A. Kauppinen, *J. Proteome Res.* **2007**, *6*, 498 – 505.
[38] R. t'Kindt, K. Morreel, D. Deforce, W. Boerjan, J. Van Bocxlaer, *J. Chromatogr. B* **2009**, *877*, 3572 – 3580.
[39] A. D. Hegeman, *Briefings Funct. Genomics Proteomics* **2010**, *9*, 139 – 148.
[40] E. Róth, A. Berna, K. Beullens, S. Yarramraju, J. Lammertyn, A. Schenk, B. Nicolaï, *Postharvest Biol. Technol.* **2007**, *45*, 11 – 19.
[41] D. Vuckovic, I. de Lannoy, B. Gien, R. E. Shirey, L. M. Sidisky, S. Dutta, J. Pawliszyn, *Angew. Chem.* **2011**, DOI: 10.1002/

ange.201006715; *Angew. Chem. Int. Ed.* **2011**, DOI: 10.1002/anie.201006715.
[42] M. Gallagher, C. J. Wysocki, J. J. Leyden, A. I. Spielman, X. Sun, G. Preti, *Br. J. Dermatol.* **2008**, *159*, 780 – 791.
[43] V. Witte, L. Abrell, A. B. Attygalle, X. Wu, J. Meinwald, *Chemoecology* **2007**, *17*, 63 – 69.
[44] L. E. Hurd, F. R. Prete, T. H. Jones, T. B. Singh, J. E. Co, R. T. Portman, *J. Chem. Ecol.* **2004**, *30*, 155 – 166.
[45] M. V. Novotny, H. A. Soini, Y. Mechref, *J. Chromatogr. B* **2008**, *866*, 26 – 47.
[46] M. Coen, E. J. Want, T. A. Clayton, C. M. Rhode, S. H. Young, H. C. Keun, G. H. Cantor, A. L. Metz, D. G. Robertson, M. D. Reily, E. Holmes, J. C. Lindon, J. K. Nicholson, *J. Proteome Res.* **2009**, *8*, 5175 – 5187.
[47] H. A. Soini, K. E. Bruce, I. Klouckova, R. G. Brereton, D. J. Penn, M. V. Novotny, *Anal. Chem.* **2006**, *78*, 7161 – 7168.
[48] Y. Xu, S. J. Dixon, R. G. Brereton, H. A. Soini, M. V. Novotny, K. Trebesius, I. Bergmaier, E. Oberzaucher, K. Grammer, D. J. Penn, *Metabolomics* **2007**, *3*, 427 – 437.
[49] X. Zhang, K. Oakes, D. Luong, J. Wen, C. Metcalfe, J. Pawliszyn, M. Servos, *Anal. Chem.* **2010**, *82*, 9492 – 9499.
[50] E. Pionnier, C. Chabanet, L. Mioche, J. -. Le Quéré, C. Salles, *J. Agric. Food Chem.* **2004**, *52*, 557 – 564.
[51] J. C. Verdonk, C. H. R. De Vos, H. A. Verhoeven, M. A. Haring, A. J. Van Tunen, R. C. Schuurink, *Phytochemistry* **2003**, *62*, 997 – 1008.
[52] J. M. Cevallos-Cevallos, J. I. Reyes-De-Corcuera, E. Etxeberria, M. D. Danyluk, G. E. Rodrick, *Trends Food Sci. Technol.* **2009**, *20*, 557 – 566.
[53] B. Biais, J. W. Allwood, C. Deborde, Y. Xu, M. Maucourt, B. Beauvoit, W. B. Dunn, D. Jacob, R. Goodacre, D. Rolin, A. Moing, *Anal. Chem.* **2009**, *81*, 2884 – 2894.
[54] D. Vuckovic, J. Pawliszyn, *Anal. Chem.* **2011**, *83*, 1944 – 1954.
[55] B. Crews, W. R. Wikoff, G. J. Patti, H. -. Woo, E. Kalisiak, J. Heideker, G. Siuzdak, *Anal. Chem.* **2009**, *81*, 8538 – 8544.
[56] F. Michopoulos, L. Lai, H. Gika, G. Theodoridis, I. Wilson, *J. Proteome Res.* **2009**, *8*, 2114 – 2121.
[57] E. Zelena, W. B. Dunn, D. Broadhurst, S. Francis-McIntyre, K. M. Carroll, P. Begley, S. O'Hagan, J. D. Knowles, A. Halsall, I. D. Wilson, D. B. Kell, *Anal. Chem.* **2009**, *81*, 1357 – 1364.
[58] C. Wibom, I. Surowiec, L. Mörén, P. Bergström, M. Johansson, H. Antti, A. T. Bergenheim, *J. Proteome Res.* **2010**, *9*, 2909 – 2919.
[59] L. Groth, A. Jørgensen, *Anal. Chim. Acta* **1997**, *355*, 75 – 83.
[60] P. Nandi, S. M. Lunte in *Handbook of Sample Preparation* (Eds: J. Pawliszyn, H. L. Lord) **2010**, 103 – 123.
[61] A. Tolonen, M. Turpeinen, O. Pelkonen, *Drug Discovery Today* **2009**, *14*, 120 – 133.
[62] S. Ma, S. K. Chowdhury, K. B. Alton, *Curr. Drug Metab.* **2006**, *7*, 503 – 523.

