Optimization of Solid-Phase Microextraction Sampling for Analysis of Volatile Compounds Emitted from Oestrous Urine of Mares

Raimondas Mozūraitis[a,b,*], Vincas Būda[b,c], and Anna-Karin Borg-Karlson[a]

[a] Ecological Chemistry Group, Division of Organic Chemistry, Department of Chemistry, School of Chemistry and Engineering, Royal Institute of Technology, Teknikringen 36, SE-10044, Stockholm, Sweden. Fax: +46 8791 2333. E-mail: raimis@kth.se
[b] Laboratory of Chemical and Behavioural Ecology, Institute of Ecology, Vilnius University, Vilnius, Lithuania
[c] Centre for Ecology and Environmental Studies, Faculty of Natural Sciences, Vilnius University, Vilnius, Lithuania

* Author for correspondence and reprint requests

Z. Naturforsch. **65c**, 127–133 (2010); received August 20/September 20, 2009

The solid-phase microextraction (SPME) technique was applied and optimized for collection of volatile compounds emitted from oestrous urine of mares *Equs cabalus* L. (Perissodactyla, Equidae) for GC-MS analyses. Variables such as type of SPME fibre, collection time of volatiles, and addition of salt were optimized to improve the sampling efficiency in two aspects: extent and selectivity of absorption/adsorption of urine volatiles onto SPME fibres. The data revealed that the number of volatiles and the total amount represented as quantitative peak areas of the compounds trapped on fibres coated either with polydimethylsiloxane-divinylbenzene or with divinylbenzene-carboxen-polydimethylsiloxane were significantly higher compared to those coated with polydimethylsiloxane, polyacrylate, and carbowax-divinylbenzene. The polydimethylsiloxane-divinylbenzene-type of fibre coating was chosen for optimization of sampling time and effect of salt addition. Sampling periods lasted for 15, 30, 60, 120, and 240 min. The optimal collection time of volatiles from urine maintained at about 36 °C was 60 min, as the number of compounds detected with amounts sufficient for quantification did not differ significantly from those trapped during longer collection periods. No significant increase in total amount of volatiles trapped was registered after 120 min of sampling. Addition of 0.3 g NaCl to the 2-ml of samples shortened the collection period from 60 to 15 min during which almost all compounds were trapped. Addition of salt has a significant effect at all sampling periods taking into consideration the total amounts of volatiles trapped. The total intensities increased about 8, 5, 3, 3, and 2 times at collection periods of 15, 30, 60, 120, and 240 min, respectively, when compare with the ones obtained from the urine samples with no salt addition. In oestrous mare's urine, 139 ± 4 (average number ± standard deviation) volatile compounds suitable for quantitative analyses were detected compared to 45 compounds collected by the gas-tight syringe method.

Key words: Urine Volatiles, Headspace, *Equs cabalus*

Introduction

Urinary metabolites of vertebrates provide pertinent information about sexual and dominant status, health and body condition, quality of diet etc. Moreover, it is an energetically least expensive and efficient way to transfer chemical signals from one individual to another (Müller-Schwarze, 2006). Behavioural observations indicated that horses use urine volatiles to acquire biologically relevant information (Kiley-Worthington, 1987). In addition to dung piles, stallions urinate to mark their territorial borders (Klingel, 1975). Chemical signals are important for the detection of mares' reproduction status. It is assumed that the odour of a ready-to-mate mare is perceptible to a stallion from over half a kilometre (Ainslie and Ledbetter, 1980), implying that volatile sex pheromones are involved. Moreover, smearing of oestrous mare's urine onto castrated males, or non-oestrous females, elicits the mounting response and copulation by the stallions, which have previously shown no interest to unsmeared individuals (Hafez and Wierzbowski, 1961). This observation suggests that urine collected from a mare during the oestrous stage contains chemical signal(s) for the stallion.

The long-lasting goal of our project is to identify volatile chemical markers which correlate with the reproductive stage of a mare and allow rapid

0939–5075/2010/0100–0127 $ 06.00

and precise determination of the ovulation time. To achieve this goal numerous urine samples are planed to be analyze by gas chromatography-mass spectrometry (GC-MS). Surveys have shown that more than 80% of the analysis time is spent on sample collection and sample preparation. This is necessary because in most cases analytical instruments cannot handle the sample matrices directly (Vas and Vékey, 2004). Few techniques are available to collect volatiles from urine samples including the most recently developed solid-phase microextraction (SPME). The number of variables such as type of SPME fibre, collection time of volatiles, and ionic strength influence the efficiency of sampling (Pawliszyn, 1997) by SPME.

In this work we present data on evaluation and optimization of SPME as a tool for stationary headspace sampling of volatile compounds emitted from urine of mares.

Material and Methods

Urine collection

Four mares were observed for two natural oestrous cycles at stud-farm Augustinų dvaras, Ukmergė district, Lithuania. Urine samples were collected from each individual during the oestrous period when the mare was receptive to a stallion. The precise time of ovulation was determined by means of ultrasound technique. After collection, the urine was poured into glass jars, and samples were placed in a freezer at −20 °C. Samples selected for analysis were placed on dry ice in a thermostat box and transported to the analytical laboratory.

Analysis of volatiles

Urine samples collected one day before ovulation were used in the experiments. A stem mixture of urine was formed by combination of defrosted urine samples from 4 mares. 2 ml of urine from the stem mixture were transferred to a 10-ml bottle, and the bottle was covered with an aluminium foil. The sample was preheated to about 36 °C in a water bath for 15 min. The SPME technique was used for collecting compounds released from the urine samples. Before the collection periods, the routine conditioning of the SPME fibre was done at 225 °C for about 10 min in a GC injector until the purity of the fibre was satisfactory. Afterwards, the needle of a syringe was pierced through the aluminium foil and then the cleaned fibre was pushed out from the needle and placed a few mm above the urine sample. The sorption was conducted at 36 °C for certain periods of time. The analyses were performed by using a GC-MS system, including a Varian 3400 gas chromatograph and Finnigan SSQ 7000 mass spectrometer. A DB-wax silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) was used with a temperature programme of 40 °C (2 min), increased by 5 °C/min to 230 °C, and thereafter maintained constant at 230 °C for 10 min. The split/splitless injector temperature was 225 °C and the splitless period lasted for 60 s. Helium was used as the carrier gas with an inlet pressure of 70 kPa. Electron ionization mass spectra were determined at 70 eV with the ion source at 150 °C. When the sample had been injected, the desorption of the volatiles from the SPME fibre was checked by subsequent injection into a split/splitless injector of a second gas chromatograph for 60 s of splitless time and an injector temperature of 225 °C.

The data concerning volatiles collected from urine samples from different experiments were compared by calculating the peak areas from total ion chromatograms (TIC) using X-calibur programme package version 3.1 (Thermo-Finnigan).

Tests for trapping efficiency of SPME fibre coatings

Silica fibres (Supelco, Sigma-Aldrich group, St. Louis, MO, USA) coated with polydimethylsiloxane-divinylbenzene (PD), divinylbenzene-carboxen-polydimethylsiloxane (DCP), carboxen-polydimethylsiloxane (CP), carbowax-divinylbenzene (CD), polyacrylate (PA), and polydimethylsiloxane (PS) were used to collect the volatiles. Sampling periods lasted for 120 min to trap as many volatiles as possible.

Influence of sampling time and salt addition on number and total amount of compounds absorbed in SPME fibre

For evaluating the influence of sampling time on the trapping of urine volatiles the most efficient fibres coated with PD were used. Sampling periods lasted for 15, 30, 60, 120, and 240 min. To estimate the effect of ionic strength on the sampling efficiency, a saturating amount, *i.e.* 0.3 g, of NaCl was added to 2 ml of urine. Four replicates of every sample type were obtained.

Statistical analysis

The data were analyzed by Mann-Whitney U-test (Sokal and Rohlf, 1995) using the computer programme package Statistica. Significantly different values were obtained at the $P \leq 0.05$ level and indicated with different characters.

Results

Gas chromatographic conditions

The condition of gas chromatography (GC) were adjusted to achieve good separation of peaks within a reasonable analysis time, which lasted 50 min. All the injected volatiles were eluted from the column after 40 min. In addition, 10 min were required to keep the column isothermal at 230 °C to elute impurities derived from SPME fibre consequently, *i.e.* the total analysis time lasted 50 min.

After the sample had been injected, desorption of volatiles from the SPME fibre was checked by subsequent injection into a split/splitless injector of a second gas chromatograph for 60 s of splitless time and an injector temperature of 225 °C. It was found that total desorption of the compounds had occurred during the first injection.

Effect of SPME fibre coatings on trapping efficiency

Fibre types coated with PD and DCP adsorbed/absorbed (132 ± 5) and (139 ± 4) compounds, respectively, and were found to be significantly more efficient than other commercially available fibre types we have tested (Table I). In addition, the total amounts represented as quantitative peak areas of the compounds trapped were significantly higher on PD- and DCP-coated fibres compared to the PA-, PS-, CD- and CP-coated ones (Table I). PS and PA fibres preferably absorbed the non-polar compounds of higher molecular weight and more polar compounds, respectively, while fibres coated with CP were more selective for lower-molecular weight compounds (Fig. 1). Preliminary analyses of chromatographic patterns of urine volatiles obtained from mares of different reproduction statuses indicated, that differences between the samples were determined by compounds which were better trapped by fibres coated with PD; consequently the fibres of this type were selected for further testing.

Effects of sampling time and salt addition on number of compounds adsorbed on SPME fibre

No significant increase in the number of compounds adsorbed on fibres coated with PD was obtained after 60 min or longer sampling intervals of urine volatiles. 15- and 30-min collections resulted in (105 ± 4) and (113 ± 3) compounds, respectively, and these values differed significantly from the ones of longer sampling intervals (Fig. 2). In conclusion, 60 min was determined as optimal sampling period with respect to the number of quantitative peaks.

Addition of a saturating amount (0.3 g) of NaCl to 2 ml of urine allowed a significant reduction of sampling duration. Almost all compounds in quantitative amounts were detected after the shortest, *i.e.* 15-min sampling period, and duration of the optimal collection was reduced 4 times when compare to the 60-min sampling of urine volatiles without salt (Fig. 2).

Table I. Effect of SPME fibre coatings on trapping efficiency of urinary volatiles.

Type of fibre coating	Total amount[a] (mean ± SD)	Number of quantitative peaks (mean ± SD)
Polydimethylsiloxane-divinylbenzene (PD)	46[b] ± 3.7 a	132 ± 5 a
Divinylbenzene-carboxen-polydimethylsiloxane (DCP)	41 ± 6.4 ab	139 ± 4 a
Carboxen-polydimethylsiloxane (CP)	37 ± 3.3 bc	123 ± 5 b
Carbowax-divinylbenzene (CD)	31 ± 2.1 c	94 ± 6 c
Polyacrylate (PA)	2.8 ± 0.2 d	26 ± 4 d
Polydimethylsiloxane (PS)	2.9 ± 0.2 d	23 ± 3 d

[a] Sum of quantitative peak areas in the range of 3.5–40 min.
[b] "46" is the number of counts related to the abundance of the ions formed and corresponds to the amount of compounds registered at a selected time period; it has to be read as $46 \cdot 10^6$.

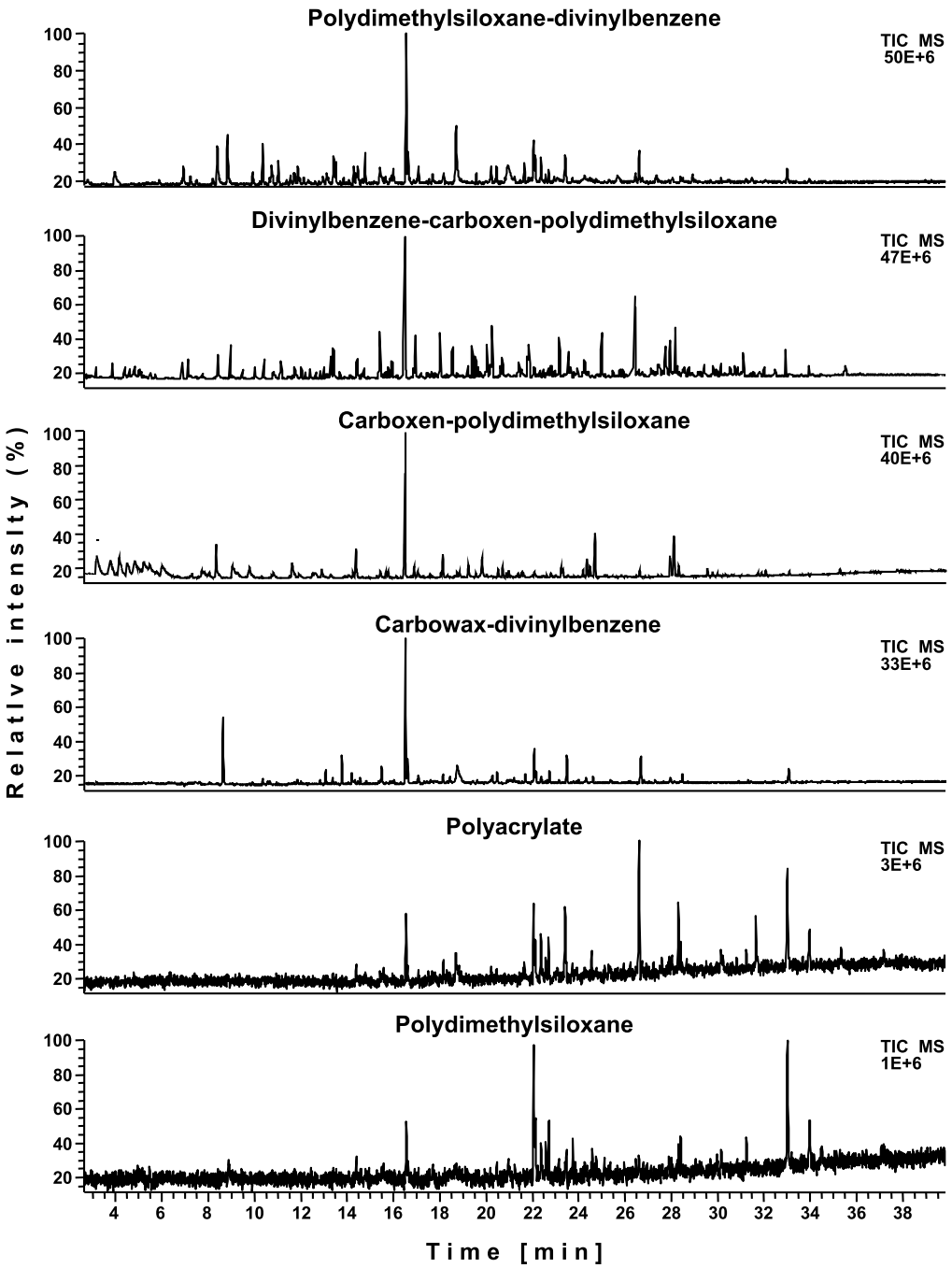

Fig. 1. Chromatographic patterns of urinary volatiles collected on six SPME fibre coatings. "TIC MS" represents total ion mass chromatograms at the range of 30–400 *m/z* obtained after a 2-h sampling period; DB-wax fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness); "50E+6" is the number of counts related to the abundance of the ions formed and corresponds to the amount of compounds registered at a selected time period; it has to be read as $50 \cdot 10^6$.

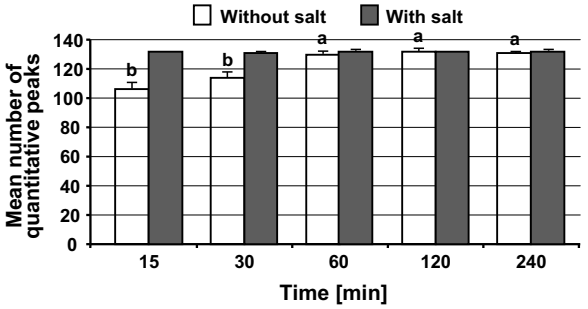

Fig. 2. Effects of sampling time and salt addition on the number of compounds adsorbed on the SPME fibre coated with polydimethylsiloxane-divinylbenzene in amounts suitable for quantitative analyses. Values represented by the columns filled with the same pattern and indicated by the same letter are not significantly different according to Mann-Whitney U-test, $P \leq 0.05$; vertical bars denote standard deviations; $n = 4$; * indicates that differences between the values obtained from samples without and with addition of salt are significant.

Influence of sampling time and salt addition on recorded amounts of urinary compounds adsorbed on SPME fibre

The longer collection periods of urine volatiles, from the samples with no and with extra salt added, resulted in an increase of the total amount of volatile compounds trapped. The minimal duration of sampling, when an increase in absorption was not significant, has been determined as 120 and 60 min in the tests without and with addition of salt, respectively (Fig. 3). In addition, an ionic strength effect was significantly pronounced at all sampling periods. It increased the total amount of volatiles trapped about 8, 5, 3, 3, and 2 times at collection periods of 15, 30, 60, 120, and 240 min, respectively, when compare with the ones obtained from the urine samples with no extra addition of salt.

Discussion

There are many variants of a sample-released airborne volatiles collection or entrainment technique (Golub and Weatherston, 1984; Heath *et al.*, 1989; Borg-Karlson, 1990), but despite many variations, the basic principle of volatiles collection is the same: the compounds are absorbed/adsorbed via an air stream (dynamic headspace) or passively (static headspace) on porous polymers, such as Porapak Q, Tenax GC, or active charcoal. After collection, the volatiles are desorbed from the polymers by heating or by rinsing the polymers with organic solvents (Borg-Karlson, 1990). Over 15 years ago, the SPME technique was developed and made commercially available (Belardi and Pawliszyn, 1989; Zhang and Pawliszyn, 1993). The advantage of SPME is that it integrates sampling, extraction, concentration and introduction processes into a single solvent-free step. This makes the procedure simple, quick, sensitive, and efficient (Vas and Vékey, 2004); unfortunately, the method needs more optimization efforts compared with other techniques as the number of variables such as type of SPME fibre, collection time of volatiles, ionic strength, and temperature influence the efficiency of sampling (Pawliszyn, 1997).

Numerous of SPME fibre types with respect to the coating material are commercially available for manual injection into a gas chromatograph (http://www.sigmaaldrich.com). The selectivity of fibres to certain chemicals is determined by polarity and thickness of a coating (Pawliszyn, 1997). We have tested six types of SPME fibres which provide capacity for collection of a wide range of volatile and semi-volatile compounds. Our data revealed that fibres covered with a mix of coatings including divinylbenzene blended with non-polar polydimethylsiloxane and porous particles of carboxen as well as a binary mix of polydimethylsil-

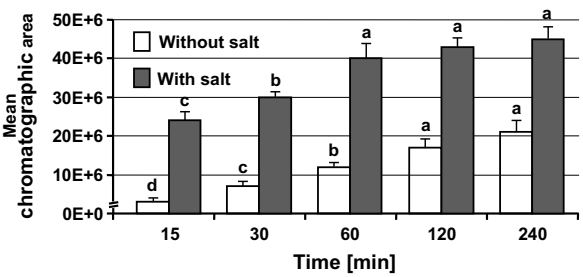

Fig. 3. Effects of sampling time and salt addition on the amount of urinary volatiles adsorbed on the SPME fibre coated with polydimethylsiloxane-divinylbenzene. Values represented by the columns filled with the same pattern and indicated by the same letter are not significantly different according to Mann-Whitney U-test, $P \leq 0.05$; vertical bars denote standard deviations; $n = 4$; differences between all values obtained from samples without and with addition of salt are significant; "50E+6" is the number of counts related to the abundance of the ions formed and corresponds to the amount of compounds registered at a selected time period; it has to be read as $50 \cdot 10^6$.

oxane and divinylbenzene were the most efficient ones of the types of fibre coatings we have tested for trapping efficiency of a wide range of volatiles released from urine of oestrous mares.

As far as we know there are no data published dealing with the application of SPME in analysis of horse urine volatiles. Literature data concerning the effect of SPME fibre coatings on the trapping efficiency of volatiles from mammals including human urine revealed that carboxen-polydimethylsiloxane coating due to the pore size of 2–20 Å and its high porosity which provides a large surface area is ideal for trapping small molecules (Mills and Walker, 2000, 2001). These fibres are highly sensitive to volatile acids and alcohols (with chain lengths < C_8) (10 ppt–1 ppm), C_2 – C_8 aldehydes (1–500 ppb), and C_3 – C_9 ketones (5 ppb–1 ppm) (Scheppers and Wercinski, 1999; Abalos *et al.*, 2000). Urine is a complex matrix, and in addition to many low-molecular weight volatile compounds, less volatile compounds could be trapped from urine headspace by the SPME method when an appropriate fibre type is chosen and the experimental conditions are adjusted. Porous particles of divinylbenzene have large pores compared to carboxen and blending with polydimethylsiloxane improves their sensitivity for larger volatile compounds (Mills and Walker, 2000, 2001).

The SPME method requires that equilibrium is reached between the fibre and head-space, thus the time of sampling affects the sensitivity of the method and is crucial for good reproducibility (Zhang and Pawliszyn, 1993). Based on the data presented in Fig. 2 and 3 which show the number and amounts of volatiles sampled as a function of time, we selected the sampling time of 60 min, without addition of salt to urine, as a compromise between sensitivity and duration of sampling. However, the sampling period could be shortened to 30 min with no loss of number of compounds suitable for quantification by addition of saturating amounts of NaCl into urine when conducting large analytical series. In that way a 30 min shorter sampling period could be used for equilibration of the headspace before sampling.

Our data demonstrated that over 140 compounds were trapped from the headspace of oestrous mares' urine by the SPME technique compare to 45 compounds collected by the gas-tight syringe method (Ma and Klemm, 1997). In conclusion, SPME proved to be a sensitive, rapid, reliable, and economic sample preparation technique prior to GC-MS analysis. Fibres covered with PD as well as DCP could be used to detect and identify urinary odours from mares and to determine oestrous-specific compounds, as well as their dynamics with respect to the ovulation period under optimized experimental conditions.

Acknowledgements

This study was supported by the grants T-19/04 and T-74/08 from Lithuanian State Science and Studies Foundation, by the grant CTS08:265 from Carl Tryggers Found, Sweden, and by a grant from the Swedish Institute via the Visby program to the Nordic-Baltic network in Ecological Chemistry. We thank Dr. Jonas Kutra, Institute of Animal Science, Lithuanian Veterinary Academy, for determination of the reproductive status of mares and assistance in collection of samples.

Abalos M., Bayon J. M., and Pawliszyn J. (2000), Development of a headspace solid-phase microextraction procedure for the determination of free volatile fatty acids in waste waters. J. Chromatogr. A **873**, 107–115.

Ainslie T. and Ledbetter B. (1980), The Body Language of Horses. William Morrow, New York, USA.

Belardi R. P. and Pawliszyn J. B. (1989), The application of chemically modified fused silica fibers in the extraction of organics from water matrix samples and their rapid transfer to capillary columns. Water Pollut. Res. J. Can. **24**, 179–191.

Borg-Karlson A.-K. (1990), Chemical and ecological studies of pollination in the genus *Ophrys* L. (Orchidaceae). Phytochemistry **29**, 1359–1387.

Golub M. A. and Weatherston I. (1984), Techniques for extracting and collecting sex pheromones from live insects and from artificial sources. In: Techniques in Pheromone Research (Hummel H. E. and Miller T. A., eds.). Springer-Verlag, Berlin, Germany, pp. 223–285.

Hafez E. S. E. and Wierzbowski S. (1961), Analysis of copulatory reflexes in the stallion. In: Fourth International Congress of Animal Reproduction, The Hague, Netherlands.

Heath R. R., Landolt P. L., Tumlinson J. H., and Calkins C. O. (1989), A system to collect volatile chemicals emitted by insects. J. Chem. Ecol. **15**, 1121–1130.

Kiley-Worthington M. (1987), The Behaviour of Horses. Allen JA, London, UK.

Klingel H. (1975), Social organization and reproduction in equids. J. Reprod. Fertil. Suppl. **23**, 7–11.
Ma W. and Klemm W. R. (1997), Variation of equine urinary volatile compounds during the oestrous cycle. Vet. Res. Commun. **21**, 437–446.
Mills G. A. and Walker V. (2000), Headspace solid-phase microextraction procedures for gas chromatographic analysis of biological fluids and materials. J. Chromatogr. A **902**, 267–287.
Mills G. A. and Walker V. (2001), Headspace solid-phase microextraction profiling of volatile compounds in urine: application to metabolic investigations. J. Chromatogr. B **753**, 259–268.
Müller-Schwarze D. (2006), Chemical Ecology of Vertebrates. Cambridge University Press, Cambridge, UK.
Pawliszyn J. (1997), Solid-Phase Microextraction: Theory and Practice. VCH, New York, USA.
Scheppers S. and Wercinski C. (1999), Solid Phase Microextraction – A Practical Guide. Marcel Dekker, New York, USA.
Sokal R. R. and Rohlf F. J. (1995), Biometry. Freeman and Co, New York, USA.
Vas G. and Vékey K. (2004), Solid-phase microextraction: a powerful sample preparation tool prior to mass spectrometric analysis. J. Mass. Spectrom. **39**, 233–254.
Zhang Z. and Pawliszyn J. (1993), Headspace solid phase microextraction. Anal. Chem. **65**, 1843–1852.