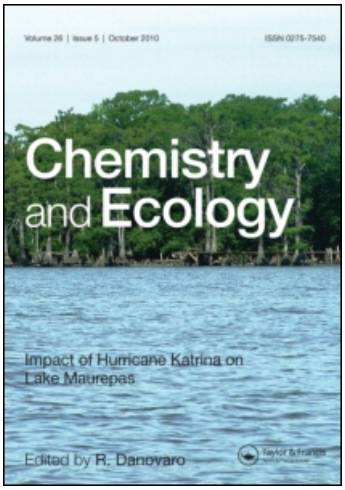

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

Solid-Phase Microextraction As A Tool for Studying Volatile Compounds in Frog Skin

Benjamin P. Smith[abc]; Claudia Alcaraz Zini[de]; Janusz Pawliszyn[d]; Michael J. Tyler[a]; Yoji Hayasaka[f]; Brian Williams[b]; Elina Bastos Caramao[e]
[a] Department of Environmental Biology, University of Adelaide, South Australia, Australia [b] Department of Soil and Water, University of Adelaide, South Australia, Australia [c] Department of Soil and Water, University of Adelaide, Adelaide, South Australia, Australia [d] Department of Chemistry, University of Waterloo, Ontario, Canada [e] Instituto de Química, Universidade Federal Do Rio Grande Do Sul, Brazil [f] Australian Wine Research Institute, South Australia, Australia

To cite this Article Smith, Benjamin P. , Zini, Claudia Alcaraz , Pawliszyn, Janusz , Tyler, Michael J. , Hayasaka, Yoji , Williams, Brian and Caramao, Elina Bastos(2000) 'Solid-Phase Microextraction As A Tool for Studying Volatile Compounds in Frog Skin', Chemistry and Ecology, 17: 3, 215 — 225
To link to this Article: DOI: 10.1080/02757540008037674
URL: http://dx.doi.org/10.1080/02757540008037674

Chemistry and Ecology, 2000, Vol. 17, pp. 215–225
Reprints available directly from the publisher
Photocopying permitted by license only

SOLID-PHASE MICROEXTRACTION AS A TOOL FOR STUDYING VOLATILE COMPOUNDS IN FROG SKIN

BENJAMIN P. SMITH[a,e,*], CLAUDIA ALCARAZ ZINI[b,c], JANUSZ PAWLISZYN[b], MICHAEL J. TYLER[a], YOJI HAYASAKA[d], BRIAN WILLIAMS[e] and ELINA BASTOS CARAMAO[c]

[a]*Department of Environmental Biology, University of Adelaide, South Australia, Australia;* [b]*Department of Chemistry, University of Waterloo, Ontario, Canada;* [c]*Instituto de Química, Universidade Federal Do Rio Grande Do Sul, Brazil;* [d]*Australian Wine Research Institute, South Australia, Australia;* [e]*Department of Soil and Water, University of Adelaide, South Australia, Australia*

(Received 23 June 2000; In final form 3 August 2000)

Solid-phase microextraction (SPME) is an effective technique for studying frog volatile secretions. Its primary advantage is in its application to sampling live animals. The ability to sample an organism over an extended period allows changes in an individual's chemical signature to be determined. The presence of eucalyptol in the skin secretion of Ewing's tree frog, *Litoria ewingi*, was used to assess the effectiveness of SPME in sampling frog volatiles. Rapid sample times coupled with the polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibre provided the best signal/noise ratio for the majority of frog volatiles analysed, and importantly resulted in the least amount of stress to the animals involved.

Keywords: Solid-phase microextraction; Frog volatile secretions

*Address for correspondence: Department of Soil and Water, University of Adelaide, Adelaide, South Australia, Australia 5005. Tel.: +618 8303 7385, Fax: +618 8303 4364, e-mail: benjamin.smith@adelaide.edu.au

INTRODUCTION

Frogs produce a remarkable array of noxious, distasteful and highly toxic compounds, which on secretion from so-called granular skin glands can serve to protect the amphibian from predators (Daly, 1998). Many of these secretions are odorous and the question has been raised as to the role olfactory signals may play in a frog's defence strategy (Williams *et al.*, 2000). Despite the interest in the effect of volatile defence secretions on amphibian behaviour, few studies have sought to determine the components responsible for the odour. A primary reason for this lies in the difficulty of sampling volatile compounds and their subsequent chemical identification. The destructive nature of available sampling techniques is also a concern, particularly in light of the world's declining frog population (Tyler, 1991).

Solvent extraction and volatile trapping are the two established techniques for sampling semiochemicals (Jones and Oldham, 1999). Solvent extraction relies on the transfer of compounds to a liquid medium based on their degree of solubility. Samples may be extracted by dipping, soaking, homogenising in solvent, or by continuous extraction in a soxhlet extractor. This method is destructive and limited as it reveals the secretion present in the gland at the time of death and cannot be used to examine the changes in the chemical signature of individuals over extended periods (Turillazzi *et al.*, 1998). Furthermore, it has been shown that in insects the ratios of volatiles obtained by this technique frequently do not correspond to the ratio emitted by the live animal (Golub and Weatherston, 1984). In addition, large solvent peaks can obscure rapidly-eluted components and, if the sample is concentrated before chromatography, components more volatile than the solvent can be partially or completely lost. Disintegration of the tissue sample may also result in unwanted reactions occurring between compounds which ordinarily would not come into contact (Bostock and Stermer, 1989; Blight, 1990; Tollsten and Bergström, 1998). For example, Tollsten and Bergström (1998) showed that intact plants of a number of *Brassica* species released a different blend of volatile chemicals compared to macerated plant material.

Volatile trapping is more amenable to sample living organisms (Golub and Weatherston, 1984; Jones and Oldham, 1999). Routinely,

dynamic analysis is employed (Jones and Oldham, 1999), although static headspace analysis is used occasionally (Jakobsen, 1997). The technique involves drawing purified air over the sample and the volatiles caught in the air stream are collected by sorbent trapping on an adsorbent matrix or by cryogenic trapping. Semiochemicals can then be removed by solvent washing, soxhlet extraction (Finnegan and Chambers, 1993), or thermal desorption (Cossé *et al.*, 1995; Agelopoulos and Pickett, 1998). Such techniques, however, are labour intensive and time consuming and not usually suitable for frequent sampling (Malosse *et al.*, 1995). Solvent removal of the trapped analytes from the matrix would encounter similar problems experienced in liquid extractions, whilst the equipment used for thermal desorption is costly. Analysis of volatiles in live frogs introduces an additional problem in that the air flowing across the animal must be kept moist to minimise desiccation, a factor that is particularly important if long sampling times are employed. Additional traps to remove the moisture prior to the introduction of the sample to the instrumention provide other sites for analyte loss, especially if the compounds of interest are low in concentration. Water molecules may also compete for sites on the adsorbent trap reducing the concentration of volatiles collected (Koziel *et al.*, 1999).

As part of our studies into the chemistry of the volatiles emitted from frogs we have investigated the use of solid phase microextraction (SPME) as an alternative to conventional sampling methods. SPME is a relatively new isolation method that has been applied to a wide range of volatile and semi-volatile organic compounds from various matrices such as air, water, and soil (Zhang and Pawliszyn, 1993). The technique has found applications in food, pesticide, and environmental fields (Pawliszyn and Smith, 1999), and has received recent attention for the sampling of insect pheromones (Malosse *et al.*, 1995; Mozuraitis, 1996; Moneti *et al.*, 1997; Monnin *et al.*, 1998). Such studies have focussed on 'contact' SPME, whereby the extracting fibre is rubbed over the exposed pheromone gland or cuticle (Malosse *et al.*, 1995; Frerot *et al.*, 1997). In the case of frogs, such a technique is ineffective due to the concurrent release of protein, lipid and mucous secretions from the frog's integumentary glands. To overcome this limitation, our study examined the use of headspace SPME and its application to the sampling of live frogs. In headspace, SPME

analytes partition between the sample, the headspace above the sample and a polymer coated fused silica fibre. They can then be thermally desorbed from the fibre to a capillary GC or HPLC column (Pawliszyn, 1997).

METHODS

The effectiveness of SPME was determined by analysing the release of eucalyptol from the skin glands of Ewing's tree frog, *Litoria ewingi* (Duméril and Bibron). Following preliminary trials employing crude hexane extractions of skin, it was observed that eucalyptol (identified using the NIST92 chemical library) was a regular (albeit trace) component of the extract. Attempts were made to absorb the secretion using three commercially available SPME fibres.

The technique was compared with the non-destructive method employed by Tyler *et al.* (1992) in which secretions are released from the frogs' skin glands during the application of an electrical stimulus to the skin surface. The secretions are then washed from the skin with water and collected for analysis. Three frogs, collected from Mannum, South Australia, Australia, were stimulated and their secretions pooled. The resultant water/secretion mixture was then extracted with an equal volume of hexane and concentrated for analysis. Analyses of the extracted secretion were preformed on a Varian 3400C gas chromatograph. The gas chromatograph was fitted with a HP-5MS silicone column (30 m × 0.25 mm × 0.25 μm) and coupled to a mass analyser (Finnigan Mat 70-TSQ mass spectrometer). The carrier gas was helium at 10 psi. Oven temperature was maintained at 40°C for 1 minute and was then programmed at 5°C minute^{-1} to 110°C and 20°C minute^{-1} to 250°C. The injector temperature was held constant at 250°C. Ionization for mass analysis was by electron impact at 70 eV and 220°C. Selective ion scanning mode was employed.

For SPME studies, eight individuals were collected from the same site at Mannum. The frogs were maintained in quarantine for one month before being transported to the Department of Chemistry, University of Waterloo, Ontario, Canada. The animals were housed in plastic aquaria (400 × 210 × 260 mm) lined with a substrate of wet newspaper. Lighting was supplied by overhead fluorescent lights and a

photoperiod of 16:8 light:dark employed. Laboratory temperature was maintained at 23°C. Frogs were fed on crickets obtained from the Department of Zoology, University of Guelph.

Three commercially available SPME fibres, 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) and 75 μm polydimethylsiloxane/carboxen (CAR/PDMS), were investigated for headspace sampling of the volatile components of the secretion. Frogs were sampled by placing them in 15 ml clear glass vials, into which stainless steel wire baffles were inserted to limit the movement of the frog and prevent it from contacting the fibre (Fig. 1). Initially larger sample chambers were used; but, the volume was reduced to facilitate mass transfer of the analytes to the fibre coating, after determining that the frogs were not unduly stressed in the smaller vial, and had adequate oxygen for the entire sampling duration. Other techniques usually employed to increase mass transfer include vial

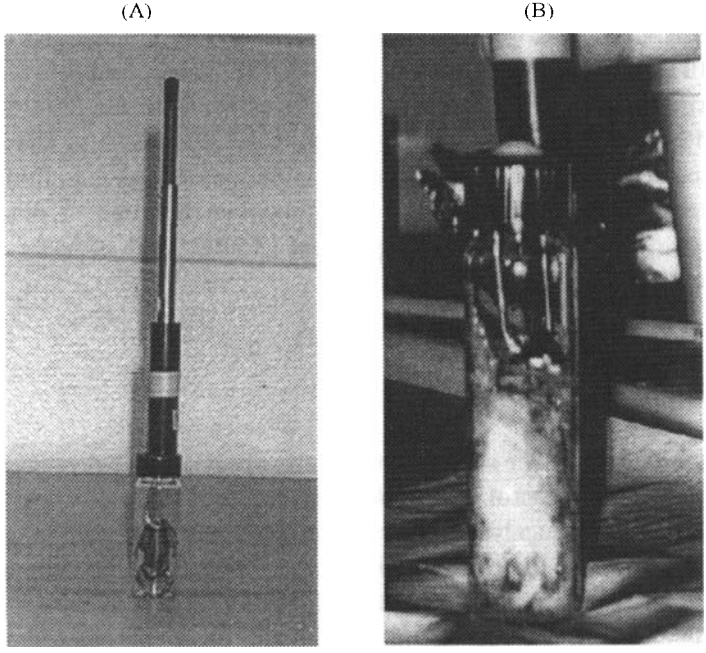

FIGURE 1 Headspace sampling of frog volatile compounds. (A) SPME fibre holder and vial with frog. (B) Close up of sample vial following frog stimulation. The foamy residue on the side of the vial is released secretion.

and/or fibre agitation or heating (Pawliszyn, 1997); both of these prove difficult when sampling volatiles from live animals.

Prior to sampling, frogs were voided of urine by applying gentle pressure to the bladder. Once the frog was enclosed in the vial, the vial was shaken vigorously for 10–20 seconds to stimulate release of its glandular secretion and the associated volatile compounds.

The headspace of the vial was then sampled at 23°C for 1 minute using the PDMS/DVB fibre and 10 minutes for both the PDMS and CAR/PDMS fibres. Sample times were based on times determined by Koziel *et al.* (1999) for fast sampling of air. Following sampling, the PDMS fibre was immediately introduced to the gas chromatograph's injector. Both the PDMS/DVB and CAR/PDMS fibre were stored on dry ice prior to analysis.

Analysis was performed on a Varian 3400C gas chromatograph coupled to an ion trap detector (Varian 4D Saturn GC/MS). The same column type and temperature programme used for the hexane extract was employed. A septum programmable temperature injector (SPI) was used and its temperature held constant at 250, 210 and 260°C for the PDMS, PDMS/DVB and CAR/PDMS fibres, respectively. A desorption time of 3 minutes was employed for both the PDMS and PDMS/DVB fibres and 5 minutes for the CAR/PDMS fibre. The mass spectrometer was operated in electron impact mode using a multiplier voltage range of 1600 to 1620 volts, axial modulation 4, filament current 25 μA, ion trap temperature 150°C and transfer line temperature 250°C.

RESULTS

Hexane extracts of the water/secretion mix obtained by Tyler's method did not yield eucalyptol. Eucalyptol was, however, isolated *via* SPME. Co-eluting with eucalyptol was the monoterpene limonene (Fig. 2). The presence of both compounds was confirmed by comparing retention times with the retention times of standard compounds (Sigma-Aldrich Canada Ltd.) under the same chromatographic conditions. Mass spectra from NIST92 and in-house libraries were also used.

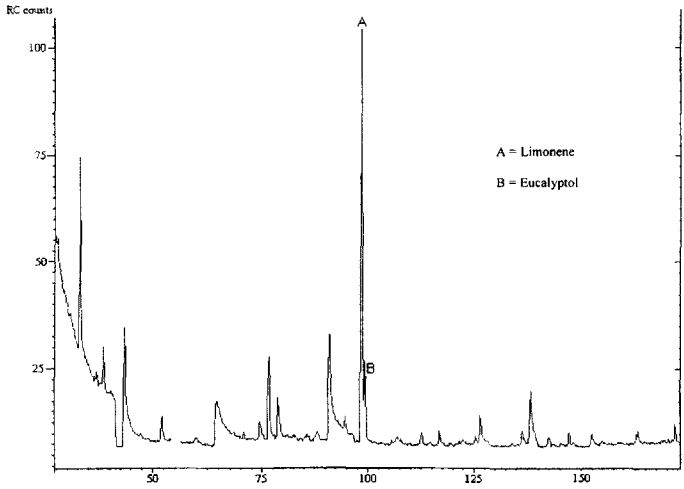

FIGURE 2 The presence of limonene and eucalyptol in the volatile profile of *L. ewingi*. Both compounds are believed to be derived from dietary sources (unpublished data), with limonene prominent in the food source of the frogs.

TABLE I Detection of limonene and eucalyptol in the volatile profile of *L. ewingi*

	PDMS/DVB 65 μm		*CAR/PDMS* 75 μm		*PDMS* 100 μm	
	Limonene	*Eucalyptol*	*Limonene*	*Eucalyptol*	*Limonene*	*Eucalyptol*
Control 1	***	***	***	***	nd	nd
Frog 1	***	***	***	***	**36**	**7**
Control 2	nd	nd	nd	nd	nd	nd
Frog 2	**20**	**15**	#	#	nd	nd
Control 3	nd	nd	nd	nd	nd	nd
Frog 3	#	#	**18**	**33**	**11**	**14**
Control 4	nd	nd	nd	nd	nd	nd
Frog 4	**280**	**68**	**60**	**26**	**84**	**19**
Control 5	9	nd	6	nd	tr	nd
Frog 5	**280**	**91**	**65**	**48**	**67**	**36**
Control 6	12	nd	15	nd	10	nd
Frog 6	**146**	**32**	**23**	**30**	**44**	**48**
Control 7	tr	nd	nd	nd	***	***
Frog 7	**41**	**14**	**34**	**26**	***	***
Control 8	tr	nd	nd	nd	***	***
Frog 8	**96**	**35**	**80**	**36**	***	***

nd Not detected.
tr Trace (S/N < 5).
*** Fibre not tested.
# Fibre damaged by frog.

Table I shows the detection of both compounds in 8 animals using the three fibres. It is clear, that under the described experimental conditions, the PDMS fibre did not perform as well as the PDMS/DVB or CAR/PDMS fibres. Using the PDMS/DVB fibre coupled with a short sample time provided the best signal/noise ratio for the majority of frog volatiles analysed.

DISCUSSION

Herpetologists have been forced to put on hold the identification of many amphibian and reptile volatile compounds, despite the recognition of the importance such chemicals play in an organism's life. For instance, Brodie and Formanowicz (1981) suggest the release of odorous secretions by amphibians during real or simulated predation may provide a defensive function, whilst there is a large body of literature on the effect of prey and other airborne odours on both lizards and snakes (*e.g.*, Cooper and Vitt, 1984; Cooper and Burghardt, 1990; Halpern *et al.*, 1997; Shivik, 1998; Roth *et al.*, 1999). The rate-limiting step for such studies has been the analytical technology.

Although Tyler's method is effective for the collection of nonvolatile compounds such as peptides and alkaloids, it is limited in its application because compounds in low concentration may be lost either during the collection or extraction process. Furthermore, washing the secretion from the skin with water decreases the ability to capture water-insoluble compounds, and may explain the lack of eucalyptol in hexane extracts of the *L. ewingi* secretion.

Although further studies are needed to determine the best sampling conditions (appropriate fibre coating, exposure time, desorption time and temperature), it is clear SPME provides an attractive tool to investigate volatile biological compounds. The ease with which SPME can be used (due mainly to its simple operation and lack of sample preparation steps) makes this technique attractive to both chemists and non-chemists. The primary advantage, however, lies in its application to the sampling of living organisms. Conventional methods can be destructive to both the target chemical and organism under study, and are severely limited when it comes to

studying changes in the chemical signature of an individual. With headspace SPME only those compounds volatile enough to interact with other organisms or the organism's physical environment are sampled. These compounds can then literally be taken from the frog's back and introduced directly to the analytical instrumentation, removing the risk that the compounds emitted are changed during the extraction process. As the frog is not harmed during this process, repeated sampling events can be conducted. The ability to sample the same animal at various life stages increases the amount of information that can be obtained and also provides information about any temporal variation of volatile compounds in the frog's skin.

The rapid rate with which a sample can be collected is of further advantage when sampling frogs. Due to the nature of frog skin, and its role as a respiratory surface, it is important the animal be kept moist to minimise desiccation. Standard techniques of volatile trapping usually employ dynamic systems in which air is drawn across the sample over time. Desiccation stress as a result of such methods may alter the chemical profile of the animal or, if humidified air is used, traps to remove the moisture could provide sites for analyte loss. Short sample times overcome these problems and negate the need for expensive experimental setups.

Besides the analytical shortfalls associated with conventional methods, consideration must be taken into account of the widespread decline in many frog species (Tyler, 1991). It is imperative that non-destructive techniques for the identification of volatiles be developed if we wish to continue to search nature's vast store of chemicals without plundering the environment. SPME fills this gap for volatile and semivolatile secretions and opens up what until now has been a poorly explored field of research.

Acknowledgments

This work was carried out while B.P.S. was a guest at the Department of Chemistry, University of Waterloo, Ontario, Canada. Financial support was provided by a travelling Fellowship from The Company of Biologists Ltd. to B.P.S. and through industry support from Hamilton Laboratories Pty Ltd. to M.J.T.. Special thanks goes to

A. Chugg, Singapore Airlines, for arranging the safe transport of the frogs to Canada and Riocell S.A. for supplying the eucalyptus trees.

References

Agelopoulos, N. G. and Pickett, J. A. (1998) Headspace analysis in chemical ecology: Effects of different sampling methods on ratios of volatile compounds present in headspace samples. *Journal of Chemical Ecology*, **24**, 1161–1172.

Blight, M. M. (1990) Techniques for isolation and characterisation of volatile semiochemicals of phytophagous insects. In: *Chromatography and Isolation of Insect Hormones and Pheromones* (Eds. McCafferty, A. R. and Wilson, I. D.), pp. 281–288, New York: Plenum Press.

Bostock, R. M. and Stermer, B. A. (1989) Perspectives of wound healing in resistance to pathogens. *Annual Review of Phytopathology*, **27**, 343–371.

Brodie, E. D. Jr. and Formanowicz, D. R. Jr. (1981) Palatability and antipredator behaviour of the treefrog, *Hyla versicolor*, to the shrew, *Blarina brevicauda*. *Journal of Herpetology*, **15**, 235–236.

Cooper, W. E. Jr. and Burghardt, G. M. (1990) Vomerolfaction and vomodor. *Journal of Chemical Ecology*, **16**, 103–104.

Cooper, W. E. Jr. and Vitt, L. J. (1984) Detection of conspecific odours by the female broad-headed skink, *Eumeces laticeps*. *Journal of Experimental Biology*, **229**, 49–54.

Cossé, A. A., Todd, J. L., Millar, J. G., Martinez, L. A. and Baker, T. C. (1995) Electroantennographic and coupled gas chromatographic electroantennographic responses of the Mediterranean fruit fly to male-produced volatiles and mango odour. *Journal of Chemical Ecology*, **21**, 1823–1836.

Daly, J. W. (1998) Thirty years of discovering arthropod alkaloids in amphibian skin. *Journal of Natural Products*, **61**, 162–172.

Finnegan, D. E. and Chambers, J. (1993) Identification of the sex pheromone of the Guernsey carpet beetle, *Anthrenus sarnicus* Mroczkowski (Coleoptera: Dermestidae). *Journal of Chemical Ecology*, **19**, 971–984.

Frerot, B., Malosse, C. and Cain, A. H. (1997) Solid-phase microextraction (SPME): a new tool in pheromone identification in Lepidoptera. *Journal of High Resolution Chromatography*, **20**, 340–342.

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

Halpern, M., Halpern, J., Erichsen, E. and Borghjid, S. (1997) The role of nasal chemical senses in garter snake response to airborne odour cues from prey. *Journal of Comparative Psychology*, **111**, 251–260.

Jakobsen, H. B. (1997) The preisolation phase of *in situ* headspace analysis: Methods and perspectives. In: *Plant Volatile Analysis*, Modern Methods of Plant Analysis 19 (Eds. Linskens, H. and Jackson, J.), pp. 1–22, Berlin: Springer-Verlag.

Jones, G. R. and Oldham, N. J. (1999) Pheromone analysis using capillary gas chromatographic techniques. *Journal of Chromatography A*, **843**, 199–236.

Koziel, J., Jia, M., Khaled, A., Noah, J. and Pawliszyn, J. (1999) Field air sampling analysis with SPME device. *Chemica Acta*, **400**, 153–162.

Malosse, C., Ramirez-Lucas, P., Rochat, D. and Morin, J.-P. (1995) Solid-phase microextraction, an alternative method for the study of airborne insect pheromones (*Metamasius hemipterus*, Coleoptera, Curculionidae). *Journal of High Resolution Chromatography*, **18**, 669–670.

Moneti, G., Dani, F. R., Pieraccini, G. and Turillazzi, S. (1997) Solid-phase microextraction of insect epicuticular hydrocarbons for gas chromatographic-mass spectrometric analysis. *Rapid Communications in Mass Spectrometry*, **11**, 857–862.

Monnin, T., Malosse, C. and Peeters, C. (1998) Solid-phase microextraction and cuticular hydrocarbon differences related to reproductive activity in queenless ant *Dinoponera quadriceps*. *Journal of Chemical Ecology*, **24**, 473–490.

Mozuraitis, R., Borg-Karlson, A.-K., Eiras, A., Witzgall, P., Kovaleski, A., Vilela, E. F. and Unelius, C. L. (1996) Solid phase microextraction technique used for collecting volatiles released by individual signalling *Bonagota cranaodes* moths. *Abstracts of the International Society of Chemical Ecology 13th Annual Meeting*, Aug. 18–22, Prague, p. 193.

Pawliszyn, J. and Smith, R. M. (Eds.) (1999) *Applications of Solid Phase Microextraction*. Cambridge: RSC Chromatography Monographs, New York: Springer-Verlag.

Pawliszyn, J. (1997) *Solid Phase Microextraction, Theory and Practice*. New York: Wiley.

Roth, E. D., May, P. G. and Farrell, T. M. (1999) Pigmy Rattlesnakes use frog-derived chemical cues to select foraging sites. *Copeia*, **3**, 772–774.

Shivik, J. A. (1998) Brown tree snake response to visual and olfactory cues. *Journal of Wildlife Management*, **62**, 105–111.

Tollsten, L. and Bergström, G. (1998) Headspace volatiles of whole plants and macerated plant parts of *Brassica and Sinapis*. *Phytochemistry*, **27**, 4013–4018.

Turillazzi, S., Sledge, M. F. and Moneti, G. (1998) Use of a simple method for sampling cuticular hydrocarbons from live social wasps. *Ethology, Ecology and Evolution*, **10**, 293–297.

Tyler, M. J. (1991) Declining amphibian populations – a global phenomenon? An Australian perspective. *Alytes*, **9**, 34–50.

Tyler, M. J., Stone, D. J. M. and Bowie, J. H. (1992) A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. *Journal of Pharmacological and Toxicological Methods*, **28**, 199–200.

Williams, C. R., Brodie, E. D. Jr., Tyler, M. J. and Walker, S. J. (2000) Antipredator mechanisms of Australian frogs. *Journal of Herpetology*, **34**, 431–443.

Zhang, Z. and Pawliszyn, J. (1993) Analysis of organic compounds in environmental samples using headspace solid phase microextraction. *Journal of High Resolution Chromatography*, **16**, C689–692.