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INTRODUCTION

In contrast to a plethora of known insect pheromones, a paucity of mammalian pheromones has been identified, two of which have been in elephants (Albone, 1984; Brown and Macdonald, 1985; Wyatt, 2003; Burger, 2005). Elephants possess one of the world's best chemosensory systems, due in no small measure to their prehensile trunk. The trunk is not only the gateway to smelling (primary olfaction), but also the means by which chemical signals are conveyed from their source to the openings of the vomeronasal organ ducts in the roof of the mouth (the flehmen response; secondary olfaction) (Rasmussen, 1999). The late L. E. L. "Bets" Rasmussen was a pioneer in the study of chemical signaling among elephants (Goodwin and Schulte, 2007). Among her many notable accomplishments are the identification of the urinary, preovulatory pheromone of the Asian elephant (*Elephas maximus*; Rasmussen et al., 1996a), and a chemical signal of musth in Asian male elephant temporal gland secretion (TGS; Rasmussen and Greenwood, 2003).

When prospecting for elephant pheromones, or those from any other mammal, four tasks must be accomplished:

(1) *extraction* of the volatile organic chemicals from the biological matrix;

(2) *separation* of the chemical components of the extraction mixture from each other;

(3) *identification* of the chemical compounds present in the mixture; and

(4) *verification* of bioactivity of the putative pheromones.

In our elephant research, and in general with similar investigations, the workhorse methodologies for *separation* and *identification* are gas chromatography (GC) and mass spectrometry (MS), respectively. *Verification* is achieved through behavioral bioassays, which in the case of elephants are quantified using flehmens and other distinctive trunk behaviors (Schulte et al., 2005; Schulte, 2006.) In this article we will discuss extraction procedures, primarily solventless ones that have been employed in the search for mammalian chemical signals.

For several years, the authors, an organic chemist (T.E.G.) and an animal behaviorist (B.A.S.), reaped the benefits of a productive collaboration and friendship with Bets Rasmussen (a biochemist). The main focus of our team was a study of chemical communication among African elephants (*Loxodonta africana*) (for example, see: Schulte et al., 2004; Bagley et al., 2006; Goodwin et al., 2006; Loizi et al., 2009). Our chemical analyses of volatile organic compounds in elephant excretions and secretions have involved solventless, and thus environmentally friendly ("green"), extraction methodologies. This report is not intended to be a comprehensive review of such procedures and mammalian applications thereof, but rather will focus on our work, along with selected examples of the techniques employed and discoveries made by other researchers in this area. (For more detailed comparisons and discussions of solventless extractions, see the following: Baltussen et al., 2002; Pillonel et al., 2002; Bicchi et al., 2004.)

GREEN CHEMISTRY

Green chemistry has been defined as "the utilization of a set of principles that reduces or eliminates the use of hazardous substances in the design, manufacture, and application of chemical products" (Anastas and Warner, 1998; p. 11). Green chemistry is increasingly being taught and practiced not only in chemical industry (Constable et al., 2007), but also in chemical education (Anastas and Kirchhoff, 2002; Goodwin, 2004). As the fifth entry in their well-known Twelve Principles of Green Chemistry, Anastas and Warner (1998; p. 30) state the following: "The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used." We were thus motivated to use various solventless sample pre-concentration techniques and subsequent analysis by GC-MS as a cornerstone of our chemical ecology research. In the sections below after a short discussion of traditional extractions with organic solvents, we highlight a

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number of recent developments in solventless extraction of organic volatiles from aqueous solutions. For each technique, we present examples of the use of that technique for the study of chemical signaling in mammals.

SOLVENT EXTRACTION OF BIOLOGICAL SOLUTIONS

Traditionally, extraction of dissolved organic chemicals from dilute aqueous biological media has been carried out using a petrochemical organic solvent and a separatory funnel, or an apparatus for continuous solvent extraction. The extracts are then concentrated by solvent evaporation, the volatiles are cryoconcentrated (cryofocused), and analysis is by GC-MS (for example, see: Whittle et al., 2000; Zhang et al., 2003). Not only are these solvent-intensive procedures less environmentally benign, but there is also a danger of losing some of the more volatile analytes when the extract is concentrated via solvent evaporation. Nonetheless, solvent extraction coupled with bioassay-guided fractionation has led to some spectacular successes, notable among which is the 15 year quest by Bets Rasmussen to identify the pre-ovulatory pheromone in female Asian elephant urine. The specific estrous chemical signal, previously known as a sexual signal in many moth species, was found to be the simple ester Z-7-dodecen-1-yl acetate **(1)** (Z7-12Ac; Rasmussen et al., 1996a, 1997). Goodwin et al. (1999) also used this technique to analyze the temporal gland secretion (TGS) of African elephants, and identified several unusual sesquiterpenes, including (E)-2,3-dihydrofarnesol **(2)**, a bumblebee pheromone, and drimane-8 ,11-diol **(3)**, previously found only in a Greek tobacco.

SOLVENTLESS EXTRACTION TECHNIQUES USED WITH MAMMALIAN SAMPLES

TRAPPING ANALYTES ON A POROUS POLYMER

An early technique for solventless extraction of volatile chemicals from a sample's headspace (the vapor phase in equilibrium with a liquid mixture) involved passage of the vapor over a porous polymer (for example, Tenax®), followed by thermal desorption, cryoconcentration, and finally analysis by GC-MS. This process has proven to be very useful in the study of biological media (for example, see: Zlatkis et al., 1973; Jorgenson et al., 1978; Schwende et al., 1986; Service et al., 2001).

In recent years, several more powerful and simpler solventless methodologies have been developed, as discussed below.

EVACUATED CANISTER CAPTURE FOLLOWED BY CRYOGENIC TRAPPING (ECC/CT)

In a 1996 publication, Bets Rasmussen, her husband Rei, and their co-workers described how a novel technique named "evacuated canister capture-cryogenic trapping" (ECC/CT), originally developed for atmospheric sampling, could be adapted for the extraction of volatile organic chemicals from biological samples (Perrin et al., 1996; Rasmussen and Perrin, 1999). The technique involves the use of evacuated, scrupulously cleaned, inert, air-tight stainless steel canisters to collect sample headspace volatiles, pressurization of the canisters, and cryo-trapping the canister vapors in a liquid nitrogen-cooled U-tube containing 60-80 mesh glass beads, followed by gentle U-tube heating to release the analytes into the GC-MS. In this 1996 report, the TGS from a male Asian elephant was analyzed.

Among many subsequent applications of the ECC/CT methodology is a study of urinary chemical signals of musth in wild elephants in Kenya (Rasmussen and Wittemyer, 2002). Male elephants, both African and Asian, undergo a periodic rut-like condition called musth. Unlike rutting, however, musth is asynchronous among any group of male elephants. Characteristics of musth include elevated serum androgens (particularly testosterone and dihydrotestosterone), heavy drainage from the temporal glands, urine dribbling, increased aggressiveness, and enhanced success in competition for breeding (Eisenberg et al., 1971; Poole and Moss, 1981; Poole, 1987; Rasmussen et al., 1996b; Schulte and Rasmussen, 1999; Ganswindt et al., 2005). The Kenya study offers the first detailed musth/non-musth comparison of urinary chemical signals in both wild and captive African elephants. Not only were major differences observed between urinary volatiles from musth and non-musth samples, but also there were remarkable similarities to results obtained in earlier studies with captive African elephants, as well as with wild and captive Asian elephants. In particular, a group of ketones and alcohols was found to occur in greater amounts in musth versus non-musth urine in both species. These compounds are likely the result of increased metabolism of fatty acids during musth, and are thus chemical signals of musth to other male and female elephants.

ECC/CT also was used to analyze volatile organic compounds from three rather unusual elephant sources: (1) an aqueous solution that African (but not Asian) elephants eject from their ears (Riddle

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et al., 2000); (2) interdigital glands (Asian, but not African; Lamps et al., 2001; Rasmussen and Goodwin, unpublished); and (3) breath (Rasmussen, 1998; Rasmussen and Riddle, 2004). In addition, Bets used ECC/CT and a complementary solventless extraction technique, solid phase microextraction (SPME), to analyze female elephant urine and TGS of young musth males as discussed below.

SOLID PHASE MICROEXTRACTION (SPME)

SPME relies on a small glass fiber coated with an adsorbant polymer that can be exposed to the headspace over an aqueous sample, or immersed directly in the aqueous solution (Pawliszyn 1997; marketed by Supelco, Inc.). SPME has been employed for an immense variety of applications, due in part to its early development and ease of use (for example, see: Theodoridis et al., 2000). Selection of a particular SPME fiber (with various absorption polymer coatings) is based upon the properties of the analytes of interest. SPME is easily automated using the versatile and rugged Combi PAL GC autosampler (CTC Analytics). SPME is a static technique (the vapor does not move across the absorbant), unlike the older Tenax® dynamic methodology mentioned earlier. Additionally, the fiber is rather fragile and has a relatively low polymer loading.

Bets used the complementary techniques of ECC/CT and SPME plus GC-MS to identify and quantitate Z7-12Ac and other compounds in the pre-ovulatory urine of Asian elephants (Rasmussen, 2001). She and her collaborators also used ECC/CT and SPME to extract the sweet-smelling compounds that characterize musth secretions by young male Asian elephants (Rasmussen et al., 2002). Additionally, the beetle pheromone frontalin, having first been detected in male Asian elephant TGS using ECC/CT (Perrin et al., 1996), was demonstrated to be a chemical message of musth (Rasmussen and Greenwood, 2003). Even more remarkable was the demonstration that the nature of this chemical signal depends upon the enantiomeric ratio of frontalin in the TGS (Greenwood et al., 2005). In this latter project, SPME was used to extract frontalin from TGS.

We employed SPME/GC-MS to analyze African elephant TGS and found, inter alia, three 2,3-

dihydrofarnesol sesquiterpene derivatives **(4, 5, 6)** that were previously unknown to science (Goodwin et al., 2002). Structural assignments were confirmed by synthesis of these compounds from farnesol. We have not yet conclusively determined whether these sesquiterpenes and those reported in our earlier paper (Goodwin et al., 1999) serve as chemical signals among African elephants. In addition to our TGS analyses, we have used SPME to analyze female African elephant urine in the search for a preovulatory pheromone (Goodwin et al., 2005).

STIR BAR SORPTIVE EXTRACTION (SBSE)

Stir bar sorptive extraction (SBSE), a more recent development than SPME, offers some distinct advantages over the earlier technique (Baltussen et al., 1999; Baltussen et al., 2002; Kawaguchi et al., 2006). In SBSE, the absorbant polymer is coated on a small stir bar which is marketed by Gerstel, Inc. as Twister®. SBSE, originally developed for immersion use, has been adapted for headspace extraction in which the stationary stir bar is suspended above the liquid matrix. Implementation of SBSE is more expensive than SPME, requiring specialized add-ons for the GC-MS, but has a much thicker polymer coating and thus can extract a larger amount of analytes. SBSE has been used to extract a variety of organic compounds from human urine (Tienpont et al., 2002), and general treatises on the use of SBSE in a search for mammalian chemical signals have been published (Soini et al., 2005a; Novotny and Soini, 2007). In a seminal series of papers, the Novotny group and their collaborators have described a number of specific SBSE applications, including the following analyses: (1) hamster urine (Soini et al., 2005b); (2) ferret urine and anal gland secretion (Zhang et al., 2005); (3) mouse urine (Novotny et al., 2007); and (4) human axillary sweat, urine, and saliva (Penn et al., 2007). Additionally, these researchers have developed a "rolling stir bar sampling technique" in which the coated stir bar is fitted into a novel holder and rolled directly across the surface of interest (Soini et al., 2006). The analytes are then desorbed into a GC-MS. For example, extraction of surface volatiles from human skin, grapefruit, human fingerprints on a mirror, and bird feathers are described (Soini et al., 2007). A notable feature of this innovation is the ability to imbed an internal standard in the stir bar coating prior to sampling, thus allowing analyte quantitation.

SAMPLE ENRICHMENT PROBE (SEP)

Burger and co-workers developed a "sample enrichment probe" (SEP) for high-capacity extraction of analytes from gaseous and aqueous samples, followed by GC-MS analysis (Burger et al., 2006a). The probe itself consists of a thin rod of inert material (usually stainless steel), fitted on one end with a short sleeve of polydimethylsiloxane rubber tubing. SEP employs a much larger volume of sorptive phase than SPME, and yields results

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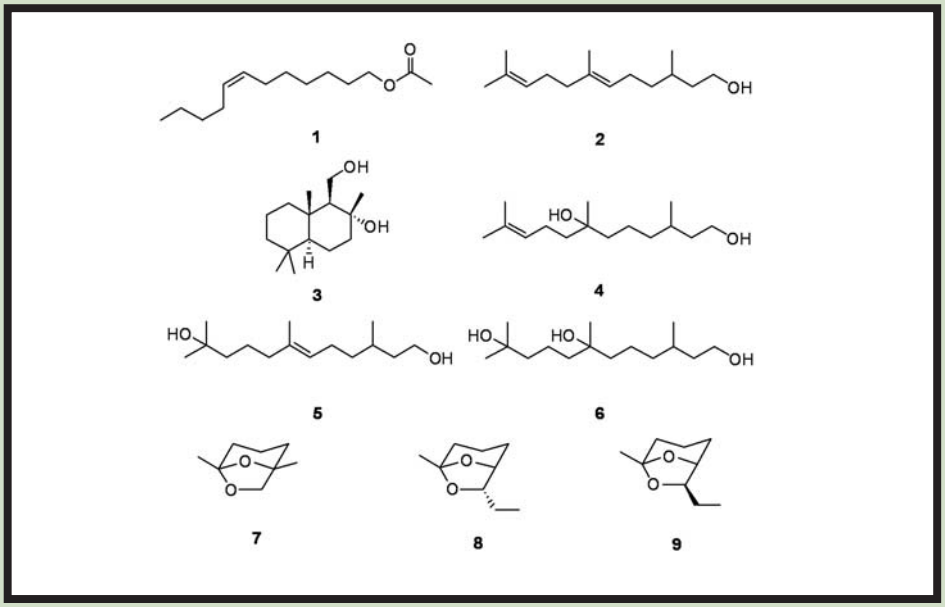
comparable to SBSE without the need for cryofocusing of analytes. Simple modifications to the GC inlet are required. This solventless extraction technique has been used successfully to analyze the urine of the cheetah, and the territorial marking liquid of the male Bengal tiger (Burger et al., 2006b, 2008). Two techniques that are similar to SEP are HCSP, an automated "high capacity sorption probe" (Pettersson et al., 2004), and SPACE ("solid-phase aroma concentrate extraction"; Ishikawa et al., 2004).

SOLID PHASE DYNAMIC EXTRACTION (SPDE)

Solid phase dynamic extraction (SPDE), the newest of the commercially available acronym trio that includes SPME and SBSE, features concentration of headspace analytes by repetitive flow back and forth over a polymer coating on the inside wall of a stainless steel syringe needle that is attached to a gas-tight syringe (Lipinski, 2001; marketed by Chromsys/Chromtech). SPDE has more absorbant polymer coating than SPME, but less than SBSE. SPME and SPDE have a larger variety of polymer coatings available than SBSE. SPDE is easily automated, as are SPME and SBSE. SPDE, unlike SPME and SBSE, is a *dynamic* technique for headspace analysis, and appears to offer some advantages for extraction of volatile organic compounds. The SPDE needle is more robust than the SPME fiber, has more extraction capacity, and for most applications can be used for hundreds of extractions before replacement. SPDE/GC-MS has proven to be useful in a variety of applications (see, for example, Musshoff et al., 2002; Bicchi et al., 2004; Ridgeway et al., 2006).

To our knowledge, we are the only group thus far to implement SPDE in the search for mammalian chemical signals. Most of our work has focused on a search for the putative preovulatory urinary pheromone in African elephants (Goodwin et al., 2007). Although male African elephants can distinguish conspecific female urine from different times in the estrous cycle (Bagley et al., 2006), specific chemical signals have not yet been verified. We employed SPDE/GC-MS to identify in female African elephant urine not only the beetle aggregation pheromones frontalin **(7)**, endo- **(8)** and exo-brevicomin **(9)**, but also their biochemical beetle precursors, thus suggesting a common beetle/elephant biosynthetic pathway (Goodwin et al., 2006). Extensive behavioral bioassays are underway to determine whether any of these compounds, or a blend of them, is functioning as a pheromone among African elephants. Most recently, we have begun to analyze volatiles in male African elephant urine to document not only musth/non-musth differences, but also how the mix of volatiles changes as young elephants mature, and as the urine ages after excretion. While elephant chemical signals remain our primary focus, our search for mammalian signals currently includes collaborative studies on lemur glandular secretions and urine, maned wolf urine, vole urine, putative interdigital glands of polar bears, and kakapo feather scents from a highly endangered, nocturnal, flightless parrot of New Zealand.



1 *= (Z)-7-dodecen-1-yl acetate,* ***2*** **=** ***2,3-****dihydrofarnesol,* ***3*** **=** *drimane-8,11-diol,* ***4*** **=** *2,3-dihydrofarnesol, 6,7-monohydrate,* ***5*** **=** *2,3-dihydrofarnesol, 10,11-monohydrate,* ***6*** **=** *2,3-dihydrofarnesol, dihydrate,* ***7*** **=** *frontalin,* ***8*** **=** *endo-brevicomin,* ***9*** **=** *exo-brevicomin.*

CONCLUSION

In this brief review, we have presented an overview of the various solventless extraction techniques that have been used to search for mammalian chemical signals. A major focus has been our own research on chemical signals in elephants, and the pioneering work of our friend and collaborator, the late Bets Rasmussen. Our search will continue to rely on chemical analyses in the laboratory, coupled with extensive behavioral bioassays with both captive and wild elephants. We hope that by learning more about these magnificent and endangered mammals, we can contribute to ensuring their long-term survival.

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