Chapter 1
Volatile Mammalian Chemosignals: Structural and Quantitative Aspects

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Abstract Current investigations of mammalian chemical signals are providing biologically useful information. Precision of profiling data has become substantially improved due to better sampling methodologies, selective detection techniques and vastly improved mass-spectrometric instrumentation. This, in turn, facilitates the use of powerful chemometric methodologies in evaluation of large data sets and a future integration of volatile profiling data into the systems biology knowledge.

1.1 Introduction

In molecular terms, we live in the complex world involving chemosignaling in many events, both internal and external. In morphologically and functionally sophisticated mammalian systems, a balanced chemical communication among their biological constituent cells is a standard of normalcy, as are the intracellular events determined through transmembrane signaling. In the "outside world", a living organism is constantly being subjected to chemical signals, both spurious and naturally programmed, which are both consciously and subconsciously perceived. The biological selectivity and sensitivity of perception are inherent and important to the processes of chemical communication both within a species and among different species. Consequently, the inherited traits for chemical communication signals (pheromones and allomones) and their receptors, and the learned responses to other chemosignals in the environment are widely and effectively utilized by different animals. While it would seem that the large number of species and their regulatory needs for hormonal and reproductive function could result in a staggering number of chemical messengers to fulfill these tasks, there are some limitations imposed by the nature of biosynthetic processes. Consequently, in the mammalian pheromones identified

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thus far, we see a great deal of structural redundancy, or at least small structural modifications, with the more scientifically explored world of insects. Naturally, this observation has been confined to the volatile molecules used in a distance communication, while the larger biomolecules, if employed in chemical communication, can present a wider range of structural/biochemical diversity to satisfy the requirements for biological selectivity.

We still know globally relatively little above the chemical nature of the individual chemosignals and the biochemical events involved in their perception. Since identification of the first definitive mammalian pheromones in the house mouse (Novotny, Harvey, Jemiolo and Alberts 1985; Jemiolo, Harvey and Novotny 1986), substantial improvements were made in bioanalytical methodologies (combined capillary gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry) to yield additional chemosignal structures. Moreover, with the more recent capabilities of quantifying precisely the ratios of different volatile components of a biological stimulus, there now appear new exciting possibilities for comparative studies of the genetic correlates of chemical communication, as exemplified by the recent studies of MHC-congenic mice, (Novotny, Soini, Koyama, Bruce Wiesler, and Penn 2006). The strategy of looking at the ratios or patterns of organic volatiles as a distinct signal interpretable by a mammal's sensory pathways has been amply precedented in modern biology: for example, in a situation of comparable complexity, cell-to-cell communication in sophisticated mammalian systems is likely based on the entire reservoir of different glycan (oligosaccharide) structures which guard a cellular function in disease or health (Lowe and Marth 2003). To interpret properly such multicomponent (pattern) signals, chemical ecologists should turn increasingly to modern chemometric procedures (Brereton 2003) that can deal effectively with complex analytical data. At a more readily understandable level, optical activity and other subtle forms of substance isomerism can play a role in conveying different messages. This has recently been shown by Rasmussen and co-workers (Greenwood, Comeskey, Hunt and Rasmussen 2005) with the utilization of frontalin in different ratios of its enantiomers. In this view, many other cases where biological responses were elicited from the racemic mixtures of putative pheromone need to be re-examined.

During the last decade, the availability of new analytical methodologies and instrumentation has revolutionized modern biology and biochemistry. Starting with the Human Genome Project and the field of genomics, new separation techniques and biomolecular mass spectrometry have paved the way to conquering the proteomics of different species, including their sophisticated posttranslational modifications and non-covalent biomolecular complexation. This quest for additional molecular information continues unabated, toward the fields of glycomics, lipidomics and metabolomics. The unprecedented wealth of molecular information can now be tied, through the advanced computational techniques (bioinformatics) into the holistic approach of systems biology (Kitano 2002) to provide an unprecedented understanding of an organism in its different conditions and environment. Such an approach and capabilities provide a unique opportunity for solving typical problems in chemical communication.

1.2 Advances in Analytical Methodologies

A search for mammalian pheromones in different laboratories during the last two decades followed the methodologies used with the studies of juvenile hormones, ecdysones and pheromones in the insect world (McCaffery and Wilson 1990). Solvent extractions and trapping of volatiles are still commonly used in this type of research, although the methodologies based on dynamic headspace extraction (Novotny, Lee and Bartle 1974) or solid-phase microextraction (SPME) using coated silica fibers (Zhang and Pawliszyn 1993) are more typically seen in today's laboratories pursuing pheromone identification through GC-MS. Solvent extraction is seemingly beneficial for handling moderately volatile compounds at relatively high concentrations (Burger, Tien, LeRoux and Mo 1996). Preconcentration of volatiles on the surface of adsorptive materials, such as organic porous polymers, with a subsequent desorption for a GC-MS analysis was for a long time a standard procedure in our laboratory, used in structural elucidation of the first mouse pheromones (Schwende, Wiesler, Jorgenson, Carmack and Novotny 1986). While this method referred to as the "purge-and-trap" or "dynamic headspace' technique, may be used with satisfaction in semiquantitative studies to observe large differences in metabolite excretion, it may not be the best approach when small quantitative differences in compounds may account for recognition of a biological message.

Current developments in analytical sampling seem to favor *absorption* rather than *adsorption* preconcentration principles. Using newer techniques in this direction, the organic polymers (absorption media) are attached to a mechanical device, such as a silica fiber (Pawliszyn 1997) or a glass-coated stir bar (Baltussen, Sandra, David and Cramers 1999) to allow sample sorption from a medium (e.g., urine or a glandular secretion). The stir bar approach has further advantages over the SPME approach in a better dynamic concentration range of the trapped organics, better preconcentration of trace volatiles, and analytical reproducibility (Bicchi, Iori, Rubiolo and Sandra 2002). In contrasting the purge-and-trap sampling with a stir bar preconcentration (Fig. 1.1) some additional advantages of the latter approach become at once evident: a system setup allows high analytical throughput and more reproducible sample transfer.

Quantitative aspects of stir bar sorptive extraction in the measurements pertaining to various applications in chemical ecology have recently been demonstrated. This novel methodology has been found highly reproducible and linear in aqueous sampling of volatile and semivolatile organic compounds from mammalian urine and tissue extracts. In comparison with the adsorbent-based preconcentration procedures, the stir bar extraction technique has shown vastly improved repeatability (Soini, Bruce, Wiesler, David, Sandra and Novotny 2005). A rolling stir bar sampling procedure has recently been found effective in the in-situ surface sampling of biological objects such as human skin (Soini, Bruce, Klouckova, Brereton, Penn and Novotny 2006) or bird feathers. As demonstrated with nearly 200 human volunteers, whose skin volatile profiles were recorded as "odor signatures" of individuality (examples shown in Fig. 1.2) (Penn, Oberzaucher, Grammer, Fischer, Soini, Wiesler, Novotny, Dixon, Xu and Brereton 2006), a high degree of analytical reproducibility

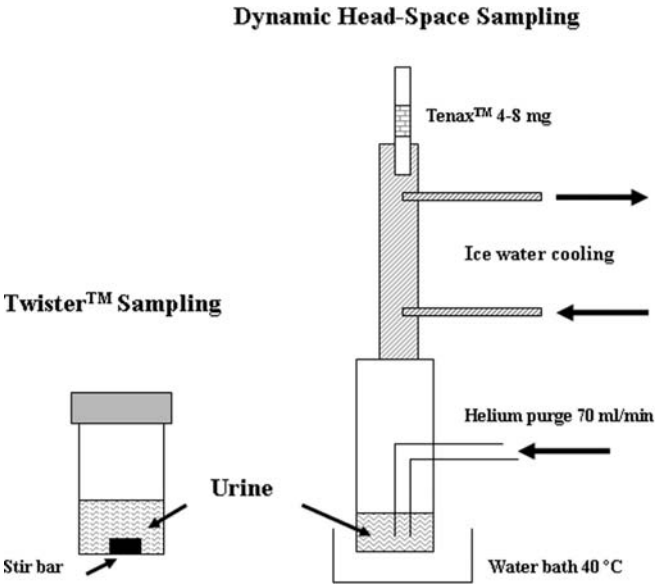

Fig. 1.1 Aqueous Twister™ and purge- and-trap Tenax™ sampling devices for trapping volatiles from urine

can be ascertained through the use of internal standards embedded onto the stir bar surface before sampling. This sampling variant provides opportunities for sample acquisition in a location geographically distant from the analytical laboratory.

Since the first uses of GC-MS in pheromone research about four decades ago, the instrumentation has made significant gains in performance and sensitivity. Highly efficient capillary columns are almost exclusively used, while sophisticated and automated inlet systems ensure the analytical reliability required for the meaningful comparative profile studies. The structural elucidation (solute identification) often remains the most difficult task requiring a chemical knowledge. The newer and more affordable high-resolution mass spectrometers can be most useful in aiding the structural identification. This can be exemplified by the case of implicating a possible structure of 2-*sec*-butyl-4,5-dihydrothiazole (a male mouse pheromone) from its MS fragmentation pattern and nominal molecular weight ($m/z = 143$) with a conventional quadrupole instrument, as compared to its unequivocal identification through the exact molecular mass ($m/z = 143.0819$) on a high-resolution time-of-flight mass spectrometer (Fig. 1.3). Naturally, additional spectrometric techniques based on IR and NMR can provide further, structurally important data whenever applicable.

Element-specific detection combined with capillary GC has become a key technique in the chemical communication studies of our laboratory. An effective detector of this type is based on the microwave plasma emission (Wylie and Quimby 1989), with a tunable selectivity for several elements and a prominent sensitivity for sulfur-containing compounds, which is significantly greater than

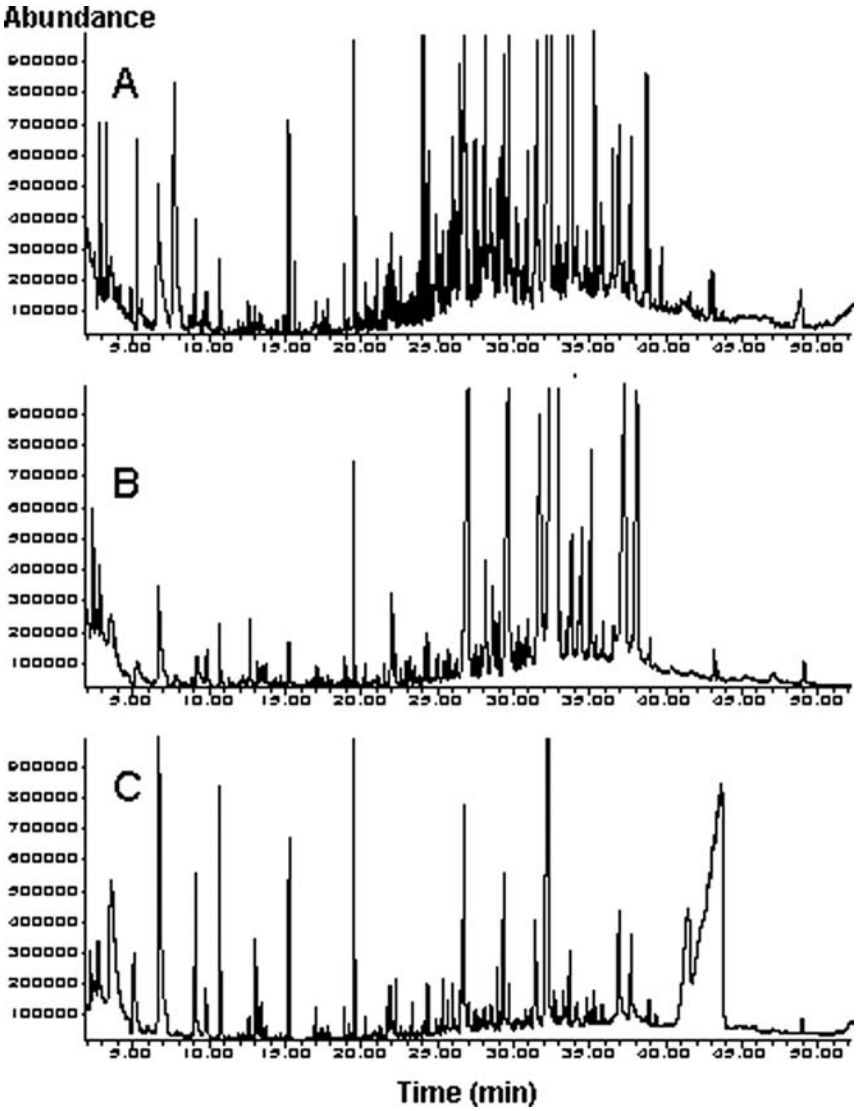

Fig. 1.2 A comparison of total ion chromatograms (GC-MS TIC) of selected axillary skin surface compounds for A: a female , B: a male (1), C: a male (2) human subjects

that of ordinary GC-MS. As many such compounds, including certain known chemosignals, are particularly odoriferous, sulfur-sensitive detection remains an important tool. Representative applications of the element-specific detection include mouse urinary profiles (Novotny et al. 2006), male-female comparisons of the ferrets (Zhang, Soini, Bruce, Wiesler, Woodley, Baum and Novotny 2005), junco songbird preen oils (Soini, Bruce, Klouckova, Brereton, Penn and Novotny 2006)

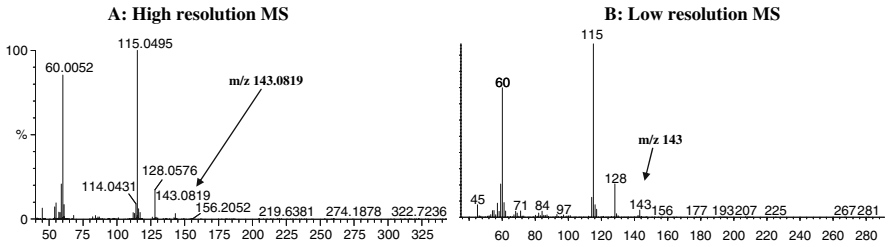

Fig. 1.3 Illustration of the mass spectra for the GC-MS TIC compound eluting at retention time of 23.4 min (2-sec-butyl-4,5-dihydrothiazole). A: high resolution MS spectrum, B: low resolution MS spectrum

and sulfur-containing human skin volatiles (Soini, Bruce et al. 2006). Using the sulfur detection mode, we have also demonstrated large quantitative and qualitative differences in male and female cat urinary volatile constituents containing sulfur (Soini, Wiesler and Novotny 2006).

The recent advances in sampling reproducibility and the availability of high-throughput GC-MS instrumentation have made it now feasible to conduct biological observations and experiments at large scale, at which hundreds of specimens can be quantitatively profiled under different sets of circumstances. This has recently been demonstrated in the studies of individual and gender "fingerprints" in human body odor involving a repeated collection of axillary sweat samples from 197 adults and their subsequent GC/MS analysis (Penn et al. 2006). To assist such large-sale investigations, it has been essential to implement new methodologies for peak detection and data set matching and computer-aided pattern recognition (Dixon, Brereton, Soini, Novotny and Penn 2007; Dixon, Brereton, Soini, Novotny and Penn 2007; Penn et al. 2007).

1.3 Chemosignaling Diversity in Mammalian Species: Different Structures or Their Proportions?

Genetic and biochemical diversities in different species have their reflections in their use of chemical communication: Are they gregarious or solitary animals? Nocturnal scant chemical evidence thus far, we know that the compounds both "chemically sophisticated" and simple can serve as chemical messengers in mammals when perceived in a proper behavioral context. Examples of some sophistication are the stereospecifically determined chemosignals in the house mouse (Novotny, Xie, Harvey, Wiesler, Jemiolo and Carmack 1995) and the Asian elephant (Greenwood et al. 2005), while the behaviorally fascinating mammary pheromone of the rabbit happens to be a relatively simple organic compound (Schaal, Coureaud, Langlois, Giniès, Sémon and Perrier 2003). The biosynthetic pathways leading to what we know today are the behaviorally distinct chemosignals in both insects and mammals, which are seemingly preserved across different species. Thus, the terpenic structures

such as α- and β- farnesene are pheromones in the house mouse (Novotny, Harvey and Jemiolo 1990), ovulatory Asian elephant (Goodwin, Eggert, House, Weddell, Schulte and Rasmussen 2006) and several insect species (Bowers, Nault, Webb and Dutky 1972), while (Z)-7-dodecen-1-yl acetate is the pre-ovulatory pheromone of female Asian elephant (Rasmussen, Lee, Roelofs, Zhang and Daves 1996) and several moth species alike. The mouse pheromone, dehydro-*exo*-brevicomin (Novotny, Schwende, Wiesler, Jorgenson and Carmack 1984) differs only in the presence of its double bond from the pheromone of the bark beetle and Asian elephant (brevicomin).

While 2,5-dimethylpyrazine has previously been implicated as the puberty-delay primer pheromone in the house mouse (Jemiolo and Novotny 1994), we find many other pyrazine derivatives in additional species: in deermice *Peromyscus californicus* (Jemiolo, Gubernick, Yoder and Novotny 1994), *Peromyscus maniculatus* (Ma, Wiesler and Novotny 1999) and hamsters (*Phodopus campbelli)* (Soini, Wiesler, Apfelbach, König, Vasilieva and Novotny 2005). Interestingly, substituted pyrazines are absent in *Phodopus roborovski*, which feature uniquely alkylquinaxolines instead.

While the earlier studies in chemical ecology of mammals were preoccupied with relatively gross measurements (e.g., "presence" or "absence" of a chemical/pheromone in an olfactory stimulus), the new quantitative capabilities make it now imperative to evaluate more accurately the volatile compound ratios or patterns under different biological circumstances.

1.4 Genetic Comparisons

Mice have often been referred to as the reproductively most successful mammals on Earth, while their sophisticated chemical communication systems are viewed as highly important in this success (Bronson 1979). Yet within the *Mus* genus group, there is a wide geographical distribution of behavioral and reproductive attributes that justifies comparative studies of different mouse types (Patris and Baudoin 2000). While the phylogeny is well established (Kikkawa, Miura, Takahama, Wakana, Yamazaki, Moriwaki, Shiroishi and Yonekava 2001), there have been no consistent chemical investigations which would characterize their genetic differences in terms of chemical signaling. For example, two groups which differ substantially in their nesting behavior and breeding are *Mus musculus domesticus* (common house mouse, which has a polygamous mating system) and *Mus spicilegus* (mound-building mouse, which is monogamous, Patris and Baudoin 1998). In a recent collaborative effort (Soini, Wiesler, Bruce, Koyama, Ferón, Baudoin and Novotny, 2006), we have observed some fundamental differences between the two mouse groups (Fig. 1.4): the key male pheromone of the house mouse, a thiazoline derivative, is totally absent in the urine of *M. spicilegus* (as is its characteristic smell), while the mound-building male mouse appears to feature different volatiles of its own, which are also under testosterone control.

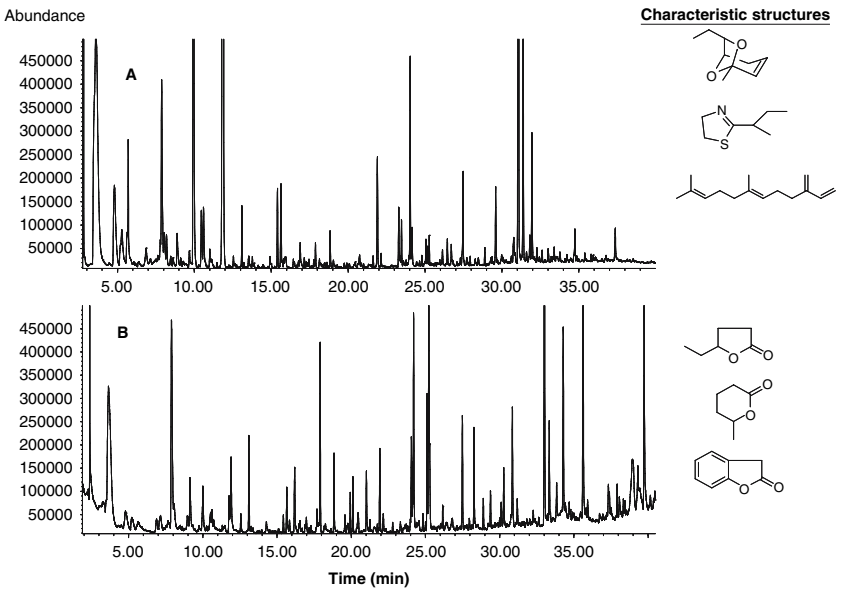

Fig. 1.4 Comparative male mouse urinary volatile profiles for (A) Mus domesticus and (B) Mus spicilegus by GC-MS with characteristic chemical structures

The house mouse has been an extremely important species due to its wide utilization in biomedical experimentation. It has also been the most documented case for studying the pheromonal effects in both behavioral terms (Hurst and Beynon 2004) and chemistry (Novotny 2003). Among the most recent investigations of the genetic influences on the mating behavior, there has been a renewed interest in a role of the major histocompatibility complex (MHC) (Penn and Potts 1998; Beauchamp and Yamazaki 2003). Our recent data (Novotny et al. 2006) for a large group of mouse chemosignaling compounds provide evidence that even minute genetic variations in MHC can be reflected in the quantitative differences of selected volatile profile constituents of the mouse urine. The results also indicate that concentrations of these compounds are not solely determined by this gene complex, but can be linked to other gene regions (for different background strains). While *Mus domesticus* is an attractive model for additional gene-behavior studies, some recent studies suggest that the MHC-dependent mating preferences may exist in fish, lizards, birds, and even humans (reviewed in Penn 2002).

1.5 Conclusions

Whereas the role of olfaction in chemical ecology is gaining increased attention, new bioanalytical methodologies and instrumentation provide unprecedented opportunities besides the structural elucidation of pheromones and other chemosignals,

their release into the environment and perception at the level of olfactory neurons. Precise comparative chemical measurements can also be helpful in phylogeny studies and developmental biology.

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