THE QUANTITATIVE ANALYSIS OF SEMIOCHEMICALS

by

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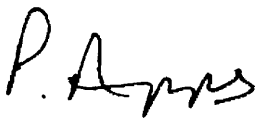
DECLARATION

I declare that the work presented here is original, that except where otherwise acknowledged is my own and that it has not been submitted to any other University.



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SUMMARY

Social odours have a major influence on the organization of mammalian societies but our understanding of their chemistry has been hampered by shortcomings in analytical techniques which have been borrowed from other fields of odour analysis. The known properties of mammalian odours suggest that an appropriate analytical system would need to be able to deal quantitatively with nanogram components from milligram specimens. The dynamic solvent effect (DSE), a novel sampling system for gas-liquid chromatography which allows the exploitation of the quantitative analytical capacity of capillary columns and the associated detectors, has been developed and tested to this end. The DSE traps gas-borne volatiles on a film of solvent held in dynamic equilibrium between evaporation and capillarity in a porous bed. A theoretical model of the DSE is developed which predicts that whether a compound will be trapped depends on its partition ratio above the solvent film.

DSE sampling is carried out on sintered glass beds, deactivated by a novel method using silicon and ethene, and held in tubes 120 mm long and 1,4 mm in internal diameter. Transfer of the sample to a capillary column is by a heatable, stainless steel and silica inlet. The stages in the evolutionary development of the concentrators and inlet were tested with a new diagnostic test mixture for solvent effect inlets.

When sampling from solvent, gaseous or aqueous specimens the precision, sensitivity and freedom from artifacts of the DSE are as good as, or better than, other sampling systems reported in the literature.

Ancillary techniques such as solvent and gas purification, glassware design and reagent handling were adapted to the needs of the DSE.

DSE sampling was tested against selected biological problems. The defensive secretions of two heteropteran bugs; *Thaumastella namaquensis* and *T. elizabethae* were analysed in order to clarify their systematics. Extremely complex and, in some cases, unexpected patterns of stasis and change were demonstrated when the temporal changes in volatile emissions from single scent marks of brown hyaenas (*Hyaena brunnea*) were followed for up to nine days. Among the complex mixture of volatiles emitted by male *Mastomys natalensis* and *M. coucha* one compound; 3-nonene-2-one was shown to be specific to *M. coucha*. The body odours of male laboratory mice (*Mus musculus*) were shown to change with social status. Three compounds; benzaldehyde, 2-nitrophenol and 2-methylpropanoic acid were identified as candidate aggregation pheromones in the bont tick *Amblyomma hebraeum* by sampling from groups of ticks while they fed. An attempt to identify the compound(s) causing the unpleasant body odour of schizophrenics was unsuccessful due to inadequate detector sensitivity. Major volatile constituents were identified from the anal gland secretions of *Proteles cristatus, Cynictis penicillata, Atilax paludinosus, Ictonyx striatus* and *Hystrix africaeaustralis*.

A comparison of the performance of the DSE with published results from other sampling systems suggests that it is the most powerful available sampling technique for the quantitative analysis of mammalian odours by gas-liquid chromatography.

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OPSOMMING

Die organisasie van soogdiergemeenskappe word baie deur sosiale reukafskeidings beïnvloed. A.g.v. tekortkominge van die analitiese tegnieke wat verkry is uit ander gebiede van reukanalisie, is kennis van die chemie van hierdie afskeidings gebrekkig. Die bekende eienskappe van soogdierreuke dui daarop dat 'n toepaslike analitiese metode benodig word wat komponente in nanogram-hoeveelhede uit milligram-hoeveelede monster kan hanteer. Die dinamiese oplosmiddeleffek (DO) is ontwikkel as 'n monsteringstegniek vir gasvloeistofchromatografie. Hierdie tegniek maak ten volle gebruik van die kwantitatiewe analitiese moontlikhede van kapillêre kolomme en geassosieerde detektors. Die DO akkumuleer gasvervoerde verbindings uit die dampfase deur 'n dinamiese ewewig tussen die dampfase en 'n oplossmiddel op 'n poreuse bed. 'n Teoretiese model van die DO dui daarop dat die mate waarin 'n verbinding gekonsentreer word, afhang van die partisieverhouding tussen die vloeistof en gas.

Die DO word uitgevoer op gesinterde glas in 'n glasbuis (120 mm x 1,4 mm binnedeursnee). Hierdie buis word gedeaktiveer met silikon en eteen. Die monster word oorgedra na die kolom d.m.v. 'n vlekvryestaal en silika-inlaat wat verhit kan word. 'n Nuwe diagnostiese toetsmengsel is gebruik gedurende die ontwikkeling van die inlaat.

Die presisie van die DO vir opgeloste (beide water of organiese oplosmiddels) of gasvervoerde verbindings dui, op sensitiwiteit en afwesigheid van artefakte, wat net so goed of beter is as ander monsteringstegnieke wat bekend is uit die literatuur.

Bykomstige tegnieke soos oplosmiddel- en gassuiwering, die ontwerp van glasware en die hantering van reagense is aangepas vir die behoeftes van die DO.

Die DO monsteringstegniek is getoets op 'n aantal geselekteerde biologiese probleme. Die beskermingsafkskeiding van twee stinkbesies nl. *Thaumastella namaquensis* en *T. elizabethae* is geanaliseer om die sistematiek te verklaar. Komplekse en onverwagte patrone van statiese en veranderende samestellings is gevind toe die vlugtige bestandele van enkele reukmerke van die bruin hyena (*Hyaena brunnea*) oor nege dae bepaal is. In die komplekse mengsel van vlugtige bestandele wat deur manlike *Mastomys natalensis* en *M. coucha* afgegee word, is 3-noneen-2-oon net in *M. coucha* gevind. Daar is aangetoon dat die liggaamsreuk van manlike laboratoriummuise (*Mus musculus*) met sosiale status verander. Benzaldehied, 2-nitrophenol en 2-methylpropanoësuur is as kandidaat aggregasie feromone in die bontbosluis, *Amblyomma hebraeum* geïdentifiseer, deur 'n groep voedende bosluise te monster. 'n Poging om die verbinding(s) wat die onaangename liggaamsreuk by skisofrene veroorsaak, te analiseer was a.g.v. onvoldoende detektorsensitiwiteit onsuksesvol. Die vlugtige komponente van die anaalklierafskeidings van *Proteles cristatus*, *Cynictis penicillata*, *Atilax paludinosus*, *Ictonyx striatus* en *Hystrix africaeaustralis* wat in hoë konsentrasie voorkom is geïdentifiseer.

'n Vergelyking van die werksverrigting van die DO met gepubliseerde resultate van ander monsteringstegnieke, dui daarop dat dit die kragstigste beskikbare monsteringstegniek vir die kwantitatiewe analise van soogdierreuke vir gasvloeistofchromatografie is.

ACKNOWLEDGEMENTS

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Three people deserve special thanks; they are Egmont Rohwer for persuading me that a bed with a hole in it was worth trying, Keith Lawson for the idea of making the holes by horizontal sintering and Willie Viljoen for his expert running and interpretation of the mass spectra, from very difficult material, which appear in the following pages.

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Chapter 1

INTRODUCTION

Numquam efficietur

"But, independently of these obvious reasons, scent is affected by causes into the nature of which none of us can penetrate. There is a contrariety in it that ever has puzzled, and apparently ever will puzzle, the most observant sportsman ..... and therefore, in ignorance of the doubtless immutable, though to us inexplicable, laws by which it is regulated, we are contented to call it "capricious"." (Hutchinson 1909 p 102).

In the majority of mammals, apart from those which are permanently aquatic, signals transmitted by odours play an important, if not dominant, role in communication (Brown & Macdonald 1985) Dominance hierarchies, spatial organization, foraging patterns, mate choice, sexual maturation, mother-infant interactions, individual recognition and food choice are all affected by airborne, organic volatiles transmitted from one animal to another (Mugford & Nowell 1970; Müller-Schwarze 1974; Mykytowyzc 1974; Stoddart 1974; Henry 1977; Lenington 1983; Posadas-Andrews & Roper 1983; Cox 1984; Novotny, Harvey, Jemiolo & Alberts 1985; Halpin 1986; Vandenbergh & Coppola 1986; Galef, Mason, Preti & Bean 1988).

The growing fund of information on the behaviour of mammals in the wild has confirmed Mech & Peters' (1977 p 326) view; "It should be clear ..... that such detailed information about chemical communication automatically lends a great deal of insight into the social system of the species. In fact, it appears that one could hardly claim to understand a mammal's social system until that person gains detailed knowledge about the species' olfactory signalling."

Progress in the chemistry of mammalian social odours has not kept pace with the expansion of knowledge about their biology (Albone 1984 pp 9-11). While many factors have contributed to this state of affairs (Thiessen 1977; Mykytowycz 1979; Albone 1984 pp 5-11) I believe that the most important one is simply that the available analytical techniques have been inadequate to address successfully the quantitative chemical subtleties of mammalian odour signals. As will be discussed later, mammalian chemical signals present unique combinations of analytical problems which are considerably more challenging than those faced in "insect pheromone research, clinical

chemistry, flavour and perfume chemistry, environmental chemistry and natural products chemistry" (Albone 1984 p 9). The work reported here was undertaken in response to these analytical shortcomings.

Gas-liquid chromatography on open tubular columns is the most powerful available technique for separating the components of small quantities of complex mixtures of organic volatiles. If the performance of the ancillary instrumentation (Lee, Yang & Bartle 1984 pp 100-173) complemented that of the columns the analysis of mammalian social odours would be a matter of routine. Evidently this complementarity has not been achieved. A particularly weak link in the chromatographic chain is due to the techniques whereby the material to be separated is introduced to the column. Pretorius & Bertsch (1983a) have described sample introduction as the "Achilles heel" of chromatography and commented that "the performance of a lamentable proportion of otherwise excellent capillary columns is ruined by poor sample introduction".

The aim of the work reported here was to develop a novel sampling technique for capillary gas-liquid chromatography and to determine to what extent it enabled the potential of capillary column separations to be more fully utilized in the analysis of mammalian semiochemicals.

A point of departure from the types of sampling technique for gas-phase volatiles which had already been developed was provided by the concept of trapping volatiles on an evaporating film of organic solvent held in dynamic equilibrium between evaporation and capillary rise in a porous bed. This process became known as the dynamic solvent effect. It was hoped that a technique aimed from its inception at the quantitative analysis of odour signals might be more successful than one borrowed from another field.

The information available on the chemical properties of mammalian social odours has been limited by the shortcomings in the available analytical techniques. Because the analytical performance levels necessary for quantitative semiochemistry could not be defined *a priori* (that is from the known properties of the semiochemicals themselves) no amount of "in principle" argument or testing with synthetic mixtures could demonstrate that the goal had been achieved. Testing against real semiochemical problems was an inescapable requirement.

Scope of the Investigation

Both of the fields on which this thesis draws are large, diverse, and fragmentary. The review of chromatography by Poole & Schuette (1984) contains 658 references in the chapter on inlet techniques alone, and in the two volume work on "Social Odours in Mammals" edited by Brown and Macdonald (1985) the contributions of 13 authors cover 882 pages. To keep the present work within practical limits it was necessary to draw rather definite, but inevitably arbitrary, lines around the area it was intended to cover. Consideration has been given only to true odours, that is airborne volatiles, the mechanisms of chromatography have been considered only in so far as they contributed to the development of the dynamic solvent effect, no bioassays of candidate semiochemicals were carried out, and no direct experimental comparisons were made between the dynamic solvent effect and other sampling systems.

Volatility

Since gas-liquid chromatography is a vapour-phase technique it cannot be directly employed for the analysis of nonvolatile compounds. It is for this reason that only cases of true odour communication are considered here.

Chromatographic mechanisms

The mechanisms of chromatography have been dealt with repeatedly in the literature. Treatments (in order of increasing sophistication) are provided by Albone (1984 pp 279-285), Willard, Merrit, Dean & Settle (1981 pp 431-486) and Lee *et al.* (1984). The mechanism of solvent effect inlet systems for capillary gas-liquid chromatography is dealt with in a thesis submitted by Lawson in 1987, and a series of short papers (Pretorius & Bertsch 1983b; Pretorius, Lawson, Apps & Bertsch 1983; Pretorius, Lawson & Bertsch 1983a, b; Pretorius, Phillips & Bertsch 1983a, b, c; Pretorius, Rohwer & Lawson 1983; Pretorius, Rohwer, Lawson & Apps 1984; Pretorius, Lawson, Rohwer & Bertsch 1984; Pretorius & Lawson 1986a, b; Pretorius & Lawson 1987). Rather than repeating or anticipating this other work only those aspects of chromatographic mechanism directly relevent to the design of dynamic solvent effect sampling systems are presented here.

Bioassay

In the sense in which it is used here bioassay is simply the testing of an animal's responses to natural or artificial odours. While the "acid test" in a semiochemical investigation is the demonstration of appropriate behavioural or physiological responses to experimentally manipulated signals, the design and interpretation of bioassays are

not usually simple matters (Mackintosh 1985).

For example, despite its users' arguments to the contrary (Mykytovycz 1979), I remain unconvinced of the value of bioassays based on the sniffing of column effluents by restrained animals. There are problems with the validity of responses to consecutive, short bursts of single odours presented out of context. Experience with sniffing the outlet of capillary columns reveals an astonishing variety of odour qualities, ranging from the appetizing to the repulsive, in materials whose overall odour often bears no relationship to those of its separated volatile constituents. A test animal similarly sniffing at a column outlet might be presented with odour sensations associated with food, predators or sex when the original specimen was supposedly a territorial marker. Any or all of these sensations might produce a change in heart rate and even if a change is registered its significance in terms of the animal's "emotional" state is by no means obvious.

Even under reasonably "natural" conditions some aspects of an animal's response to an odour may be unexpected. In *Tupaia belangeri* the heart rates of animals introduced to a cage decrease faster if the cage has been marked by a dominant male than if the cage is clean (Von Holst 1985). As Johnston (1977) repeatedly stresses, findings with caged animals may not extrapolate to animals in the wild. In 2-choice bioassay tests dimethyl disulphide has been demonstrated to be a potent attractant for male hamsters (*Mesocricetus auratus*) (O'Connell, Singer, Pfaffman & Agosta 1979; but see also Johnston 1977). Dimethyl disulphide alone is approximately half as attractive as the vaginal discharge in which it occurs, which itself has very persistant attractant effects. Changes in the frequency with which females mark with vaginal discharge as they pass through four-day oestrous cycles led Johnston (1977) to suggest that it serves as an attractant of males to females in pro-oestrus who are about to become sexually receptive. Singer, Macrides & Agosta (1980) assigned this function specifically to dimethyl disulphide. However, dimethyl disulphide is one of the more widespread of the volatile components of mammalian secretions (see Chap 15 and references cited there) and it must be presumed that male hamsters are exposed to dimethyl disulphide from sources other than hamster vaginal secretion. What keeps them from continual and fruitless searching for receptive females ?, perhaps some of the other components of the secretions carry hamster/non-hamster messages. *M. auratus* males are attracted almost as much by *M. brandti* vaginal discharge as by that from females of their own species when each is presented separately, but in a 2-choice test males of both species prefer the odours of conspecific females (Johnston 1977). In addition to the ecological work called for by Johnston a bioassay of the attractiveness to male hamsters of anal gland secretion from e.g. porcupines (*Hystrix africaeaustralis*), or even yellow mongooses

(*Cynictis penicillata*), would be interesting.

Some bioassays show poor repeatability between laboratories; compare McKenna Kruse & Howard (1983) with Goodwin, Gooding & Regnier (1979), Johnston (1977) with O'Connell *et al.* (1979) and Singer, Agosta, O'Connell, Pfaffman, Bowen & Field (1976), and Michael & Bonsall (1977) with Goldfoot, Kravetz, Goy & Freeman (1976) (Bonsall & Michael 1980; Keverne 1983 pp 85-87; Albone 1984 pp 229-230).

A minimum, necessary condition for a valid bioassay is an understanding of the effects, under natural conditions, of the natural signal on the response being measured, be it heart rate, hormone levels or scent marking. To obtain even this baseline information in convincing detail is a major undertaking; see, for example, the work by Rasa (1973) on dwarf mongooses (*Helogale undulata rufula*), by Macdonald (1979) on urine marking in red foxes (*Vulpes vulpes*), by Epple and her co-workers on marmosets and tamarins (review Epple & Smith III 1985) and by Von Holst's group on tree shrews (review Von Holst 1985). Similar work was patently impractical within the scope of a method development project.

Comparisons of performance

Thiessen's (1977) warning against "the man of one method" notwithstanding, the diversity of alternative sampling techniques for capillary chromatography (see Chap 6) renders a standardized comparison among all of them practically impossible. A comparison between the dynamic solvent effect and the most commonly used technique for airborne volatiles; adsorption-desorption on Tenax, would have been interesting. Nevertheless, such a comparison would have been of doubtful value if the Tenax system had not received the same substantial investment in optimization as did the dynamic solvent effect. Comparison between the performance of the dynamic solvent effect and that of other systems was, therefore, based on published information.

Throughout the development and testing the viewpoint was adopted that neither the hardware, its operation or the experimental procedures should be any more elaborate than was necessary to solve the problem in hand.

Approach

Problems in mammalian semiochemistry can be approached from a variety of different angles depending on the interests of the investigator (Thiessen 1977; Albone 1984 pp 5-13). What answers are obtained, if any, will depend on the approach. An analytical chemist's "answer" might well be the identification, confirmed by synthesis, of one component of a mammalian secretion (e.g. Burger, Le Roux, Spies, Truter & Bigalke 1978; Maurer, Grieder & Thommen 1979) while that of a biologist might be a demonstration that animals spend longer sniffing one type of odour than they do another (e.g. Blizard & Perry 1979; Roeder 1980). Advocates of either approach can with some justification direct at their counterparts the sceptic's question "So what ?".

Claesson & Silverstein (1977) call identifying everything the "brute force approach". Paradoxically, in the investigation they cite as an example; the identification of isovaleric acid as an active constituent of male pronghorn (*Antilocapra americana*) subauricular secretion (Müller-Schwarze, Müller-Schwarze, Singer & Silverstein 1974) not every component of the secretion was eluted as a recognisable peak, far less identified, because packed columns were used and only compounds in the microgram range were identified.

One variant of the "identify everything" approach has been clearly set out by Burger, Le Roux, Bigalke & Novellie (1979); "Whilst continuing the investigation along classical lines by isolation and bioassay of the major components of exocrine secretions, an alternative approach, based on the recognition of chemical or physical similarities between the secretions employed by different species for the transmission of similar information, is being investigated. This approach necessitates the identification of all the components that may play a part in the function of a particular secretion as well as comparison of the secretions of as many related and unrelated species as possible". This approach requires not only that every component of a particular species' odour signals be identified but also that the process be repeated for several species. Quite apart from the biological difficulties inherent in recognising when truly similar information is being transmitted, and the untested assumption that this will involve similar chemicals, the analytical input required renders such an approach impractical.

The view that it might even be possible to identify every component of a mammalian odour remained tenable only as long as separations were carried out on packed columns. With these low-resolution separations even a "complex" chromatogram contained only a few tens of peaks so that their exhaustive identification did not appear impractical.

This picture changed sharply with the advent of high-resolution, capillary separations. Andersen & Bernstein (1975) separated skunk (*Mephitis mephitis*) anal gland secretion into three fractions on a packed column and identified all of them. Later, with a capillary column they identified 24 compounds (Andersen, Bernstein, Caret & Romanczyk 1982) but reported that capillary separations of the secretion had yielded some 160 peaks. Odours which appear simple when analysed at low sensitivity may reveal a daunting complexity when detection limits are lowered. For example the odour of the anal gland secretion of *Ictonyx striatus* separates into 15 peaks on a capillary column when 10 cm^3 is sampled and detection is by mass spectrometric total ion monitoring (Chap 15) (Fig. 1.1 a). When the same volume is sampled and detection is by the more sensitive flame ionization detector it becomes apparent that several minor components were previously undetected (Fig. 1.1 b) and when 150 cm^3 is sampled the chromatogram's complexity makes impractical any attempt to identify everything in it (Fig. 1.1 c).

Given that a full analysis of an odour is impractical or impossible due simply to the number of compounds involved, it becomes desirable to restrict analytical attention to those components of the odour which are known to have semiochemical significance. Accepting for the sake of the present discussion that a relevant bioassay is actually available (see above) there is a spectrum of methods which have been applied to the assignment of semiochemical activity to a particular component or set of components.

A broad fractionation based on e.g. volatility (Epple, Golob & Smith 1979) or chemical character (Hesterman, Goodrich & Mykytowycz 1981) may be applied and followed up by progressively finer splitting until a single active component is isolated or, more likely, activity is lost. This method depends on all the active components having in common the property distinguishing a given fraction.

Narrow fractionation proceeds directly from the whole odour to bioassay of "single" components (Singer *et al.* 1976; O'Connell *et al.* 1979). If semiochemical activity depends on a synergy between two or more compounds this approach is almost certain to be unproductive. The prospect of "testing all combinations of the fractions" (Claesson & Silverstein 1977) is hardly an attractive one when a separation has yielded a 100 peak chromatogram. The closest practical approach may be to systematically subtract various components.

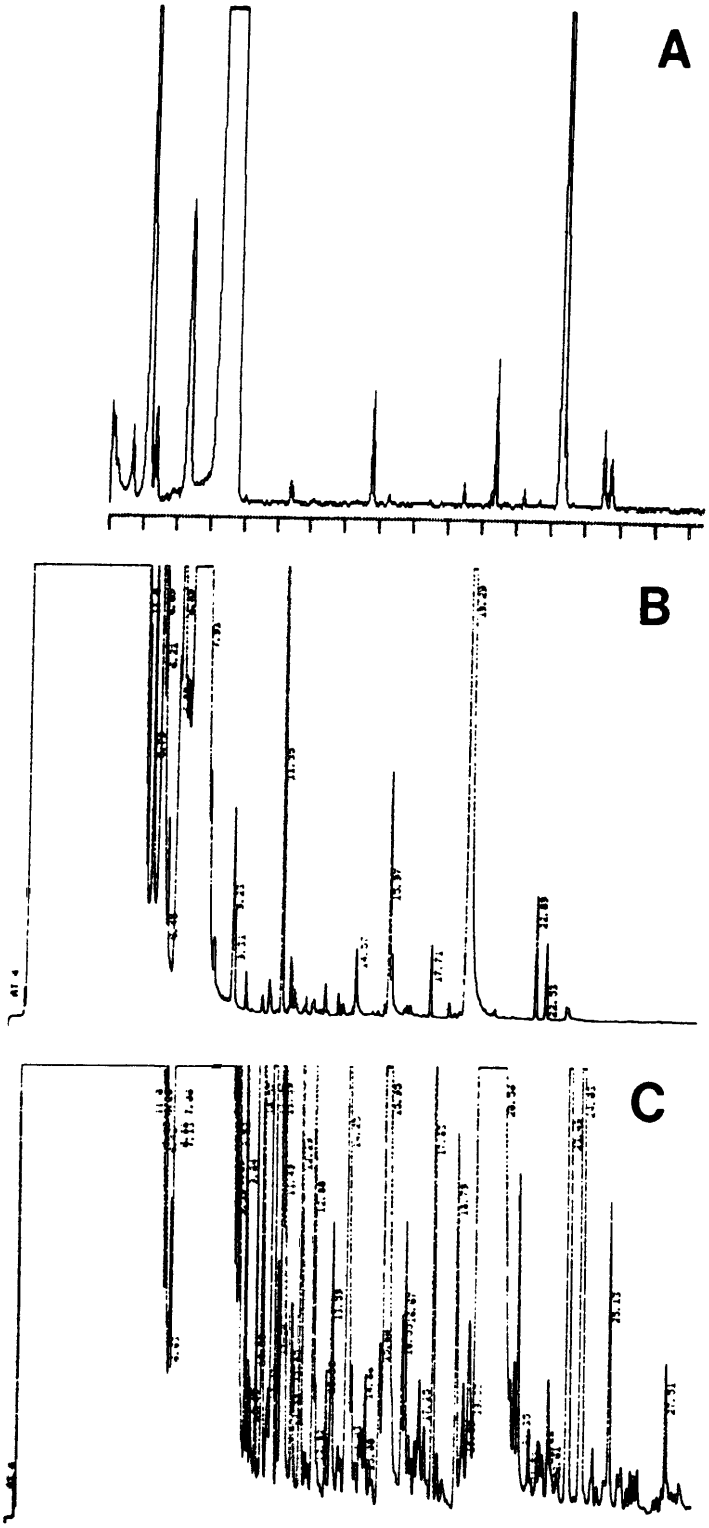


Fig. 1.1 Series of chromatograms of headspace volatiles from the anal gland secretion of a male *Ictonyx striatus* to illustrate increasing complexity with increasing analytical sensitivity. A: 10 cm^3 headspace, mass spectrometer total ion monitor detection; B: 10 cm^3 head space, detection by flame ionization detector (FID) at a sensitivity of 4 x 10^{-11} A mv^{-1} full scale deflection; C: 150 cm^3 headspace, FID detection at 4 x 10^{-11} A mv^{-1}.

An extension of narrow fractionation is to monitor the responses of an animal exposed to the column effluent (Goodrich, Hesterman, Shaw & Mykytowycz 1981). Like the last method its validity depends on semiochemical activity residing in single compounds but it suffers the additional drawback that the putative signals are presented out of context (see above).

All the above methods have been labelled "response guided" by Albone (1984 p 5), who seriously questions their general validity in work on mammals. He suggests as a complementary approach a "chemical image strategy"; "a valuable alternative approach might be the holistic one in which the nature of the entire chemical image presented by one animal to another is surveyed". Such an approach inevitably incorporates exhaustive identification, though it does not rely on intermediate bioassays. In practise it would probably come to resemble Burger *et al.*'s (1979) comparative, "identify everything" approach. The most attractive aspect of a chemical image strategy is its independence from any assumptions about the nature of the chemical signal.

This advantage is shared by what may be called the "chemical image differentiation" approach. This approach, which has been adopted in section 2, may be summarized as follows:-

That animals are able to discriminate between one class of odours and another, e.g. between the odours of different individuals, of sexually receptive and non-receptive females, or of scent marks of different ages, can be rigorously demonstrated by observation under natural conditions, or by experiment.

These discriminations must be based on some characteristic of the odours which differs more between classes than within them.

If odours from each class are appropriately sampled, and separated by high resolution gas chromatography the series of chromatograms should exhibit features (presence or absence of peaks, patterns of relative peak areas etc) which similarly differ between, and not within, classes.

The differentiating peaks are then strong candidates for the role of chemical signals and identification, synthesis and bioassay can be confined to them rather than exhaustively to the whole chromatogram.

Chemical image differentiation is by no means a new approach; it was used in 1969 by Brownlee, Silverstein, Müller-Schwarze & Singer. They concentrated their attention on one component of the black-tailed deer's (*Odocoileus hemionus columbianus*) tarsal gland scent on the basis of its being much more abundant in the male than in the female

(Claesson & Silverstein 1977). More recently the differentiation approach has been used with considerable success by Novotny's group; mouse urine components whose production was sex-, or hormone-dependent being selected for identification, synthesis and bioassay (Novotny, Schwende, Weisler, Jorgenson & Carmack 1984; Novotny, Harvey, Jemiolo & Alberts 1985; Schwende Weisler, Jorgenson, Carmack & Novotny 1986).

An approach to the biology of chemical communication which is implicit in the concept of "chemical ecology" and which reached something of an apogee in Stoddart's (1980) book "The Ecology of Vertebrate Olfaction" is that chemical signals operate, and should be understood, at the population or species level (see e.g. Whitten & Bronson 1970 p 320; Mykytowycz 1974; Stoddart 1974 p 300, 1976 p 42, 1980 pp 85, 106, 123, 141-142, 148; Rogers & Beauchamp 1976 p 182; Vandenbergh & Coppola 1986; pp 84-85). This is in contrast to developments in theoretical biology which have stressed the role of the individual (e.g. Williams 1966; Hamilton 1971; Trivers 1972; Maynard Smith 1974, 1982; Dawkins 1982; Lomnicki 1982), and to the results of field studies of mammalian societies which have revealed striking intra-specific variability and the importance of differences between individuals in determining structure and process at higher levels (Simpson 1973; Slater 1981; Macdonald & Moehlman 1982; Macdonald 1983; Rood 1983). Behavioural evidence points to an important role for olfaction in individual recognition and its consequences for social organisation (Rasa 1973; Gorman 1976; Roeder 1980, Halpin 1986). Clearly the chemical analysis of semiochemicals must also be carried out at the individual level, that is to say, with samples from single animals.

Terminology

Three problems of terminology need to be dealt with at the outset. "Pheromones" were defined by Karlson & Lüscher (1959) as "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example a definite behaviour or a developmental process." This definition was formulated in the context of insect chemical communication where, at that time at least, there were good grounds for believing that single compounds were involved, that "release" was an appropriate term for the ways in which the responses were elicited and where it appeared that, while the insect was signalling, the pheromone was the only compound being released into the surroundings. Even for insects this simple picture has now become much more complex. Many pheromones have been demonstrated to be multicomponent blends whose precise quantitative composition is critical for biological activity (Descoins & Frerot 1979; Mustaparta 1979; Ritter 1979 pp 8-11; Roelofs 1979; Silverstein 1979; Tamaki 1979). Whether or not a chemical releases a response can depend on

time of day (Brown 1972). There are cases in which an eliminatory product such as faeces contains a signalling compound (e.g. Persoons & Ritter 1979) and at least one case, the honeybee (*Apis mellifera*) where large quantities of apparently inactive material are continually released.

The methods used in insect semiochemistry were soon applied to mammals (Brownlee *et al.* 1969) and since then the word "pheromone" has been applied to almost any substance not obviously eliminatory which mammals shed into their environment (Goodrich & Mykytovyc 1972; Burger, Le Roux, Garbers, Spies, Bigalke, Pachler, Wessels, Christ & Maurer 1976; Fass & Stevens 1977; Burger, Le Roux, Spies, Truter & Bigalke 1981). With forgivable, and only slight, overstatement Beauchamp, Doty, Moulton & Mugford (1976 p 148) and Bronson (1976 p 123) referred to such indiscriminate use of the term as a bastardization. Whether or not the pheromone concept is appropriate in mammalian biology has been discussed many times (Beauchamp *et al.* 1976; Bronson 1976; Bronson, Burghardt, Stürckow & Wilson 1970; Mykytowycz 1979; Stoddart 1976 pp 2-3; Thiessen 1977; Yamazaki, Yamaguchi, Boyse & Thomas 1980), but always with the limitation that too little is known about mammalian chemical communication for such discussions to be firmly empirically based.

While I find the biological arguments against regarding mammalian chemical communication as pheromonal more convincing than those in favour, the strongest objection to the indiscriminate use of the term adopted in the literature of chemistry and reproductive physiology is a semantic one. If "pheromone" continues to be used loosely (or is redefined (Thiessen 1977)) as a synonym for "chemical signal" or "semiochemical" a need will arise to coin a new term for those compounds which actually are pheromones according to the original definition. A situation will then have arisen in which "pheromone" means one thing to an entomologist and another to a mammalogist and where true pheromones are labelled as such in insects but called something else in mammals.

In some cases "pheromone" may indeed be the appropriate term but if the word is to retain any value its use should be deferred until conformity with the original definition has been demonstrated.

Besides "pheromone" at least 21 other terms have been used as labels for various classes of chemical communication (*sensu lato*) (Claesson & Silverstein 1977; Macdonald & Brown 1985). In mammals the practical difficulties of applying these classifications, or even of recognizing when "communication" is taking place (Burghardt 1970), have restricted their application and are likely to continue to do so until much more is known about the modes of operation of mammalian semiochemicals.

To avoid fruitless semantic controversy the term "semiochemical" is employed for a substance which transfers information from one animal to another. Where substances are released into the environment but their semiochemical activity has not been demonstrated terms such as "scent" and "odour" are used.

The nomenclature of chromatography is scarcely, if at all, clearer than that of chemical communication. One reason for this may be that new terms have been coined on an *ad hoc* basis in a field in which rapid progress has been made by several groups working simultaneously. For example; what Jennings, Freeman & Rooney (1978) call "the solvent effect" is what Grob (1983) calls "phase soaking" and what Pretorius & Lawson (1986b) call "stationary-phase swelling".

While a terminology based on mechanism rather than effect is desirable its application presupposes that the mechanisms are understood, a goal which, despite Grob's (1987 p vi) optimism, has not yet been reached.

An attempt has been made by Pretorius & Bertsch (1983a) to develop a classification system for gas chromatographic sample introduction which would allow an "unambiguous description" of a particular separation. While the proposed system can, in principle, accommodate any possible sampling method the full, formal classification of some techniques is inconveniently lengthy. For example the dynamic solvent effect sampling and subsequent transfer to a capillary column of the volatiles from a portion of the anal-gland contents of some mammal is, formally:-

During accumulation of volatiles

sample pre-treatment:	sample splitting
sample inlet geometry:	perforated packed bed
primary operational modes:	splitting/non-splitting, vapourizing, solvent focussing
secondary operational modes:	solvent splitting, solute non-splitting, slow vapourizing, isothermal solvent film

During transfer of volatiles to column

sample pre-treatment:	none
sample inlet geometry:	perforated packed bed, dynamic solvent effect inlet
primary operational modes:	non-splitting vapourizing solvent focussing stationary phase focussing
secondary operational modes:	slow vapourizing isothermal sample film.

Such terminology is, self-evidently, inappropriate to concise descriptions of methods and discussion of results and mechanisms.

Grob's (1987) terminology is pictorially descriptive but consists mainly of a labelling of specific phenomena which cannot easily be generalized to circumstances other than liquid injections into smooth-walled, open-tubular inlets. Since the dynamic solvent effect can operate only in a porous packed bed Grob's terminology is not straightforwardly applicable to it.

The term "dynamic solvent effect" is employed hereafter as a workable condensation of the Pretorius-Bertsch formal description above.

The third problem of terminology is introduced by the different ways in which biologists and chemists use the word "sample". Most biologists would understand a sample to be a (usually representative) subset of a population (Runyon & Haber 1980 p 7). Most chemists would regard a sample as something which was to be analysed, and only in certain cases would such a "sample" be used to estimate the parameters of a population (Bertsch 1981; Reid 1980; Woodbridge & McKerrell 1980; Beyermann 1984 pp 91-130). In chromatography "sample" has acquired the additional connotation as that material (possibly somehow derived from an initial "sample") which is actually introduced to the gas chromatograph.

These three uses of the term are so well entrenched, and so well understood within each field, that to propose any alternative would be futile. In an attempt to reduce possible confusion the following terms have been adopted. A "sample" is the material which is transferred to the separating column (usual chromatographic usage). The material from which the sample is obtained is termed a "specimen" (to distinguish it from a biological "sample"). "Sampling" is the process which turns a specimen into a sample.

Arrangement and Presentation

The thesis falls into two parts. The first; Chaps 2-7, concerns the dynamic solvent effect's mechanism, the development of hardware and operating procedures, and its performance with synthetic mixtures. The second is a series of studies of particular biological problems selected as examples of different types of analytical challenge.

Chapter 2

TARGETS OF SAMPLING SYSTEM PERFORMANCE

ANALYTICAL CHALLENGES POSED BY AIRBORNE VOLATILES

Two fields of analysis which are concerned principally with airborne volatiles are air pollution and flavours and fragrances. Due to commercial, medical and environmental interest these have received an enormous research and technical input. Reviews are provided by the chapters by Bertsch, Shibamoto, Jennings, Verzele & Sandra, and Rapp in Jennings (1981).

Complexity

In both these fields samples are, at least potentially, complex mixtures covering a wide range of chemical types. For example 680 volatile compounds have been identified in coffee (Van Straten, Maarse, Beauveser & Visscher 1983), 218 in cognac (Ter Heide, Valois, Visser, Jaegers & Timmer 1978) and 40-136 per study in city air (Lee *et al.* 1984 p 313).

Diversity

Natural flavours and fragrances are approximately as chemically diverse as mammalian odours (see below) but some unusual compounds are found among synthetic fragrances e.g. the nitro-musks (Wood 1982). Air pollutants are chemically more diverse than either natural fragrances or mammalian odours because they include such anthropogenic compounds as halocarbons and polyaromatic hydrocarbons. On the other hand their dominant components are hydrocarbons which present no special analytical difficulty (Bertsch 1981).

Concentration

Air pollutants have been measured at low parts per trillion concentrations and in quantities of 3 pg (R. L. Grob & Kaiser 1982 p 168), although typical concentrations are in the parts per billion range (Bertsch 1981). The concentration target levels for flavour analysis are similar to those for mammalian odours since in both cases the ultimate aim is to match the performance of the mammalian nose (Table 2.1). Target concentration limits for air pollutants should be below those at which biological effects are produced.

Lability

There are probably no material differences in component lability between flavours, air pollutants and mammalian volatiles (Devitofrancesco, Furnari, Bacaloni & Petronio 1981; Rudolph, Ehhalt, Khedim & Jebsen 1981; Shibamoto 1981).

Interfering components

In both flavour and fragrance, and air pollution work the compounds of interest may make up only a small proportion of the total volatiles present. For example, only 3-4% of the absolute of jasmine is made up by its three fragrance impact compounds (Demole 1982). A similar situation may occur in cases of air pollution by particular, highly toxic compounds (Bertsch 1981 pp 99-104). In these situations the volatile components which are not of direct interest may interfere with the analysis of those that are, the most common problem being that a major "unimportant" component coelutes with a minor one. A particularly troublesome interfering volatile which is of almost universal occurrence is water (see p 148).

Specimen size

In flavour research specimens are typically in the tens of grams to tens of kilograms range (e.g. Lund & Dinsmore 1978; Noble 1978; Rapp & Knipser 1979; Buttery, Seifert, Guadagni & Ling 1969); Moshonas & Shaw 1983; Ho, Lee & Jin 1983) and extend up to hundreds of kilograms e.g. Ter Heide *et al.* (1978) extracted the trace components from 465 l of cognac, and Kami (1983) steam distilled 230 kg of alfalfa. The "micro" scale in plant volatile work deals with specimens in the 0,2-0,5 g range (Bicchi & Sandra 1987). In pollution studies volatiles may be sampled from tens of litres or cubic metres of air (Bertsch, Zlatkis, Liebich & Schneider 1974; Lewis & Jackson 1982).

ANALYTICAL CHALLENGES POSED BY MAMMALIAN ODOURS

Paradoxically the analytical problems posed by semiochemical materials can be fully understood only once they have been overcome. What follows is, therefore, a summary of the properties of odour secretions based on analyses already carried out, and on the characteristics of the chemical signal's receptor; the mammalian olfactory system.

Complexity

It is, I think, generally accepted that mammalian odours are complex mixtures of volatiles (which is not equivalent to saying that all mammalian semiochemicals are necessarily also complex). The number of compounds identified from different sources varies from 1 in the striped hyaena (*Hyaena hyaena*) (Wheeler, Von Endt & Wemmer 1975), 2 in the reindeer (*Rangifer tarandus*) preorbital gland (Andersson 1979), 3 in the sternal gland of *Galago crassicaudatus* (Crewe, Burger, Le Roux & Katsir 1979), 12 in wolf (*Canis lupus*) urine (Raymer, Wiesler, Novotny, Asa, Seal & Mech 1984), 63 in wolf anal sac (Raymer *et al.* 1985), 57 in rabbit (*Oryctolagus cuniculus*) faeces (Goodrich *et al.* 1981) and 61 in mouse urine (Shwende, Wiesler, Jorgenson, Carmack & Novotny 1986), to 133 in human whole-body volatiles (Ellin, Farrand, Oberst, Crouse, Billups, Koon, Musselman & Sidell 1974).

Much of this apparent variation in odour complexity, and the simplicity of some secretions, can be attributed to differences in analytical methods and approach. The influence of the analytical method is particularly evident in the case of the analysis of the skunk's (*Mephitis mephitis*) anal gland secretion; where packed column chromatographic analyses identified 8 compounds high resolution capillary methods identified 25 (Andersen *et al.* 1982). In most, if not all, cases several compounds are unidentified, in Raymer *et al.*'s (1984) chromatograms of wolf urine volatiles there are more than 60 unidentified peaks.

Concentration

Where they have been reported concentrations of volatiles in mammalian secretions vary from the parts per million to the percentage level, and quantities per gland or per scent mark are usually in the micro- or milligram range. Crewe *et al.* (1979) found benzyl cyanide, 2-(*p*-hydroxyphenyl)-ethanol and *p*-hydroxybenzyl cyanide to make up 8 %, 17 % and 56 % respectively of the galago's (*Galago crassicaudatus*) sternal gland secretion, and that one scent mark contained 0,5 µg of benzyl cyanide. Wellington, Byrne, Preti, Beauchamp & Smith III (1979) quantified 30 compounds with concentrations above $30{:}10^6$ in guinea pig (*Cavia* sp.) anal gland secretion. Muscone makes up 0,5-2 % of musk (Albone 1984 p 85). The components of pronghorn subauricular scent identified by Müller-Schwarze *et al.* (1974) occurred in 10-100 µg quantities per gland. Aliphatic acids are present in $16\text{-}6000{:}10^6$ concentrations in dog anal sac secretion (Preti, Muetterties, Furman, Kennelly & Johns 1976).

These data are without doubt biased towards more abundant components; compounds at lower levels being undetected or unquantifiable.

There appear to be no data in the literature on the vapour-phase concentrations of mammalian odourants except those of Wellington *et al.* (1979) which are expressed in terms of mg g^{-1} of secretion, and cannot be converted to more meaningful units.

Chemical diversity

Volatiles from mammals contain as wide a range of chemical types as those from other sources (Wheeler 1977; Albone 1984 pp 32-33, 74-134; see also Chaps 10, 11, 12, 14, and 15). With such a diversity it cannot be safely assumed that any particular class of organic volatile is absent from an unanalysed specimen. Compounds as different as aliphatic acids and amines may occur together (Albone & Perry 1975; Preti *et al.* 1976).

Lability

Some of the compounds identified from mammalian secretions are thermolabile, e.g. dimethyl trisulphide (Singer *et al.* 1976) and hydroxyketones/lactols (Schwende *et al.* 1986). It must be assumed that there are other, even more thermolabile, compounds which have escaped detection altogether or have been detected only in the form of their unrecognised breakdown products.

Interfering compounds

Although much less information is available, at least some types of mammalian odours have minor components which are active. For example, feeding by snowshoe hares (*Lepus americanus*) is suppressed by 3-propyl-1,2-dithiolane which is a minor component of stoat (*Mustela vison*) anal gland secretion but not by 2-propylthietane which is a major component (Sullivan & Crump 1984). The semiochemically active compound dimethyl disulphide is a minor volatile among those from golden hamsters (Singer *et al.* 1976). The presence of water is a possibility; mammalian odour sources may be dry, e.g. some scent marks, wet, e.g urine, or warm and wet, e.g. whole mammals.

Specimen size

In contrast to the situation in other fields much biological material is available in strictly limited quantity. An aardwolf (*Proteles cristatus*) anal gland pasting contains approximately 5 mg of secretion (Chap 15). The total body weight of a Thaumastellid bug is 0,3-0,8 mg (Chap 9). One "female equivalent" of golden hamster vaginal discharge is 20 mg of secretion, which contains 5 ng of semiochemically active dimethyl disulphide (O'Connell *et al.* 1979). Tree shrew scent marks contain 2-20 mg of secretion (Von Holst 1985).

Mammalian olfactory thresholds

The sensitivity limits for the mammalian nose are extremely low (Table 2.1). Fortunately the assumption that successful work on mammalian semiochemistry demands an analytical system with similar capabilities is unsupported by both theory and practice. First, it is unlikely that a communication system would normally operate near the limits of detection of its signals. An analogy may be drawn from auditory communication. The threshold of human hearing is a sound pressure of 0 dB, normal speech has a sound pressure of 6 dB -- six orders of magnitude higher than the threshold (Geldard 1960 p 104). A semiochemical example is provided by the golden hamster (*Mesocricetus auratus*). Dimethyl disulphide is a component of the female's vaginal discharge to which males are extremely sensitive; 50 fg is an overestimate of the minimum detectable quantity. Nevertheless there are 5 ng of dimethyl disulphide in the 20 mg of secretion which can be wiped from a female hamster's perineum (O'Connell *et al.* 1979). Secondly, if the assumption were true there would have been negligible progress in the analysis of mammalian chemical signals. Even under favourable conditions one nanogram of a compound is needed for its GC-MS identification; to accumulate that quantity from a solution with a concentration of $1:10^{14}$ would require a 100 l specimen. If such a volume of urine were required from a single mouse its collection, at approximately 2 cm^3 per day, would take 137 years.

Thus, in terms of specimen concentration, sample complexity, component chemical diversity, sample lability and the presence of interfering compounds, similar analytical problems are posed by air pollutants, flavours and fragrances, and mammalian odours. From the point of view of analytical technique the most important difference between work on semiochemicals and that on air pollution or flavours etc is the limited size of the specimens.

Table 2.1 Olfactory detection limits of airborne volatiles for some species of mammals.

Species	Odorant	Threshold	Reference
rat	C_5-C_7 alkanes	$1:10^{12}$-$1:10^{9}$	Goff 1961*
rat	octanol	$5:10^{9}$	Bennet 1968*
rat	cyclohexanone	$2:10^{8}$	Davis 1973
dog	fatty acids	$1:10^{12}$-$1:10^{11}$	Neuhaus 1953*
dog	fatty acids	$<1:10^{12}$-$1:10^{7}$	Moulton *et al.* 1960*
dog	alpha-ionone	$2:10^{14}$	Moulton 1977
human	heptanol	$1:10^{8}$	Stone 1963*
	heptanone	$8:10^{9}$	
	octanone	$6:10^{9}$	
	ethyl valerate	$2.5:10^{9}$	
human	amyl acetate	$3:10^{10}$	Davis 1973
human	cyclohexanone	$6:10^{11}$	
human	isovaleric acid	$1:10^{9}$	Amoore 1982**
	1-pyrroline	$2:10^{9}$	
	trimethylamine	$1:10^{9}$	
	isobutaraldehyde	$5:10^{9}$	
	androstenone	$2:10^{10}$	
	ω-pentadecalactone	$2:10^{8}$	
	l-carvone	$6:10^{9}$	
	1,8-cineole	$1:10^{8}$	
human	butyric acid	$4:10^{9}$	Punter 1983***
	isohexanoic acid	$8:10^{9}$	
	2,4-dichlorophenol	$3:10^{11}$	
	2,4,6-trichlorophenol	$2:10^{11}$	
	isoamyl acetate	$13:10^{9}$	

* cited in Davis 1973; ** these compounds are eight of Amoore's primary odourants; *** examples selected from an extensive list.

ESTABLISHED TECHNIQUES FOR MAMMALIAN ODOUR ANALYSIS

The techniques which have been applied to the analysis of mammalian odour volatiles cover a surprisingly restricted subset of the very wide range which has been developed.

Sampling

When glandular secretions have been analysed they have usually been squeezed, scraped, sucked or swabbed from their sites of production (Claesson & Silverstein 1977). On the other hand Wheeler *et al.* (1975) analysed striped hyaena scent marks, Kalina & Adams (1984) intermandibular gland marks from the mouse deer (*Tragulus napu*), Yarger, Smith III, Preti & Epple (1977) the scent marks of saddle-back tamarins (*Saguinus fuscicollis*) and Mills, Gorman & Mills (1980) anal gland pastings from brown hyaenas (*Hyaena brunnea*). Urine has usually been collected after production but Raymer *et al.* (1984) catheterized drugged wolves. An elegant urine collection method is that of Jorgenson, Novotny, Carmack, Copland, Wilson, Katana & Whitten (1978) in which fox (*Vulpes vulpes*) scent marks are collected, already frozen, from fresh snow. Faeces have been investigated only rarely and have then been obtained *via* the natural route (Goodrich *et al.* 1981).

With few exceptions volatiles have been separated from the specimen matrix by one of two methods; extraction (with or without simultaneous distillation), and gas stripping. Solvent extractions have sometimes been made specific to one chemical class by adjustment of pH (Maurer *et al.* 1979; Wellington *et al.* 1979). Gas stripping of volatiles ("dynamic head-space") has been widely applied to glandular secretions (e.g. Wellington *et al.* 1979), urine (Goodwin, Gooding & Regnier 1979; Jorgenson *et al.* 1978; Schwende *et al.* 1986), faeces (Goodrich *et al.* 1981) and, in one case, to whole mammals (Von Stralendorff 1982). Heating and pH adjustment during stripping have been employed (Bailey, Bunyan & Page 1980)

In a few cases raw secretion has been injected into the gas chromatograph (Preti *et al.* 1976; Crewe *et al.* 1979; Burger, Le Roux, Spies, Truter, Bigalke & Novellie 1981; Gorman, Kruuk & Leitch 1984).

The chromatography of intractable compounds such as fatty acids has been facilitated by derivatization (Hefetz, Ben-Yaacov & Yom-Tov 1984). Goodwin *et al.* (1979) took the unusual course of combining gas stripping with "on-adsorbent" derivatization.

The traditional approach to the problem of restricted specimen size has been to pool material from large numbers of individual animals. Chow, Wang & Lin (1975) extracted 100 000 whole dog ticks (*Rhiphicephalus sanguineus*) and Goodrich & Mykytowycz (1972) used material from 1 450 rabbits in one study. On a smaller scale, recent work by Novotny's group (e.g. Novotny, Schwende, Wiesler, Jorgenson & Carmack 1984, Andreolini, Jemiolo & Novotny 1987) on laboratory mice has employed urine specimens accumulated from groups of 3-10 mice, sometimes over periods of 2-3 days.

Separation

Both packed, and open tubular columns have been used. Among the latter wall-coated columns have been the most common, although support coating has found occasional application (e.g. Goodrich *et al.* 1981). Stationary phases in both packed and open tubular columns have included glycols (Andersen *et al.* 1982; Schwende *et al.* 1986), methylsilicones (Brinck, Gerell & Odham 1978; Crump 1980a, b) and phenylmethylsilicones (Andersen & Bernstein 1975). Ellin *et al.* (1974) used three different liquid, and one solid phase to separate different classes of human organic volatiles. The polar phases such as SP 1000, Carbowax and FFAP have been used more widely than the non-polar silicones except for derivatized samples and the anal gland secretions of mustelids.

Detection

As in the case of general gas chromatographic analysis the FID has served as a universal detector for separated odour volatiles. Of the specific detectors the flame photometric in the sulphur mode, and the thermionic in the nitrogen mode have also been employed (Liebich, Zlatkis, Bertsch, Van Dahm & Whitten 1977; Jorgenson *et al.* 1978; Schwende *et al.* 1986).

Identification

It is only very rarely that mass spectrometry (MS) has not been used for identification. Gorman, Nedwell & Smith (1974) based identifications only on retention times, and Andersen & Bernstein (1975) used nuclear magnetic resonance (NMR) and infra-red adsorption spectroscopy (IR). Usually identifications have been based on low-resolution spectra, but high resolution techniques have sometimes also employed (Preti *et al.* 1976; Liebich *et al.* 1977). Mass spectra have, quite frequently been supplemented by IR and NMR.

In exceptional cases "wet" chemistry has been applied to fractions trapped at the column outlet; Andersen & Bernstein (1975) used the melting points of sulphide derivatives to confirm the identification of thiols and Macdonald, Krantz & Aplin (1984) employed a colour reaction between cholesterol and antimony trichloride.

ASSESSMENT OF ESTABLISHED TECHNIQUES FOR ODOUR ANALYSIS

It is apparent from their appearance in the literature that the techniques reviewed above have been successful. This is not to say, however, that alternative techniques might not have worked better.

Sampling

"It must be emphasized that no step in the entire analytical process is more critical than that of sample preparation. All too frequently the investigator irretrievably compromises his results even before beginning the analysis." (Jennings 1981 p 513)

Since mammalian odour signals are dilute mixtures of gas-phase volatiles sampling by solvent extraction must inevitably cause distortion of the quantitative volatile profile. This problem is compounded if the material which is extracted differs from that which normally emits the signal e.g. if gland contents rather than real scent marks are used. Selective extraction procedures implicitly pre-judge the nature of the chemical signal.

Injection of raw secretion may be expected to bias the chromatographic profile towards its less volatile components which would normally have low emission rates from the secretion and thus low concentrations in its odour.

If gas stripping is used to separate volatiles and matrix the chromatographic profile will reflect more closely that of the "real" odour than if extractions or direct injections are employed. Nevertheless the limitations on the preferred choice of odour source still apply. Care should also be exercised in the adjustment of pH or use of heat to increase the yield of volatiles because these can unpredictably influence the relative quantities of some components (Wellington *et al.* 1979). For rigorous quantitation the effects of humidity need also to be taken into account (Regnier & Goodwin 1977; see also Chap 10).

Thermal desorption from adsorbents such as Tenax might be expected to cause breakdown of thermally labile compounds. Schwende *et al.* (1986) report just such an occurrence with hydroxyketones/lactols from mouse urine but Singer *et al.* (1976) successfully recovered dimethyl trisulphide from hamster vaginal discharge.

Except in special cases pooling has a serious practical drawback; the required number of individual animals may exceed the supply. Goodrich & Mykytovycz's (1972) sample size of anal glands of the European rabbit exceeds the total population of its relative *Bunolagus monticularis* (Duthie 1988) and even for more common species there are formidable difficulties in acquiring hundreds of individuals. The exceptions are animals which are very common pests, as is the rabbit, those which can be bred in large numbers, such as laboratory rodents, and those which are commercially exploited.

A second disadvantage of pooling is that inter-individual differences are obscured. For example, Liebich *et al.* (1977), on the basis of work with *Mus musculus* urine accumulated into a 15 cm^3 pool, reported that "The profiles of volatile components in urines of different mice of the same strain and sex under the same nutritional conditions are subject to very small qualitative and quantitative variations." whereas findings presented in Chapter 12 show very marked inter-individual differences in chromatographic odour profile in the same species when individual mice were used as odour donors.

Differences in volatile production associated with changes in e.g. physiology may be successfully studied with pooled specimens by grouping animals in similar physiological condition. This approach has been used by Novotny's team in work on mouse semiochemicals (e.g. Novotny, Jemiolo, Harvey, Wiesler & Marchlewska-Koj 1986; Andreolini *et al.* 1987; Jemiolo, Andreolini, Wiesler & Novotny 1987) but its requirement for uniform groups restricts its practical application to inbred laboratory rodents (Epple, Alveario, Golob & Smith III 1980 p 737).

On the other hand pooling may be deliberately employed to <u>eliminate</u> the effects of individual variation. Epple *et al.* (1979) combined scent marks from 3 males of each of the subspecies *Saguinus fuscicollis fuscicollis* and *S. f. illigeri* for behavioural testing of subspecific odour differentiation, and from 5 males of each subspecies for chromatographic analysis. In the investigations of *Thaumastella* (Chap 9) and porcupine (*Hystrix africaeaustralis*) (Chap 15) secretions, specimens with marked differences were combined to yield samples in which all the volatiles were present in quantities large enough for GC-MS identification, thus reducing the number of GC-MS runs required.

Appropriately applied, most sampling systems will provide some information about what is in an odour source but if the aim of the investigation is to understand a chemical signalling system it is necessary to keep in mind that what is in an odour source is not necessarily the same as what comes out of it.

This is most clearly exemplified by a series of investigations of the role of aliphatic acids as "copulins" in rhesus monkeys (*Macacca mulatta*) (Michael & Keverne 1968; Michael, Keverne & Bonsall 1971; Michael & Bonsall 1977; Bonsall & Michael 1980, Albone 1984 pp 229-230; Epple 1985 pp 762-763). On the basis of male sexual activity odour cues were implicated as one channel for signals of female receptivity. Short-chain fatty acids occur in the females' vaginal fluid, and when applied to ovariectomized females increase their attractiveness to males. When vaginal lavages were analysed for fatty acids their concentrations were found to fall during the periovulatory period when the females are most attractive and receptive. These contrasting findings were reconciled by demonstrating that at mid-cycle the vaginal fluid with its complement of acids passes more rapidly onto the perineum where presumably it is more accessible to males. The total concentration of fatty acids on the perineum was found to increase at that time but the analyses were based on sampling by swabbing and extraction and the results were erratic. There was not, and as far as I know has not been, an analysis of the actual signal perceived by the males; the odour of a female rhesus monkey.

Separation

It is now firmly established that packed columns are markedly inferior to capillaries for the analysis of complex samples (Hermann & Seiber 1981 p 177; Jennings 1981 pp 511-512; Lin 1981 p 125; Shibamoto 1981 pp 474-476; Lee *et al.* 1984 pp 1-13). The use of packed columns for analyses of mammalian, and indeed most other, odours may actually generate misleading results (Preti, Smith & Beauchamp 1977), and must now be considered inappropriate.

Detection

Although it is the most sensitive of the general gas chromatographic detectors (Lee *et al.* 1984 pp 126-152) the FID is still three orders of magnitude less sensitive than a male hamster or a dog (*Canis familiaris*) (Moulton 1977; O'Connell *et al.* 1979).

The testing of analytical systems for mammalian odours has received scant attention. Raymer *et al.* (1985) reported that, with Tenax trapping and capillary column separation of gas-stripped volatiles from wolf urine, "Peak areas resulting from replicate analyses of the same samples indicate a maximum of 15% variation with less than 10% being typical." However, Schwende, Jorgenson & Novotny (1984), working in the same laboratory on mouse urine, commented "While analyzing data from replicate chromatograms of the same sample, it became apparent that correlation coefficients of better than 0.90 were not consistently obtained. The source of error was attributed to certain "unreliable" mixture components (most likely those which suffer from an irreversible adsorption in the GC system), a problem typically met with in multicomponent trace organic analysis."

PERFORMANCE OF CHROMATOGRAPHIC SYSTEMS

The performances of the various components of a chromatographic system which are reported in the literature and elsewhere often fall into one of three classes:-

- optimistic calculated values without experimental verification, based on assumptions that every component of the system works perfectly (Noij, Van Es, Cramers, Rijks & Dooper 1987; Pretorius & Lawson 1987).

- "one-off" successful results of doubtful replicability (Lipsky 1983 and references therein).

- results applicable to only one analytical problem, and sometimes obtained after laborious optimization (Grob 1973; Grob & Grob 1974; Grob, Grob & Grob 1975; Grob & Schilling 1983; Grob & Zürcher 1976).

Obviously it is unrealistic to expect these best case performances to be routinely achievable over a wide range of analytical problems. Consequently the following summary of system performance is based on practical laboratory experience rather than published figures.

It is convenient to work backwards through the analytical system from identification to sampling.

The mass spectrometer is the most sensitive of the instruments for the identification of gas-chromatographically separated compounds. Under favourable conditions; a well-tuned machine receiving sharp peaks from a capillary column, and with a skilled operator, an identifiable spectrum can be obtained from 1 ng of material. Ten ng can be considered as a quantity from which identifiable spectra should be routinely obtained.

When coupled to a capillary column a flame ionization detector can be expected to yield a definite peak from 10^{-11} g of material. It should be pointed out that such peaks are well above the impractical 2:1 signal:noise ratio which is often quoted as a sensitivity limit. Ten picogram peaks are, however, very small (see Fig 6.5) and 10^{-10} g represents a more realistic limit for routine work.

Capillary columns can easily deliver 10^{-11} g peaks to the detector. This performance is, however, attainable only with the most tractable of compounds. Reversible adsorptive activity towards polar compounds reduces their detectability by distorting their peak shapes and irreversible adsorption causes permanant losses of some components. A well-deactivated column can elute sharp peaks of approximately nanogram quantities of even intractable compounds such as alcohols and phenols. Average columns should be capable of an equivalent performance with 10 ng. Unfortunately the column is often the weak link in the chromatographic chain and tens of nanograms of some compounds may disappear without trace (pp 116, 292).

Thus the separation and detection/identification steps of high resolution gas chromatographic analysis form a rather well integrated system whose limits of operation lie reliably between 10^{-10} and 10^{-8} g. A comparison of this limit with the known, and anticipated, properties of mammalian chemical signals (above) suggests that they should be accessible to analysis provided that the performance of the sampling techniques matches that of the rest of the system.

The modest progress which has been made in the field of analytical mammalian semiochemistry suggests that this requirement has not been fulfilled. An examination of the performances of various sampling systems (Chap 6) confirms this view.

The necessary performance of a sampling system which does fulfill the requirement can be concisely defined:-

- to sample 1-10 ng quantities of any sort of organic volatiles from the gas phase at concentrations at or below a few parts per billion, and

- to deliver the sample to a capillary column in a way which does not compromise the column's ability to, in turn, deliver the separated components to the detector as sharp, symmetrical peaks, and

- to yield quantitative results with coefficients of variation of less than 10%.

While the fulfilment of these requirements can be demonstrated with synthetic specimens of known composition, only successful analyses of real specimens can demonstrate that the requirements were, in fact, appropriate and that, using the correct techniques, problems of mammalian semiochemistry are actually accessible to routine instrumental analysis.

Chapter 3

MODEL MECHANISMS FOR SOLUTE FOCUSSING BY THE SOLVENT EFFECT

INTRODUCTION

The peculiar chromatographic properties of mechanically stable, evaporating solvent films were first recognized by Deans (1971) who provided both experimental results and a fundamental model of the mechanisms involved. This work was re-examined and extended by Pretorius and co-workers (Pretorius & Bertsch 1983b; Pretorius, Lawson, Apps & Bertsch 1983; Pretorius, Lawson & Bertsch 1983a, b; Pretorius, Phillips & Bertsch 1983a, b, c; Pretorius, Rohwer & Lawson 1983; Pretorius, Rohwer, Lawson & Apps 1984; Pretorius, Lawson, Rohwer & Bertsch 1984; Pretorius & Lawson 1986a, b; Pretorius & Lawson 1987). At the same time splitless, cold-on-column and retention gap techniques were being investigated in painstaking detail, especially by the Grobs. These techniques all employ smooth-walled, open-tubular, "inlets" of capillary dimensions, in which the mechanical behaviour of liquid films is markedly different to that in porous beds (Grob 1981; Saravelle, Munari & Trestianu 1983; Pretorius, Lawson, Apps & Bertsch 1983). Since the dynamic solvent effect can only operate on mechanically stable solvent films, whose theoretical properties have already been modelled in detail, I shall present here only a basic summary of the mechanisms of solute focussing by the static solvent effect and, with that as a basis, a similarly brief consideration of the dynamic solvent effect.

THE STATIC SOLVENT EFFECT

Consider a tube with a porous layer on its inside wall, in which a pure liquid is held as a mechanically stable film by capillary attraction. Along the axis of the tube is an open channel down which flows a stream of gas at a velocity sufficiently low that the shape of the liquid film is not altered by shear forces. The liquid will evaporate into, and be carried away by, the stream of gas at a rate governed by the liquid's vapour pressure and the volume flow rate of the gas. The mass rate of removal of the solvent vapour is given by:

$$E_{lm} = (P_v \cdot V_g \cdot M_l) / (R \cdot T) \qquad [1]$$

The mass rate at which liquid evaporates from the film must obviously be the same as the mass rate of removal of the vapour. The evaporation rate in terms of liquid volume is then:

$$E_{lv} = E_{lm} / p_l \qquad [2]$$

$$E_{lv} = (M_l \cdot V_g \cdot P_v) / (R \cdot T \cdot p_l) \qquad [3]$$

Provided that equilibrium between the gas and liquid phases is quickly established the gas will become saturated with vapour after passing over only a short length of the film. Evaporation will then be confined to the part of the film first encountered by the gas - the upstream edge of the film.
Because the film is held in place by the capillarity of the porous layer in which it lies, evaporation at the upstream edge removes liquid from that area alone. As a result of this removal the position of the film's edge moves downstream. The film therefore becomes shorter but does not move in bulk.

The rate at which the upsteam edge moves at a given evaporation rate is determined by the volume of liquid held in unit length of the film, which is given by:

$$A_l = Q_l / l_f \qquad [4]$$

A_l is simply the cross sectional area of the liquid annulus, and the rate of downstream movement of the position of the upstream edge of the film is the volume evaporation rate divided by the area of the liquid annulus:

$$U_l = E_{lv} / A_l \qquad [6]$$

$$= (P_v \cdot V_g \cdot M_l) / (R \cdot T \cdot p_l \cdot A_l) \qquad [7]$$

Now consider the behaviour of a volatile solute dissolved in the liquid film. Being volatile it will partition between the gas and the liquid and will consequently be carried chromatographically downstream at a velocity determined by its partition ratio and the gas phase velocity:

$$v_s = U_g \; / \; (k_s + 1) \qquad [8]$$

If, at the upstream edge of the film the solute is moving downstream faster than the position of the film's upstream edge i.e. if:

$$v_s > U_l \qquad [9]$$

the solute concentration at the upstream edge of the film will fall as the solute is moved forward and ultimately stripped from the film by the gas.

If, on the other hand, the solute moves downstream more slowly than the film's edge i.e. if:

$$v_s < U_l \qquad [10]$$

at the upstream edge of the film the solvent is being removed from the solution by evaporation faster than the solute is being removed by chromatographic migration. The solute's concentration consequently rises. Solute left behind by the liquid film is in a region of infinite phase ratio and therefore it moves forward at the gas velocity and re-equilibriates with the liquid film. These solutes are focussed by the solvent effect.

Inequality [10] can be rewritten from equation [7] as:

$$v_s < (P_v \; . \; V_g \; . \; M_l) \; / \; (R \; . \; T \; . \; p_l \; . \; A_l) \qquad [11]$$

The linear flow rate of the gas phase is its volume flow rate per unit area of the gas channel's cross section:

$$U_g = V_g \; / \; A_g \qquad [12]$$

Equation [11] then becomes:

$$v_s < (P_v \; . \; U_g \; . \; A_g \; . \; M_l) \; / \; (R \; . \; T \; . \; p_l \; . \; A_l) \qquad [13]$$

The area of the gas channel and that of the liquid annulus determine the phase ratio:

$$\beta = A_g \; / \; A_l \qquad [14]$$

Substituting in equation [13] then gives:

$$v_s < (P_v \cdot U_g \cdot M_l \cdot \beta) / (R \cdot T \cdot p_l) \qquad [15]$$

Substituting from equation [8] in equation [15]:

$$U_g / (k_s + 1) < (P_v \cdot U_g \cdot M_l \cdot \beta) / (R \cdot T \cdot p_l) \qquad [16]$$

$$1 / (k_s + 1) < (P_v \cdot M_l \cdot \beta) / (R \cdot T \cdot p_l) \qquad [17]$$

$$(k_s + 1) > (R \cdot T \cdot p_l) / (P_v \cdot M_l \cdot \beta) \qquad [18]$$

Consider now the partitioning of the solvent between the liquid and gas phases. For dilute solutions the concentration of solvent in the liquid phase is the solvent density. The concentration in the gas phase is the mass rate of solvent evaporation into the volume of gas sweeping the film in unit time (equation [1]).

$$k_l = (p_l \cdot R \cdot T) / (P_v \cdot M_l \cdot \beta) \qquad [19]$$

Equation [18] can now be rewritten:

$$(k_s + 1) > k_l \qquad [20]$$

$$k_s = K_s / \beta \qquad [21]$$

$$k_l = K_l / \beta \qquad [22]$$

The condition for solute focussing by the solvent effect then becomes:

$$(K_s + \beta) > K_l$$

CONSEQUENCES OF SOLUTE FOCUSSING BY THE STATIC SOLVENT EFFECT

As the upstream edge of the liquid film moves downstream solutes for which the condition for solvent effect focussing is met accumulate in a short band at the upstream edge of the film. Within the limits imposed by the usual chromatographic band spreading processes the length of the band of concentrated solutes is small compared to the length of the film, and the solute focussing mechanism continues to operate until nearly all the film has evaporated. At this stage the length of the film has become the same as that of the concentrated band so that the solutes are no longer chromatographically retarded by a film of liquid lying downstream of them. The last traces of the liquid film therefore consist of a concentrated solution of the

solutes occupying a short region of the porous layer at the position previously occupied by the downstream end of the liquid film. It is the high concentration and small volume of the solution which makes possible its efficient transfer to a capillary column, and it is in this respect that static solvent effect focussing is useful as a sampling technique for large, dilute, liquid specimens.

SOLUTE ESCAPE

If there are solutes in the downstream edge of the liquid film they will eqilibriate with the gas stream and be carried away by it. This applies even to solutes for which the conditions for solute focussing by the solvent effect are fulfilled because, as soon as the solutes move downstream of the film, where the phase ratio is infinite, they begin to move at the velocity of the carrier gas. This process of solute loss is known as solute escape. In the present work the term is restricted to the process just described. The extent to which solutes are lost by escape has been treated theoretically by Pretorius, Lawson & Bertsch (1983b).

SOLUTE LAGGING

Although solutes which are left behind by the liquid film are in a region of infinite phase ratio they will not necessarily move forward with the velocity of the carrier gas. If the solute molecules interact with the surface of the inlet, which is inevitable in a real system (pp 56, 156), their forward movement is retarded and they may actually be left behind by the downstream movement of the evaporating edge of the liquid film. Solutes to which this happens are said to be lagging. The process has been modelled for theoretical, ideal conditions by Pretorius, Phillips & Bertsch (1983c). The consequence of lagging is that the solutes, rather than being concentrated into a short band, are spread along the length of the porous layer originally occupied by the liquid film. The extent of the spreading depends on the strength of the interactions between the solutes and the inlet surface.

Pretorius, Phillips & Bertsch (1983c) calculated that lagging would be significant only for solutes with molecular weights in excess of about 500. However, the assumption on which the calculation was based, that there is no interaction between the solutes and the inlet surface is impossible to fulfill. In practise even *n*-hexane, used as a solvent, lags and to ensure the elution of sharp peaks from the column it is necessary to overcome lagging by heating the inlet at the completion of the solvent effect (pp 56, 156).

GAS-BORNE VOLATILES AND THE SOLVENT EFFECT

If the gas contains volatiles before it encounters the liquid film, in other words if it is a gas-phase specimen or the head-space of a specimen, the vapours will impinge on, and dissolve in, the upstream edge of the film. They will then behave in the same way as solutes originally in the film. Pavelka (1964) used a film of solvent to trap volatiles from the gas phase but without exploiting solute focussing by the solvent effect. Grob (1975) demonstrated the focussing of low-boiling volatiles from 1 cm^3 of the headspace of a spice by a 2 µl film of hexane on a capillary column. Jennings (1979) trapped food headspace volatiles on a refluxing film of Freon 12. Roerade & Blomberg (1983) mentioned the possibility of using the solvent effect to focus gas phase volatiles, but presented no experimental results. Pretorius & Bertsch (1983b) provided a theoretical treatment but their requirement that the carrier gas be saturated with solvent to prevent evaporation of the solvent film is impractical and incompatible with sampling from live animals and any specimen which cannot be enclosed and pressurized. Recognizing these drawbacks Pretorius & Lawson (1987) considered theoretically a more versatile case in which the solvent film is allowed to evaporate during sampling. Neither of these theoretical papers included any experimental findings.

LIMITS TO STATIC INLET SIZE

The static solvent effect inlets of randomly-packed, porous particles advocated by Pretorius, Lawson, Apps & Bertsch (1983) need to have an overall volume approximately ten times as large as the volume of the liquid sample. Thus for a 1 cm^3 sample, 10 cm^3 of packing is required. This is sufficient to fill an ordinary packed column of 2 mm i.d. 3.2 m long. To non-selectively deactivate such a volume of packing well enough to handle low-nanogram quantities of intractable compounds such as alcohols, amines and fatty acids is probably impossible.

In addition, static solvent effect inlets for volumes in excess of a few tens of microliters are prohibitively bulky. If the packed beds are increased in diameter to allow them to be shorter and more compact the linear flow rates of the carrier gas become so low that band spreading by diffusion may be expected to become significant, as it does in beds of adsorbent (Grob & Habich 1985).

The largest liquid samples handled on a routine basis with a static solvent effect inlet were those of 20 µl used to test the properties of the static model of the dynamic solvent effect concentrator (Chap 4). Experience with these inlets suggests an optimistic upper limit to sample size of 100 µl for reliable operation (Lawson 1987 Chap 12).

At temperatures around 27°C approximatley 2 µl of *n*-hexane are evaporated from a film by 1 cm^3 of gas (p 170) (the values calculated by Pretorius & Lawson (1987) are optimistic). With a maximum inlet capacity of 100 µl of solvent the maximum volume of a head space specimen from which volatiles could be accumulated on a solvent film would be approximately 50 cm^3. While this would be sufficient in certain cases (see e.g. the analysis of *Ictonyx* anal gland secretion Chap 15) it is unsatisfactorily restrictive and does not allow for consumption of solvent while the sample is dried. Cooling the solvent film in order to increase its lifetime (Pretorius & Lawson 1987) does not provide a practical solution for biological specimens since it merely increases the amount of condensed water which must subsequently be evaporated from the sample.

It was apparent that a solution to these particular shortcomings of static solvent effect inlets would be provided by a means of enabling large volumes of liquid, either solvent-matrix specimens or pure solvent, to be handled on small, porous beds. Since the volume of even a modest target for liquid volume; 1 cm^3 was equal to the total volume of the largest bed which could be expected to operate reliably measures such as increasing the bed porosity would obviously be ineffective.

It was recognized that the introduction of extra liquid to the bed while the solvent effect was in progress might provide an appropriate solution, as long as the introduction could be carried out in such a way that the solvent effect was not disrupted. Introduction of the extra liquid at the top of the bed would be expected to be unsatisfactory. Although a zone of increased solute concentration would be produced by solvent evaporation the solutes migrating chromatographically away from that zone would not be followed by the rear edge of the film until sample introduction was complete. Consequently they would escape in large quantities. Upstream addition has in fact been employed as a means of increasing sample size by slow, on-column or splitless injection, but it works well only for heavy solutes which are strongly stationary-phase focussed, e.g. the lightest solute in Vogt, Jacob, Ohnesorge & Obwexer's (1979) test mix was *n*-octadecane.

On the other hand the introduction of the extra liquid at the bottom of the bed would, in effect, result in the laying down of a fresh liquid film in front of any escaping solutes. At the same time capillarity in the packed bed would draw the extra liquid towards the evaporation zone which would, under conditions of dynamic equilibrium between evaporation and capillarity, be fixed in space as long as the supply of liquid continued. When the supply stopped the evaporation zone would begin its downstream migration and the evaporation of the

film would be completed under static solvent effect conditions. It was this process which formed the basis of what was to become known as the dynamic solvent effect.

THE DYNAMIC SOLVENT EFFECT

Rather than attempting to duplicate the sophisticated treatment of the theory of the dynamic solvent effect which is being prepared by Pretorius (unpubl.) I shall present a basic model of the mechanism which I hope will be sufficient to illustrate the processes involved, and their outcome in terms of solute focussing.

In the case of the static solvent effect, when solvent evaporates from the upstream edge of the film the edge moves downstream while there is no bulk movement of the liquid in the body of the film. In the case of the dynamic solvent effect the upstream edge of the liquid film lies at the upstream end of the porous layer holding the film. The downstream end of the film is in contact with a source of whatever liquid it is composed of. Now when gas is passed over the film, liquid removed by evaporation from its upstream edge is replaced by liquid drawn by capillary attraction from the liquid source. Thus there is a continuous bulk movement of liquid towards the evaporation zone, which retains its position at the upstream end of the porous layer until the source of liquid is removed. Solutes are carried towards the evaporation zone by this bulk movement of liquid, and in the opposite direction by the gas. Solutes whose net upstream velocity is higher than their net downstream velocity accumulate in the evaporation zone.

Because the porous layer acts principally as a conduit for the transport of liquid from the source to the evaporation zone there is no need for the size of the porous bed to be increased in order to accomodate large volumes of liquid. Thus, at least in principle, the dynamic solvent effect offers a means of overcoming one of the main shortcomings of the static solvent effect; the inability to handle liquid volumes above a few tens of microlitres.

Consider a tube with a porous layer on its inside wall and an open channel along its axis. If the tube is dipped into a liquid which wets the porous layer the liquid will rise into it (passage of liquid into the axial channel is ignored for present purposes).

The height to which the liquid will rise against gravity is given by (Levich 1962 p 383):

$$h = (2 \cdot \gamma) \ / \ (\rho_l \cdot g \cdot r_h) \qquad [1]$$

If the porous layer can be approximated by a random packing of solid, spherical particles:

$$r_h = \varepsilon / [S_p \cdot (1 - \varepsilon)] \qquad [2]$$

$$S_p = [6(1-\varepsilon)] / d_p \qquad [3]$$

and from [1], [2] & [3]:

$$h = [12 \cdot \gamma \cdot (1-\varepsilon)^2] / (\rho_l \cdot g \cdot \varepsilon \cdot d_p) \qquad [4]$$

For *n*-hexane, the most commonly used solvent, γ = 20 dynes cm^{-1}, ρ = 0,7 g cm^{-1} and for randomly packed spheres ε = 0,35.

If the mean size of the particles is 0,1 mm:

$$h = [12 \cdot 20 \cdot (1-0,35)^2] / (0,7 \cdot 981 \cdot 0,35 \cdot 0,01)$$

$$\approx 376 \text{ mm}$$

This is 6-7 times as high as the liquid film rises when the dynamic solvent effect is used for sampling. Consequently the effects of hydrostatic pressure on the behaviour of the film can be neglected in the following model.

When there is no pressure gradient, as here, the velocity with which a liquid enters a pore due to capillarity is given by (Levich 1962 p 383):

$$U_{pp} = (r_h \cdot \gamma) / (4 \cdot \eta \cdot L) \qquad [5]$$

The speed with which a liquid front is drawn into a porous bed is reduced by the tortuosity of the channels in which it flows:

$$U_{pb} \approx U_{pp} / \tau = (r_h \cdot \gamma) / 4 \cdot \eta \cdot L \cdot \tau) \qquad [6]$$

The volume rate at which the liquid flows into the porous layer depends on the cross sectional area of the liquid annulus:

$$V_{pb} = (r_h \cdot \gamma \cdot A_l) / (4 \cdot \eta \cdot L \cdot \tau) \qquad [7]$$

The volume of liquid held in the porous layer depends on the length of wetted bed and the liquid annulus cross sectional area:

$$Q_l = l_f \cdot A_l \qquad [8]$$

When gas is passed down the axial channel the liquid which has risen into the bed will evaporate into the gas. As in the case of the static film (equation [3], p 30) evaporation will be confined to the upstream edge of the film and its rate will be given by:

$$E_{lv} = (M_l \cdot V_g \cdot P_v) / (R \cdot T \cdot p_l) \qquad [9]$$

If the rate of evaporation is greater than the rate at which liquid flows into the porous layer there will be a decrease with time in the volume of liquid in the layer and from equation [8] the length of the film will decrease (A_l is fixed by the geometry of the porous layer).

Since:

$$l_f = L / \tau \qquad [10]$$

and from equation [7] the volume of liquid entering the bed is inversely dependent on the length of the pores through which the liquid has to permeate, the shortening of the film allows a faster flow rate into the porous layer. As the film shortens a point is reached at which:

$$V_{pb} = E_{lv} \qquad [11]$$

and no further change in film volume, film length or liquid flow rate occurs. The upstream edge of the liquid film will now hold a fixed position within the porous layer, and liquid is supplied to it by capillarity at a rate given by equation [7], and removed from it by evaporation at a rate given by equation [9].

In this equilibrium condition:

$$V_{pb} = (M_l \cdot V_g \cdot P_v) / (R \cdot T \cdot p_l) \qquad [12]$$

The average linear velocity of the upward movement of the liquid is related to the volume flow of liquid by equations [6] and [7]:

$$U_{pb} = (M_l \cdot V_g \cdot P_v) / (R \cdot T \cdot p_l \cdot A_l) \qquad [13]$$

so that, as is intuitively obvious, the liquid in a dynamic solvent film flows upstream at the same linear velocity as that with which the upstream edge of the equivalent static film moves downstream.

Under equilibrium conditions solutes are moved chromatographically away from the evaporation zone, and towards it by the liquid flow.

The rate of downstream movement is given by:

$$v_{sd} = U_g / (k_s + 1) \qquad [14]$$

and the rate of upstream movement by:

$$v_{su} = U_{pb} / [(1 / k_s) + 1] \qquad [15]$$

Solutes for which:

$$v_{sd} > v_{su} \qquad [16]$$

experience a net downstream movement and are stripped from the liquid film (compare equation [9], p 31).

Solutes for which:

$$v_{sd} < v_{su} \qquad [17]$$

experience a net upstream movement towards the evaporation zone, where they accumulate. This is the condition for solute focussing by the dynamic solvent effect (compare equation [10], p 31).

From equations [14] and [15] inequality [17] can be rewritten as:

$$U_g / (k_s + 1) < U_{pb} / [(1 / k_s) + 1) \qquad [18]$$

$$U_{pb} / [(1 / k_s) + 1] = (U_{pb} \cdot k_s) / (k_s + 1) \qquad [19]$$

Inequality [18] then becomes:

$$U_g < (U_{pb} \cdot k_s) \qquad [20]$$

$$k_s > U_g / U_{pb} \qquad [21]$$

now:

$$U_g = V_g / A_g \qquad [22]$$

and so from equation [13]:

$$k_s > V_g / [(M_l \cdot V_g \cdot P_v \cdot A_g) / (R \cdot T \cdot p_l \cdot A_l)] \qquad [23]$$

$$ks > 1 / [(M_l \cdot P_v \cdot \beta) / (R \cdot T \cdot p_l)] \qquad [24]$$

$$k_s > (R \cdot T \cdot p_l) / (M_l \cdot P_v \cdot \beta) \qquad [25]$$

As in the case of the static film (equation [19], p 32):

$$k_l = (p_l \cdot R \cdot T) / (P_v \cdot M_l \cdot \beta) \qquad [26]$$

so that [25] can be rewritten:

$$k_s > k_l \qquad [27]$$

from which it follows that:

$$K_s > K_l$$

Thus the condition for solute focussing by the dynamic solvent effect is somewhat more stringent than that for focussing by the static solvent effect.

THE DYNAMIC SOLVENT EFFECT: A DESCRIPTIVE MODEL

A schematic representation of the dynamic solvent effect in operation is shown in Fig 3.1 (Apps, Pretorius, Lawson, Rohwer, Centner, Viljoen & Hulse 1987).

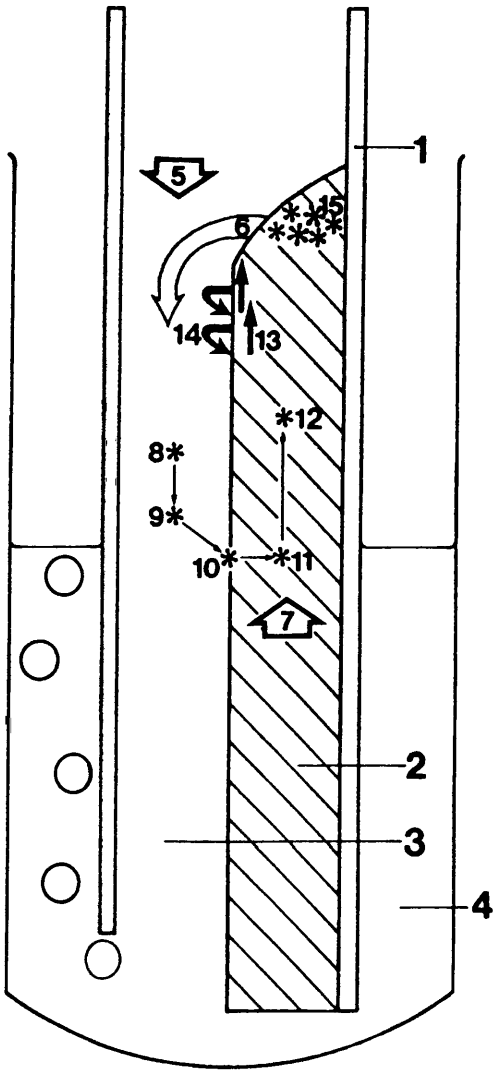


Fig. 3.1 Schematic representation of the operation of the dynamic solvent effect. 1) glass tube; 2) sintered porous bed; 3) gas channel; 4) solvent. When gas is passed down the tube (5) evaporation of solvent at the top edge of the bed (6) causes an upward movement of solvent driven by capillary rise (7), solutes in the gas phase (8) are carried downwards by the gas (9), impinge (10) on and dissolve (11) in the solvent and are carried upwards by it (12). Exchange of solute between gas and liquid results in a chromatographic transport of solute. As long as the rate of upward transport (13) is greater than the rate of downward transport (14) solutes accumulate in the evaporation zone (15).

SYMBOLS, Chapter 3

A_g = cross sectional area of gas channel

A_o = cross sectional area of liquid annulus

d_p = mean particle diameter in a porous material

E_{lm} = mass rate of solvent evaporation

E_{lv} = volume rate of solvent evaporation

g = acceleration due to gravity

h = height of a liquid column

k_l = partition ratio of solvent between itself and the gas

k_s = partition ratio of solute between liquid and gas

K_l = partition coefficient of solvent between itself and the gas

K_s = partition coefficient of solute between liquid and gas

l_f = length of liquid film

L = length of a pore

m_l = mass of solvent

M_l = molecular weight of solvent

p_l = density of solvent

P_v = partial pressure of a vapour

Q_l = volume of liquid

r_h = hydraulic radius

R = universal gas constant

S_p = specific surface area of a porous solid

T = temperature

U_f = velocity of upstream edge of film

U_g = linear velocity of gas

U_{pb} = linear velocity with which a liquid permeates a porous solid

U_{pp} = linear velocity with which a liquid permeates a pore

V_g = volume flow rate of gas

V_{pb} = volume rate at which liquid flows into a porous solid

v_s = chromatographic velocity of solute plug (static film)

v_{sd} = chromatographic downstream velocity of a solute plug (dynamic film)

v_{su} = chromatographic upstream velocity of a solute plug (dynamic film)

β = phase ratio

η = viscosity

ε = porosity of a porous solid

γ = tortuosity of the pores in a porous solid

γ = surface tension of a liquid

Chapter 4

DEVELOPMENT OF THE DYNAMIC SOLVENT EFFECT SAMPLING SYSTEM

The simple, ideal models of the static and dynamic solvent effects in porous beds presented in Chapter 3 suggested that they could provide the basis of powerful sample introduction techniques for capillary chromatography. Nevertheless, experience with static solvent effect inlets demonstrated quite clearly that the ideal performance of the models was not readily achieved in practise. The reality of dynamic solvent effect focussing was soon demonstrated with crude, preliminary systems (p 60) but for the dynamic solvent effect to work in practise as an analytical tool required the development of hardware and operating procedures compatible with capillary gas-liquid chromatography.

All developmental testing was carried out with well defined, synthetic specimens in order to obtain as clear a picture as possible of the properties of various alternative systems and their responses to changes in conditions.

DIAGNOSTIC TEST MIXTURE FOR SOLVENT EFFECT INLETS

A test mix was required which would allow diagnosis of the causes of unsatisfactory performance as well as providing a representative test of inlet performance for a variety of compounds. Fulfillment of the following requirements was therefore necessary:-

- the test solutes must have a range of vapour pressures from close to, to well below that of the solvent,
- a representative range of polarities and functional groups must be included,
- the test must include a series of probes for mechanical disruption of the sample film,
- all components of the mix should be widely separated by a 20 m methyl silicone column so that peak identities can be confidently assigned even if some components suffer retention shifts.
- none of the components should cause concentration overloading of the column.

None of the commercial test mixes (Scientific Glass Engineering 1984 p 38; Alltech 1987 p 28; Hewlett Packard 1988 pp 180-184; Supelco 1988 p 72), or the Grob mix (Grob, Grob & Grob 1978) are able to satisfy these requirements and a new mix was developed empirically. Its components were; C_8 to C_{15} *n*-alkanes, 2,6-dimethyl-4-heptanone, *para*-cresol, 2,6-dimethyl aniline and *n*-decanol dissolved in *n*-hexane. The 2,6-dimethyl-4-heptanone contained an impurity which eluted just after the pure compound. This Test Mix underwent two modifications. *n*-Tridecane was replaced by the methyl ester of *n*-decanoic acid as a probe for the splitting of methyl ester peaks and, later, linalool was substituted for *n*-undecane as a representative of a major class of flavour compounds.

The Test Mix concentration was adjusted according to the volume used so that approximately 3-4 ng of each component was transferred to the column.

Since inlets, and not columns, were under test, fast temperature programmes were usually employed: $10^{o}C\ min^{-1}$ was standard. System verification during applications work usually employed the same programme as the analytical runs in order to provide a retention standard from the series of alkanes.

Departures from ideal peak shape for the Test Mix components were exhibited under a variety of circumstances presented throughout the following pages. For convenience they, and their causes, where known, are summarized here, the numbers refer to Figure 4.1.

1. Solvent tail; due to dead volumes, adsorptive or absorptive materials exposed to sample (see e.g. Pretorius, Apps, Rohwer & Lawson 1985), stationary phase in inlet, (rarely) poorly coated columns.

2. Baseline hump; due to adsorbed solvent driven off inlet surface by heating - visible only if heating is late.

3. Shifts in retention; due to strong, uniform retention in inlet coupled to late inlet heating.

4. Symmetrical broadening; due to poor stationary phase focussing (Pretorius, Rohwer & Lawson 1983) and/or adsorption in inlet, stationary phase in inlet.

5. Splitting of polar compound peaks; due to late heating of inlet.

6. Broadening and extra retention of alcohol; found only with dicumyl peroxide cross-linked columns. Not an inlet effect.

7. Methyl ester splitting; mechanism unknown.

8. Splitting of late-eluting alkanes; due to early heating of inlet.

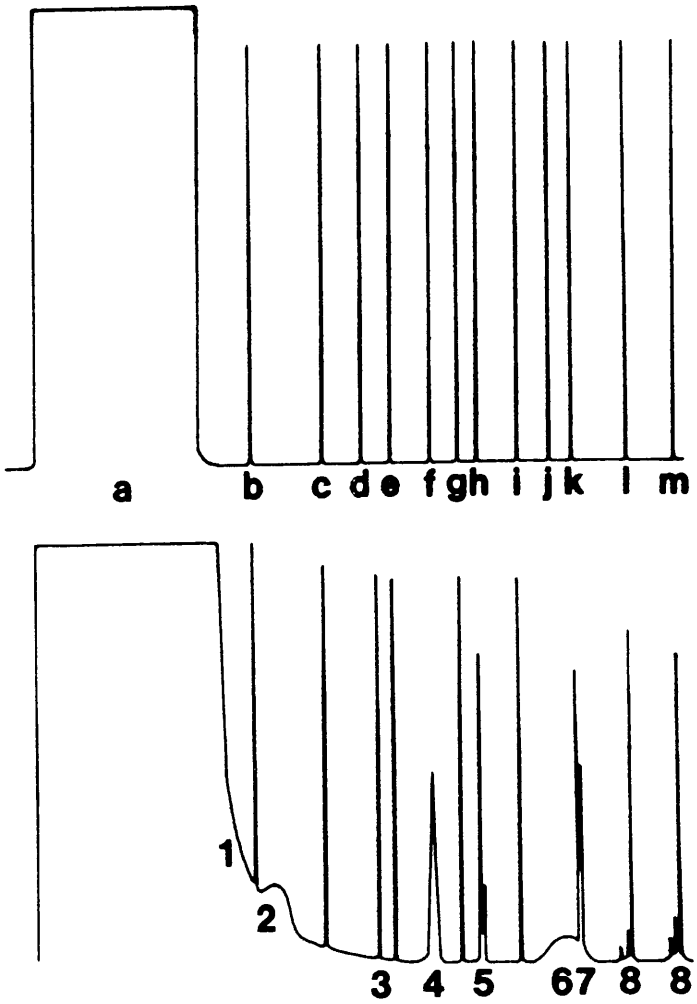


Fig. 4.1 Hand drawn chromatograms to illustrate the diagnostic qualities of the Test Mix. Top: perfect performance; bottom: compilation of results of various hardware and procedural shortcomings. a: *n*-hexane solvent; b: *n*-octane; c: *n*-nonane; d: 2,6-dimethylheptan-4-one; e: *n*-decane; f: *p*-cresol; g: *n*-undecane; h: 2,6-dimethylaniline; i: *n*-dodecane; j: *n*-decanol; k: methyldecanoate; l: *n*-tetradecane; m: *n*-pentadecane. Numbers refer to those in the text.

The synthetic test mix which accurately predicts the performance of a chromatographic system with all possible types of real samples has not yet been developed. In the development of the mixes used in this study priority was given to the inclusion of probes for the sub-optimal performance of the solvent effect *per se*. This approach allowed diagnosis of the causes of degraded performance and thus more rapid optimization than would have been possible with any of the

established test mixtures.

The importance (and difficulty except with hindsight) of matching test mixes to samples is illustrated by the need to include a methyl ester as a probe for a specific problem; peak splitting of this class of compound, which affected none of the components of the original Test Mix. The substitution of methyl decanoate and linalool for tridecane and undecane was made feasible by a decreasing need, in the light of accumulated experience, for the components of the original test which were diagnostic of shortcomings in inlet performance.

STATIC SOLVENT EFFECT INLET WITH A PERFORATED PACKED BED

As a model for the transfer of the contents of dynamic solvent effect concentrators to the capillary column a static solvent effect inlet was constructed and various factors affecting its performance were investigated. The geometry of the static solvent effect inlet was a slightly idealised version of that of the dynamic solvent effect concentrators (see later). The performance of the static solvent effect inlet provided an indication of the potential performance of the complete dynamic solvent effect system.

CONSTRUCTION OF PACKED BED INLETS

The packed bed inlet consisted of a 4 mm o.d., 1,4 mm i.d. borosilicate glass tube with a 50 mm long, concentric layer of 105-125 µm borosilicate glass chips sintered onto its inside wall so as to leave a central, axial channel 0,4 mm across (Fig 4.2). The channel was formed by sintering the glass chips into place around a thin-walled nickel tube which was then dissolved away with a hot 1:1 mixture of 20% hydrochloric and nitric acids. The perforated bed was washed with 100 cm^3 of freshly distilled water and dried overnight at 250°C in air. One end of the glass tube was pulled down to an o.d. of 1,0 mm for later connection to the column, and the tube was filled to a pressure of 200 KPa with semiconductor grade monosilane from which silicon was deposited by heating slowly to 400°C and holding at that temperature overnight (Pretorius, Du Toit & Purnell 1981; Bertsch, Pretorius & Van Niekerk 1982).

In order to keep thermal inertia to a minimum a coil of resistance wire was wound directly onto the glass tube containing the perforated bed and fixed into position with polyimide resin. The heaters dissipated 36 W. The maximum temperature to which the bed was heated was controlled by an indicating temperature controller through feedback from a thermocouple glued to the outside of the tube with

polyimide. The heaters were able to raise the temperature of the bed from 40°C to 250°C within 80 s.

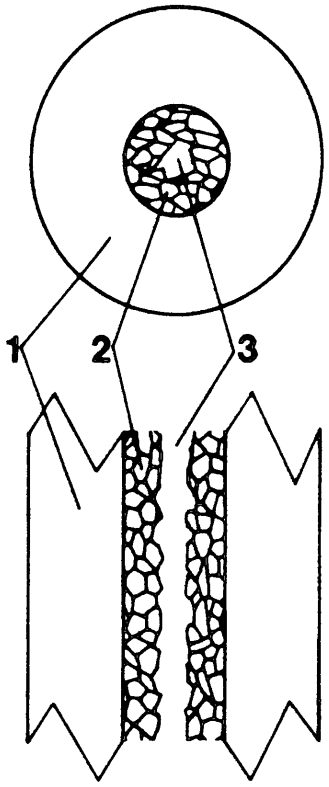


Fig. 4.2 Vertical and horizontal sections through the perforated packed bed of a static solvent effect inlet. 1: wall of borosilicate glass tube; 2: porous bed of sintered borosilicate glass chips; 3: axial channel.

The perforated packed bed was mounted in a quarter inch Swagelok union modified to act as a septum injection port with carrier gas input and septum purge (Fig 4.3). The Swagelok was clamped into the packed column inlet block of a Varian 3700 gas chromatograph and connected to the column by a heat-shrink polytetrafluoroethylene joint which was kept at 200°C to avoid adsorption of solvent (Pretorius *et al.* 1985). The column was 19 m x 0,3 mm x 0,4 µm methyl silicone, its temperature was programmed at 10°C min^{-1} from 50°C to 220°C after elution of the solvent peak (usually aproximately 6,5 min). Hydrogen was used as carrier gas with a linear flow through the column of 50 cm s^{-1}. The FID's output was fed to a Varian 4270 integrator.

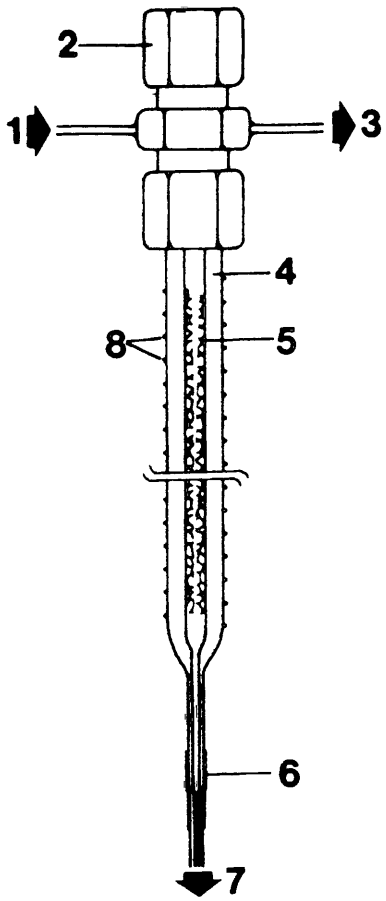


Fig. 4.3 Method of installation of a static solvent effect inlet. 1: carrier gas inlet; 2: 1/4" Swagelok fitting modified to act as injection port; 3: septum purge outlet; 4: borosilicate glass tube; 5: sintered borosilicate glass bed; 6: heat-shrunk polytetrafluoroethylene connection; 7: flow to column; 8: heater coils.

EXPERIMENTAL

The silicon-coated beds were tested either without further treatment or after modification by the following processes:-

- D_4 (octamethylcyclotetrasiloxane) deactivation (Bertsch, Pretorius & Van Niekerk 1982)
- Silyl 8 deactivation according to the manufacturer's instructions
- water washing by slowly running 50 cm^3 of freshly distilled water through the bed while it was immersed in an ultrasonnator bath
- on-line water washing after installation by injecting 20-30 µl of distilled water and allowing it to run through the bed and column.

Beds were tested with 20 µl injections of 2:10^7 Test Mix, giving 3-4 ng of each component.

Water washed beds were further tested for the effects of various inlet heating schedules on peak shape.

The quantitative precision of the inlets was tested by a series of 30 injections of 20 µl of 2:10^7 Test Mix onto a water-washed, conditioned bed operating with an optimum heating schedule *viz:* initial temperature of inlet 50°C, ballistic heating to 250°C after 5 minutes. Peak areas, percentage areas and heights were recorded by the integrator. Peak widths at half height were calculated on the basis of Gaussian peak shapes and were used to calculate Trenzhal numbers for each pair of alkane peaks. Standard deviations and coefficients of variation were calculated.

RESULTS

Surface treatments (See below: CONCENTRATOR DEACTIVATION)

Inlet heating

When an "inactive" water-washed, silicon-surfaced (see below) inlet was not heated all the peaks on the test chromatogram were broadened. The polar compound peaks were most strongly affected. In addition the DMA and decanol peaks were tailed and the decanol peak showed retarded elution (Fig 4.4).

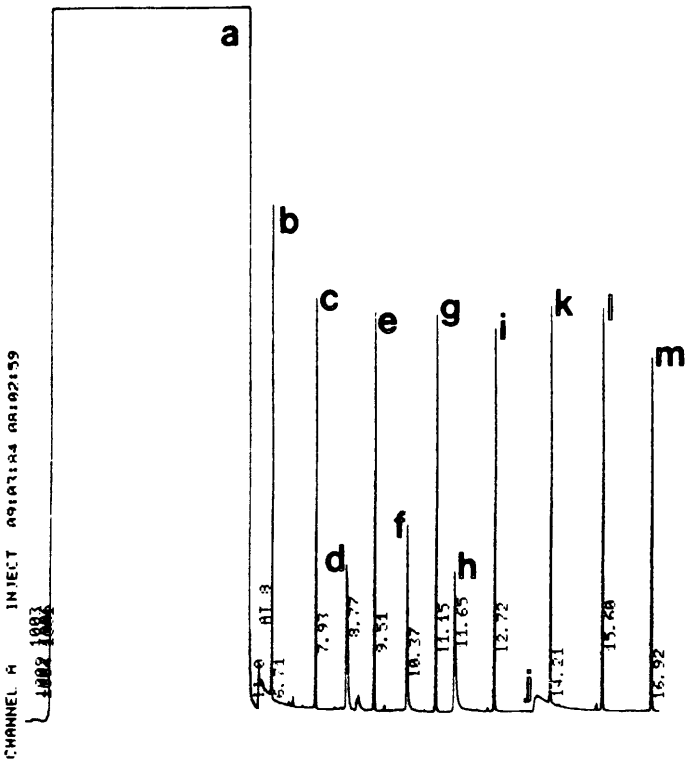


Fig. 4.4 Chromatogram of 20 µl of Test Mix using a perforated packed bed static solvent effect inlet with water-washed silicon deactivation. The inlet was not heated during transfer of the solutes to the column. Peak identities as in Fig 4.1, except k: *n*-tridecane.

When the inlet heater was switched on as the solvent peak returned to baseline the alkane peaks were sharp but some of the polar compound peaks were split. A small hump on the baseline appeared just after the solvent peak. This was shown by mass spectrometry to be *n*-hexane. Progressively earlier heating resulted in the earlier elution of the second section of each split peak until the two sections co-eluted and sharp symmetrical peaks were obtained. Late eluting peaks were initially less widely split and coalesced sooner than did early eluters. With earlier heating the baseline hump moved nearer to, and finally disappeared into, the solvent peak (Fig 4.5).

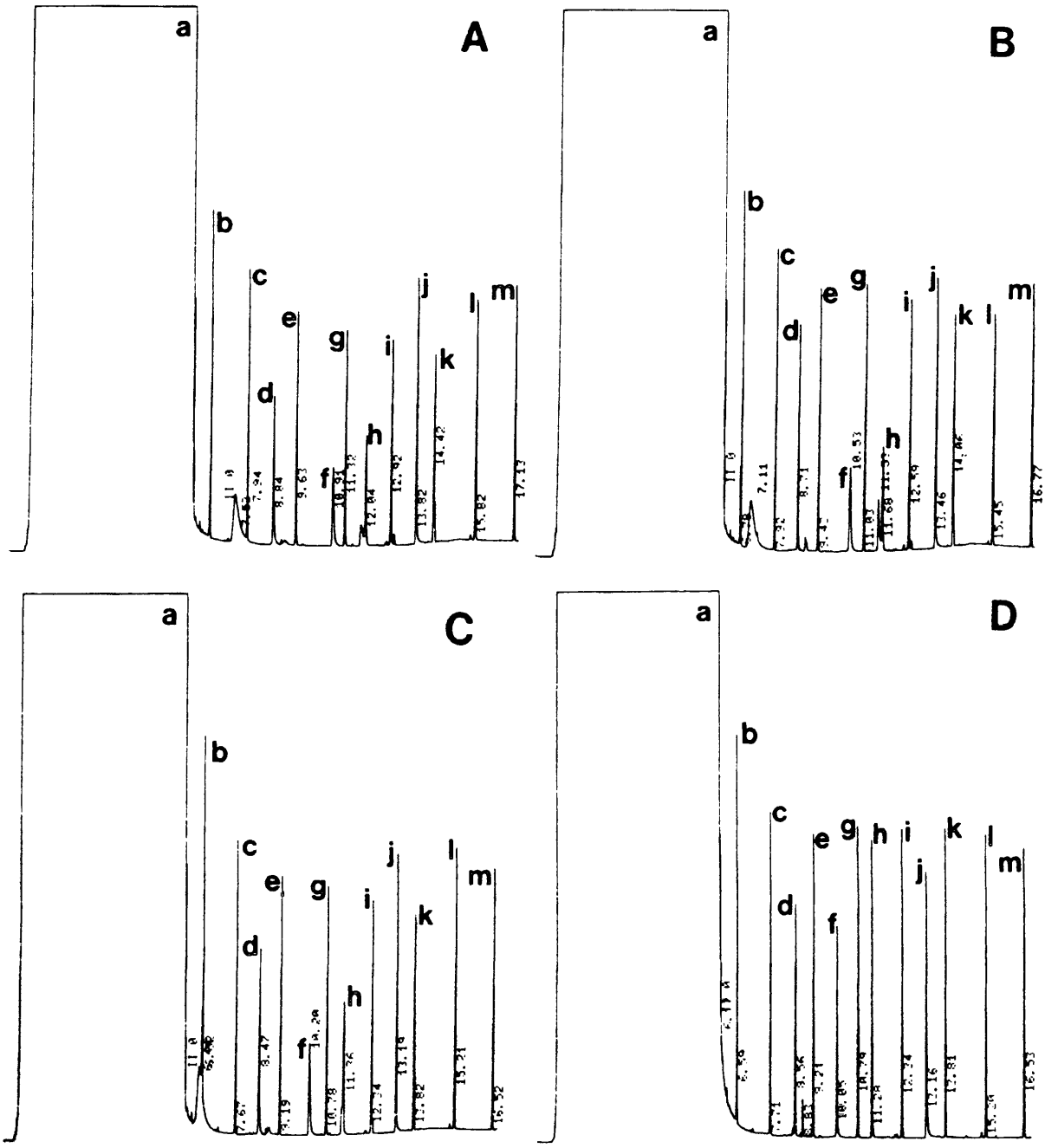


Fig. 4.5 Chromatograms of 20 µl of Test Mix using a perforated packed bed static solvent effect inlet. The time after injection at which the inlet was heated was; A: 6,5 min; B: 6,0 min; C: 5,5 min; D: 5,0 min. Peak identities as in Fig 4.1, except k: *n*-tridecane.

As the heating time was made even earlier distortion of the leading edges of the late-eluting alkane peaks became apparent (Fig 4.6).

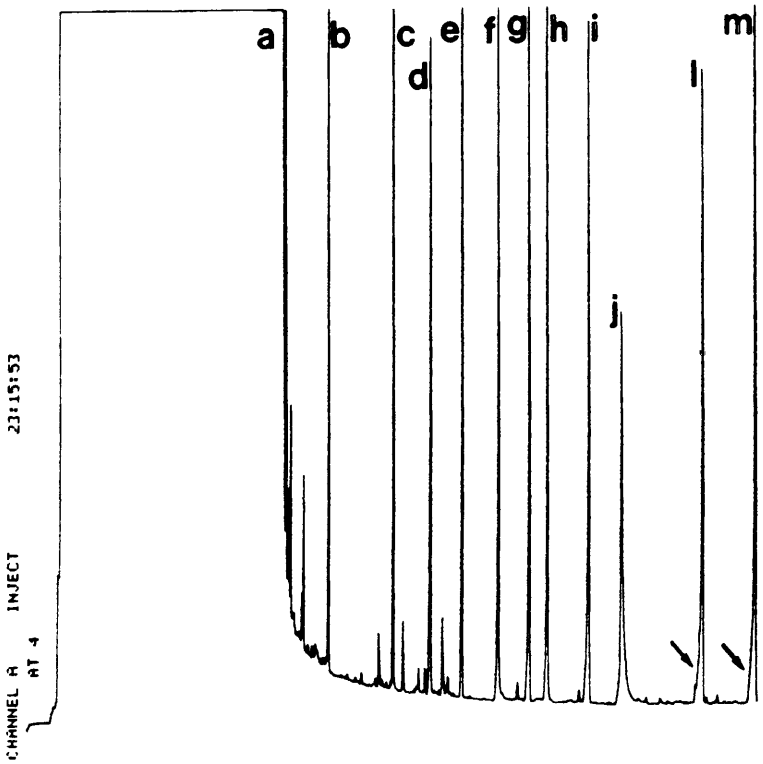


Fig. 4.6 Distortion of leading edges of late-eluting alkane peaks due to early heating of a packed bed, static solvent effect inlet.

Heating to different maximum temperatures

When the bed was heated to only 100°C the decanol peak was slightly broadened compared to when the bed was heated to 250°C. None of the other peaks was affected. Heating to 150°C or 200°C yielded peaks as narrow as those from heating to 250°C (Fig 4.7).

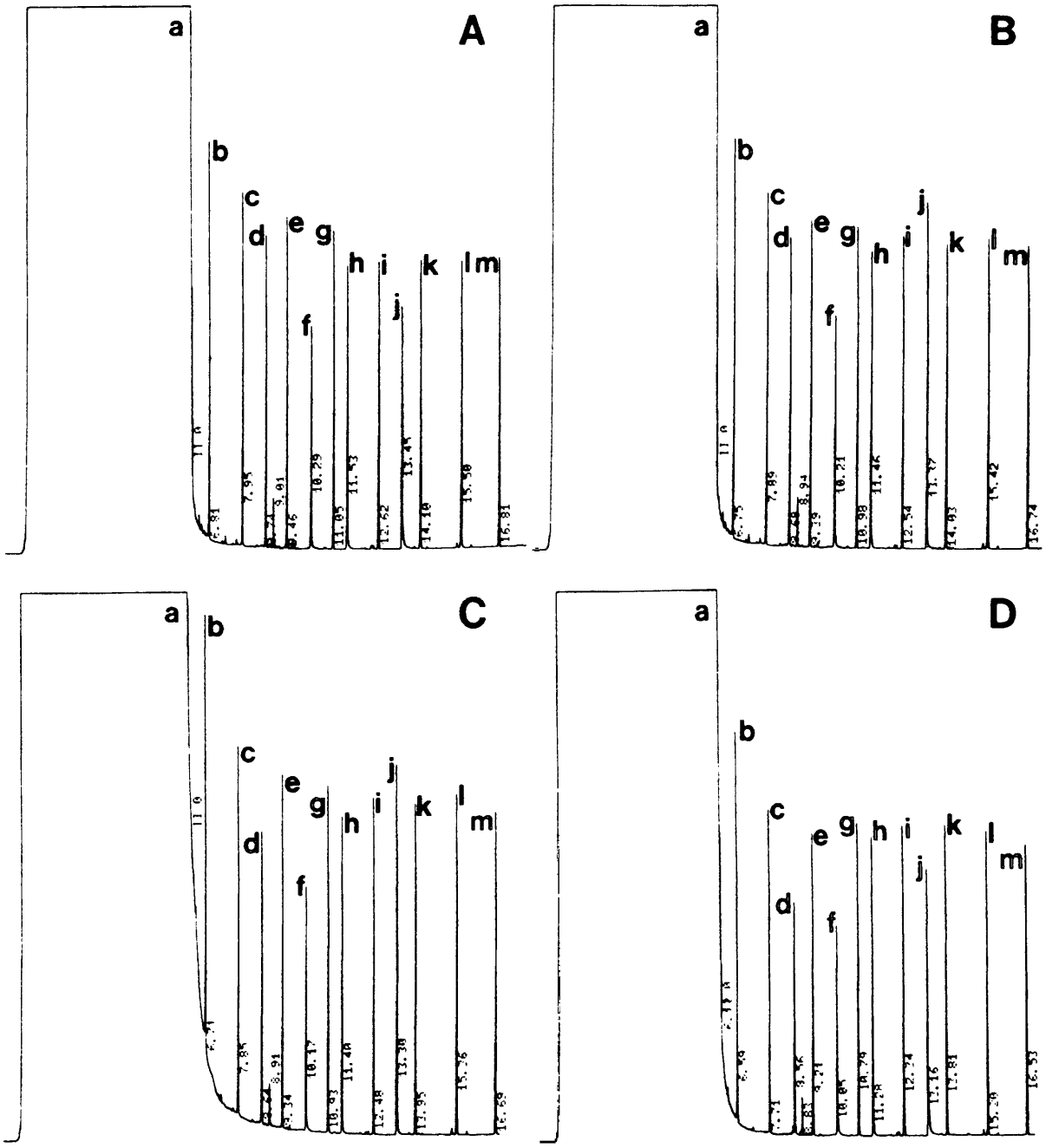


Fig. 4.7 Effect on peak shapes of heating a static solvent effect inlet to different temperatures during transfer of the solutes to a capillary column. A: 100°C; B: 150°C; C: 200°C; D: 220°C. Peak identities as in Fig 4.1, except k: *n*-tridecane.

Precision

Figures for the quantitative precision of chromatographic runs using the packed bed inlet are given in Table 4.1.

Table 4.1 Coefficients of variation of peak areas and percentage areas for 20 µl injections of a 2:10 ^{7} Test Mix onto a perforated, packed bed, static solvent effect inlet. Inlet heating was time-cued.

Compound	% coefficient of variation	
	Area	% area
n-octane	2,7	0,8
n-nonane	2,8	0,6
2,6-dimethyl-4-heptanone	6,2	3,6
n-decane	3,0	0,5
p-cresol	4,2	1,0
n-undecane	3,0	0,5
2,6-dimethylaniline	3,0	0,5
n-dodecane	3,0	0,5
n-decanol	4,4	1,2
n-tridecane	3,0	0,5
n-tetradecane	3,0	0,5
n-pentadecane	2,8	0,6

Resolution

The Trenzhal numbers obtained when using the static solvent effect inlet were between 26,7 and 33,5 for the 7 pairs of alkanes in the Test Mix, those from split injections onto the same column were between 26,6 and 38,5.

DISCUSSION

The widths of peaks leaving the column when the inlet was not heated, and the period over which the time of heating could be varied while still affecting peak splitting, confirm the earlier finding (Pretorius, Rohwer, Lawson & Apps 1984) that, in the absence of bed heating, solutes leave a packed bed inlet as wide bands. The packed bed used here delivered alkanes with band widths of less than 100 s and polar compounds with band widths of 100 s or more when unheated. This clearly refutes statements (Grob & Grob 1978; Saravelle, Munari & Trestianu 1987; Grob 1985) that solutes must enter the column as very narrow bands if good resolution is to be achieved.

The shapes of solute bands leaving packed bed inlets were investigated directly by K. Lawson and H.W. Viljoen (Lawson 1987 Chap 5). Alkane bands were approximately Gaussian in shape while *n*-decanol eluted as a wide, almost flat-topped band. In their experiments inlet heating narrowed the bands but did not affect their shape - a result which can be reconciled with the present findings on peak splitting only by invoking differences in bed geometry and surface between the loose-packed Chromosorb they used and the silicon deactivated, sintered, perforated bed employed here.

That narrow peaks and high resolution can be achieved when the solute bands leaving the inlet are at least several seconds wide is due largely to the effects of the stationary phase focussing which occurs as the solute bands enter the column; a phenomenon which has been considered in detail by Pretorius, Rohwer & Lawson (1983), Pretorius & Lawson (1986a, b) and Pretorius, Lawson & Rohwer (1986).

An additional focussing mechanism affected only those peaks which eluted close behind the solvent. The octane and nonane peaks were extremely narrow (0,85 s for octane) because they moved all the way through the column on the trailing edge of a ramp of increasing retention generated by the heavy loading of the stationary phase by solvent (Harris 1973; Pretorius & Lawson 1986b).

Splitting of the late-eluting alkane peaks due to early heating is interpreted as being due to condensation on the column of sufficient sample to form lenses which were blown along the column by the carrier gas. This is equivalent to attempts to carry out the solvent effect in the presence of stationary phase - a procedure which is known to be liable to cause severe peak distortion (Grob 1983; Takeoka & Jennings 1984).

The peak distortion which accompanies it makes incorrect heater timing a major cause of unsatisfactory performance with packed bed, solvent effect inlets. Fortunately the dependence of the pattern of peak distortion on the heater timing enables straightforward, empirical optimization. The procedure used to determine the correct heating time was as follows. A volume of $2{:}10^7$ Test Mix equal to that of the sample to be analysed was injected and the inlet heated as the solvent peak returned to baseline. The width in time of the top of the solvent peak was measured and a second injection was made with the heater switched on 60 % of the solvent peak width after injection. The resulting chromatogram was inspected for evidence of early or late heating and the heating time adjusted accordingly in 30 s increments. Once the heater time was set for a particular sample volume under a given set of conditions no re-adjustment was necessary.

The hexane which caused the baseline hump when heating of the inlet was late was presumably reversibly adsorbed onto the bed surface at 50°C and driven off by the rise in temperature. A similar-looking hump, with no comment on its significance, appears in chromatograms run by Grob & Kuhn (1984) using ballistic heating of a "retention gap" inlet. The Chrompack thermodesorption unit generates a double set of solvent humps (Kirschmer & Oehme 1984). That even a volatile solvent like *n*-hexane remains in the inlet after the evaporation of the main film is in sharp contrast to the theoretical prediction of Pretorius, Phillips & Bertsch (1983c) that only solutes with molecular weights above 500 would lag. Interactions between the inlet surface and the sample components, which are inevitable in a real system, account for the discrepancy.

Although these static solvent effect inlets were designed and tested primarily as models for their dynamic counterpart the qualitative and quantitative performances they delivered in their own right were superior to those of other inlet systems for large liquid specimens (Lawson, Pretorius & Apps 1987), even so-called "universal" systems from commercial sources (Saravelle *et al.* 1987).

One outstanding advantage of "cold" sampling techniques is that thermolabile compounds are exposed to temperatures no higher than are necessary for their passage through the column. The heating of the bed which is necessary for the efficient operation of a packed-bed solvent effect inlet potentially exposes the solutes to a temperature of 250°C. Peak width, however, was almost independent of maximum temperature, indicating that all the test compounds left the bed at temperatures below 150°C and that all except decanol left at below 100°C. Only compounds which combine thermolability with high polarity will be adversely affected by bed heating.

SOLVENT PEAK MONITORING

The successful operation of a packed-bed, solvent effect inlet depends on (among other things) the time at which the inlet is heated having a fixed relationship to the time at which solvent evaporation is completed (p 51). Consequently, successful operation of a solvent effect inlet with time-cued heating depends on the solvent evaporation time remaining constant from sample to sample. This in turn demands constant conditions of temperature and carrier gas flow, and the injection of a fixed sample size. These requirements might be expected to fall away if the time at which the inlet was heated was determined by directly monitoring the evaporation of the solvent peak.

EXPERIMENTAL

To test the practicability of such an approach an adjustable splitter (designed by Rohwer* pers. comm.) was installed between a standard, static solvent effect inlet and a capillary column (Fig 4.8). A fraction of the inlet effluent was diverted to an auxilliary detector and the inlet was heated when this detector showed that solvent evaporation was complete. The amount of inlet effluent diverted was adjusted by controlling the pressure of the splitter's make-up gas. Two types of auxilliary detector were used; one in which the splitter effluent was burned in air so that completion of solvent evaporation was signalled by a change in flame colour from blue to colourless and one in which the splitter effluent was fed to an FID.

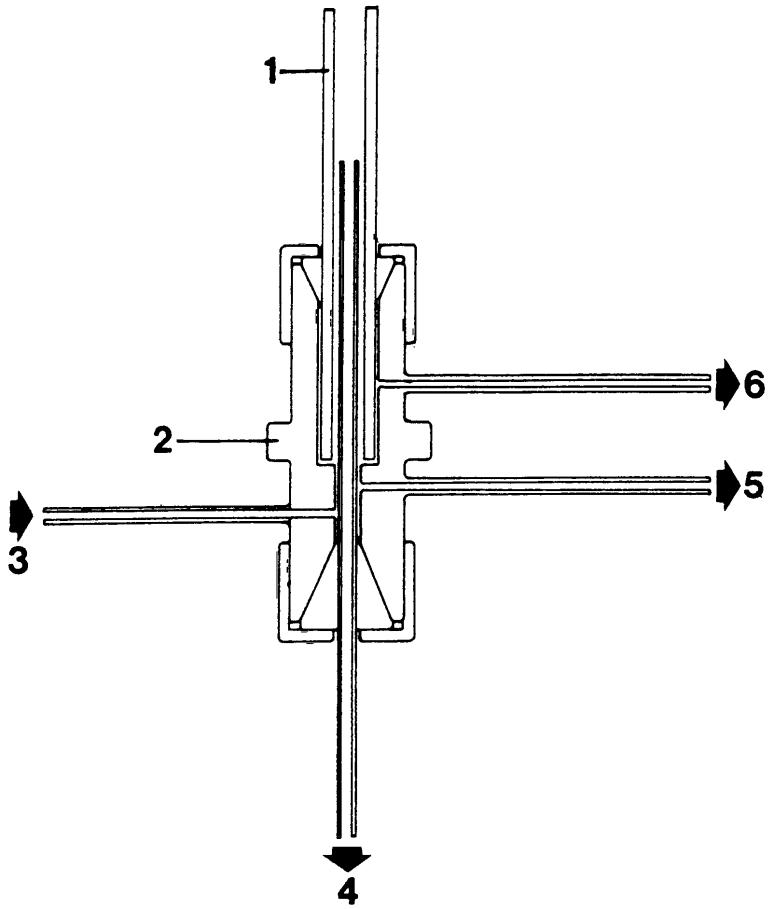


Fig. 4.8 Schematic vertical section through a splitter used to divert an adjustable fraction of the effluent from a static solvent effect inlet to a flame-based detector in order to monitor the evaporation of the solvent from the sample. 1: outlet of inlet; 2: 1/16" Swagelok union; 3: pressure controlled gas supply; 4: column; 5: vent; 6: to flame detector.

*Prof. E.R. Rohwer, Institute for Chromatography, University of Pretoria

RESULTS

The quantitative and qualitative performance of the static solvent effect inlet with heating cued by either of the solvent peak monitors was consistently poorer than with time-cued heating (Table 4.2, Fig 4.9). In addition the monitor split ratio was extremely difficult to adjust and control due to the very small pressure drop between the splitter lumen and the head of the column.

Table 4.2 Coefficients of variation of peak areas and percentage areas for 20 µl injections of a 2:10 ^{7} Test Mix onto a perforated, packed bed, solvent effect inlet. Inlet heating was cued by a solvent peak monitor. Compare Table 4.1.

Compound	% coefficient of variation	
	Area	% area
n-octane	4,9	1,8
n-nonane	8,4	5,1
2,6-dimethyl-4-heptanone	9,2	6,0
n-decane	8,7	5,4
p-cresol	5,9	6,6
n-undecane	7,0	3,7
2,6-dimethylaniline	3,0	3,8
n-dodecane	3,6	0,5
n-decanol	6,4	8,1
n-tridecane	1.3	2,8
n-tetradecane	1,6	4,1
n-pentadecane	1,9	4,4

DISCUSSION

Even if the technical difficulties of set-up and adjustment are overcome the success of a solvent effect inlet with heating cued by a solvent peak monitor depends on the assumption that the correct time to heat such an inlet is, in fact, after the completion of solvent evaporation. Although this assumption has not been systematically investigated there is some evidence to suggest that it may be unjustified. Lawson (1987 Chap 10) found that the column's stationary phase recovered within milliseconds from the swelling induced by saturated solvent vapour. For stationary phase swelling to have been effective in focussing the octane and nonane peaks (p 55) they must have been transferred to the column along with the last of the solvent film. This suggests that heating should begin just before solvent evaporation is complete. With this, and its poor practical

performance, in mind the solvent peak monitor was abandoned.

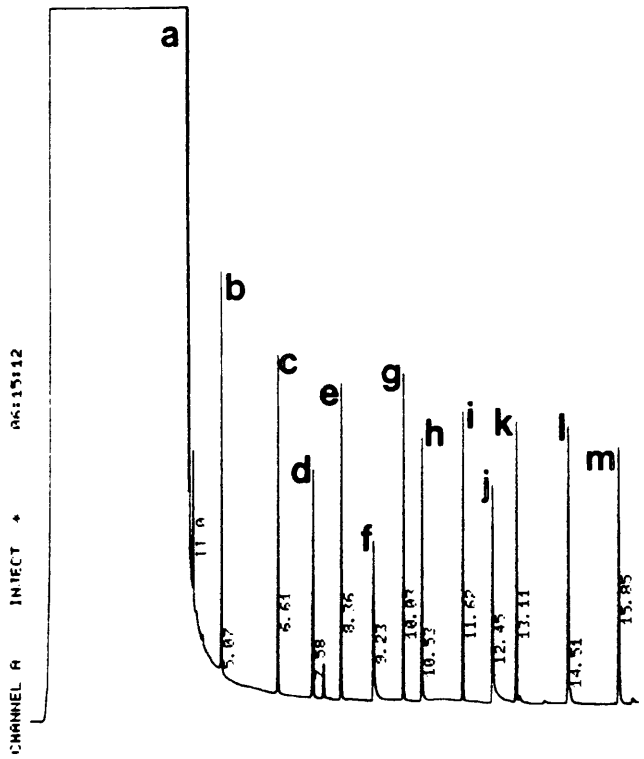


Fig. 4.9 Chromatogram from 20 µl of 2:10 ^{7} Test Mix in *n*-hexane sampled by the static solvent effect with inlet heating cued by a solvent peak monitor. Peak identities as in Fig 4.1, except k: *n*-tridecane.

DYNAMIC SOLVENT EFFECT CONCENTRATOR GEOMETRY

Like its static counterpart the dynamic solvent effect can, in theory, occur within a wide range of concentrator geometries and gas flow rates. Nevertheless its practical usefulness depends on optimization of concentrator geometry within the constraints imposed by compatibility with capillary columns.

CONSTRUCTION AND EXPERIMENTAL

Designs of concentrator which represent significant stages in the development of the standard type are shown in Fig 4.10.

1. The dynamic solvent effect was first carried out on a 50 mm long bed of 125-150 µm Chromosorb G-HP held in place by a small glass sinter in the drawn down tip of a 4 mm i.d. glass tube. The solvent was *n*-hexane and the specimens were either a subliming iodine crystal or a solution of iodine.

2. A 1 cm^3 liquid specimen of diesel in octane at a concentration of $1:10^8$ was concentrated on a 22 x 2,2 mm bed of 125-150 µm Chromosorb W-H.P. held in a glass tube between two 150 µm glass sinters. A chromatogram was run on a 20 m x 0,3 mm x 0,4 µm methyl silicone column with a temperature programme of $5^oC\ min^{-1}$ from 100°C for both bed and column and a detector sensitivity of 10^{-12} x 32.

3. Concentrators with loose packings of porous particles; 53-63 µm borosilicate glass sintered, crushed and sieved to approximately 500 µm, 100-200 µm Spherisorb or 63-125 µm Fractosil 25 000, held in place with small plugs of glass wool in 1,8 mm i.d. glass tubes, were tested for height of solvent rise against perforated beds (q.v.) of the same dimensions under the same conditions of temperature (20-25°C) and gas flow (20 $cm^3\ min^{-1}$) with *n*-hexane as solvent.

4. Concentrators of 1,8 mm i.d. with porous beds perforated by (more or less) central, axial gas channels were produced by sintering borosilicate glass chips into glass tubes around a variety of formers which were subsequently removed (see "Concentrator Production"). Only rough comparisons of height of solvent rise against gas flow were made since these concentrators suffered various shortcomings which would have precluded their practical application.

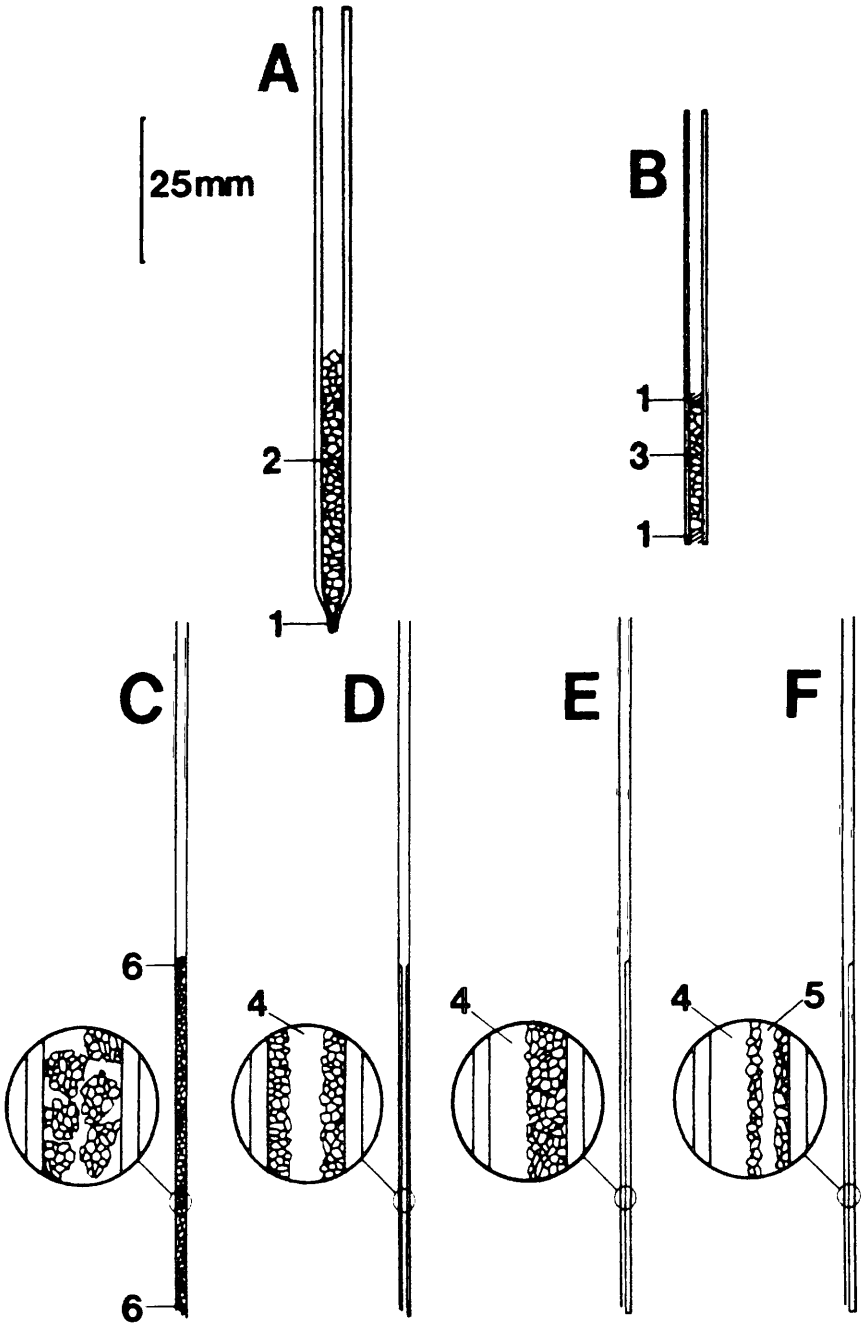


Fig 4.10 Stages in the evolution of concentrators for the dynamic solvent effect. A: diatomaceous earth bed held by a glass sinter in the drawn down tip of a borosilicate glass tube; B: diatomaceous earth bed held by two sinters without restricting diameter of tube; C: porous particles held between glass wool plugs; D: porous annulus of sintered glass chips around an axial gas channel; E: porous layer of sintered glass chips on one side of the tube, gas channel on the other; F: the same as E except that the porous layer is itself axially perforated by an open channel. 1: glass sinter; 2: 125-150 µm Chromosorb G-HP; 3: 125-150 µm Chromosorb W-HP; 4: gas channel; 5: liquid channel; 6: glass wool.

5. Concentrators of 1,8 or 1,4 mm i.d. with the porous bed up one side and the gas channel up the other were produced by sintering a layer of borosilicate glass chips onto one side of a glass tube (see "Concentrator Production"). The effect of bed particle size on the height of solvent rise was investigated as follows. A set of concentrators with bed particles in the size ranges 45-53, 53-63, 63-75, 75-105, 105-125, 150-180 and 180-210 µm were produced with a constant bed:channel cross-sectional area ratio of approximately 2:1. Two concentrators with each particle size were produced. No surface modification was carried out. Charcoal-filtered nitrogen was blown into each bed at 20 cm^3 min^{-1} at a temperature of 25°C and the height of rise of an *n*-hexane film was measured against a millimeter scale (Fig 4.11).

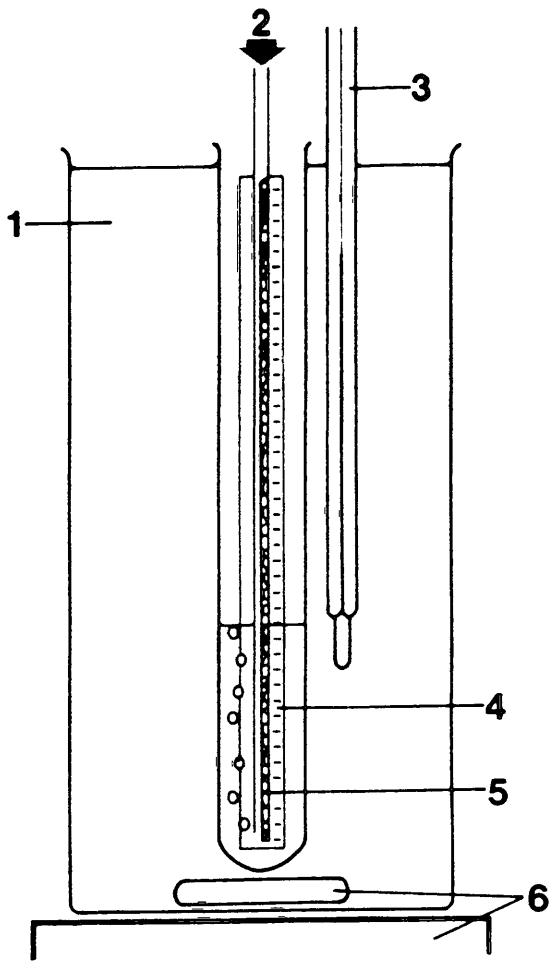


Fig 4.11 Apparatus used to measure the height to which films of solvent rose into dynamic solvent effect concentrators under various conditions. 1: water bath; 2: gas flow; 3: mercury-in-glass thermometer; 4: millimeter scale; 5: dynamic solvent effect concentrator 6: heater and stirrer.

6. The effect of sampling temperature and gas flow rate on the height to which the solvent film rose in the concentrators' beds was investigated as follows.

Concentrators with bed particles of 53-63 µm and 125-150 µm were produced with bed:channel cross sectional area ratios of 2:1. The glass surface was not modified. Using the same apparatus as that used for the effect of bed particle size on film height the height of films of *n*-hexane was measured at flow rates between 11 and 115 $cm^3\ min^{-1}$ at a temperature of 26°C and at temperatures between 10 and 59°C with a flow rate of 20 $cm^3\ min^{-1}$ on both concentrators. On the 125-150 µm particle concentrator the height of methanol films was measured for flow rates between 13 and 103 $cm^3\ min^{-1}$ at a temperature of 25°C and at temperatures between 15 and 52°C with a flow rate of 20 $cm^3\ min^{-1}$.

7. A single concentrator in which the lateral porous bed was axially perforated by a 0,4 mm solvent channel was made by horizontal sintering around a thin-walled nickel tube which was subsequently dissolved away with acid (see "Concentrator Production"). Height of solvent rise against various gas flows was measured with *n*-pentane as solvent.

RESULTS

1. From either iodine vapour or solution crystals could be grown on the top of the bed. With *n*-hexane solvent the gas flow rate was limited to 3-4 $cm^3\ min^{-1}$ for a film height of 50 mm.

2. The chromatogram of the diluted diesel specimen is shown in Figure 4.12.

3. In concentrators of 1,8 mm i.d. none of the porous particle fillings allowed film heights of greater than 10 mm with *n*-hexane solvent at flow rates of 15-20 $cm^3\ min^{-1}$.

4. Concentrators with centrally perforated beds typically held films of *n*-hexane more than 50 mm high against gas flows of 15-20 $cm^3\ min^{-1}$.

5. Laterally perforated concentrators held films of *n*-hexane between 20 and 60 mm high depending on their bed particle sizes (Fig 4.13).

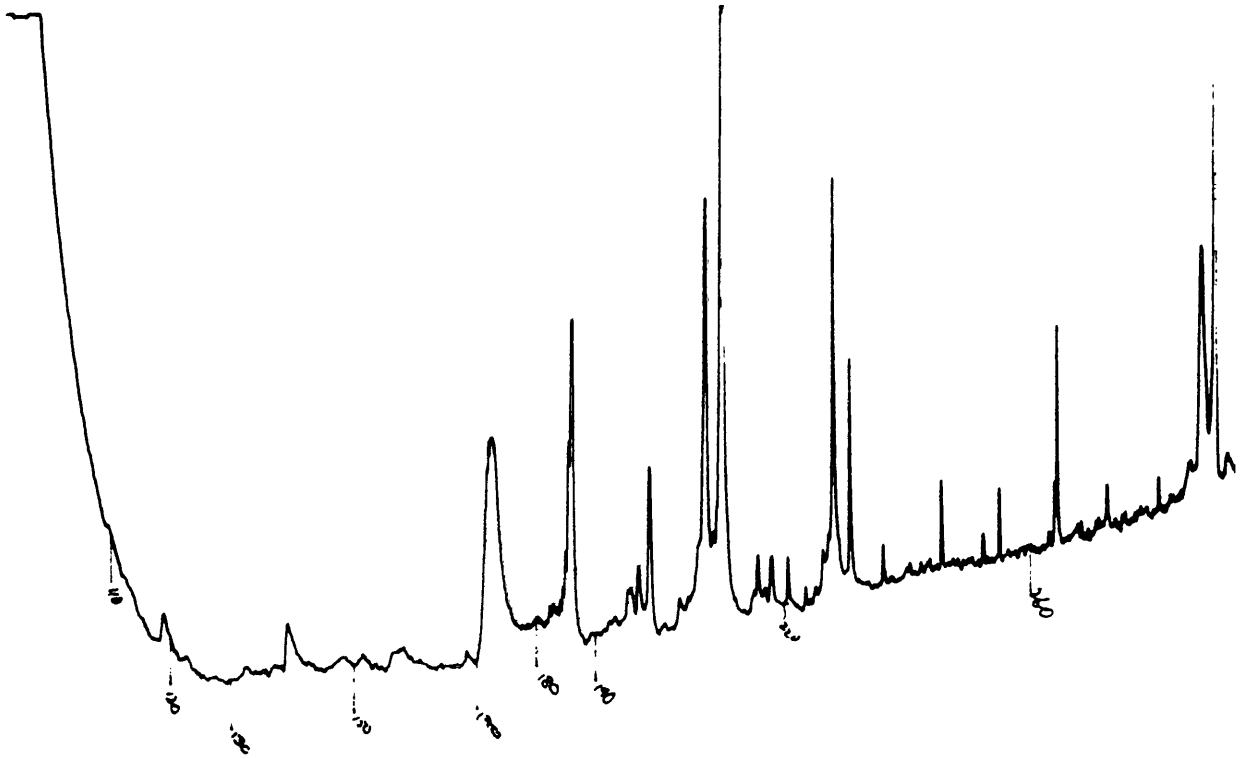


Fig. 4.12 The first chromatogram run using a dynamic solvent effect inlet system. The specimen was 1 cm^3 of a solution of diesel in octane with a concentration of $1:10^8$. For analytical conditions see text.

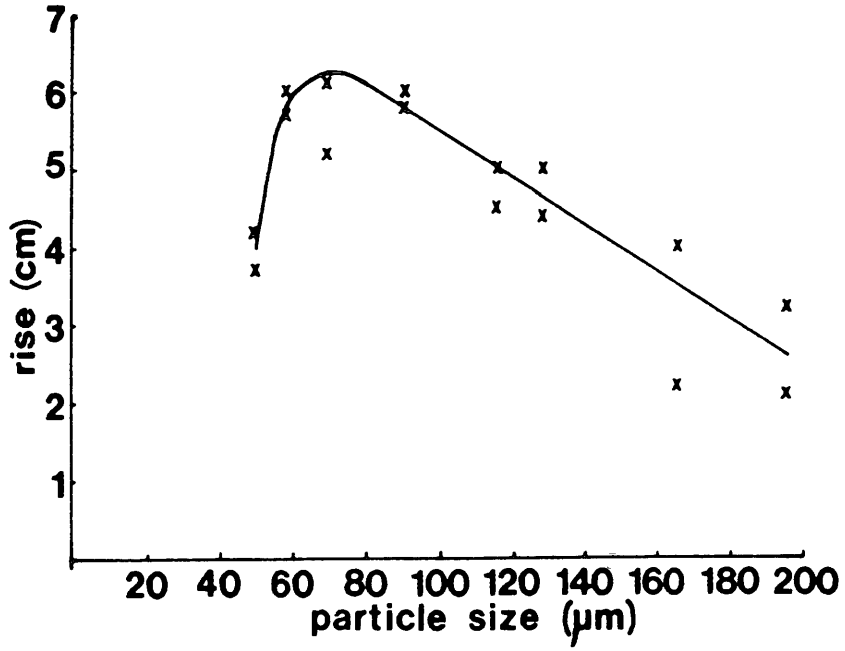
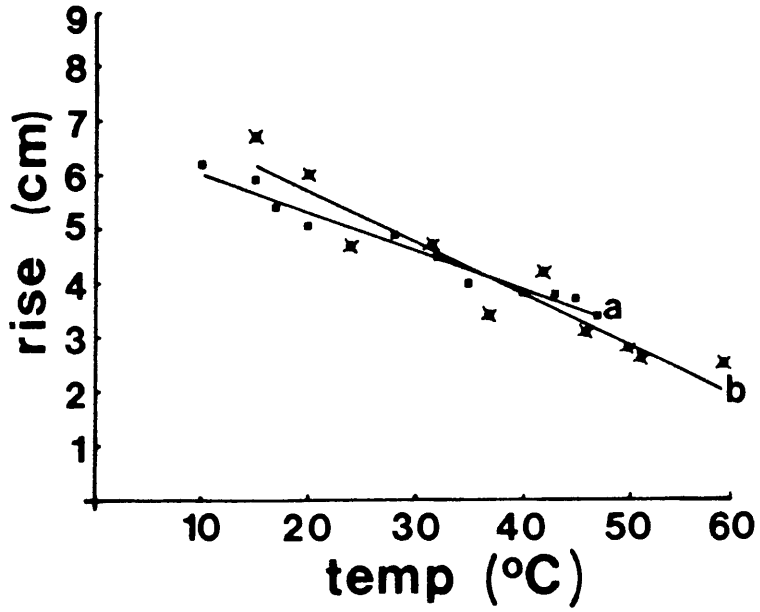


Fig. 4.13 The effect of bed particle size on the height to which a film of *n*-hexane rises into a dynamic solvent effect concentrator.

6. The height of rise of both methanol and hexane films was inversely dependent on temperature and gas flow rate (Figs 4.14 and 4.15). The effect was approximately the same for both solvents. The hexane film in the 53-63 μm bed was marginally more sensitive to changes in temperature and less sensitive to changes in gas flow rate than the same solvent in the 125-150 μm bed.



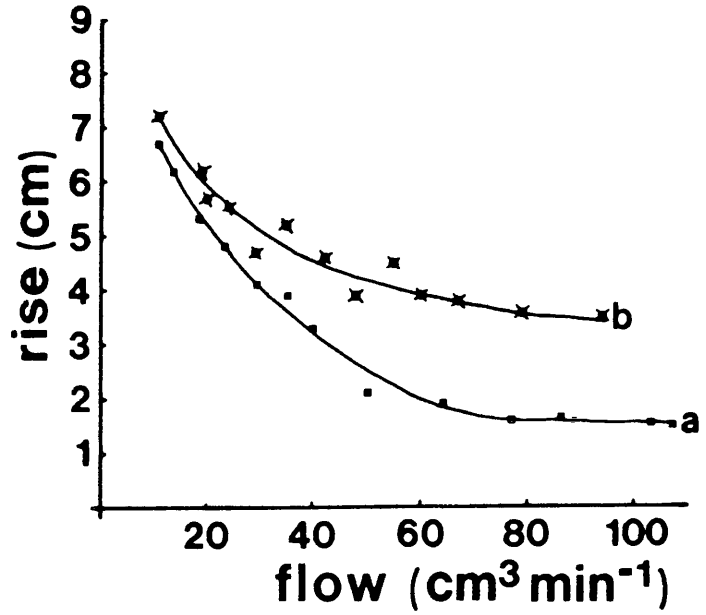


Fig. 4.14 The effect of temperature and gas flow rate on the height to which *n*-hexane rises into a dynamic solvent effect concentrator with beds of a: 125-150 μm particles and; b: 53-63 μm particles.

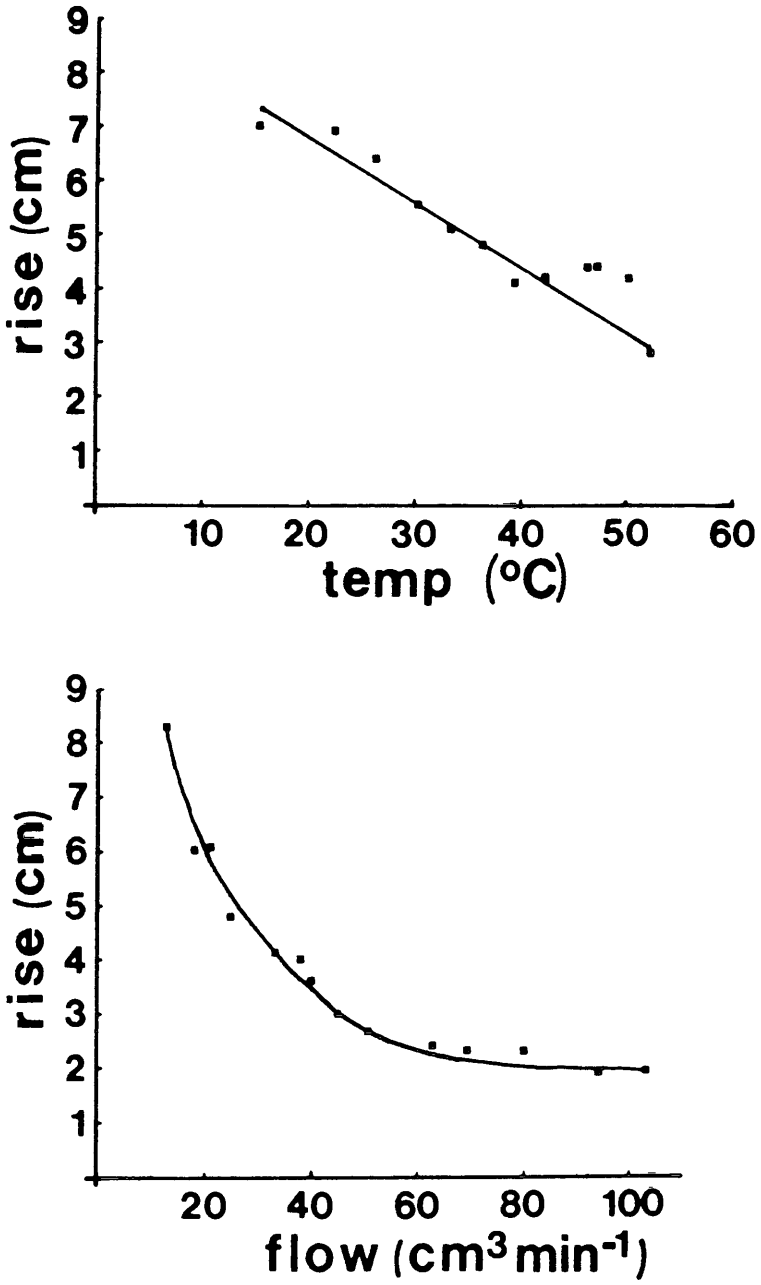


Fig. 4.15 The effect of temperature and gas flow rate on the height to which methanol rises into a dynamic solvent effect concentrator with a bed of 125-150 µm particles.

7. The double channel concentrator held a 40 mm high film of *n*-pentane against a gas flow of 1 500 cm^3 min^{-1}.

DISCUSSION

The two loose-pack Chromosorb concentrators are mainly of historical interest. The growth of an iodine crystal on the bed with the constricted lower end was the first demonstration of the dynamic solvent effect in practise. The chromatogram of the diesel specimen, with its obvious shortcomings, was the first produced using a dynamic solvent effect concentrator/inlet system (see Figure 5.18 for a comparison).

The limited film heights (i.e. gas flow capacities) in loose packed concentrators were due to a number of factors. A liquid rising against a gas stream in a loose packed bed is confined to the intra-particle and smallest inter-particle pores because it is blown out of the larger pores by the gas. In a random packing of irregular or spherical particles the area of contact between adjacent particles is small, so that liquid passing from one particle to the next must pass a "bottleneck". The mean diameter of the intra-particle pores in which most of the liquid flow must take place is 0,065 µm in Spherisorb and 2,2 µm in Fractosil compared to 20 µm for the wetted pores in an axially perforated, sintered bed of 100 µm particles. The resistance to the flow of fluid through a pore is a fourth power inverse function of the pore size (Streeter 1966 p 220). Thirdly the area of liquid exposed to shear from the downward moving gas (see later) is much larger in a loose packed bed than in a perforated one; e.g. in concentrators with an i.d. of 2mm and a bed 5 cm long the liquid-gas interface has an area of approximately 6 000 mm^2 for 100 µm porous spheres *versus* 10 mm^2 for a lateral-hole, perforated bed.

The film holding abilities of loose-packed concentrators are also compromised by the need to use plugs of e.g. glass wool or sintered glass in order to hold the bed in place. Such plugs may well be even less efficient in the transport of liquid than are the beds they support.

In perforated concentrators the presence of an open gas channel allowed liquid to flow upwards through all the pores of the bed with only the liquid at the bed-channel interface subject to shear from the gas flow. In many ways the bed was equivalent to a single porous particle with the gas flowing over only one face. The large size of the "particle" allowed larger intra-particle pores and thus much lower resistance to flow as discussed above. Beds of grain size between 53 and 105 µm held the highest films. Standardized concentrators for analytical work employed particles at the upper end of, and just above, this size range because specific surface area, and thus adsorptive activity, increases with decreasing particle size (Holland 1973 p 163).

The importance of gas-liquid shear in limiting the height of rise of solvent films was established by the difference between 125-150 and 53-63 µm beds in the effects of temperature and gas flow rate on the height of the solvent films that they held. Increases in both temperature and gas flow rate increase the rate of solvent evaporation which, as the basic model (Chap 3) predicts, led to a decrease in solvent film height in both beds. When the increase in evaporation was due to a rise in temperature the effect was more marked for the 53-63 than for the 125-150 µm bed because the resistance to liquid flow is higher in beds of smaller particles. In contrast, an increase in evaporation rate caused by faster gas flow is accompanied by a more marked reduction in film height in the coarser of the two beds, in direct opposition to the predictions of the basic model. Thus there must be, in addition to flow resistance in the bed, some factor which affects liquid held in coarse beds more than that held in fine beds, and which is due to an increase in gas flow rate; this factor is undoubtedly mechanical shear. The modelling of its influence on a stream of liquid whose free surface lies at various, unmeasured, depths in pores of widely varying shapes and sizes will not even be attempted.

The high gas volume flow rates supported by the doubly perforated concentrator demonstrate the importance to the concentrator's sampling rate of resistance to liquid flow in the bed. Nevertheless the practical advantages of a doubly perforated design are limited by the need for equilibrium between liquid film and gas to be reached within the length of the film. In a 1,8 mm i.d. concentrator at a gas flow rate of 1 500 cm^3 min^{-1} the linear gas flow rate is 400 cm s^{-1}. The plate height due to mass transfer resistance in the gas phase is then approximately 160 mm for a solute with a molecular weight of 200 (Lee *et al.* 1984 p 18). This is about 3 times the length of the solvent film and, consequently, for specimens in a gas matrix a very high sampling rate will be counteracted by inefficient accumulation. On the other hand high liquid flow rates with low gas flow rates are generated by sampling near the boiling point of the solvent. Such a situation occurs when very volatile solvents such as *n*-pentane, dichloromethane or Freon 11 are used for head-space sampling at room temperature or when specimens in a solvent matrix are sampled near their boiling point. Even in these cases the film holding abilities of the doubly perforated concentrator may be regarded as something of an overkill. The influence of mechanical shear on solvent rise in both loose-packed and singly-perforated beds suggests that, at very high gas flow rates, the surface of the liquid in a doubly perforated concentrator is carried downwards while the solvent film is maintained by rise through the liquid's channel. Under these conditions no solvent effect focussing can occur. In addition such doubly-perforated beds proved extremely difficult to make.

PRESSURE DROP

The presence of an open gas channel in the perforated concentrators was expected to decrease the pressure drop necessary to generate a workable gas flow.The working pressure drop through a trap has an influence on the type of sampling plumbing, joints and gas supply which can be employed with a given sampling system. A comparison was made of the pressure drops across four types of traps; a dynamic solvent effect concentrator, two sizes of adsorption trap and an open-tubular, fused silica trap.

EXPERIMENTAL

The dynamic solvent effect concentrator was of standard design with a tube i.d. of 1,4 mm, a bed length of 6 cm, a bed:channel ratio of 1:1 and a bed particle size of 105-125 µm. *n*-Hexane was used as solvent.

A "micro" adsorption trap was made by filling 90 mm of a 1,4 mm i.d. glass tube with 20 mg of 35-60 mesh Tenax-GC held in place by two small glass wool plugs.

A "macro" adsorption trap was made from a 100 mm length of 6 mm o.d., 4,8 mm i.d. stainless steel tube containing 200 mg of Tenax-GC between glass wool plugs.

The fused silica, open-tubular trap was simulated by a 1 m length of uncoated 0,32 mm i.d. fused silica tubing.

Each trap type was connected to a source of pure air which was mass flow controlled between 2 and 60 $cm^3\ min^{-1}$. The pressure immediately upstream of the trap was measured with a water manometer for the dynamic solvent effect concentrator and the macro adsorption trap, by a water manometer and a mercury manometer for the micro adsorption trap and by a mercury manometer and a Borden gauge for the fused silica, open-tubular trap. No single pressure gauge could cover the required pressure range with sufficient accuracy. Gas flow rate was measured directly downstream of each trap with a bubble flow meter which was disconnected while pressure readings were taken.

Pressure drops were measured for a number of flow rates between 2 and 60 $cm^3\ min^{-1}$ for each trap and the equation of the line of best fit to flow *vs* pressure was calculated.

RESULTS

There were marked differences in the resistance to gas flow of the different traps.

In the equation:

$$F = P/R$$

where: F = volume flow rate ($cm^3\ min^{-1}$)
P = pressure drop (mm water)
R = resistance

R = 0,22 for the dynamic solvent effect concentrator;
R = 0,83 for the macro adsorbent trap;
R = 21,00 for the micro adsorbent trap;
R = 639,0 for the fused silica, open-tubular trap.

DISCUSSION

Adsorbent traps of micro dimensions have been used for e.g. wine flavour analysis (Williams & Strauss 1977), human odours, fruit and vegetable odours, and plant taxonomy (Murray 1977) at flow rates of up to 40 $cm^3\ min^{-1}$ with two traps in series during sampling, and of 40 $cm^3\ min^{-1}$ during desorption from single traps. Murray (1977) mentions that stoppers used in the sampling plumbing needed to be of small diameter to resist the back pressure from the traps, and that micro-scale traps were unsuitable for high-volume sampling of air pollutants.

Macro adsorption traps have been employed in work on insect pheromones (e.g. Byrne, Gore, Pearce & Silverstein 1975) and air pollution (e.g. Jonnson & Berg 1980; Bertoni, Bruner, Liberti & Perrino 1981). For the latter application standardized traps are commercially available (e.g. Supelco 1988 pp 227-231). In these applications sampling is usually carried out by sucking gas through the trap and the maximum available pressure drop of 1 atmosphere is sufficient to generate flows of tens of litres per minute. The use of macro-scale traps packed with 80-100 mesh molecular sieve 5Å has been reported to limit gas flows to a maximum of 50 $cm^3\ min^{-1}$ (Deprez, Franzman & Burton 1986)

Fused silica, open-tubular traps are a recent development advocated by Grob & Habich (1985) to improve the desorption step's compatibility with capillary columns. This type of trap has been applied by Burger & Munro (1986) to insect pheromones, mammal urine and flavours. Although sampling flow rates were not reported, problems with the

production of 100 cm3 min-1 flow rates by syringe pump were experienced during testing.

The very low back pressures generated during dynamic solvent effect sampling allow considerable versatility in the design of sampling plumbing. Ground glass joints, even those with small areas of contact (see e.g. Chaps 11 and 12), may be sealed with distilled water rather than with potentially contaminating grease (Fig 4.16). Connections between components may be made with push fit, thin-walled polytetrafluoroethylene sleeves which are more easily manipulated and less adsorptively and catalytically active than metal compression fittings. Whole live animals may be sampled without exposing them to pressures outside the normal range of variation in atmospheric pressure.

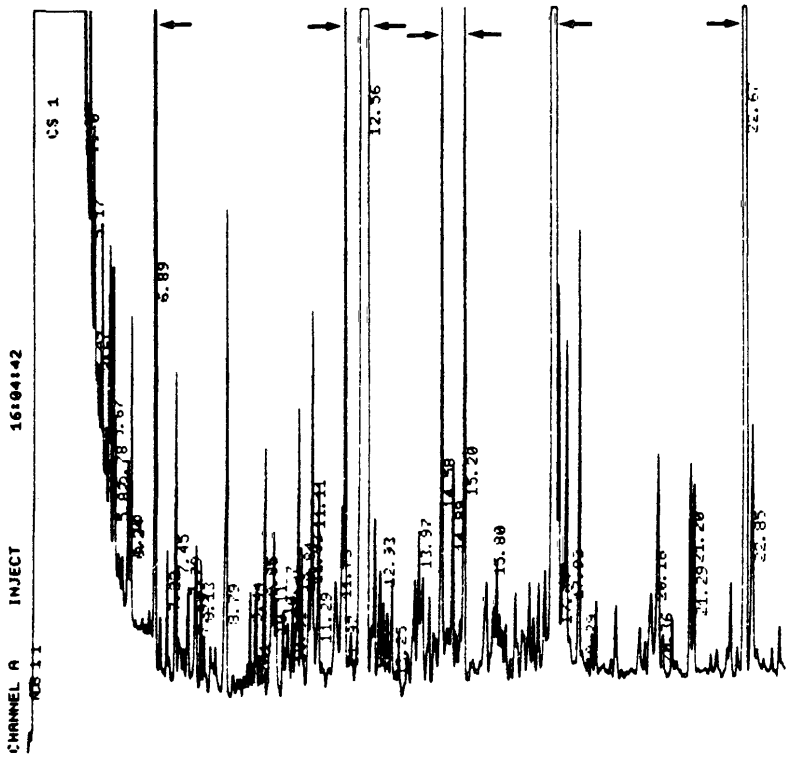


Fig. 4.16 Contamination caused by sealing a ground glass joint with silicone grease. Arrowed peaks are the major contaminants.

When using high-resistance traps, pressure-sensitive specimens may be kept at atmospheric pressure by sucking gas over them and through the trap (assuming that sufficient flow can be generated by a one atmosphere pressure drop). Such vacuum-driven systems suffer from the disadvantage that leaks suck contaminants into the system - a considerably greater practical problem than the loss of sample caused by leaks in a positive pressure system.

Of the three other traps investigated here only the macro adsorbent trap, which is incompatible with the transfer of volatiles direct to capillary columns (Grob & Habich 1985; Werkhoff & Bretschneider 1987a, b), has a gas flow resistance comparable to that of the dynamic solvent effect concentrator. The micro adsorbent trap, and especially the fused silica, open-tubular trap generate back pressures which are likely to restrict their versatility.

CONCENTRATOR BLANK PRODUCTION

Having established that concentrators with perforated beds performed best in terms of film holding at various gas flows it was necessary to develop a reliable method for their production. From previous experience sintered borosilicate glass chips were known to provide mechanically and thermally stable porous solids whose properties could be adjusted by altering particle sizes and sintering conditions. The problem was, therefore, to produce a sintered glass bed with an axial hole inside a glass tube of dimensions compatible with transfer of samples to capillary columns.

CONCENTRATOR TUBES

In static solvent effect inlets packed beds between 1 and 3 mm i.d. had proved to be compatible with capillary columns (Lawson 1987 Chap 5) (p 47). The need to heat solvent effect inlets at the completion of the solvent effect had also been established (Lawson, Pretorius and Apps 1987), (p 51), indicating the desirability of thin-walled tubes to reduce thermal inertia. The total solvent capacity of the concentrator; 20 µl, represented a compromise between short solvent evaporation times and the necessary accuracy of heater timing, and demanded a bed approximately 50 mm long. The tube on which the concentrator was based was thus required to be of 1-3 mm i.d., thin-walled and sufficiently in excess of 50 mm in length to allow convenient manipulation, especially when inserting it into and removing it from the dynamic solvent effect inlet of the gas chromatograph (Chap 5). Fortuitously Capilette blood sampling capillaries provide a cheap source of such tubes. The 100/200 µl capacity tubes are 125 mm long, 1,8 mm i.d. and 2,5 mm o.d., the 50/100 µl tubes are 125 mm long, 1,4 mm i.d. and 2mm o.d.

SINTERING

Of the three variables; temperature, time and pressure which affect the sintering process only temperature and time could be conveniently varied in the present case. Increases in either time or temperature produced beds which were stronger, less porous and more subject to shrinkage and distortion. Sintering 75-105 µm or 105-125 µm particles at 780°C for 30 min produced beds which were at least adequately porous, strong enough to endure the minor stresses imposed on their lower ends by insertion into the dynamic solvent effect inlet and free of significant distortion. Since 780°C is substantially higher than the softening point of borosilicate glass (650°C) it was necessary to keep the concentrators free of mechanical stress while sintering.

PERFORATION

Perforation of the concentrator beds could be achieved by sintering them around a former which was subsequently removed to leave the open channel. Unfortunately none of the techniques tested was fully satisfactory. Steel wire formers removed by stretching as for nickel columns (Pretorius, Rohwer, Apps, Lawson & Giesel 1984) caused breakage of the sinter and contamination of the glass by metal ions revealed by coloured stains. The use of organic formers removable by oxidation, including cotton thread, cocktail sticks, pencil leads and cactus spines, failed because organic vapours emitted by the formers prevented sintering by contaminating the bed surface, and because the concentrators were distorted by movement of the formers as they carbonized. Formers of thin-walled, electroformed nickel tube allowed distortion-free sintering but the subsequent removal of the nickel using hot acid (p 47) was too time consuming for the production of what was, at the time, considered to be an item with a limited lifetime.

An alternative approach (Lawson* pers. comm.) was to sinter the bed onto only one side of the tube, leaving an open gas channel up the other side. This was achieved by spreading a layer of glass chips along one side of the concentrator tube and then sintering in a horizontal position. An even layer of chips was produced by vibrating the tube with an engraving tool. For purposes of standardization a fixed 30 mm length of tube was filled with vibrated-down chips which were then spread along one side of 60 mm of tube. This produced concentrators in which the porous bed and the gas channel each occupied half the cross sectional area of the bottom 60 mm of the tube.

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The double perforated bed was produced by arranging a nickel tube to run through a horizontally spread bed.

Because the thin walls of the Capilette tubes were inconveniently fragile a 40 mm length of 2 mm o.d., 1 mm i.d. glass tube was micro-glassblown onto the top end to facilitate such operations as flame sealing during deactivation treatments (q.v.) and connection to sampling plumbing.

CONCENTRATOR DEACTIVATION

The criterion set for adsorptive and absorptive activity of inlet systems was that undistorted peaks should elute from the separating column. It is important to note that this does not necessarily imply the absence of interactions between the sample components and the inlet.

Borosilicate glass, from which the concentrators were constructed, has been widely employed for the production of capillary columns. Deactivation of packed bed inlets and their associated plumbing was attempted with a variety of techniques adapted from those reported as useful for column deactivation. The first experimental work in this area was carried out on static solvent effect inlets of various types.

EXPERIMENTAL

The following types of treatment were tested; Grob acid leaching and high temperature persilylation; room temperature silylation with e.g. dimethyldichlorosilane (DMDCS); Carbowax deactivation; D_4 deactivation of leached glass; pyrolytic deposition of silicon from silane; and silicon-ethene treatment.

a) Acid leaching and high temperature persilylation.
Grob, Grob, Blum & Walther's (1982) procedure was followed with minor adaptations to the differences in geometry between columns and inlets.

b) Room temperature silylation.
Plain glass, or acid leached, inlets were soaked in a 10% solution of dimethyldichlorosilane (DMDCS) in dry chloroform, then rinsed with methanol and conditioned under gas flow at 220°C

c) Carbowax deactivation.
A diatomaceous earth packed bed 50 mm long and 3mm i.d. was heavily coated with Carbowax 1000. An inlet was connected to the downstream end of this bed. Carbowax was bled off the bed and through the inlet with a 5 cm^3 min^{-1} flow of nitrogen at 260°C (Aue, Hastings & Kapila 1973). Inlets were conditioned overnight at 240°C before testing.

d) D_4
Inlets were acid leached with 20% hydrochloric acid at 160°C overnight, and dehydrated at 260°C overnight. Methanol was introduced, flushed through the inlet and left as an adsorbed film by evaporating away the liquid under vacuum. D_4 (octamethylcyclotetrasiloxane) was then flushed through the inlet with nitrogen and the inlet was flame sealed under vacuum and baked overnight at 400°C (Blomberg, Markides & Wannman 1981; Bertsch *et al.* 1982). Rinsing with a series of methanol, dichloromethane and hexane preceded conditioning and testing.

e) Silicon deposition
The basic procedure was adapted from that of Bertsch *et al.* (1982). Inlets were evacuated and filled with semiconductor grade monosilane at a pressure of 200 KPa. Amorphous silicon was deposited by heating the inlets to 400°C. The evenness of silicon deposition was improved if the inlets were buried in alumina powder in a muffle furnace whose temperature was increased slowly (aproximately 4 hours to reach 400°C). The silicon-surfaced beds were tested either without further treatment or after modification by the following processes:

- D_4 treatment as above
- Silyl 8 treatment according to the manufacturer's instructions
- water washing by running 50 cm^3 of distilled water through the bed while immersed in an ultrasonnator bath or by injecting 20-30 µl of distilled water onto a bed after installation and allowing it to run through the bed and column.

f) Silicon-ethene deactivation.
This technique was applied only to dynamic solvent effect concentrators. An apparatus (Fig 4.17) was constructed with which concentrators could be evacuated, filled with monosilane, heated, evacuated, filled with ethene and flame-sealed under vacuum without exposing the surface of the deposited silicon to air or moisture.

The procedure was as follows. With 2-way valves A and B connected to vacuum the open end of a concentrator blank was sealed into the compression fitting of valve A. The furnace was then lifted around the concentrator which was evacuated at a temperature of 400°C for 15 min. The furnace was then lowered and the concentrator, when it had cooled, was filled with silane at 200 KPa by turning valve A. Valve A was then set at an intermediate position to seal the concentrator

against both silane and vacuum, the furnace was raised and the concentrator was again heated to 400°C. After 15 min valve A was turned to vacuum and the hydrogen and excess silane in the concentrator pumped away. The pump was protected by the hot tube which pyrolized excess silane to silicon and hydrogen. Vacuum at 400°C was maintained for 15 min, the furnace was then lowered and ethene at a pressure of 150 KPa was admitted to the concentrator through valve B. Valve A was turned to the intermediate off position and a Dewar flask of liquid nitrogen was raised around the concentrator. With the ethene frozen down the concentrator was flame sealed near the valve. Silicon-coated, ethene-filled concentrators were irradiated with a dosage of 100 MRad of gamma rays from a cobalt 60 source. After irradiation they were opened at both ends, rinsed with acetone and conditioned for at least 30 min at 220°C.

Variations on this procedure which were tested were to heat the concentrators to up to 700°C for up to 7 days instead of irradiating them, or to irradiate with 5, or 10, instead of 100 MRad of gamma rays.

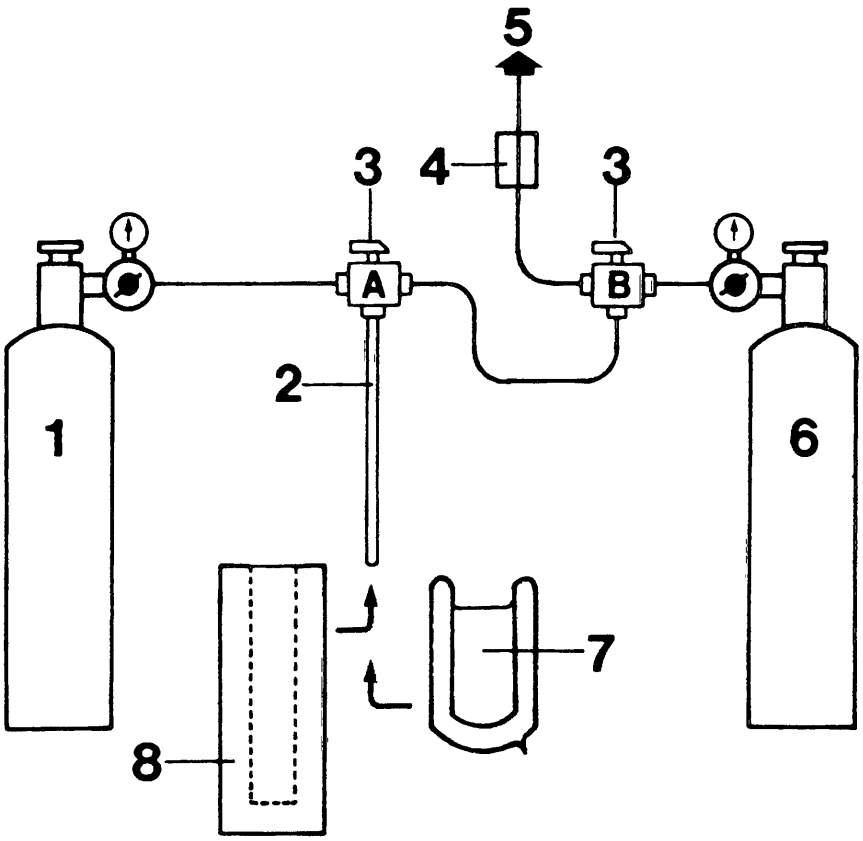


Fig. 4.17 Apparatus used for silicon-ethene deactivation of dynamic solvent effect concentrators. 1: monosilane; 2: dynamic solvent effect concentrator; 3: two-way valve; 4: heater; 5: to rotary vacuum pump; 6: ethene; 7: liquid nitrogen; 8: tube furnace. For details of operation see text.

RESULTS

a) The Grob procedure for columns proved technically unsuitable for packed bed inlets. Due to the high pressures developed during leaching, thin walled inlets (e.g. concentrators) were susceptible to bursting unless enclosed in a thick walled tube or autoclave. Efficient rinsing, or uniform dehydration of beds, especially perforated ones, was also difficult. Consequently, although inlets with acceptable performance were produced, high temperature leaching and persilylation were not considered suitable as routine methods.

b) DMDCS treatment of either leached or plain glass was unsuccessful (Fig 4.18).

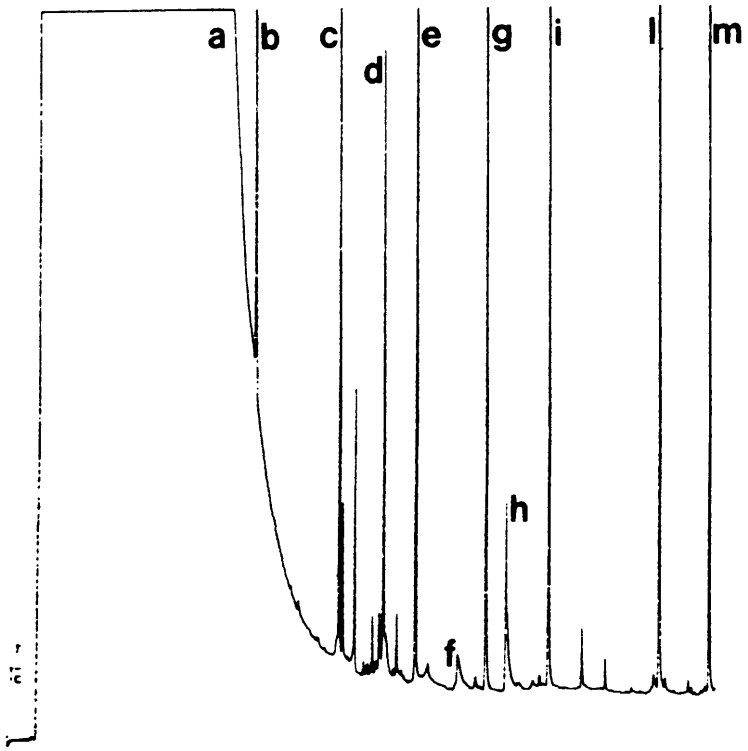


Fig. 4.18 Chromatogram from 20 µl of 2:10 ^{7} Test Mix (without *n*-tridecane) in *n*-hexane sampled on a dynamic solvent effect inlet deactivated with dimethyldichlorosilane. Peak identities as in Fig 4.1.

c) Carbowax treatment was partially successful. Surface activity was acceptable but the solvent peak was badly tailed and the baseline was unstable (Fig 4.19).

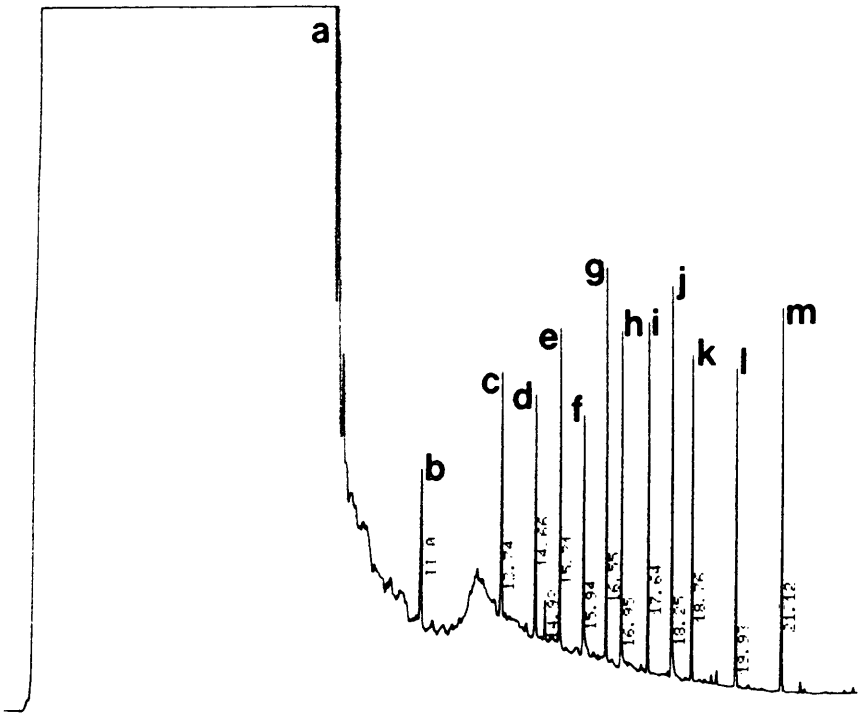


Fig. 4.19 Chromatogram from 20 µl of 2:10 ^{7} Test Mix in *n*-hexane sampled on a static solvent effect inlet deactivated with Carbowax. Peak identities as in Fig 4.1, except k: *n*-tridecane.

d) D_4 treatment caused even more serious solvent peak tailing and baseline instability (Fig 4.20).

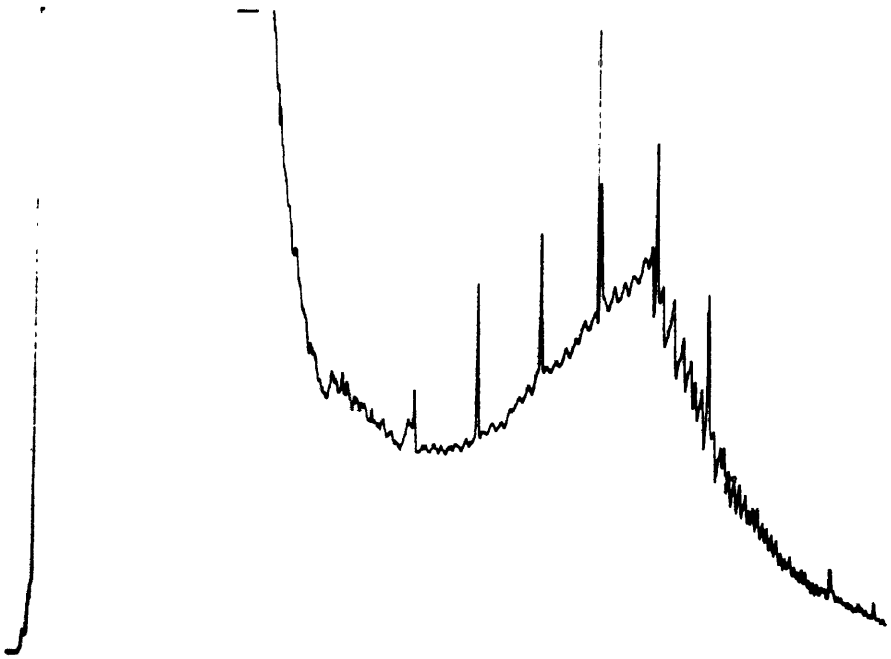


Fig. 4.20 Chromatogram from 20 µl of 2:10 ^{7} Test Mix in *n*-hexane sampled on a static solvent effect inlet deactivated with octamethylcyclotetrasiloxane (D_4).

e) The performance of silicon-coated beds was strongly influenced by subsequent surface treatments:-

(i)"Bare" silicon. A perforated, static bed coated only with silicon yielded poor results (Fig 4.21). Although the alkane peaks were sharp, of the polar compounds only dimethylaniline was eluted satisfactorily, though with extra retention, the others showed severe peak distortion and loss of area.

(ii) D_4 treatment of silicon. The results were the same as when bare glass was subjected to this treatment (Fig 4.20).

(iii) Silyl 8 treatment of bare silicon. Satisfactory peak shapes were obtained after conditioning the treated bed overnight at 220°C (Fig 4.22) but the deactivation was unstable and peak shapes deteriorated rapidly. Re-treatment was only temporarily effective. Use of excess Silyl 8 caused distortion of some peaks.

(iv) Water washing. After conditioning under carrier gas flow at 220°C for approximately 36 hours water-washed, silicon-surfaced beds yielded excellent peak sharpness for every component of the original Test Mix and the Grob mix (Fig 4.23). The surface was slightly unstable in that adsorptive activity towards alcohols was exhibited after 3 days at 220°C but its deactivation could be regenerated by injecting 20-30 µl of distilled water and reconditioning (Fig 4.24).

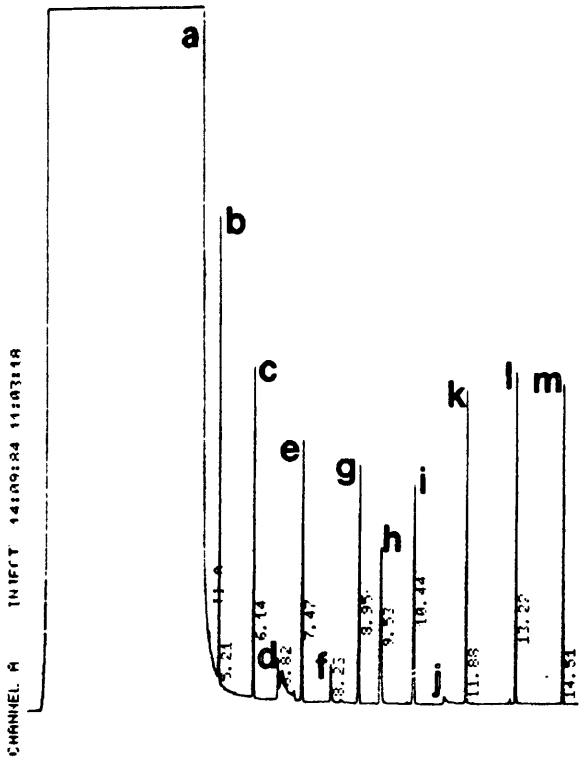


Fig. 4.21 Chromatogram from 20 µl of 2:10 ^{7} Test Mix in *n*-hexane sampled on a static solvent effect inlet deactivated with "bare" silicon. Peak identities as in Fig 4.1, except k: *n*-tridecane.

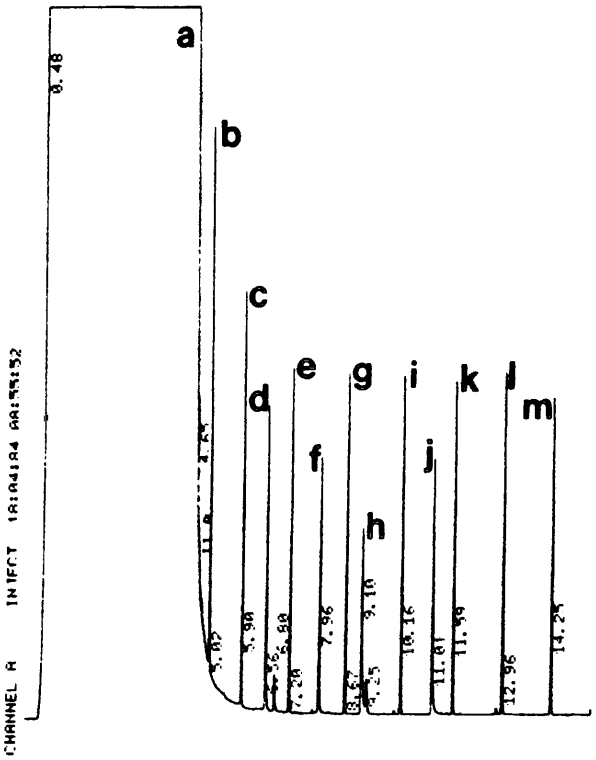


Fig. 4.22 Chromatogram from 20 µl of 2:10 ^{7} Test Mix in *n*-hexane sampled on a static solvent effect inlet deactivated with Silyl 8-treated silicon (N.B. splitting of DMA peak caused by slightly late inlet heating). Peak identities as in Fig 4.1, except k: *n*-tridecane.

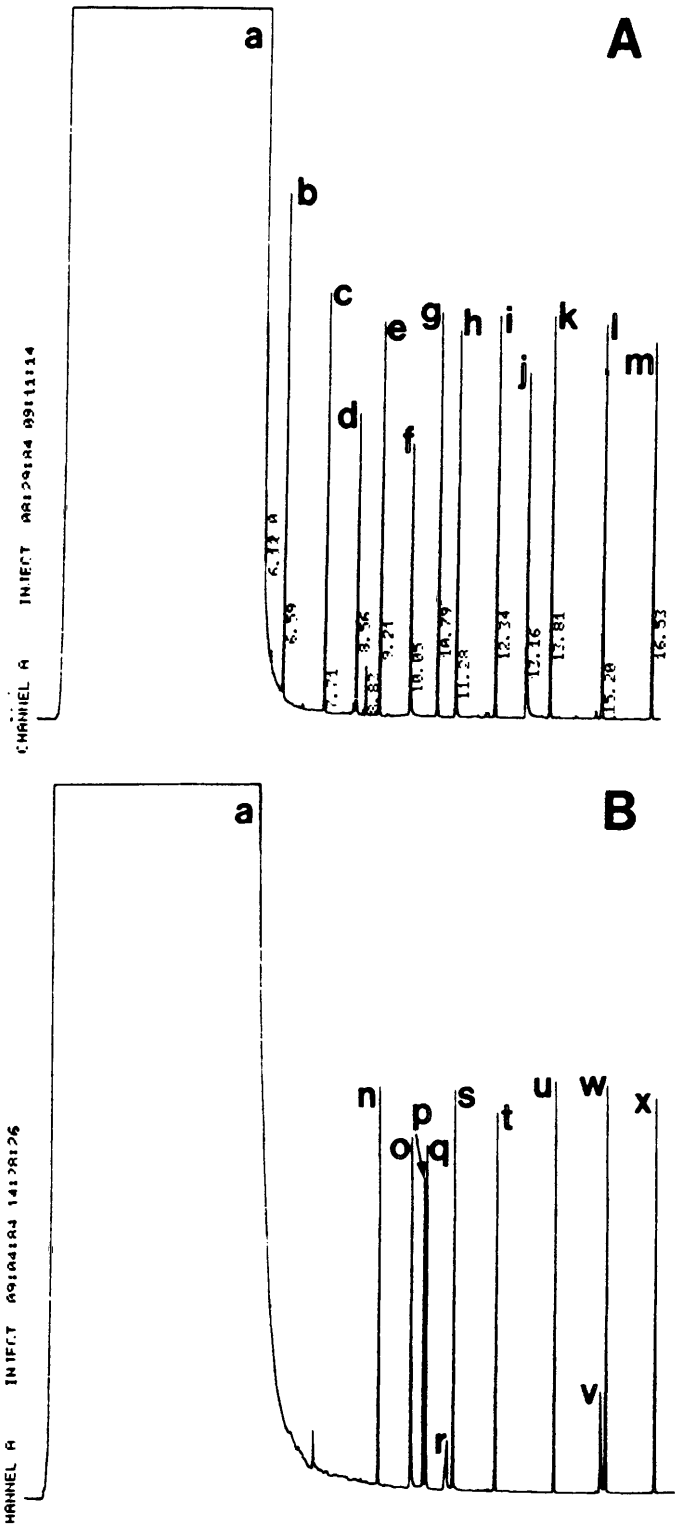


Fig. 4.23 Chromatograms from; A: 20 µl of 2:10 ^{7} Test Mix in *n*-hexane and; B: 20 µl of 1:10 ^{7} Grob mix sampled on a static solvent effect inlet deactivated with water-washed silicon. Peak identities as in Fig 4.1, except k: *n*-tridecane and; n: *n*-decane; o: *n*-octanol; p: *n*-nonanal; q: 2,6-dimethylphenol; r: ethylhexanoic acid; s: 2,6-dimethylaniline; t: *n*-dodecane; u: methyl decanoate; v: dicyclohexylamine; w: methyl undecanoate; x: methyl dodecanoate.

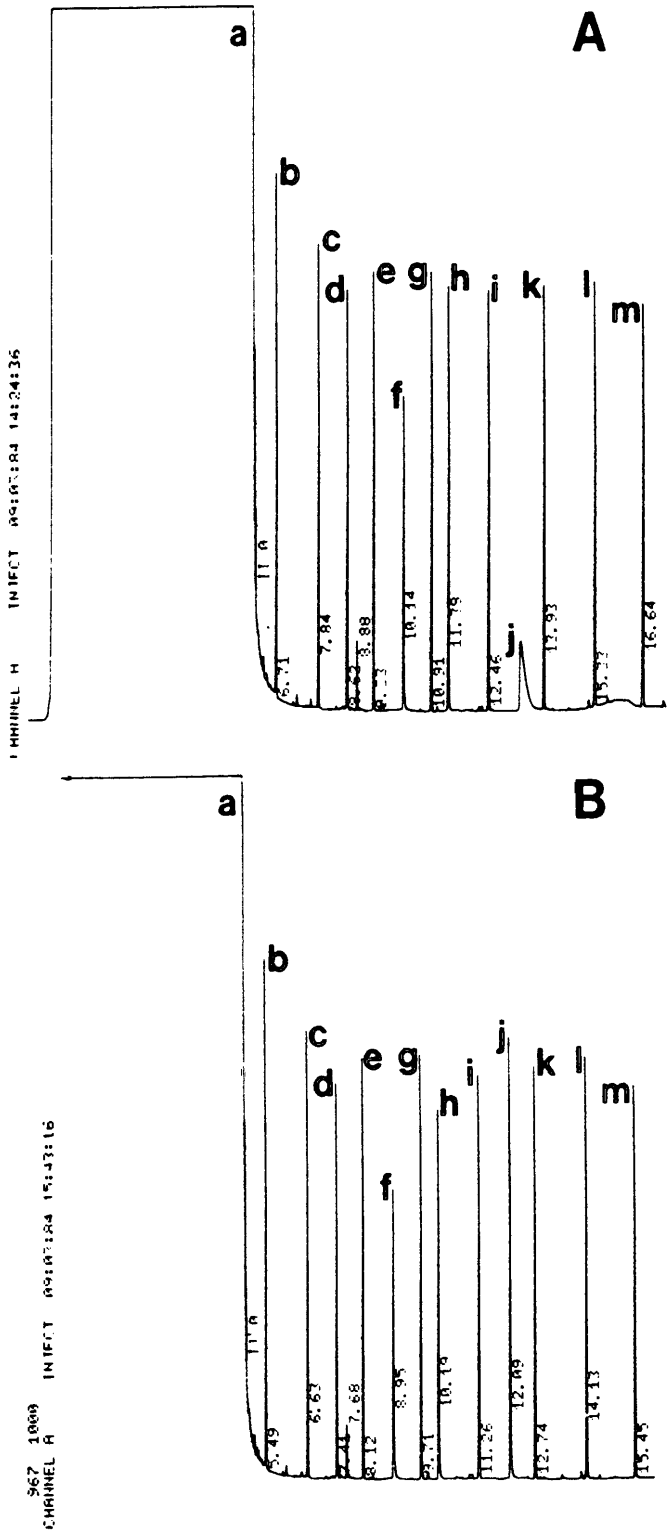


Fig. 4.24 Deterioration and regeneration of the inactive surface of a water-washed, silicon-surfaced static solvent effect inlet. a: adsorption of *n*-decanol after 3 days at 220°C; b: regeneration of surface by washing and conditioning. Peak identities as in Fig 4.1, except k: *n*-tridecane.

Water-washed concentrators caused splitting of fatty acid methyl ester peaks which was unresponsive to changes in inlet heating (Centner* pers. comm.).

f) Silicon-ethene. This treatment yielded concentrators which were less active than the columns with which they were tested (Fig 4.25).

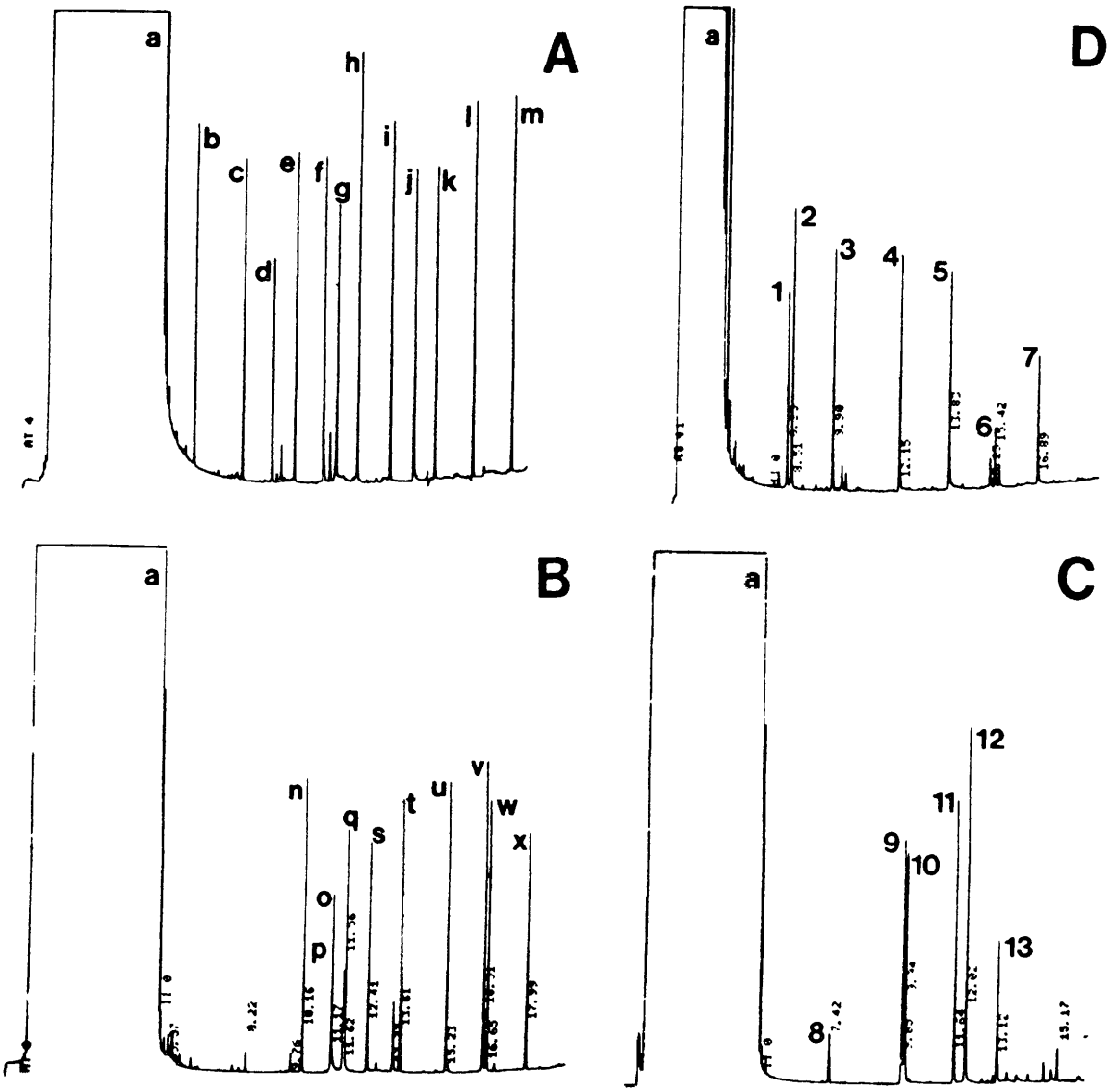


Fig. 4.25 Tests of system adsorptive activity using silicon-ethene deactivated dynamic solvent effect concentrators. A: Test Mix, 300 µl $1:10^{8}$ solution, approx 3 ng per peak, peak identities as in Fig 4.1 except g: linalool; B: Grob mix nominal 10 ng of each component in 20 µl, peak identities as in Fig 4.23; C: phenols, nominal 10 ng of each in 20 µl, 1: phenol; 2: 2-chlorophenol; 3: *p*-cresol; 4: 2,4-dichlorophenol; 5: 2,6-dimethylphenol; 6: *p*-nitrophenol; D: 1-4 ng carbonyl compounds, 7: *n*-heptanone; 8: *n*-heptanal; 9: 2,6-dimethylheptan-4-one; 10: *n*-nonanal; 11: *n*-decanal; 12: *n*-undecanal; 13: *n*-dodecanal.

*Marc Centner, formerly Institute for Chromatography, University of Pretori

Heat treated silicon-ethene concentrators were initially very inert but deteriorated over 3-4 runs to the level of "bare" silicon. When opened heat treated beds had a strong, tarry smell and if they had been over-filled with ethene a dark deposit was visible. The first run with unconditioned, heat treated concentrators yielded very dirty chromatograms (Fig 4.26). Five or 10 MRad irradiation also produced only temporary deactivation, though with no tarry smell or visible deposit.

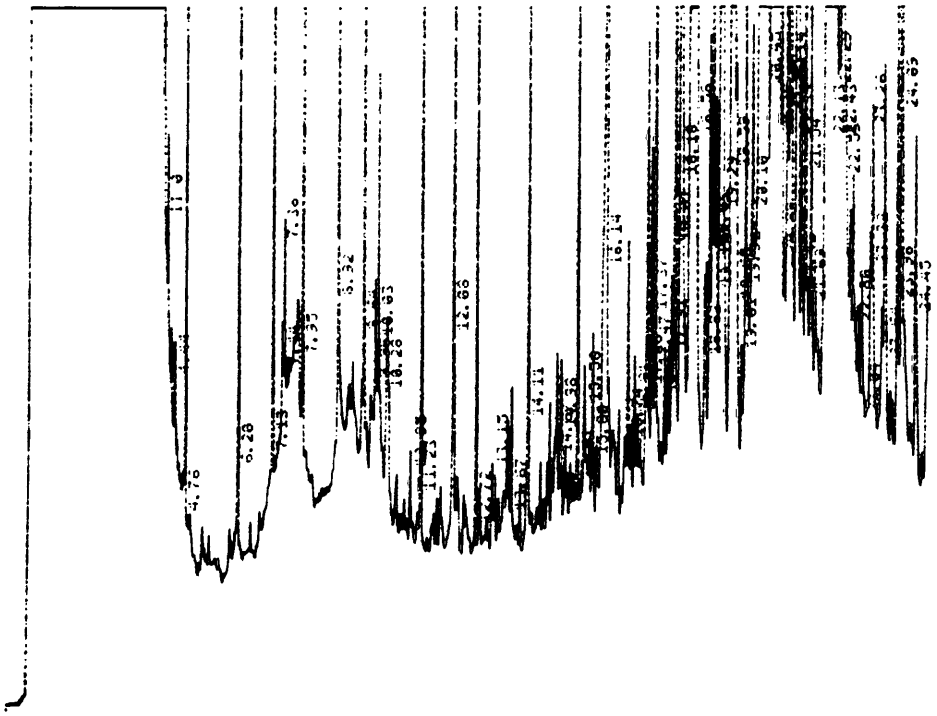


Fig. 4.26 Chromatogram from the first run with a heat-treated silicon-ethene surfaced dynamic solvent effect concentrator.

The silicon-ethene deactivation proved to be extremely robust. In no case was a concentrator treated with this method discarded due to deterioration of its deactivation. Lifetimes of months and several hundred samples were typical but a detailed investigation of this aspect was impossible due to the very rapid deterioration of the available columns. The most common cause of concentrator failure was breakage, and a few were discarded because they had become contaminated with solid particles from specimens.

DISCUSSION

It should be noted at the outset that the results presented here do not necessarily imply that that any of the methods tested are fundamentally unsuitable for the deactivation of packed bed, solvent effect inlets. Given enough time for optimization of procedures any or all of them could well have proved as effective as the method which was finally adopted.

Methods involving the deposition of silicones (e.g. D_4, DMDCS, Silyl 8) suffered from the common fault of causing solvent peak tailing (D_4, DMDCS) or distortion of solute peaks (D_4, Silyl 8, DMDCS). With Silyl 8 the problem occurred only when excess reagent was used, with DMDCS tailing was not serious but with D_4 both solvent peak tailing and peak broadening were completely unacceptable. Kong, Woolley, Fields & Lee (1984) report that the use of D_4 to deactivate narrow-bore columns can lead to their blockage through the cross-linking of D_4 which has pooled into lenses. It seems likely that D_4 and the other silicone reagents have a similar tendency to pool in the narrower channels of a packed bed, and then to crosslink to form uneven, retentive silicone layers. Solvent effect focussing is inefficient in the presence of stationary phase (Grob 1983), and is likely to be more so if the phase is unevenly distributed. The poor performance of Carbowax treated beds was probably due to a similar cause since this procedure is known to produce a retentive surface layer (Grob & Schilling 1987).

The highly adsorptive nature of the unwashed silicon surface suggests that it was not, in fact, elemental silicon. Amorphous silicon deposited by pyrolysis of monosilane at 400^{o}C contains approximately 2 % hydrogen (Scott 1984 p 124) covalently bonded to structural defects and the film surface (Hirose 1984 p 118; Scott 1984 p 144). The Si-H bonds in silanes are susceptible to oxidation to silica (Stone 1962 p 7) and thin layers of oxide on clean silicon reach a thickness of approximately 10 Å after 10 min in air at room temperature (Meyer & Sparnaay 1975 p 393). The presence of such a surface layer of silica would account for the activity shown by "bare silicon" surfaces.

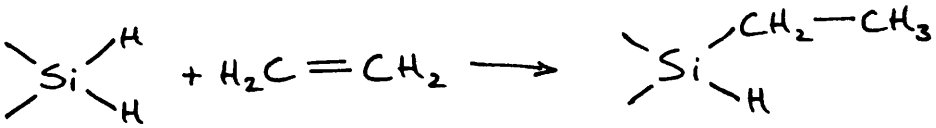
Silanes are also susceptible to base-catalysed hydrolysis (Stone 1962 p 19). Although the water used for bed washing was neutral to litmus it had been distilled from alkaline permanganate and collected in glass vessels and could, therefore, have contained catalytic levels of alkali. If the silane model of the silicon surface is correct water washing would have led to very high, and uniform, populations of surface silanols. Roughening of the silicon surface by etching of structural defects may have occurred to a limited extent (Chenevas-Paule 1984 p 261). Water is known to have a deactivating effect through its displacement of less polar molecules from active sites (Teranishi, Mon, Robinson, Cary & Pauling 1971). On the surface of a

water-washed, silicon-surfaced inlet strong binding of water to silanols is implied by the deactivation's stability to heat and its regeneration by washing and conditioning. Further light is thrown on the nature of the well-conditioned, washed surface by the shapes of peaks when the bed was not heated. All the polar compound peaks are broadened due to slow evaporation but only the decanol peak exhibits much adsorptive tailing. Plainly the washed, conditioned surface is inactive to all except very polar compounds and its activity towards these can be overcome by modest heating.

Although water-washed, silicon-surfaced beds produced excellent results in static solvent effect systems (pp 54, 81) their consistent production proved problematical (possibly because the catalytic effects of *p*H were not appreciated at the time). Two other factors also pointed to the unsuitability of water-washed silicon as a deactivation for dynamic solvent effect concentrators. First, most biological specimens contain water which would have disrupted the conditioning of the hydrated silicon surface. Second was the intractable splitting of methyl ester peaks by a mechanism which is still unknown. Consequently water washing was abandoned, and ethene treatment adopted, as a means of modifying the silicon surface.

The following is tentatively proposed as a model for the modification of the surface properties of silicon by irradiation in an atmosphere of ethene.

Silanes form addition products with alkenes in free radical initiated reactions which are catalysed by peroxides, metals, amines, UV light and gamma rays (MacDiarmid 1968 p 213). While the nature of an ethene treated silicon surface is not exactly known the model of an hydrogenated silicon surface as a silane suggests that addition products are present:



Whether all surface Si-H bonds are replaced by Si-C is unknown, steric hinderance may well influence the extent of this reaction, or of subsequent ones such as hydrolysis or oxidation.

The Si-C bond is thermally stable and susceptible to oxidation and hydrolysis only under extreme conditions never likely to occur in gas-liquid chromatography (MacDiarmid 1968 pp 351-367) - properties which are consistent with the long lifetimes of the concentrators, conferred by the stability of the 100 Mrad irradiated silicon-ethene surfaces.

The short lifetime of 5, and 10 MRad irradiated concentrators suggests that a low percentage of surface Si-H bonds had been replaced by Si-C, leaving the rest to oxidize or hydrolyze to adsorptive siloxyl and silanol sites as the concentrator was used. The tarry smell, dark deposits, dirty chromatograms and short lifetimes of heat treated silicon-ethene concentrators all suggest that gas phase polymerization of the ethene, rather than reaction with surface Si-H, groups was occurring (MacDiarmid 1968 p 216).

Chapter 5

TRANSFER OF SAMPLE FROM CONCENTRATOR TO COLUMN

Having effected the accumulation of a sample on a concentrator it was, of course, necessary to transfer it to a capillary column for high resolution separation. That, in theory, solute focussing by the solvent effect was not critically dependent on such factors as gas flow, temperature and inlet geometry suggested that the contents of a loaded concentrator could be transferred to a capillary column by carrying out the solvent effect in the static mode on the concentrator bed itself.

The requirement was for an inlet which would direct carrier gas through the concentrator and onto the column, and into which concentrators could be interchangeably inserted. At the minimum this would consist of a port for insertion and removal of concentrators, a carrier gas input and some type of seal between the concentrator and the head of the column. The inlet would, of course need to be chromatographically "invisible" i.e. free of adsorptive or absorptive activity and making a negligible contribution to peak width. Insertion and removal of concentrators without subjecting them, or the column, to mechanical stress was necessary.

The design of the concentrators (Chap 4) exerted certain constraints on the design of the inlet. The shape of the bottom end of the concentrator was aimed at unimpeded solvent flow during sampling, consequently any connection to the column requiring a narrowing of the concentrator tip was impractical. To minimize thermal inertia the walls of the concentrators were very thin (0,3 mm); consequently cone joints could not be ground into them. The design of the seal between concentrator and column had also to allow for deviations from perfect roundness and straightness in the concentrators caused by unavoidable shrinkage and distortion during sintering of the packed bed.

ALL-GLASS, SINGLE STAGE INLET (LONG SEAT)

CONSTRUCTION AND DEACTIVATION

Inlets were constructed from borosilicate glass using conventional, and micro glassblowing (Fig. 5.1). The ground glass joint was attached after the rest of the inlet had been coated internally with silicon by pyrolysis of silane. An electrical heater was wound onto the inlet body and secured by polyimide as for the static solvent effect inlet (Chap 4). Connection of the inlet to the column was by a heated polytetrafluoroethylene joint (Pretorius *et al.* 1985).

OPERATION

With the carrier gas supply turned off the top of the inlet was removed and a loaded concentrator was dropped into the seat. The top was quickly replaced and carrier gas flow restored. The heating of the inlet was timed from when the carrier gas was switched on.

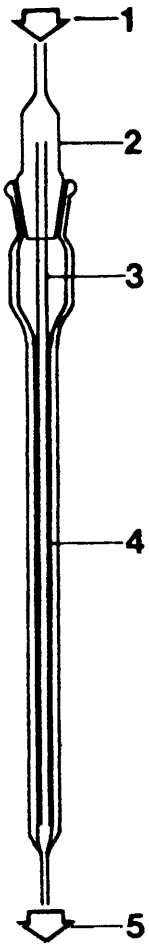


Fig. 5.1 All-glass, single stage, dynamic solvent effect inlet with long seat. 1: carrier gas input; 2: B7 ground glass stopper; 3: dynamic solvent effect concentrator; 4: glass tube with internal diameter a light sliding fit to the outside of the concentrator; 5: to column.

EXPERIMENTAL

The performance of the inlets was tested with water-washed, silicon-surfaced concentrators loaded by micro-syringe with 20 µl of 2:10 ^{7} Test Mix. The column was 22 m x 0,3 mm x 0,4 µm methyl silicone with hydrogen as carrier gas at a linear flow velocity of 40 cm s^{-1}. Detection was by FID at a sensitivity of 4 x 10^{-11} A mv^{-1} full scale deflection.

PERFORMANCE AND DISCUSSION

With an appropriate heating schedule (Chap 4) the long-seated inlet delivered acceptable solute peak shapes, but the tail of the solvent peak obscured the *n*-octane peak and returned to baseline only after the elution of the *n*-decane peak (Fig. 5.2). The extended solvent tail was ascribed to the lack of gas flow between the concentrator and the seat and in the space inside the ground glass cap; solvent vapour entering these areas during insertion of the concentrator subsequently re-entering the carrier gas stream by the relatively slow process of diffusion.

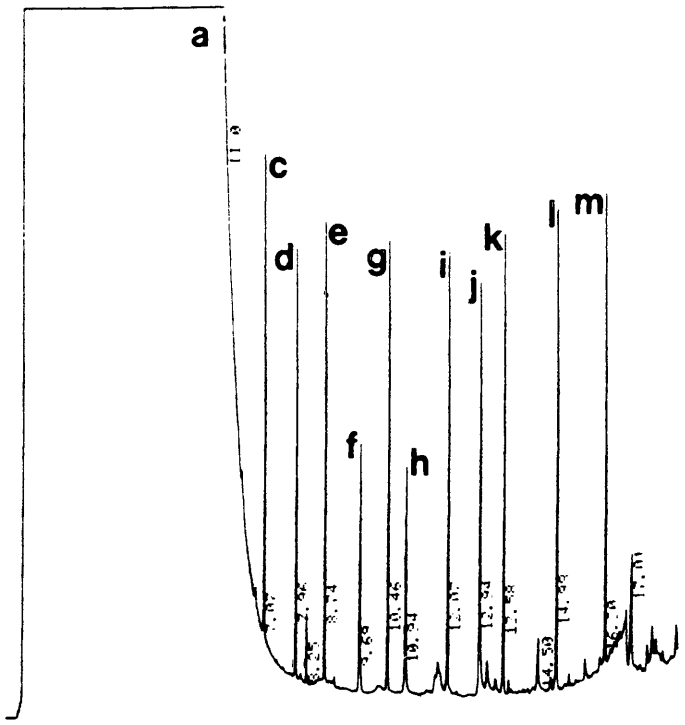


Fig. 5.2 Chromatogram from 20 µl of 2:10 ^{7} Test Mix with sample introduction by an all-glass, single stage, dynamic solvent effect inlet with a long seat. a: *n*-hexane solvent; b: *n*-octane; c: *n*-nonane; d: 2,6-dimethylheptan-4-one; e: *n*-decane; f: *p*-cresol; g: *n*-undecane; h: 2,6-dimethylaniline; i: *n*-dodecane; j: *n*-decanol; k: methyldecanoate; l: *n*-tetradecane; m: *n*-pentadecane.

ALL-GLASS, SINGLE STAGE INLET (SHORT SEAT)

CONSTRUCTION

The inlet was modified by shortening the seat and adding a gas outlet which allowed carrier gas to purge the space between the concentrator and the inlet body (Fig. 5.3). The shorter seat was a less effective seal than its longer prototype and, as a consequence, more gas flowed between it and the concentrator, purging any solvent vapour more quickly onto the column. With a flow of 30 cm^3 min^{-1} through the gas outlet the volume inside the inlet was purged approximately 10 times per minute. The clearance between the inlet and the concentrators also provided some tolerance in their straightness.

OPERATION and EXPERIMENTAL procedures were the same as for the long-seated inlet

PERFORMANCE AND DISCUSSION

The modifications improved the tail of the solvent peak to an acceptable level (Fig. 5.4). The *p*-cresol, DMA, and decanol peaks were severely broadened and/or split. As predicted from the behaviour of a static solvent effect inlet (Chap 4) the shape of these peaks was improved by earlier heating of the inlet, but before the DMA and decanol peaks reached acceptable sharpness peak splitting diagnostic of too early heating became apparent (Fig. 5.5). There was no heating time with which all peaks in the Test Mix could be satisfactorily eluted.

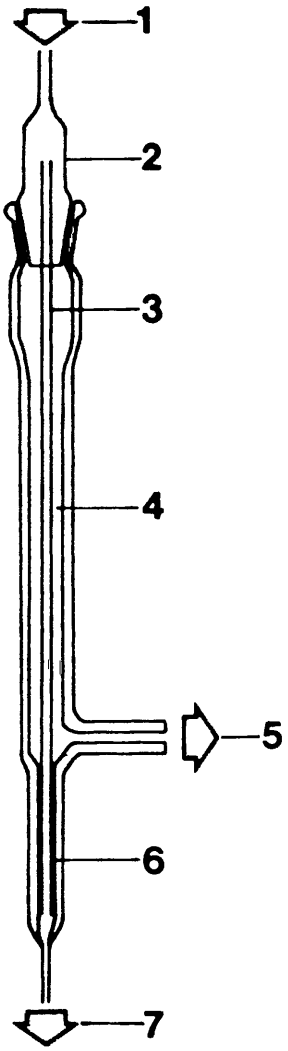


Fig 5.3 All-glass, single stage, dynamic solvent effect inlet with short seat. 1: carrier gas input; 2: B7 ground glass stopper; 3: dynamic solvent effect concentrator; 4: clearance between inlet body and concentrator; 5: purge outlet; 6: tube with internal diameter a light sliding fit to the outside of the concentrator; 7: to column.

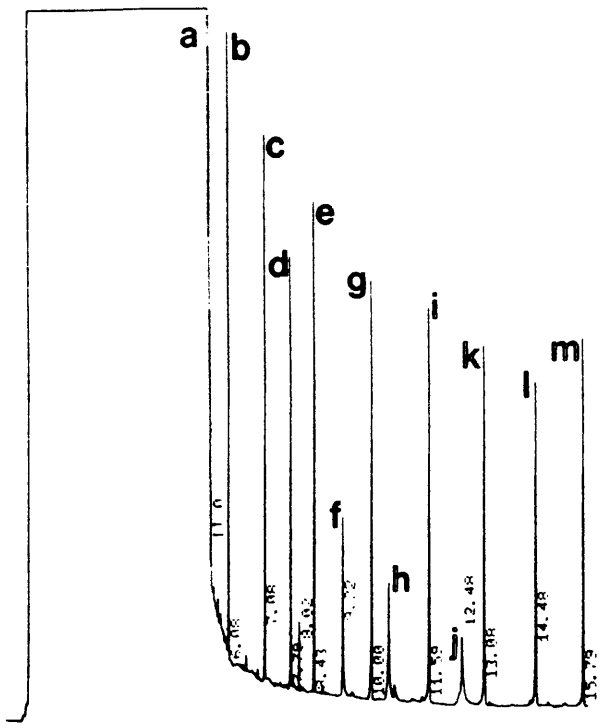


Fig. 5.4 Chromatogram from 20 µl of 2:10 ^{7} Test Mix with sample introduction by an all-glass, single stage, dynamic solvent effect inlet with a short seat. Peak identities as in Fig 5.2. Heating time 4,8 min.

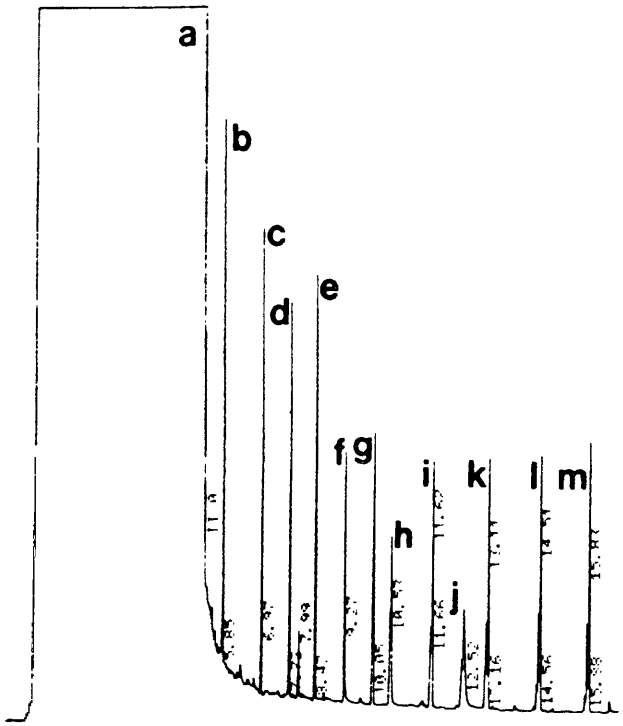


Fig 5.5 Peak distortion caused by early heating of an all-glass, single stage, dynamic solvent effect inlet with a short seat. Peak identities and injection volume as in Fig 5.4, heating time 3,5 min.

This intractable peak splitting may have been due to uneven heat flow to the concentrator. Heat transfer was mainly by radiation where the concentrator was not in contact with the inlet walls and by conduction through glass in the area of the seat. Under conditions of even heat flow over the length of the concentrator's packed bed, provided by the long seat of the earlier prototype, the solute peaks were eluted without distortion.

The disappointing results of trying to carry out the static solvent effect on a concentrator bed prompted the development of an inlet in which the whole of the sample was transferred from the concentrator to a perforated packed bed of the same design as the highly successful static solvent effect inlet.

ALL-GLASS, TWO STAGE INLET

CONSTRUCTION AND DEACTIVATION

Inlets (Fig. 5.6) were made from borosilicate glass by conventional and micro-glassblowing. The perforated packed beds were constructed as described in Chap 4 except that a tube of 2,6 mm o.d was used to further reduce thermal inertia. The ground glass joint was attached after the rest of the inlet had been coated internally with amorphous silicon by thermal decomposition of silane. Deactivation of the silicon was by water washing, as for the static inlets (Chap 4).

One heater was wound onto the upper stage of the inlet and another onto the lower stage. The heaters were independently controlled from thermocouples.

Connection to the column was by a heated polytetrafluoroethylene joint (Pretorius *et al.* 1985).

OPERATION

With the carrier gas supply turned off the ground glass top of the inlet was removed and a loaded concentrator was dropped into the inlet seat. The top was then replaced, the carrier gas switched on and the upper stage of the inlet rapidly heated to 250^{o}C to evaporate the concentrator's contents. The sample vapour condensed in the perforated packed bed of the lower stage of the inlet, whose temperature was controlled by the GC oven. After a solvent evaporation time determined as for the static solvent effect (Chap 4) the lower stage of the inlet was heated to 250^{o}C.

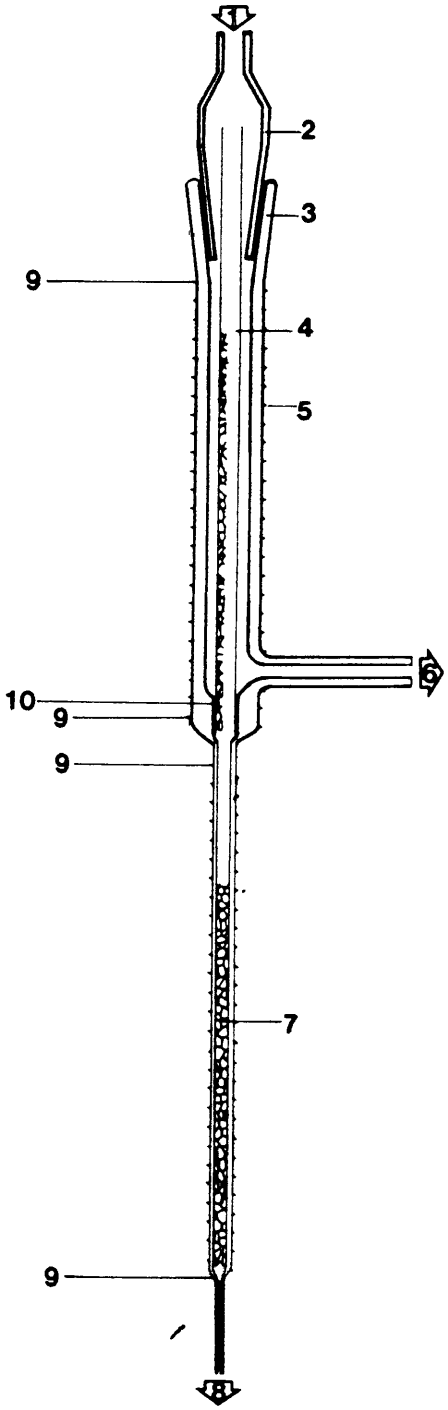


Fig 5.6 All-glass, two stage, dynamic solvent effect inlet. 1: carrier gas input; 2: glass stopper; 3: B7 ground glass joint; 4: dynamic solvent effect concentrator; 5: glass tube with loose fit to concentrator; 6: purge outlet; 7: perforated, sintered, porous bed; 8: to column; 9: heater coils; 10: tube with internal diameter a light sliding fit to the outside of the concentrator.

EXPERIMENTAL

The performance of the two stage inlet was tested with silicon-surfaced, water-washed concentrators loaded in each of three ways;

- by microsyringe injection onto the concentrator bed of 20 µl of a $2:10^7$ solution of Test Mix,
- by dipping the concentrator into a $2:10^7$ solution of Test Mix,
- by dynamic solvent effect concentration of 1 cm^3 of $1:10^8$ Test Mix.

The chromatographic conditions were the same as those used in the testing of the single-stage inlets.

RESULTS

A series of runs with syringe loading of the concentrator yielded qualitative and quantitative performances approaching those of the static solvent effect inlet (Chap 4) (Fig. 5.7, Table 5.1).

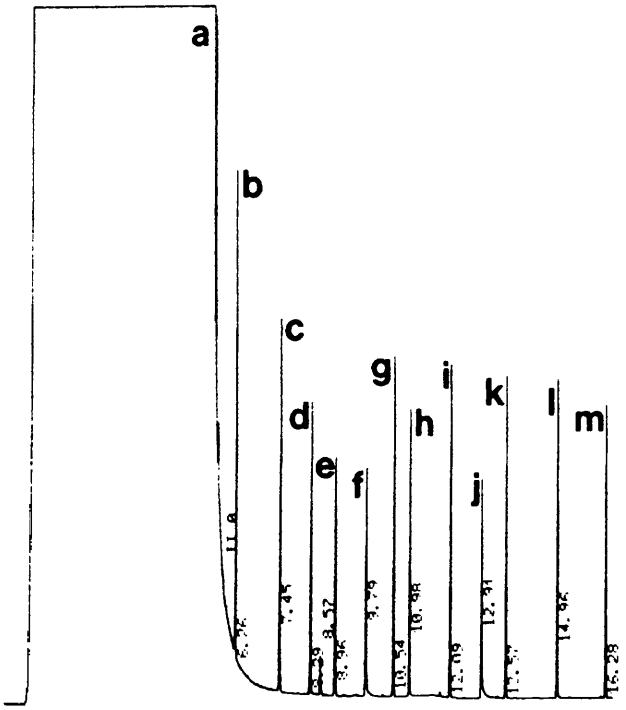


Fig. 5.7 Chromatogram from 20 µl of $2:10^7$ Test Mix with sample introduction by an all-glass, two stage, dynamic solvent effect inlet. Peak identities as in Fig 5.2.

Table 5.1. Coefficients of variation of peak areas and peak percentage areas for components of 20 µl of 2:10 ^{7} Test Mix loaded by microsyringe onto a dynamic solvent effect concentrator and transferred to a capillary column by a two-stage inlet.

Compound	% coefficient of variation	
	Area	% area
n-octane	2,4	0,8
n-nonane	2,6	0,7
2,6-dimethyl-4-heptanone	4,1	1,9
n-decane	2,7	0,6
p-cresol	5,0	2,9
n-undecane	2,6	0,6
2,6-dimethylaniline	3,5	1,1
n-dodecane	2,6	0,7
n-decanol	6,8	4,7
n-tridecane	2,5	1,1
n-tetradecane	2,6	2,1
n-pentadecane	2,4	1,2

Unfortunately it proved impossible to obtain similar results with either dip or dynamic solvent effect loading of the concentrators. In the latter case it was impossible find a setting for the heating time.

DISCUSSION

The importance of heating a packed bed, solvent effect inlet at the correct time with respect to the evaporation of the solvent has already been discussed in relation to the static solvent effect (Chap 4). In the case of the two-stage inlet, with dip or dynamic solvent effect loading of the concentrators, it appears that the volume of solvent delivered to the lower stage perforated packed bed was extremely sensitive to variations in operating conditions. This was partly because the technique for "topping up" concentrators (Chap 7) had not been developed when these experiments were carried out. In addition slight changes in the initial temperature (room temperature) and rate of heating of the upper stage of the inlet, and in carrier gas flow rate would have had marked effects on the volume of solvent condensing in the lower stage of the inlet. In some cases hexane vapour could be smelled at the purge outlet and solute peaks were reduced in area by up to 60%, indicating back flow and loss of sample resulting from increases in pressure in the inlet due to rapid evaporation of large volumes of solvent.

These operational shortcomings, coupled with the fragility of the all-glass inlet clearly precluded its use in a routine analytical system.

METAL SINGLE STAGE INLET

The unsatisfactory performance of the all-glass, two-stage inlet led to a return to a single stage system. In order to simplify alterations to the geometry of the seat, and to increase the inlet's mechanical robustness, a metal body into which a glass seat was sealed by a compression fitting was designed. This inlet design underwent a series of changes as follows:-

First prototype

The dimensions of the inlet were appropriate to a concentrator outer diameter of 2,4 mm (Fig. 5.8). The seat was of borosilicate glass.

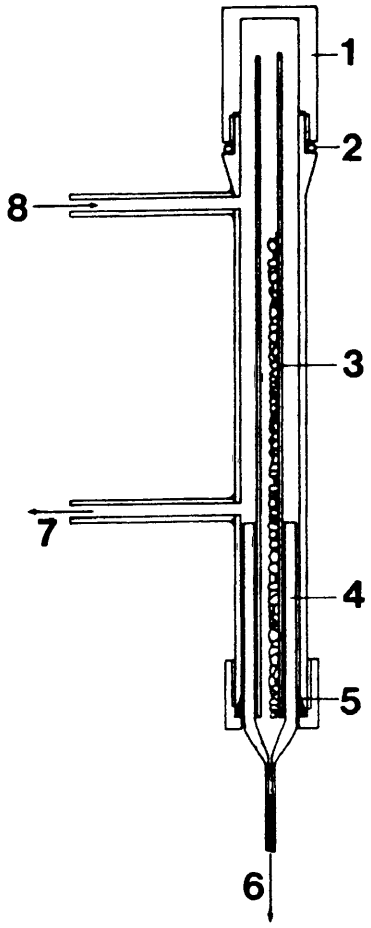


Fig 5.8 Metal, single stage dynamic solvent effect inlet. 1: screw cap; 2: sealing ring; 3: dynamic solvent effect concentrator; 4: glass seat with bore a light sliding fit to the outside of the concentrator; 5: compression fitting; 6: to column; 7: purge outlet; 8: carrier gas input.

PERFORMANCE

The polar component peaks of the Test Mix were symmetrically broadened (Fig. 5.9). Unlike the all-glass, single-stage inlet the metal inlet's performance depended on heating time in the same way as that of the model, static solvent effect inlet (Chap 4).

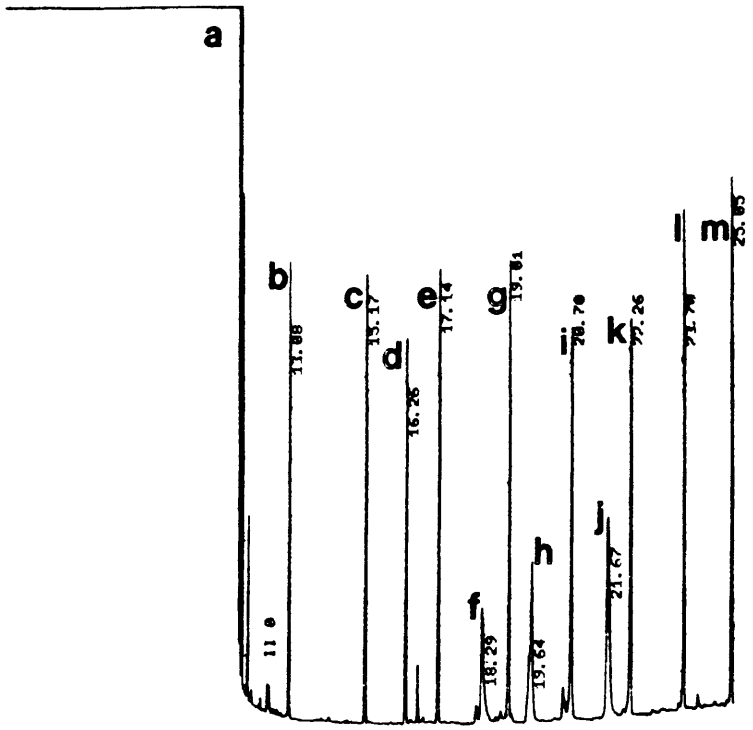


Fig 5.9 Chromatogram from 20 µl of 2:10 ^{7} Test Mix with sample introduction by a metal, single stage, dynamic solvent effect inlet. Peak identities as in Fig 5.2.

Scaled down version

The dimensions of the inlet were scaled down to match a concentrator o.d. of 2,0 mm. Restrictions on the availability of glass tube of suitable dimensions for the seat dictated the use of soda-lime glass.

PERFORMANCE

Chromatograms showed solvent tailing, and contaminant peaks which were not due to gas or solution contamination (Fig. 5.10). Replacement of the polytetrafluoroethylene cap seal with a lead washer, or introduction of carrier gas through the top of the cap resulted in cleaner chromatograms and sharper solvent tails (Fig. 5.11).

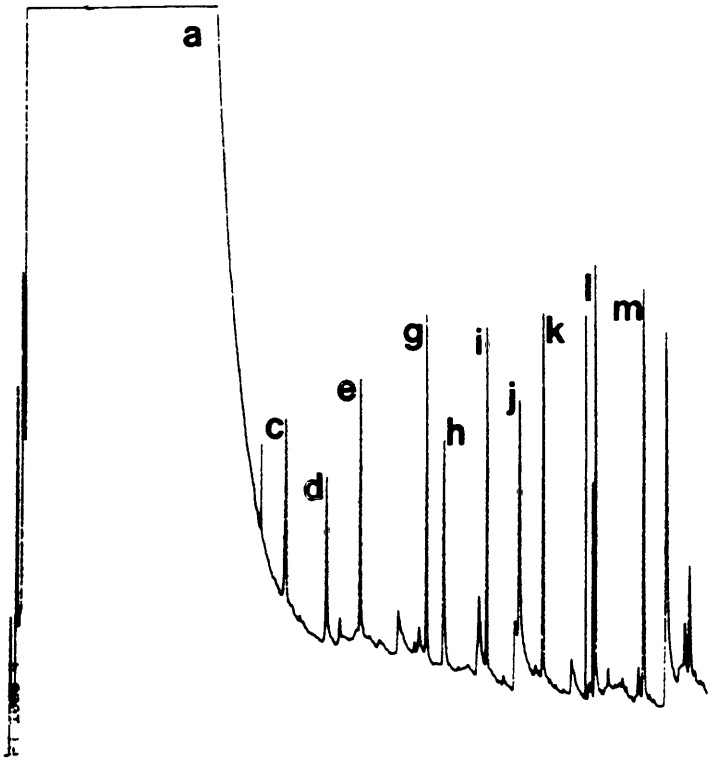


Fig 5.10 Contamination and solvent peak tailing caused by a loose-fitting screw cap on a metal, single stage, dynamic solvent effect inlet. Peak identities as in Fig 5.2.

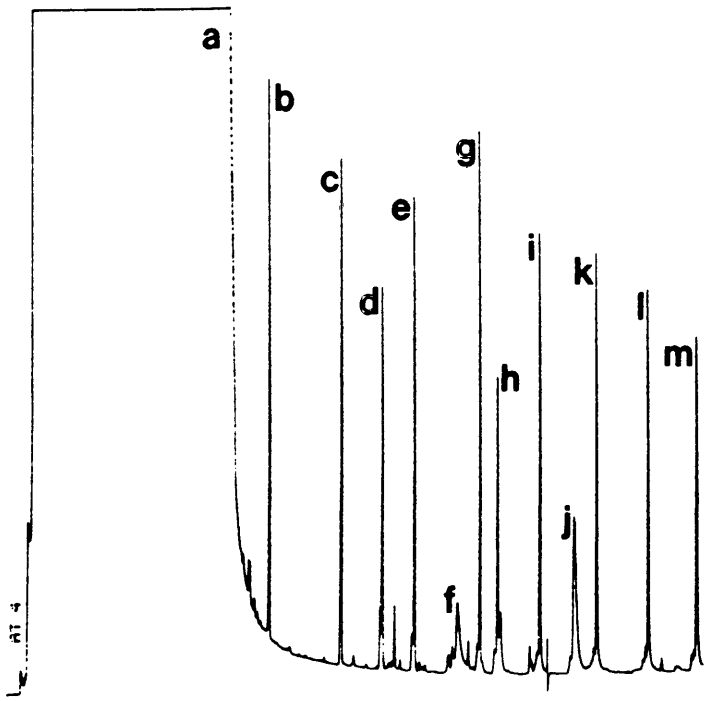


Fig 5.11 Improvement to contamination and solvent peak tail when the polytetrafluoroethylene sealing ring of a metal, single stage, dynamic solvent effect inlet was replaced by a lead washer. Sample and peak identities as in Fig 5.10.

Re-directed gas input

The carrier gas input line was extended so that carrier gas was introduced inside the inlet cap above the top of the inserted concentrator (Fig. 5.12).

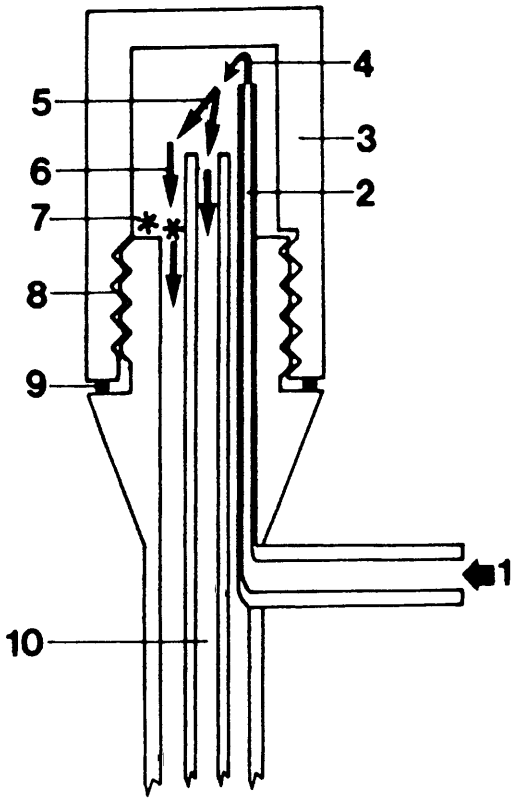


Fig 5.12 Modification to a metal, single stage, dynamic solvent effect inlet to redirect the carrier gas input away from the screw cap seal and threads. 1: carrier gas input; 2: extension tube; 3: carrier gas released into inlet above the top of the concentrator; 5: carrier gas splits between purge flow and flow through concentrator; 6: purge flow carries away; 7: contaminants from; 8: cap threads and; 9: sealing ring; 10: concentrator.

PERFORMANCE

Apart from that derived from the sample no contamination was evident and the solvent peak was sharp. The symmetrical broadening of the dimethylaniline and decanol peaks disappeared but that of the *p*-cresol peak persisted despite changes in heating schedule, use of different solvents, addition of chasers to the sample, washing the concentrators with either water or dilute hydrochloric acid and depositing the silicon at different temperatures (Fig. 5.13). When concentrators deactivated with the, then newly developed, silicon-ethene process

were used in soft glass seats the shape of the *p*-cresol peak changed from symmetrically broadened to classic, adsorptively tailed (Fig. 5.14). This tailing disappeared if the soft glass seat of the inlet was heated with a hot air gun while the inlet was being heated. Use of a "one-off" borosilicate glass seat specially pulled down to a nominal 2 mm i.d., and acid leaching of the soft glass, also improved the shape of the *p*-cresol peak (Fig. 5.15).

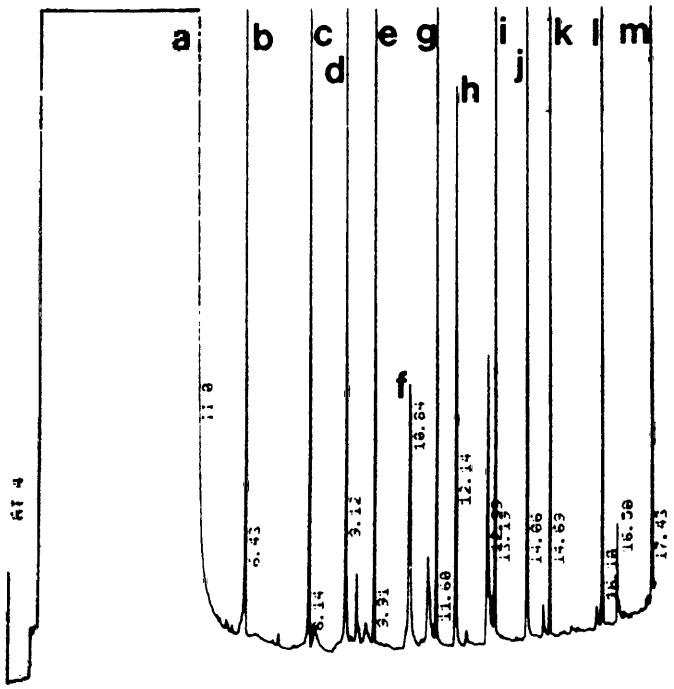


Fig 5.13 Chromatogram from 20 µl of 2:10 ^{7} Test Mix with sample introduction by a scaled down, metal, single stage, dynamic solvent effect inlet with redirected carrier gas input. Concentrator deactivated with water-washed silicon. Peak identities as in Fig 5.2.

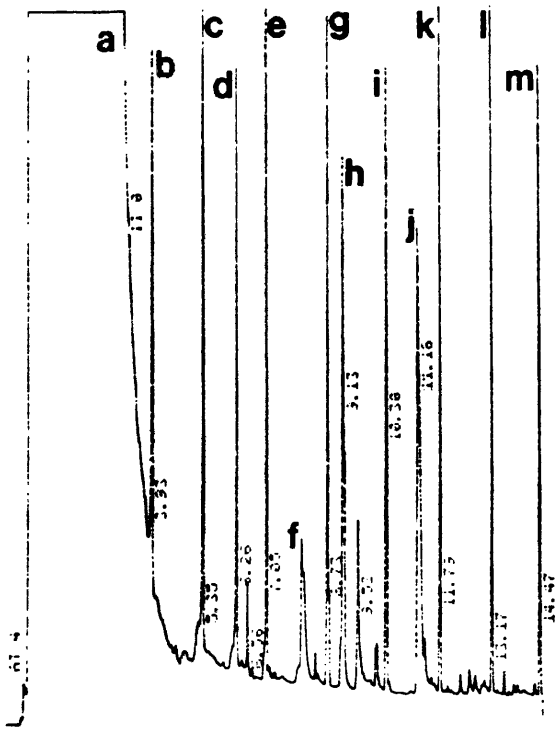


Fig 5.14 Chromatogram from 20 µl of 2:10 ^{7} Test Mix with sample introduction by a scaled down, metal, single stage, dynamic solvent effect inlet. Concentrator deactivated with silicon-ethene, soft glass seat. Peak identities as in Fig 5.2.

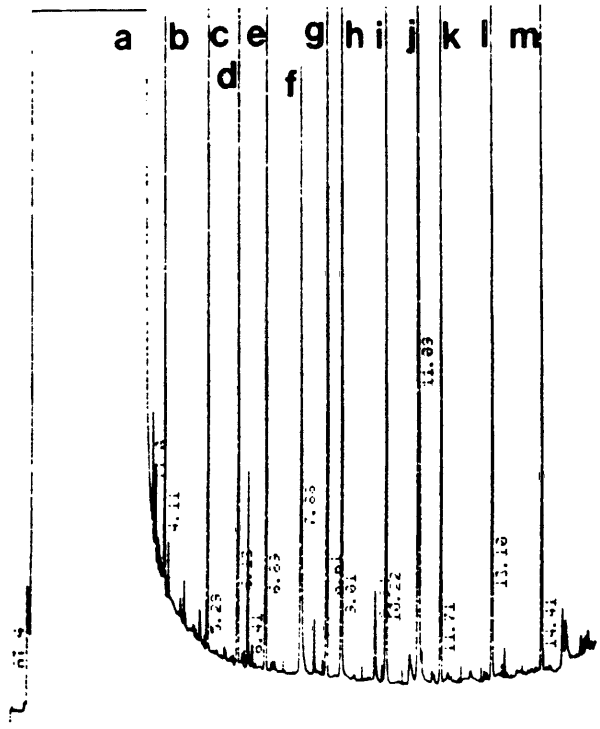


Fig. 5.15 Chromatogram from 20 µl of 2:10 ^{7} Test Mix with sample introduction by a scaled down, metal, single stage, dynamic solvent effect inlet. Concentrator deactivated with silicon-ethene, borosilicate glass seat heated with hot air during solute transfer to column. Peak identities as in Fig 5.2. Compare with Fig 5.14.

Modification of compression fitting for the seat

So that the heater element of the inlet could be wound to its lower end the compression fitting for the seat was designed to work with an internal nut (Fig. 5.16). At the same time silica tube of nominal 2 mm i.d. became available so that it was possible to produce silica seats with press fit polyimide connectors (Rohwer, Pretorius & Apps 1986) to the silica legs of the newly developed "hybrid" columns (Rohwer & Pretorius 1987). The connection to the column lay within the heatable zone of the inlet.

The inlet was tested with silicon-ethene surfaced concentrators.

PERFORMANCE

For all components of the Test Mix the dynamic solvent effect inlet provided peak shapes which approached those from the model, static solvent effect inlet (Chap 4). All components of the linalool-substituted Test Mix were eluted as sharp, symmetrical peaks (Fig. 5.17, see also Fig. 4.25), as were a very wide range of compounds encountered in various natural samples (see applications section). A 1 cm^3 specimen of diesel in hexane at a concentration of $1:10^8$ produced a much more satisfactory chromatogram than was obtained on the first attempt with a dynamic solvent effect system (compare Figs 4.12 and 5.18). See Chap 6 for a more detailed treatment of the system's performance.

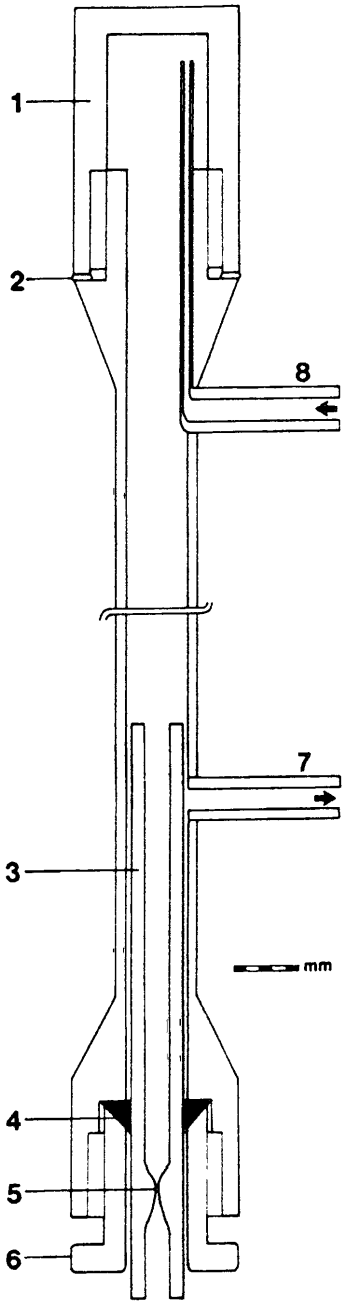


Fig. 5.16 Metal, single stage, dynamic solvent effect inlet with redirected carrier gas input, silica seat and connection to column within heatable zone of the inlet. 1: screw cap; 2: sealing ring; 3: silica seat; 4: graphite ferrule; 5: taper for press fit connection to column; 6: compression nut; 7: purge flow; 8: carrier gas input.

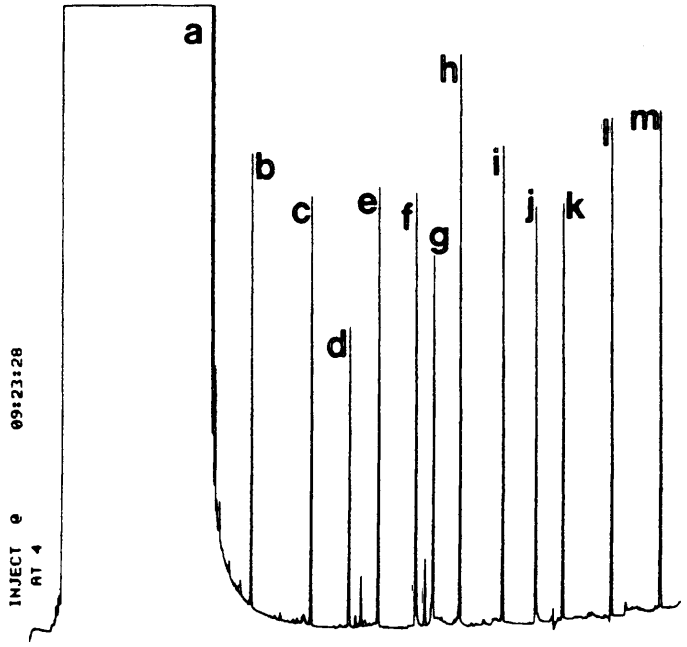


Fig. 5.17 Chromatogram from 300 µl of $1:10^{8}$ Test Mix sampled by the dynamic solvent effect with sample introduction by a metal, single stage, dynamic solvent effect inlet with redirected carrier gas input, silica seat, internal nut compression fitting and press-fit connection to column. Concentrator deactivated with silicon-ethene. Peak identities as in Fig 5.2, except g: linalool.

DISCUSSION

The metal inlet performed much more satisfactorily than its all-glass predecessor (compare Figs. 5.4 and 5.17). This was probably due to a more even transfer of heat to the concentrator in the metal version. In the metal inlet the inner walls were oxidised stainless steel - a better radiator of heat than glass - and the seat was in less intimate thermal contact with the heater element.

The symmetrical broadening of the polar compound peaks when 2,4 mm o.d. concentrators were used was interpreted as being due to longtitudinal diffusion due to relatively slow linear gas flow rates through the concentrators. For DMA and decanol this interpretation is supported by the success of the reduction in concentrator diameter as a solution to the problem. In the case of the *p*-cresol peak adsorptive activity in the concentrator and on the inlet seat must also have played a role.

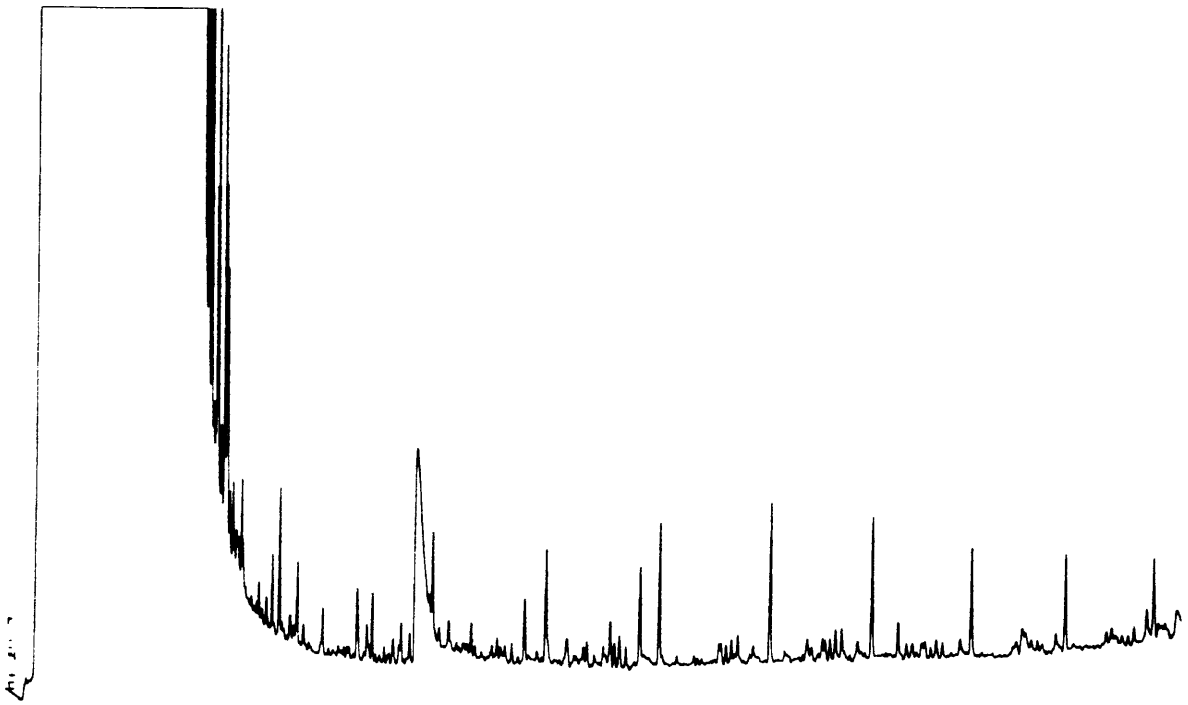


Fig. 5.18 Chromatogram of diesel sampled by the dynamic solvent effect from a 1 cm^3 solution in *n*-hexane at a concentration of $1:10^8$ using a laterally perforated, silicon-ethene deactivated concentrator and transferred to the column by a metal, single stage inlet with redirected carrier gas input, silica seat, internal nut compression fitting and press-fit connection to the column. Column 25 m x 0,3 mm x 0,4 µm methyl silicone, temperature programme $10^oC\ min^{-1}$, FID sensitivity 1 x 10^{-11} A mv^{-1}.

The scaled down prototype of the metal inlet had been made to extremely loose tolerances. Consequently there was a large dead volume around the cap threads which provided a path for diffusion of contaminants from the seal to the carrier gas. When the gas input was redirected to the inside of the cap the gas which passed this contaminant source subsequently passed out through the purge rather than passing through the concentrator and on to the column.

Plainly soda-lime glass was not a suitable material for the seat. Its surface is basic and distortion of the acidic *p*-cresol peak was to be expected. Because soda-lime and silica seats were not interchangeable between standard and internal-nut compression fittings the relative contributions of seat temperature and surface activity to the peak distortion cannot be assessed.

Although silica provides an extremely inert surface its high melting point makes it much more difficult to work than borosilicate glass, which from the point of view of activity would be suitable for routine analyses. Unfortunately light-walled, borosilicate glass with a nominal 2 mm i.d. was not commercially available when this development work was carried out.

Chapter 6

THE PERFORMANCE OF DYNAMIC SOLVENT EFFECT SAMPLING

QUANTITATIVE PERFORMANCE

Performance targets were based on a survey of the literature, the performance of the available columns and detectors, and the results of pilot experiments. So that the performance attained would not reflect a "best case", applicable only to specimens containing high concentrations of chromatographically tractable compounds, a general requirement was set; that performance tests would be carried out using test specimens containing a range of chemical types, of each of which less than 10 ng would be present in each sample.

Quantitative performance has three aspects; accuracy, precision and sensitivity. Which of these is most important will depend on the type of problem under investigation.

Accuracy is the departure of a measured from a calculated value, and can only be measured if the calculated value is error free, or has a known sampling distribution. Attempts to make up standard specimens with low parts per billion concentrations, which were accurate to within the 1-2 % limits needed for this investigation proved unproductive. Adsorption, the effects of temperature on density, limited volumetric accuracy and evaporation of volatile solvents all contribute to uncertainty in the concentration of a standard (e.g. Barrat 1981; Ioffe & Vitenberg 1983 p 234; Huynh & Vu Duc 1985; Hammers & Bosman 1986) (see Tables 6.1, 6.2, 6.12 and Discussion). Nor was it possible to measure the concentration of a standard independently since even the best of the alternative methods yield sampling variations as large, or larger than, that provided by the dynamic solvent effect (Table 6.19 and Discussion). Consequently only precision, and not accuracy, of dynamic solvent effect sampling was investigated.

The target for precision was set at coefficients of variation of less than 10 % for peak areas, peak area ratios and peak percentage areas.

Because sensitivity in terms of minimum detectable quantity proved not usually to be a critical factor in applied analyses (p 289), and because in any case it is highly dependent on the criteria adopted for "detection", it was not investigated in detail.

Three specimen types were investigated; liquid specimens in a matrix compatible with direct dynamic solvent effect sampling (*n*-hexane), specimens in a gaseous matrix and specimens in a liquid matrix incompatible with direct dynamic solvent effect sampling (water).

METHODS

Chromatographic conditions

All samples were run on a 25 m x 0,3 mm x 0,4 µm methyl silicone column. Unless otherwise stated the initial temperature of inlet and column was 50°C, the inlet was ballistically heated to 220°C after a solvent evaporation time determined for each concentrator (Chap 4), and the column temperature was programmed at 10°C min^{-1} after 4 min. Detection was by FID at a sensitivity of 10^{-11} A mv^{-1} and chromatograms, peak areas and peak percentage areas were recorded on a Varian 4270 integrator with a full scale deflection of 4 mv for the specimens in liquid matrices and 1 mv for the specimens in a gas matrix.

Accuracy and precision

i) Specimens in a liquid matrix compatible with dynamic solvent effect concentration

Solutions of Test Mix, with linalool substituted for *n*-undecane, were made up in *n*-hexane. Three sampling procedures were applied; first 300 µl of $1:10^8$ solution was measured by Drummond Microcap into a solvent holder and concentrated to dryness, and the concentrator was topped up with *n*-hexane. Secondly 200 µl of *n*-hexane was measured by Microcap into a solvent holder and 20 µl of a $1:10^7$ Test Mix was added before concentration to dryness and topping up. Thirdly 10 cm^3 of $1:10^8$ solution was prepared and sampling was carried out direct from the bulk without measuring into other containers. The sampling time was 15 min; the time taken to concentrate a measured 300 µl specimen to dryness. The concentrators were not topped up before the samples were run.

In all cases sampling was carried out in a room whose temperature varied between 26 and 27°C using 15 cm^3 min^{-1} of palladium cell purified hydrogen.

ii) Specimens in a gas matrix

Low concentrations of a complex mixture of hydrocarbons in air were generated by passing a 10 cm^3 min^{-1} flow of charcoal-filtered air over the surface of approximately 10 cm^3 of white petroleum jelly in a 20 cm^3 tube (Fig. 6.1), at room temperature (26-27°C). The air flow was maintained for one week, with hydrocarbon concentrations monitored daily, to allow the system to stabilize before serial sampling was carried out. Sampling was carried out by passing the hydrocarbon loaded air through a dynamic solvent effect concentrator, with *n*-hexane as solvent, for 10 min. One series of five samples was taken on each of three concentrators. The samples were analysed under the same conditions as those from liquid specimens (above) except that the starting temperature of the temperature programme was 40°C.

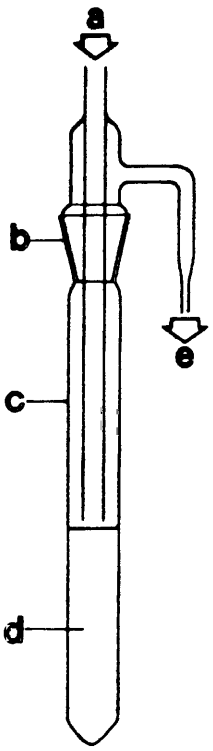


Fig. 6.1 Apparatus used to generate ppb concentrations of complex mixtures of airborne hydrocarbons. a: 10 cm^3 min^{-1} charcoal-purified air; b: 14/23 ground glass joint; c: 20 cm^3 tube; d: approximately 10 cm^3 of white petroleum jelly; e: gas flow to dynamic solvent effect concentrator.

iii) Specimens in a liquid matrix incompatible with dynamic solvent effect concentration

Major problems were encountered in attempting to make up aqueous solutions of volatile organics which were both dilute and stable enough to provide a useful and reliable measure of the performance of dynamic solvent effect sampling (see discussion). The standard which was finally employed contained a series of aldehydes and ketones; (concentrations in ppb) 2-heptanone (40), heptanal (16), 2,6-dimethyly-4-heptanone (16), nonanal (48), decanal (16), undecanal (24) and dodecanal (8) in distilled water, which had been purged of organic volatiles by vigorous boiling and sparging with charcoal-filtered nitrogen.

Each specimen was a 5 cm^3 aliquot of the stock standard measured into a 10 cm^3 "mini" bubbler (Fig. 7.1 C) using a glass pipette. Both pipette and bubbler were rinsed with 5 cm^3 of the standard immediately before each specimen was measured. The test compounds were purged from the water with a 10 cm^3 min^{-1} flow of palladium-purified hydrogen for 10 min, and trapped by the dynamic solvent effect using *n*-hexane as solvent, at a temperature of 30-30,6°C. No drying was necessary. Separations were carried out under the standard conditions (above) except that the starting temperature was 40°C. A series of five samples were run on each of three concentrators.

A further series of five samples, for which the pipette and bubbler were not rinsed, was run on one concentrator.

Data Analysis

Standard deviations of peak areas and peak percentage areas were calculated from integrator reports from 5 consecutive runs, the coefficient of variation is the standard deviation divided by the mean. Standard deviations and coefficients of variation were calculated for the ratios of areas between pairs of peaks. Differences in detector response to different compounds were assumed to be constant.

Coefficients of variation of peak area ratios (internal standard precision) were also calculated for the data from the study of linearity (below).

Detection limit

From the results of the investigation of linearity of peak area against sampling time (qv) a minimum detectable concentration limit of $3{:}10^{12}$ was calculated for a 200 µl solvent specimen. A solution of linalool-substituted Test Mix in *n*-hexane was made up to this concentration and sampled for 15 min with a 10 cm^3 flow of palladium cell purified hydrogen at a temperature of 30-30,6°C. Solutions with concentrations of 6, 10, 20, 30 and $50{:}10^{12}$ were also sampled under the same conditions. Samples were run with a detector sensitivity of 10^{-12} A mv^{-1} with the integrator set for a 4 mv full scale deflection. A solvent blank was run under the same conditions and peak identities were based on retention times from a 20 µl specimen with a concentration of $1{:}10^8$.

Linearity of peak areas vs specimen concentration

Five cm^3 of Test Mix at a concentration of $2{:}10^7$ was made up in *n*-hexane. For each specimen approximately 0,5 cm^3 of solution was decanted into a solvent holder which had been rinsed with the same solution to reduce adsorptive activity. Each specimen was sampled for 10 min (approx. 200 µl) with a 15 $cm^3 min^{-1}$ flow of palladium cell purified hydrogen at a temperature of 28-29°C. One specimen was sampled on each of three concentrators. The residue of the Test Mix was then diluted by making up to approximately the original volume with *n*-hexane and another set of three specimens was sampled and run. To minimize exposure of the Test Mix to adsorptive glass surfaces the quantity of solution and of added hexane was determined gravimetrically rather than volumetrically. This process was repeated until the chromatographic peaks were less than 2000 counts in area (approx. 1 ng). Peak areas were plotted against calculated concentrations and the straight line giving the best, least-squares fit was calculated using the computer programme CURFIT.

RESULTS

Precision and accuracy

i) Specimens in a DSE-compatible liquid matrix

Concentrating 300 µl, $1:10^8$ specimens to dryness yielded peak areas and percentage areas with coefficients of variation of less then 10 % for all test compounds except *p*-cresol (Table 6.1).

Table 6.1 Coefficients of variation of peak areas and peak percentage areas for dynamic solvent effect sampling of 300 µl of $1:10^8$ Test Mix (approx. 3 ng per peak) in *n*-hexane, concentrated to dryness (procedure 1).

Compound	% coefficient of variation	
	Area	% area
n-octane	1,14	2,20
n-nonane	1,30	2,97
dimethylheptanone	3,06	3,05
n-decane	1,80	2,80
p-cresol	23,68	20,99
linalool	8,37	6,36
dimethylaniline	4,56	1,90
n-dodecane	0,81	3,23
methyl decanoate	2,55	2,31
n-tetradecane	0,97	3,27
n-pentadecane	6,98	5,95

With the second sampling procedure; dilution of 20 µl of $1:10^7$ solution with 200 µl of *n*-hexane immediately before sampling, the precision fell far short of the 10% co-efficient of variation target for peak areas of *p*-cresol, linalool and pentadecane, and for peak percentage areas of *p*-cresol and pentadecane (Table 6.2).

Table 6.2 Coefficients of variation of peak areas and peak percentage areas for dynamic solvent effect sampling of 200 µl of Test Mix diluted to $1:10^8$ in *n*-hexane immediately before sampling (sampling procedure 2) (approx. 2 ng per peak).

Compound	% coefficient of variation	
	Area	% area
n-octane	4,56	6,63
n-nonane	4,90	6,89
dimethylheptanone	7,59	5,91
n-decane	3,28	7,22
p-cresol	31,66	23,38
linalool	13,00	5,64
dimethylaniline	9,02	8,52
n-dodecane	2,71	7,44
methyl decanoate	5,81	5,04
n-tetradecane	2,39	8,10
n-pentadecane	26,01	17,20

Timed sampling from a bulk specimen (procedure 3) yielded extremely high precision of peak areas and percentage areas for all peaks on each of the three concentrators (Table 6.3), with all peaks in the Test Mix eluting with satisfactory sharpness and symmetry except *n*-decanol, towards which the column was very active (Fig. 6.2).

Table 6.3 Coefficients of variation of peak areas and peak percentage areas for dynamic solvent effect sampling for 15 min from a bulk $1:10^8$ Test Mix in *n*-hexane (procedure 3) (approx 3 ng per peak).

Compound	% coefficient of variation					
	Bed Q2		Bed Q3		Bed Q4	
	Area	%area	Area	%area	Area	%area
n-octane	1,95	1,40	3,70	2,98	3,92	3,08
n-nonane	1,35	0,63	1,89	0,89	2,77	1,70
dimethylheptanone	1,17	0,48	2,01	0,99	2,51	1,35
n-decane	1,29	0,46	1,99	0,96	2,69	1,57
p-cresol	0,91	1,06	2,30	0,89	2,81	3,10
linalool	1,30	0,49	2,37	0,90	1,96	0,75
dimethylaniline	0,81	0,61	1,79	1,70	1,14	0,55
n-dodecane	1,27	0,41	2,80	1,77	2,88	1,80
methyl decanoate	1,21	0,54	2,21	1,00	1,91	0,68
n-tetradecane	0,71	0,34	1,72	1,06	1,10	1,40
n-pentadecane	0,05	0,86	2,70	2,86	1,71	2,40

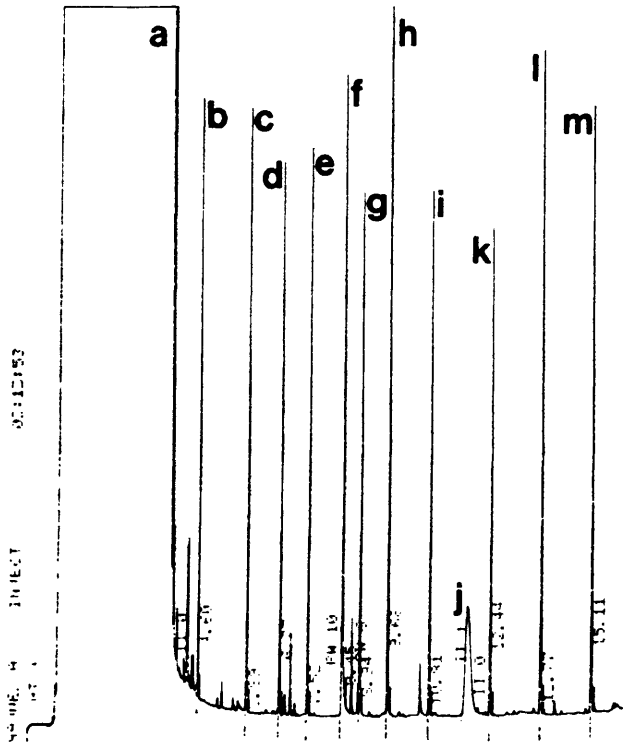


Fig. 6.2 Chromatogram of sample accumulated from a $1:10^8$ hexane solution of Test Mix by dynamic solvent effect sampling for 15 min at a temperature of 26-27°C with a gas flow rate of 15 cm^3 min^{-1}. Each peak represents approximately 3 ng. a: *n*-hexane; b: *n*-octane; c: *n*-nonane; d: 2,6-dimethylheptan-4-one; e: *n*-decane; f: *p*-cresol; g: linalool; h: 2,6-dimethylaniline; i: *n*-dodecane; j: *n*-decanol; k: methyldecanoate; l: *n*-tetradecane; m: *n*-pentadecane. For analytical conditions see text.

With the exception of the tetradecane:pentadecane ratio, the precision of peak area ratios was good for non-polar components, less good but within target levels for pairs with a polar member, and unacceptable for the decane:*p*-cresol ratio when sampling procedure 1 was followed (Table 6.4).

Table 6.4 Coefficients of variation of ratios of peak areas for dynamic solvent effect sampling of 300 µl of $1:10^8$ Test Mix in *n*-hexane (sampling procedure 1).

Compounds	% coefficient of variation
octane:nonane	0,85
nonane:dimethylheptanone	2,44
nonane:decane	1,22
decane:dodecane	0,78
dodecane:methyl decanoate	2,25
dodecane:tetradecane	0,23
tetradecane:pentadecane	7,63
decane:*p*-cresol	24,56
dimethylaniline:dodecane	4,59
decane:linalool	8,33
linalool:dodecane	8,22

Using the second procedure peak area ratio precision was good for pairs of non-polar compounds but poor for pairs with a polar member (Table 6.5).

Table 6.5 Coefficients of variation of ratios of peak areas for dynamic solvent effect sampling of 200 µl of Test Mix diluted to $1:10^8$ in *n*-hexane immediately before sampling (sampling procedure 2).

Compounds	% coefficient of variation
octane:nonane	0,89
nonane:dimethylheptanone	5,01
nonane:decane	1,90
decane:dodecane	0,78
dodecane:methyl decanoate	3,87
dodecane:tetradecane	0,95
tetradecane:pentadecane	20,30
decane:*p*-cresol	33,07
dimethylaniline:dodecane	7,82
decane:linalool	11,61
linalool:dodecane	10,80

Timed sampling (procedure 3) yielded very precise peak area ratios (Table 6.6).

Table 6.6 Coefficients of variation of peak area ratios for dynamic solvent effect sampling for 15 min from a bulk 1:10^8 Test Mix in *n*-hexane.

Compounds	% coefficient of variation		
	Bed Q2	Bed Q3	Bed Q4
octane:nonane	0,90	2,17	1,49
nonane:dimethylheptanone	0,39	0,15	0,42
nonane:decane	0,34	0,65	0,26
decane:dodecane	0,32	0,91	0,40
dodecane:methyl decanoate	0,43	0,80	1,44
dodecane:tetradecane	0,42	2,60	3,09
tetradecane:pentadecane	0,50	1,91	1,59
decane:*p*-cresol	1,48	1,71	4,50
dimethylaniline:dodecane	0,81	3,40	2,16
decane:linalool	0,22	1,05	1,20
linalool:dodecane	0,27	1,56	1,33

The coefficients of variation of peak area ratios over the two orders of magnitude of concentration covered by the investigation of linearity are shown in Table 6.7.

Table 6.7 Coefficients of variation of peak area ratios for dynamic solvent effect sampling from liquid specimens over a concentration range from 2:10^9-200:10^9 (approx 0,5-50 ng per peak).

Compounds	% coefficient of variation		
	Bed Q2	Bed Q3	Bed Q4
octane:nonane	----	13,73	1,90
nonane:dimethylheptanone	9,50	12,58	9,76
nonane:decane	1,52	1,52	0,68
decane:dodecane	3,40	2,18	4,90
dodecane:methyl decanoate	4,66	3,17	7,16
dodecane:tetradecane	4,41	6,28	4,68
tetradecane:pentadecane	6,95	14,96	2,10
decane:*p*-cresol	4,50	3,60	8,16
dimethylaniline:dodecane	8,86	12,95	2,65
decane:linalool	----	----	----
linalool:dodecane	14,13	1,57	2,77

ii) Airborne hydrocarbons

Despite the complexity of the mixture of hydrocarbons (Fig. 6.3) and the small quantities involved (0,6-2,0 ng) the target of coefficients of variation below 10 % was met in all cases (Tables 6.8, 6.9)

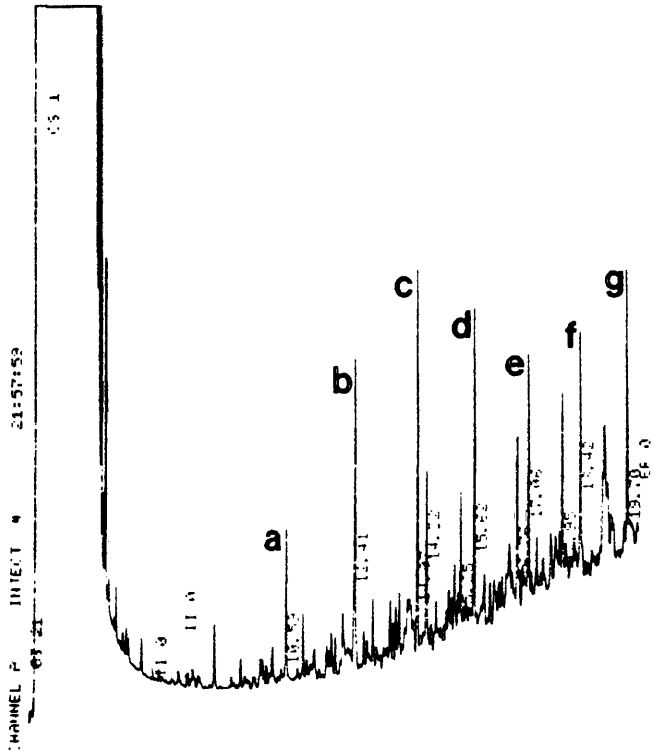


Fig. 6.3 Chromatogram of airborne hydrocarbons sampled by the dynamic solvent effect for 10 min with an air flow rate of 10 cm^3 min^{-1}, using *n*-hexane as solvent at a temperature of 26-27°C. a: *n*-decane; b: *n*-undecane; c: *n*-dodecane; d: *n*-tridecane; e: *n*-tetradecane; f: *n*- pentadecane; g: *n*-hexadecane. For analytical conditions see text.

Table 6.8 Coefficients of variation of peak areas and peak percentage areas for a mixture of airborne hydrocarbons sampled by the dynamic solvent effect. Retention times (T_r) correspond to those in Fig. 6.3. Concentrations are approximate, based on an assumption of no sampling losses.

T_r	Conc. v/v,ppb	% coefficient of variation					
		Bed Q2		Bed H5		Bed H7	
		Area	%area	Area	%area	Area	%area
10.59	1,3	4,69	1,79	2,46	2,12	0,63	2,77
12.41	2,9	4,14	2,00	2,44	1,56	1,07	2,66
14.07	2,6	3,48	1,74	2,40	1,74	0,95	2,23
14.32	1,3	3,60	1,58	1,92	1,87	0,67	2,80
15,25	0,7	2,70	3,26	2,07	1,70	1,34	4,08
15.62	1,8	4,24	1,89	1,96	1,73	1,84	1,69
16,76	0,9	3,17	2,19	1,58	2,59	1,63	1,89
17.06	1,4	4,57	2,19	2,70	1,23	9,26	7,38
17.95	1,8	3,23	3,06	3,13	1,78	1,39	1,85
18.42	1,5	3,72	1,96	3,12	1,68	1,39	3,18
19.70	1,4	6,30	4,07	7,21	6,33	3,10	3,32

Table 6.9 Coefficients of variation of peak area ratios for dynamic solvent effect sampling of a mixture of airborne hydrocarbons. Peaks are labelled by their retention times in Fig. 6.3.

Peaks	% coefficients of variation		
	Bed Q2	Bed H5	Bed H7
10.59:12.41	1,26	2,82	0,51
12.41:14.07	1,42	1,94	0,70
14.07:14.32	0,62	0,66	0,69
14.32:15.25	2,13	2,60	1,40
15.25:15.62	2,38	2,47	2,67
15.62:16.76	1,13	1,40	0,47
16.76:17.06	1,66	3,13	6,91
17.06:17.95	2,44	2,65	7,91
17.95:18.42	1,24	1,14	3,18
18.42:19.70	3,23	4,58	6,54

iii) Aqueous aldehydes and ketones

In most cases the coefficients of variation of the various statistics were below the 10% target level. The exceptions were the peak areas of *n*-decanal and *n*-dodecanal on concentrator Q2, the areas of *n*-decanal, *n*-undecanal and *n*-dodecanal on concentrator H7, the percentage area of *n*-dodecanal on concentrator H7 and the *n*-decanal:*n*-undecanal ratio on concentrator H7 (Tables 6.10, 6.11). For all the test components the peaks were sharp and symmetrical (Fig. 6.4).

Table 6.10 Coefficients of variation of peak areas and percentage areas for an aqueous solution of carbonyl compounds sampled by the dynamic solvent effect (glassware rinsed with specimen solution).

Compound	% coefficient of variation					
	Bed Q2		Bed Q3		Bed H7	
	Area	%area	Area	%area	Area	%Area
n-heptanone	3,87	4,02	3,94	2,79	6,25	3,20
n-heptanal	3,22	2,83	2,43	1,79	7,63	7,17
dimethylheptanone	4,30	5,66	3,88	2,83	6,14	2,96
n-nonanal	3,92	1,27	5,29	2,31	8,84	3,02
n-decanal	10,51	7,96	5,22	2,05	16,18	8,70
n-undecanal	9,68	6,25	8,48	6,53	10,14	8,99
n-dodecanal	10,88	8,06	9,61	7,58	12,94	11,44

Table 6.11 Coefficients of variation of peak area ratios for aqueous carbonyl compounds sampled by the dynamic solvent effect (glassware rinsed with specimen solution).

Compounds	% coefficients of variation		
	Bed Q2	Bed Q3	Bed H7
heptanone:heptanal	3,68	2,96	2,17
heptanal:dimethylheptanone	5,83	2,92	2,34
dimethylheptanone:nonanal	5,67	3,75	5,55
nonanal:decanal	7,71	1,09	6,73
decanal:undecanal	9,69	7,10	15,82
undecanal:dodecanal	3,35	3,46	3,63

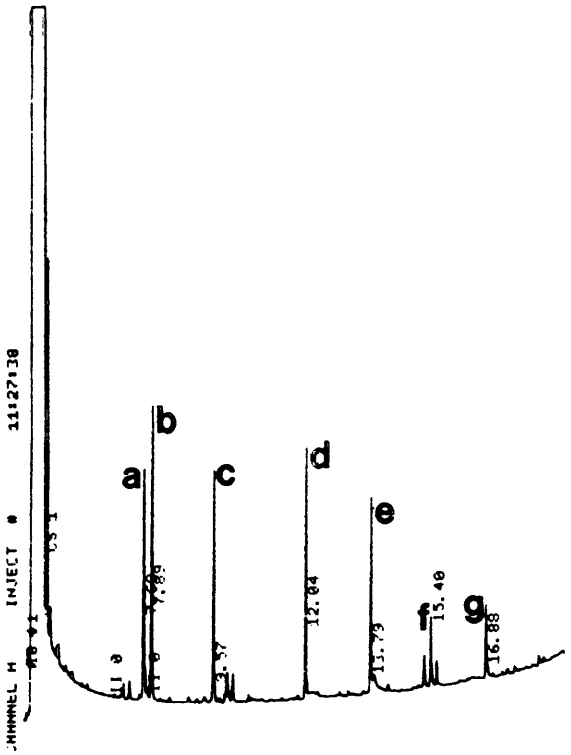


Fig 6.4 Chromatogram of carbonyl compounds sampled from 5 cm^3 of aqueous solution by gas sparging at 10 cm^3 min^{-1} for 10 min at 30-30,6 °C and trapping by the dynamic solvent effect using *n*-hexane as solvent. a: *n*-heptan-2-one; b: *n*-heptanal; c: 2,6-dimethylheptan-4-one; d: *n*-nonanal; e: *n*-decanal; f: *n*-undecanal; g: *n*-dodecanal. For analytical conditions see text.

When the rinsing of the pipette and mini-bubbler with specimen solution was omitted the precision of the peak areas was so poor for one series of five samples on one concentrator (Table 6.12) that no further samples were run or calculations carried out.

Table 6.12 Coefficients of variation of peak areas for aqueous carbonyl compounds sampled by the dynamic solvent effect (glassware not rinsed with specimen solution).

Compound	% coefficient of variation Bed Q2
n-heptanone	6,36
n-heptanal	11,55
dimethylheptanone	6,70
n-nonanal	17,93
n-decanal	28,65
n-undecanal	27,02
n-dodecanal	43,25

Inter-concentrator differences

When the mean peak areas for each concentrator are compared, and the coefficients of variation calculated for all 15 samples from each specimen type, differences between concentrators become apparent (Tables 6.13, 6.14 and 6.15).

Table 6.13 Comparison of mean peak areas of each component of a $1:10^8$ solvent-matrix Test Mix sampled from the bulk, on each of three dynamic solvent effect concentrators, and overall coefficients of variation of peak areas from five samples on each of three concentrators. Mean peak areas within a row bearing the same, lower case superscript are significantly different at P<0,05, mean peak areas within a row bearing the same, upper case superscript are different at P<0,01 (t test).

Compound	Mean peak area (N = 5)			CV%
	Bed Q2	Bed Q3	Bed Q4	
n-octane	6266^{AB}	5362^{A}	5336^{B}	8,50
n-nonane	8343^{AB}	6623^{Aa}	7233^{Ba}	10,14
dimethylheptanone	8395^{AB}	6639^{Aa}	7336^{Ba}	10,18
n-decane	8833^{A}	6881^{A}	7760^{A}	10,72
p-cresol	12460^{aA}	9667^{AB}	11404^{aB}	10,84
linalool	8530^{A}	6685^{A}	7657^{A}	10,37
dimethylaniline	11524^{A}	8497^{A}	10670^{A}	12,94
n-dodecane	8743^{Aa}	7013^{Ab}	8005^{ab}	9,52
methyldecanoate	8044^{Aa}	6466^{AB}	7745^{aB}	9,69
n-tetradecane	11185^{Aa}	9206^{AB}	11671^{aB}	10,38
n-pentadecane	10591^{Aa}	8124^{AB}	11115^{aB}	13,67

Table 6.14 Comparison of mean peak areas of each component of a mixture of airborne hydrocarbons sampled on each of three dynamic solvent effect concentrators and overall coefficients of variation of peak areas from five samples on each of three concentrators. Mean peak areas within a row bearing the same, lower case superscript are significantly different at P<0,05, mean peak areas within a row bearing the same, upper case superscript are different at P<0,01 (t test).

Peak	Mean peak area (N = 5)			CV%
	Bed Q2	Bed H5	Bed H7	
10.59	1667	1669	1670	2,85
12.41	3956	3829	3900	2,99
14.07	4150	4195	4172	2,30
14.32	2040	2060	2042	2,25
15.25	1135	1156^{a}	1089^{a}	3,25
15.62	3322	3406	3352	2,87
16.76	1539	1607	1568	2,77
17.06	2450	2573	2580	6,25
17.95	1647	1722	1693	3,14
18.42	2768	2986	2857	4,80
19.70	2812	3157	2987	7,30

Table 6.15 Comparison of mean peak areas of each component of the headspace of an aqueous solution of carbonyl compounds sampled on each of three dynamic solvent effect concentrators and overall coefficients of variation of peak areas from five samples on each of three concentrators. Mean peak areas within a row bearing the same, lower case superscript are significantly different at P<0,05, mean peak areas within a row bearing the same, upper case superscript are different at P<0,01 (t test).

Compound	Mean peak area (N =5)			CV%
	Bed Q2	Bed Q3	Bed H7	
n-heptanone	4924	4695	5465	8,09
n-heptanal	6996	6912	7193	4,99
dimethylheptanone	6942^{B}	6335^{a}	5311^{aB}	12,06
n-nonanal	6711^{a}	6085	5161^{a}	12,32
n-decanal	6530^{b}	5580^{a}	3762^{ab}	24,48
n-undecanal	1912	1849	1457	14,83
n-dodecanal	3478^{B}	3891^{A}	1492^{AB}	38,11

Detection limit

In the chromatogram resulting from dynamic solvent effect sampling of a solution with a calculated concentration of $3:10^{12}$ there were peaks whose retention times corresponded to those of *n*-nonane, *n*-decane and *n*-dodecane (Fig. 6.5). However, peaks with the same retention times also appeared in the chromatograms of the "pure" solvent in which the solution had been made up (Fig. 6.6). The *n*-nonane and *n*-decane peak areas were only loosely related to the specimen solution's calculated concentration, although that of *n*-dodecane showed an approximately linear relationship (Fig. 6.7). In all chromatograms the peaks corresponding to Test Mix components were smaller than those due to unidentified contaminants.

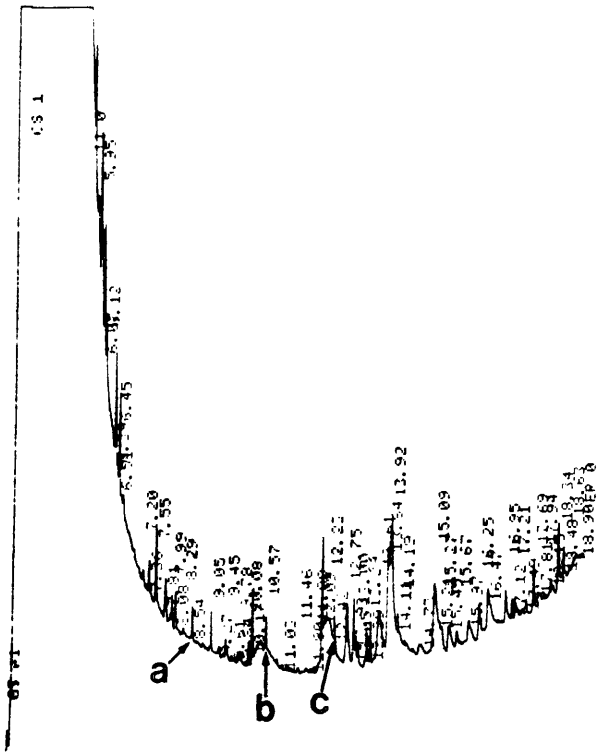


Fig. 6.5 Chromatogram of $3:10^{12}$ solution of test mix in *n*-hexane sampled by the dynamic solvent effect for 15 min. with a gas flow of 10 cm^3 min^{-1} at a temperature of 30-30,6°C. Detector sensitivity 10^{-12} x 4 A mv^{-1} full scale deflection. Peak identities based on retention times; a: *n*-nonane; b: *n*-decane; c *n*-dodecane. For analytical conditions see text.

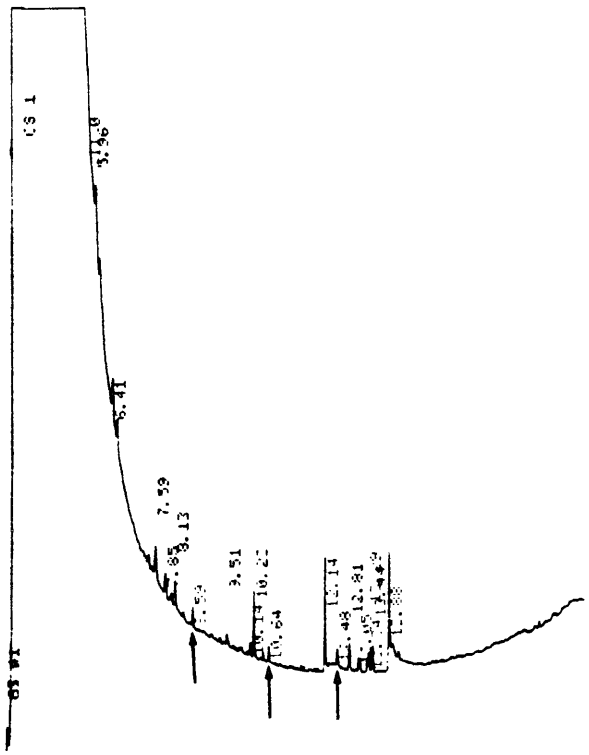


Fig. 6.6 Chromatogram of "pure" *n*-hexane sampled and analysed as in Fig. 6.5.

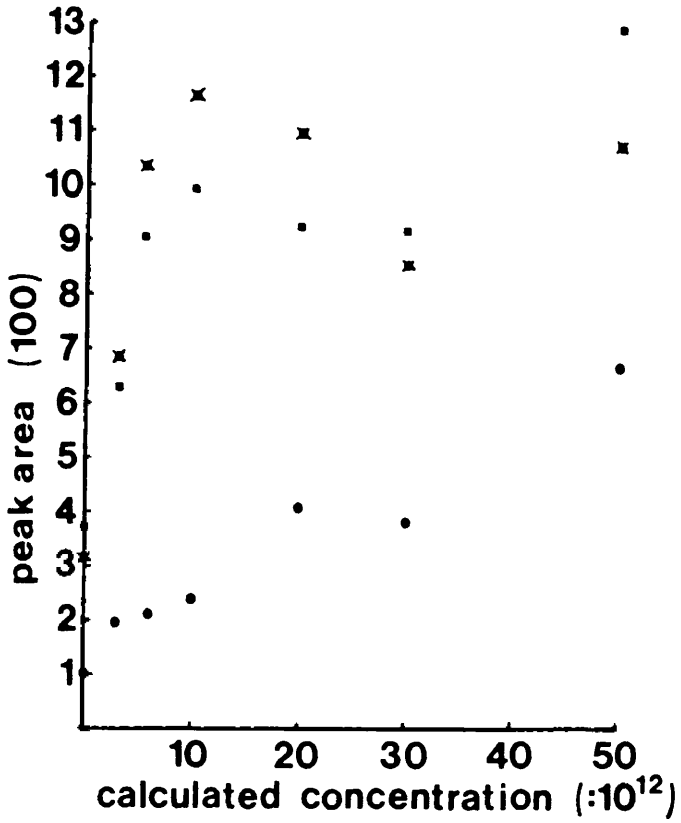


Fig. 6.7 Relationship of peak area to calculated specimen concentration for ppt solutions of test mix in *n*-hexane sampled by the dynamic solvent effect; squares: *n*-nonane; crosses: *n*-decane; circles: *n*-dodecane.

Linearity

All components of the Test Mix exhibited almost perfect linearity of peak area against calculated specimen concentration (Table 6.16, 6.17, 6.18).

Table 6.16 Equations of lines of best, least-squares fit to the relationship between peak area and specimen concentration for dynamic solvent effect sampling of specimens in a solvent matrix. Concentrator Q2.

Compound	Equation of line	r^2	P%
n-octane	*----------	------	---
n-nonane	y = 305 x + 902	0,9999	0,1
dimethylheptanone	y = 323 x + 629	0,9993	0,1
n-decane	*----------	------	---
p-cresol	y = 595 x + 207	0,9989	0,1
linalool	y = 381 x + 24	0,9993	0,1
dimethylaniline	y = 525 x + 83	0,9999	0,1
n-dodecane	y = 407 x + 180	0,9987	0,1
methyl decanoate	y = 393 x - 53	0,9984	0,1
n-tetradecane	y = 536 x - 17	0,9988	0,1
n-pentadecane	y = 468 x + 258	0,9996	0,1

* these peaks were obscured by contaminants

Table 6.17 Equations of lines of best, least-squares fit to the relationship between peak area and specimen concentration for dynamic solvent effect sampling of specimens in a solvent matrix. Concentrator Q3.

Compound	Equation of line	r^2	P%
n-octane	y = 240 x + 1707	0,9986	0,1
n-nonane	y = 350 x + 1707	0,9985	0,1
dimethylheptanone	y = 372 x + 1251	0,9991	0,1
n-decane	*----------	------	---
p-cresol	*----------	------	---
linalool	y = 422 x + 636	0,9996	0,1
dimethylaniline	y = 569 x - 193	0,9996	0,1
n-dodecane	y = 444 x + 677	0,9996	0,1
methyl decanoate	y = 421 x + 486	0,9997	0,1
n-tetradecane	y = 562 x + 639	0,9997	0,1
n-pentadecane	y = 483 x + 690	0,9997	0,1

*these peaks were obscured by contaminants

Table 6.18 Equations of lines of best, least-squares fit to the relationship between peak area and specimen concentration for dynamic solvent effect sampling of specimens in a solvent matrix. Concentrator Q4.

Compound	Equation of line	r^2	P%
n-octane	*----------	------	---
n-nonane	y = 272 x + 1312	0,9990	0,1
dimethylheptanone	y = 290 x + 1016	0,9995	0,1
n-decane	*----------	------	---
p-cresol	y = 538 x + 613	0,9993	0,1
linalool	y = 337 x + 420	0,9998	0,1
dimethylaniline	y = 475 x + 652	0,9999	0,1
n-dodecane	y = 389 x + 465	0,9998	0,1
methyl decanoate	y = 378 x + 187	0,9997	0,1
n-tetradecane	y = 523 x + 49	0,9997	0,1
n-pentadecane	y = 459 x + 309	0,9997	0,1

* these peaks were obscured by contaminants

DISCUSSION

The external standard method of quantitation involves dividing the area of an experimental peak by the area of a peak obtained from a known quantity of the same compound (Lee *et al.* 1984 p 224). Therefore, the coefficient of variation of the calculated mass will be the same as that for the peak area (Tables 6.3, 6.8, 6.10). Internal standardization involves comparison of the areas of two peaks on the same chromatogram (Lee *et al.* 1984 pp 222-223), in this case the coefficients of variation of peak area ratios (Tables 6.6, 6.9, 6.11) provide a direct measure of the highest available precision. In practice the precision of this technique will probably be limited by errors in the addition of the internal standard rather than by analytical variation.

Peak area provides a measure of the mass of a component sampled, and thereby a measure of its concentration in the specimen. Peak percentage area provides a measure of the components' contribution to the total volatiles. Neither of these provide any more than ordinal data on the contribution of a particular component to a mixture's odour, or the magnitude of its biological effects. Even assuming that gas phase volatiles have been sampled, thereby excluding the effects of different volatilities and partition coefficients, there is no straightforward relationship between the composition of a mixture of volatiles and the intensity or quality of its odour or the magnitude of its other biological effects (Engen 1970; Amoore 1982; Albone 1984 pp 34-39).

The importance of precise analytical quantitation is that real biological differences should not be obscured by analytical noise, and that spurious differences should not be due to analytical artifacts.

If the widely accepted view that mammalian chemical communication is based on changes in relative concentrations of the components of complex mixtures (Beauchamp *et al.* 1976; Gorman 1980; Albone 1984 pp 6-7) is correct then high precision of peak area ratios, even between compounds of contrasting chemical character (see e.g. the decane:*p*-cresol, dimethylaniline:dodecane and alkane:linalool ratios in Table 6.6) may well be the critical factor in an analytical approach to mammalian semiochemistry.

In the case of solvent-matrix specimens the analytical precision was influenced by the sampling procedures applied. Four factors were expected to exert an important influence on performance:-

- errors in measuring specimen volume,
- adsorption of solutes onto the Microcaps used for measurement,
- adsorption of solutes onto the solvent holders,
- errors in judging when the specimens had been concentrated to dryness.

These four factors were eliminated in the case of sampling from a bulk specimen (procedure 3), although a fifth factor; variations in evaporation rate due to temperature fluctuations was then expected to operate.

Errors in volume measurement probably affected procedures 1 and 2 approximately equally since both involved three Microcap measurements (3 x 100 µl or 2 x 100 µl + 1 x 20 µl).

Errors in judging the end point of concentration to dryness would be expected to have affected procedure 2, with its smaller total volume, more than procedure 1. Like errors in volume measurement, end point errors would be expected to have equal affects on all the test components.

Adsorption effects would have been expected to be stronger in procedure 2, with its smaller quantity of each test component, and for the polar components of the Test Mix (*p*-cresol, dimethylaniline and linalool).

Comparison among the results from methods 1,2 and 3 shows clearly that adsorption had a much stronger effect on performance than did volume errors, that volume errors due to end point judgement were more serious than those due to Microcap measurement and that both of these had stronger effects than variations in evaporation rate.

Once variable adsorption onto containers had been eliminated by sampling from a bulk specimen the coefficients of variation of peak areas were independent of the nature of the test component. The concentrators must, therefore, have been effectively free of adsorptive activity.

Adsorption on containers was also a major cause of variability in sampling from aqueous solutions. When the headspace of an aqueous solution of aldehydes and ketones was sampled a very marked improvement in precision was achieved by simply rinsing the pipette and micro-bubbler with specimen solution. This points clearly to an important, possibly dominant, role for adsorption on glassware in limiting sampling system performance. Adsorption is also indicated by the tendency of precision to be better for lower molecular weight compounds and for compounds present in higher concentrations. To what extent the variability seen when the glassware had been rinsed was due to residual adsorptive activity is uncertain. That adsorption had not been completely eliminated by rinsing is suggested by comparison with the good performance achieved with airborne hydrocarbon specimens generated in a continuous-flow system, where the effects of adsorption were eliminated. The adsorptive activity of containers can be neutralized by rinsing until the surface is in equilibrium with the specimen solution but then, during sampling, adsorbed material will bleed back into the specimen when its concentration falls as volatiles are purged from it.

Some concentrators performed better than others; Q2 was the best of the five involved in testing. What characteristics of a concentrator influence its quantitative performance are, at present, unknown. Small differences in the distribution of the glass chips making up the porous bed may well influence the flow, and retention characteristics of the solvent film held in it (see also p 146). Ageing may also have affected concentrator performance; concentrators H5 and H7 had already been heavily used for sampling brown hyaena pastings while Q2, Q3 and Q4 were new at the start of the quantitative investigation.

Although it is very convenient, the use of petroleum jelly as a source of airborne hydrocarbons suffers the disadvantage that the true concentration of each component in the vapour phase is unknown. Generation of accurately known concentrations of gas phase volatiles in the ppb range presents formidable problems. Of the techniques available the use of diffusion/permeation tubes appears to be the most

accurate (Barrat 1981). However even at 50 ppm the calibration, by weight loss, of such a device is "tedious and time consuming" (Namiesnik, Torres, Kozlowski & Matheiu 1981). At ppb levels it would be practically impossible -- an emission rate yielding 1 ng in a 100 cm^3 specimen sampled at 10 cm^3 min^{-1} would give a weight loss of 1 mg over a period of 19 years. Serial dilution of a more concentrated vapour provides a solution to the weighing problem, but the accuracy with which the final concentration is known will be limited by the cumulative inaccuracies of gas flow measurement and regulation at each dilution step (Bertoni *et al.* 1981). Crisp (1980) suggests that diffusion standards should be calibrated analytically; a procedure which is neatly circular. Lieber & Berk (1984) generated ppb "gas phase" standards by injecting a calculated volume of liquid onto adsorbent traps, but still obtained accuracies of no better than 6 % above calculated levels.

Thus it appears that measurement of the accuracy of dynamic solvent effect sampling of airborne volatiles will have to await the development of more accurate methods of generating such specimens.

Examination of chromatograms from the various test specimens strongly suggests that a major limiting factor in the system's performance was the quality of the column. This was unquestionably the case for the *n*-decanol component of the Test Mix, which suffered such serious peak distortion that it had to be excluded from the data analysis (Fig. 6.2). In the analyses of airborne hydrocarbons, and the testing of detection limits, the settings of the electrometer and integrator were dictated by baseline drift due to erratic column bleed, which in some cases at least was associated with anomalous peak areas (Figs 6.3, 6.5)

The composition of the liquid specimens in an aqueous matrix did not conform to the requirement of chemical diversity among the test components (see Introduction). A more satisfactory situation from this point of view would have been the sampling of an aqueous Test Mix. In preliminary testing, however, serious practical difficulties came to light. The hydrocarbon constituents of the mix have such low partition coefficients between air and water that they were lost from aqueous solutions during storage and manipulation (Hammers & Bosman 1986), while the polar components have such high partition ratios that they could not be recovered by sparging from dilute solutions. In more concentrated (ppm) solutions the ionizable *p*-cresol and dimethylaniline appeared to interact. Aldehydes and ketones provided a workable compromise between these extremes, medium- and long-chain alcohols might also have been suitable but their peak shapes would have been degraded by the column.

It is apparent from Tables 6.13, 6.14 and 6.15 that the use of a single concentrator for repeated sampling provides substantially better precision than if three are used. Only in the case of concentrators Q2, H5 and H7, used for sampling airborne hydrocarbons did the coefficients of variation of peak area remain below the 10 % target level for all components of the test when the results from three concentrators were pooled.

It is not known whether the differences between specimen types in inter-concentrator variability were due to the specimen or to different sets of concentrators having had to be used for each specimen type. In the second case it should be possible to select sets of matched concentrators for applications demanding very high precision but where the serial use of a single concentrator is impossible (see Chap 10 for an example).

In the case of the solvent matrix specimen the pooled coefficient of variation was, like the coefficients of variation within the five samples on each concentrator, independent of the nature of the test component; indicating that the concentrators were uniformly free of adsorptive activity,

The only applied work in which any quantitative aspect of sample composition could be expected to remain constant was that on the brown hyaena scent pasting (Chap 10). In that case the coefficients of variation in peak area ratios for a group of unidentified peaks, sampled on seven different randomly chosen concentrators, varied between 3,3 and 8,0 %, despite an increase in sampling time from 4 min to 1 h, and 300 fold decrease in the emission rates of the volatiles as the pasting aged.

With the available data it is not possible to calculate a definite limit for the minimum detectable concentration. Clearly the solvent contained some background level of nonane, decane and dodecane (or compounds which co-eluted with them) so that the calculated concentrations are lower than the real ones by at least this background level. The slope of the peak area *vs* concentration line for the dodecane peak (the only one which was approximately linear) gives a peak area increment of 10 as equivalent to a concentration increment of 1 ppt. Extrapolation of peak area calibrations at the 1-5 ng level gives an area increment of 10 as equivalent to a concentration difference of 2,5 ppt. On this basis the background level was approximately 10-25 ppt for dodecane and approximately 35-90 ppt for octane and nonane, and these levels then represent the minimum detectable concentrations. It is possible, however, that each addition of stock solution to, and each sampling from, the ppt specimen was accompanied by its contamination with hydrocarbon vapours from the laboratory air. For the actual addition to the specimen to

be double what was calculated at each stage would require an airborne concentration of only approximately $0{,}5{:}10^{9}$ of each contaminant. In such a case the area *vs* concentration line would be less steep, and the minimum detectable concentration lower, but the practical impossibility of handling such dilute solutions in a normal laboratory would be simultaneously established.

The linearity of peak area *vs* specimen concentration for dynamic solvent effect sampling effectively matches that of other sampling systems and should prove adequate for all practical work. In fact, given the strongly non-linear relationship between odourant concentration and perceived intensity, and the probably irregular relationship between odour quality and odourant composition (Engen 1970; Amoore 1982; Shirley 1984 pp 268-275) rigorous analytical linearity may well be one of the less important aspects of work on semiochemicals and flavours.

Table 6.19 is a compilation of reports of the precision of sampling techniques for gas chromatography. It is based on a systematic survey, from indexed titles, of the main chromatographic literature (Journal of High Resolution Chromatography and Chromatography Communications, Journal of Chromatography, Chromatographia, Journal of Chromatographic Science, Riva Symposia, Advances in Chromatography) and a less systematic coverage of the applied literature. Only those reports which included all the information necessary for a comparative assessment of quantitative performance have been included.

Table 6.19 Quantitative performance of a variety of sampling methods for gas chromatographic analysis. Techniques are abbreviated as follows: ads: adsorption; ct: cold-trapping; d: derivatization; di: distillation e: extraction; ECD: electron capture detection; FID: flame ionization detection; hs: headspace; HPLC: high performance liquid chromatography; MS: mass spectral detection; Nafion: water subtraction by porous polymer; NPD: nitrogen-phosphorus detection; oc: on-column injection; p: purging; ptv: programmed temperature vapourizer; SIM: specific-ion monitoring; sl: splitless injection; sp: splitting; TEA: thermal energy analyser detection; TIM: total ion monitoring; vi: valve inlet.

	Matrix	Solutes	Technique	Concentration or mass	%CV	Reference
1	Solvent	Alkanes	ptv FID	10%	0,2-14	Loyola *et al.* 1987
2	Solvent	C_{18}-C_{32} alkanes	ptv FID	250ng:20µl	0,9-5,2	Vogt & Jacob 1985
		Cl-compounds	" ECD	1ng:1µl	2,4-7,0	
		P-pesticides	" NPD	1ng:5µl	1,0-2,1	
3	solvent	mixed	oc sl FID	10-40ng:1µl	0,5-4,2	Snell *et al.* 1987
4	solvent/ gas	halocarbons	ads* ct TIM	200ng:1µl	3-44,4	Pankow 1983
5	solvent	Varian mix	vi oc FID	400ng:1µl	0,05-1,9	Steele & Vassilaros 1983
6	solvent	C_8-C_{10} alkanes	vi ct sp FID	500ng:0,5µl	3-6	Jacobsson & Berg 1982

7	solvent	C_{10}-C_{28} alkanes	ptv FID	240ng:0,2µl	0,7-5,7	Poy *et al.* 1982
8	solvent	C_{10}-C_{18} alkanes	oc FID	44ng:0,4µl	0,5-1,2	Schomburg *et al.* 1982
9	solvent	alkanes	sp FID	160-1600ng	1,7-33,1	Reglero *et al.* 1986
	"	"	sp ptv FID	"	0,9-6,23	"
10	solvent	Cl-pesticides	vi ECD	16 ppt	4,4	Zlatkis *et al.* 1984
	"	"	"	40 ppt	2,2-9,5	"
11	solvent	PCBs PBBs	ads ct TIM	75ng:100µl	10-19	Hart & Pankow 1987
12	solvent	C_{10}-C_{32} alkanes	oc/sl FID	0,002%	2-3,1	Schomburg *et al.* 1983
	"	"	sp FID	0,1%	1,5	"
13	solvent	PAH	ads ct FID	0,4-1µg:100 µl	1,2±0,6	Kirschmer & Oehme 1984
14	solvent	*n*-eicosane	oc FID	15ng:1µl	1,6-4.8	Ogden & McNair 1983
15	solvent	C_9-C_{36} alkanes	oc FID	3,6-10,3%	0,4-9,4	Steverink & Steunenberg 1983
16	solvent	phenols PAH	sp MS	30ng:1µl	13-41	Colby *et al.* 1983
17	solvent	C_{18}-C_{32} alkanes	sp/sl FID	250ng:20µl	0,8-5,2	Vogt *et al.* 1979

18	solvent	C_{10}-C_{32} alkanes	sl/oc FID	$2:10^5$,0,2-0,5 µl	0,1-1,5	Schomburg 1985
19	air	C_{14} C_{16}-ac	hs ads e sl FID	4,8±3,5ng:600cm^3	10-19	Du *et al.* 1987
20	air	solvents	vi FID	7-529ppm,28 µl	2,2-11,3	Radell & Rea 1983
21	air	hydrocarbons	ct FID	32-6680ppt,300cm^3	1,5-8,4	Schmidbauer & Oehme 1986
22	air	pesticides	ads NPD/ECD	0,5ng:5l	3,4-12,4	Lawrence 1987
	"	amphetamine	ads/d SIM	50ng	13,3	"
23	air	halocarbons	ads* e sp ECD	0,5-0,05ng	0,4-3,9	Leiber & Berk 1984
24	aqueous	wine flavour	p ct sp FID	400ppm	2-12	Etievant *et al.* 1986
	"	"	p e sp FID	"	4-14	"
	"	"	hs sp FID	"	1-35	"
25	water	hydrocarbons	hs ct sl FID	0,1-15 µg:50cm^3	8,6-27	Drozd *et al.* 1978
26	water	alcohols/ carbonyls	e ads ct FID	0,13ng:100 µl	4,5-9,8	Lee *et al.* 1978
27	water	pollutants	p ads* FID	640ng:40cm^3	1,4-18	Otson & Williams 1982

28	water	aromatics	p Nafion ct FID	27-100ng:5cm^3	0,4-2,6	Cochran 1987
	"	"	"	260-500ng:5cm^3	2-2,6	"
29	water	Cl-phenols	d e sl ECD	1-100ng:100cm^3	1-8,9	Abrahamson & Xie 1983
	"	"	"	0,1-0,5ng:100cm^3	10-20	"
30	water	halocarbons	hs sp ECD	0,5-3,0 µgl^{-1}	5,5-7,1	Kolb *et al.* 1983
31	water	wide range	ads e FID	100 µgl^{-1}	2-22	Giabbai *et al.* 1983
32	water	phenols	di/e sp FID	20 ppb	2,4-12	Rijks *et al.* 1983
33	water	pesticides	HPLC vi ECD	0,1ng:1cm^3	1,9-32	Noroozian *et al.* 1987
	"	"	"	2,5ng:1cm^3	1,1-48	"
34	water	range	p Nafion ct FID	100ng:8cm^3	1,3-26	Noij *et al.* 1987
35	water	non-polar	p ads* ct FID	100ngl^{-1}	3-250	Hammers & Bosman 1986
36	water	pyridines	di e SIM	1 µg:500cm^3	2-3,9	Tsukioka & Murakami 1987
37	water	halomethanes	p ads* FID	100-120ng:20cm^3	4-23	Cailleux *et al.* 1987
38	water	hydrocarbons	p ads* FID	0,15-15 µg:15cm^3	6,4-14,3	Belkin & Eposito 1986

39	urine/ plasma	drugs	e d spl SIM	2-500ng:1cm^3	2-5	Marunaka *et al.* 1987
40	water	pollutants	p ads SIM	200ng:5cm^3	0,8-31	Lopez-Avila *et al.* 1987
41	water	flavour test	p ads* ct FID	100 ng	1,7-6,8	Werkhoff & Bretschneider 1987
42	solid	aromatics	p ads* ct FID	800-935ng	0,8-2,8	Venema 1986
43	sediment	PCBs	e oc/sl ECD	250-375 µgl^{-1}	1,8-9	Onuska *et al.* 1983
44	meat	nitrosamines	di/e TEA	5-20ppb	3,3-25	Gavinelli *et al.* 1986

* the adsorbent used was Tenax

During the compilation of Table 6.19 it became apparent that quantitative precision is not an aspect of analytical performance which has received general attention (Werkhoff & Bretschneider 1987a). Indeed it was the exception, rather than the rule, that this type of data accompanied, or even followed, descriptions of sampling techniques. For example, the series of papers by the Grobs on their closed-loop stripping apparatus contains no figures for precision (Grob 1973; Grob & Grob 1974; Grob, Grob & Grob 1975; Grob & Zürcher 1976). Even among those papers where precision was reported its interpretation was confounded by a lack of information on the composition of the test specimens employed. The works by Schomburg, Husmann & Rittmann (1981), Haynes & Steimie (1987), Grob (1985 pp 206, 249) and Yang, Brown & Cram (1978) are examples of, otherwise detailed, reports of high precision results from which the quantitative compositions of the test specimens were omitted, and which are, as a consequence, of no value in the present discussion.

Although dynamic solvent effect sampling has been successfully applied to a variety of analytical problems e.g. air pollution and flavours, the following comparison of the precision of dynamic solvent effect sampling with that of the methods in Table 6.19 is restricted to a consideration of their suitability for work on mammalian semiochemicals and on other materials posing similar analytical problems.

Of the 44 investigations in Table 6.19, 41 achieved precision for equivalent specimens equal to or better than that obtained in the present case by dynamic solvent effect sampling.

Results from sampling solvent specimens are only indirectly applicable to semiochemicals, which will nearly always be sampled from aqueous or gaseous matrixes. Nevertheless they do provide a measure of performance for compounds which are difficult to incorporate in other types of specimen.

Among the investigations of sampling from solvent specimens only two (11, 16) report coefficients of variation consistently larger than those from the dynamic solvent effect (Table 6.3). However, among the other 15 studies, 11 employed only hydrocarbons, and one only halocarbons (with mass spectrometric detection), which are so chromatographically tractable that their sampling behaviour throws hardly any light on likely performances with more polar compounds. Two of the remaining studies involved pesticides or their analogues and in both cases specific detectors, which are unsuitable for semiochemicals, were employed.

Although Steele & Vassilaros (1983, No 5) used a test mix as challenging as that employed for the dynamic solvent effect they did so at such high concentrations; approximately 400 ng per component, that any effects of adsorbtive activity would have been effectively masked.

A better performance was reported by Snell, Danielson & Oxborrow (1987, No 3) who also used a challenging test mix and, with quantities of solute only ten times as large as those employed in the present study, achieved coefficients of variation almost equal to those from the dynamic solvent effect.

Of the five studies of airborne volatiles four were as precise as dynamic solvent effect sampling of airborne hydrocarbons (Table 6.8). Two of these four used specific detectors. Schmidbauer & Oehme (1986, No 21) cold-trapped light hydrocarbons and used a potassium carbonate drying tube which would remove fatty acids and phenols as well as water. Radell & Rea (1983, No 20) sampled difficult solvent vapours in a process monitoring application, but their method is restricted to small sample volumes, and therefore high concentrations, by the use of a valve inlet with no focussing step.

All 18 of the investigations of sampling from aqueous specimens report at least some figures for precision which are better than those obtained for the dynamic solvent effect (Table 6.10). Eleven of these (24, 25, 27, 28, 30, 34, 35, 37, 38, 40, 41) employed a purging or headspace step, which would be necessary if the techniques were to be used for quantitative semiochemistry. Two of these eleven (28, 34) included cold trapping, and a drying step which removed medium-polar solutes and hydrocarbons above *n*-decane (Noij *et al.* 1987). A further five used hydrocarbon and halocarbon test compounds which throw no useful light on the performance to be expected with less tractable substances.

Etievant, Maarse & Van den Berg (1986, No 24) tested five different sampling techniques, of which four could be used for semiochemistry, with synthetic wine flavour specimens. Unfortunately the concentration of their specimens ($400:10^6$) was four orders of magnitude higher than that of the aqueous solution of carbonyls used in the present study so that the suitability of any of their techniques for semiochemistry must remain in doubt.

Gas purging and trapping on Tenax were used for sampling an aqueous mixture of pollutants, including some ketones and an alcohol, by Otson & Williams (1982, No 27). Although their test mix covered a wide range of volatilities and included some difficult compounds each component was present in quantities too large to be considered useful for semiochemical analysis.

From 5 cm^3 specimens Lopez-Avila, Wood, Flanagan & Scott (1987, No 40) purged and trapped 250 ng of pollutant test compounds, including some ketones. The precision of their method was good, though rather variable, but its dependence on single ion, mass spectrometric monitoring is likely to restrict its applicability.

Werkhoff & Bretschneider (1987b, No 41) investigated a purge-adsorb-thermally desorb-cold trap system for flavour compounds in water. Their adsorbent was Tenax and cold trapping was at an elevated flow rate to overcome the incompatability between analytical and desorption flow rates. The high precisions they report, and the quality of the chromatograms they present, suggest that a similar system might be useful for some types of semiochemical analysis, providing that the specimen size and solute abundance can be reduced by two orders of magnitude.

Of the three studies on sampling from solids only Venema's (1986, No 42) involved a non-specific detector, and the quantities and type of solutes do not suggest that the method would be suitable for work on semiochemicals.

Lee, Nurok & Zlatkis (1978, No 26) are the only group in Table 6.19 to report coefficients of variation from a sampling system designed, like the dynamic solvent effect to handle small quantities of biological materials. Their transevaporator operates in two stages. First the lighter volatiles are purged from the specimen and collected on Tenax, then the less volatile, and more polar solutes are collected on glass beads by an extraction-readsorption process. It is a pity that precision is not reported for the Tenax mode since this would be the more useful for work on semiochemicals. The adsorption mode yielded excellent results with very small quantities of intractable solutes. Even to elute 0,13 ng of butanol as a recognisable peak requires, apart from anything else, an uncommonly well-deactivated column.

It is of considerable interest that in only eight of the 44 studies (2, 10, 14, 22, 23, 26, 29, 33) were the quantities of solute similar to, or smaller than, those used to test the dynamic solvent effect. In six of these cases (2, 10, 22, 23, 29, 33) an electron capture detector, which is both selective and approximately three orders of magnitude more sensitive than an FID, was used.

The work by Du, Löfstedt & Löfqvist (1987, No 19) on acetate moth pheromones closely approaches the performance of the dynamic solvent effect in terms of high precision with small quantities of test compounds. If their method of adsorption onto glass wool is adaptable to the wide range of compounds found in mammalian secretions it will probably prove to be more than adequately precise for work on mammalian chemical signals.

Lee *et al.* (1978, No 26) used their transevaporator in the glass bead mode to recover very small quantities (0,13 ng) of ketones and alcohols from aqueous specimens. In view of their use of very active silica adsorbents and a dynamically coated stainless steel capillary column this performance must be regarded as incredibly good.

CONCENTRATOR CAPACITY

No trap can continue to accumulate sample indefinitely. To discover whether the upper limit for capacity of dynamic solvent effect sampling lay within a range likely to have practical significance, and if it did where the upper limit was, the time over which specimens were sampled was varied.

METHODS

A $1:10^8$ solution of Test Mix in *n*-hexane, with linalool substituted for *n*-undecane, was sampled for different periods ranging from 2-80 minutes. The sampling gas was palladium cell purified hydrogen at a flow rate of 10 cm^3 min^{-1}, the temperature during sampling was 30-30,6°C. Chromatograms were run under standard conditions (p 110) and peak areas were plotted against sampling time.

An equivalent series of samples were run using ppb concentrations of airborne hydrocarbons from petroleum jelly.

RESULTS

For the liquid specimens the relationships of peak area to sampling time showed negative deviations from linearity whose strength increased with the volatility of the Test Mix component (Fig. 6.8). A maximum of accumulation for *n*-octane was reached after approximately 40 min, *n*-nonane, *n*-decane and *n*-dodecane continued to accumulate slowly after that time, and *n*-tetradecane and *n*-pentadecane continued to accumulate even at 80 min, the longest sampling period investigated.

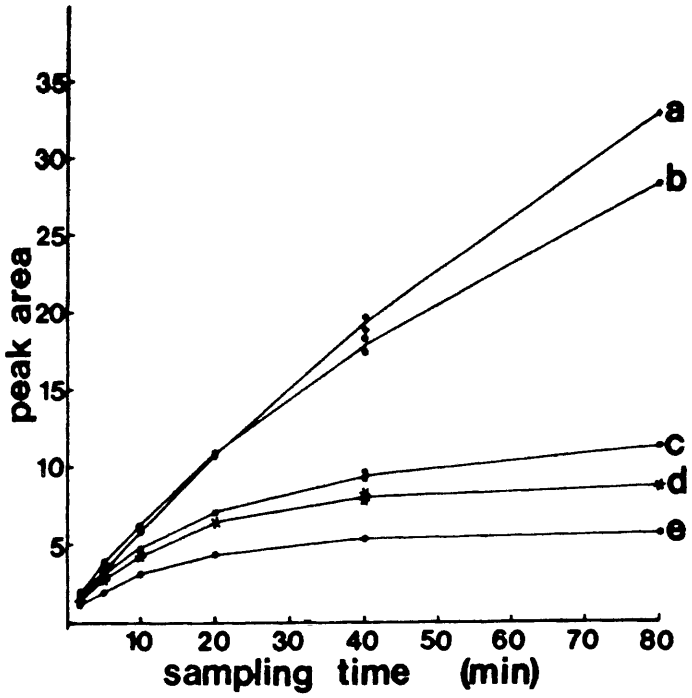


Fig 6.8 Relationship of peak area to time for which a $1:10^8$ solution of test mix in *n*-hexane was sampled by the dynamic solvent effect with a gas flow rate of 10 cm^3 min^{-1} at a temperature of 30-30,6°C;
a: *n*-pentadecane; b: *n*-tetradecane; c: *n*-decane; d: *n*-nonane;
e: *n*-octane.

In contrast when gas phase volatiles were sampled peak areas increased approximately linearly with time all the way up to the maximum 80 min sampling time (Fig. 6.9).

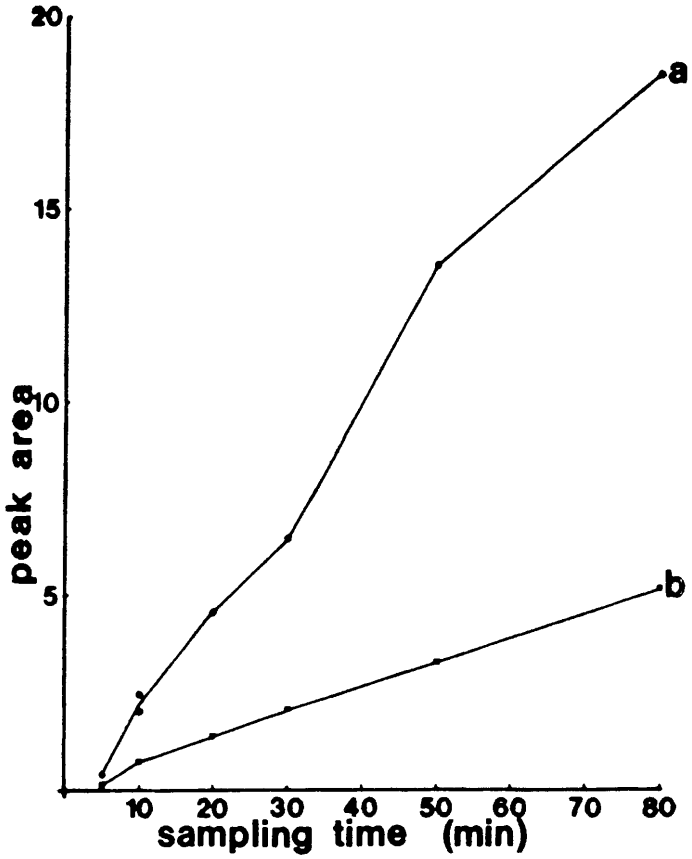


Fig. 6.9 Relationship of peak area to time for which ppb airborne hydrocarbons were sampled by the dynamic solvent effect using *n*-hexane as solvent with a flow rate of 15 cm^3 min^{-1} at a temperature of 30-30,6°C. a: *n*-pentadecane; b: *n*-decane.

DISCUSSION

The results for solvent-matrix specimens are consistent with there being a rate of loss of accumulated material which increases with the amount accumulated. Whether the loss rate is a fixed proportion of the accumulated amount is unknown. Plainly under these circumstances an equilibrium will sooner or later be established when the rate of accumulation and the rate of loss are equal. That the more volatile components of the Test Mix are more strongly affected supports the finding from the effect of sampling flow rate (p 154) that the main route of solute loss is via the gas phase.

For each solute there will be a maximum quantity which can be accumulated from a solution of a given concentration under a given set of conditions. So long as the loss rate depends only, or mainly, on the amount accumulated, the ratio of maximum quantity to concentration will be independent of specimen concentration. For *n*-octane in the present case:-

$$\text{max accumulated} = \text{max peak area/peak area ng}^{-1}$$
$$= 2{,}4 \text{ ng}$$

$$\text{specimen concentration} = 1{:}10^{8}$$

$$\text{accumulation ratio} = \text{max accumulated/specimen concentration}$$
$$= 2{,}43 \times 10^{-9}/10^{-8}$$
$$= 0{,}24$$

In other words a maximum of 0,24 ng of *n*-octane can be accumulated for every part per billion of solvent specimen concentration.

This, in turn, can be used to calculate a minimum detectable concentration (MDC) when the minimum detectable quantity (MDQ) of the chromatographic detector is known, e.g. for an FID:-

$$\text{MDQ} = 5 \times 10^{-12} \text{ g}$$

$$\text{Accumulation ratio} = 5 \times 10^{-12}/\text{MDC}$$

$$\text{MDC} = 5 \times 10^{-12} / 0{,}24$$
$$= 2{:}10^{12}$$

(see Detection Limit pp 125-126, 132).

It can be seen from Fig. 6.9 that different sampling times yield different values for the ratios of peak areas, although these differences are fairly small for adjacent members of an homologous series. Therefore it is important that, whenever possible, quantitative work is carried out under a fixed set of sampling conditions.

It appears that, when sampling gas phase volatiles, solutes of vapour pressure similar to *n*-decane will be quantitatively trapped even over long sampling periods. The longest sampling time applied in practice was one hour, solvent consumption and purity being the limiting factors (Chap 7). Over the usual 10-20 min sampling period accumulation will probably be quantitative for most compounds which can be satisfactorily resolved from the solvent in the separation step.

The marked difference between solvent and gas matrix specimens in this respect can be ascribed to two effects of the different routes by which the solutes in each phase reach the evaporation zone at the top of the concentrator bed. The major route for solute loss from a concentrator is via the gas leaving its bottom end (pp 33, 154). In the case of solvent matrix specimens the liquid at the bottom of the bed with which this gas is in equilibrium necessarily has the same concentration as the specimen. Thus even in the absence of any other mechanism there is a finite minimum rate of loss even of those solutes for which the conditions for solvent effect focussing (Chap 3) are met. If this were the only factor operating, however, the accumulation rate would be lower than otherwise, but still constant.

To account for losses which increase with time, and therefore with the amount accumulated, the following is proposed as a tentative model. Solutes in the gas phase are transported directly to the evaporation zone and their remaining there depends on the fulfillment of the conditions for solvent effect focussing (Chap 3) only in that area and immediately below it. Solutes in the liquid phase must travel the whole length of the concentrator bed to reach the zone of accumulation, and will experience different rates of upward migration at different points if the geometry of the bed and gas channel change. If this is the case there will be localized accumulations of solute just below any "bottlenecks" in the bed. These accumulations, being lower down the bed than the main body of solute in the evaporation zone are more susceptible to band spreading losses from the bottom of the concentrator.

To produce a precisely even bed of particles of exactly the same length and thickness in every concentrator was practically impossible, and this tentative model may account for the specimen-related differences in inter-bed quantitative variation (pp 123-124).

The effect on trapping efficiency of losses of sample has been most intensively investigated in connection with the use of adsorbents for sampling from gas phase specimens. Downstream losses of sample, termed breakthrough, have been considered to be significant at various proportions of the inlet concentration, or of the quantity trapped (Namiesnik *et al.* 1981), and experiments have been conducted with a range of different compounds, making comparisons difficult.

On Tenax, the most widely used of the adsorbents (Table 6.19), breakthrough volumes of hydrocarbons in the C_6 - C_{10} range vary from 59-1560 l per gram of adsorbent (Van der Straeten, Van Langenhove & Schamp 1985; Supelco 1986). Williams & Strauss (1977) present chromatograms showing an unmeasured fraction of breakthrough of ethyl decanoate after purging 30 l of gas through a 100 mg Chromosorb trap. The open-tubular traps tested by Grob & Habich (1985) showed

breakthrough of wine volatiles after passage of only 4 cm3 of headspace. With an improved design on the same principle Burger & Munro (1986) recorded breakthrough volumes of up to 500 cm^3 for *n*-octane.

Since, in the testing of dynamic solvent effect sampling of airborne hydrocarbons no recognisable sample losses occurred, the maximum volume of gas sampled, 800 cm^3 may be taken as a conservative minimum value for the breakthrough volume of *n*-decane. This compares closely with Burger & Munro's (1986) open-tubular traps. It is very much less than can be expected from traps containing hundreds of milligrams of Tenax but when very small Tenax traps are used breakthrough volumes of a few litres may be encountered. For example; Jemiolo *et al.* (1987) reported poor quantitation of early eluting constituents when volatiles from mouse urine were purged by 100 cm^3 min^{-1} of helium, for an unspecified time, onto 4 mg of Tenax.

LOSSES DURING DRYING

Many biological materials have a high water content. As a result, when volatiles are sampled from biological specimens, the water vapour content of the carrier gas approaches saturation. Although water does not accumulate chromatographically on films of non-polar solvent the cooling, caused by solvent evaporation (Pretorius, Apps, Rohwer & Lawson 1984), at the top of a dynamic solvent effect concentrator bed is sufficient to cause condensation of water droplets onto the walls of the concentrator. If this water is transferred to a capillary column it forms liquid lenses which travel slowly down the column, degrading chromatographic performance, damaging the stationary phase and often extinguishing the flame of the FID. It was standard practise when sampling wet biological specimens to remove condensed water by running a 10-15 cm^3 min^{-1} flow of palladium cell purified (therefore very dry) hydrogen through the concentrator for periods of 5-15 minutes.

Plainly, losses of accumulated material could be expected to occur during the drying step. The extent of such losses was investigated by simulating the drying of test mix samples.

METHODS

Concentrator Q2 (from the liquid specimen reproducibility study) was loaded with *n*-hexane, and 3 µl of a test mix containing 3-4 ng of *n*-octane, *n*-nonane, *n*-decane, *n*-tetradecane and *n*-pentadecane was injected into the tube above the bed by 5 µl microsyringe. The samples were run immediately, or after "drying" steps of various lengths using 6, 10 or 15 cm^3 min^{-1} flows of palladium cell purified hydrogen at a temperature of 30,0-30,6°C. The quantity of test solutes remaining after drying was measured by integration of peak areas and losses were calculated as percentages of the peak areas with no drying.

RESULTS

As predicted "drying" for any length of time led to reductions in peak areas. Losses increased with test component volatility, with drying time and, most markedly, with gas flow rate (Figs. 6.10 and 6.11).

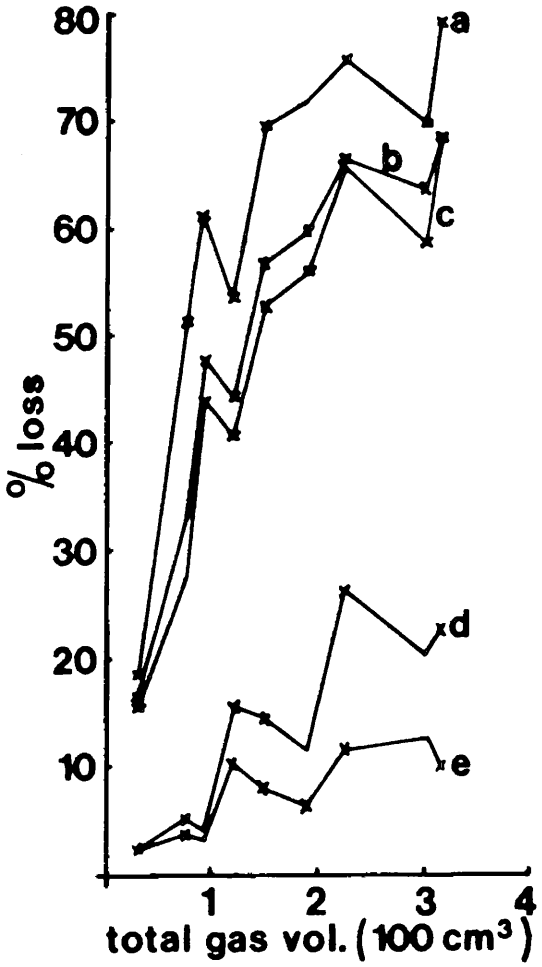


Fig. 6.10 Relationship of losses in solute peak area to time for which dynamic solvent effect samples were dried at a flow rate of 15 cm^3 min^{-1}. a: *n*-octane; b: *n*-nonane; c: *n*-decane; d: *n*-tetradecane; e: *n*-pentadecane.

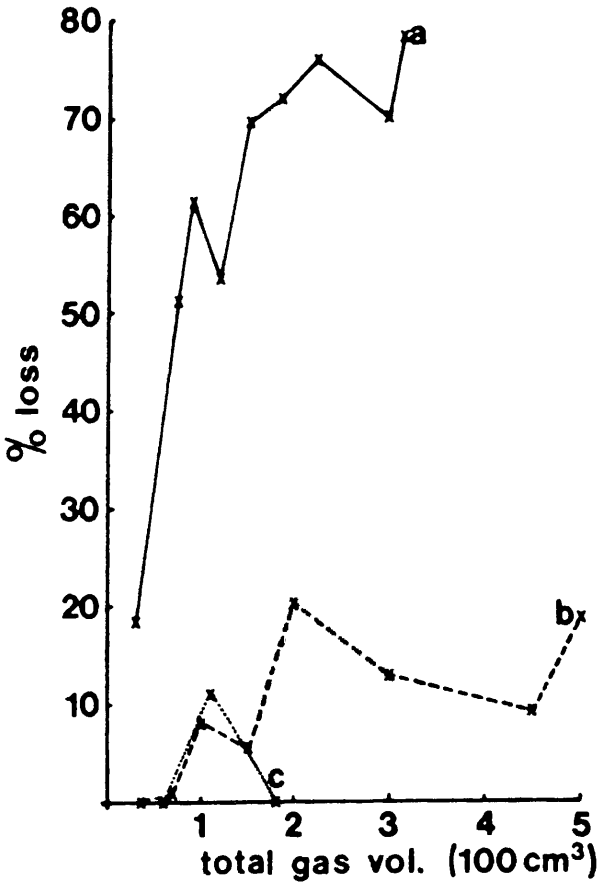


Fig. 6.11 Relationship of loss of *n*-octane peak area to time for which dynamic solvent effect samples were dried at flow rates of a: 15 cm^3 min^{-1}; b: 10 cm^3 min^{-1}; c: 6 cm^3 min^{-1}.

DISCUSSION

In contrast to the high reproducibility in normal dynamic solvent effect sampling, peak areas from replicate samples in this experiment showed erratic variability. This variability, which accounts for the deviations in the graphs of Figs 6.10 and 6.11, is ascribed to shortcomings in the syringe loading of the beds with test mix. Fortunately no important trends are obscured and the conclusions are unaffected.

Results presented earlier in this chapter show that sampling, and by analogy drying, at flow rates of 15 cm^3 min^{-1} generates extremely precise and accurate quantitative results. Therefore losses during drying, provided that a standard procedure is followed will affect only the sensitivity of the analysis, which in most cases (Chap 16) is not the factor limiting analytical success.

If quantitative comparisons are to be made between samples from specimens with different water contents, a standardized drying time, long enough to dry the wettest sample, should be applied to all of them so that losses are the same in each case.

If the volume of gas needed to remove a given quantity of water is independent of flow rate then the improvement in component retention due to slow drying flow rates is achieved with a cost in time - 10 minutes drying at 15 cm^3 min^{-1} becomes 15 minutes at 10 cm^3 min^{-1}. It is probable, however, that drying proceeds more effectively at low flow rates, due to reduced evaporative cooling (Pretorius, Apps, Rohwer & Lawson 1984) and improved equilibrium between water drops and gas, in which case the cost in time would be reduced. A drying flow of 10 cm^3 min^{-1} offers losses of less than 10 % with drying times normally between 10 and 15 minutes, drying flow rates of 15 cm^3 min^{-1} are costly in terms of sample loss and their employment in some of the work reported later was on the basis of rule of thumb optimization based on the rapid equilibrium assumption of static solvent effect theory (Chap 3).

The removal of water from wet specimens is an almost universal problem in gas chromatography with capillary columns. Without a drying step the use of cold traps is practically impossible - even if the trap does not block with ice the column suffers from the effects of condensed water droplets (Teranishi, Mon, Robinson, Cary & Pauling 1971, Cochran 1987). In evaluating a commercial head-space-cryofocussing system Kolb, Liebhart & Ettre (1986) denied that water condensation was a problem. However, the samples they analysed on WCOT, liquid stationary phase columns; chewing gum, cheese and tea leaves can hardly be considered challenging in this respect, and the only aqueous specimen examined contained only one solute and was analysed on a PLOT alumina/potassium chloride column.

Although water can successfully be removed by supplementary trapping the selectivity of these techniques is limited, so that other components tend to be lost from the sample along with the water. The use of Nafion tube leads to losses of hydrocarbons heavier than decane and of compounds of even moderate polarity such as ketones (Noij *et. al.* 1987). Condensing out the water between the specimen and a cold, or adsorbent, trap has also been reported to lead to losses of volatiles (Noij *et al.* 1987), although other workers state that this is not the case (Werkhoff & Bretschneider 1986a), a disparity which is resolved by Liebich & Al-Babbili's (1975) finding that the effects of a condenser depend on its temperature. Using a purge-condenser-adsorbent sampling system Raymer *et al.* (1985) successfully analysed a variety of polar, and high boiling compounds from wolf (*Canis lupus*) anal gland secretion, although the fatty acids which they detected by extraction were conspicuously absent from the head space samples.

The use of silica gel or anhydrous salts to remove water will certainly lead to the loss from the sample of polar volatiles, e.g. the potassium carbonate used by Schmidbauer & Oehme (1986) will remove all fatty acids and phenols.

If wet gas is passed into an adsorbent trap even hydrophobic materials such as Tenax can accumulate enough water to cause problems on capillary columns. Methods for removing this water have included centrifugation and vacuum dehydration (Pankow & Isabelle 1982) and, most commonly, purging with fast flows of dry gas (e.g. Williams & Strauss 1977; Lee *et al* 1978; Langlois, Mielle & Etievant 1984). The effects of purging on the recovery of volatiles appear not to have been investigated.

Belkin & Eposito (1986) prevented water accumulation on Tenax by keeping the traps warm and diluting the sampling gas flow 50:50 with dry helium. No losses of solutes resulted; recoveries were around 100%.

OPTIMUM GAS FLOW RATE FOR SAMPLING FROM LIQUID SPECIMENS

The sensitivity of solute loss to gas flow rate during simulated drying (above) suggested that a similar sensitivity might apply to sampling from specimens in a solvent matrix. In addition, since the rate of solvent evaporation depends on gas flow rate, there is the possibility of an optimum sampling rate, in terms of solute accumulation in unit time, being available. These aspects were investigated as follows.

METHODS

Specimens of linalool-substituted Test Mix at a concentration of $1:10^8$ in *n*-hexane were sampled at gas flows between 1,9 and 16,7 $cm^3\ min^{-1}$. The sampling time was adjusted such that at each flow rate a total of 100 cm^3 of gas was passed through the concentrator. For convenience in adjustment, charcoal-filtered nitrogen controlled by a metal-diaphragmed mass-flow controller was used in preference to palladium cell purified hydrogen. Chromatograms were run under standard conditions (p 110), and peak areas and accumulation rate were plotted against sampling gas flow rate.

RESULTS

Peak area showed a steady decrease with increasing gas flow rate, an effect which was stronger for the more volatile components of the Test Mix (Fig. 6.12).

For the *n*-pentadecane peak accumulation rate increased with flow rate throughout the tested range, but for the *n*-octane peak there was a maximum in accumulation rate at a flow rate of approximately 10 $cm^3\ min^{-1}$ (Fig. 6.13).

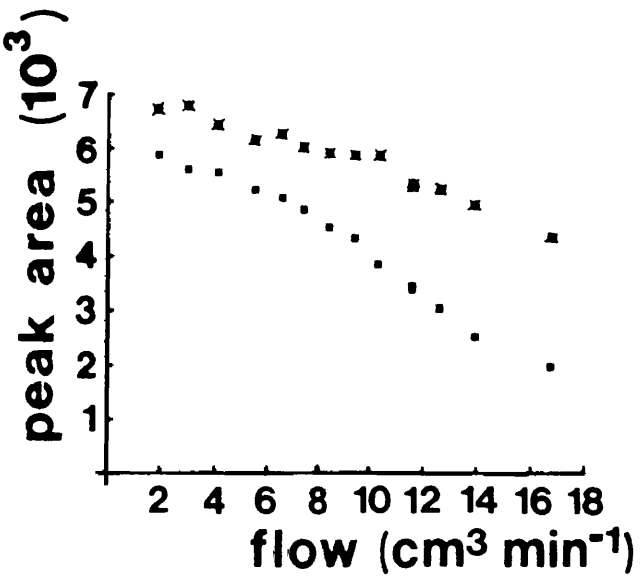


Fig. 6.12 Effect of gas flow rate, during dynamic solvent effect sampling from $1:10^8$ solvent matrix specimens, on solute peak areas; solid sqares: *n*-octane; crosses: *n*-pentadecane.

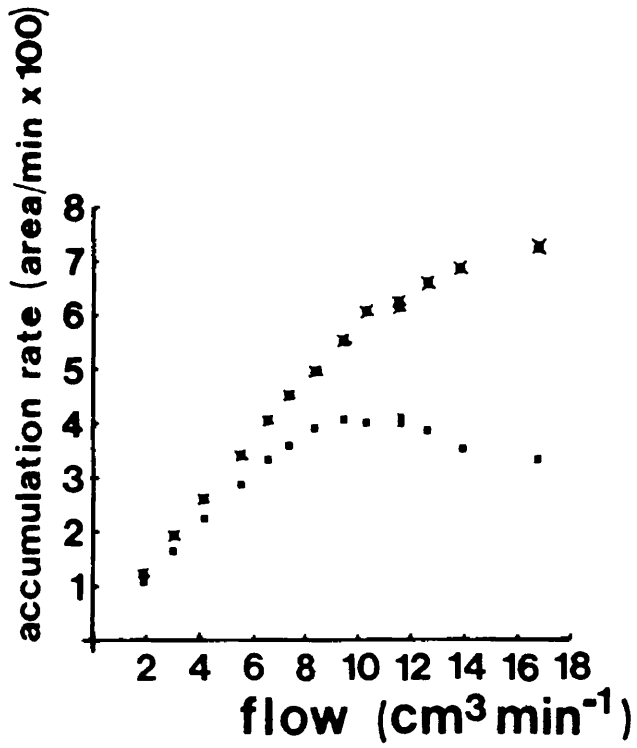


Fig. 6.13 Effect of gas flow rate, during dynamic solvent effect sampling from solvent matrix specimens, on rate of accumulation of solute; solid sqares: *n*-octane; crosses: *n*-pentadecane.

DISCUSSION

The greater sensitivity to changes in sampling flow of the peak areas of the more volatile Test Mix components, and the absence of a peak in *n*-pentadecane accumulation rate within the tested range of flow rates both indicate that, as expected, losses of material from the concentrator are due to transport in the gas, rather than the liquid, phase.

That the accumulation rate for *n*-octane peaked at $10cm^3\ min^{-1}$ is in agreement with the results from simulated drying (p 150).

The optimum flow rate for sampling from a given specimen will depend on its composition and the needs of the analysis. Sensitivity will be increased, at a cost in time, by sampling at low flow rates. Sensitivity in unit time will be maximized for volatile components at flow rates lower than for those less volatile. Examination of Fig. 6.13 suggests that if a wide range of components are of interest, as they usually will be in work on semiochemicals, flow rates of 10-15 cm^3 may be employed without serious costs in either time or inefficient accumulation.

INLET HEATING

An advantage of analysis by capillary chromatography is that it is a "gentle" technique. A compound may be carried through a column at a temperature well below its boiling point, in an inert atmosphere with exposure only to chemically unreactive surfaces and stationary phases. This advantage is lost if the transfer of the sample to the column is accompanied by high temperatures. A "cold" inlet, which can efficiently transfer samples to a column without heating, is the ideal in this respect. Such an inlet's operating temperature will be the same as that of the column during sample transfer.

METHODS

To investigate the contribution of heating a dynamic solvent effect inlet to the elution of sharp peaks from the column the temperature to which the inlet was heated was progressively lowered. In each case the peak shapes were compared with those produced under standard conditions. Each sample was 20 µl of $2:10^{7}$ linalool-substituted Test Mix loaded into the concentrator by "topping up" (Chap 7). The separation conditions were the standard ones.

RESULTS

The lowest temperature to which the inlet could be heated while every component of the Test Mix was still eluted from the column as a sharp peak was 120°C. At 100°C the *p*-cresol and *n*-decanol peaks showed slight losses of height which became more marked as the temperature was reduced further. Not until the temperature to which the inlet was heated was reduced to 65°C did the linalool and dimethylaniline peaks show signs of tailing. When the inlet was unheated the *p*-cresol, linalool, dimethylaniline, decanol, methyl decanoate, *n*-tetradecane and *n*-pentadecane peaks were tailed but the alkanes up to *n*-dodecane and dimethylheptanone continued to be eluted as sharp peaks (Fig. 6.14).

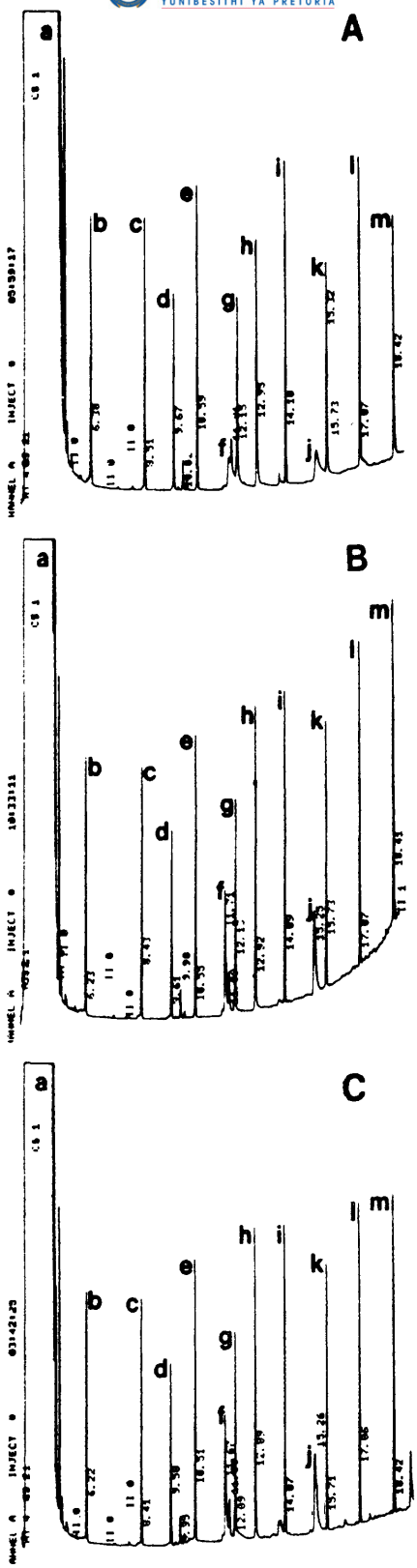


Fig. 6.14 Effects on peak shape of heating a dynamic solvent effect inlet to different maximum temperatures during the transfer of solutes to a capillary column; A: inlet unheated; B: inlet heated to 65°C; C: inlet heated to 220°C. a: *n*-hexane; b: *n*-octane; c: *n*-nonane; d: 2,6-dimethylheptan-4-one; e: *n*-decane; f: *p*-cresol; g: linalool; h: 2,6-dimethylaniline; i: *n*-dodecane; j: *n*-decanol; k: methyldecanoate; l: *n*-tetradecane; m: *n*-pentadecane.

DISCUSSION

While the dynamic solvent effect inlet cannot strictly be considered to be a "cold" one, the temperatures to which it needs to be heated in order to produce sharp peaks of even adsorptively active compounds are similar to those at which the compounds elute from capillary columns. Thus a compound which can be eluted from a column without suffering thermal degradation can be safely transferred to the column by a dynamic solvent effect inlet.

In their analysis of mouse urine volatiles Schwende *et al.* (1986) found a series of compounds which were interpreted as being the products of thermal degradation of hydroxyketones/lactols. Since they used Tenax as an adsorbent, and desorbed the trapped volatiles at 250^{o}C this interpretation seemed likely to be correct. Nevertheless, in the work on mouse semiochemicals reported in Chapter 12, the same series of compounds was found, despite the use of a dynamic solvent effect inlet, and their occurrence was independent of the rate at which the inlet was heated and its maximum temperature. One peak (No 2 Fig. 12.2) did, however, show distortion typical of degradation on the column which, on equivocal mass spectral evidence, was accompanied by the release of carbon dioxide and which was not, therefore, a dehydration reaction.

LOSS OF LOW-BOILING COMPONENTS

The selectivity of dynamic solvent effect sampling for solutes which satisfy the conditions for solvent effect focussing (Chap 3), and the presence in a dynamic solvent effect sample of approximately 20 µl of solvent, place a lower limit on the volatility/molecular weight of solutes which can be successfully analysed when a particular solvent is used.

For reasons of availability and convenience of handling at laboratory temperatures of up to 30°C (see Chap 7) *n*-hexane was used almost exclusively in the work reported here and in other applications of dynamic solvent effect sampling.

METHODS

No systematic investigations were undertaken. The lower limit of volatility/molecular weight of compounds which could be accumulated on, and chromatographically separated from, *n*-hexane when a methyl silicone column was used for analysis can be defined from various components of the mixtures of volatiles which were sampled during applications of the dynamic solvent effect.

RESULTS

n-Octane is accumulated and clearly resolved, *n*-heptane is not. Where between these two the cut-off point occurs is unknown.

Esters with a total of six carbon atoms e.g. ethyl butyrate are accumulated and well resolved.

Ketones and aldehydes with six carbon atoms are accumulated and resolved, those with five are not.

The lightest accumulated alcohol is hexanol.

All aromatic hydrocarbons except benzene itself are accumulated and resolved.

All phenols are accumulated and clearly resolved.

Dimethyl disulphide is accumulated and clearly resolved from the solvent peak.

Both butanoic and 2-methylpropanoic acid accumulate and resolve from the solvent but their peaks are, of course, badly front tailed.

In general compounds with retention indices on methyl silicones of above about 750 can be made to generate distinct peaks when accumulated on an *n*-hexane film.

What few samples were run with *n*-pentane and *n*-heptane as solvent suggest that one carbon unit can be subtracted or added respectively from the results for *n*-hexane.

DISCUSSION

With respect to losses of low-boiling solutes, sampling procedures which culminate in the injection of a solvent-matrix sample will perform approximately the same as the dynamic solvent effect. Solutes lighter than the solvent may, however be present and under favourable conditions (Miller & Jennings 1979) may elute as well-shaped peaks.

Solvent-free sampling systems i.e. those using thermal desorption from cold traps or adsorbents, can be expected to be more effective than is the dynamic solvent effect in dealing with lighter compounds. This expectation is borne out in practice; with appropriate procedures even methane can be trapped and analysed (Ellin *et al.* 1974).

The most widely used adsorbent for airborne volatiles is Tenax (Table 6.19). It has been used for 1,3-dichloropropene (Leiber & Berk 1984), chlorobromomethane (Adlard & Davenport 1983) and trichlorofluoromethane (Bayer & Black 1987) but Jonsson & Berg (1980) found it to be inadequately retentive of dichloro- and dibromoethane. Hexanol was the lightest alcohol, and hexyl acetate the lightest ester identified in Tenax-trapped volatiles from roses by Yao-zu, Zhao-lin & Hai-quan (1987). The lower limit of volatility for Tenax sampling has been described by the manufacturers of a competing product as "a linear C_6 backbone or equivalent structure" (Supelco 1986 p 6). Bertsch, Anderson & Holzer (1975) reported that substances lighter than benzene suffered heavy losses. Claesson & Silverstein (1977) report using Tenax to accumulate C_9-C_{11} ketones and Singer *et al.* (1976) successfully analysed for dimethyl disulphide. The earliest eluting compound (on a polar column) which Jemiolo *et al.* (1987) identified and quantitated using Tenax trapping of mouse volatiles was ethylcyclohexene. Albone's (1985 pp 176-178) compilation of urine volatiles includes acetone, propanal, ethanol, dimethyl disulphide and toluene as the lightest members of their classes which have been analysed by the use of Tenax.

Whether or not dynamic solvent effect sampling's selectivity against volatile compounds will present practical difficulties depends at the most basic level on whether those compounds are present in the specimen. In any particular case their presence can only be confirmed or denied by a suitable analysis, but some general trends can be predicted from the results of analyses which have aimed to identify "everything" in a mixture of volatiles (Table 6.20).

Table 6.20 Number of compounds, out of the total identified in each study, which would be detectable by dynamic solvent effect sampling with *n*-hexane as solvent and using a methyl silicone capillary column for separation.

Odour source	Compounds detectable	Reference
human whole-body	74/135	Ellin *et al.* 1974
bitch urine	8/15	Schultz *et al.* 1985
red deer tail gland	27/28	Bakke & Finkenschou 1983
Mephitis anal gland	19/25	Andersen *et al.* 1982
dog anal sac	6/12	Preti *et al.* 1976
human urine	59/83	Albone 1984 pp 176-178
rabbit *	58/71	Goodrich *et al.* 1981
red fox *	8/8	Jorgenson *et al.* 1978
mouse *	58/61	Schwende *et al.* 1986

* volatiles trapped on Tenax

Dynamic solvent effect sampling is almost certain to allow the loss of some components of "typical" mammalian odours. In this respect however it compares only slightly unfavourably with Tenax when applied to non-human volatiles (see also Chap 9).

The use of more volatile solvents will allow a more complete analysis of an odour mixture but may be impractical for reasons of solvent purity, high ambient temperatures or sample throughput being much slower if chromatograph ovens have to be cooled to below the boiling point of very volatile solvents.

If a chemical image differentiation strategy (Chap 1) is adopted the total loss of even a large number of an odour's most volatile components may very well be irrelevant. As long as the compounds which differentiate between two chemical signals appear on the chromatogram the absence of other compounds which are common to both, or vary at random, may actually simplify the analysis.

Chapter 7

ANCILLARY TECHNIQUES

CLEANING GLASSWARE AND SAMPLING PLUMBING

Glassware exposed to ordinary room air became detectably contaminated within minutes. It could be cleaned by the standard method of soaking in chromic acid, rinsing with distilled water and oven drying. To prevent recontamination it was held at 250°C until use. A more convenient method was to rinse with distilled water and acetone, bake at 450°C to oxidise organic contaminants, and hold at that temperature until use. Whenever possible glassware was used immediately it had cooled to room temperature. Containers which had to remain cool for any time e.g. those used for collecting specimens in the field were capped while still warm and kept closed until use. For critical applications at high sensitivity, or when working in "dirty" air, blanks were run of the complete sampling system and procedure.

Polytetrafluoroethylene seals and connector sleeves were held at 250°C between uses. Calibrated glassware, e.g. pipettes and measuring cylinders, is damaged by heating to 450°C; it was cleaned with chromic acid and held at 250°C.

DEACTIVATION OF SAMPLING GLASSWARE

Reversible and irreversible adsorption of specimen components on the glass surfaces to which they are exposed during sampling is a potential cause of quantitative errors (Chap 6) or, at the trace level, complete loss of components.

Although high temperature persilylation treatments such as are used for column deactivation (Grob, Grob & Grob 1979a, b; Grob *et al.* 1982) are effective for containers, the high temperatures and pressures involved make them unsuitable for routine use. Only room temperature treatments with e.g. dimethyldichlorosilane can be applied on a routine basis. These treatments were found to be suitable only for sampling system components which were exposed only to the gas phase. Silicone contaminants appeared in chromatograms when silicone deactivated glassware was in contact with liquid specimens or with solvents. Presumably these silicones had been liberated from the glass surface.

Silicone deactivated glassware cannot be held at 250oC for more than a few days without the development of a cloudy film of silica though oxidation and hydrolysis of the silicone.

In the case of specimens in an aqueous matrix -- which make up the bulk of biological materials -- it can be expected that water will displace polar organic molecules from active sites on glass surfaces. Using standardized glassware of minimum volume for a given specimen size and keeping transfer lines as short as possible also help to reduce adsorptive losses. In some cases sampling glassware was specially designed to meet these requirements (Fig. 7.1).

Adsorption of solutes onto container surfaces was one of the main contibutors to variability in quantitative analyses (Chap 6). The deactivation of glassware is an area in which further investigation is required.

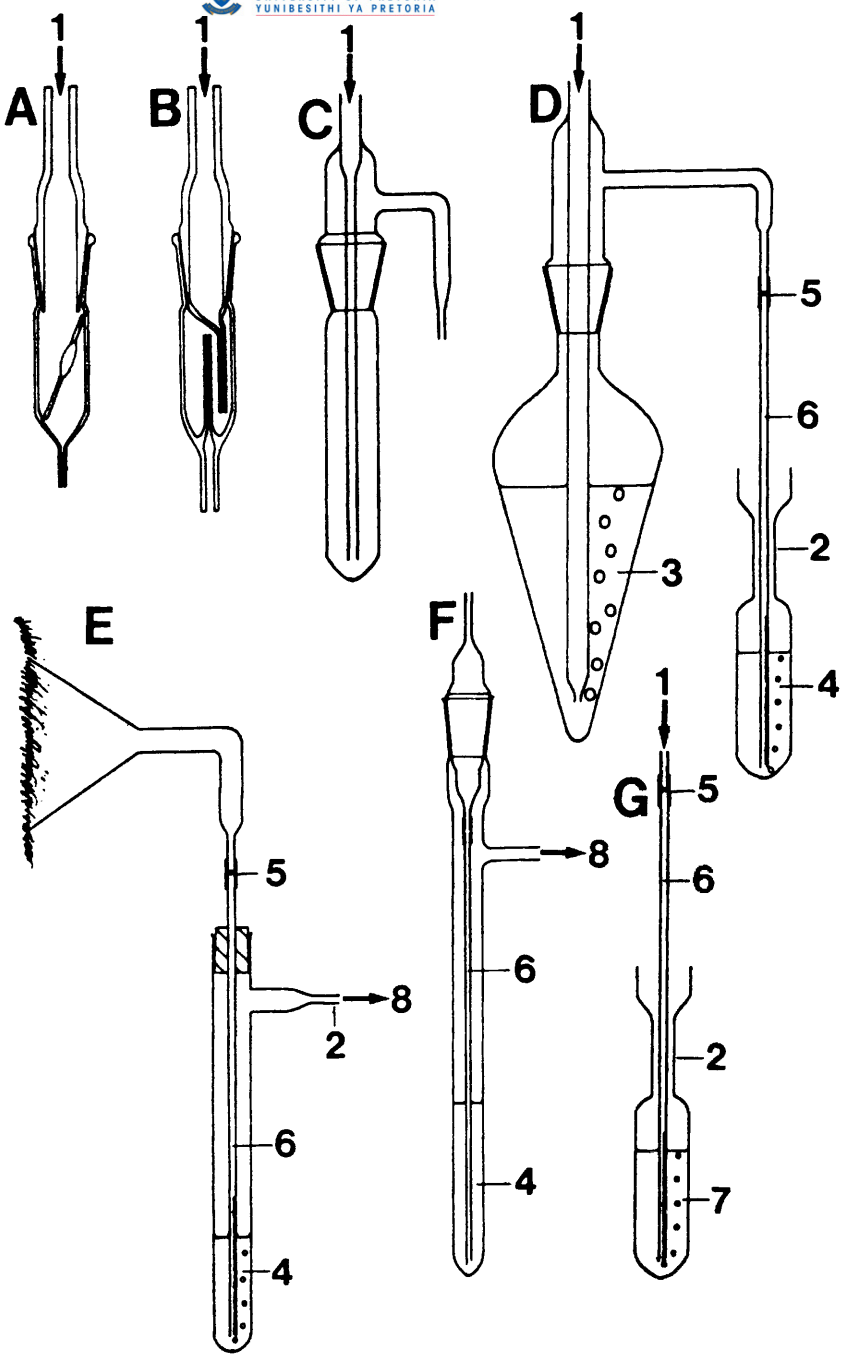


Fig. 7.1 Examples of "plumbing" used for dynamic solvent effect sampling from specimens of different types and volumes. A: for cohesive, solid samples e.g. scent marks on substrates or live insects, joint sizes 5/13 upwards; B: for friable solids and less than 1 cm^3 of liquids, joint sizes 5/13 - 10/19; C: for larger quantities of friable solids and 1-20 cm^3 of liquids, joint sizes 10/19 - 19/26; D: for friable solids and liquid volumes above 20 cm^3, joint size 10/19 upwards; E: for sampling from surfaces of e.g. large mammals, concentrator less well protected than in D; F: for vacuum sampling from ambient air with concentrator contained within, and protected by, the solvent holder tube, joint size 10/19; G: for specimens in a solvent matrix. 1: pure gas input; 2: constrictions to prevent back-diffusion of contaminants; 3: liquid-matrix specimen; 4: pure solvent; 5: polytetrafluoroethylene connector sleeve; 6: dynamic solvent effect concentrator; 7: solvent-matrix specimens; 8: mass-flow controlled vacuum.

STANDARDIZED CONCENTRATOR LOADING

The relationship between the time taken for the solvent to evaporate and the time at which a solvent effect inlet should be heated (Chap 4) demands that the volume of solvent introduced to the inlet varies only within rather narrow limits. The porous bed of a given concentrator would hold a fixed amount of solvent if allowed to fill itself to capacity, and the evaporation time of that amount of solvent could be used to fix the inlet heating time for that concentrator. However, the concentrator bed would contain less than its maximum capacity of sample after sampling under conditions in which the solvent did not rise to the top of the bed (Chap 4) or, especially with very volatile solvents, if transfer of the loaded concentrator from sampling site to chromatograph was not quickly carried out. If a partly-filled concentrator is introduced to the inlet the solvent's evaporation is completed earlier than expected and the heating of the inlet is correspondingly late.

Partly filled concentrators were "topped up" by touching the lower end of the porous bed to the top of a short piece of capillary tube (e.g. a 20 µl Microcap) whose lower end was immersed in a small quantity of pure solvent (Fig. 7.2). This allowed the porous bed to fill to capacity while avoiding the flooding of the gas channel with solvent which occurred if the concentrator was dipped directly into the solvent.

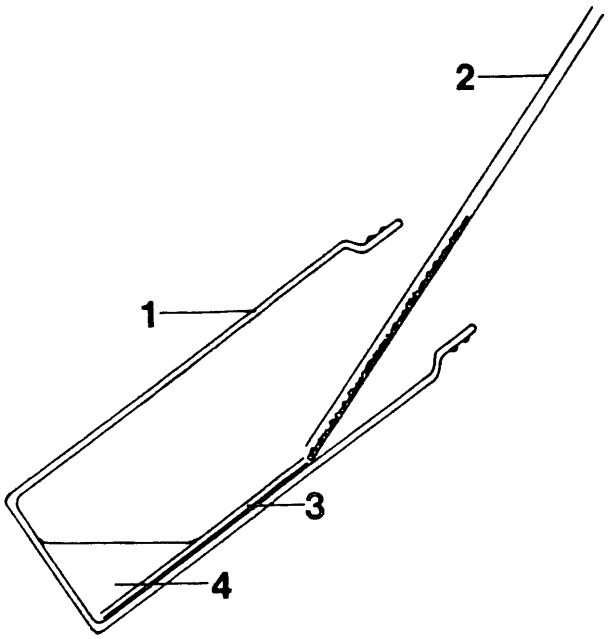


Fig. 7.2 Method of "topping up" dynamic solvent effect concentrators to ensure consistent sample volumes. 1: screw-top bottle; 2: dynamic solvent effect concentrator; 3: thin glass tube e.g. 10 µl Microcap; 4: 1-2 cm^3 of pure solvent.

Reduction of reagent volume

CARRIER GAS

Less carrier gas will be consumed for a given sample if the partial pressures of the solutes in the gas phase are raised. This can most straightforwardly be achieved by heating the specimen but, unless the concentrator is also heated, which will increase the volume of solvent consumed, the concentrator will then operate as a cold trap which, with most biological samples, will lead to the condensation of large amounts of water. This water must then be evaporated away - a process which consumes more gas.

Altering the properties of the specimen matrix by e.g. changing pH or salt concentration may increase the partial pressures of some of its solutes (Ioffe & Vitenberg 1983 pp 104-105). Although these manipulations are sometimes valuable in qualitative analyses they are unsuitable for the quantitative work for which the dynamic solvent effect is intended. In addition they involve the use of extra reagents which must, in their turn, be rigorously purified.

SOLVENT

Solvent consumption during sampling depends only on evaporation rate. A lowering of evaporation rate may be achieved by cooling the solvent, using a less volatile solvent or using a solvent with a smaller molar volume. For general practise all three of these can be rejected as solutions to the problem of reagent purity. Cooling the solvent has the same effect on the operation of the concentrator as warming the specimen, and the same drawbacks. In general only solutes less volatile than the solvent can be analysed with the use of the dynamic solvent effect for sampling. The use of a less volatile solvent results in the loss from the sample of a wider spectrum of non-focussed volatiles. Such losses may be tolerated only under special circumstances (see Discussion). A decrease in molar volume is accompanied by an increase in polarity (Przybytek 1980). Polar solutes accumulate unwanted water chromatographically and are incompatible with columns coated with non-polar stationary phases.

Solvent recycling

Under normal operating conditions the solvent evaporated at the upstream edge of the solvent film is carried away by the sampling gas and is lost to atmosphere. Re-cycling this solvent by recondensation provides a means of reducing this loss and thus limiting the volume of solvent consumed.

EXPERIMENTAL

In the prototype apparatus shown in Fig. 7.3 the effectiveness of solvent recycling in reducing solvent consumption was tested for *n*-pentane, dichloromethane, diethylether and *n*-hexane.

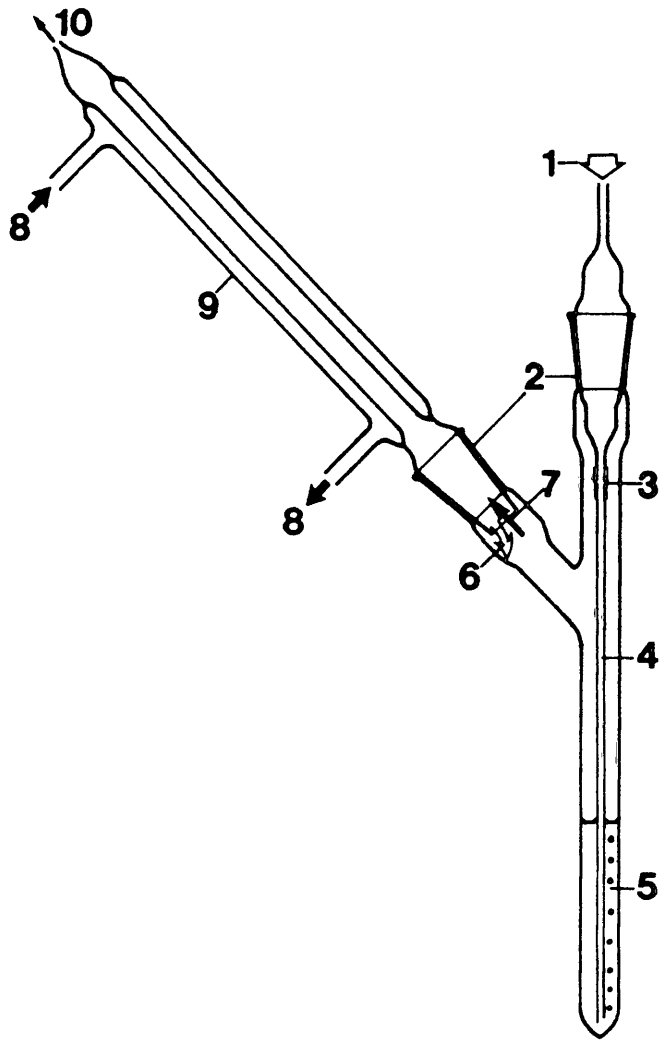


Fig. 7.3 Prototype apparatus used to recycle solvent by recondensation during sampling by the dynamic solvent effect. 1: gas-borne volatiles; 2: 10/19 ground glass joint; 3: polytetrafluoroethylene sleeve connector; 4: dynamic solvent effect concentrator; 5: pure solvent; 6: condensed solvent; 7: solvent vapour; 8: cooled air; 9: condenser; 10: constriction to prevent back-diffusion of contaminants.

Approximately 5 cm3 of the solvent was poured into the tube and with the concentrator in place, but no gas flowing, the height of the solvent surface was marked on the outside of the tube. A supply of 15 cm^3 min^{-1} of pure nitrogen was then connected to the concentrator and solvent was allowed to evaporate, without the condenser being cooled. At intervals of a few minutes the gas supply was briefly disconnected and the height of the solvent surface was marked. After at least 1 cm^3 of solvent had been consumed, and the tube topped up if necessary, the condenser was cooled by a flow of air which had passed through a liquid nitrogen cold trap and the consumption of solvent was recorded as before.

RESULTS

For all four solvents re-cycling markedly reduced the solvent consumption rate (Table 7.1).

Table 7.1 The effect of solvent recycling by recondensation on solvent consumption rate

	Consumption cm^3 $hour^{-1}$	
Solvent	Not recycled	Recycled
diethyl ether	5,44	0,32
dichloromethane	2,54	0,34
n-pentane	4,24	0,14
n-hexane	1,40	0,07

DISCUSSION

The results presented in Table 7.1 slightly overestimate the solvent consumption rates since about 0,2 cm^3 of solvent was necessary to wet the inside of the condenser and some loss of solvent was due to fluctuations in the condenser's temperature caused by an unreliable supply of compressed air.

Three main benefits accrue from the use of solvent recycling. With volatile solvents (e.g. pentane, diethyl ether and dichloromethane) and sampling times up to about an hour, solvent purity standards are relaxed, due to the smaller quantity of solvent consumed, to levels which can be reliably reached (see below). Secondly, sampling times may be extended without the need for solvents to be further purified. Thirdly, solvents such as hexane may be warmed during sampling to avoid, or decrease, the condensation of water from hot, wet specimens without a resultant increase in solvent consumption.

For specimens whose sampling consumed up to 1 cm^3 of solvent, and this is the great majority, solvent recycling is unnecessary. The more complex apparatus with its longer setting up time should be reserved for especially challenging sampling problems.

Reduction of Contaminant Concentration

CARRIER GAS

At a maximum acceptable level of any accumulated contaminant of 10^{-11}g and a standard sampling rate of 15 cm^3 min^{-1} the maximum acceptable concentration of any accumulated contaminant is:

$$10^{-11}/15.t \quad g.cm^{-3}$$

where t = sampling time in minutes.

For e.g. octane vapour this is a volume:volume concentration of $(1,3/t):10^{11}$, which is far below quoted purities for any commercially available compressed gas. Even if gas of sufficient purity was available in cylinders it could not be safely assumed that it would remain free of contamination during its passage through regulators and transfer lines.

SOLVENT

For a maximum acceptable level of any accumulated contaminant of 10^{-11} g the maximum acceptable concentration of that contaminant is $10^{-11}/v$ where v is the volume in cm^3 of solvent consumed. With flow rates of 15 cm^3 min^{-1} at sampling temperatures of 20-25°C consumptions were of the order of 5 cm^3 h^{-1} for *n*-pentane, 1,5 cm^3 h^{-1} for *n*-hexane and 0,1 cm^3 h^{-1} for *n*-heptane and *iso*-octane, requiring purities of 2, 7, and 100 : 10^{12} respectively for one hour sampling times.

EXPERIMENTAL

In addition to continual monitoring during developmental and analytical programmes the following experiments were performed.

The purity of cylinder air, high purity cylinder nitrogen, charcoal-filtered nitrogen, and hydrogen purified by passage through a specially constructed palladium cell (Fig. 7.4) were tested. In all cases the cylinder regulators had neoprene diaphragms and the transfer lines were of flamed-out stainless steel. The flow rates of all gasses except palladium-purified hydrogen were adjusted by metal-diaphragmed mass flow controllers, upstream of the charcoal trap if there was one. The flow of palladium purified hydrogen was controlled by the temperature of the palladium cell.

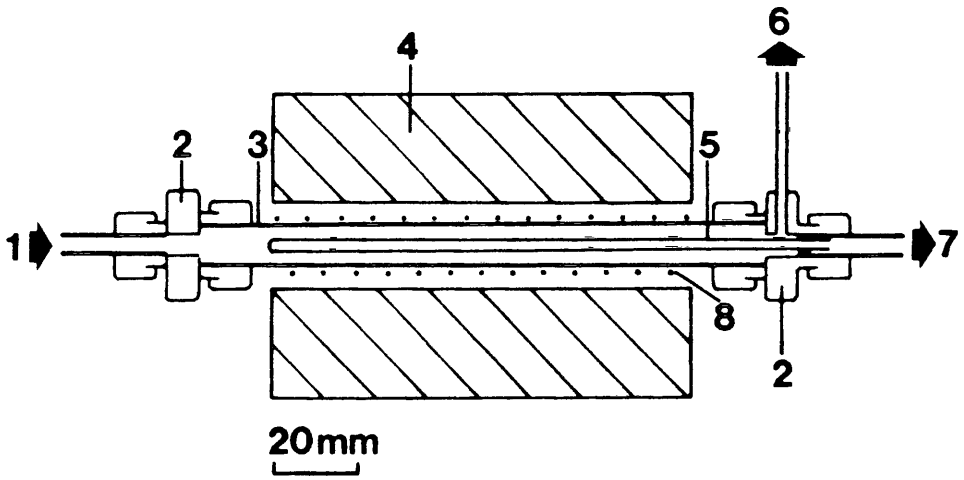


Fig. 7.4 Semi-diagrammatic section through a palladium cell hydrogen purifier. 1: commercial hydrogen input; 2: 1/8"- 1/4" stainless steel Swagelok; 3: 1/4" stainless steel tube; 4: insulation; 5: palladium-silver alloy tube; 6: purge outlet (2 cm^3 min^{-1}); 7: pure hydrogen outlet.

For one hour 30 cm^3 min^{-1} of each gas was blown through an empty concentrator tube in a liquid nitrogen cold trap. The concentrator tube was dropped into a dynamic solvent effect inlet which was immediately heated ballistically to 220°C, and a chromatogram was run on a 20 m methyl silicone column with a detector sensitivity of 1 x 10^{-11} A mv^{-1} fsd.

The following commercial solvents were tested by either "topping up" concentrators with 20 µl samples or sampling them as solvent specimens at approx 25°C with a gas flow rate of 15 cm^3 min^{-1} of palladium-purified hydrogen:

n-pentane (Merck, GR)
n-hexane (Merck, GR)
n-hexane (Burdick and Jackson, distilled in glass)
n-hexane (BDH, pesticide residue grade)
carbon disulphide (Merck, spectroscopic)
methanol (Merck, LichroSolve)
acetonitrile (Merck, LichroSolve)

n-Hexane was subjected to a number of experimental purification treatments.

Distillation was carried out through a Raschig ring column generating 4-5 plates.

Liquid-solid, frontal elution chromatography was carried out by running distilled hexane through a 250 mm x 10 mm column of W 200 neutral aluminium oxide (activity 1).

Frontal elution gas-solid chromatography, using either aluminium oxide W 200 or activated charcoal packings, was carried out on 1m x 17 mm columns. The carrier gas was charcoal-purified nitrogen which was saturated with hexane vapour at 40°C and passed through the columns at a flow rate of 250 cm^3 min^{-1} and a temperature of 180°C. Between runs the columns were backflushed at the same flow rate at 270°C. The eluting hexane vapour was condensed directly into storage containers at approximately -10°C.

To assess the effect of different storage conditions on the deterioration of solvent purity alumina-purified *n*-hexane was stored in flame-sealed, soda-lime glass vials, borosilicate glass vials with polytetrafluoroethylene-lined screw caps, silicon coated borosilicate glass tubes or aluminium bottles with aluminium foil-lined screw caps. Storage was at room temperature, in daylight or darkness and at -4°C in darkness.

Solvent purity was screened with 20 µl "topped up" (Chap 7) samples and batches which passed this test were run as solvent specimens with a 20 min concentration period at 20-25°C (room temperature) using palladium-purified hydrogen at 15 cm^3 min^{-1}.

RESULTS

Figure 7.5 shows chromatograms of impurities in the various gasses. Some background contamination was present. Neither bottled gas was pure but charcoal filtration removed contamination almost to the level achieved by palladium purification of hydrogen. Charcoal traps were, however, subject to unpredictable breakthrough of contaminants (Figure 7.6).

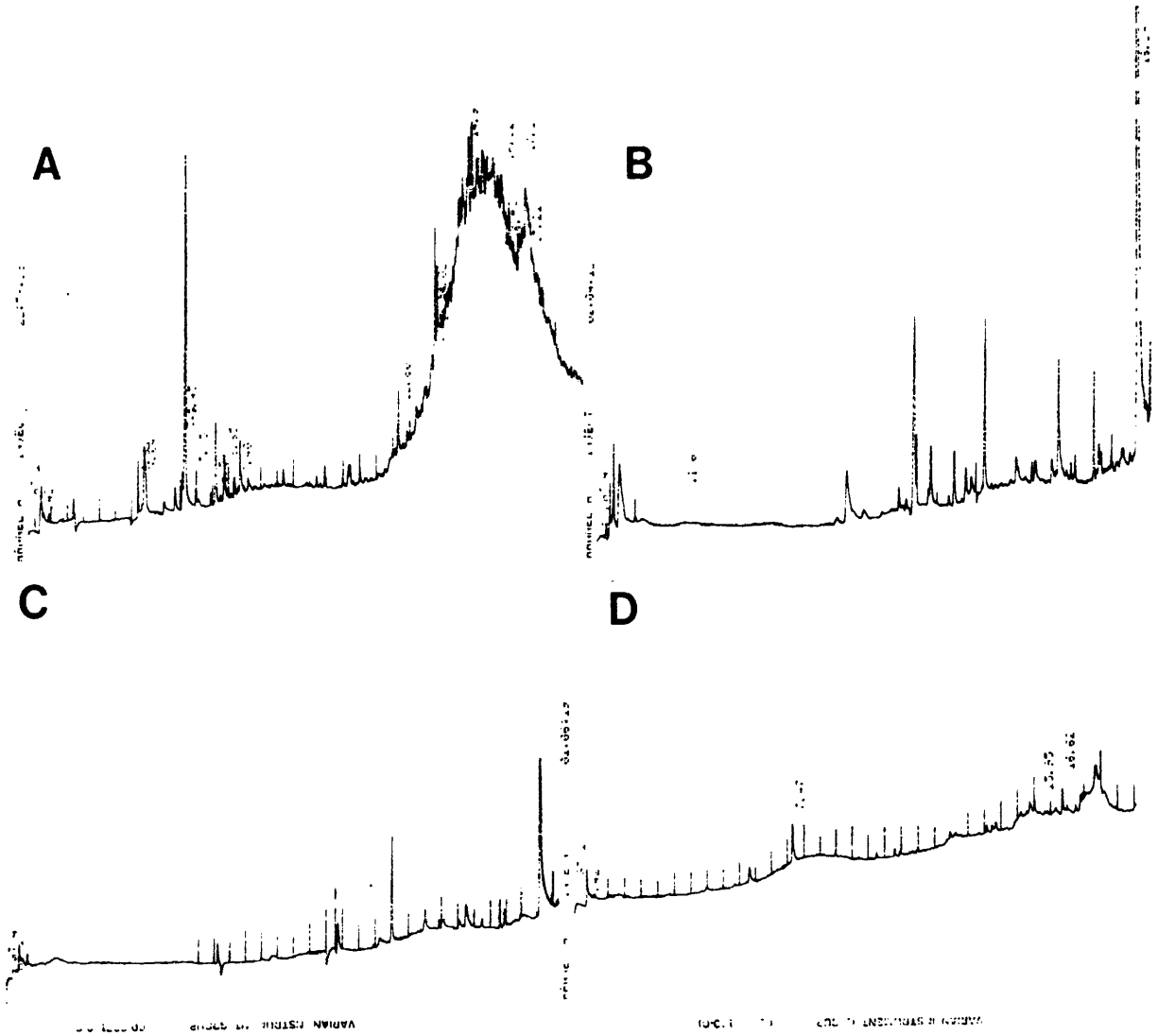


Fig. 7.5 Impurities cold-trapped from 1800 cm^3 of various gasses. A: industrial compressed air; B: high purity nitrogen; C: charcoal-filtered nitrogen; D: palladium cell purified hydrogen. For analytical conditions see text.

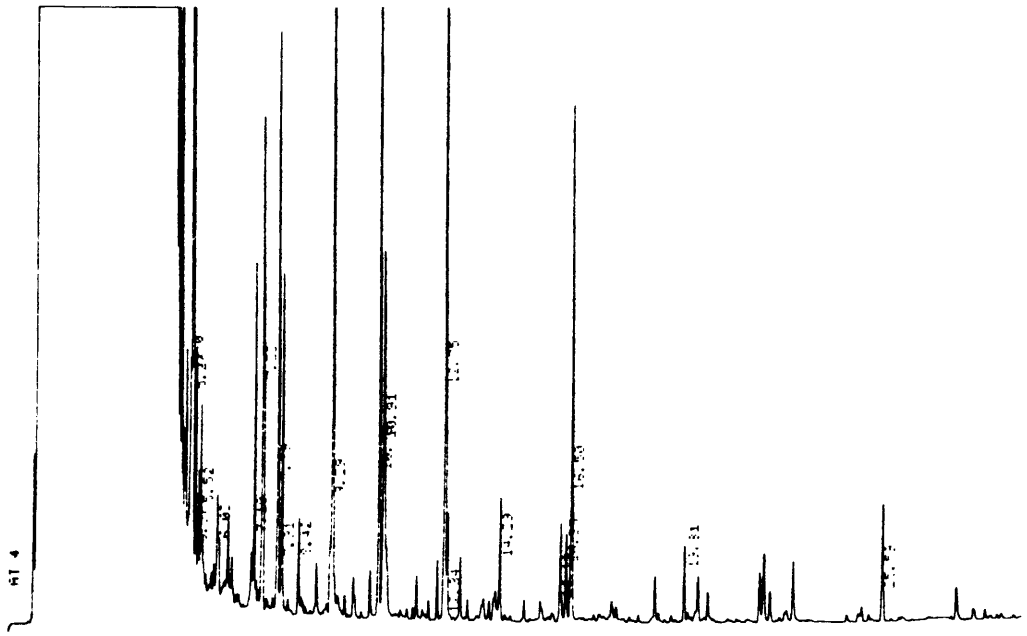


Fig. 7.6 Example of contamination from breakthrough on a charcoal trap. 15 cm^3 min^{-1} of high purity nitrogen sampled for 20 min with alumina-purified *n*-hexane as solvent. Separation on a 20 m x 0,3 mm x 0,4 µm methyl silicone column, initial temperature 40 °C for 6 min then programmed at 10°C min^{-1}. Sensitivity 10^{-11} x 4 A mv^{-1} full scale deflection. The peak at 16,5 min represents approximately 5 ng.

All commercial solvents revealed unacceptable levels of impurities (Figure 7.7).

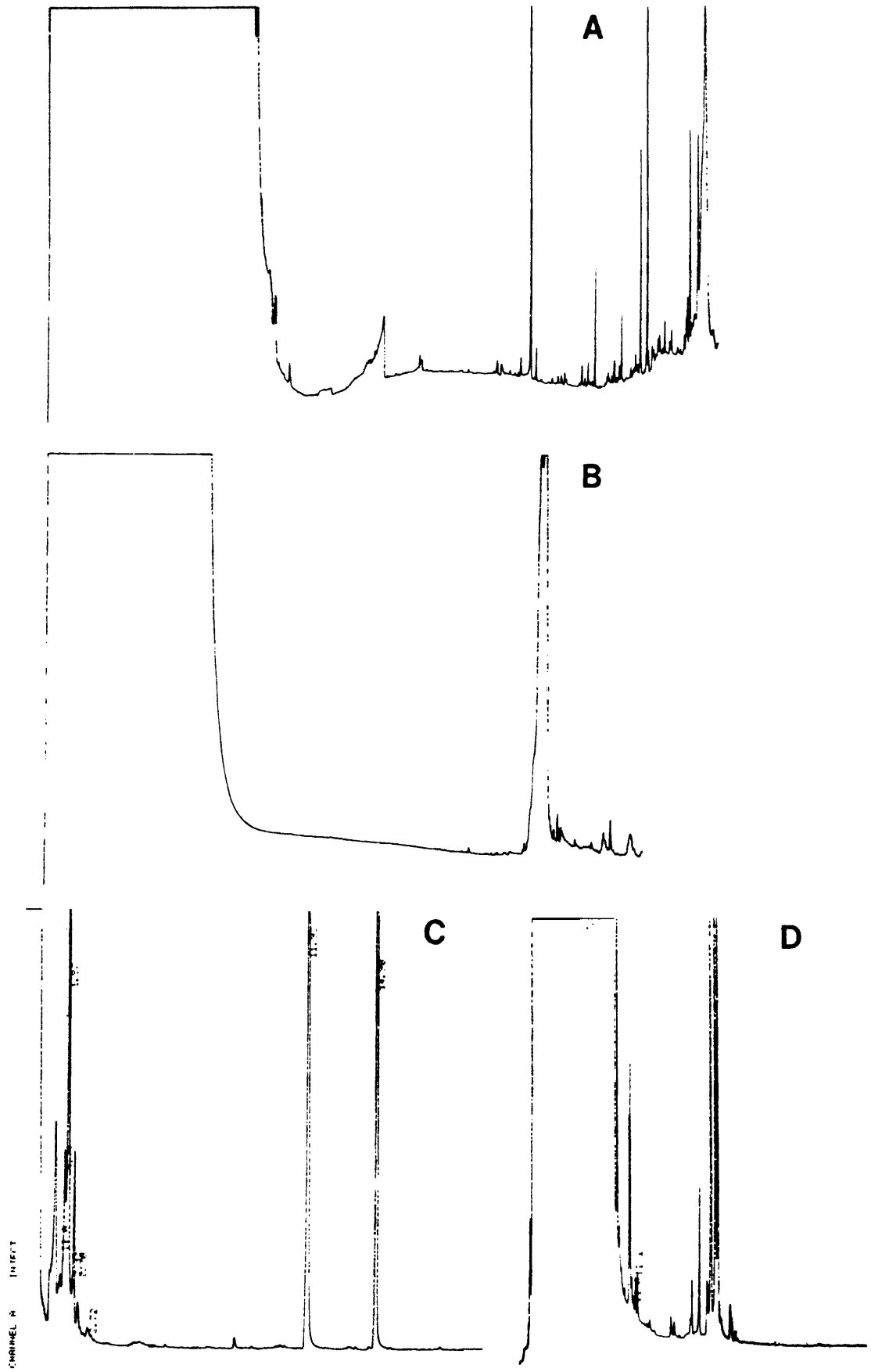


Fig. 7.7 (continued on next page)

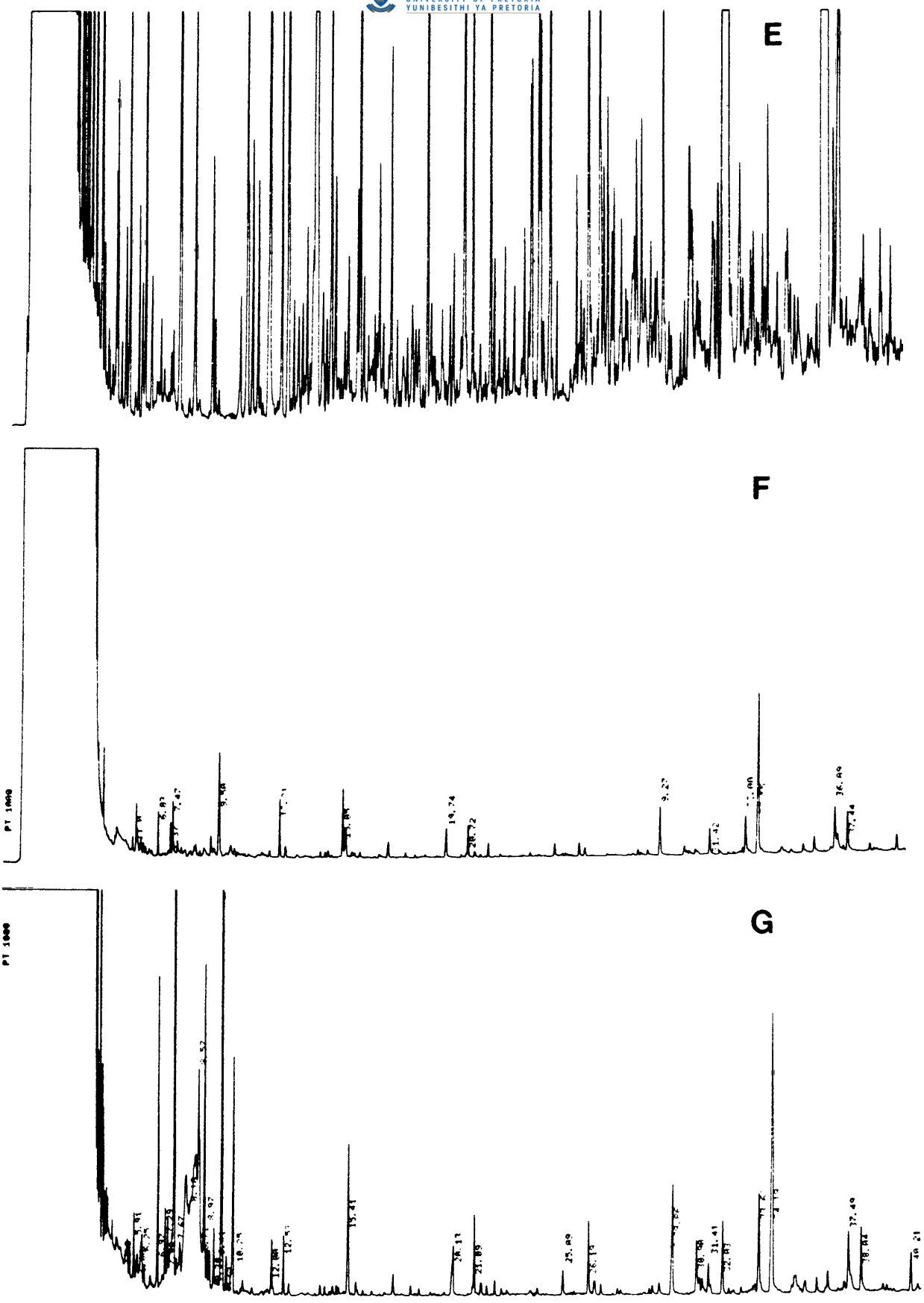


Fig. 7.7 Chromatograms of impurities in commercial solvents. A: Merck LichroSolve acetonitrile; B: Merck LichroSolve methanol; C: BDH pesticide residue grade *n*-hexane; D: Merck analytical (GR) *n*-hexane; E: Merck analytical (GR) *n*-pentane; F: Burdick and Jackson "Distilled in Glass" *n*-hexane; F: Merck spectroscopic grade carbon disulphide. A-E sampled for 30 min with a 15 cm^3 min^{-1} flow of palladium cell purified hydrogen at a temperature of 25-28°C, F 20 µl, and G 30 µl, sampled by topping up a concentrator.

Freshly distilled *n*-hexane was barely pure enough for dynamic solvent effect applications (Fig 7.8).

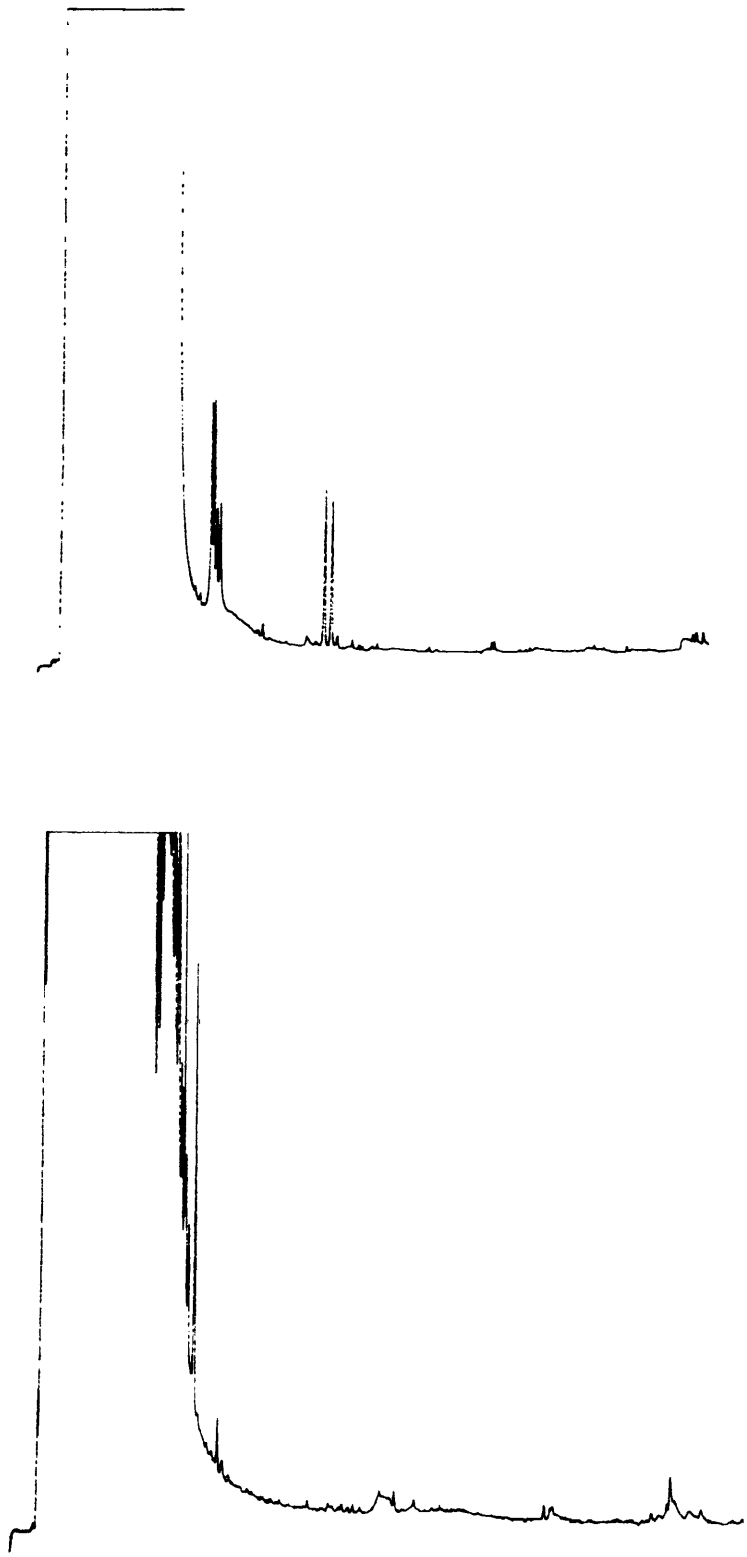


Fig. 7.8 Chromatograms of impurities in 1 cm^3 specimens of fractionally distilled *n*-hexane sampled by the dynamic solvent effect with 15 cm^3 min^{-1} of palladium cell purified hydrogen for approximately 1 hr.

After liquid-solid frontal elution the hexane was dirtier than when it started. Gas-solid frontal elution on activated charcoal suffered the same problem although it became less serious after the column had been continuously backflushed for 144 h (Fig 7.9).

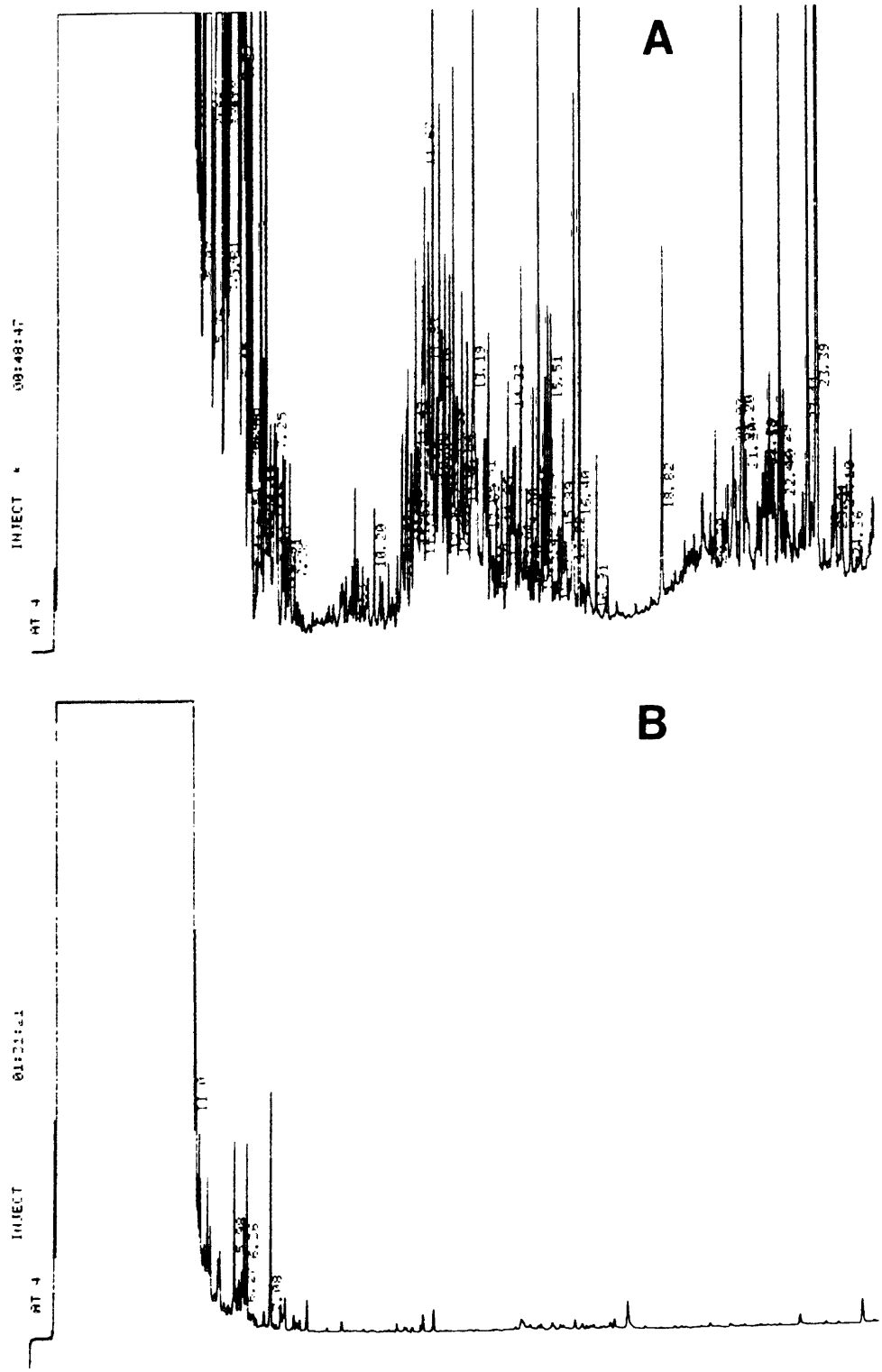


Fig. 7.9 Chromatograms of impurities in *n*-hexane after "purification" by vapour phase frontal elution on activated charcoal. A: after backflushing of charcoal for 18 hours; B: after backflushing of charcoal for 6 days. Both specimens sampled for 15 min with a 15 cm^3 flow of palladium cell purified hydrogen. Separation conditions as in Fig. 7.6.

Gas-solid frontal elution on alumina yielded solvents with impurities detectable only at the ppt ($1:10^{12}$) level (Fig 7.10). The purification process was reliable in practise and less labour intensive than distillation.

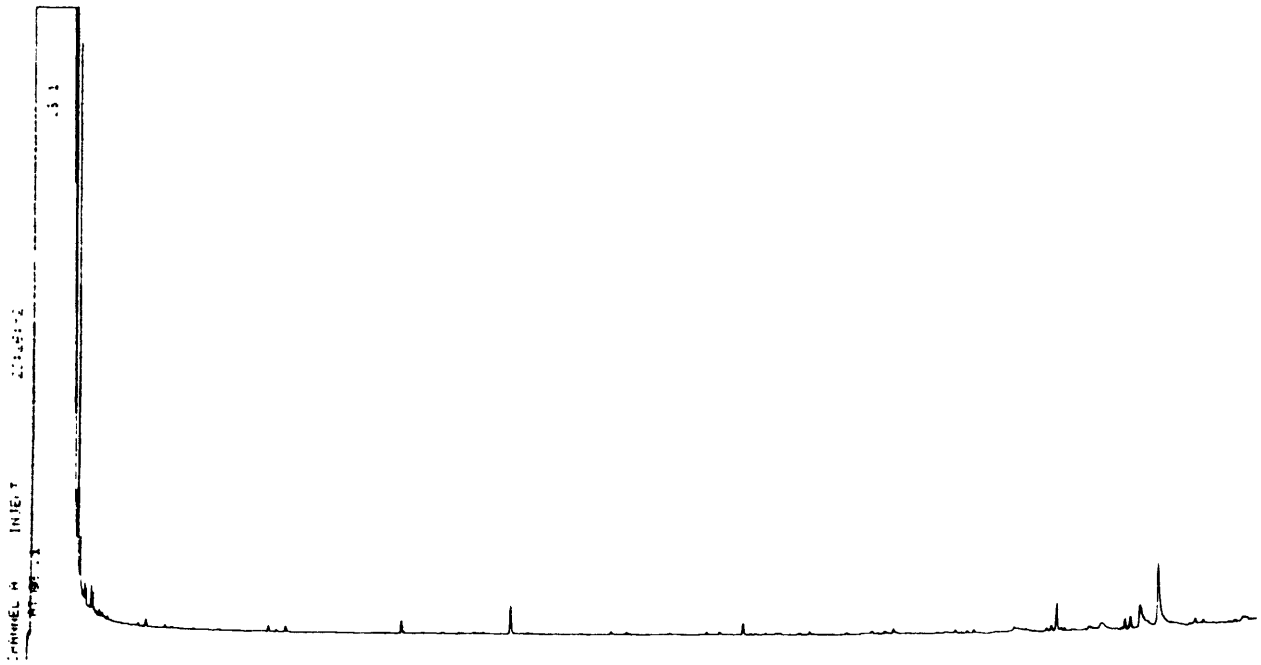


Fig. 7.10 Chromatogram of impurities in 1 cm^3 of *n*-hexane purified by vapour phase frontal elution on activated alumina. Sampling by the dynamic solvent effect with a 15 cm^3 min^{-1} flow of palladium cell purified hydrogen for approximately 1 hr. Separation conditions as in Fig. 7.6, N.B. chart speed reduced for first 4 min.

Solvent deterioration presented serious practical difficulties. In both borosilicate and soda-lime glass containers at room temperature a group of contaminant peaks (Fig 7.11) appeared after one or a few days storage. The rate of deterioration was very variable - one batch of hexane in a borosilicate glass bottle stayed pure for a week whereas other samples developed impurities at the ppb level (10^{-9}) within 24 h. The rate and pattern of contamination appeared to be independent of storage in darkness *versus* daylight. Different patterns of contamination were developed by hexane in a silicon-surfaced tube, hexane in a borosilicate glass bottle kept at -4°C for 3 days and hexane in an aluminium container (Fig 7.11).

Mass spectrometry of the contaminants from solvent in a soda-lime glass vial revealed that they were a series of straight chain and branched ketones and aldehydes.

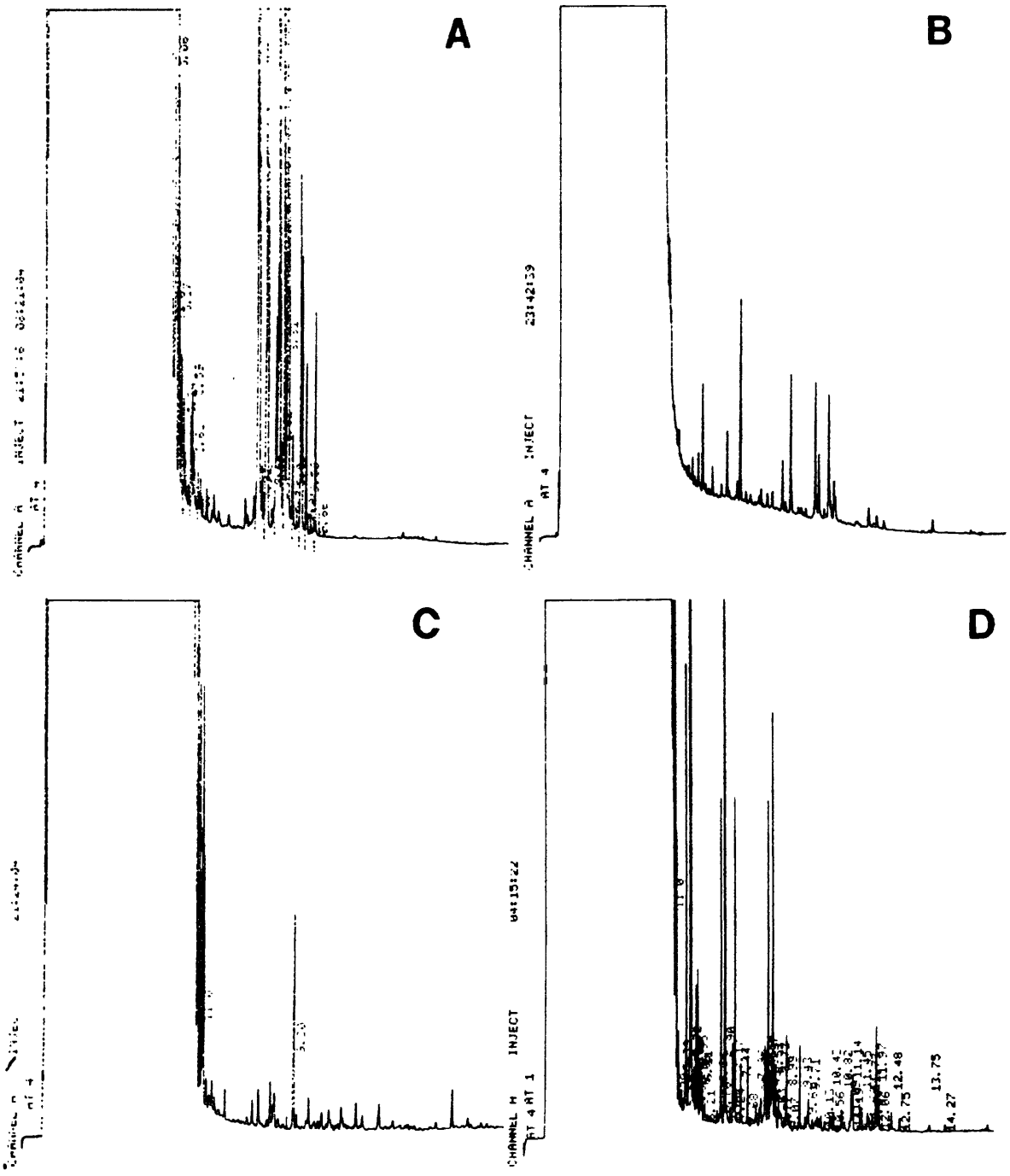


Fig. 7.11 Deterioration in purity of *n*-hexane stored in: A: a flame-sealed, soda-lime glass vial for 2 days at room temperature; B: a screw-topped, borosilicate glass bottle for 2 days at room temperature; C: a silicon-surfaced glass tube for 4 days at room temperature; D: an aluminium container for 8 days at room temperature.

DISCUSSION

Although there is no fundamental upper limit to specimen size with dynamic solvent effect systems, a practical upper limit is set by the specimen size at which reagent impurity peaks interfere significantly with those of the specimen components (but see also Chap 6). Significant interference of an impurity with the peak from a specimen component of interest depends on the type of analysis. Even if the impurity and the specimen component co-elute, quantitative work will be only marginally affected so long as the contribution of the impurity to total peak area is acceptably small. The 10^{-11} g upper acceptable limit for contaminants set in the introduction is based on a 1 % contribution to the area of a 1 ng peak. If the contaminant's contribution to the peak area is significant it can be eliminated by blank run subtraction. In qualitative analyses involving mass spectrometry good spectra of specimen components which co-elute with impurities may be obtained if the impurity contributes only a small part to the peak or if its identity is already known so that its spectrum can be subtracted from that of the combined peak.

Partial co-elution presents more serious problems for quantitation since only that part of the impurity peak which contributes to the area of the specimen component peak should be subtracted. This will vary with minor changes in peak shape, size and resolution. Small contaminant peaks are not a problem and for qualitative analyses partial co-elution presents no difficulty if background subtraction is used to "clean up" combined mass spectra. A blank should be run in order to locate those peaks which are due to impurities.

Impurities which do not co-elute with specimen components may still be a source of difficulty. If an impurity is present in sufficiently high concentration it may accumulate on the solvent film in quantities sufficient to alter its chromatographic properties; e.g. 2% of octane in hexane will completely fill the concentrator bed with octane during an analysis which consumes 1 cm^3 of hexane. Impurities which accumulate to the microgram level may cause broadening of peaks which elute just before them and sharpening, sometimes with retention shifts, of those which elute just after them (Harris 1973; Grob 1983; Grob & Schilling 1983). These phenomena can also occur with specimen components which are present in high concentration (Miller & Jennings 1979).

The more complex is the mixture of volatiles in a specimen the greater is the chance that a given impurity will affect the analysis of at least one of them. In the extreme case of specimens being monitored for a single component the level of impurities found in commercial solvents and gasses may be acceptable if there is no serious co-elution. On the other hand mammalian chemical signals and flavour

volatiles are usually very complex so that reagents must be pure across the full spectrum of impurity retention times.

Impurities which occur in both solvents and gasses are more likely to accumulate to significant levels. This situation is most likely to occur if a common source of contamination e.g. dirty room air has affected several components of the sampling and solvent purification hardware. Those background peaks which are due to airborne contaminants may appear larger in experimental chromatograms than in blank run chromatograms. This is because if the specimen has been exposed to the air it will itself carry a load of the contaminants which, during sampling, will be emitted into the purified sampling gas and subsequently accumulated. This situation was particularly apparent during work on mice (Chaps 11 and 12) which, for obvious reasons, could not be kept in sealed containers.

A class of impurities which may be tolerated in high concentrations are those which do not accumulate on the solvent film during sampling. This includes any compound which is a gas at the sampling temperature, of which the the gasses used for purging are a special example. Compounds such as methanol will not accumulate on non-polar solvent films and may be tolerated at concentrations below those at which they affect the retentive properties of the film.

Specimen components accumulated on a dynamic solvent film are held near the film's evaporating edge as a concentrated solution. Under these conditions reactions with reagent impurities may proceed more readily than they would in the more dilute, bulk solutions usually employed in gas chromatography.

Although, in the tests reported here, bottled gas passed through neoprene-diaphragmed regulators was not found to be seriously contaminated, long term experience, both with sampling and with other applications, showed that its purity could not be relied upon. The use of such gas for sampling would have demanded the frequent and time consuming running of system blanks. Additionally the passage of contaminated gas through regulators and transfer lines would have demanded their cleaning or replacement before sampling could recommence.

With the exception of live animals all specimens were sampled using an inert gas to avoid reactions between gas and sample. Despite the disadvantage of its combustibility hydrogen was usually employed since it could be reliably purified by passage through the palladium cell. Due to its mechanism of operation; the diffusion of atomic or ionic hydrogen (Fast 1976), such a cell, if it passes gas at all, can pass only completely pure hydrogen; no breakthrough of contaminants is possible.

Obviously whole live animals e.g. honey bees *Apis mellifera* (Centner *et al.* 1988) and small rodents (Chaps 11 and 12) could not be sampled using hydrogen. Charcoal-filtered cylinder air was employed. Charcoal traps were reconditioned every day and a blank was run at least once a day to check for breakthrough. Air flow was controlled by a metal diaphragmed mass flow controller upstream of the charcoal trap.

Mammalian chemical signals operate over a restricted temperature range whose upper and lower limits are set by the environmental conditions in which they occur. Consequently the use of elevated temperatures to reduce the consumed volume of purging gas was of restricted applicability. One exception was the case of the schizophrenic odour (Chap 14); the component of interest was present in extremely low concentrations and was apparently strongly adsorbed onto the hair specimen. Distortions in the quantitative emissions of other volatiles from the hair were of no importance since the investigation's only aim was to identify the odourous compound.

The use of a less volatile solvent when only high-boiling specimen components are of interest presupposes that the nature of the compounds of interest has been determined. For the majority of analyses this was not the case - one of the aims of most of them being to identify which volatiles were present. The work by Centner on honey bees (Centner *et al.* 1988) is an exception in which the identities of the compounds of interest were known in advance.

Liquid-solid frontal elution on alumina columns is recommended by Engelhardt (1972) for the purification of a range of solvents, including hexane. Its lack of success in the present case may have been due to more exacting standards of purity or to some defect in the procedure. It is perhaps significant that the liquid-solid chromatographic apparatus was not automatically shielded from atmospheric contamination as is its gas-solid counterpart. Regeneration of the adsorbent in a liquid-solid system would require off-line treatment rather than the simple backflushing employed with the gas-solid system.

Gas-solid frontal elution on alumina was adopted as the standard method for purification of alkane solvents. Due to the problems of storage (q.v.) a batch (10-20 cm^3) of solvent was freshly prepared each day, and the column was backflushed overnight to be ready for purification the following morning. Production of a day's batch of solvent took about 90 min, by tapping off the solvent as it was eluted a specimen could be sampled after approximately 45 min, of which 25 min were needed to cool the column oven. This method proved more reliable, and less costly in time, than distillation as a pre-analysis treatment or blank run subtraction and similar post-analysis data manipulations.

Alkanes are subject to atmospheric oxidation at room temperature by reactions which are free-radical initiated, catalysed by metal salts and autocatalytic (Coffey 1964 p. 330). The deterioration of pure hexane was consistent with such a mechanism. Metal ions are present in higher concentration in soda-lime than in borosilicate glass (Adams 1972) and are absent from amorphous silicon - accounting for the differences in deterioration rate in different containers. The variability in deterioration rate in a given type of container would be expected for an autocatalytic process, especially if trace levels of ketones or peroxides were present in freshly purified solvent. In contrast to the rapid deterioration of pure solvent test mixtures containing the free radical inhibitor *p*-cresol (Ingold 1970) were stable for months. The presence of oxygen in solvent which had been purged with nitrogen during purification and subsequently, in the case of vials at least, sealed under vacuum is unexpected. Nevertheless only approximately 1,4 ng of oxygen is necessary to form 100 ng of ketone so that ppb concentrations of oxygen would have been sufficient to generate the observed levels of impurities.

The stability of purified solvent is an area requiring further detailed study. Containers which are free of catalytic activity are required. Free radical inhibitors and reducing agents may prove practical so long as they do not accumulate on the solvent film during sampling, a requirement which demands that they be either more volatile than the solvent or that they be completely insoluble in it. It may be that solvents other than *n*-alkanes may prove less susceptible to deterioration.

Chapter 8

THE APPROACH TO THE TESTING OF DYNAMIC SOLVENT EFFECT SAMPLING AGAINST REAL BIOLOGICAL PROBLEMS

The known properties of mammalian odour secretions (Chap 2) and the performance of the dynamic solvent effect with synthetic specimens (Chap 6) strongly suggested that it could provide a suitable sampling technique for mammalian semiochemicals. To establish that this was in fact the case it was necessary to test the dynamic solvent effect's performance with real, biological problems.

It may be as well to reiterate here the aims of these tests: to determine to what extent the dynamic solvent effect can provide a sampling technique for quantitative, high resolution gas chromatography, which is sensitive and precise enough to make problems in mammalian semiochemistry accessible to routine, instrumental chemical analysis.

The minimum requirement for this aim to be achieved was that systematic differences be found between the chromatographic profiles of odours known to differ on biological grounds. This is the third step in the "chemical image differentiation" approach introduced on p 9. Identification of the components of the profile accounting for the differences was, strictly, outside the aim of the testing. It was, however, included in each case in order to round off the analytical aspects of the chemical image differentiation approach, to establish the compatibility of dynamic solvent effect procedures with GC-MS and to allow comparison of results with those from published work.

The test cases were selected according to the following criteria:

There was a requirement for strong evidence, either directly empirical or *a priori*, that there was a real odour difference involved in a biological phenomenon. Since animals can readily be trained to discriminate between different "irrelevant" odours (e.g. Bowers & Alexander 1967; Dagg & Windsor 1971) this is not equivalent to a demonstration of semiochemical activity. All that was required was clear biological evidence of an odour difference, whose detectability using dynamic solvent effect sampling could be tested.

So that odours could be unequivocally assigned to one class or another clear cut biological differences, with which the odour was associated, were required. To assist in this respect the tests were conducted, whenever possible, with the collaboration of researchers having experience with the biological aspects of the particular problem.

In order to make the testing as general as possible a range of biological phenomena were investigated (mate recognition, behavioural status etc) using different specimen types (secretions, scent marks, whole animals etc). It was hoped that this diversity would generate examples of different types of analytical challenge.

In order to avoid simply duplicating, with a new method, work carried out by other groups, problems which had already been sucessfully investigated were not re-addressed.

Availability of material necessarily dominated other considerations. Within the framework of development and testing of the dynamic solvent effect it was not practical to spend any but the shortest periods collecting material from the field. The test cases were thus restricted to investigations which could be carried out with material from captive animals, sometimes belonging to collaborators' research colonies, or from those captured by other researchers for their own purposes.

Although not semiochemistry in the strict sense, another facet of the chemistry of mammalian secretions was included in the testing. This was the surveying of the identities of the volatile components of secretions from a variety of species. As argued in Chapter 15 this type of investigation is complementary to semiochemical studies and the results of such work constitute a substantial proportion of the mammalian chemistry literature.

The anticipated quantitative complexities of mammalian semiochemicals were the analytical challenge at which the development of the dynamic solvent effect was directed. Nevertheless two of the test cases involved arthropods rather than mammals. The project on Thaumastellid systematics (Chap 9) was undertaken as an example from a field in which chromatographic analysis has been widely employed but in which there has been little attention directed towards the biological significance of the chemical differences. This contrasts with the situation for inter-specific odour differences in small mammals (Chap 11) for which there is a wealth of biological, but almost no chemical, data. The investigation of tick aggregation pheromones (Chap 13) was considered interesting in that it combined quantitative changes in odour profile with a very straightforward bioassay against which the

analytical results could be explicitely examined, a situation which is not to be expected among mammals (see pp 3-5).

That the biological work was required to fit within the framework of the testing of the dynamic solvent effect placed constraints on the experimental designs employed. One of these was that smaller sample sizes were employed than would have been the case had the same studies been undertaken in their own right. It is perhaps appropriate to repeat here that the aim of the applications studies was to demonstrate that biological problems of various types are accessible to investigation using the dynamic solvent effect.

A test could be considered to have been successful if dynamic solvent effect sampling and capillary chromatography yielded:-

1. chromatograms of high quality to show that the performance of the dynamic solvent effect was not being significantly adversely affected by the sample itself,

2. similar chromatograms from similar materials,

3. trends in chromatographic results consistent with associated biological changes,

4. correlations between biological and chromatographic data.

An additional criterion of success was that dynamic solvent effect sampling be compatible with ancillary analytical techniques, the two of interest being being mass spectrometry and column effluent sniffing.

In the present context any biological insight which emerged from the analytical results can be considered as a bonus.

Chapters 9-14 each consider a particular biological problem and Chapter 15 combines examples of qualitative investigations of the identities of the major volatile components from a selection of mammalian secretions. Appended to each chapter is a discussion of the analytical challenges posed by the biological problem and the contribution of the dynamic solvent effect to their solution.

Chapter 9

THE CHEMISTRY OF THE DEFENSIVE SECRETION OF *Thaumastella namaquensis* AND *T. elizabethae*.

The Heteroptera (true bugs) are an example of a group of animals in which morphological and chemical taxonomy are known to be strongly correlated (Calam & Youdeowei 1968). The aim of this investigation of the chemistry of the defensive secretions of *Thaumastella namaquensis* and *T. elizabethae* was to throw light on the uncertain taxonomic status and systematic relationships of the Thaumastellidae (Stys 1964; Schaefer & Wilcox 1971). The results presented here supplement other research on Thaumastellid sytematics by D. Jacobs, Department of Genetics, University of Pretoria, who collaborated in this study.

Whether or not the defensive secretions of Heteroptera are true semiochemicals is a moot point. They are typically powerful contact irritants and poisons (Remold 1963) - effects which cannot be considered semiochemical in nature - but are also long-range repellants (Remold 1963) and can thus be considered to be allomones. In some species, e.g. *Podisus maculiventris*, the "defensive" secretion actually acts as a sex attractant (Aldrich, Kochansky & Abrams 1984) and in others, e.g *Carlisis wahlbergi*, as an alarm signal (*pers. obs.*). In the two *Thaumastella* species considered here the function of the "defensive" secretion has not been investigated. The minute size of the insects (approximately 2,5 mm long) suggests that their secretion would be of limited value for defence.

The analysis of the *Thaumastella* secretions is included here as a test of the versatility of dynamic solvent effect sampling.

METHODS

Specimens of *T. elizabethae* collected at Gifberg in Namaqualand and of *T. namaquensis* collected near Springbok, Namaqualand by Jacobs were maintained on a diet of seeds and water. Volatiles from the defensive secretion were sampled by the dynamic solvent effect as follows. Individual insects were gently aspirated into small sampling chambers made by necking down one end of a 1 cm piece of 1,4 mm i.d., 2 mm o.d. borosilicate glass tube. Active-charcoal-filtered air was passed through the chamber and into a dynamic solvent effect concentrator at a flow rate of 20 cm^3 min^{-1} (Fig 9.1). *n*-Hexane was used as solvent. After 1 min a stream of hot air was passed momentarily over the

sampling chamber. This induced the insect to release its defensive secretion and quickly killed it by thermal shock. Sampling was continued for a further 5 min to ensure transport of the volatiles out of the sampling chamber and into the concentrator.

Gas chromatography-flame ionization detector (GC-FID) analyses of the defensive secretion volatiles were carried out on a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet and a 20 m x 0,3 mm x 0,4 µm methyl silicone capillary column. The carrier gas was hydrogen with a linear velocity of 55 cm s^{-1}. The starting temperature of both inlet and column was 40°C, the inlet was heated ballistically to 220°C after 2,2 min and the column temperature was programmed at 5°C min^{-1} after 7 min. The detector sensitivity was 4 x 10^{-11} A mv^{-1} full scale deflection. Gas chromatography-mass spectrometry (GC-MS) analyses were carried out under equivalent conditions on a modified Varian 1400, using helium as carrier gas, with an open split interface to a VG Micromass 16F spectrometer operating in the electron impact mode. The source temperature was 220°C and the electron energy was 70eV.

Where possible compounds were identified by comparison of their mass spectra with those in the libraries or the literature. Where necessary identities were confirmed by retention indices.

Seven *T. namaquensis* and 5 *T. elizabethae* were examined individually by GC-FID. Four individual *T. namaquensis* and four, pooled *T. elizabethae* were examined by GC-MS. Quantitative comparisons were based on the GC-FID analyses.

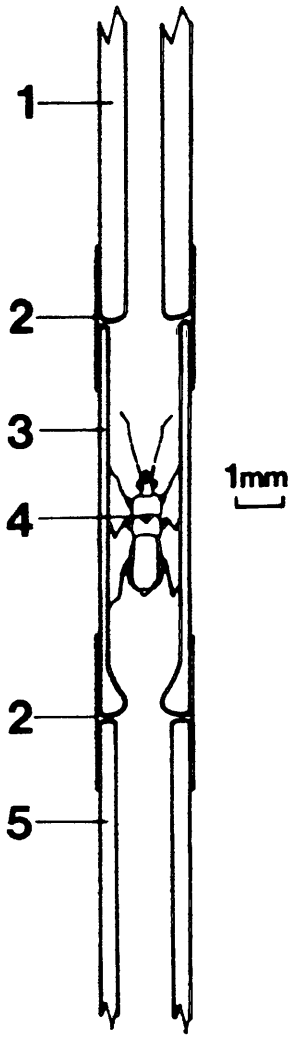


Fig. 9.1 Micro-chamber used for sampling volatiles from Thaumastellid bugs. 1: outlet of activated charcoal filter; 2: polytetrafluoroethylene sleeve connector; 3: 2 mm o.d., 1,4 mm i.d. borosilicate glass tube; 4: bug; 5: top end of dynamic solvent effect concentrator.

RESULTS

Individuals of both species proved very reluctant to emit their defensive secretion except under extreme stress. The secretion was sometimes emitted as a fine spray in sufficient quantities to wet the walls of the sampling chamber; it was a colourless liquid which evaporated within 10-20 s. The secretion's odour closely resembled that of green coriander.

The defensive secretion of both species contained a complex mixture of volatiles (Fig 9.2). Identities were assigned to sixteen compounds, another five remain unidentified. The mass spectra of the unidentified compounds are;

Unknown 1: 41 100%; 70 65%; 39 58%; 69 39%; 81 37%; 68 35%; 27 29%; 29 27%; 91 22%; 53 22%; 79 21%; 66 21%; 40 19%; 94 17%; 77 15%; 55 15%; 65 14%; 109 14%; 67 9%; 51 9%; 54 8%; 42 8%; 92 7%; 120 7%; 95 6%; 82 4%; 107 4%; 136 1%,

Unknown 2: 69 100%; 70 83%; 55 72%; 41 57%; 29 23%; 67 21%; 81 17%; 111 17%; 43 16%; 39 16%; 27 13%; 54 12%; 40 12%; 57 11%; 110 11%; 68 10%; 79 8%; 53 7%; 42 6%; 83 5%; 91 4%; 82 4%; 80 4%; 56 4%; 109 4%; 77 3%; 123 2%; 136 1%;

Unknown 3: 43 100%; 54 32%; 41 22%; 55 21%; 67 20%; 68 15%; 40 12%; 29 12%; 81 11%; 82 10%; 39 10%; 57 9%; 27 8%; 96 7%; 95 6%; 83 6%; 71 6%; 69 6%; 110 6%; 42 5%; 138 3%; 109 3%; 156 2%;

Unknown 4: 43 100%; 69 51%; 54 45%; 55 43%; 41 31%; 79 30%; 110 23%; 80 19%; 81 18%; 67 17%; 29 15%; 68 12%; 39 10%; 94 8%; 93 8%; 27 8%; 82 7%; 70 7%; 66 7%; 78 6%; 57 6%; 40 6%; 95 5%; 91 5%; 42 5%; 164 5%; 113 5%; 107 5%; 77 4%; 71 4%; 53 4%; 135 3%; 122 3%; 121 3%; 123 2%;

Unknown 5: 41 100%; 55 60%; 70 33%; 39 30%; 81 30%; 67 29%; 83 28%; 27 27%; 29 23%; 123 22%; 69 20; 68 17%; 95 13%; 43 13%; 53 12%; 82 11%; 84 11%; 79 10%; 108 10%; 40 10%; 109 9%; 94 7%; 152 1%.

All 21 of these compounds were found in *T. namaquensis* and nineteen of them occurred in *T. elizabethae* (Table 9.1). In both species there was substantial quantitative variation among individuals (Table 9.2), which extended to variable presence or absence of some components (Table 9.1).

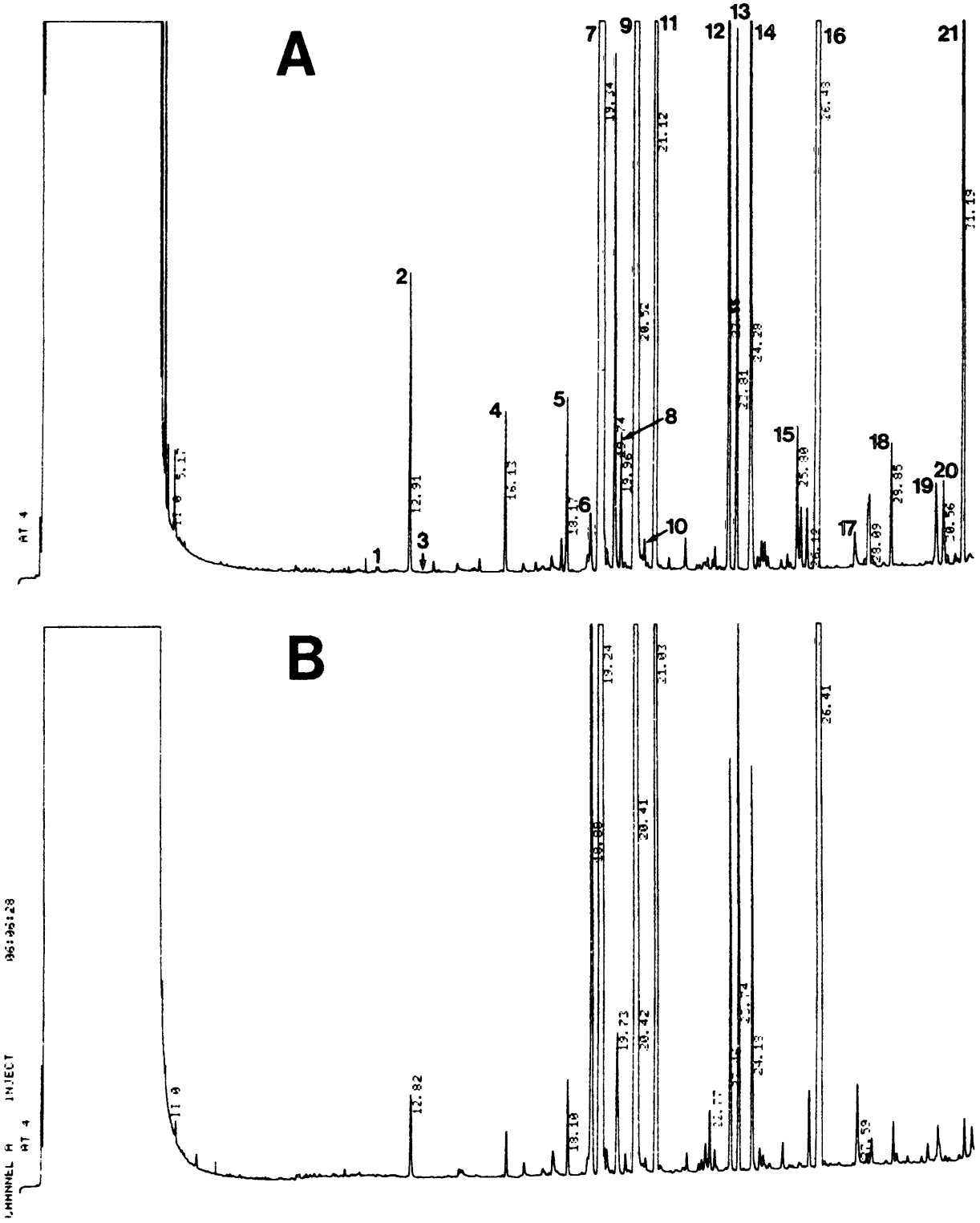


Fig. 9.2 Chromatograms of volatiles from defensive secretions of; A, *Thaumastella namaquensis* and B, *T. elizabethae*. Secretion was sampled from single insects by the dynamic solvent effect and separated on a 20 m x 0,3 mm x 0,4 µm methylsilicone column with a temperature programme of 5°C min^{-1}. Detection was by FID at a sensitivity of 4 x 10^{-11} A mv^{-1}. Peak numbers correspond to identities in Table 9.1.

Table 9.1 Compounds from the defensive secretion of *Thaumastella namaquensis* and *T. elizabethae* and their occurrence in each sampled individual. - absent; o less than 0,5 ng present; + more than 0,5 ng present. Compounds marked with an asterix are unique to the Thaumastellidae.

	T. namaquensis							*T. elizabethae*				
Compound	n1	n2	n3	n6	n7	n10	n12	ec	ed	ee	ef	eg
Hexyl acetate	-	-	-	-	-	o	-	-	-	-	o	-
2-octenal	o	+	+	+	+	+	o	+	+	o	+	+
(E,E)-2,4-hexadienyl acetate	-	-	-	-	-	+	-	o	o	-	o	-
2-nonenal	o	+	+	+	+	o	o	o	+	o	o	o
dodecane	o	+	+	+	+	o	o	o	+	o	o	+
unknown 5	o	+	o	o	o	+	o	o	o	o	o	+
2-decenal	+	+	+	+	+	+	+	+	+	+	+	+
methoxyphenylethanone*	+	+	+	o	o	+	o	-	-	-	-	-
unknown 1	+	+	+	+	+	+	o	+	+	+	+	+
tridecene*	o	+	o	+	o	o	o	o	o	-	o	o
tridecane	+	+	+	+	+	+	+	+	+	+	+	+
unknown 3	+	o	+	+	+	+	o	o	o	o	o	+
tetradecane	+	+	+	+	+	+	o	+	+	+	+	+
unknown 2	+	+	+	+	+	+	o	+	+	o	o	+
pentadecene*	o	+	+	o	+	o	o	o	o	o	-	o
pentadecane	+	+	+	+	+	+	+	+	+	+	+	+
unknown 4	+	o	o	o	o	o	o	+	o	o	-	+
hexadecane*	o	o	+	o	+	o	o	o	o	o	-	o
heptadecadiene*	o	o	+	o	+	o	-	o	o	o	-	o
heptadecene*	o	o	+	o	+	o	-	-	-	-	-	-
heptadecane*	+	+	+	+	+	+	o	o	o	o	-	o

Table 9.2 Mean ± standard deviation of peak areas for volatile components of defensive secretion of *Thaumastella namaquansis* and *T. elizabethae*. Compounds marked with an asterix are unique to the Thaumastellidae

Compound	*T. namaquensis*	*T. elizabethae*
Hexyl acetate	28 ± 76	100 ± 223
2-octenal	6437 ± 6392	3008 ± 3561
(E,E)-2,4-hexadienyl acetate	4562 ± 12070	120 ± 214
2-nonenal	4417 ± 4866	1177 ± 2165
dodecane	8310 ± 10719	1851 ± 2324
unknown 5	1283 ± 1804	7623 ± 16488
2-decenal	997355 ± 833958	785500 ± 929619
methoxyphenylethanone*	3094 ± 3120	0
unknown 1	85176 ± 71797	166079 ± 110710
tridecene*	1199 ± 2032	90 ± 74
tridecane	472823 ± 624846	243002 ± 317617
unknown 3	18383 ± 20584	3137 ± 6678
tetradecane	13124 ± 10289	16592 ± 15477
unknown 2	19964 ± 13492	81994 ± 8431
pentadecene*	2838 ± 3684	70 ± 45
pentadecane	310751 ± 277402	672156 ± 482100
unknown 4	1366 ± 3133	9344 ± 18665
hexadecane*	1797 ± 2867	120 ± 84
heptadecadiene*	864 ± 1683	1315 ± 1747
heptadecene*	1199 ± 1960	0
heptadecane*	26738 ± 31877	100 ± 71

DISCUSSION

The significance of the defensive secretion chemistry of *T. namaquensis* and *T. elizabethae* for Thaumastellid systematics is discussed by Jacobs, Apps & Viljoen (in prep.).

ANALYTICAL CHALLENGES AND SOLUTIONS

It cannot be said that, in general, the secretions of heteropterans present serious analytical challenges. Apart from the volatile acids found in e.g. the Triatominae (Schofield 1979) and Coreidae (Calam & Youdeowei 1968; De Lange & Van Rensburg 1982) the secretions' components; aldehydes, hydrocarbons, esters and terpenoids, are chromatographically tractable and in most cases free from interfering

substances such as water and dissolved solids. From several species pure secretion may be obtained in microliter quantities e.g. 20 µl from *Elasmopoda valga* (Burger, Munro, Röth, Geertsema & Habich 1986), 15-20 µl from *Amorbus* sp. and *Mictis* sp. (Waterhouse & Gilby 1964) and 0,6-2,3 mg from *Chelinidea vittiger* (McCullough 1973). This is reflected in the analytical techniques which have been employed. Among 42 papers searched for mass spectra 39 reported successful analyses using packed column gas chromatography, and only five using capillary columns (De Lange & Van Rensburg 1982; Hanssen & Jacob 1982; Aldrich *et al.* 1984; Aldrich, Lusby, Kochansky & Abrams 1984; Gough, Games, Staddon & Olagbemiro 1985; Burger *et al.* 1986).

Thaumastella namaquensis and *T. elizabethae*, with body weights of 0,6-0,85 mg and 0,3-0,5 mg respectively are the smallest heteroptera whose defensive secretions have been analysed. The next smallest is *Oxacarenus hyalinipennis*, with body weights of 1,3±0,3 mg in males and 2,9±0,5 mg in females, which were analysed individually using on-column injection of whole gland complexes on packed columns (Olagbemiro & Staddon 1983). Even with the small *Oxacarenus* the sensitivity of packed column separation with FID detection was sufficient for inter-individual comparison, although in GC-MS analyses some spectra were reported as being "weak" (Olagbemiro & Staddon 1983). In a subsequent study in the same laboratory a large pool of 4000-5000 individuals was used, but this was in order to carry out UV and NMR investigations of farnesene isolated by liquid-solid column chromatography (Knight, Rossiter & Staddon 1984). Parenthetically it may be noted that Brill & Bertsch (1986) analysed cuticular hydrocarbons from pieces of ants with total body weights around 0,4 mg, but were able to do so only under conditions approaching those of pyrolysis.

Perhaps surprisingly, in the light of these earlier results, the analysis of Thaumastellid secretions did require rather high sensitivity. Although the major components of each insect's secretion were always present in tens or hundreds of ng, inter-individual comparison of the presence or absence of particular components required that a compound present in 100 ng quantities in one insect be detected at sub-nanogram levels in another (Fig 9.3). Without the high sensitivity provided by dynamic solvent effect sampling and high resolution separation on capillary columns the seven compounds unique to the Thaumastellidae (Table 9.2) may have remained unidentified. For none of the seven compounds was the mean peak area equivalent to more than approximately 1,5 ng, although in some individuals quantities were larger; up to approximately 5 ng for the most abundant of the seven, *n*-pentadecene, which might well have been sufficient for identification by less sensitive techniques.

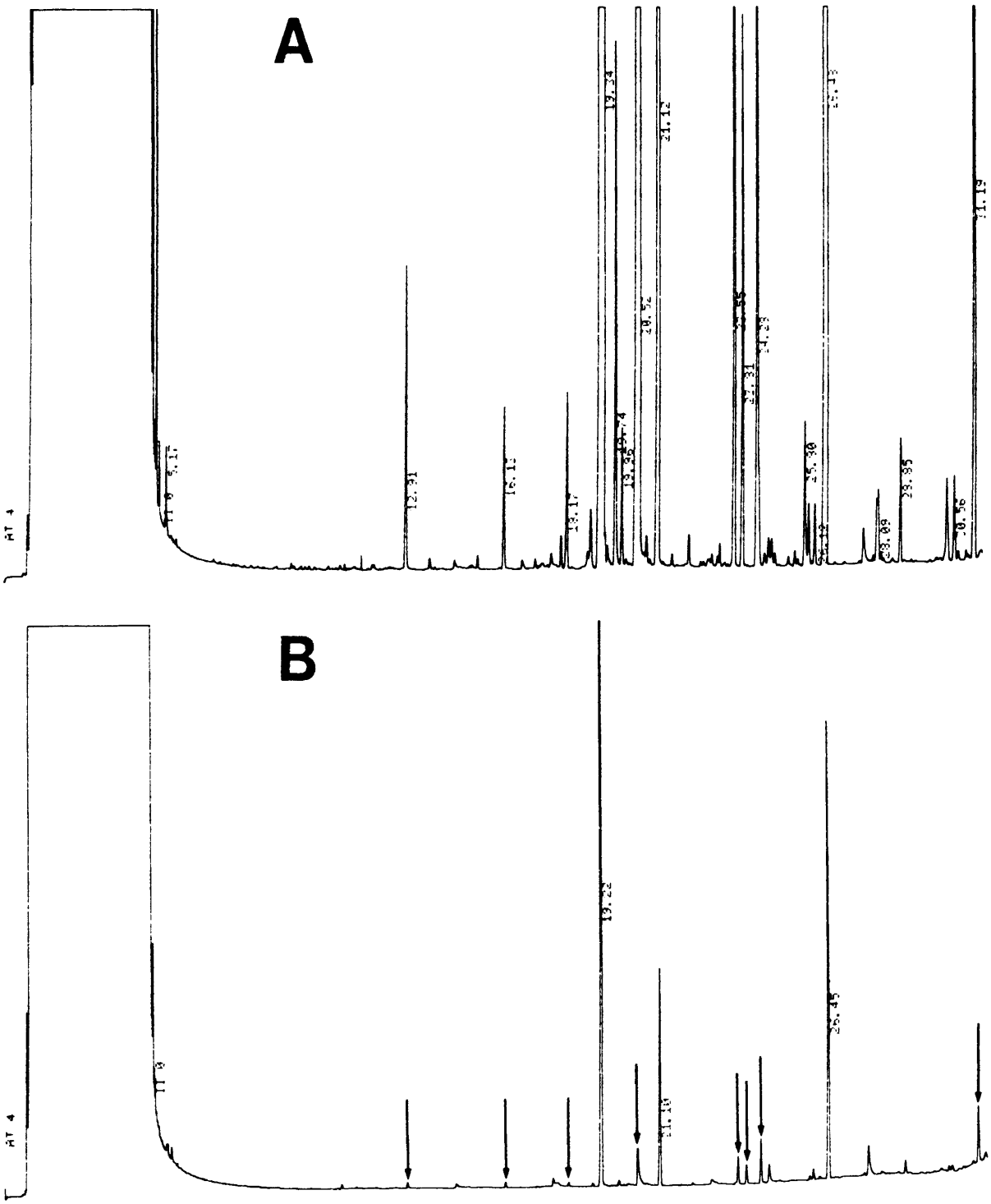


Fig. 9.3 Chromatograms of volatiles from the defensive secretion of two *Thaumastella namaquensis* to illustrate the role of analytical sensitivity in inter-individual comparisons. The large peaks at retention times 12.91, 16.13, 18.17, 20.52, 23.55, 23.81, 24.28, and 31.19 in A are reduced to traces (arrowed) in B which would have been undetected in a less sensitive analysis. Conditions as for Fig. 9.2.

Both species are scarce, only a few dozen of each ever having been collected, so that pooling of large numbers of individuals was impossible. For GC-MS analysis with sensitivity limits some 100 times higher than GC-FID it was necessary to run 4 *T. namaquensis* as individuals and 4 *T. elizabethae* as a pooled sample before satisfactory spectra were obtained for all the major peaks.

Probably in part because the secretions are readily available in liquid form there have been only a few studies based on head space analyses. Schofield (1979) cold-trapped isobutyric acid from groups of six species of Triatominae and Aldrich *et al.* (1984) used Ambersorb activated charcoal to trap volatiles from the secretion of single male *Podisus maculiventris*, in which the "defensive" secretion acts as a sex attractant. The so-called "head space" analysis by Hanssen & Jacob (1982) was actually a solvent extraction of the secretion emitted by 50-60 bugs.

The selectivity of dynamic solvent effect sampling against compounds not accumulated on the solvent film (Chap 6) did not preclude successful analysis in the case of the Thaumastellids, although other heteroptera do produce compounds which would be undetected using hexane as solvent; e.g isobutyric acid (Schofield 1979), acetic acid (De Lange & Van Rensburg 1982), ethanal, butanal, butanol, (Burger *et al.* 1986) butenal, propenal and butan-2-one (Calam & Youdeowei 1968).

The question of whether or not dynamic solvent effect sampling and capillary separation represent useful advances in the analysis of heteropteran secretions can only be answered by a direct comparison of different analytical techniques applied to the same material.

Although it is no reflection on the performance of the sampling system it would have been preferable, given the small number of specimens available, to have been able to sample without killing the insects. However they consistently failed to emit their defensive secretion under anything less than fatal stress.

Chapter 10

INVESTIGATION OF PATTERNS OF VOLATILE EMISSIONS FROM THE SCENT MARKS OF THE BROWN HYAENA (*Hyaena brunnea*)

INTRODUCTION

One of the most important aspects of communication by scent marking is that messages can be transmitted through time. A mark deposited by an animal may continue to emit odour signals for days, weeks or months after the animal has left. This property is not shared by other channels of communication except those visual signals which involve modifications to the surroundings (Gosling 1985 pp 574-575). The effects of a mark's age on its signalling properties have been investigated in, for example, genets (*Genetta genetta*) (Roeder 1978, 1980, 1983), tamarins (*Saguinus fuscicollis*) (Epple, Beauchamp & Wojcieschowski-Metzler 1980), guinea pigs (*Cavia porcellus*) (Wellington *et al.* 1983) and bush babies (*Galago crassicaudatus*) (Katsir & Crewe 1980; Clark 1982) by presenting scent marks to animals and recording investigation and other responses. However, no investigation of the chemical changes during ageing of natural scent marks has been undertaken.

Working with radioactively labelled phenylacetic acid as an "active" component of model scent marks Regnier & Goodwin (1977) established that the emission rate of this compound depended on the mark's substrate, the chemical properties of its matrix and the humidity of its surroundings. The presence of a matrix buffered the effects of substrate and humidity.

There has, to date, been no direct study of the volatiles actually emitted by any real scent mark. Thus there are two major gaps in our knowledge of the chemistry of mammalian odour communication; the composition of the volatiles actually emitted by natural marks, and the changes in composition of those volatiles as the mark ages. Since the function of a scent mark is to communicate by the emission of volatiles over a more or less extended period these gaps must be filled if the ways in which scent marks fill their unquestionably important role in the organization of mammalian societies are to be understood.

It was the aim of this study to determine how useful dynamic solvent effect sampling and high resolution gas chromatography could be in filling the gaps.

The scent mark used in this investigation was that of the brown hyaena (*Hyaena brunnea*). The hyaena employs its complex, eversible anal pouch, and a specialized and elaborate behaviour to deposit, usually on a single grass stem, a mark consisting of two discrete components. The marks are known as pastings. The two components are a thin smear of black, aqueous, apocrine secretion and, about 12 mm below it, a blob of white, lipid-rich, sebaceous secretion. To the human nose the two components differ in quality, intensity and persistance of odour. GLC analysis has revealed that the composition of both components is specific to the individual which produced them (Mills *et al.* 1980).

The brown hyaena's scent mark provides a natural mark which is:-

- visually conspicuous and thus easily collected,
- easy to collect intact and uncontaminated,
- a combination of two discrete, chemically distinct and functionally specialized components.

The behavioural and ecological role of scent marking by brown hyaenas has been intensively investigated and the scent marks have been subject to conventional chemical analysis (Mills *et al.* 1980; Gorman & Mills 1984).

Brown hyaenas are scavengers which forage solitarily within group territories ranging in size from 5,5-48,9 km^2 in the Transvaal (Skinner & Van Aarde 1987) to 235-480 km^2 in the Kalahari (Mills 1982) and 107-220 km^2 in the southern Namib Desert (Goss 1986). The hyaenas scent mark throughout their territories. In the Kalahari the pastings are deposited at a rate of 2,2-4,5 per km travelled, each animal producing approximately 29 000 annually. The standing crop of active pastings within one Kalahari territory was at least 20 000 (Mills *et al.* 1980; Gorman & Mills 1984).

The function of pasting in relation to the brown hyaena's ecology has been discussed in detail by Mills *et al.* (1980) and Gorman & Mills (1984). Two major roles were proposed; communication within a group informing one group member of another's recent movements, and communication between groups to inform non-resident hyaenas that an area is occupied. The black paste seems suited to short-term, intra-group signalling and the white paste to long-term demarcation of an occupied area.

Gosling (1982) has argued convincingly that a uniquely cheat-proof means of territorial marking is provided by a system of scent matching. An intruding animal may recognise the holder of a territory by matching the odour of an animal it meets with that of scent marks it has encountered in the immediate vicinity. Scent marks would be expected to be distributed in such a way as to increase their probability of being encountered by an intruder. The pastings of brown hyaenas are indeed distributed in this fashion (Gorman & Mills 1984).

Gosling's model requires that those aspects of a scent mark's odour which identify its producer remain stable with time, the Gorman-Mills model of movement records requires that some aspect of the pastings' odour change perceptibly with time.

The relationships between an odour's chemical composition and its perceived quality, and between its concentration and its perceived intensity are far from simple (Amoore 1982; Albone 1984 pp 34-39; Shirley 1984). Nevertheless with the simplifying assumptions that the odour quality of a mixture will change if, and only if, its composition changes, and that perceived odour intensity changes in the same direction as odourant concentration, it is possible to make testable predictions about the emission of odour volatiles from the pastings of brown hyaenas.

- the emission rates, or the relative emission rates, of some components will remain constant with time,

- the emission rates, or the relative emission rates, of some components will change with time,

METHODS

Pastings were obtained from brown hyaenas kept in three, mixed-sex groups, in large pens with natural vegetation at the De Wildt Cheetah Research and Breeding Station, Transvaal. The hyaenas were observed from outside their pens and when a pasting was seen to be deposited it, and the grass stem to which it was adhering, were collected and cooled in liquid nitrogen as quickly as possible. The age of the pasting was measured as the time since it was deposited less the time it spent in liquid nitrogen. Only pastings deposited on clean substrates were used for quantitative analyses. Pastings which overlay other, older marks were used for qualitative identification by gas chromatography - mass spectrometry.

In the laboratory the pastings' volatile emissions were sampled as follows:

Examination of Temporal Changes in Emission Rates of Volatiles

While still as cold as possible the black and white pastes were separated by cutting the stem on which they had been deposited. Each paste was transferred to a small sampling chamber through which 10 cm^3 min^{-1} of dry, charcoal-filtered air was continuously passed (Fig 10.1). The timing of the paste's ageing recommenced when the frost cleared from its surface. Thereafter the paste was kept at a temperature of 29-30°C in an incubator. Volatiles emitted from the paste were sampled by the dynamic solvent effect from the stream of air passing through the chamber, using *n*-hexane as solvent. The length and spacing of sampling periods were arranged, in the light of pilot experiments, to provide a workable compromise between sampling time and sensitivity as the paste aged.

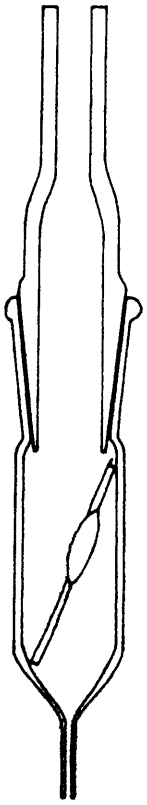


Fig. 10.1 Sampling chamber used for brown hyaena pastings, based on a 10/19 ground glass joint.

Effect of Humidity on Emission of Volatiles

A complete pasting was mounted in a sampling chamber which allowed either dry or moist air to be passed over the pasting (Fig 10.2). The moist air had been bubbled through 100 cm^3 of water which had been purged of volatile organics by vigorous boiling and to which potassium permanganate had been added to inhibit microbial growth. By trapping the water vapour on anhydrous calcium chloride the moist air was found to have a relative humidity of 96 %.

After mounting in the chamber the pasting was allowed one hour to stabilize under dry air flow. The pasting's emissions of volatiles into dry air were then sampled for 15 min. The air flow through the chamber was then immediately reversed and the volatiles emitted by the pasting into moist air were sampled for 15 min. The air flow was then switched back to dry air until the next sampling session.

Identification of Volatiles

Palladium cell-purified hydrogen was passed over six pastings of various ages at a flow rate of 15 $cm^3\ min^{-1}$ for 30 min.

Volatiles sampled by the dynamic solvent effect were separated by capillary gas-liquid chromatography using a 25 m x 0,3 mm x 0,4 µm methyl silicone column in a Varian 3700 chromatograph fitted with a dynamic solvent effect inlet. The carrier gas was hydrogen with a linear velocity of 50 cm s^{-1}. A flame ionization detector was used with a sensitivity of 10^{-11} A mv^{-1}, the detector signal was fed to a Varian 4270 integrator set for a full scale deflection of 1 mv. The initial temperature of both inlet and column was 40°C, the inlet was heated ballistically to 220°C after 3 min and the column temperature was programmed at 5°C min^{-1} to 220°C after 7 min.

Gas chromatography - mass spectrometry was carried out under equivalent conditions using a Varian 1400 GC with an open-split interface to a VG Micromass 16F mass spectrometer. The carrier gas was helium, the ion source temperature was 220°C and the ionization energy was 70 eV. Identifications were based on mass spectra library searches, where possible identities were confirmed by retention indices.

The rates of emission of volatiles were calculated by dividing the area of the corresponding peak (arbitrary units) by the sampling time in minutes. The mean emission rate for a sampling period was taken as the instantaneous emission rate at the mid-point of the sampling session. The detector response factor was assumed to be the same for all compounds, an approximation which generates only systematic errors.

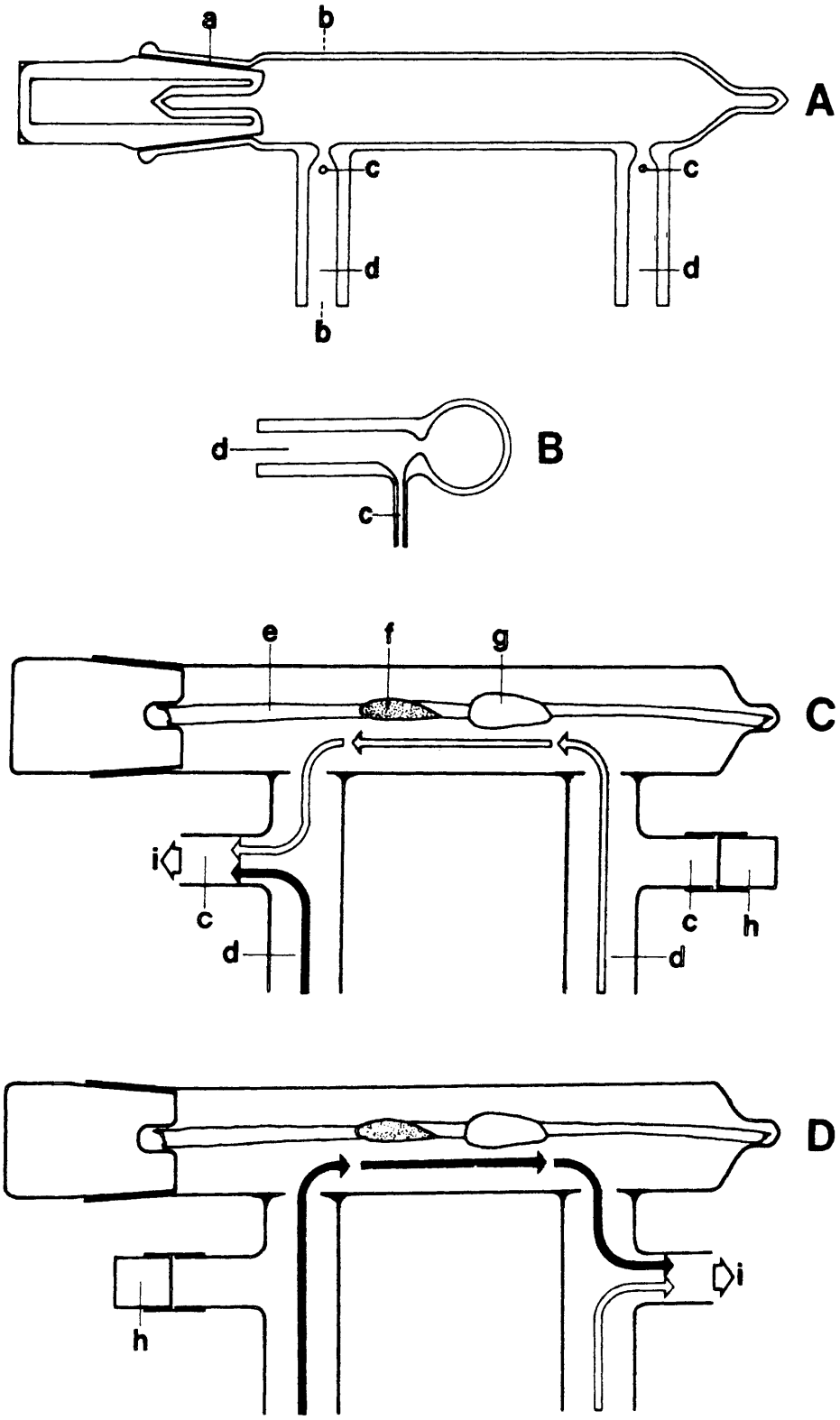


Fig. 10.2 Chamber used for sampling volatiles from brown hyaena pastings exposed to either dry or moist air. A: plan view; B: section through b-b; C: pasting exposed to dry air (open arrows); D: pasting exposed to moist air (solid arrows). Equal volumes of dry and moist air flow to the concentrator irrespective of the conditions to which the pasting is exposed. a: 7/16 ground glass joint; c: outlet; d: gas inlet; e: grass stem; f: black paste; g: white paste; h: glass stopper; i: gas flow to dynamic solvent effect concentrator.

RESULTS

Unless warmed very slowly, a process which was incompatible with accurate measurement of ageing, the pastings were liable to shatter when removed from liquid nitrogen temperatures. More than half the collected pastings were rendered useless for sampling in this way and in some cases the fragmentation was so vigorous that none of the pasting remained on the grass stem.

As will become evident later, and can be seen in Figs. 10.3 and 10.4 there was a very marked variability in the properties of different marks, even those deposited within a short time of one another by one animal. Consequently it has not been possible to compile a set of data which can be taken as representative of the properties of the pastings. The results presented here therefore illustrate only certain aspects of the volatile emission behaviour of individual marks.

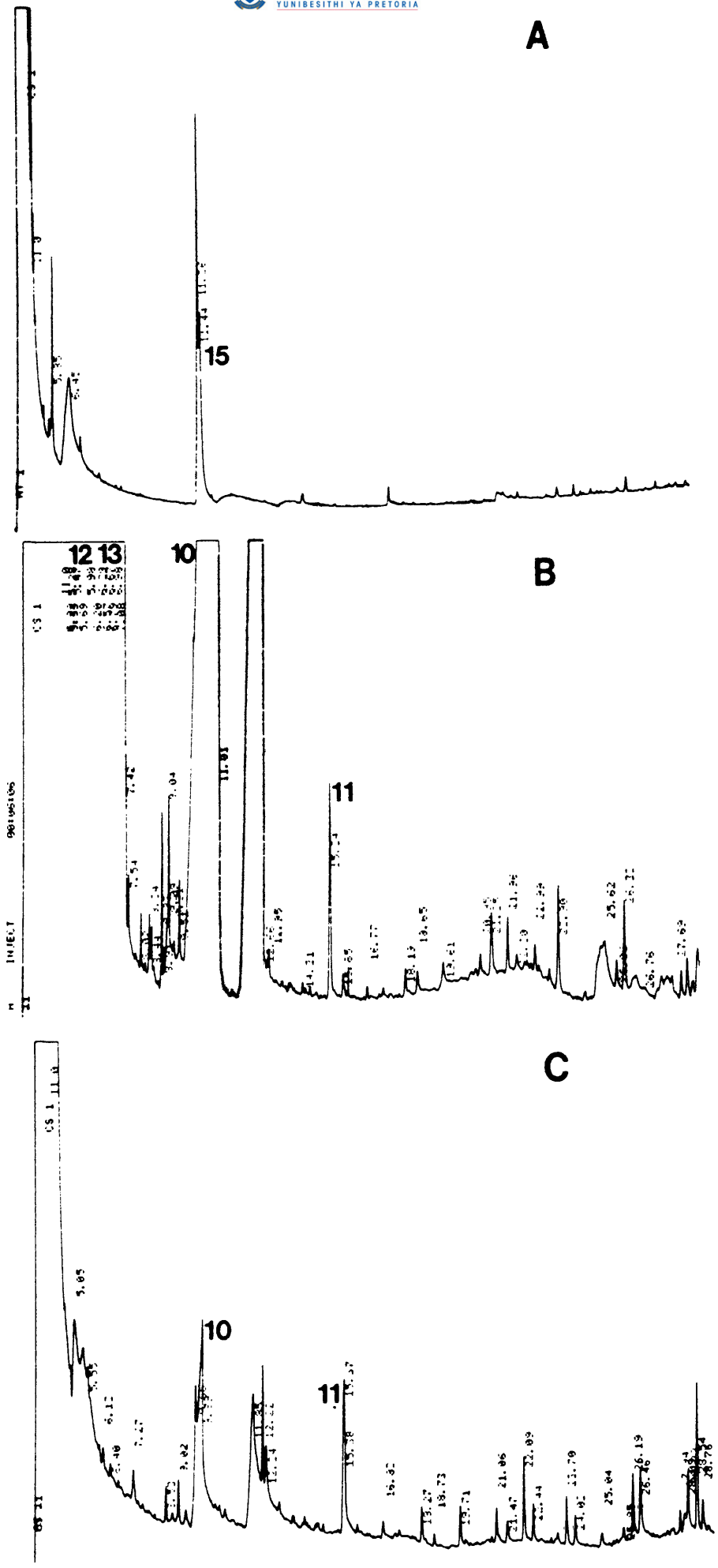


Fig. 10.3 (continued on next page)

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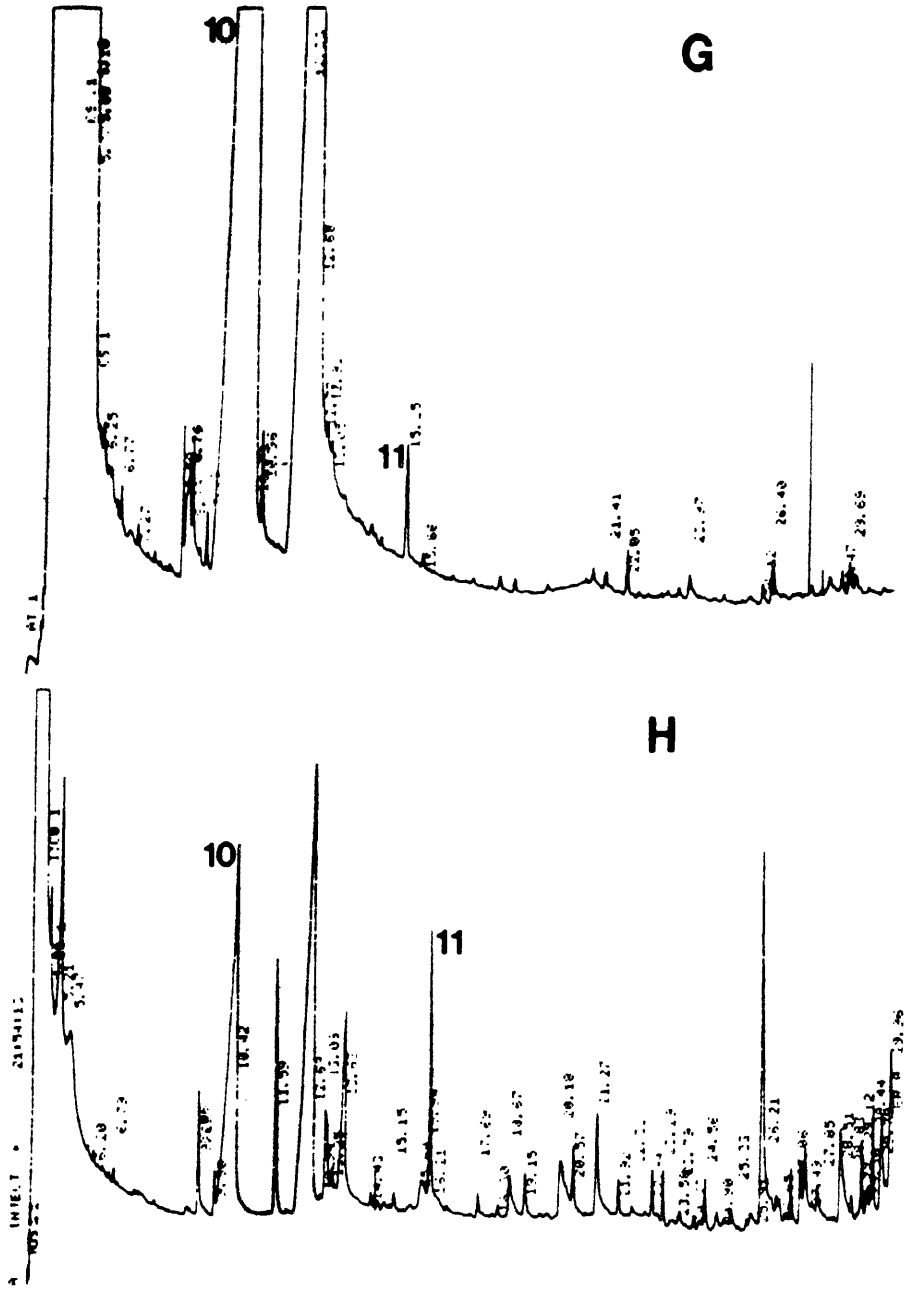

Fig. 10.3 Chromatograms of volatiles from brown hyaena pastings. All pastings were sampled by the dynamic solvent effect at a flow rate of 10 cm^3 min^{-1} and a temperature of 30°C with *n*-hexane as solvent. Separation was on a 25 m x 0,3 mm x 0,4 µm methyl silicone column with an initial temperature of 40°C held for 6 min then programmed at 5°C min^{-1}. The detector sensitivity was 1 x 10^{-11} A mv^{-1} except for E where it was 4 x 10^{-11} A mv^{-1}. A: black paste, sampling time 4 min; B: white paste, 10 min; C: black paste, 20 min; D: white paste, 20 min; E: black paste, 25 min; F: white paste, 15 min; G: white paste, 10 min; H: white paste, 15 min. C and D, and E and F are separated parts of single marks. Peak numbers refer to Table 10.1 and to discussion in the text.

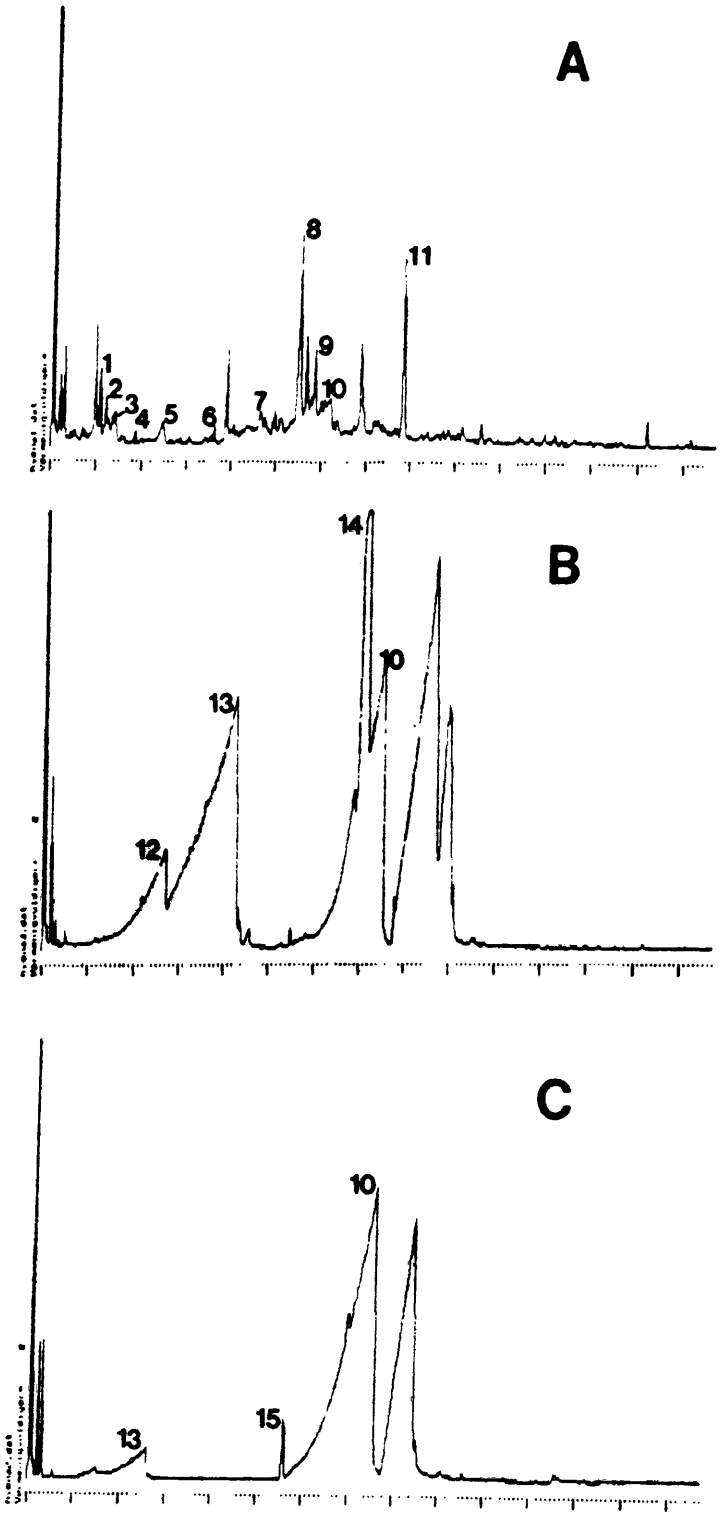

Fig. 10.4 Total ion chromatograms of volatiles from brown hyaena pastings. A: whole mark overlying older material; B: white paste, female; C: white paste, male. For analytical conditions see text. Peak numbers refer to Table 10.1 and to discussion in text.

Composition

The components of the pastings' emissions for which tentative identifications are available are given in Table 10.1. The dominant components of 6/8 of the white pastes, 4/6 of the black pastes and 0/1 of the whole marks investigated were fatty acids. These acids were emitted at rates of up to 60 ng min^{-1}, rendering the volatiles chromatographically intractable to a degree which was unusual even among mammalian odours.

Table 10.1 Tentative identifications of volatiles from pastings of the brown hyaena (*Hyaena brunnea*). Peak numbers correspond to Fig. 10.4.

Peak	Compound
1	2-butyltetrahydrofuran
2	3-hexanone
3	4-methyl-3-pentenal
4	hexanal
5	2-hexenal
6	heptanal
7	benzaldehyde
8	6-methyl-5-hepten-2-one
9	octanal
10	hexanoic acid
11	nonanal
12	2-methylpropanoic acid
13	butanoic acid
14	5-thiomethylpentane-2,3-dione
15	2-methyl-2,5-cyclohexadiene-1,4-dione

Effect of Humidity

After stabilizing in dry air a whole pasting was sampled in dry air and immediately afterwards in moist air. After 23 hours in dry air it was again sampled in dry and in wet air. As can be seen from Fig. 10.5 an increase in humidity was accompanied by a decrease in the emission rates of the pasting's major, fatty acid components. Among the minor components two increased in emission rate in moist air, six decreased in emission rate in moist air and 13 increased in emission rate on one day and decreased on the other. There were three pairs of peaks within which the relative rates of emission were independent (within a precision level of 20%) of the humidity and of the one day increase in the age of the mark.

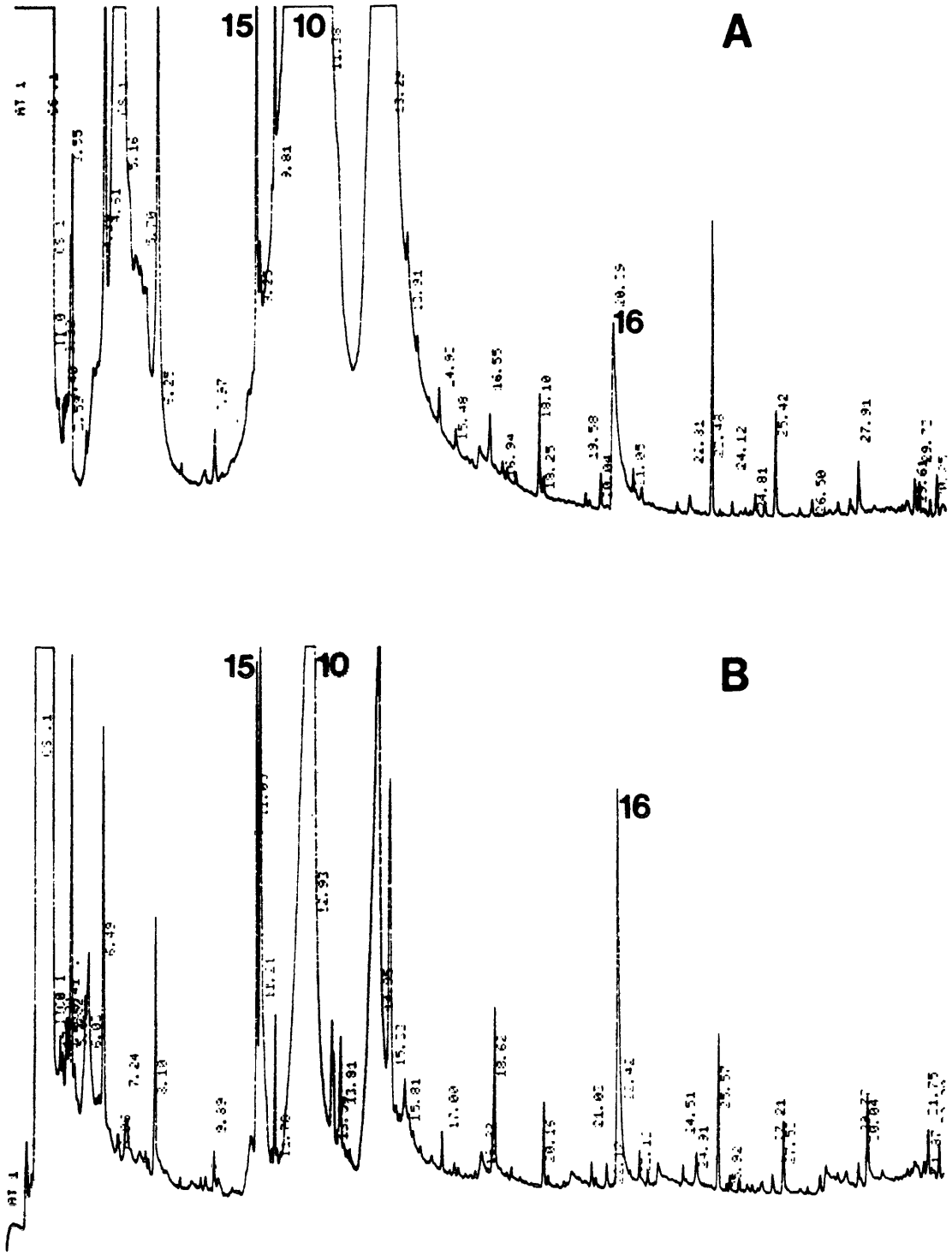

Fig. 10.5 (continued on next page)

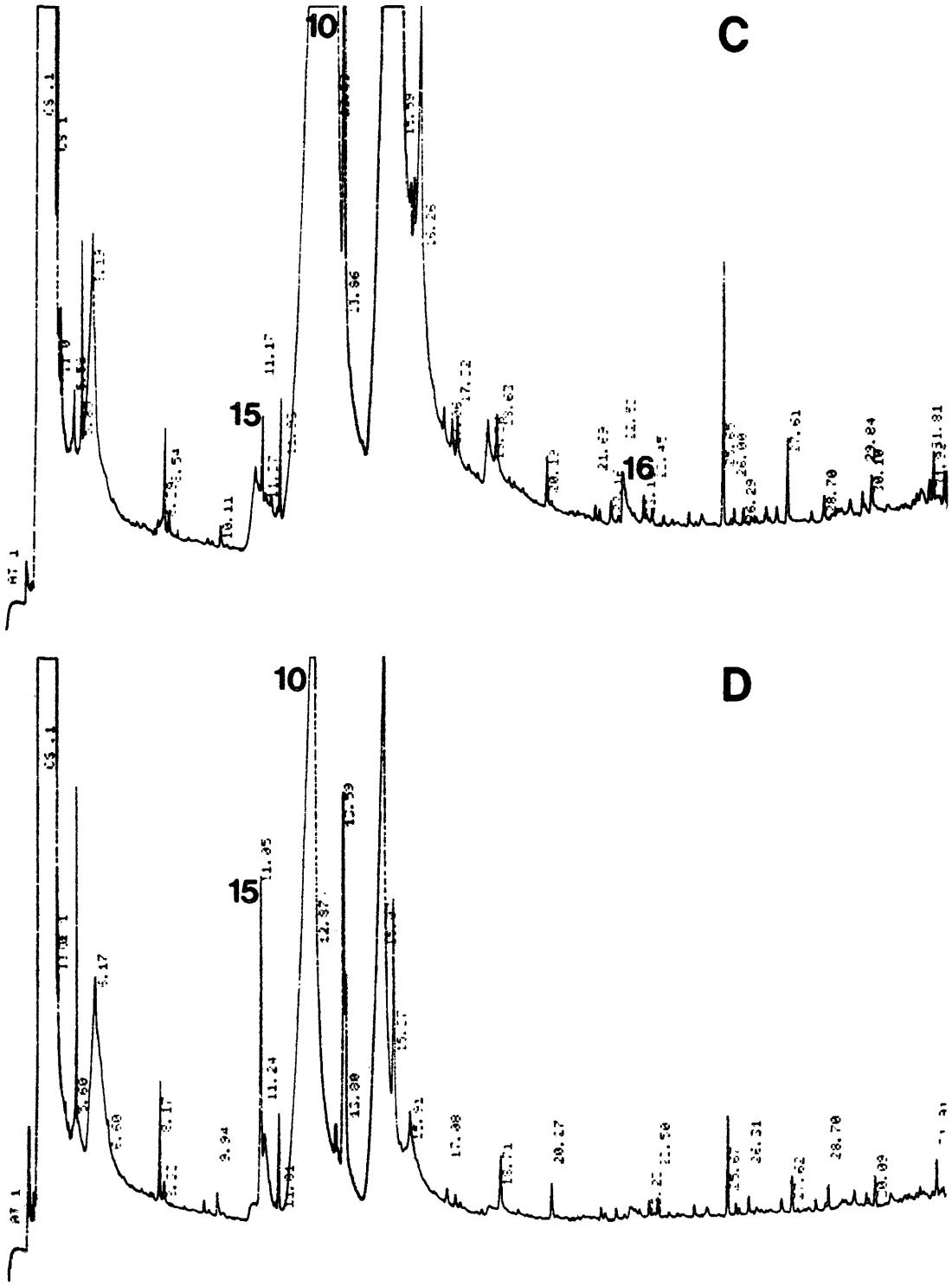

Fig. 10.5 Chromatograms of volatiles emitted from a whole brown hyaena pasting in dry air (A and C) and moist air (B and D). For analytical conditions see text.

Effect of Ageing

Changes in appearance

Apart from a slight dulling of the, initially glossy, surface the black pastes underwent no noticeable changes in appearance during ageing. White pastes which were aged in clean, dry air began to turn pale grey on their second day. At the same time the paste began to take on a foamy appearance. but without a noticeable change in volume, due to the internal generation of gas bubbles. The grey colour and the foamy appearance persisted and after 4-5 days the initially soft, creamy paste had hardened into a material resembling grey meringue.

Emissions decreasing with time

As expected the most common behaviour was for the emission rate to decrease with time. The decrease was, at least approximately, exponential (Fig. 10.6).

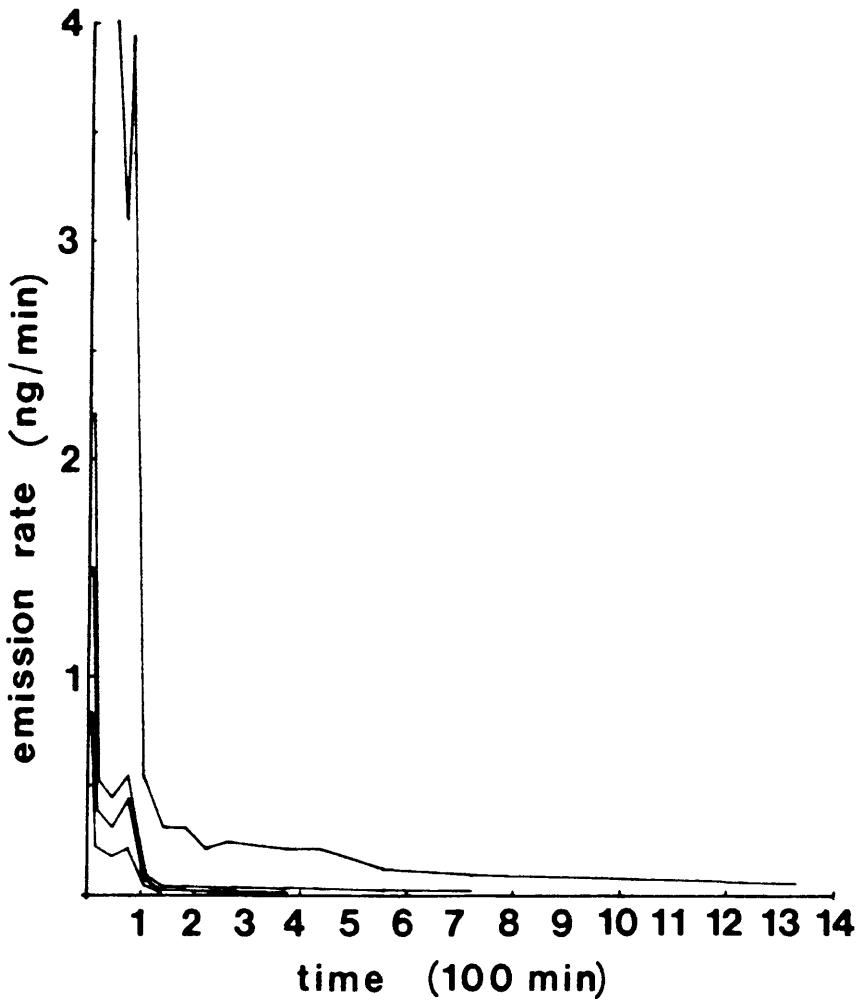

Fig. 10.6 Plots of emission rate *vs* time for volatiles from brown hyaena scent marks exhibiting approximately exponential decreases. From highest to lowest the lines correspond to peaks i, j, c and d in Fig. 10.9. For analytical conditions see text.

Fluctuating emissions

The emission rates of two peaks (15 and 16 Fig. 10.3) from one black, and one white, paste fluctuated widely with time (Fig. 10.7). In the case of the black paste the fluctuations were superimposed on trends of declining emission rate. Emissions from the white paste showed no declining trend. The retention time of peak 15 corresponded to that of peaks from other marks identified by GC-MS as 2-methyl-2,5-cyclohexadien-1,4-dione.

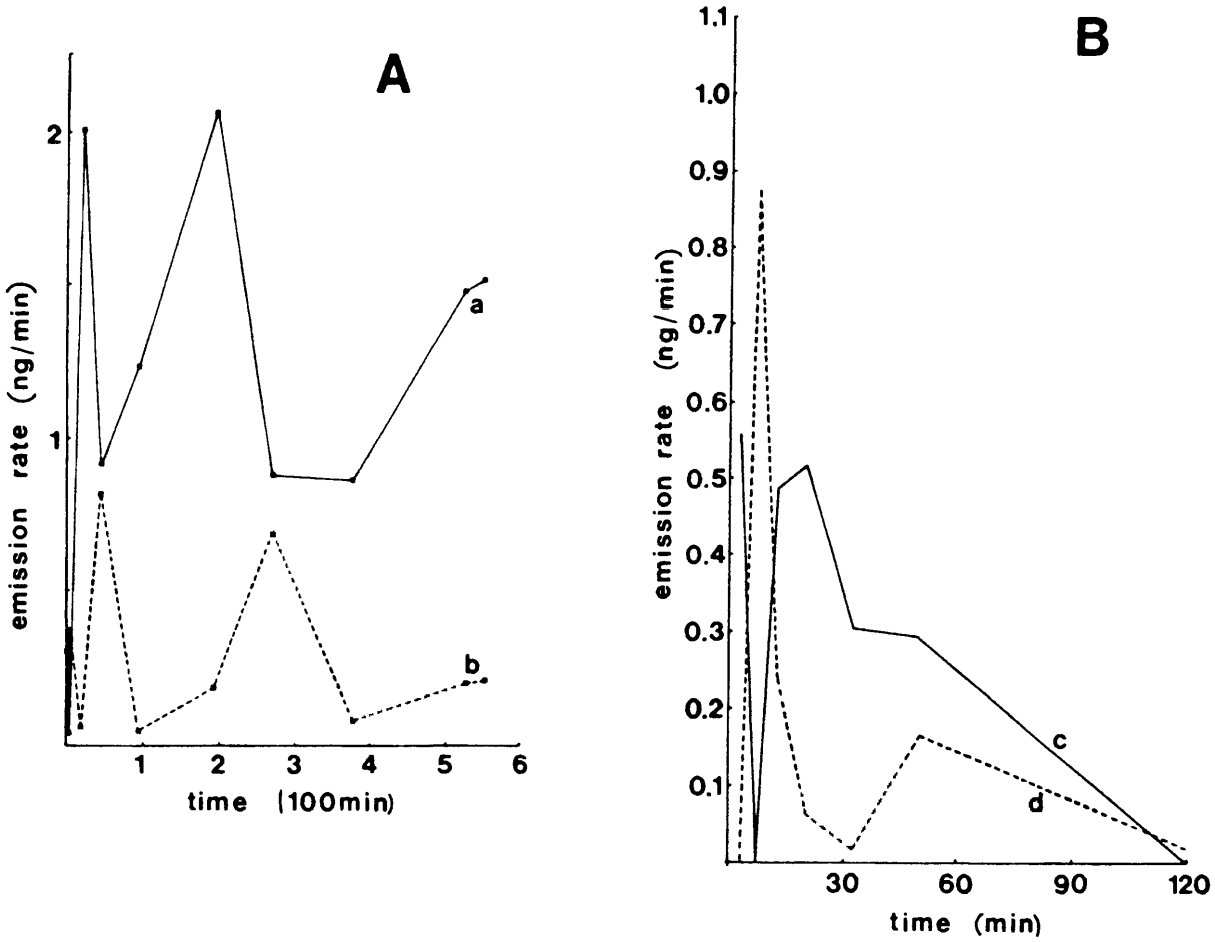

Fig. 10.7 Plots of emission rate *vs* time for volatiles from brown hyaena scent marks exhibiting fluctuating emission rates. A: white paste; B: black paste. a: peak 15 Fig. 10.3; b: peak 16 Fig. 10.3; c: peak 15 Fig. 10.3; d: peak 16 Fig. 10.3. For analytical conditions see text.

Emissions increasing with time

In one case the peak corresponding in retention time to no. 16 in Fig. 10.3 showed a fluctuation emission rate superimposed on a trend of increasing emission rate (Fig. 10.8).

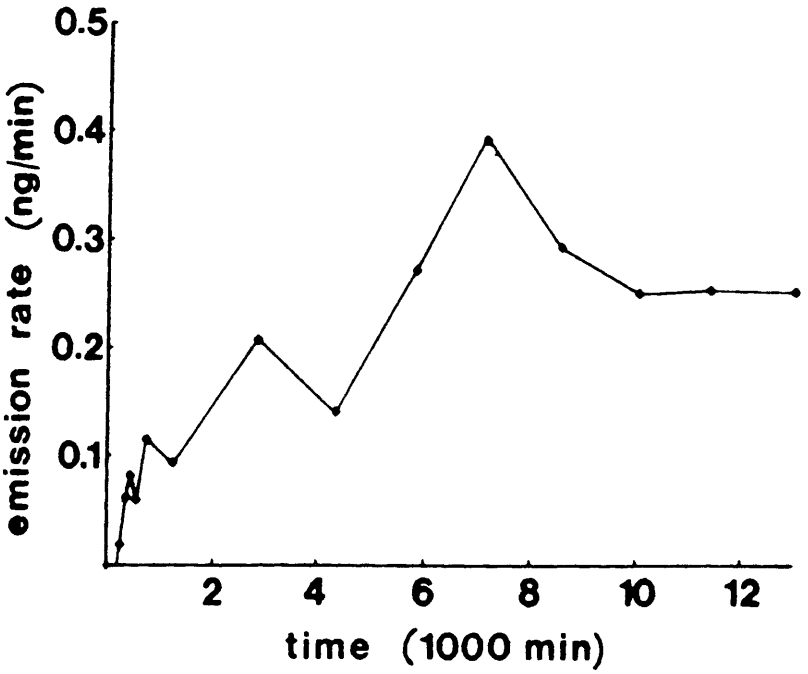

Fig. 10.8 Plots of emission rate *vs* time for a volatile compound from a brown hyaena white paste showing emissions fluctuating about an increasing trend. The corresponding peak is 16 in Fig. 10.3. For analytical conditions see text.

Relative emission rates remaining constant with time.

From one white paste a group of peaks were obtained whose individual emission rates declined exponentially with time but whose emission rates relative to one another remained constant with time (Figs. 10.9, 10.10; Table 10.2). The same group of peaks also occurred in a chromatogram from the black component of the same pasting, but their ageing was not followed. Among the volatiles from the white paste there was (at least) one pair of peaks whose emission rates relative to each other remained constant but whose ratios to the components of the main group varied with time (Figs. 10.9, 10.10; Table 10.2).

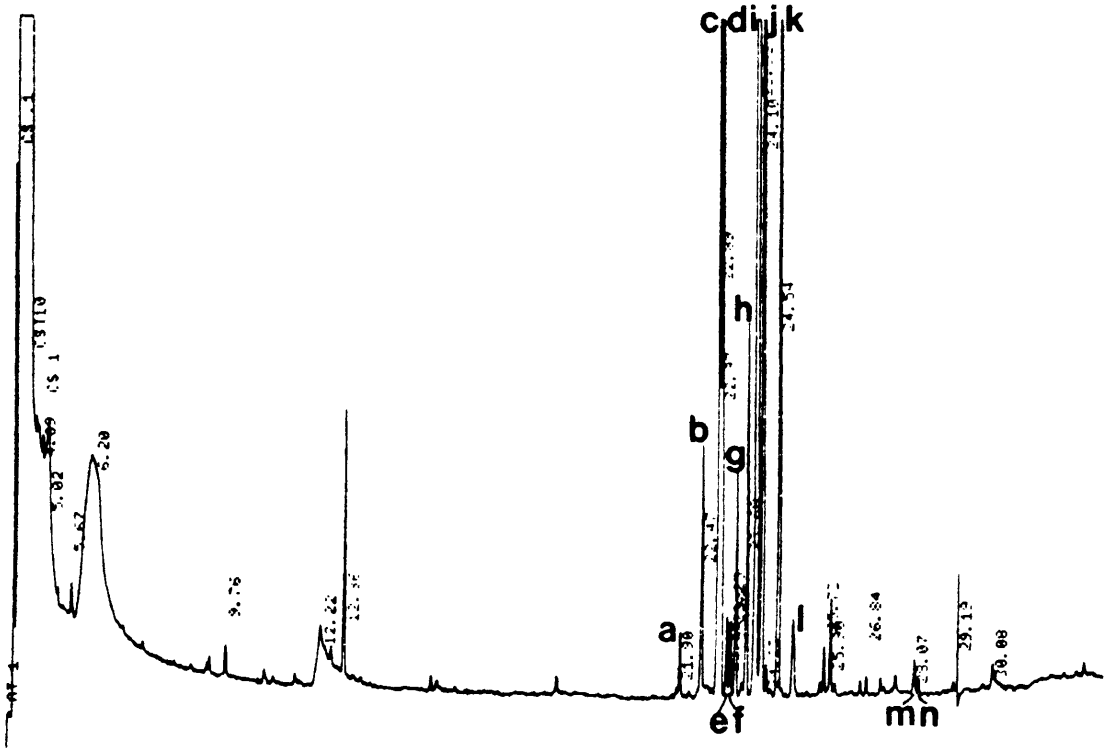

Fig. 10.9 Chromatogram of volatiles from the white paste of a brown hyaena scent mark. Peak numbers correspond to Table 10.2. For analytical conditions see text.

Table 10.2 Equations of lines of best least squares fit, to ln-ln transformations of decreases with time in emission rates of volatiles from brown hyaena (*H. brunnea*) pastings, and coefficients of variation of peak areas normalized against peak i (Fig. 10.9). If two compounds maintain a constant ratio of emission rates the gradients of the corresponding lines are equal and the coefficent of variation of their relative peak areas is small. Peak numbers correspond to Fig. 10.9.

Peak no.	Equation of line	CV of ratio to area of peak i
c	y = 9,53 - 0,99x	7,5%
d	y = 8,97 - 1,01x	5,8%
i	y = 12,07 - 1,04x	------
j	y = 10,04 - 1,02x	3,3%
k	y = 9,00 - 0,91x	8,0%
l	y = 6,34 - 0,69x	47,1%
m	y = 6,02 - 0,50x	108,8%
n	y = 5,16 - 0,47x	119,1%

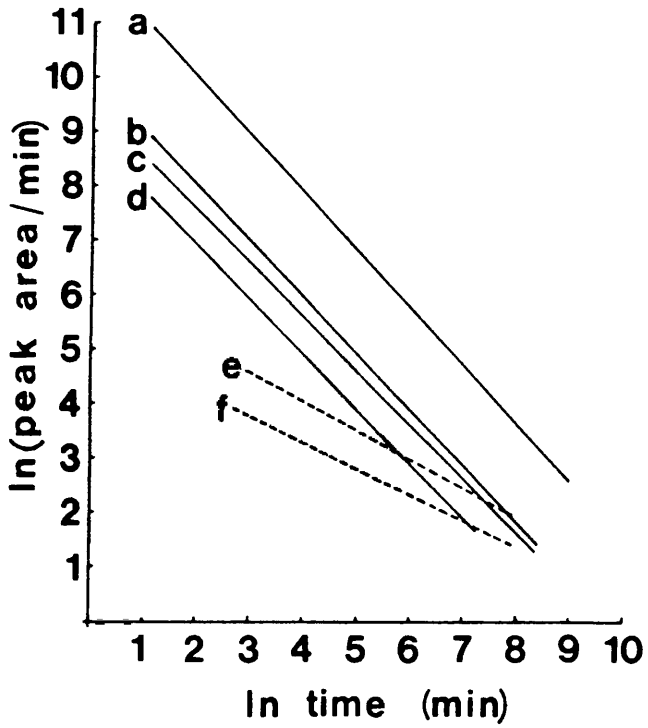

Fig. 10.10 Lines of best, least-squares fit to ln-ln transformations of emission rates *vs* time for volatiles from the white paste of a brown hyaena scent mark. Corresponding peaks in Fig. 10.9 are; line a: peak i; line b: peak j; line c: peak k; line d: peak d; line e: peak m; line f: peak n. The equations of the lines are given in Table 10.2. Lines which are parallel are from components which maintain constant ratios of emission rates. For analytical conditions see text.

Such a group of peaks, with emissions in excess of 1 ng per sampling session, was obtained from only one pasting. Other pastings yielded groups of peaks but their emission rates, even from fresh pastes, were so low that their quantification was impractical.

DISCUSSION

Appearance

Under natural or semi-natural conditions the appearance of the white paste of brown hyaena pastings does not change in the same way as it did under the controlled conditions used for sampling. The darkening of the paste continues until it is almost black, there is no foaming and instead of maintaining its original shape and volume and becoming hard the paste shrinks but remains slightly soft. Clearly the artificial conditions of the experiments affected the marks' behaviour and some of the results may, therefore, be analytical artifacts.

Composition

An interesting feature of the composition of the brown hyaena's pasting was the presence of 5-thiomethylpentane-2,3-dione, which has been reported from the striped hyaena (*Hyaena hyaena*) (Wheeler *et al.* 1975). This compound was present in only one of three brown hyaena pastings used for qualitative analysis and of the two of these which were fresh marks it was absent from the one produced by a male. It is perhaps significant that Wheeler *et al.* collected pastings from only a single, female striped hyaena.

Extracts of both black and white pastes from two Kalahari brown hyaenas were analysed by Mills *et al.* (1980). The separation was carried out under conditions similar to those employed here; a 25 m methyl silicone column with a temperature programme of $5^{o}C\ min^{-1}$, but no identifications were carried out. The most obvious difference between their results and those presented here is the relatively low contribution made by more volatile components to the extracts as compared to the head space samples. This difference is expected, although not to the extent shown in the case of the black paste. In the chromatograms which Mills *et al.* present there are no peaks with the front-tailed shape characteristic of fatty acids on non-polar phases. A discussion of the causes of these differences can only be speculative, and they may well represent only analytical differences or an extension of the variability between different marks noted here.

Effect of humidity

Regnier & Goodwin's (1977) experimental work with a single, radio-labelled, "active" component of artificial marks established unequivocally that in such a system an increase in ambient humidity increased the volatile emission rate. Obviously the behaviour of the brown hyaena's pasting is considerably more complex than this. The volume of the pasting compared to the thin grass stem on which it is deposited suggests that the effects of the pasting's matrix would dominate those of the substrate. The matrix of the white paste is apparently mostly lipid and polysaccharide, while that of the black paste (based on the histochemistry of the tissue which produces it) is more complex, and is dominated by polysaccharides and lipo-fuschin (Mills *et al.* 1980). In the white paste at least a substantial part of the matrix consists of the fatty acids which are the dominant volatile emissions. Fatty acids are volatile only when unionized, and the degree to which they are ionized is sensitive to pH and the presence of water. Thus, when the mixture of fatty acids in a paste is exposed to an increase in humidity the degree to which they are ionized may be expected to rise and their volatilities to fall as a

result. In such a complex mixture these changes may well have interactive effects on the behaviour of both the acids and the minor components dissolved in them.

Whether the bulk of the paste, or only a thin, outer layer, is affected by changes in humidity is uncertain. All emissions must, of course, take place from the surface and it may well be that the properties of the surface alone determine the volatile emission patterns. Such a situation would be expected to affect the sensitivity and the speed of response of the emissions to rapid changes in ambient conditions such as occur when an animal breathes on, or licks, a mark.

Effects of Ageing

That the emission rates of most of the volatiles should decrease exponentially with time is to be expected. Even in the absence of any other changes the resulting decrease in the intensity of the mark's odour would provide an indication of the time since the mark was deposited.

The narrow limits within which the relative emission rates within some groups of peaks remain constant, despite 300 fold decreases in the absolute rates, suggest that the aspects of the mark's odour for which they account will also remain unaltered with time. If this is the case such components of the marks may well provide the persistant identity labelling of the mark required by Gosling's (1982) scent matching hypothesis.

When two or more sets of components maintain through time ratios of emission rates which are constant within the sets but differ between them the odour of the mark carries at least two persistant labels (perhaps for example individual identity and group membership). In addition the changes with time in the inter-set relative emission rates will generate changes in the quality of the odour superimposed on the decline in its intensity resulting from the overall decrease in volatile emission rates.

The case of increasing emission rate was possibly due to formation of increasing concentrations of the compound concerned. This formation may have been by chemical transformations within the pasting, by atmospheric oxidation or by bacterial action. It is also possible that the increasing emission rate was due to some change in the pasting's matrix which increased the vapour pressure of the compound. Such a change could simply have been a decrease in matrix volume brought about by evaporation of water and fatty acids.

Components for which the changes with time in emission rates are in opposite directions must have relative emission rates which vary particularly strongly with time. Therefore the presence of components whose emission rates increase with time could generate more distinct changes in odour quality, and thus provide a more readily perceptible indication of a scent mark's age, than would different rates of decrease in emission rate alone.

All the changes with time in emission rate discussed so far have been unidirectional. If they provide a measure of a mark's age they must do so in the same way as an hourglass or a stopwatch. The age indicating properties of the brown hyaena's pasting may be more complex than this. If the fluctuations in measured emission rate shown in Fig 10.6 really do reflect fluctuations in emission rate for the compounds concerned, these cyclical fluctuations could cause repeated. rapid changes in odour quality and / or intensity. Such cyclical changes could enhance the precision of measurement of the mark's age in the same way as the minute hand of a clock provides more precise measurement of time than does the hour hand alone.

Figure 10.6 gives the impression that the emission rates of the two compounds involved are, to an extent, complementary, one increasing while the other decreases. What the mechanism of such a system could be is unknown; only one of the compounds involved, 2-methyl-2,5-cyclohexadiene-1,4-dione, has been identified.

It must be kept in mind that the complex volatile emission patterns observed here were displayed by pastings aged at uniform temperature in clean, dry air. In the wild, pastings would be subject to fluctuating temperature and humidity (not least while being sniffed), contamination by airborne dust, and continual innoculation with airborne microorganisms. None of these processes can be thought likely to simplify the emission pattern but whether the additional complexity increases or decreases the information content of the emissions is a matter for conjecture.

The chemical evidence presented here is compatible with both Gosling's (1982) scent matching hypothesis and Mills *et al.*'s (1980) (Gorman & Mills 1984) movement record model. The changes with time in emission pattern, which could indicate a pasting's age to a hyaena able to decode them, are unexpectedly complex. In addition the pastings emit a number of compounds whose information content has not been discussed here, and almost certainly some which have not even been detected. An extremely sophisticated series of bioassays would be necessary to demonstrate how much information is available to a hyaena sniffing a pasting, and which of the pasting's components carry that information.

Even in the present context of testing the performance of a new sampling system it would be tempting to dismiss the variability among pastings as an analytical shortcoming, were it not for the corroborating observation that the pastings also vary erratically in another property not directly connected with volatile emissions. Under 254 µm UV light some white pastes fluoresce a pale turquoise while others do not fluoresce at all. Some black pastes show a blue surface fluorescence while others do not and one mark contained clumps of pale blue fluorescing, rounded granules approximately 1 mm across.

Plainly a full understanding of the extremely complex chemistry of the brown hyaena's scent marks will require quantitative analysis of its volatile emissions to be integrated with other analytical approaches.

ANALYTICAL CHALLENGES AND SOLUTIONS

The brown hyaena's pastings presented three major analytical difficulties. The presence of large quantities of volatile fatty acids presented particular problems because these compounds overload methyl silicone phases, producing wide, front-tailed peaks which obscure those due to other compounds and, in extreme cases cause retention shifts in other peaks. That the development of the dynamic solvent effect has, hitherto, been focussed on its compatibility with non-polar solvents and stationary phases must be regarded as a practical shortcoming. The problem of the fatty acids was exacerbated by their tendency to suffer adsorption in systems which are not very well deactivated, a category into which the diculmyl peroxide cross-linked column with which this investigation was conducted unfortunately fell. Although dynamic solvent effect sampling is compatible with derivatization techniques (Centner *et al.* 1988) impurities in the reagents tend to interfere with the sub-nanogram quantitation of a mixture's minor components.

The very small emissions of the pastings' non-fatty-acid components tested the working sensitivity limits of the detector and column (Chap 2), not to mention those of the dynamic solvent effect. Whether the yield of volatiles would have been increased by a faster sampling gas flow rate is an open question. The presence of such large quantities of volatile acids was a complicating factor because it precluded the use of the higher sensitivity settings of the gas chromatograph.

The extreme qualitative and quantitative variability among the pastings also presented difficulties. It was impossible to predict accurately the sampling time necessary to provide a working compromise between the detection of minor components, overloading with fatty acids and resolution in terms of the pasting's age. In this respect it was advantageous that the operating procedures for the dynamic solvent effect are independent of the size of the sample.

Linearity of peak area against solute quantity, precision of relative peak areas for dissimilar solutes over a range of concentrations and independence from sampling time of the accumulation rate of airborne solutes (Chap 6) were features of the dynamic solvent effect which proved important in this investigation.

Chapter 11

CHROMATOGRAPHIC ANALYSIS OF SPECIES SPECIFIC ODOUR PROFILES IN *Mastomys natalensis* AND *M. coucha* (RODENTIA: MURIDAE)

INTRODUCTION

There is now such a diversity of demonstrations of specific and sub-specific odour discrimination by mammals that it can begin to be regarded as a general phenomenom. In addition to the references on rodents cited later see Müller-Schwarze & Müller-Schwarze (1975); Mech & Peters (1977); Epple *et al.* (1979); Von Holst (1985 p 206) and Halpin (1986).

Recognition of, and preference for, conspecific odours is widespread among rodents. A preference for the odour of conspecifics over that of other species has been demonstrated in *Mus musculus* (Bowers & Alexander 1967), *Peromyscus* spp. (Moore 1965; Doty 1972, 1973; Drickamer 1984), lemmings (Huck & Banks 1979), *Cavia* spp (Beauchamp, Criss & Wellington 1979; Wellington, Byrne, Preti, Beauchamp & Smith III 1979), *Meriones* spp (Dagg & Windsor 1971), *Apodemus sylvaticus* (Stoddart & Smith 1984), *Microtus* spp (De Jonge 1980) and *Mastomys* spp (Apps, Terblanche & Gordon unpubl.). Similar discrimination occurs at the subspecies level in *Spalax ehrenbergi* (Nevo, Bodmer & Heth 1976) and *Clethrionomys* (Godfrey 1958).

On this evidence semiochemicals are potentially important contributors to the recognition of conspecific mates and to the maintenance of species gene pools. Two findings are of particular relevance here: in *Peromyscus californicus* and *P. eremicus* only mice from sympatric populations prefer the odour of their own species over that of the other (Carter & Brand 1986) and in house mice (*Mus musculus*) odour preferences contribute to ethological isolation between adjacent demes (Cox 1984).

Although the demonstration of odour preferences is a straightforward, but nonetheless powerful, technique it suffers from the disadvantage that the odour differences between individuals can be demonstrated only by the use of other animals. This introduces a number of variables e.g reproductive condition, learning, diet and social status which may not be rigorously controllable, may vary with the odour difference and may themselves be relevant to mate recognition. In addition preference studies allow only a ranking of odour signals.

An instrumental technique for odour analysis can yield data which are rigorously quantitative and independent of the interfering variables affecting the preferences of test animals. Chemical identification of relevant compounds for bioassay is also possible. The aim of this investigation was to establish the validity of such an analytical approach by studying odour differences between the cryptic species *Mastomys natalensis* and *Mastomys coucha*.

M. natalensis and *M. coucha* are abundant in southern Africa and of considerable medico-zoological importance (Keogh & Price 1981). The two species differ in karyotype; (*M. coucha* 2n = 36, *M. natalensis* 2n = 32), haemoglobin electromorphs, courtship behaviour and sperm morphology but are indistinguishable on external morphology (Lyons, Green, Gordon & Walters 1977; Gordon 1978, 1984; Green, Keogh, Gordon, Pinto & Hartwig 1980; Lyons, Gordon & Green 1980; Smithers 1983 p 253-256; Gordon & Watson 1986). Although the two species are widely sympatric and produce viable, though infertile, hybrids when made to interbreed in captivity no wild hybrids have been found. In this respect *Mastomys coucha* and *M. natalensis* are typical members of an order whose taxonomy and systematics are complicated by uniform external morphology; leading to the description of numerous cryptic, sibling species.

The habits and habitats of most rodents have led them to rely to a large extent on auditory and olfactory signals for social and reproductive communication (Stoddart 1974) and it is to these signals that attention should be directed if rodent species characteristics are to be understood (Lambert, Michaux & White 1987). In *Mastomys* differences in olfactory preferences have been demonstrated and although both species show conspecific odour preferences *M. coucha* is much less discriminating than *M. natalensis* (Apps, Terblanche & Gordon unpubl.). Whether this asymmetry is a property of the chemical signal or of the receiver's responses provided a model problem for the analytical approach.

METHODS

While other investigators of the chemistry of rodent semiochemicals have restricted their attention to either urine (Liebich *et al.* 1977; Schwende, Jorgenson & Novotny 1984; Novotny *et al.* 1986) or glandular secretions (Stoddart 1977) the problem of pre-judging the source of the species-specific odours in *Mastomys* was avoided by sampling the whole-body odours of live animals.

Volatiles were sampled by the dynamic solvent effect. Each mouse was transferred from its home cage to a 500 cm^3, wide-necked glass jar and charcoal filtered air was blown through the jar, over the mouse and through a dynamic solvent effect concentrator at a flow rate of 20 cm^3 min^{-1} for 20 min (Fig 11.1). *n*-Hexane was used as solvent in the concentrator. Condensed water was removed from the concentrator by a 10 cm^3 min^{-1} flow of palladium cell purified hydrogen for 15 min. Volatiles accumulated from the mouse's odour were quantitatively analysed by high resolution, gas-liquid chromatography using a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet. The column was 25 m x 0,3 mm x 0,4 µm methyl silicone with hydrogen as carrier gas at a linear velocity of 50 cm s^{-1}. Detection was by FID at a sensitivity of 10^{-11} A mv^{-1} and the signal was fed to a Varian 4270 integrator with a full scale deflection of 1 mv. The starting temperature was 40°C for inlet and column, the inlet was heated ballistically to 220°C after 2.2 min and the column was temperature programmed at 5°C min^{-1} after 6 min. A blank of the complete analytical and sampling system was run every day.

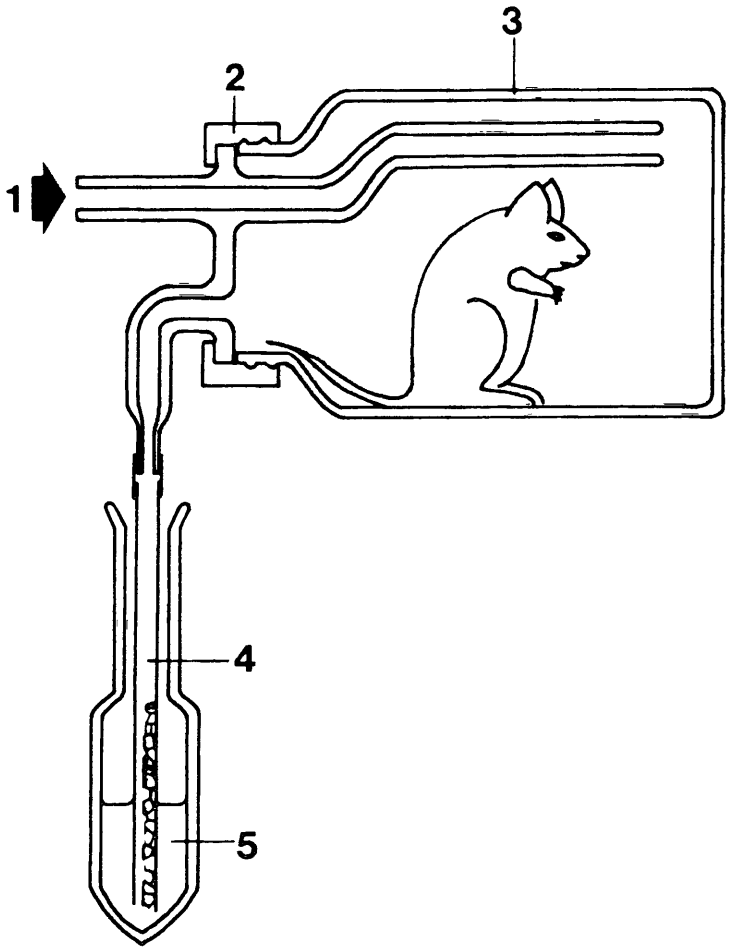

Fig. 11.1 Apparatus used to sample whole-body volatiles from *Mastomys*. 1: 20 cm^3 min^{-1} charcoal-filtered air; 2: screw cap; 3: 500 cm^3 glass jar; 4: dynamic solvent effect concentrator; 5: *n*-hexane solvent.

A total of 17 adult male mice; 6 *M. coucha*, 10 *M. natalensis* and one hybrid, were used as odour donors. Most of the donors were sampled three times, one of the *M. coucha* was sampled four times and six of the *M. natalensis* were sampled twice each. At least one day elapsed between successive samples from the same animal. All the mice were colony bred; all the *M. coucha*, four of the *M. natalensis* and the hybrid at the Transvaal Museum (TM) and six of the *M. natalensis* at the South African Institute for Medical Research (SAIMR). The TM animals were housed separately while those from the SAIMR were housed in an all male group. All animals were supplied with rat pellets and water *ad lib.*

The series of chromatograms was examined for peaks whose areas differed consistently between, but not within, species. The compound represented by the peak which most nearly fulfilled this criterion was identified by gas chromatography-mass spectrometry using a Varian 1400 chromatograph with open split interface to a VG Micromass 16F mass spectrometer operated in the electron impact mode with an electron energy of 70 eV and a source temperature of 200°C. Analytical conditions were equivalent to those above except that the sampling time was extended to 40 min and the chromatographic carrier gas was helium.

Since, for both species, the distribution of peak areas was heavily skewed the significance of the difference between the species was tested by the Mann-Whitney U test using the mean peak area for each individual.

RESULTS

The whole body odours of both species yielded very complex chromatograms after high resolution separation (Fig. 11.2).

Inspection of the chromatograms revealed only one peak, arrowed in Fig. 11.2, whose area was species specific. This peak was absent from, or small in, chromatograms of *M. natalensis* volatiles and was always present, sometimes as the major component, in chromatograms of *M. coucha* volatiles. The overlap between *M. coucha* and *M. natalensis* in the area of this peak was small and was reduced still further if its percentage contribution to the total peak area was considered (Fig. 11.3). The mean and standard deviation of the peak area for *M. natalensis* was 148 $\pm$ 486 and for *M. coucha* 8599 $\pm$ 9630. The peak was absent from chromatograms of the odour of the single hybrid. The inter-specific difference was significant at the 0,1% level.

The species specific compound was identified as 3-nonene-2-one.

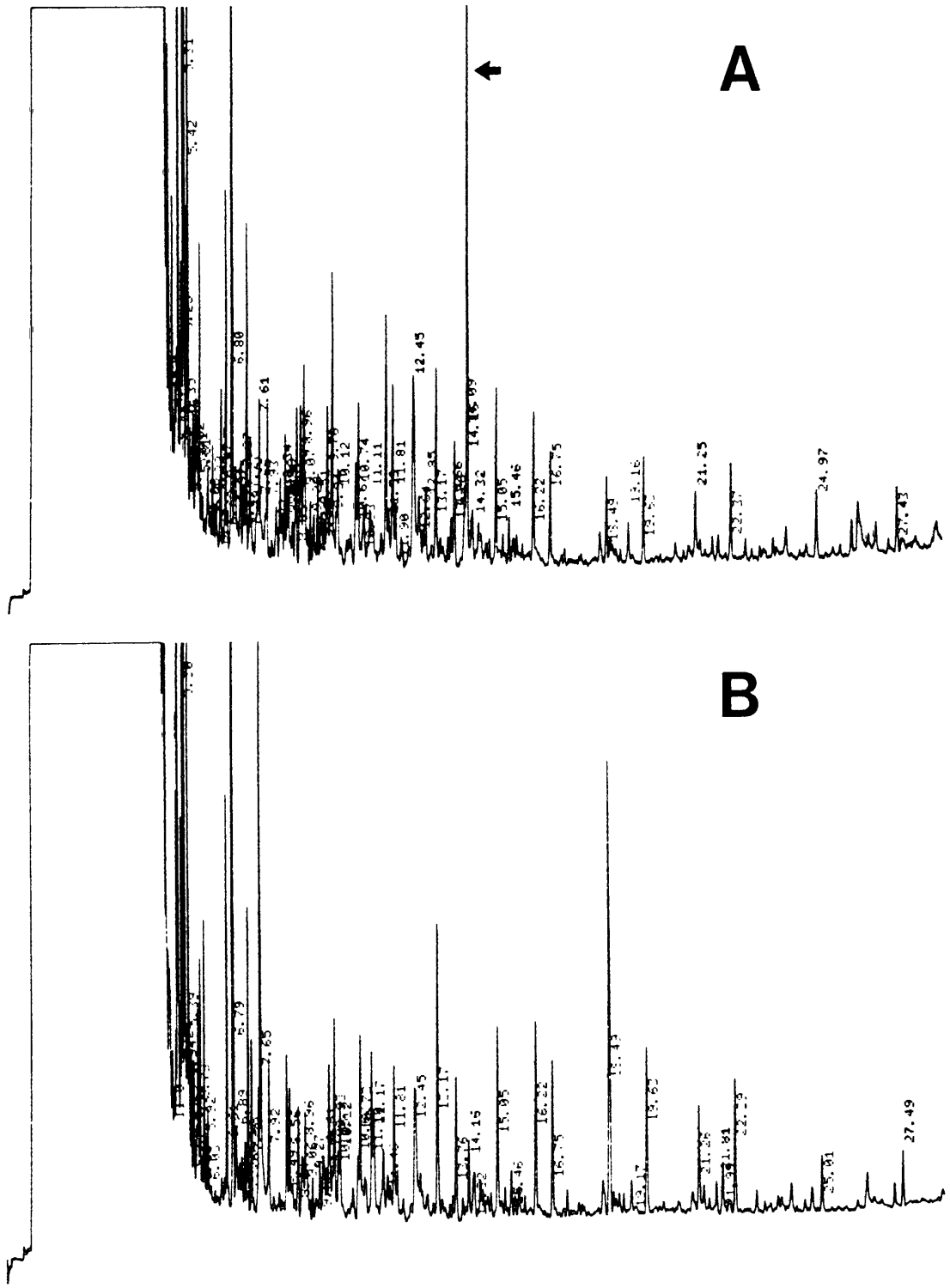

Fig. 11.2 Chromatograms of whole-body volatiles from A: male *Mastomys coucha*, and B: male *M. natalensis*. Volatiles were collected by dynamic solvent effect for 20 min at a flow rate of 20 cm^3 min^{-1} and separated on a 25 m methyl silicone capillary column with a temperature programme of 5°C min^{-1}. Detection was by FID with a sensitivity of $1x10^{-11}$ A mv^{-1} full scale deflection. The peak due to the species-specific compound 3-nonene-2-one is arrowed.

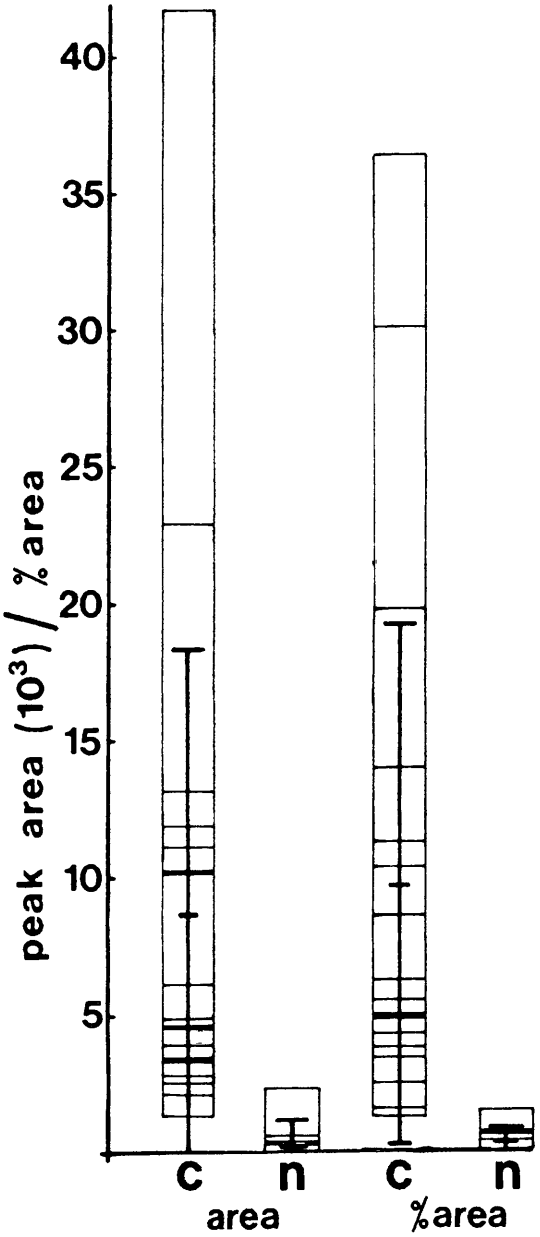

Fig. 11.3 Areas and percentage areas of 3-nonene-2-one peaks in chromatograms of whole-body volatiles from male *Mastomys coucha* (c) and *M. natalensis* (n). The heavy, vertical bar spans one standard deviation either side of the mean (truncated at zero), the light, horizontal bars are real values from each sample and the light, vertical bars span the range of real values. In 23 samples from *M. natalensis* the peak area was zero.

DISCUSSION

The possibility that the observed differences in chromatographic profile are due only to analytical variation must be acknowledged. Nevertheless neither the magnitude or the type of differences encountered can be considered likely to have arisen from this source. Dynamic solvent effect sampling has been shown to yield coefficients of variation of better than 10% for nanogram quantities of gas-phase volatiles (Chap 6) whereas the differences considered here range over 3 orders of magnitude for 0-22 ng quantities.

The area of the 3-nonene-2-one peak is a measure of that compound's concentration in the air around the odour donor, which is very probably not linearly related to the odour which would be perceived by another rodent. Even for humans there is no quantifiable relationship between the odour quality of a complex mixture and the relative concentrations of its components (Engen 1970; Amoore 1982). Any attempt to relate the quantitative composition of an odour to its quality and intensity as perceived by a non-human animal should certainly be regarded as premature. In the following discussion it is assumed only that perceived odour and chromatographic odour profile are ordinally related.

The quantitative chemistry of the species specific odour is consistent with the results of odour preference tests (Apps, Terblanche & Gordon unpubl.). Female *M. natalensis* show a stronger preference for conspecific male odour than do female *M. coucha*. If, as seems reasonable, the scatter of odour intensities generated by the males of each species reflects the range of odour intensities over which a male is recognized as conspecific, the overlap between the recognition range of female *M. coucha* and the odour range of male *M. natalensis* is much greater than the corresponding overlap for female *M. natalensis* and male *M. coucha*. Thus a male *M. coucha* is more likely to fall outside the conspecific recognition range of a female *M. natalensis* than is a male *M. natalensis* to fall outside the conspecific recognition range of a female *M. coucha* (Fig 11.3). This asymmetry in the properties of the odour signal is sufficient to account for the asymmetry of the females' odour preferences.

That such a small chemical difference should be associated with species specific odour preferences is at first sight surprising. Nevertheless in extracts of rodent secretions Stoddart (1977) also found interspecific differences to be small compared to inter-generic, sexual and age differences.

The absence from all the *M. natalensis* chromatograms of any other peak as large as that for 3-nonene-2-one in the *M. coucha* chromatograms, and the occasional presence of 3-nonene-2-one in *M. natalensis* demonstrates that the difference between the species is a quantitative rather than a qualitative one. In the latter case the occurrence of a compound unique to *M. natalensis*, and possibly chemically related to 3-nonene-2-one, might be expected.

Although the precise origin of the odour difference was not investigated, environmental influences can be ruled out since all subjects received the same diet and, except for the SAIMR *M. natalensis* were housed under uniform conditions. Because both social status (Apps, Rasa & Viljoen 1988; Chap 12) and reproductive condition (Jemiolo *et al.* 1987) have analytically detectable effects on the odours of laboratory mice, only adult males which, except for those from SAIMR, were individually housed were used in this investigation. Thus some aspect of odour production presumably is under genetic control. In house mice single gene differences at the T and the H_2 loci affect not only odour but also odour preference (Yamazaki *et al.* 1980; Lenington 1983; Drickamer & Lenington 1987) and in the H_2 case the odour differences are analytically detectable (Schwende *et al.* 1984). In both these cases the odour differences form the basis of selection of mates carrying "good genes".

The interpretation of the significance of the wide scatter of peak areas within each species is complicated by an almost equally wide range of peak areas in replicate samples from given individuals e.g. among the *M. coucha* the two highest and the third lowest levels of 3-nonene-2-one were produced by one individual and among the *M. natalensis* one individual produced both the highest and the equal lowest levels. While such fluctuations in odour emission may be under voluntary control it seems more likely that they are related to episodic changes in hormone levels; quantitative fluctuations in odour are also found in male laboratory mice (Apps, Rasa & Viljoen 1988; Chap 12) where testosterone secretion is known to be pulsatile (Bartke & Dalterio 1975). What the consequences of odour fluctuations are for recognition of conspecifics will depend to a large extent on the nature of the discrimination effected by the females; if, for example, the discrimination is based on whether or not the contribution of 3-nonene-2-one to the male's odour exceeds a certain threshhold then differences, both within and between individuals, almost as wide as those recorded here may be of no significance.

Since the principal aim of this study was to examine the validity of an instrumental, analytical approach to problems of mammalian species-specific odour signals it is of interest to compare the performance of the analytical system with that of *Mastomys* females presented with odours from males of both species in a 2 choice olfactorium. Of 7 female *M. coucha*, 5 sniffed more at the odour of a homospecific male but in only 3 cases was the difference significant ($P<0,05$). Of 4 female *M. natalensis*, 4 sniffed more at male *M. natalensis* odour and 3 of these differences were significant. If those 3-nonene-2-one peak areas for one species which fall within the range of peak areas for the other species are taken as cases of incorrect identification then 2 out of 19 samples from *M. coucha* and 1 out of 24 samples from *M. natalensis* were misidentified. If the "true" odour of each individual is taken as the mean of its sample peak areas then no individuals of either species were misidentified. This leads to the rather surprising, but nonetheless encouraging, conclusion that odour analysis by dynamic solvent effect sampling and capillary gas chromatography is actually more reliable in recognising to which species a *Mastomys* male belongs than are conspecific females in a two choice olfactorium.

Because they were so similar in the two cases the analytical challenges posed by the investigations of rodent whole-body volatiles are both considered at the end of Chapter 12.

Chapter 12

QUANTITATIVE CHROMATOGRAPHIC PROFILING OF ODOURS ASSOCIATED WITH DOMINANCE IN MALE LABORATORY MICE

INTRODUCTION

When male laboratory mice are kept in groups they form dominance hierarchies (Uhrich, 1938) which are established by overt fighting but maintained by more subtle "psychological" cues (Bronson & Eleftheriou 1965). Compared to subordinates, dominant mice have higher serum testosterone levels, higher rates of testosterone synthesis, metabolically more active testes (McKinney & Desjardins 1973), heavier preputial and ventral prostate glands (Brain 1972), lighter and less active adrenal glands (Archer 1970) and lower peripheral corticosterone levels (Nock & Leshner 1976). Labov (1981) reported heavier testes in dominant mice but Brain (1972) found no difference in testes weight, or in either body weight or readiness to attack strangers.

Only dominant males are able to accelerate puberty in young females (Lombardi & Vandenbergh 1977), an effect which varies in proportion to injections of supplementary testosterone (Lombardi, Vandenbergh & Whitsett 1976). The aversiveness of male urine to other males is also increased if the urine donor's testosterone levels are high (Jones & Nowell 1974). High levels of testosterone lead to short attack latencies (Van Oortmerssen, Dijk & Schuurman 1987; Whalen & Johnson 1987) and to a more markedly sex-biased selection of opponent (Whalen & Johnson 1987).

The urine of dominant males stimulates more attacks against standard opponents than does urine from subordinate males (Mugford & Nowell 1970). The odour of dominant males alone is sufficient to accelerate puberty in females (Vandenbergh 1969). Aggressive TA females prefer the odour of non-aggressive TNA males, and vice versa (Sandnabba 1986b). Genetically and environmentally based aggressiveness have equivalent effects on urine's aversiveness to males and its attractivenes to females (Sandnabba 1986a, b, c).

Taken together these findings indicate that dominant male mice emit odours which are different to those of subordinate mice in their physiological and behavioural effects on other mice. An analysis of these differences in odour would provide insight into the chemical signals involved in these physiological and behavioural effects. The aim of the present study was to demonstrate that dynamic solvent

effect sampling makes status-associated odour differences among individual mice accessible to quantitative analysis by high resolution gas chromatography.

Urine has been the focus of nearly all investigations of the source and nature of rodent semiochemicals (Brown 1985 p 317). However, olfactory investigation between mice involves intensive sniffing of the perianal region, the face and the general body surface, as well as the urinogenital opening. To avoid pre-judging the issue of the source of the odour differences between dominant and subordinate mice odour volatiles from live mice were sampled.

Because male mice are apt to inflict serious wounds on one another a relatively non-aggressive strain, NMRI, was employed in order to limit injuries. Only the three dominants kept together in the second phase suffered puncture wounds. On the other hand, the NMRI mice formed very labile dominance relationships and were much more difficult to rank than are those of more aggressive strains.

METHODS

Eighteen 3 week old, male NMRI mice from the HA Grove Animal Research Centre, Pretoria were divided randomly into six triads, each housed in a 420 mm x 240 mm x 100 mm polycarbonate cage on commercial cat litter, with shredded paper bedding, and food and water *ad lib*. The mice were kept in a mixed small animal room without temperature or humidity control and with a 12D:12L photoperiod.

In order to detect any "home cage" effects the residents of three of the cages were exchanged when the mice were 7 weeks old so that each cage contained one original resident and two other mice from two different cages. All mice were then marked with aqueous picrate or coloured ink, which was renewed as necessary.

From an age of 11 weeks the mice were observed daily in their home cages and each was assigned a dominance rank on the basis of which individual was the winner of spontaneous aggressive interactions. When some of the hierarchies had apparently stabilized (age 19 weeks) the mice were ranked independently by the author and A. Rasa and the whole body volatiles from each mouse were sampled on the day it was ranked (two cages were ranked and sampled each day). The mice were then re-sorted so that each cage contained three mice of equal rank. After a further 7 weeks the mice (aged 26 weeks) were again independently ranked and their whole-body volatiles sampled. Because, in the first session, the agreement between status on the basis of

winning and chromatographic odour profiles was not significant the mice were also ranked on the basis of numbers of aggressive interactions initiated. In addition, each member of each triad was transferred to one section of a cage divided into three segments by wire mesh and with the floor covered by a sheet of paper. The pattern of urine marking by each mouse was visualized by UV light (Desjardins, Maruniak & Bronson 1973).

Volatiles were sampled by the dynamic solvent effect. Each mouse was transferred from its home cage to a 500 cm^3, wide-necked glass jar and charcoal filtered air was blown through the jar, over the mouse and through a dynamic solvent effect concentrator at a flow rate of 20 cm^3 min^{-1} for 20 min (Fig 12.1). *n*-Hexane was used as solvent in the concentrator. Condensed water was removed from the concentrator by a 15 cm^3 min^{-1} flow of palladium cell purified hydrogen for 10 min. Volatiles accumulated from the mouse's odour were quantitatively analysed by high resolution, gas-liquid chromatography using a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet. The column was a 25 m x 0,3 mm x 0,4 µm methyl silicone with hydrogen as carrier gas at a linear velocity of 50 cm s^{-1}. Detection was by FID at a sensitivity of 10^{-11} A mv^{-1} and the signal was fed to a Varian 4270 integrator with a full scale deflection of 1 mv. The starting temperature was 40°C for inlet and column. The inlet was heated ballistically to 220°C after 2,2 min and the column was temperature programmed at 5°C min^{-1} after 6 min. A blank of the complete analytical and sampling system was run every day.

Chromatograms of the whole body volatiles were examined for features which varied in parallel with the dominance ranks of the mice. These features were then quantified in terms of peak areas and their ranking was compared to the dominance ranking using the binomial statistical test.

Where possible, the major components of the whole-body odours and those components most closely related to dominance status were identified by gas chromatography-mass spectrometry. A Varian 1400 GC with dynamic solvent effect inlet was coupled by open split interface to a VG Micromass 16F spectrometer operating in the electron impact mode with an electron energy of 70 eV and a source temperature of 220°C. Helium was used as carrier gas while other chromatographic conditions were equivalent to those above.

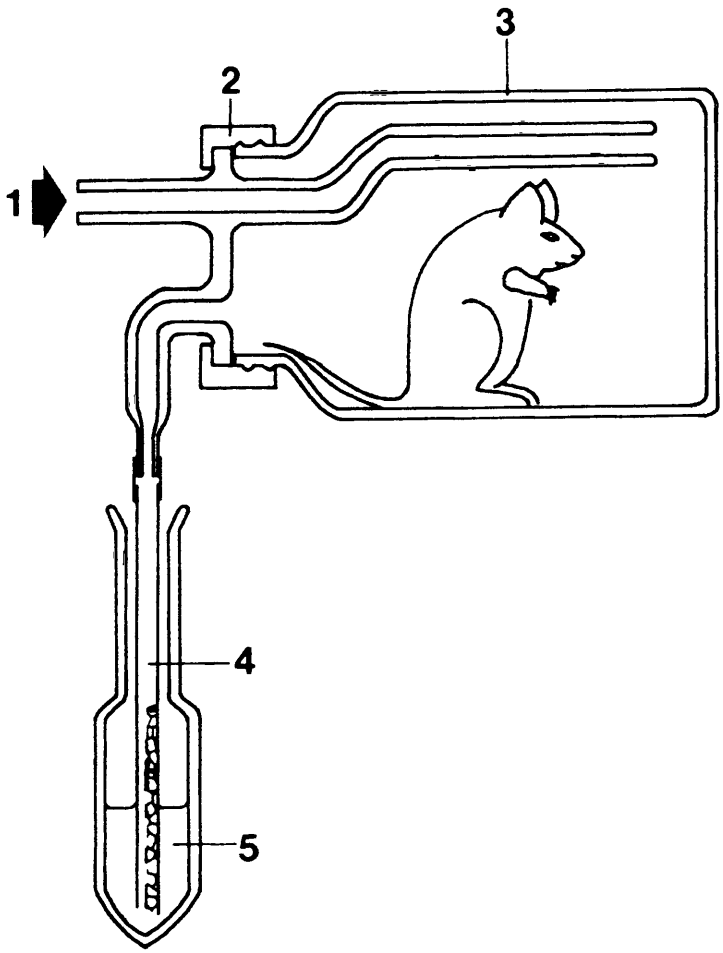

Fig. 12.1 Apparatus used to collect whole-body volatiles from live mice. 1: 20 cm^3 min^{-1} charcoal-filtered air; 2: screw cap; 3: 500 cm^3 amber glass jar; 4: dynamic solvent effect concentrator; 5: *n*-hexane; 6: glass flange.

RESULTS

The whole-body odours of the mice yielded chromatograms of moderate complexity which differed both quantitatively (total volatiles, peak areas) and qualitatively (area ratios, percentage areas) between mice. Examination of the chromatograms revealed eight peaks whose areas were markedly variable between mice (Fig 12.2). There were clear differences between mice of different ranks within a cage (Fig 12.2) but also between mice of the same rank in different cages (Fig 12.3). Of the eight peaks one (2) could not be quantified due to severe distortion. Among the remaining seven there were four (1, 3, 4 and 5) which consistently varied in parallel with one another and which were present as discrete peaks in all chromatograms. The chromatographic profiles were ranked in ascending order of the areas of these four peaks.

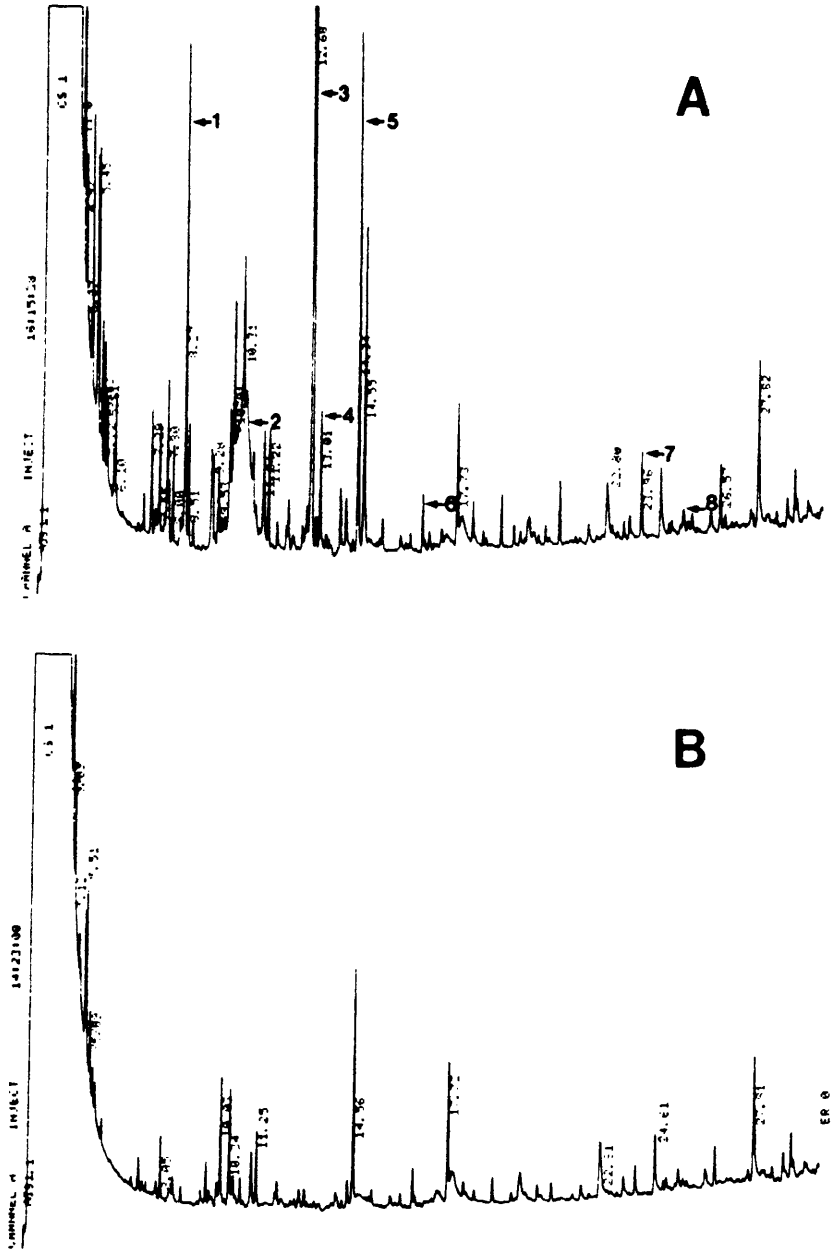

Fig. 12.2 Chromatograms of whole body volatiles from; A: mouse jY in cage c and B: mouse iB in cage c (second sampling session). Mouse jY was dominant and mouse iB was subordinate. The arrowed peaks are those which showed the most marked inter-individual variation. For analytical conditions see text.

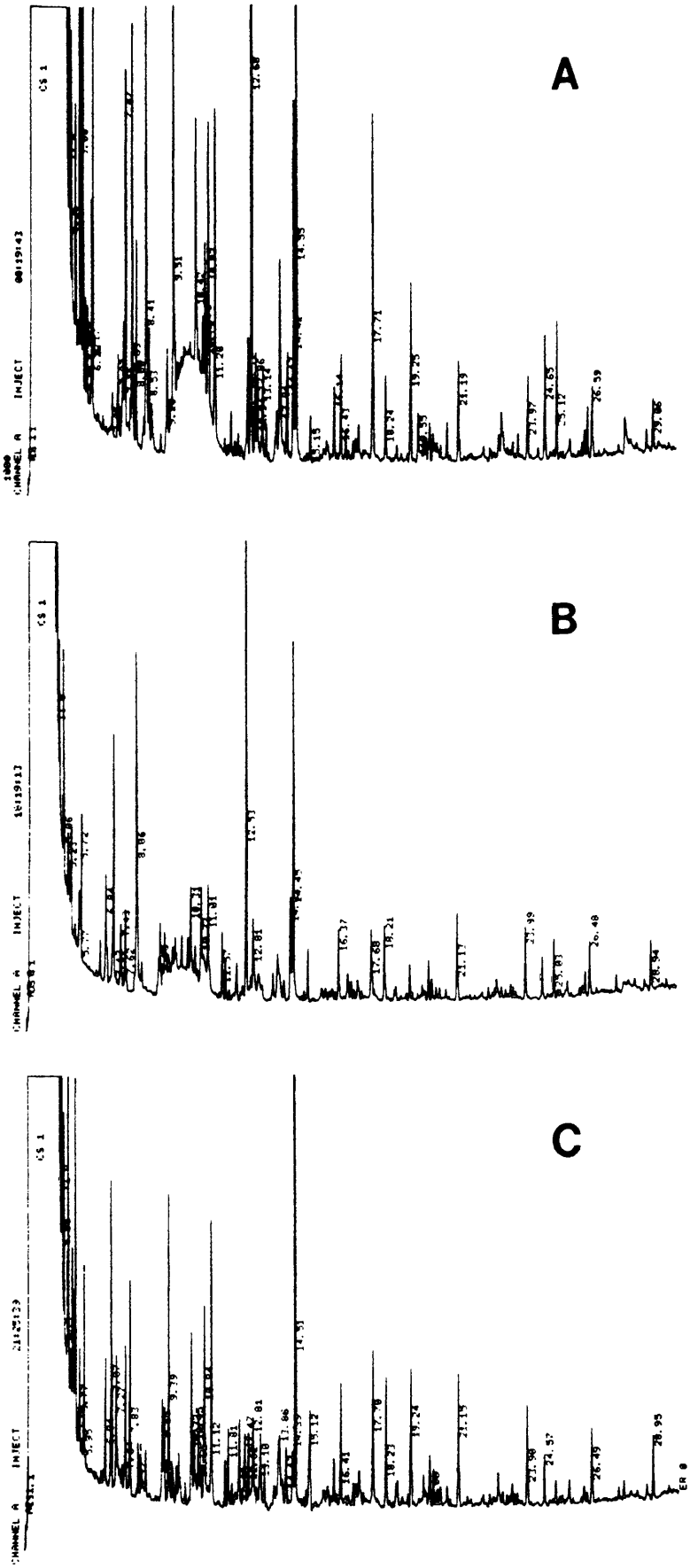

Fig. 12.3 Chromatograms of whole-body volatiles from three dominant male mice; A: mouse eG in cage e; B: mouse cY in cage c; C: mouse aB in cage a. First sampling session. For analytical conditions see text.

The winner ranks and the odour profile ranks for the first sampling session are compared in Table 12.1 and the extent of agreement among them is summarized in Table 12.2. The chromatographic profile ranks did not agree significantly often with the behavioural ranks.

Table 12.1 Rankings of 5 triads and one pair of male NMRI mice based on won encounters observed independently by the author (PA) and A. Rasa (AR) and on chromatographic odour profiles (Chrom). First sampling session. Mice are identified by their (lower case)cage letter and their (upper case) marking colour initial.

Cage	Wins AR	Wins PA	Init	Mark	Chrom
a	aB.aY	aB.aY			aB.aY
c	cY.cW.cR	cY.cR.cW			cY.cR.cW
d	dY.dR.dG	dY.dR.dG			dG.dR.dY
e	eG.(eY.eR)	eG.eR.eY			eG.eR.eY
i	iY.iG.iB	iY.(iG.iB)			iG.iB.iY
j	jB.jR.jY	jB.jR.jY			jR.(jB.jY)

Table 12.2 Numbers of cages in which the ranking method in the column heading assigned the same mouse to dominant status as the ranking method in the row heading i.e. AR's ranking on the basis of wins agreed in 6 cases with PA's and in three cases with the chromatographic odour profile. First sampling session. Abbreviations follow Table 12.3.

	Wins AR	Wins PA	Init	Mark	Chrom
Chrom	3	3			
Mark					
Init					
Wins PA	6				
Wins AR					

For the second sampling session the winner ranks, the initiator ranks, the scent marking ranks, and the odour profile ranks are compared in Table 12.3 and the agreement between them is summarized in Table 12.4. The odour profile ranks agreed significantly often with; P.A.'s winner rank (P<5%), the aggression initiator rank (P<1%) and the scent marking ranks (P<5%). The cage in which one mouse had died was excluded from the statistical analysis.

Table 12.3 Rankings of 5 triads and one pair of male NMRI mice based on won encounters observed independently by PA and AR, numbers of aggressive encounters initiated (Init), scent marking patterns (Mark) and on chromatographic odour profiles (Chrom). Second sampling session. Mice are identified by their (lower case) cage letter and their (upper case) marking colour initial.

Cage	Wins AR	Wins PA	Init	Mark	Chrom
a	eG.aB.cY	eG.aB.cY	eG.cY.aB	eG.aB.cY	eG.(aB.cY)
c	cR.iB.jY	iB.cR.jY	(cR.iB).jY	cR.iB.jY	iB.(jY.cR)
d	jB.iY.dG	jB.iY.dG	jB.iY.dG	jB.dG.iY	jB.(dG.iY)
e	dR.eY.cW	dR.eY.cW	eY.dR.cW	(eY.dR).cW	eY.(dR.cW)
i	dY.eR.iG	dY.eR.iG	dY.iG.eR	(eR.dY.iG)	dY.(eR.iG)
j	jR.aY	aY.jR	jR.aY	aY.jR	jR.aY

Table 12.4 Numbers of cages in which the ranking method in the column heading assigned the same mouse to dominant status as the ranking method in the row heading i.e. AR's ranking on the basis of wins agreed in 4 cases with PA's and in 4 cases with the chromatographic odour profile. Second sampling session. Abbreviations follow Table 12.3.

	Wins AR	Wins PA	Init	Mark	Chrom
Chrom	4	4	6	4	
Mark	5	5	5		
Init	5	4			
Wins PA	4				
Wins AR					

Those components of the odour profile which were identified are given in Table 12.5.

Table 12.5 Identities of volatile compounds from the whole body odour of male mice, based on mass spectra and chromatographic retention time.

Compound	Peak no.	Reference*
limonene		0
3,4-dehydro-*exo*-brevicomin	3	2
methyl benzenamine	4	2
m-cresol		2
nonanal		0
2-*sec*-butyl-4,5-dihydrothiazole	5	1,2
decanal		0
indole		0
copaene		0
alpha-gurjurene		0
carophyllene	7	2

* 0; not previously reported, 1; Liebich *et al.* 1977; 2; Schwende *et al.* 1986.

DISCUSSION

Of the 8 compounds identified here from the odour volatiles of male mice 4 have been previously reported. Liebich *et al.* (1977) found 2-isopropyl-4,5-dihydrothiazole and 2-(*sec*-butyl)-4,5-dihydrothiazole to be sex-dependent components of male mouse urine. Two testosterone-dependent urine volatiles were identified as 2-(*sec*-butyl)-dihydrothiazole and 7-*exo*-ethyl-5-methyl-6,8-dioxabicyclo-[3.2.1]-3-octene (*exo*-3,4-dehydrobrevicomin) by Schwende *et al.* (1986) and Novotny, Schwende, Weisler, Jorgenson & Carmack (1984). Peak numbers 1 and 2 in Fig 2 were two of four peaks with fragments of m/z 126, 111 and 97 in their mass spectra which, like those with this fragmentation pattern found by Schwende *et al.* (1986), remain unidentified.

When presented together 2-(sec-butyl)-4,5-dihydrothiazole and *exo*-3,4-dehydrobrevicomin produce the Whitten effect (stimulation of oestrus in crowded females) (Jemiolo *et al.* 1986) and increase the attractiveness to females of the urine of castrated males (Jemiolo *et al.* 1985). The same two compounds promote inter-male aggression when mixed with castrate urine (Novotny *et al.* 1985).

The area of a chromatographic peak is a measure of that compound's concentration in the air around the odour donor, which is very probably not linearly related to the odour intensity perceived by a rodent. In humans perceived odour intensity is a logarithmic function of concentration for single-component odours, in mixtures perceived intensity is additive (Amoore 1982). Odour quality is more reliably discriminated than intensity (Engen 1970) but even for humans there is no quantifiable relationship between the odour quality of a complex mixture and the relative concentrations of its components. Any rigorous attempt to relate the quantitative composition of an odour to its quality and intensity as perceived by a non-human animal should certainly be regarded as premature. In the following discussion the only assumption made is that changes in perceived odour are ordinally related to changes in chromatographic odour profile.

Different measures of "dominance" typically show considerably less close agreement than was achieved here (Benton & Dalyrymple-Alford 1981). However, since odour is known to signal dominance status between mice (Vandenbergh 1969; Sandnabba 1986a, b, c) a closer agreement between chromatographic odour profile and social rank would have been expected.

There are (at least) four possible reasons for the overall lack of significant agreement between the chromatographic and the winner ranks. First, the observed differences among profiles may have been only analytical artifacts. Secondly the differences may merely have been biological "noise" with no significance for social status. Thirdly the chromatographic profiles may have been related to a real behavioural-physiological condition which was not itself closely related to dominance. Fourthly chromatographic profile, but not wins in aggressive encounters, may be a reliable indicator of social dominance.

The possibility that the observed differences in chromatographic profile are due only to analytical variation can be dismissed. Neither the magnitude or the type of differences encountered can be considered likely to have arisen from that source. Dynamic solvent effect sampling has been shown to yield coefficients of variation of better than 10% for nanogram quantities of gas-phase volatiles (Chap 6) whereas the differences considered here range over two orders of magnitude for 0.1-45 ng quantities. Where differences between mice were within the limits of sampling precision the mice were considered to be tied in chromatographic rank. In addition, it should be noted that the odour of every mouse which yielded a dominant profile was both stronger and more typically "mousey" than that of mice which yielded submissive profiles.

The consistency of the differences between mice of different status argues against the possibility of biological noise. A biological noise explanation is also incompatible with the strong association between initiator, and scent marking rank, and chromatographic rank. Given the pervasive physiological effects of changes in status it is not surprising that dominant and subordinate mice should emit different odours, and the occurence of a compound in the odour of mice in a particular behavioural or physiological state is only weak evidence that the compound acts as a chemical signal. Strong evidence of signalling role depends on bioassay. Two of the compounds found to increase in concentration with higher, or rising, dominance rank; *exo*-3,4-dehydrobrevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole have already been demonstrated to induce the Whitten effect (Jemiolo *et al.* 1986) and to promote fighting among males (Novotny *et al.* 1985).

The other two possibilities are inter-related and by no means mutually exclusive. On present evidence it is not possible to reject either of them.

To rank the mice on the basis of won encounters proved extremely difficult because, as was plain from both the nature of the interactions between the mice and the continual changes in rank in some cages, the dominance hierarchies were extremely labile. That stable hierarchies had not formed in some cages was borne out by the mice's scent marking. In only three of the cages (A, C and D) was there the clear-cut difference between urine spots and puddles recorded by Desjardins *et al.* (1973) and even in these only one of the three mice urinated in puddles; all other scent mark rankings had to be based on differences in the density of urine spots. This situation contrasts sharply with that described by Desjardins *et al.* "...in every instance the same differential type of urinary marking pattern [spots *vs* puddles] emerges immediately after the establishment of a stable social hierarchy". This aspect of the hierarchies tends to support the fourth possibility.

The failure of the mice to form stable hierarchies is probably a strain characteristic. NMRI are a non-aggressive strain and except in the case of cage A, with its three previous dominants, bite wounds and the accompanying stress which mediates an increase in submissiveness (Nock & Leshner 1976), were conspicuously absent. As Kaufmann (1983) has pointed out; "stable dominant-subordinate relationships depend for their existence on the submissiveness of the subordinate individuals."

The perfect agreement between aggression-initiator, and chromatographic, ranks reveals that an odour profile of type A (Fig 12.2) is related to the status of "challenger" rather than "dominant". Such profiles will, therefore, be obtained from mice which are rising in status but from only some of those which are already dominant. Sandnabba (1986c) arrived at a similar conclusion on the basis of behavioural experiments. The case of cage D in the first sampling session further illustrates this point. The chromatographic, and winner ranks were exactly opposite but two days after sampling the mouse which had ranked highest on odour profile had risen to highest in winner rank.

Although the physiological basis for parallels between odour profile and rising social rank is unknown it is tempting to speculate that it might be related to episodic fluctuations in testosterone level (Bartke & Dalterio 1975) - a mouse whose testosterone levels are rising being both odourous and aggressive.

There was no evidence of home cage effects; a mouse which remained in its original cage was no more likely to be dominant than were those which were introduced, nor did permanent residence affect a mouse's odour profile.

If the difference in scent marking between dominant and subordinate mice made dominants more likely to urinate in the sampling chamber this would probably have contributed to the difference between their odour profiles. Nevertheless subordinate mice which urinated during sampling did not yield dominant's odour profiles. The odour profile obtained under the sampling conditions employed here probably reflects the odour of a mouse in its home territory.

That *exo*-3,4-dehydrobrevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole elicit aggression from other mice when painted together, in castrate urine, onto castrated males (Novotny *et al.* 1985) is at first sight incompatible with the present findings that these two compounds are related to dominant status. It would be expected that dominant-smelling mice would be avoided, or deferred to, by others (Kaufmann 1983). However the tests conducted by Novotny *et al.* were carried out in the home cage territories of isolated, trained fighters who would, in fact, be expected to attack dominant or challenger intruders. Sandnabba (1986c) found that genetically aggressive males were actually attracted to the odours of other aggressive males. It would be interesting to know how subordinate males painted with urine, dehydrobrevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole are treated by other subordinates.

CONCLUSIONS

By analysis of whole-body volatiles using dynamic solvent effect sampling and capillary chromatography differences in odour profiles between individual mice can be quantified. Among triads of male NMRI mice, where dominance hierarchies are extremely labile, chromatographic odour profile correlates more closely with aggressiveness (numbers of encounters initiated) than with dominance (number of encounters won).

ANALYTICAL CHALLENGES AND SOLUTIONS

The analytical problems and methods were so similar in Chapters 11 and 12 that they can be considered together.

Both rodent species generated complex odours requiring high resolution separation. Although the range covered by the chemical differences under investigation was wide; approximately 0-21 ng per sample for 3-nonene-2-one in *Mastomys* and 0-45 ng for total volatiles in the house mice the high precision of DSE sampling was neccesary for the quantitative separation of intermediate cases. The highest mean individual 3-nonene-2-one level for *M. natalensis* was only 0,7 ng less than the lowest for *M. coucha* and the mouse dominance rankings were in some cases decided on differences of <0,25 ng of particular components.

Although discriminant function analysis or other formal pattern recognition techniques proved unneccesary in the present two studies their availability, preferably in a form suitable for data acquisition direct from the chromatographic integrator, would be desirable if other, similar investigations were to be undertaken.

The use of live rodents as specimens generated specific requirements for the sampling hardware and procedure. They were the bulkiest specimens for which enclosed sampling containers were used, requiring a container large enough for them to move freely and with a wide enough opening for their convenient insertion and removal. The use of 500 cm^3 jars with glass flange closures (made possible by the low gas flow resistance of the concentrators, Chap 4) was adopted in the interests of economy; a ground glass cone and socket of equivalent size costing R60. As well as acting as sampling carrier gas the air flow through the jar had also to provide the respiratory needs of the live animal specimen. Consequently the air flow rate was set at 20 $cm^3\ min^{-1}$ rather than the standard sampling flow of 15 $cm^3\ min^{-1}$. At the higher flow rate none of the mice ever showed any sign that the

air flow rate was inadequate, justifying the loss in accumulation efficiency (Chap 6) caused by the higher flow rate.

The distorted shape and increased width of peak 2 in the chromatograms of laboratory mouse volatiles are symptomatic of breakdown on the column. If degradation had occurred in the inlet the peak would have eluted sharp from the column, and the problem would then have been unrecognized. This interpretation is supported by the peak's retaining its shape even when the inlet was unheated during sample transfer.

Chapter 13

AGGREGATION PHEROMONES OF THE BONT TICK *Amblyomma hebraeum*: IDENTIFICATION OF CANDIDATES FOR BIOASSAY

INTRODUCTION

The bont tick *Amblyomma hebraeum* is a major pest of livestock in Africa; as well as causing injury and loss of condition through its ectoparasitic habits it is also a vector of heartwater fever.

The economic importance of soft and hard ticks has led to intensive investigations of their biology, including a series of studies of chemical communication associated with reproduction. The use of phenolic compounds as attractants of males to feeding females appears to be general among ixodid ticks (Wood, Leahy, Galun, Prestwich, Meinwald, Purnell & Payne 1975; Gothe 1987). In three species; *Amblyomma hebraeum, A. maculatum* and *A. variegatum* feeding adult males produce a chemical signal which attracts other adult males, adult females and nymphs (Gladney, Grabbe, Ernst & Oehler 1974; Rechav, Parolis, Whitehead & Knight 1977; Schöni, Hess, Blum & Ramstein 1984). Such an attractant has obvious potential as a component of a tick control formulation. Rechav & Whitehead (1978) report the successful use of mixtures of acaricide with extracts of several hundred male ticks for control of *A. hebraeum* on cattle.

Using elegant on- and off-host bioassays Rechav, Whitehead & Knight (1976) and Rechav *et al.* (1977) established that the *A. hebraeum* attractant is volatile and that the attractiveness of males increases after 4-5 days feeding, reaching a plateau after 8-9 days. The chemical identity of the signal was not established.

The approach used in the present study was to use dynamic solvent effect sampling of volatile compounds emitted by male *Amblyomma hebraeum* while actually feeding on a host. Those compounds whose temporal patterns of emission corresponded to the reported changes in the attractiveness of male ticks to their conspecifics were identified as candidates for bioassay. This chapter reports the identity of three such compounds. Their bioassay was beyond the scope of this study.

METHODS

Two adult, male, California White rabbits (*Oryctolagus cuniculus*) were used as hosts. On both rabbits a circular patch of hair about 25 mm across on each flank was clipped very short. A glass cup 20 mm across and 20 mm deep with a screw top which held a perforated polytetrafluoroethylene disc was glued over each clipped patch with cyanoacrylate adhesive (Fig 13.1). The following day six adult, male ticks were introduced into one cup on each rabbit, the other cup serving as a control. Removal of the glass cups and the ticks by the rabbits' grooming was limited (though usually not prevented) by fitting the rabbits with cloth jackets. Water and rabbit pellets were available *ad lib.*.

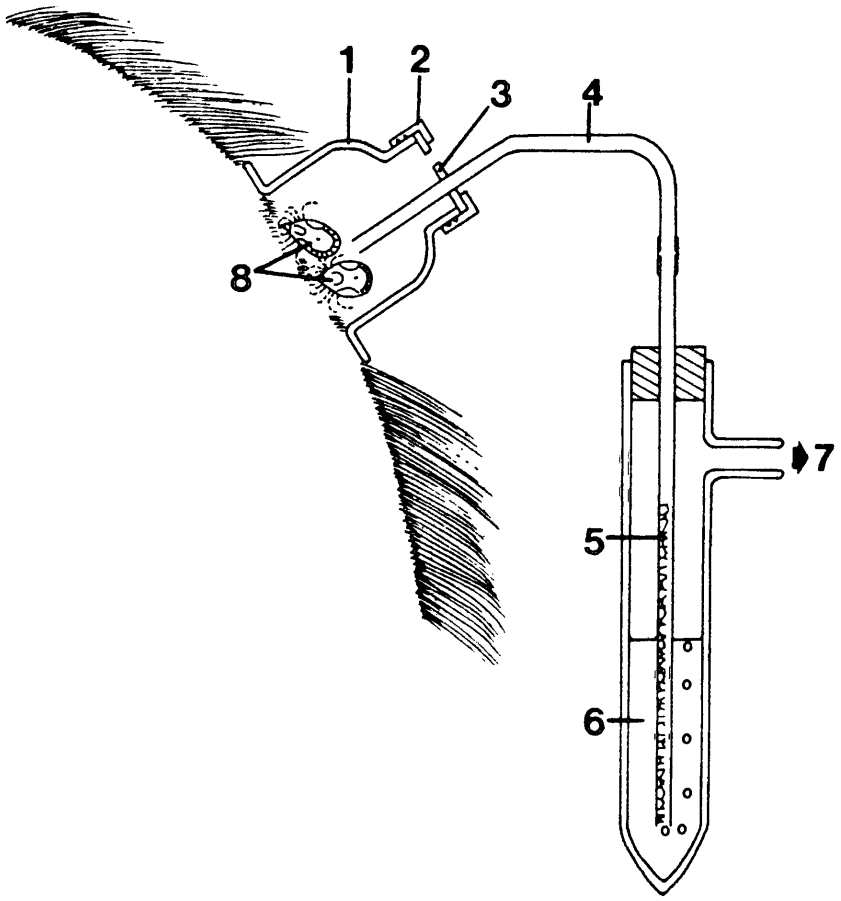

Fig. 13.1 Apparatus used for sampling volatiles from ticks feeding on their host. 1; glass cap attached to host's skin with cyanoacrylate adhesive; 2: screw cap; 3: perforated polytetrafluoroethylene disc; 4: deactivated glass tube; 5: dynamic solvent effect concentrator; 6: *n*-hexane solvent; 7: vacuum mass-flow controlled at 15 cm^3 min^{-1}; 8: ticks.

Each day samples of volatiles from the ticks and from the control cups were obtained as follows: a dimethyldichlorosilane deactivated glass tube was inserted through one of the holes in the lid of the cup and the other end was connected to a dynamic solvent effect concentrator with *n*-hexane as solvent. Air was drawn from the cups and through the concentrators at a flow rate of 15 cm^3 min^{-1} for 15 min for each sample (Fig. 13.1). Condensed water was evaporated from the concentrator by a 15 cm^3 min^{-1} flow of palladium purified hydrogen for 5 min.

Volatiles accumulated in the concentrators were separated using a dynamic solvent effect inlet installed in a Varian 3700 gas chromatograph and connected to a 25 m x 0,3 mm x 0,4 µm methyl silicone column. Detection was by flame ionization at a sensitivity of 10^{-11} A mv^{-1} , the signal was fed to a Varian 4270 integrator set for 4 mv full-scale deflection. The inlet was held at 40°C for 2,5 min, then heated ballistically to 220°C, the column was held at 40°C for 6 min and then programmed to 220°C at 5°C min^{-1}.

The areas of peaks which satisfied the criteria for potential pheromones by appearing on chromatograms from tick samples and not on chromatograms from controls, and increasing in area after 4 or more days feeding by the ticks were identified by gas chromatography - mass spectrometry using a Micromass 16F mass spectrometer with an electron energy of 70 eV and a source temperature of 200°C. Identities were confirmed by comparison of retention times with those of reference compounds.

RESULTS

In only two cases did the glass cups and the ticks survive the rabbits' grooming long enough for the ticks to begin signalling. On the chromatograms of volatiles from feeding ticks three peaks showed a marked, progressive increase in area after 4-6 days from the introduction of the ticks to the host (Fig. 13.2). These peaks were identified by GC-MS as; 2-methylpropanoic acid, benzaldehyde and 2-nitrophenol. Other peaks which appeared only in chromatograms from tick infested skin and which showed transient increases were identified as a series of short to medium chain fatty acids (Table 13.1).

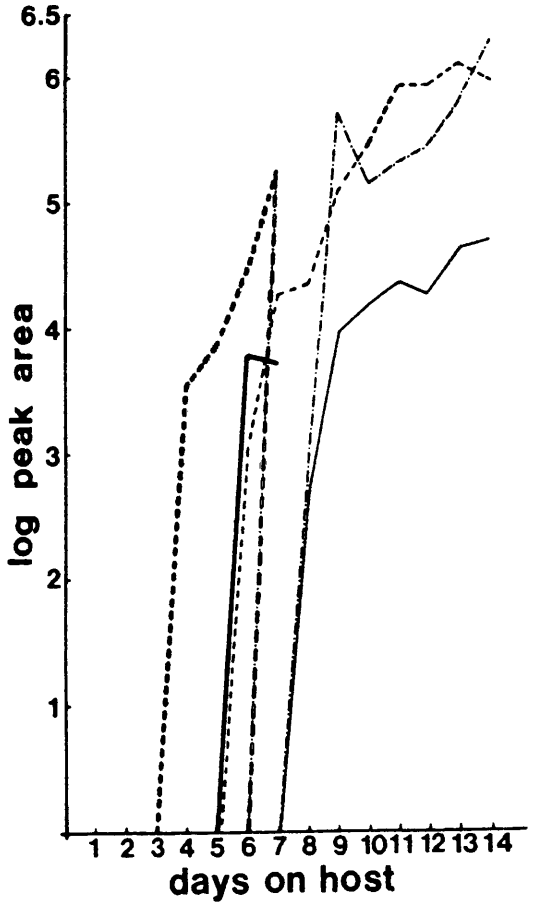

Fig. 13.2 Changes with time of emission of volatiles by feeding, male *Amblyomma hebraeum*. The heavy and light lines are two different series. The dashed lines plot 2-nitrophenol emissions, the solid lines plot benzaldehyde emissions and the dashed/dotted lines plot 2-methyl propanoic acid emissions in each series.

Table 13.1 Fatty acids emitted intermittently by six male *Amblyomma hebraeum* while feeding on a rabbit host and the days after attachment when they were detectable by dynamic solvent effect sampling and capillary gas-liquid chromatography.

Acid	Days detectable
n-butanoic	9, 11, 14
2-methylbutanoic	11, 14
n-pentanoic	11, 12, 13
methylpentanoic	13, 14
n-hexanoic	13, 14
n-heptanoic	14
n-octanoic	14
n-nonanoic	14

DISCUSSION

The compounds accumulated from tick infested skin were not produced by the skin rather than by the ticks (a possibility which would not exclude possible attractant effects). They disappeared rapidly after the removal of the ticks while the inflamed appearance of the skin remained the same. Additionally none of the three candidate signal compounds were collected from an area of skin to which three ticks remained attached for six days after they had died.

The first detection of 2-nitrophenol was after 4 days from one series and after 6 days from the other. The first detection of 2-methylpropanoic acid was after 6 and 8 days, and the first detection of benzaldehyde after 7 and 8 days. Rechav *et al.* (1977) first detected aggregation to males after they had fed for 5 days. The apparent delay in detection of volatiles in one series may have been due to slow attachment of the ticks; in pilot experiments some ticks were still not attached 24 h after their introduction to the host.

There was no definite plateau in the emission of the three candidate signal compounds, while biossays (Rechav *et al.* 1977) showed a levelling off in aggregation after 8 days. This may have been due to a plateau of response to increasing signal strength, to a logarithmic relationship of response to signal intensity or to the failure of some ticks (approximately 10%) to respond to signals of any strength.

Rechav (1978) suggested that the aggregation signal in *Amblyomma hebraeum* was a mixture of two or more components and Rechav, Norval & Oliver (1982) demonstrated the existence of species-specific and non-specific aspects of the aggregation response in *A. hebraeum* and *A. variegatum*. Using extracts of 100 fed males Schöni *et al.* (1984) identified and bioassayed the volatile aggregation signal of *A. variegatum* which employs 2-nitrophenol as a long range (100-200 mm) attractant. Mounting and clasping are stimulated by nonanoic acid and methyl salicylate in *A. variegatum* while methyl propanoic acid and benzaldehyde have been found in *A. hebraeum*. The *A. variegatum* aggregation signal thus has a non-species-specific, long range component and a species-specific short range and contact component. In addition male *A. hebraeum* intermittently produce small amounts of nonanoic and other medium chain acids. This situation is compatible with Norval & Rechav's (1979) report of a weak response by *A. variegatum* to fed, male *A. hebraeum* in a bioassay based on attachment, and with the occurrence of sterile interspecific matings between these two species when no conspecific partners are available (Rechav *et al.* 1982). An analagous case of generalized long-range attraction coupled with species-specific short range signals has been described for *Hyalomma dromedarii* and *H. anatolicum excavatum* by Khalil, Sonenshine, Sallam & Homsher (1983).

On an experimental scale Gladney *et al.* (1974) attracted female *A. maculatum* to acaricides by using extracts of fed males, and the similar use of extracts of fed, male *A. hebraeum* has been sucessfully demonstrated by Rechav & Whitehead (1978). Synthetic, rather than natural attractants must be available for such an approach to tick control to be commercially viable. The identification provided here of three potential components of such a synthetic attractant is an additional step towards its formulation and large scale application.

ANALYTICAL CHALLENGES AND SOLUTIONS

No information was available on the rates at which ticks emitted their semiochemicals. Rechav (1983) found that male *Hyalomma marginatum rufipes* were attracted to 10 ng of 2,6-dichlorophenol at a range of 20 mm. Schöni *et al.* (1984) extracted aggregation pheromones from *A. variegatum* in microgram quantities, but Sonenshine, Silverstein, Layton & Homsher (1974) obtained only 2 ng of 2,6-dichlorophenol per tick from females of *Dermacentor variabilis* and *D. andersonii*. Kellum & Berger (1977) found up to 50 ng of 2,6-dichlorophenol in female *A. maculatum* and *A. americanum*. Khalil, Nada & Sonenshine (1981) established an optimum of 3,1 µg of 2,6-dichlorophenol for the attraction of male *Hyalomma dromedarii* to engorged females, Leahy & Booth (1983) found 10 ng of the same substance to attact the most males of *A. americanum* to filter paper discs and recorded significant attraction down to 0,1 ng. Males of *Dermacentor parumapertus*, *Hyalomma anatolicum excavatum* and *A. americanum* all respond to 1 ng of 2,6-dichlorophenol on filter paper (Leahy & Booth 1983).

On the basis of these findings it was anticipated that it might be necessary to quantify emissions of less than one nanogram in the presence of compounds accumulated from the host's skin and the room air. These latter two sources of interference precluded an increase in sampling time since they would then simply have accumulated in larger quantities. In any case the rabbits could not reliably be restrained for longer than about 30 min.

That the final emission rates of 2-methylpropanoic acid, 2-nitrophenol and benzaldehyde were respectively approximately 950, 600 and 25 ng per 15 min sampling period by no means removed the need for high sensitivity; an accurate tracking of emission rates for comparison with published bioassays required the detection of much smaller quantities when the ticks first began signalling.

The high final emission rates placed demands on the linearity of the analytical system. With hindsight it can be seen that the use of a polar column could have improved linearity by providing better peak shapes for free acids than did the methyl silicone column actually employed (Fig. 13.3).

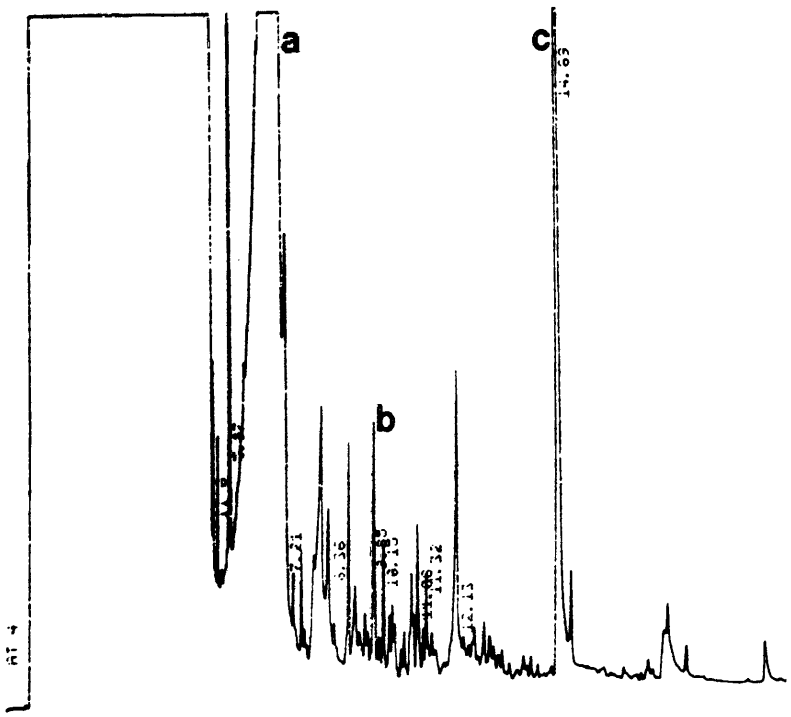

Fig. 13.3 Chromatogram of volatiles emitted by 6 male *Amblyomma hebraeum* after 9 days feeding on a rabbit. For analytical conditions see text. a: 2-methylpropanoic acid; b: benzaldehyde; c: 2-nitrophenol.

Work on tick chemical signals employing conventional GC techniques has used large numbers of ticks; ranging from hundreds (e.g. Schöni *et al.* 1984) to hundreds of thousands (e.g. Chow *et al.* 1975). Since ticks are routinely raised on live mammal hosts a technique whose sensitivity allows the use of only six ticks for each sample has obvious ethical advantages.

No other study of semiochemicals emitted by ticks while actually feeding has been found in the literature. Two investigations used headspace techniques for the collection of volatiles from ticks which had been removed from their host. Sonenshine *et al.* (1974) trapped volatiles from female *Dermacentor variabilis* and *D. andersonii* on Porapack Q over periods of 24-72 h and Brill & Solomon (1979) used a cold trap to collect volatiles from *A. hebraeum* over a period of 4

days. In both cases the trap contents were recovered by solvent extraction and used for bioassay rather than chemical analysis.

The main practical shortcoming of the sampling system employed here was its fragility; movement by the rabbits could break both the glass connector tube (Fig 13.1 4) and the concentrator. A sampling system in which the concentrator was protected by the solvent holder (e.g. Fig 7.1 F) would have been more satisfactory.

Chapter 14

ATTEMPTED IDENTIFICATION OF THE COMPOUND RESPONSIBLE FOR THE DISTINCTIVE ODOUR OF SCHIZOPHRENICS

INTRODUCTION

Rather than attempt to review a literature with which I am unfamiliar I have summarized here the chapters on schizophrenia from Sarason & Sarason's (1984) general text book. Similar ground has been covered by Garfield (1983a, b).

Schizophrenia is a diagnosis applied to a wide range of behaviour disorders whose general symptoms include thought disturbances, abnormal speech, unusual activities, delusions, hallucinations, inappropriate emotions and disrupted social relationships. It is the most serious, and the most common of mental illnesses; 1-2% of the world's population are affected, schizophrenics occupy 40% of the beds in American mental hospitals, and schizophrenics spend longer in hospital than patients suffering other mental illnesses.

Because of the diversity of its symptoms schizophrenia has been classified into five types; disorganized schizophrenics suffer profuse delusions and hallucinations and behave "childishly"; catatonics have disturbed motor activity, either prolonged immobility or hyperactivity; paranoid schizophrenics experience delusions and frequently feel persecuted. Residual schizophrenia is a persistance of disturbance following an episode of active psychosis, and undifferentiated cases are those which cannot be satisfactorily classified.

Except that it has both genetic and environmental components, which will surprise few biologists, the etiology of schizophrenia is unknown. In the light of its diversity this suggests that schizophrenia may include several conditions with different causal mechanisms and origins.

The Schizophrenic Odour

Schizophrenics are reputed to have a distinctive, unpleasant body odour. Smith & Sines (1960) were able to train rats to distinguish the odour of ether extracts of schizophrenics' sweat from the odour of ether extracts of the sweat of normal persons. A human panel was also able to discriminate, though less reliably than the rats. In 1969 Smith, Thompson & Koster identified *trans*-3-methyl-2-hexenoic acid as a compound peculiar to schizophrenics' sweat. Addition of this compound to normal sweat produced an odour which a human panel judged to be identical with that of schizophrenics - it was not tested on rats. Perry, Melançon, Lesk & Hansen (1970) failed to duplicate Smith *et al.*'s (1969) findings and suggested that the presence of *trans*-3-methyl-2-hexenoic acid was an analytical artifact.

The work described here is a continuation of an investigation into the nature of the schizophrenic odour which has been going on intermittently at the Institute for Chromatography since the early seventies. The original aim of the study was to identify those at risk of developing schizophrenia, an objective which proved unattainable because the odour occurs only sporadically among diagnosed schizophrenics and the strength of the odour correlates with the severity of the patients symptoms (Smith & Sines 1960). At the stage of the investigation reported here it was hoped that the identification of the odour might contribute to an understanding of the mechanism of schizophrenia, or that it might provide a diagnostic aid for the classification of symptoms, the prognosis and appropriate therapy being rather different for each class. The odour with which this investigation was concerned was especially associated with the patients' hair and had been described as resembling the smell of a wet dog. The experimenter was familiar with the odour as a result of earlier work.

METHODS

Four male schizophrenia patients at Weskoppies Hospital were selected as subjects on the basis of Dr P. Roos' and other hospital staff's assessment of their body odours. The patients were requested not to wash their hair for one week before hair specimens were collected, otherwise diet, daily activities and treatment were undisturbed.

Specimens of approximately 10 cm^3 of loosely packed head hair were obtained from three of the patients. The fourth had washed his hair on the previous day. Specimens were kept in glass stopperd, glass containers at liquid nitrogen temperature until immediately before analysis.

Approximately 5 cm3 of each specimen was loosely packed into a 50 cm3 flask and, to increase the yield of volatiles, moistened with approximately 0,5 cm^3 of water freshly distilled from alkaline permanganate. The flask was warmed to approximately 70°C while the volatiles were purged from the hair with a 15 cm^3 min^{-1} flow of palladium cell purified hydrogen for 20 min and trapped by the dynamic solvent effect using *n*-hexane as solvent. Samples were dried for 15 min with a 15 cm^3 min^{-1} flow of palladium cell purified hydrogen. Control specimens from the experimenter and another member of the laboratory staff, who had not washed their hair for ten days and one week respectively, were similarly sampled.

GC-FID analyses were carried out on a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet with a 25 m x 0,3 mm x 0,4 µm methyl silicone capillary column. The carrier gas was hydrogen with a linear velocity of 55 cm s^{-1}. The starting temperature of both inlet and column was 40°C, the inlet was heated ballistically to 220°C after 3 min and the column temperature was programmed at 4°C min^{-1} after 7 min. The detector sensitivity was 16 x 10^{-11} A mv^{-1} full scale deflection.

Approximately 90% of the column effluent was split away from the FID to a sniffer port. Descriptions of the odours issuing from the port were recorded on tape and subsequently transcribed to the chromatogram. Particular attention was paid to the recognition of any odours which resembled that of schizophrenics.

Mass spectral analyses were carried out on the remaining half of each 10 cm^3 hair specimen with equivalent sampling and chromatographic conditions to those above, using a Varian 1400 GC with open-split interface to a VG Micromass 16 F mass spectrometer operating in the electron impact mode. The source temperature was 220°C and the electron energy was 70 eV. Special attention was paid to the areas of the chromatogram where distinctive odours had been recorded.

RESULTS

What is meant here by the "schizophrenic odour" is distinctively animalic in character with sour and aldehydic notes. Its comparison to the smell of a wet dog is fairly close, and it also resembles the odour of the hair of some very heavy smokers. The wet dog character of the odour is shared by the coat of the brown hyaena (*Hyaena brunnea*). The only odour of which I have experience which is a very close match is that of the Cape hunting dog (*Lycaeon pictus*).

Of the three patients from whom hair specimens were obtained only one had what I recognize as the schizophrenic odour. The odour was strong enough to be perceptible at a distance of about a metre.

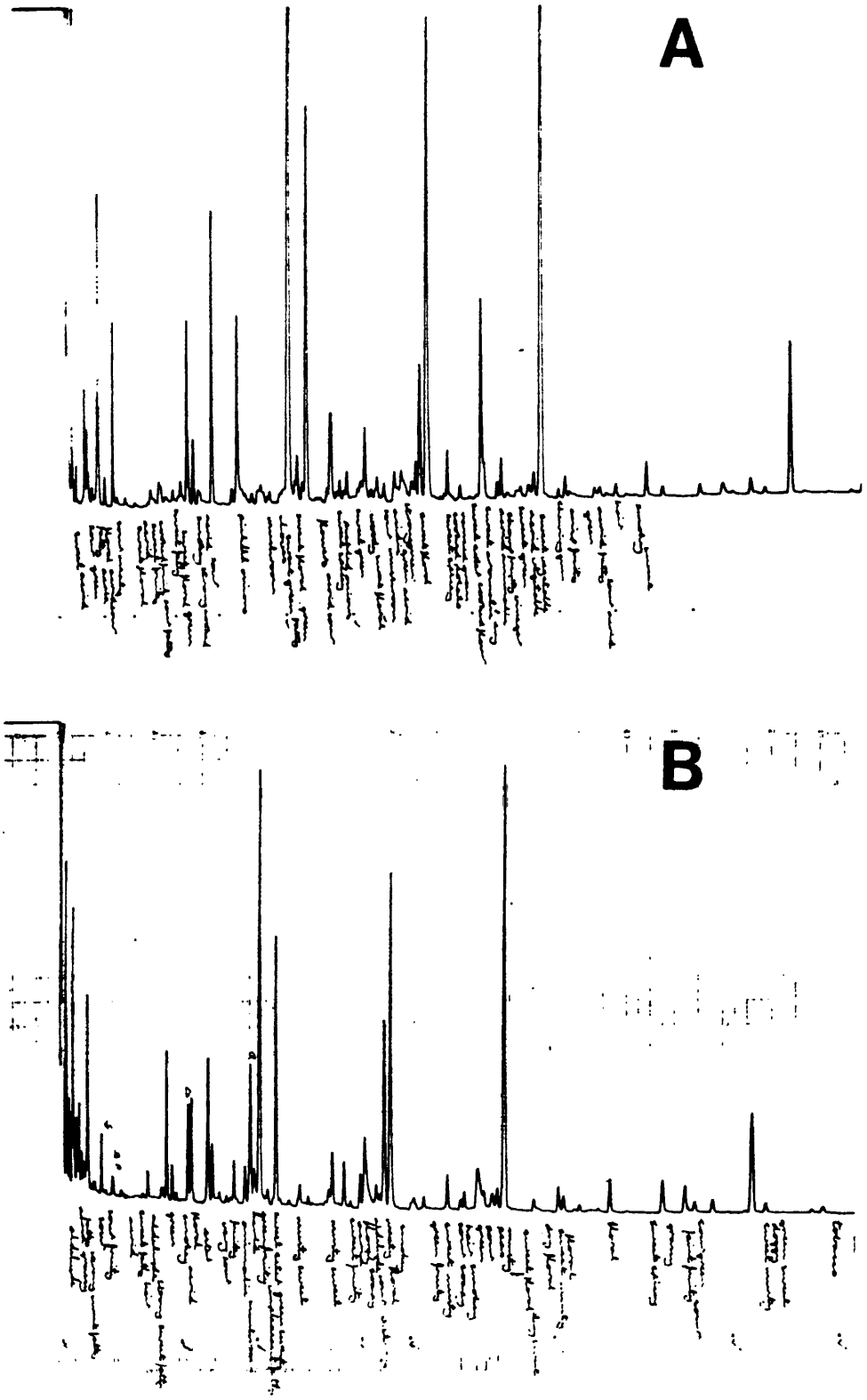

Fig. 14.1 (continued on next page)

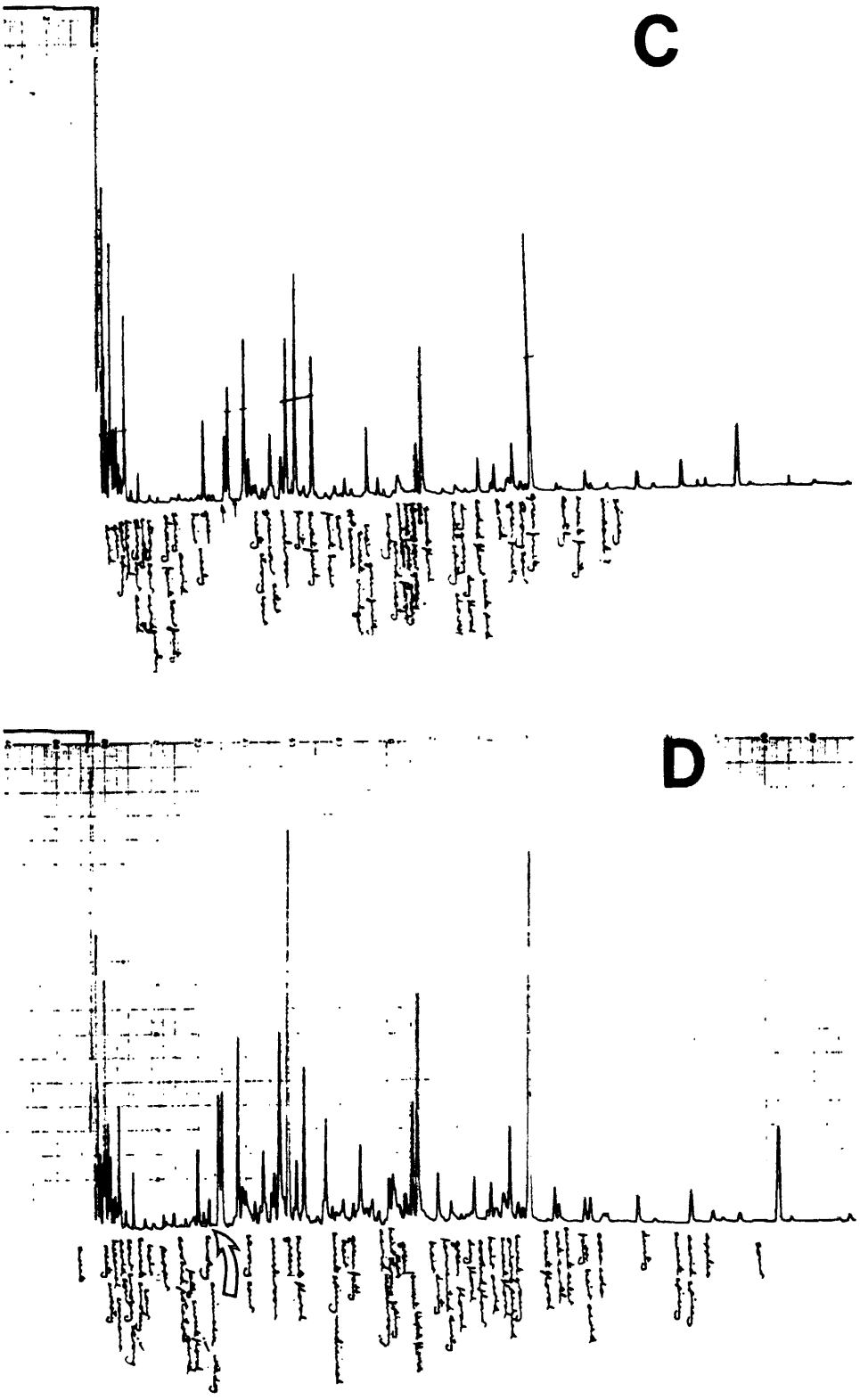

Fig. 14.1 Aromagrams of volatiles from the hair of A: a control subject; B: a control subject; C: a "non-odorous" schizophrenic; C: an "odorous" schizophrenic. The chromatographic traces are from an FID at a sensitivity of 16 x 10^{-11} A mv^{-1}, approximately 90 % of the column effluent was split to a sniffer port, descriptions of the odours were recorded on tape and transcribed to the chromatogram, the peak whose elution coincided with detection of a strong, animalic odour is arrowed in D. For analytical conditions see text.

Volatiles from all three schizophrenics and both controls yielded odours with a generally hairy/smokey/aldehydic character when sniffed at the column outlet (Fig. 14.1). Although these odours were more common among volatiles from the schizophrenics they were not close enough in character to the schizophrenic odour to be considered distinctive.

In addition the separated volatiles from the odourous patient exhibited one particularly strong, repulsive and distinctively animalic odour which did not occur in either of the other schizophrenics or the controls.

Comparison of the otherwise rather similar chromatograms revealed a peak which was unique to the odourous schizophrenic and whose elution coincided with the detection of the distinctive odour. This peak was identified by mass spectrometry as methoxybenzene, the odour of an authentic sample of which bears no resemblance whatsoever to the odour of schizophrenics.

In the area of interest the FID chromatogram shows some very small peaks which might be associated with the odour. However none of these peaks were even detected by the mass spectrometer, far less identified.

An attempt to collect a larger hair specimen from the odorous patient was unsuccessful because his hair had been cut short in the interim and washed the day before collection. Unfortunately the patient died shortly afterwards.

Trans-3-methyl-2-hexenoic acid was not identified among the volatiles from any of the schizophrenics or either of the controls.

Thus the schizophrenic odour remains unidentified.

DISCUSSION

Odours are notoriously difficult to describe, and to recognize from other people's descriptions. Smith & Sines (1960) describe the schizophrenic odour as "intense" and " heavy, unpleasant and slightly pungent". This is not the same as my description of the odour and I doubt that I would recognize the odour from their description. It is likely that different odours are involved. The same authors mention another case, of an odour which was "skunk-like", this could well be the same as the animalic component I recognize.

Although the actual cause of the schizophrenic odour was not identified *trans*-3-methyl-2-hexenoic acid was eliminated as a cause of what was recognized here as the schizophrenic odour. The quality and strength of the odour suggest that it may be a sulphur compound. The frequently occurring hairy/smokey/aldehydic impressions among the volatiles from schizophrenics may also contribute to the overall schizophrenic odour. In addition there is the possibility that different types of schizophrenia are associated with different malodours.

Not everyone is able to smell anything distinctive about schizophrenics (Perry *et al.* 1970; pers. obs.), an observation which has an interesting relationship to the labelling as hallucinations of schizophrenics' reports that they can smell foul odours coming from their bodies.

ANALYTICAL CHALLENGES AND SOLUTIONS

In cases such as this where the biologically relevant concentration of a substance is extremely low a successful analysis is mainly dependent on obtaining specimens large enough to supply sufficient of the compound for detection. In the present case the specimen size was adequate for sniffer detection, barely adequate for FID and completely inadequate for MS. For mass spectrometric detection much larger specimens, possibly pooled from several patients, will be necessary.

Two aspects of the difficulties of working with human subjects are apparent in this study. About lack of subject compliance nothing need be said except that it is not confined to the mentally ill. The second aspect is that of intra-class variation among subjects. To have applied the method of chemical image differentiation (p 9) would have required large groups of both normals and schizophrenics in order to be able to recognize biological noise and eliminate it from the data. Only in exceptional circumstances is it possible to keep groups of 10 or more human subjects under conditions as uniform as those which can be readily imposed on laboratory rodents (see Chaps 11 and 12). Consequently environmental variation is superimposed onto individuality as a source of noise. In this respect human subjects probably resemble wild animals.

These difficulties are more than compensated for by the possibility of using the human nose as a specific detector tuned for the odour feature under investigation. To adopt an eqivalent strategy with animals is probably impossible.

Chapter 15

QUALITATIVE INVESTIGATIONS OF VOLATILES FROM MAMMALIAN SECRETIONS

Of the 291 species of land mammal indigenous to the southern African subregion only the bontebok (*Damaliscus dorcas dorcas*) (Burger *et al.* 1976, 1977), the black-backed jackal (*Canis mesomelas*) (Burger & Munro 1986), the civet (*Civettictis civetta*) (Maurer *et al.* 1979; Jacob & Schliemann 1983; Von Saldern, Schliemann, Kayanja & Jacob 1987), the springbok (*Antidorcas marsupialis*) (Burger *et al.* 1978, 1981), the grysbok (*Raphicerus melanotis*) (Burger *et al.* 1981), the bushbaby (*Galago crassicaudatus*) (Crewe *et al.* 1979; Katsir & Crewe 1980) and the small- and large-spotted genets (*Genetta genetta* and *G. tigrina*) (Jacob & Schliemann 1986) have been the subjects of studies of the chemistry of their odourous secretions. Consequently there is a very wide field in which even the most basic of information on chemical communication is lacking. Although, in isolation, reports on the identities of the major volatile components of a glandular secretion, or of urine or faeces, cannot be expected to yield any profound biological insights, such information does have value. It provides an overview of secretion chemistry, allowing any chemical anomalies to be identified for future, intensive study and it allows taxonomic trends to be recognised. It is also of considerable value to the analyst to know in advance the major chemical features of his material.

VOLATILE COMPONENTS OF THE ANAL GLAND SECRETION OF THE AARDWOLF (*Proteles cristatus*).

INTRODUCTION

The aardwolf (*Proteles cristatus*) is the only member of the family Protelidae (Meester, Rautenbach, Dipenaar & Baker 1986). Although it shares many features with the Hyaenidae and has often been classified in that family it is much smaller (9-11 kg) than the hyaenas and is entirely insectivorous, specializing on termites, especially *Trinervitermes* spp. (Kruuk & Sands 1972; Cooper & Skinner 1979; Smithers 1983; Richardson 1987b). Its distributional range is divided into two main areas; one in eastern and north-eastern Africa and the other in the southern African subregion. Aardwolves live in monogamous pairs which defend territories of 1-3 km^2, whose size appears to be determined by the density of termite nests (Richardson 1987b). Territories are demarcated by scent marks produced by wiping the secretion of the well developed anal gland onto grass stems and other vegetation (Smithers 1983; Richardson 1985). It has been suggested by Richardson (1987a) that pastings may also be used by both sexes to attract mates, and by males to intimidate other males in competition for mating opportunities.

METHODS

Adult aardwolves were captured at Benfontein game farm in the northern Cape Province, South Africa. The animals were anaesthetized with ketamine hydrochloride and, by careful pressure, their anal glands were everted and the contents expressed directly into clean glass vials with polytetrafluoroethylene caps. Fresh scent marks were collected into similar vials by clipping the grass stems on which they had been deposited. All material was stored at -10°C until analysis.

Volatiles were sampled from approximately 5 mg blobs of anal gland contents of about the same volume as the real scent marks, which were sampled whole. Palladium cell purified hydrogen was passed over the specimens at a flow rate of 15 cm^3 min^{-1} and a temperature of 22-23°C. Volatiles were accumulated by dynamic solvent effect using alumina-purified *n*-hexane as solvent. Sampling periods of 5-20 min were used for gas chromatography-flame ionization detector (GC-FID) analyses and 30 min for GC-mass spectrometer (GC-MS) analyses.

GC-FID analyses were carried out on a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet and a 25 m x 0,3 mm x 0,4 µm methyl silicone capillary column. The carrier gas was hydrogen with a linear velocity of 55 cm s^{-1}. The starting temperature of both inlet and column was 40°C, the inlet was heated ballistically to 220°C after 2,2 min and the column temperature was programmed at 5°C min^{-1} after 7 min. The detector sensitivity was 4 x 10^{-11} A mv^{-1} full scale deflection. GC-MS analyses were carried out under equivalent conditions on a modified Varian 1400, using helium as carrier gas, with an open split interface to a VG Micromass 16F spectrometer operating in the electron impact mode. The source temperature was 220°C and the electron energy was 70eV.

Compounds were identified by comparison of their mass spectra with those in libraries or the literature. Where necessary identities were confirmed by retention indices and syntheses of authentic compounds.

RESULTS

The anal gland of the aardwolf contains a bright or pale orange paste which at room temperature has the consistency of soft butter. The scent marks are blobs or smears of waxy material about 10 mm long varying in colour from yellow to dark brown. Pale scent marks, but not anal gland contents, darken after 1-3 days exposure to air. When fresh the odour of the anal gland contents is dominated by a penetrating, metallic scent. Cheesy and faint, sweet, musky odours are just distinguishable. After 10-20 min exposure to air the smell changes to one in which the cheesy and musky scents are dominant but after an hour the original smell returns and persists for at least 6 months. The odour of scent marks resembled that of fresh/aged gland contents. To the human nose there were only minor differences in odour between material from males and females.

The volatiles emitted in the largest quantities by both gland contents and scent marks were fatty acids (Figure 15.1, Table 15.1). In addition there was a fairly complex mixture of minor components dominated by series of esters of the fatty acids. The only other identified compounds were hexanol and indole.

Even within the small number of specimens analysed there were marked differences in the occurrence and relative areas of various peaks (e.g. Figure 15.1). Whether these differences reflect individual identity or are related to e.g. sex or reproductive condition is, at this stage, uncertain. The proposed role of scent marking in reproductive competition among males (Richardson 1987a) requires that male scent marks be systematically different from those of females.

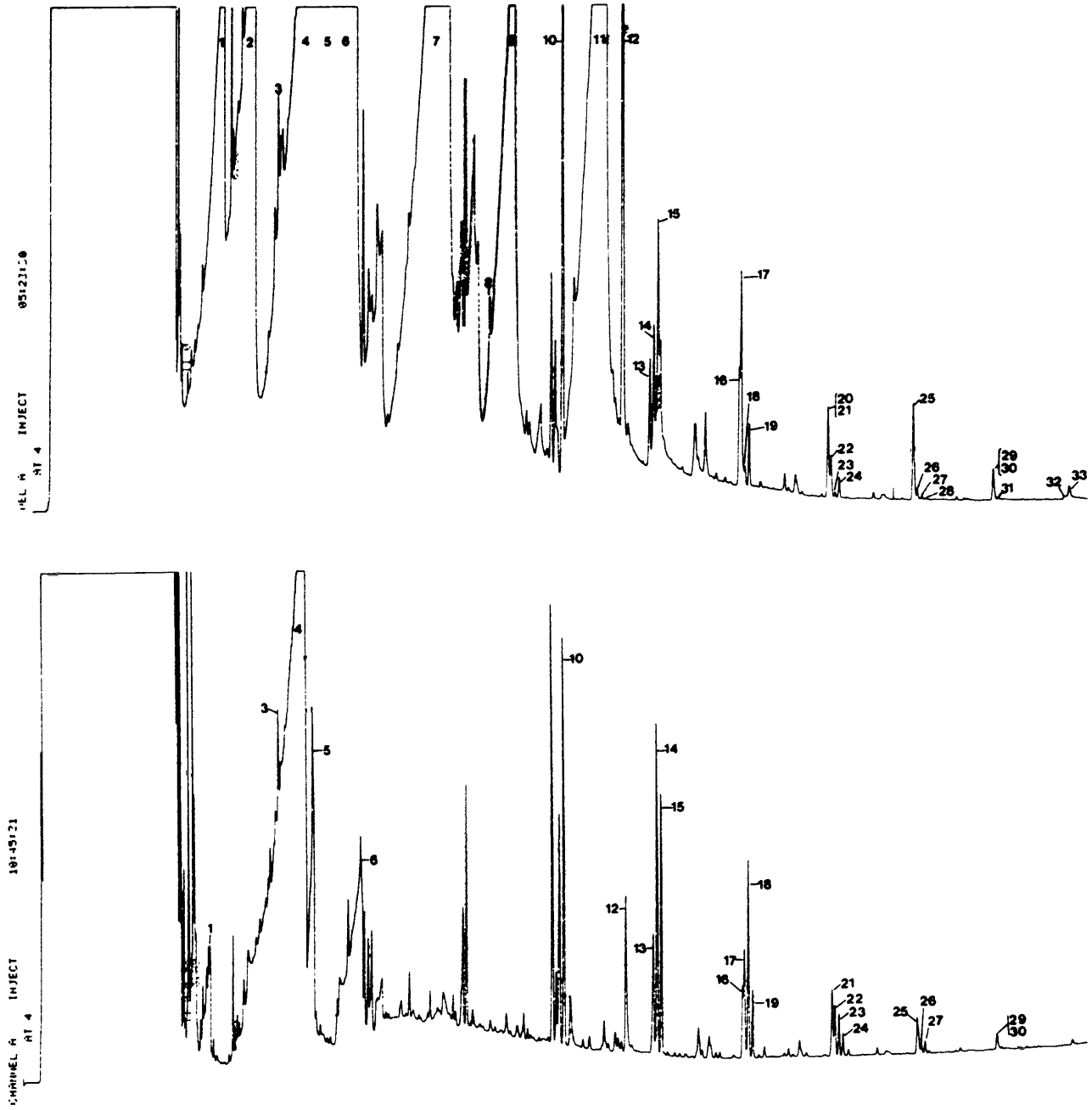

Fig. 15.1 Chromatograms of volatiles from anal gland contents of; A: male, and B; female aardwolf (*Proteles cristatus*). Column; 25 m x 0,3 mm x 0,4 µm methyl silicone, 40°C for 6 min then 5°C min^{-1} to 220°C. Sampling by dynamic solvent effect, 15 cm^3 min^{-1} for; A 20 min, B 10 min, 22-23°C, *n*-hexane as solvent. Detection by FID, sensitivity 4 x 10^{-11} A mv^{-1} full scale deflection. Peak numbers correspond to identities in Table 15.1.

Table 15.1 Volatile compounds from the anal gland secretion of the aardwolf (*Proteles cristatus*) identified by high resolution gas chromatography - mass spectrometry. Peak numbers correspond to Fig. 15.1.

Peak	Compound
1	Methylpropanoic acid
2	Butanoic acid
3	Hexanol
4	2-methylbutanoic acid
5	Pentanoic acid
6	2-methylpentanoic acid
7	Hexanoic acid
8	Ethyl heptanoate
9	Heptanoic acid
10	Ethyl octanoate
11	Octanoic acid
12	Indole
13	Butyl heptanoate
14	Propyl octanoate
15	Ethyl nonanoate
16	Hexyl hexanoate
17	Pentyl heptanoate
18	Butyl octanoate
19	Ethyl decanoate
20	Heptyl hexanoate
21	Hexyl heptanoate
22	Pentyl octanoate
23	Butyl nonanoate
24	Ethyl undecanoate
25	Heptyl heptanoate
26	Hexyl octanoate
27	Pentyl nonanoate
28	Butyl decanoate
29	Heptyl octanoate
30	Hexyl nonanoate
31	Pentyl decanoate
32	Octyl octanoate
33	Heptyl nonanoate

DISCUSSION

The colour of aardwolf anal gland secretion has been variously reported as dark brown, yellowish ochre and orange (Smithers 1983 p 349); a variability which may be accounted for by the marked differences in colour, noted here, between anal gland contents and scent marks. Although there was no systematic difference in volatile components between scent marks and gland contents the spontaneous darkening of scent marks suggests that some chemical changes are undergone by the gland secretion shortly before its deposition as a scent mark.

The rather rapid changes in the odour of the anal gland contents, and presumably in that of very fresh scent marks, have the potential of acting as short-term indicators of an animal's movements while the extreme persistence of the odour might be expected to enhance its effectiveness in demarcating territories.

Volatile fatty acids have a widespread, if somewhat sporadic occurrence in mammalian secretions (Albone 1984). Whether or not they occur generally in the anal gland secretions of hyaenids is uncertain; Wheeler *et al.* (1975) reported only 5-thiomethylpentane-2,3-dione and one unidentified volatile component from *Hyaena hyaena*, to which Wheeler (1977) added a macrocyclic lactone. Fatty acids are present in large quantities in the headspace of anal gland pastings from brown hyaenas (Chap 10).

The occurrence of a complex series of short and medium chain esters is unusual in mammalian scent secretions. C_{16} and C_{18} acetates have been reported from bank vole (*Clethrionomys glareolus*) urine (Brinck & Hoffmeyer 1984) and long chain esters and sqalene make up 96% of the scent mark of the saddle back tamarin (*Saguinus fuscicollis*) (Yarger *et al.* 1977). Esters similar to those from the aardwolf have been reported only from rabbit (*Oryctolagus cuniculus*) faeces (Goodrich *et al.* 1981) and the anal sac of the wolf (*Canis lupus*) (Raymer *et al.* 1985). Esters may well be more widespread; sample pre-treatments involving acidic or basic extractions (e.g. Maurer *et al.* 1979) are likely to lead to their loss by hydrolysis

No sulphur compounds were identified although the metallic component of the odour suggests that they may be present. They certainly do not occur in the high concentration (1%) reported for 5-thiomethylpentane-2,3-dione in the anal gland secretion of *H. hyaena* (Wheeler *et al.* 1975).

Indole occurs in the anal gland secretion of *Mustela* spp., but not in other Mustelidae (Brinck, Erlinge & Sandell 1983) and is a minor component in the anal sac of the wolf (Raymer *et al.* 1985). It has also been found in the anal gland secretions of the porcupine (*Hystrix africaeaustralis*), the yellow mongoose (*Cynictis penicillata*) (this chapter) and the guinea pig (*Cavia aperea* and *C. porcellus*) (Wellington *et al.* 1979).

A scent mark which acts to demarcate a territory would be expected to have an individually distinct odour. It has been suggested that individual differences in mammalian odours are based on differences in the relative concentrations of components of complex mixtures (Gorman 1980); if this is the case in the aardwolf the series of esters in its anal gland secretion seem ideally suited to such a role.

VOLATILE COMPONENTS FROM THE ANAL GLANDS OF THE YELLOW MONGOOSE *Cynictis penicillata*.

INTRODUCTION

The yellow mongoose is widespread throughout the southern African subregion except in desert, mountain and forest habitats. They are colonial, diurnal viverrids which are predominantly insectivorous but occasionally take seeds or small, vertebrate prey. Their general biology has been reviewed by Smithers (1983).

Like other viverrids the yellow mongoose posesses a well-developed complex of glands around the anus (Macdonald 1985b). The products of these glands are employed in scent marking throughout the animals' home ranges and in allomarking between colony members (Earle 1981; Wenhold* pers. comm.)

Some of the volatile components of the anal glands of a male and a female yellow mongoose are reported here. The material was made available by P. Taylor, Transvaal Museum.

METHODS

Anal gland secretion was obtained from one mongoose of each sex collected by Taylor. (Transvaal Museum numbers TM 39215 (female) and TM 39216 (male)). The contents of the anal sacs and adherant material from the surrounding area were collected in glass-stoppered, borosilicate glass tubes and held in liquid nitrogen until analysis.

Palladium cell purified hydrogen was passed over the secretion at a flow rate of 15 cm^3 min^{-1} and entrained volatiles were sampled by the dynamic solvent effect using *n*-hexane as solvent. Sampling periods of 5 min were used for gas chromatography - flame ionization detector (GC-FID) analyses and of 20 min for gas chromatography - mass spectrometry (GC-MS) analyses. Subtraction of volatile fatty acids during sampling was employed to facilitate identification of some minor components.

GC-FID analyses were carried out on a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet and a 25 m x 0,3 mm x 0,4 µm methyl silicone capillary column. The carrier gas was hydrogen with a linear velocity of 55 cm s^{-1}. The starting temperature of both inlet and column was 40°C, the inlet was heated ballistically to 220°C

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after 2,2 min and the column temperature was programmed at 4°C min^{-1} after 7 min. The detector sensitivity was 4 x 10^{-11} A mv^{-1} full scale deflection. GC-MS analyses were carried out under equivalent conditions on a modified Varian 1400, using helium as carrier gas, with an open split interface to a VG Micromass 16F spectrometer operating in the electron impact mode. The source temperature was 220°C and the electron energy was 70eV.

RESULTS

The peri-anal area of both the male and the female yellow mongoose was thinly covered by a dark brown, waxy material with an odour, neither strong nor unpleasant, closely resembling that of dried beef. The anal sacs contained a milky fluid with the sour, cheesy smell of short-chain fatty acids. Anal gland secretion from both sexes yielded complex mixtures of volatiles of diverse chemical character (Table 15.2, Figure 15.2).

Table 15.2 Identifications of some volatile components of the anal gland secretion of the yellow mongoose (*Cynictis penicillata*). Peak numbers correspond to Fig. 15.2.

Peak	Compound
1	dimethyl disulphide
2	hexanal
3	butanoic acid
4	3-methylbutyl acetate
5	pentanoic acid
6	2-methylbutanoic acid
7	hexanoic acid
8	benzaldehyde
9	dimethyl trisulphide
10	1-methylpropyl propanoate
11	benzeneacetaldehyde
12	3-(methylthio)-1-propanol
13	*p*-cresol
14	indole
15	propyl butanoate
16	3-methyl-1-butyl propanoate
17	3-methylbutyl 2-methylpropanoate
18	2-methylbutyl 2-methylpropanoate
19	nonanal
20	2-methylbutyl 2-methylbutanoate
21	2-methylbutyl 3-methybutanoate
22	decanal

Fig. 15.2 Chromatograms of volatiles from the anal gland secretion of A: male, and B: female yellow mongooses (*Cynictis penicillata*). For analytical conditions see text. Peak numbers correspond to Table 15.2.

DISCUSSION

Gorman *et al.* (1974) found a mixture of short chain fatty acids in the anal scent pockets of the small Indian mongoose (*Herpestes auropunctatus*) and Gorman (1976) established that differences in the ratios of these acids could form the basis of individual odour recognition. A mixture of heavier, branched acids was found in *Herpestes ichneumon* by Hefetz *et al.* (1984). The fatty acids of the yellow mongoose resemble those of *H. auropunctatus* rather than *H. ichneumon*, although the apparent differences among the three species may be to some extent due to differences in methods of collection and analysis of secretion.

In neither *H. auropunctatus* or *H. ichneumon* were compounds other than fatty acids identified, although Hefetz *et al.* record the presence of unidentified compounds in *H.ichneumon*.

Although one individual of each sex is too small a base for definite conclusions it appears that the inter-sexual differences in *Cynictis* volatiles are more complex than they are in *Ichneumon* where a single compound; 2,4,6,10-tetramethylundecanoic acid is found in males and not in females (Hefetz *et al.* 1984).

The series of esters from the yellow mongoose recalls that from the aardwolf (above). In the yellow mongoose the members of the series differ in chain length, and the presence and position of methyl branching while in the aardwolf the differences are in chain length and the position of the ester linkage. In aardwolves the ester series are similar in the two sexes while in the yellow mongoose they are more clearly represented in the female. In both species too little is known about the relationship of scent marking to movement patterns for the significance of these comparisons to be understood.

IDENTIFICATION OF VOLATILE COMPONENTS OF THE ANAL GLAND SECRETION OF THE WATER MONGOOSE (*Atilax paludinosus*).

INTRODUCTION

Water mongooses are relatively primitive, medium-sized viverrids which are widely distributed in well-watered habitats throughout sub-Saharan Africa. They feed mainly on aquatic crustacea, amphibia, small rodents and insects. They are crepuscular and solitary but it is not known whether or not they are territorial. Wild water mongooses defaecate in middens, and in captivity they also scent mark with urine, anal gland secretion and cheek gland secretion. In females the frequency of marking with the anal gland is dependent on reproductive condition (Smithers 1983 pp 497-503; Baker 1987).

Advantage was taken of the availability of a specimen by analysing the volatile components of its anal gland secretion.

METHODS

Secretions from the anal gland of an adult, female water mongoose collected by P. Taylor, Transvaal Museum were collected into a glass-stoppered, borosilicate glass tube by squeezing and scraping. The secretion was kept in liquid nitrogen until analysis.

The volatiles from approximately 0.5 cm^3 of the secretion were sampled by the dynamic solvent effect using a 15 $cm^3\ min^{-1}$ flow of palladium cell purified hydrogen, and *n*-hexane as solvent. The sampling time was 10 min for gas chromatography - flame ionization detector analyses and 30 min for gas chromatography - mass spectrometry analyses.

GC-FID analyses were carried out on a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet and a 25 m x 0,3 mm x 0,4 µm methyl silicone capillary column. The carrier gas was hydrogen with a linear velocity of 55 cm s^{-1}. The starting temperature of both inlet and column was 40°C, the inlet was heated ballistically to 220°C after 2,5 min and the column temperature was programmed at 5°C min^{-1} after 6 min. The detector sensitivity was 4 x 10^{-11} A mv^{-1} full scale deflection. GC-MS analyses were carried out under equivalent conditions on a modified Varian 1400, using helium as carrier gas, with an open split interface to a VG Micromass 16F spectrometer operating in the electron impact mode. The source temperature was 220°C and the electron energy was 70eV.

Compounds were identified by comparison of their mass spectra with those in libraries or the literature.

RESULTS

The female water mongoose's anus was enclosed by a cup-shaped pouch (the *kommietjie* of its Afrikaans name) which opened between two adposable labia. The interior of the pouch was moist and apparently glandular, under firm pressure it exuded a milky, rather viscous fluid. The anal pouch also received the tiny openings of the anal sacs which lie on either side of the anus. The fuid in the sacs was brown and watery and when the sacs were squeezed it was suddenly ejected as a thin jet over a distance of about 1 m. Approximately 2 cm^3 of the combined secretions of the pouch and the anal sacs were collected from the one animal.

The odour of the anal gland secretion was distinctive, mildly unpleasant and strongly animalic. The whole of the animal's body surface emitted a similar odour.

The volatiles from the anal gland secretion were a rather complex mixture (Fig. 15.3). Several of the minor components were identified on the basis of mass spectrum library searches (Table 15.3) but the mass spectra of several other peaks, including three of the major ones, yielded no good fits in library searches and were not amenable to interpretation (Table 15.4).

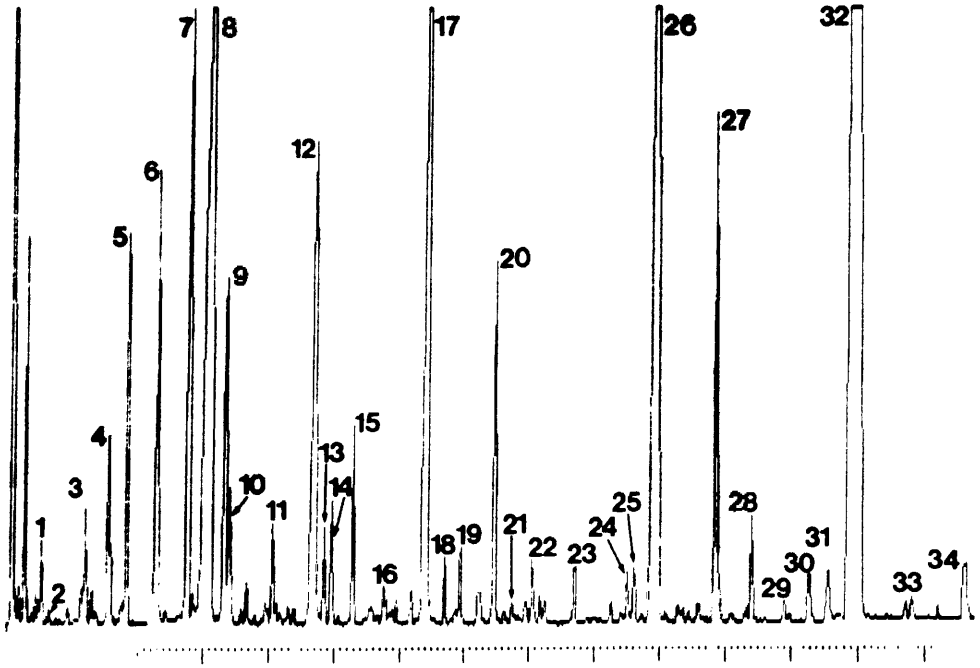

Fig. 15.3 Total ion chromatogram of the volatiles from the anal gland secretion of a female water mongoose (*Atilax paludinosus*). For analytical conditions see text. Peak numbers correspond to Tables 15.3 and 15.4.

Table 15.3 Tentative identifications of volatile compounds from the anal gland secretion of a female water mongoose (*Atilax paludinosus*). Peak numbers correspond to Fig. 15.3.

Peak	Identification
1	dimethyl disulphide
2	3-methyl-2-pentanone
3	2-hexanone
4	octane
5	unknown
6	unknown
7	unknown
8	4-heptanone
9	3-heptanone
10	3-ethylheptane
11	nonane
12	benzaldehyde
13	2-ethylhexanal
14	dimethyl trisulphide
15	4-ethyloctane
16	2-pentyl furan
17	unknown
18	unknown
19	unknown
20	3-nonanone
21	nonanal
22	undecane
23	unknown
24	decanal
25	unknown
26	unknown
27	3-undecanone
28	unknown
29	unknown
30	unknown
31	unknown
32	unknown
33	unknown
34	unknown

Table 15.4 Mass spectra; m/z (intensity), of unidentified volatile components of the anal gland secretion of a female water mongoose (*Atilax paludinosus*). Peak numbers correspond to Fig. 15.3.

Peak	Mass spectrum
5	43(100) 86(18) 71(16) 41(9) 55(8) 70(4) 39(4) 42(4) 85(2) 99(1) 114(1) 141(1)
6	55(100) 41(62) 69(45) 84(39) 70(38) 56(29) 29(20) 43(15) 39(14) 42(13) 97(11) 57(9) 67(7)68(5) 71(5) 83(5) 98(4) 126(1)
17	43(100) 57(61) 86(43) 71(23) 29(19) 41(19) 58(11) 27(11) 55(9) 85(6) 39(6) 40(5) 42(6) 56(5) 99(2) 112(1) 141(1)
18	55(100) 70(71) 41(38) 56(36) 69(32) 98(21) 29(19) 43(17) 57(17) 97(17) 39(11) 83(11) 84(9) 112(9) 54(8) 71(8) 126(1) 142(1) 159(1)
19	69(100) 55(73) 84(69) 70(67) 41(62) 43(54) 56(30) 83(27) 57(20) 29(17) 85(16) 42(14) 67(11) 97(10) 125(5) 126(3) 112(1) 140(1) 154(1)
23	72(100) 43(56) 57(51) 41(28) 28(234) 71(15) 27(13) 109(3) 128(1) 145(1)
25	43(100) 71(71) 114(32) 57(23) 58(21) 41(20) 85(20) 29(14) 72(14) 55(12) 59(9) 99(3) 127(1) 137(1) 151(1) 181(1)
26	43(100) 86(71) 71(45) 57(22) 41(19) 29(14) 55(12) 85(12) 27(8) 58(8) 99(5) 113(2) 127(1) 170(1)
28	86(100) 43(94) 71(45) 57(34) 41(22) 55(16) 29(17) 85(13) 58(9) 99(8) 39(7) 56(6) 113(1) 156(1) 169(1) 195(1)
29	72(100) 57(49) 43(41) 41(27) 55(19) 29(17) 71(14) 85(9) 69(9) 73(9) 109(3) 96(2) 97(2) 110(2) 128(2) 137(1)
30	86(100) 43(81) 71(41) 57(27) 41(21) 55(19) 110(17) 81(12) 29(11) 99(9) 69(9) 58(8) 85(8) 95(5) 111(2) 151(2) 123(1) 169(1)
31	43(100) 71(87) 57(46) 114(31) 41(28) 58(26) 55(18) 29(17) 142(15) 59(13) 72(13) 99(8) 39(7) 56(7) 82(2) 127(4)
32	86(100) 43(80) 71(39) 41(21) 55(13) 29(12) 85(10) 58(9) 99(6) 39(6) 42(6) 87(5) 113(1)
33	86(100) 43(72) 71(34) 57(33) 41(20) 55(15) 29(10) 85(9) 99(9) 99(8) 39(5) 42(5) 72(5) 97(3) 109(2) 120(1) 140(1)151(1) 177(1) 207(1)
34	86(100) 43(42) 71(29) 57)26) 41(14) 55(14) 99(12) 29(6) 69(6)85(6) 87(5) 109(4) 138(4) 123(2) 151(2) 179(2) 197(1) 226(1) 256(1)

DISCUSSION

Baker (1987 p 62) reports that the anal gland scent marks of captive water mongooses were black and oily. This suggests that the dark secretion of the sacs is mixed with that of the pouch or that the pouch secretion undergoes a colour change just before deposition, as does the anal gland secretion of the aardwolf (above). Baker also reports the forcible ejection of black, strong-smelling liquid from the anal sacs of animals under stress.

Dimethyl disulphide is a fairly widespread component of mammalian odours. It has been reported from the anal glands of *Mustela vison* (Sokolov, Albone, Heap, Kagan, Vasilieva, Roznov & Zinkevich 1980), *Canis familiaris* (Schultz, Kruse & Flath 1985), *Cynictis penicillata* (above) and *Hystrix africaeaustralis* (below), the faeces of *Oryctolagus cuniculus* (Goodrich *et al.* 1981), the vaginal secretion of *Mesocricetus auratus* (Singer, Macrides & Agosta 1980) and human urine (Liebich & Al-Babbili 1975).

Benzaldehyde is equally widespread; it occurs in the urine of *Vulpes vulpes* (Jorgenson *et al.* 1978), the anal sacs of *Canis lupus* (Raymer *et al* 1985), human urine (Liebich & Al-Babbili 1975) the urine of *Mus musculus* (Schwende *et al.* 1986) and the anal glands of *H. africaeaustralis* (below), *C. penicillata* (above) and *Hyaena brunnea* (Chap 10).

Apart from its occurrence in *C. penicillata* (above) and *H. africaeaustralis* (below) dimethyl trisulphide has been reported from *M. auratus* vaginal exudate (Singer *et al.* 1980). This apparent rarity might be a consequence of its thermal lability.

2-pentyl furan occurs in *O. cuniculus* faeces (Goodrich *et al.* 1981) and it and other alkyl furans occur in human urine (Albone 1984 p 177). Other furans are very widespread.

The absence of fatty acids from the volatiles emitted by the water mongoose's anal gland secretion is surprising. These compounds are widespread among carnivore anal gland secretions (Gorman *et al.* 1974; Preti *et al.* 1976; Sokolov *et al.* 1980; Albone 1984 pp 142-159; Hefetz *et al.* 1984; see also Chap 10 and this Chapter). An exception to this generalization is provided by the sulphur-rich secretions of the anal glands of mustelids (Andersen & Bernstein 1975; Brinck, Gerell & Odham 1978; Crump 1980a, b; Andersen *et al.* 1982; Brinck *et al.* 1983; Crump & Moors 1985; see also *Ictonyx* below). Although they were present in the secretion itself, Raymer *et al.* (1985) recovered no volatile fatty acids from the head-space of wolf anal-sac

secretion, a finding which may have been an artifact of the sampling method (p 152). Information from only one animal is too small a base for any conclusions about the possible significance of the absence of volatile acids from the odour volatiles of the water mongoose's anal gland secretion.

The similarities among the mass spectra of peaks 17, 26, 28, 30, 32, 33, and 34 point to fairly close stuctural relationships. That this group of compounds is an unusual one is suggested by the absence of even roughly similar spectra in the libraries. Unfortunately the spectra are uninformative as to molecular weight, functional group or presence of hetero-atoms. The use of such techniques as Fourier transform infra-red spectroscopy may well prove necessary if these compounds are to be identified, from this point of view it is fortunate that three of them (peaks 17, 26 and 32) are major components of the odour volatiles.

VOLATILE COMPONENTS OF THE ANAL GLAND SECRETION OF THE STRIPED POLECAT *Ictonyx striatus*

INTRODUCTION

The striped polecat *Ictonyx striatus* is a small, African mustelid which in appearance, habits and ecology resembles the skunks of North America. Its biology has been authoritatively reviewed by Smithers (1983). Like the North American skunks *Mephitis mephitis* and *Spilogale interrupta*, *Ictonyx* is equipped with a well developed anal gland whose foul-smelling secretion is used for defence. The chemistry of this secretion has not previously been reported. The identification of the major volatile components which give the secretion its defensive properties is dealt with here.

METHODS

Anal gland secretion was obtained from an adult male *Ictonyx* obtained as a fresh road casualty in the northern Cape Province, South Africa and kept deep-frozen until analysis. The anal gland was excised and its secretion expelled into a 10 cm^3 glass tube. Volatiles were accumulated from 10 cm^3 of the head space above the secretion by dynamic solvent effect sampling using *n*-hexane as solvent at 25°C. Accumulated volatiles were separated and identified by gas chromatography - mass spectrometry. A dynamic solvent effect inlet was used to transfer the volatiles to a 25 m x 0,3 mm x 0,4 µm methyl silicone capillary column in a Varian 1400 gas chromatograph. The carrier gas was helium with a linear flow rate of 30 cm s^{-1}. The injector was held at 40°C for 7 min then heated ballistically to 220°C. The column was held at 40°C for 10 min then programmed at 4°C min^{-1} to 180°C. The column was coupled by an open-split interface to a VG Micromass 16 F mass spectrometer operating in the electron impact mode with an electron energy of 70 eV and a source temperature of 220°C.

Components were identified by comparison of their mass spectra with library material or with published spectra, or by mass spectral interpretation. Where possible chromatographic retention data were used for confirmation.

RESULTS

The anal gland secretion of the male *Ictonyx striatus* consisted of a light, mobile, yellow oil and a colourless, translucent aqueous phase. The secretion emitted a strong, penetrating, metallic odour.

Fifteen clearly resolved peaks appeared on the total ion chromatogram (Fig. 15.4), of which 12 were identified (Table 15.5).

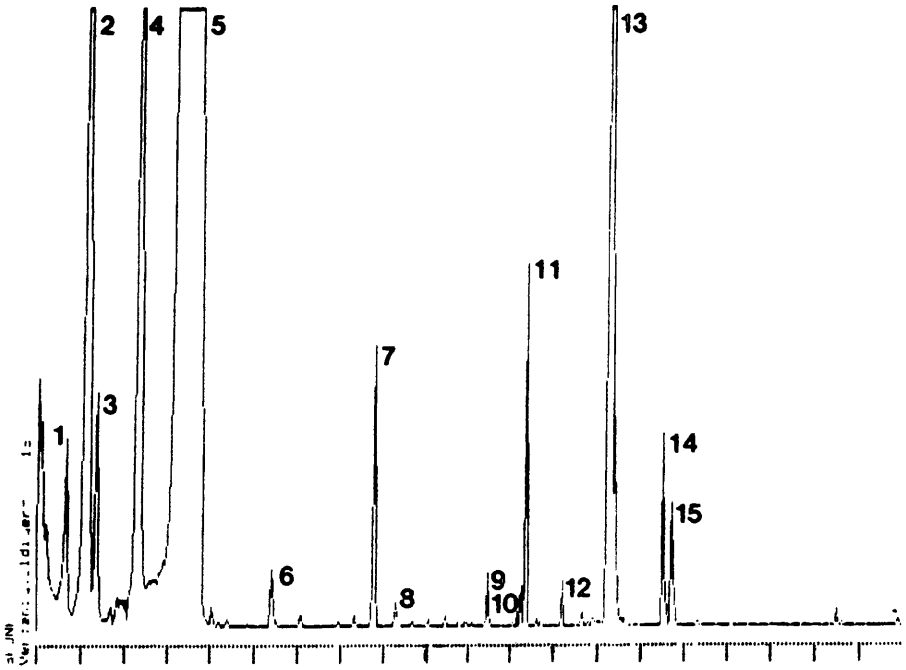

Fig. 15.4 Total ion chromatogram of volatiles from 10 cm^3 of headspace above the anal gland secretion of a male *Ictonyx striatus* sampled by the dynamic solvent effect. Peak numbers correspond to Tables 15.5 and 15.6. For analytical conditions see text.

Table 15.5 Volatile components identified from the anal gland secretion of a male *Ictonyx striatus*. Peak numbers correspond to Fig. 15.4.

Peak	Identification	Criterion*
1	2-methylthiacyclobutane	L
2	2,2-dimethylthiacyclobutane	P a
3	3-methyl-1-butanethiol	L
4	1-pentanethiol	L
5	2-ethylthiacyclobutane	P b,d
6	2-ethyl-3-methylthiacyclobutane	P c
7	4-methyl-3-heptanone	P e
8	propylthiacyclobutane isomer	I
9	methyldithiacyclopentane isomer	I
10	a ketone	I
11	1,2-dithiacycloheptane	P b
12	2-methyl-1-decene	L
13	ethyldithiacyclopentane isomer	I
14	a ketone	I
15	a ketone	I

* L = fit from mass spectra library; P = published spectrum, a: Brinck, Erlinge & Sandell 1983; b: Brinck, Gerell & Odham 1978; c: Crump & Moors 1985; d: Sokolov, Albone, Flood, Heap, Kagan, Vasilieva, Roznov & Zinkevitch 1980; e: Stenhagen, Abrahamson & McLafferty 1974; I = mass spectrum interpretation.

DISCUSSION

The occurrence in other mustelids of the compounds identified in *Ictonyx striatus* is summarised in Table 15.6.

Table 15.6 Occurrence among Mustelidae of compounds found in the anal gland secretion of *Ictonyx striatus*. Compound numbers correspond to Fig. 15.4 and Table 15.5.

Species	Compound*											
	1	2	3	4	5	6	7	8	9	11	12	13
Mustela erminea	-	o	-	-	x	-	-	o	-	-	-	-
Mustela nivalis	-	o	-	-	-	-	-	-	-	-	-	o
Mustela putorius	-	o	-	-	-	-	-	o	-	-	-	-
Mustela putorius furo	-	o	-	-	-	o	-	o	-	-	-	-
Mustela vison	-	x	-	-	x	-	-	-	-	x	-	o
Mephitis mephitis	-	-	x	-	-	-	-	-	-	-	-	-

* x = same isomer; o = different isomer; - = absent

The presence of sulphur compounds is expected since these are usually strong-smelling and are important components of the anal gland secretions of other mustelids. The major, volatile component of the *Ictonyx* secretion; 2-ethylthiacyclobutane, made up 90 % of the headspace volatiles. This compound was found as a minor component of the anal gland secretion of mink (*Mustela vison*) by Sokolov *et. al.* (1980 compound V) but was then incorrectly identified as a dimethylthiacyclobutane isomer (compare Brinck *et al.* 1983 compound 5). The offensive, odorous compounds of the anal gland of the striped skunk (*Mephitis mephitis*) are trans-2-butene-1-thiol, 3-methyl-1-butanethiol and trans-2-butenylmethyldisulphide (Andersen & Bernstein 1975). Of these only 3-methyl-1-butanethiol was found in the striped polecat, where it was a minor component.

The presence of ketones is unusual among the mustelidae (Brinck *et al.* 1983) although they are important components of fox (*Vulpes vulpes*) urine (Jorgenson *et al.* 1978).

VOLATILE COMPOUNDS FROM THE ANAL GLAND SECRETION OF THE PORCUPINE *Hystrix africaeaustralis*

INTRODUCTION

Porcupines, with mean body weights of 17-18 kg, are the largest of Africa's rodents (Smithers 1983 pp 186-188). A good deal of the work on reproductive physiology which has been carried out on other rodents has been repeated in the case of the porcupine. They live in family groups in which only one pair breeds, a feature which influences population dynamics (Van Aarde 1987a, b). In captivity the establishment and maintenance of female reproductive cycling is dependent on cohabitation with a male, males housed in pens adjacent to those of females are ineffective in this respect (Van Aarde 1985a). These social influences on reproduction make porcupines interesting subjects for work on semiochemistry. In addition the porcupine occupies the same sub-order, the hystricomorpha, as the guinea pig (*Cavia porcellus*) which, from the point of view of semiochemistry is one of the best studied mammals (review by Macdonald 1985a).

METHODS

Anal gland secretion was collected from one adult, male and four adult, female porcupines at the breeding colony of the Mammal Research Institute, University of Pretoria. The animals were anaesthetized (Van Aarde 1985b) and a tuft of the secretion-soaked bristles which surround the anogenital region was clipped off and stored at liquid nitrogen temperature in polytetrafluoroethylene-capped borosilicate glass vials.

The volatiles from the secretions were sampled by the dynamic solvent effect using a 10 cm^3 min^{-1} flow of palladium cell purified hydrogen, and *n*-hexane as solvent. The sampling time was 10 min for gas chromatography - flame ionization detector analyses and 30 min for gas chromatography - mass spectrometry analyses.

GC-FID analyses were carried out on a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet and a 25 m x 0,3 mm x 0,4 µm methyl silicone capillary column. The carrier gas was hydrogen with a linear velocity of 50 cm s^{-1}. The starting temperature of both inlet and column was 40°C, the inlet was heated ballistically to 220°C after 2,5 min and the column temperature was programmed at 5°C min^{-1} after 7 min. The detector sensitivity was 4 x 10^{-11} A mv^{-1} full scale deflection. GC-MS analyses were carried out under equivalent conditions on a modified Varian 1400, using helium as carrier gas, with an open split interface to a VG Micromass 16F spectrometer

operating in the electron impact mode. The source temperature was 220°C and the electron energy was 70eV. Because the secretion from the females was markedly variable material from all four individuals was pooled for the GC-MS run so that each peak was present in identifiable quantities.

Compounds were identified by comparison of their mass spectra with those in libraries or the literature.

RESULTS

The bristles around the anus of porcupines of either sex are soaked with a, usually whitish, secretion with a strong, characteristic odour in which the sour smell of volatile fatty acids predominates. The general odour of the animals and their living quarters is similar to, and probably derived from, that of the anal gland secretion.

There were very obvious differences in secretion chemistry between the male and the females and among the females (Fig. 15.5. Table 15.7)

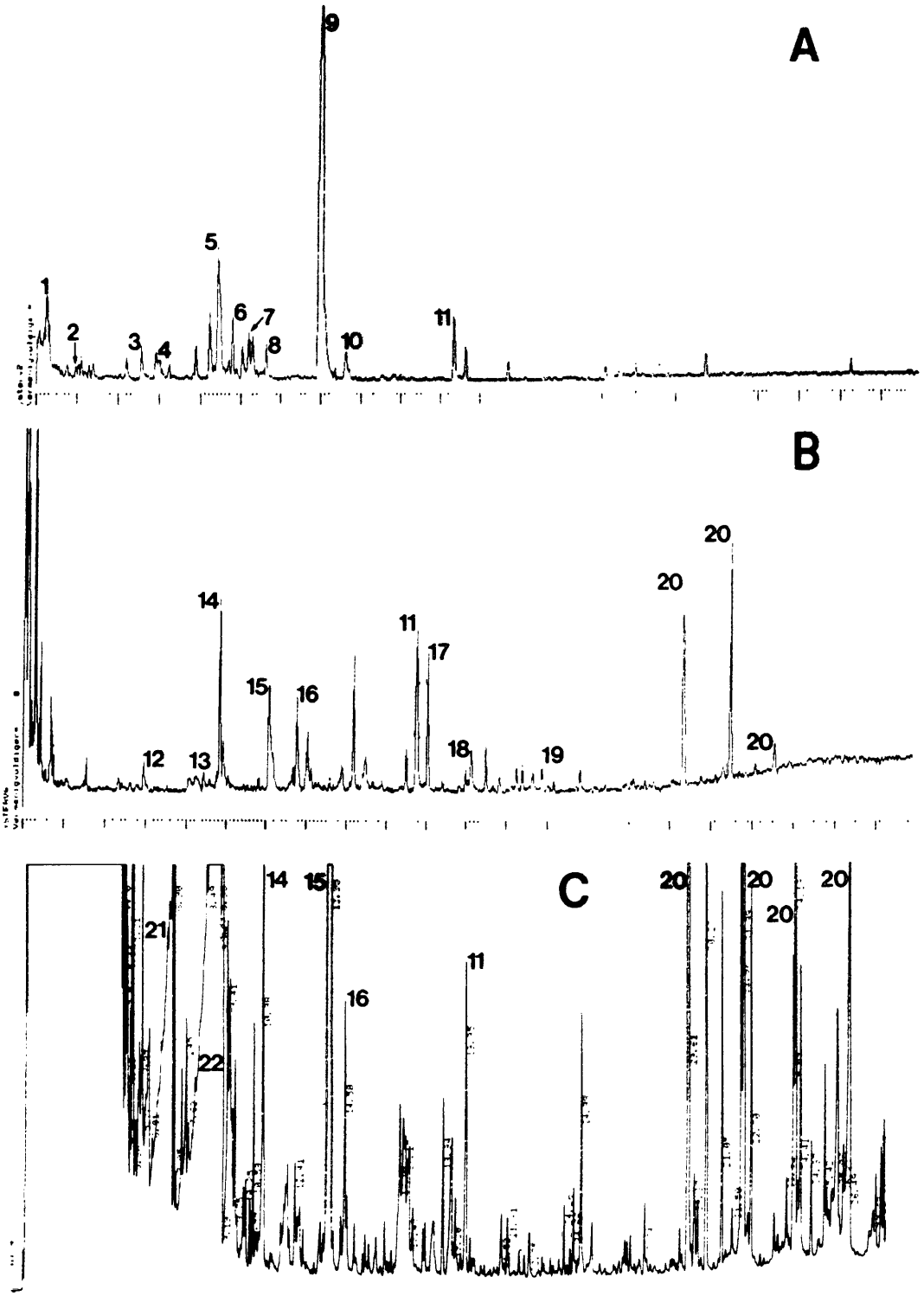

Fig. 15.5 (continued on next page)

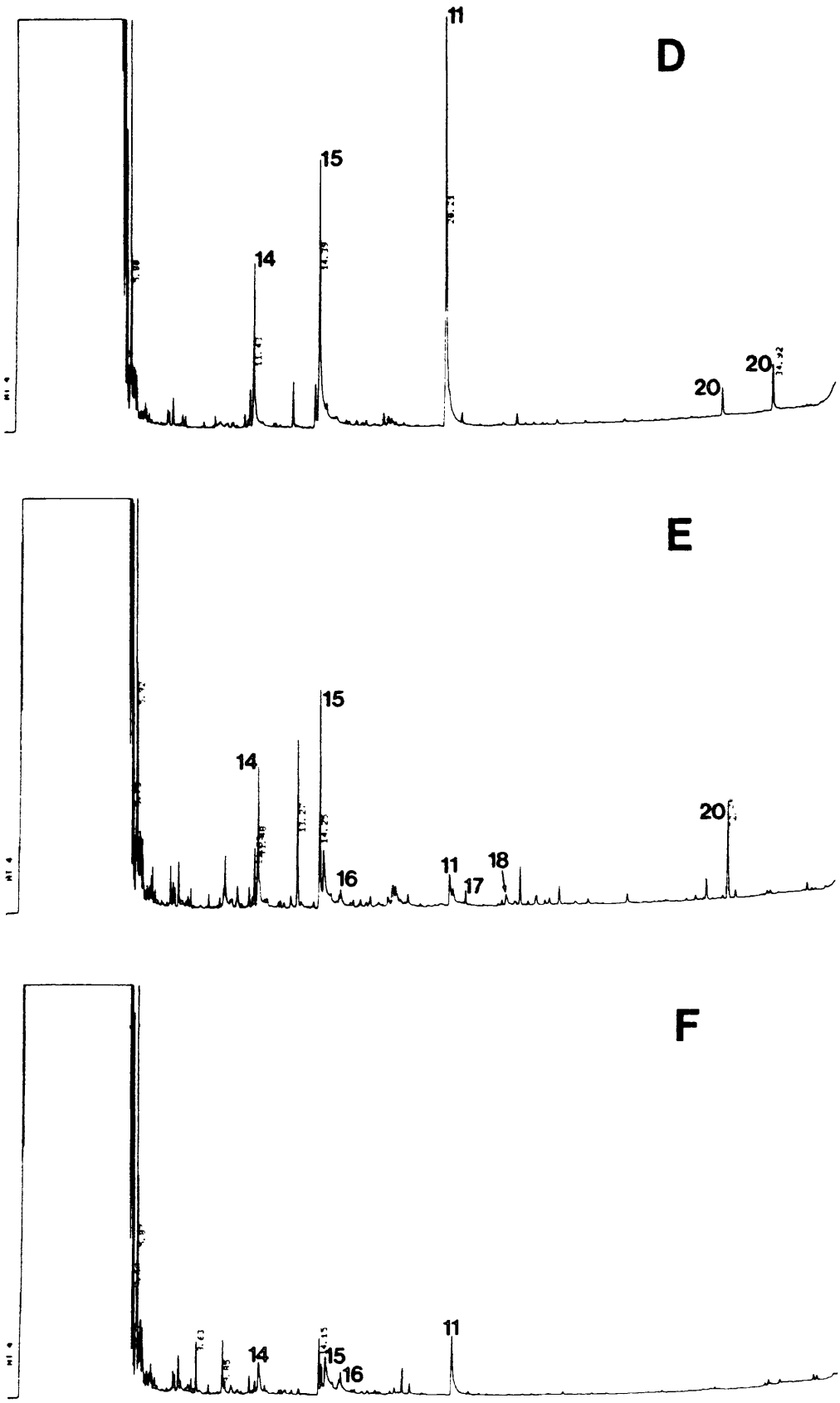

Fig. 15.5 (continued on next page)

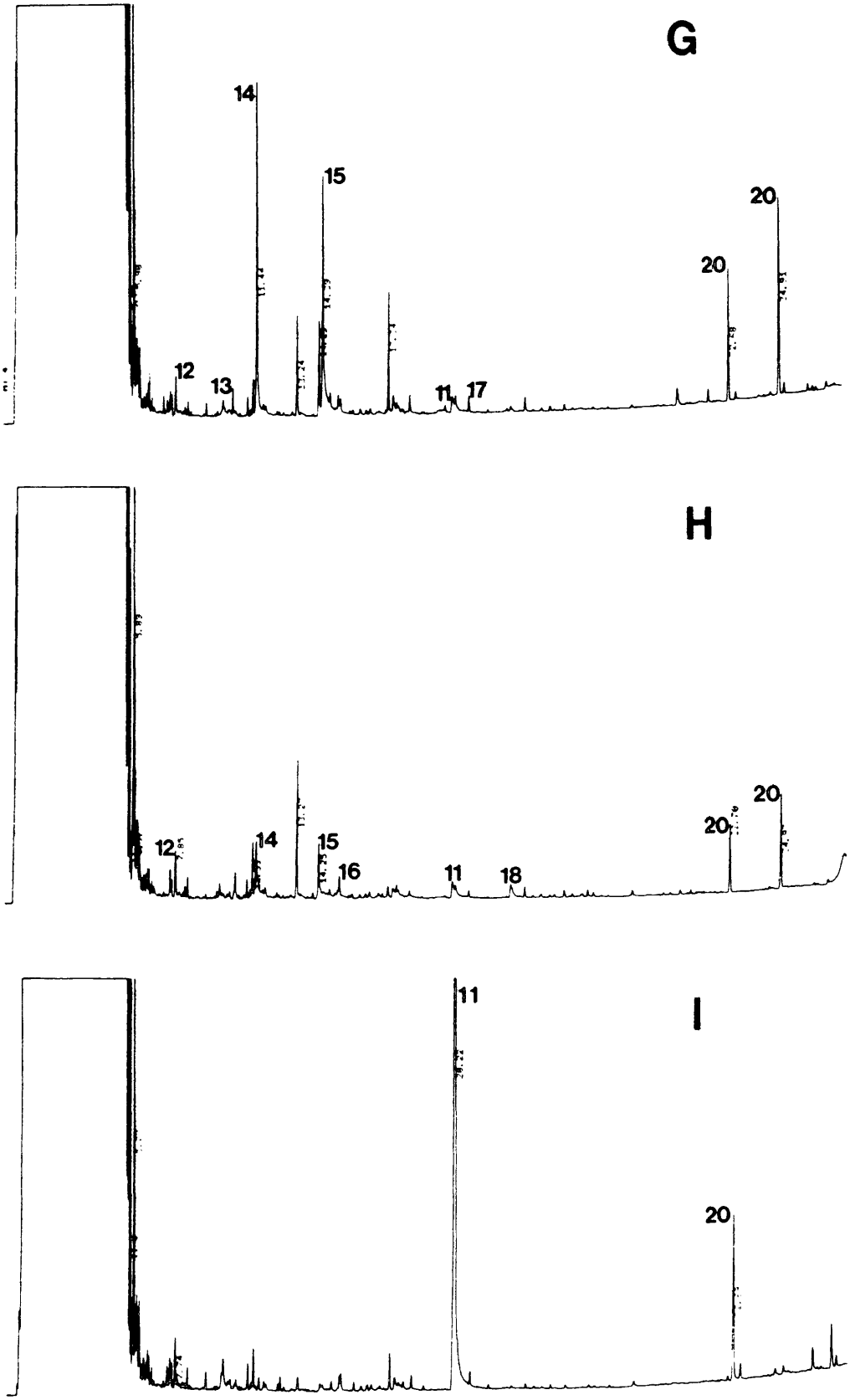

Fig. 15.5 Chromatograms of volatiles from anal gland secretions of porcupines (*Hystrix africaeaustralis*). A: secretion pooled from four adult females; B: adult female; C: adult male; D, E, F, imperforate, adult females; G: oestrous female; H: female with vaginal plug; I: lactating female. For analytical conditions see text. Peak numbers correspond to Table 15.7.

Table 15.7 Compounds provisionally identified among the volatile components of the anal gland secretions of porcupines (*Hystrix africaeaustralis*). Peak numbers correspond to Fig. 15.5.

Peak	Compound
1	dimethyl disulphide
2	3-hexanone
3	3-methylhexanal
4	*n*-heptanal
5	benzaldehyde
6	dimethyl trisulphide
7	phenol
8	3-octanal
9	*p*-cresol
10	phenylethanol
11	indole
12	2-heptanone
13	isocyanobenzene
14	2-octanone
15	acetophenone + *p*-cresol
16	2-nonanone
17	2-undecanone
18	2-methylindole
19	2-tridecanone
20	long chain fatty acid methyl esters
21	butanoic acid
22	pentanoic acid

DISCUSSION

The occurrence of fatty acids, phenols, dimethyl disulphide, dimethyl trisulphide, benzaldehyde, indole, and carbonyl compounds among mammalian secretions has been discussed earlier in this chapter.

2-phenylethanol was found in rabbit faeces volatiles by Goodrich *et al.* (1981), and *p*-hydroxyphenylethanol in the chest gland secretion of *Galago crassicaudatus* (Crewe *et al.* 1979). Acetophenone occurs in the urine of the red fox (Jorgenson *et al.* 1978) and the mouse (Schwende *et al.* 1986).

ANALYTICAL CHALLENGES AND SOLUTIONS

The aim of the investigations reported in this chapter was only to produce lists of the major volatile constituents of the secretions involved; this presented less challenging problems than the other investigations in which quantitative analyses, in some cases of minor components, were necessary, and correspondingly less light is thrown on the properties of the dynamic solvent effect as a sampling system.

What is clearly established is the compatibility of dynamic solvent effect sampling with gas chromatography - mass spectrometry.

The benefits of a sampling system able to handle intractable compounds such as fatty acids without the need for derivatization is apparent in the cases of the aardwolf, the yellow mongoose and the porcupine. Derivatization of the relatively large quantities of fatty acids would probably have compromized the analysis of some of the other components, either through their chemical degradation or as a result of co-elution with impurities from the reagents involved.

The importance of the dynamic solvent effect's compatibility with the resolving power of capillary columns is demonstrated by the series of esters in the anal gland secretion of the aardwolf. The Kovats Indices of adjacent members of each group of esters differ by only about 4 units (Jennings & Shibamoto 1980). If the peaks in each group had not been as well resolved the details of their structural relationships would have been impossible to unravel.

The successful elution of thermally unstable dimethyl trisulphide (porcupine, yellow mongoose and water mongoose) demonstrates the virtually "cool" operation of the dynamic solvent effect inlet.

Due to their limited value the generation of reports like those presented here is viable only if it can be achieved with a relatively small cost in analytical input. This demands, among other things, an analytical system which is flexible enough to run different types of sample without extensive re-optimization of procedures and conditions. That dynamic solvent effect sampling coupled with capillary GC-MS fulfills these conditions can be seen from the small number of man days and analytical runs necessary for this type of project (Table 15.8).

Table 15.8 Analytical* input to qualitative investigations of mammalian secretion chemistry using dynamic solvent effect sampling and gas chromatography-mass spectrometry. The number of man-days has been rounded up.

	Analytical Input		
	Number of runs		
Investigation	GC-FID	GC-MS	Man-days
Ictonyx	3	1	2
Proteles	7	2	2
Cynictis	4	4	2
Hystrix	5	3	3
Atilax	1	1	1

* does not include time for interpretation and checking of spectra etc.

Chapter 16

AN ASSESSMENT OF THE DYNAMIC SOLVENT EFFECT FOR MAMMALIAN SEMIOCHEMISTRY

This discussion complements that in Chap 6 which considered the quantitative performance of the dynamic solvent effect in comparison to that of other systems over a wide range of specimen types. The discussion has been divided into two parts; first a consideration of the performance of the dynamic solvent effect in its application to semiochemical problems and secondly a comparison of the dynamic solvent effect with the general performance of other systems which have been employed in work on mammalian semiochemistry.

PERFORMANCE OF THE DYNAMIC SOLVENT EFFECT

Minimum detectable quantity

In only one investigation, that of the schizophrenic odour (Chap 14) was the "active" component present at levels too low to be detected as a definite peak by flame ionization or mass spectrometer detectors. That this was a shortcoming of the detectors rather than the sampling system was demonstrated by the the detection of the compound concerned by a more sensitive detector - the human nose.

Specimen size

In all the applications the specimen sizes were biologically realistic. Samples were successfully obtained from single small rodents (Chaps 11 and 12), single insects (Chap 9) and single scent marks (Chap 10 and Chap 15, aardwolves). The groups of six male *Amblyomma hebraeum* used in Chap 13 are well within the range of numbers which aggregate normally and proved in any case to be uneccessarily large. Pooled specimens were employed only to reduce the number of mass spectrometer runs necessary to identify the components of highly variable samples (Chap 9 and Chap 15, porcupines)

In most cases the volume of gas used for sampling was larger than would be sniffed by a mammal. The clear exception was the case of the anal gland secretion of *Ictonyx striatus* (Chap 15) where only 10 cm^3 of gas was used. The volume of a sniff by a German Shepherd dog, which is probably a reasonable approximation to one by a brown hyaena,

was taken as 60 cm3 by Moulton (1977). For mammals of aardwolf size (9 kg Smithers 1983 p 348) sniff volumes of 5 cm^3, for small carnivores around 1 cm^3, and for small rodents considerably less than 1 cm^3 seem likely. Thus the gas volumes used for dynamic solvent effect sampling range from approximately equal to a single sniff e.g. 5 minutes sampling at 10 cm^3 min^{-1} from a fresh hyaena pasting (Chap 10) to at least two orders of magnitude above the volume of a sniff e.g. 20 min sampling at 20 cm^3 min^{-1} from mice (Chaps 11 and 12).

Usually mammals take more than one sniff at an interesting odour source. For example, male golden hamsters sniff for approximately half of a two minute trial at a bottle containing an anaesthetized female (Johnston 1977). Male European rabbits (*Oryctolagus cuniculus*) sniff at the urine of females in bouts about 7,5 s long (Bell 1985). Genets (*Genetta genetta*) sniff up to 62 times in 15 min at scent marks (Roeder 1980). Bush babies (*Galago crassicaudatus*) sniff for 1-4 min at scent marks (Clark 1982). Priming semiochemicals in rodents may require exposure for periods of hours (Brown 1985).

Although it is not known how soon during a sniffing bout an animal is able to decipher the information available from a mark it appears that the the gas volumes needed for dynamic solvent effect sampling may not be many times greater than those used by the animals themselves.

Sampling time and sampling rate

A decrease in sampling time would have been helpful in the investigation of the ageing of hyaena scent marks (Chap 10) since shorter sampling periods would have allowed better resolution of the age of the marks. In the three cases where samples were taken from live animals (Chaps 11, 12 and 13) shorter sampling periods would have reduced disturbance of the subjects.

It is not clear to what extent a decrease in sampling time could have been achieved by an increase in sampling gas flow rate above the 10-20 cm^3 min^{-1} allowed by the dynamic solvent effect (Chap 6). If the emission rates of volatiles are limited by some property of their source then attempting to carry them away faster by increasing the sampling rate will, plainly, be ineffective.

When whole live animals are enclosed during sampling (Chaps 11 and 12) the sampling gas must supply their respiratory needs. While 20 cm^3 min^{-1} of air was evidently sufficient for mouse-sized rodents larger animals would probably need a supplementary supply. In such a case it would be necessary to vent the additional air, or use it to provide parallel samples, so that the gas flow through the concentrator was kept within working limits.

Precision

The precision of dynamic solvent effect sampling was evidently adequate for all the problems investigated. Although in the investigation of male mouse hierarchies there were some pairs of mice whose chromatographic profiles differed by less than 10 % it is probable that these pairs actually had equal social rank, in all cases the profile of the dominant mouse was clearly distinguishable from that of the subordinates.

In the case of the brown hyaena scent mark (Chap 10) a precision of better than 8 % coefficient of variation in peak area ratios was maintained down to 0,1 ng quantities. Below these levels precision declined, although this may as well have been a feature of the scent marks themselves as a reflection of the performance of the sampling system.

Among the 43 samples taken from male *Mastomys* there were only three cases in which the area of the species-specific 3-nonene-2-one peak of an individual of one species fell within the peak area range of the other species. The 3-nonene-2-one peak area varied widely among replicate samples from single individuals, possibly as a result of biological variation.

Differential sensitivity; the ability to distinguish between odourants of different concentrations, is expressed as a Weber fraction; the smallest detectable change in concentration as a fraction of the lower of the two concentrations distinguished. In humans the values obtained for Weber fractions differ considerably between investigators. Values of a few tens of percent are typical but Cain (1977) achieved a Weber fraction of 4,2 % for *n*-butanol by careful control of stimulus noise. (Engen 1970; Cain 1977 and references cited). For the laboratory rat Davis (1973) gives a Weber fraction of 200 %, a value about which Cain (1977) has expressed reservations.

From this limited information it appears that dynamic solvent effect sampling is actually considerably better than are mammals in detecting changes in the concentrations of single odourants, a finding which is supported by the comparison between the abilities of chromatographic analysis and conspecific females in discriminating between male *M. coucha* and *M. natalensis* (Chap 11).

No information is available on the ability of man and other mammals to detect changes in the relative concentrations of the components of mixed odourants.

Even if it were to be demonstrated that mammals can detect differences too small to be accessible by dynamic solvent effect sampling, it would be difficult to believe that a chemical signalling system would be so subtly based. In the cases investigated so far biological variation has covered orders of magnitude, rather than percentage, differences in concentration (Wellington *et al.* 1979; Bailey, Bunyan & Page 1980; Von Stralendorff 1982; Raymer *et al.* 1984, 1985; Epple & Smith III 1985 p 788).

Thermal decomposition

In only one case was there evidence of a compound's thermal decomposition during analysis; one peak in the chromatograms of mouse volatiles (Chap 12) was badly distorted. The distortion persisted, however, when the dynamic solvent effect inlet remained unheated during transfer of the sample to the column. If thermal decomposition was the cause of the distortion it must have been occurring on the column rather than in the inlet. Dimethyl trisulphide, which is known to be thermally labile (Singer *et al.* 1976; O'Connell *et al.* 1979) was successfully eluted as a sharp peak from samples of volatiles from yellow mongoose (*Cynictis penicillata*), porcupine (*Hystrix africaeaustralis*) and water mongoose (*Atilax paludinosus*) anal gland secretions (Chap 15).

Adsorptive activity

The airborne chemical signals whose analyses have been reported in the last seven chapters contained free fatty acids, phenols, aldehydes, ketones, alcohols, esters, sulphur compounds, terpenes and aromatic and hetrocyclic compounds. All of these could be eluted as sharp peaks at the 1-10 ng level providing a newly installed column was used. All cases of peak distortion due to adsorptive activity were successfully overcome by changing a deteriorated column for a new one without changing the dynamic solvent effect concentrator or any other part of the sampling system. This establishes unequivocally that column deterioration (associated with the use of dicumyl peroxide as a stationary phase cross-linking agent) was the limiting factor in system performance.

Specimen manipulations

Only in the case of the attempted identification of the schizophrenic odour (Chap 14) were the specimens manipulated (addition of water and increase in temperature) in order to increase the yield of volatiles.

COMPARISON OF THE DYNAMIC SOLVENT EFFECT WITH OTHER SAMPLING SYSTEMS USED FOR MAMMALIAN ODOURS

As already argued in Chap 2 odour signals should be analysed as such, that is as dilute mixtures of airborne volatiles. The discussion which follows has, therefore, been restricted to sampling techniques compatible with this type of material.

Throughout the discussion it should be borne in mind that, in the main, the investigations cited from the literature differ from those involving the dynamic solvent effect in that testing of the sampling system was not among their stated aims. Consequently the results obtained do not necesarily represent the limits of performance of the techniques involved. An additional complication is that comparisons between results from different animals may not be valid - some species are undoubtedly smellier than others.

Specimen size

The possibility of whole-body sampling from small mammals was raised in 1977 by Claesson & Silverstein, and again by Albone in 1984. Nevertheless the only other study I am aware of in which whole-body volatiles were sampled is that by Von Stralendorff (1982) on *Tupaia belangeri*. The animals were confined overnight (12 h) in glass sleeping boxes and volatiles were collected on Grob-type charcoal traps (Grob & Zürcher 1976). Each GC separation used all of the 3 µl of dichloromethane used to elute the volatiles from the trap. Von Stralendorff's sampling rate is stated as 60 $cm^3\ h^{-1}$ but this is almost certainly an error, a flow of 1 $cm^3\ min^{-1}$ of air would be inadequate for even a sleeping mammal, and the Grob volatile trapping system is designed to work at flow rates around 1 l min^{-1}.

Interestingly the compound which Von Stralendorff identified as an active component of the tree shrew's odour; 2,5-dimethylpyrazine, has also been found to be a constituent of female mouse urine which delays puberty in other females (Novotny, Jemiolo, Harvey, Wiesler & Marchlewska-Koj 1986).

Whole-body volatiles have also been collected from humans. The sampling flow rates; 2000-4000 l h^{-1}, quantities of volatiles involved; 0,6-470 µg h^{-1}, and the scale of the apparatus; a 971 l stainless steel and glass tank, are hardly comparable with a mouse in a 500 cm^3 bottle, but the human system might well be adaptable to use with other large mammals (Ellin, Farrand, Oberst, Crouse, Billups, Koon, Musselman & Sidell 1974).

The 20 minute sampling periods made possible by the dynamic solvent effect's compatibility with the sensitivity limits of capillary columns and gas chromatography detectors offer valuable advantages over the 12 hour periods used by Von Strallendorff to load Grob-type charcoal traps. At the least, the overnight absence from a group of one of its members could affect the social correlates of the odour changes under investigation. Unless, as Von Strallendorff did, sampling is co-ordinated with a subject's sleeping habits confinenment for periods of several hours in a relatively small container is undesirable from the point of view of stress to the animal.

Sampling from the odours of single individuals has been carried out in a number of cases e.g. fox urine volatiles (Jorgenson *et al.* 1978), guinea pig anal gland volatiles (Wellington *et al.* 1979), tree-shrew whole-body odours (Von Strallendorff 1982), rabbit faecal odours (Goodrich *et al.* 1981) and bitch vaginal volatiles (Goodwin *et al.* 1979). Among these Wellington *et al.*'s, Von Strallendorff's and Goodrich *et al.*'s work considered inter-individual variation *per se*.

In contrast, other studies have involved pooled samples when, from a biological point of view, information on individual variation could have been useful. For example the use of 15 cm^3 pooled mouse urine specimens led Liebich *et al.* (1977) to conclude that there were only small inter-individual differences among the odours of mice (compare Chap 12). Singer *et al.* (1976) pooled swabs of hamster vaginal discharge in groups of about 50, despite there being about 5 ng of the active dimethyl disulphide in each swab. Raymer *et al.* (1984) pooled wolf urine into gender-season classes and Raymer *et al.* (1985) similarly combined wolf anal sac secretions. The work by Novotny's group on mouse semiochemicals routinely employs urine collected from groups of 3-5 mice over periods of a few days (Schwende *et al.* 1984, 1986; Novotny *et al.* 1986; Andreolini *et al.* 1987; Jemiolo *et al.* 1987), even when "individual variation" is being investigated (Schwende *et al.* 1984) and despite an appreciation of at least some of the disadvantages of such a technique (Schwende *et al.* 1986). This suggests that the Tenax-based adsorption system which is used is inadequate for work on single mice.

Specimen manipulation

Heating of specimens to increase the yield of volatiles has been employed in a number of cases. For example Raymer *et al.* (1985) heated wolf anal sac contents to 50°C, Goodrich *et al.* (1981) heated rabbit faeces to 40°C and Bailey *et al.* (1980) heated fox urine to 100°C.

Precision

Replicate analyses of wolf anal sac secretion using Tenax trapping yielded "a maximum of 15 % with less than 10 % being typical" variation in peak areas (Raymer *et al.* 1985). In contrast Schwende *et al.* (1984) had to resort to deletion of "unreliable" peaks from their data in order to obtain correlations of better than 90 % between replicate chromatograms from mouse urine. The quantitative errors were ascribed to adsorptive activity. It should be noted, however, that phenol was reliably quantified and that correlations of peak area with histocompatibility genotype were still obtained.

Adsorptive activity

Published chromatograms of mammalian volatiles have been examined for the peak tailing which is characteristic of chromatographic systems in which reversible adsorption is occurring. Although peak tailing is a symptom of adsorption its interpretation with respect to inlet performance is subject to two limitations. First, any activity which is present is as likely to be due to the column as to the inlet, and secondly the apparent severity of peak tailing is strongly dependent on the quantity of the solute involved and the sensitivity settings of the gas chromatograph, neither of which are usually reported.

Goodrich *et al.*'s (1981a) analysis of rabbit faecal pellet volatiles using Chromosorb 105 adsorbent and a FFAP column yielded a chromatogram in which nearly all the peaks were more or less badly tailed, on Carbowax the peaks were more symmetrical. Interpretation of the likeliehood of adsorption is confounded by the list of identified compounds not being related to the chromatograms.

Schultz *et al.*'s (1985) chromatogram of bitch urine volatiles shows some tailing of the trimethyl amine and the ethanol peaks. Phenols from mouse urine tailed somewhat when analysed by Tenax adsorption and separation on a Ucon 50-HB-2000 column (Schwende *et al.* 1986, Fig 1 peaks 56, 58 and 61). The small amount of tailing on the peaks of fox urine volatiles analysed by Jorgenson *et al.* (1978) is approximately the same for each peak, suggesting that it was not due to adsorption. With an equivalent analytical system alcohols from wolf urine produced tailed peaks, although the phenol peak was sharp (Raymer *et al.* 1985 Fig 1 peaks 1, 26, 31, 32, 41, and 50). Peak tailing also occurs in chromatograms of mouse urine volatiles produced with the same system (Schwende *et al.* 1984) but which compounds are involved is not stated. Volatiles from tree shrews sampled with a Grob-type charcoal trap and separated on Carbowax or methyl silicone columns produced peaks which were mostly symmetrical, but tailing of a ketone peak suggests that the complete disappearence of less tractable compounds such as alcohols may account for the lack of distortion among the other peaks

(Von Stralendorff 1982). Analysis of mouse urine volatiles using Tenax and a nickel column coated with Emulphor ON 870 produced several badly tailed peaks (Liebich *et al.* 1977). Liebich & Al-Babbili (1975) subjected the Tenax adsorbent and stainless steel column system they used for urine analysis to tests with synthetic mixtures. Although they used large quantities (150 ng) of each test component peak distortion, especially of ketones, was severe and could not be ascribed to the column because it was improved by increasing to 300°C the temperature at which the mixture was desorbed from the Tenax.

Sampling time and sampling rate

The sampling times and sampling rates used with adsorbent-based systems have typically been higher than those used for dynamic solvent effect sampling. Goodrich *et al.* (1981) sampled volatiles from rabbit faeces at 40 cm^3 min^{-1} for 16 hours (40 hours for GC-MS analyses), Schultz *et al.* (1985) sampled from bitch urine for 2 hours at 16 cm^3 min^{-1}. The standard procedure developed by Novotny *et al.* (1974) and employed at Indiana involves a 100 cm^3 min^{-1} purge rate over a period of an hour. Wellington *et al.* (1979) sampled guinea pig anal gland secretion for for 2 hours at 60 cm^3 min^{-1}. The 1 cm^3 min^{-1} sampling rate reported by Von Strallendorff (1982) should probably be 1 l min^{-1} (above).

Evidently the higher flow rates possible with adsorbents have not been exploited to reduce sampling times so that from this point of view they offer no advantages over the 10-20 cm^3 min^{-1} used with the dynamic solvent effect.

Thermal decomposition

Problems with the thermal decomposition of some of the volatiles from mouse urine were experienced by Schwende *et al.* (1986). The use of 200-300°C temperatures for desorption from Tenax, or of 170°C for 30 min for desorption from Chromosorb (Goodrich *et al.* 1981) would be expected to expose sample components to more thermal stress than does the heating of a dynamic solvent effect inlet. Nevertheless Singer *et al.* (1976) successfully desorbed dimethyl trisulphide from Tenax.

CONCLUSION

If the investigations considered above, and those discussed in Chap 6, are accurate reflections of the performance of the sampling systems involved then the dynamic solvent effect provides the most powerful available sampling technique for the quantitative analysis of mammalian semiochemicals by high resolution gas-liquid chromatography.

Pulcherrime processit

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Appendix A: SOURCES OF EQUIPMENT AND MATERIALS

Alumina: M. Woelm, Eschwege, West Germany

Capilettes: Boehringer Mannheim, Mannheim, West Germany

Carbowax: Supelco, Bellefonte, Pennsylvania, USA

Dimethyldichlorosilane: Pierce, Rockford, Illinois, USA

Gases: Afrox, Germiston, South Africa

Gas chromatograph: Varian Instrument Division, Walnut Creek, California, USA

Integrator: Varian Instrument Division, Walnut Creek, California, USA

Microcaps: Drummond Scientific Co., Broomall, PA, USA

Octamethylcyclotetrasiloxane: Aldrich, Gillingham, England

Polyimide resin: Alltech, Deerfield, Illinois, USA

Polytetrafluoroethylene tube: Raychem, Menlo Park, California, USA

Silyl 8: Pierce, Rockford, Illinois, USA

Swagelok: Crawford Fitting Co., Solon, Ohio, USA

Tenax: Enka, Arnhem, The Netherlands

Appendix B: PUBLISHED PAPERS

Trace Analysis of Complex Organic Mixtures Using Capillary Gas-Liquid Chromatography and the Dynamic Solvent Effect

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Key Words:

Trace analysis
Gas chromatography
Solvent effect
Inlet
Injector
Sampling

Summary

A sampling system for high resolution gas-liquid chromatography, based on the dynamic solvent effect, is described. Volatiles are accumulated off-line in a concentrator/injector and delivered to the column using an on-line inlet. Volatiles may be accumulated from gaseous or liquid matrices; they may be transferred to these by gas sparging or solvent extraction of any type of sample. The sampling technique is quantitatively precise; *e.g.* coefficients of variation of peak percentage areas better than 5% for a range of solutes at a concentration of $2:10^7$. Examples of the application of the sampling system are presented.

1 Introduction

Gas-liquid chromatography (GLC) in capillary columns is a uniquely powerful technique for the separation of the components of mixtures of volatile compounds. The type of sample which may be directly introduced to capillary columns is restricted by their susceptibility to volume overloading [1] and to degradation of performance due to contamination by *e.g.* water or involatile residues which are often part of the sample matrix. The use of high resolution GLC on the majority of samples requires some form of sample treatment before the volatiles are introduced to the separating column. Obviously these sample treatments should be quantitative at or near the limits of sensitivity of detectors used for GLC and should not introduce artifacts. Examples of sample treatments include equilibrium headspace analysis [2], gas sparging and trapping of volatiles by cryotrapping [3], or adsorbents [4], liquid-liquid extraction [5], and liquid-solid "cleanup" extractions [6]. These have been applied with varying degrees of success to problems such as flavor analysis [2-5] and environmental monitoring [6].

The dynamic solvent effect is a versatile, alternative sampling technique which makes a wide range of samples, including especially challenging biological materials, fully accessible to reliable, quantitative analysis by high resolution, gas-liquid chromatography. The technique was first introduced in 1984 [7]. Here we describe the apparatus and its operation in the light of development work carried out since then. The fundamental basis for the operation of the dynamic solvent effect and its quantitative performance will be dealt with separately [8,9].

2 Construction

2.1 Concentrator/Injector

The dynamic solvent effect is carried out in standardized, porous beds known as concentrators. Concentrators consist of borosilicate glass tubes 12 cm long, 2 mm o.d., and 1.7 mm i.d. Into the lower 6 cm of the tube a bed of 105-

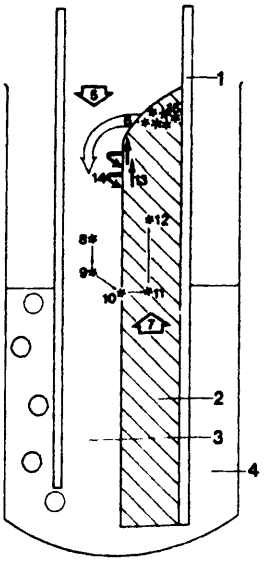

Figure 1

Vertical and horizontal sections through a dynamic solvent effect concentrator. 1) borosilicate glass tube; 2) porous bed; 3) open channel; 4) chamfered lower end of bed to improve solvent and gas flow.

125 µm borosilicate glass chips is sintered to leave half the cross section of the tube as an open channel (**Figure 1**). Sintering is carried out at 780°C for 30 min in order to preserve an open, porous structure in the beds. Concentrators are deactivated by depositing amorphous silicon on the inner surface by thermal decomposition of monosilane at 400°C [10] and treating this with ethylene [11]. After deactivation concentrators are prepared for use by opening both ends, rinsing with solvent, *e.g.* acetone, and conditioning under a flow of inert gas at 220°C for 30 min.

2.2 Inlet

Volatiles are transferred from loaded concentrators to the capillary column by use of an inlet (**Figure 2**) which replaces the conventional inlet of the gas chromatograph. The body of the inlet is stainless steel and it is mounted inside the chromatograph oven by bulkhead fittings on the carrier gas inlet and purge lines. The concentrators are a light, sliding fit in the fused silica seat so that a small flow of carrier gas purges the space between the two. The column is connected to the seat by a press-fit polyimide connection [12]. The carrier gas supply is pressure controlled and the purge flow is controlled at 30-50 ml min^{-1} by a needle valve. A 120 W electrically insulated heating element is wound evenly onto the inlet and is controlled by a temperature controller reading from a thermocouple on the inlet body. The heater is able to raise the temperature of the inlet from 40°C to 240°C in 90 s (see "Sample Injection" below). Cooling of the inlet after temperature programmed GC runs is accelerated by blowing compressed air over it.

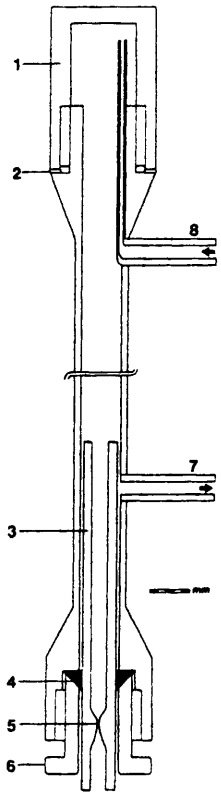

Figure 2

Vertical section of inlet for transfer of solutes from concentrator to capillary column. 1) screw cap; 2) PTFE seal; 3) silica seat; 4) graphite ferrule; 5) taper for press-fit polyimide connection to column; 6) nut; 7) purge outlet; 8) carrier gas inlet. A 120 W heater element is wound onto the inlet body. The inlet is mounted with the screw cap protruding through the roof of the gas chromatograph oven. This is most conveniently achieved by bulkhead fittings on the carrier gas inlet and purge lines mounted to the oven wall directly or via a bracket.

3 Operation

3.1 Solute Accumulation

The mechanism of solute focusing on dynamic solvent films is presented diagrammatically in **Figure 3**. The way in which it is employed depends on the nature of the sample. If the sample matrix is a solvent which is compatible with solvent effect sampling (see "Solvent Selection and Purification" below) and capillary GLC then it may be dealt with by blowing a pure gas through a concentrator while it is dipped directly into the sample. Such samples include organic solvents being monitored for purity and liquids which would otherwise be concentrated and the traditional technique of

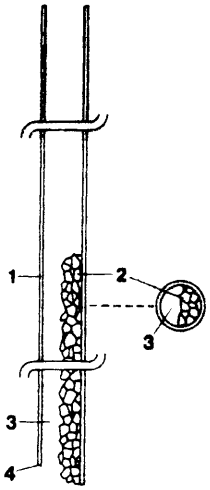

Figure 3

Schematic representation of the operation of the dynamic solvent effect. 1) glass tube; 2) sintered porous bed; 3) gas channel; 4) solvent. When gas is passed down the tube (5) evaporation of solvent at the top edge of the bed (6) causes an upward movement of solvent driven by capillary rise (7), solutes in the gas phase (8) are carried downwards by the gas (9), impinge (10) on and dissolve (11) in the solvent and are carried upwards by it (12). Exchange of solute between gas and liquid results in a chromatographic transport of solute. As long as the rate of upward transport (13) is greater than the rate of downward transport (14) solutes accumulate in the evaporation zone (15).

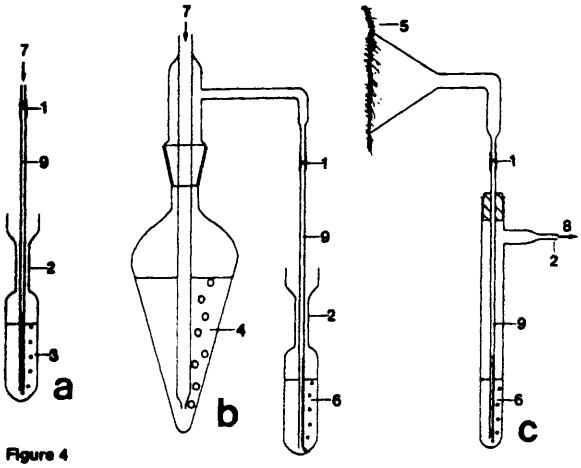

Figure 4

Examples of "plumbing" arrangements employed for accumulation by the dynamic solvent effect of solutes from different types of sample. a) direct accumulation from liquid sample; b) sparging of volatiles from liquid sample and subsequent accumulation of volatiles from headspace; c) suction sampling from material which cannot be enclosed. 1) glass butt joint with PTFE sleeve; 2) constrictions through which effluent gas flows with high linear velocity to prevent back diffusion of contaminants; 3) liquid sample with matrix compatible with solute focussing by the solvent effect; 4) liquid sample with incompatible matrix; 5) *e.g.* skin of large animal; 6) pure solvent; 7) pure gas; 8) soft vacuum; 9) concentrator.

evaporation under a stream of inert gas. If the sample matrix is a gas, as in the case of so-called headspace samples, then the sample is passed through a concentrator which is dipped into a pure solvent. If the sample matrix is incompatible with these approaches, *e.g.* solids and biological fluids, extraction into a compatible solvent or into a gas (sparging) and subsequent focusing of the extracted volatiles according to the matrix of the extract are employed.

From the point of view of purity palladium-purified hydrogen is the gas of choice for direct focusing from liquid samples or for sparging of volatiles. If hydrogen is unsuitable, purification with, *e.g.*, activated charcoal can be applied to other gases.

Samples or extracts in a gas matrix may be either blown or sucked through the concentrator. Blowing is usually appropriate when samples can be contained and slightly pressurized, *e.g.* wines, fruit juices, or small mammals. Sucking is appropriate to samples which cannot be enclosed conveniently or pressurized, *e.g.* ambient air samples or specific glandular areas of large mammals. Examples of appropriate "plumbing" systems are shown in **Figure 4**. Unless sampling is carried out in a chemically clean environment back diffusion of airborne contaminants should be prevented by arranging that the effluent gas leaves the sampling system at high linear velocity (Figure 4).

The open gas channel in the concentrator allows gas flow at extremely small pressure drops, consequently connections may be made by glass-to-glass butt joints with PTFE sleeves. These are easier to manipulate than metal fittings and do not expose sample components to metal surfaces.

At the end of the accumulation period the solutes have been focused into a narrow band near the top of the bed with approximately 20 µl of solvent in the remainder of the bed. Accumulation is terminated by removing the bottom of the bed from the liquid (sample or pure solvent) just *before* the top is disconnected from the gas so that liquid does not fill the gas channel. A loaded concentrator may be stored at low temperature or analyzed immediately.

3.2 Sample Injection

The contents of a loaded concentrator are transferred to the capillary column by carrying out the static solvent effect on the concentrator bed. The following procedure is followed; the carrier gas supply is turned off, the screw cap of the inlet is removed, the loaded concentrator is dropped into the seat, the screw cap is replaced, the carrier gas supply is turned on, and a timer is started. After a predetermined solvent evaporation time (see below) the inlet heater is turned on. Work on an on-line, static, solvent effect inlet [13] has demonstrated that it is necessary to heat a solvent effect inlet rapidly as the last of the solvent film is evaporating. The time at which the inlet is heated influences the efficiency of the subsequent chromatographic separation. If a fixed volume of solvent is introduced to an inlet under standard conditions the correct time at which to heat is fixed. When using a dynamic injector for non-critical work the time at which to heat may be determined by measuring the width in time of the solvent peak and heating at approximately 60% of that time after restoring carrier gas flow; *e.g.* with *n*-hexane as solvent and hydrogen as carrier gas, at a temperature of 40°C the solvent peak eluting from a 25 m, 0.3 mm i.d. column is about 4.5 min wide and the inlet should be heated 2.5 min after restoring carrier gas flow. For critical work we recommend the use of a diagnostic test mix containing C_8 to C_{15} alkanes, *p*-cresol, 2,6-dimethylaniline, 2,6-dimethylheptan-4-one, and *n*-decanol. Heating too early causes splitting of the late eluting alkane peaks. Heating too late causes splitting of the polar compound peaks.

3.3 Solvent Selection and Purification

The theory of the dynamic solvent effect [8] predicts that substances for which $K_x > K_o - B$ where: K_x = partition coefficient of solute between solvent and gas phase; K_o = partition coefficient of solvent between solvent and gas phase; B = phase ratio, will be accumulated. In practical terms this means that within a given chemical class solvents of higher volatility trap a wider range of compounds. Non-polar solvents are preferred since they do not trap water.

Aromatic and aliphatic hydrocarbons, esters, ketones, alcohols, and carbon disulfide have all been successfully employed as solvents or as liquid samples. Halogenated hydrocarbons are compatible with the dynamic solvent effect but tend to cause sooting of flame ionization detectors. The most generally useful solvent for the analysis of volatiles above biological materials has been found to be *n*-hexane. Other alkanes have been used in special cases.

Due to the high sensitivity of the technique solvents used to focus volatiles from gaseous matrices or to extract components from liquids and solids must be purified. The level of impurities which can be tolerated depends on the sensitivity of the analysis and the quantity of solvent consumed for each sample, which in turn depends on the sample size and, for gas samples, on the volatility of the trapping solvent. In practice solvents must be pure below the parts per billion (10^9) level. Since no commercially available solvent we have tested approaches these levels all sampling solvents are purified immediately before use by gas-solid chromatography.

3.4 Samples Containing Water

Although water does not accumulate chromatographically on non-polar solvent films it does sometimes condense in the concentrator due to cooling in the zone of solvent evaporation [14]. Condensed water may be removed by blowing dry gas through the concentrator while its lower end remains immersed in the solvent. The presence or absence of condensed water may be confirmed simply by visual inspection since the concentrators are semi-transparent.

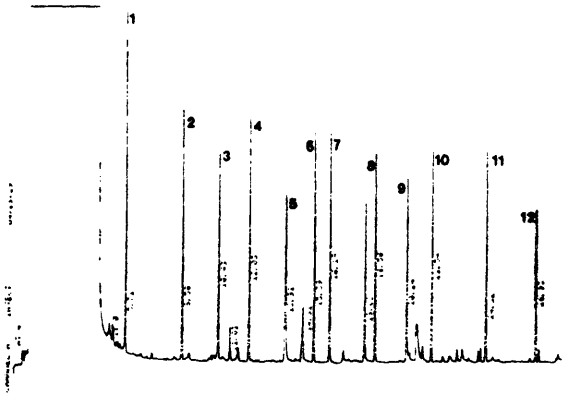

Figure 5

Test for peak distortion during transfer of solutes from concentrator to capillary column. 1) *n*-octane; 2) *n*-nonane; 3) 2,6-dimethylheptan-4-one; 4) *n*-decane; 5) *para*-cresol; 6) *n*-undecane; 7) 2,6-dimethylaniline; 8) *n*-dodecane; 9) *n*-decanol; 10) *n*-tridecane; 11) *n*-tetradecane; 12) *n*-pentadecane. Nominal 3 ng per component (other peaks are unidentified contaminants). Column 25 m × 0.3 mm × 0.4 μm SE-30, temperature program 40°C for 5 min then 5° min^{-1} to 200°C, hydrogen carrier gas with linear velocity 60 cm s^{-1}. Inlet ballistically heated to 220°C after 2.5 min solvent evaporation.

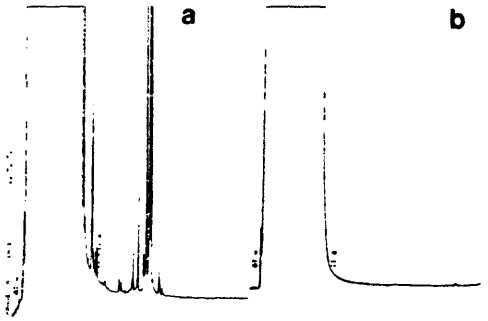

Figure 6

Comparison of impurities in: a) 20 μl of commercial "distilled in glass" *n*-hexane and b) 200 μl of *n*-hexane purified by passage through alumina. Analytical conditions as for Fig. 5 except temperature program 10° min^{-1}.

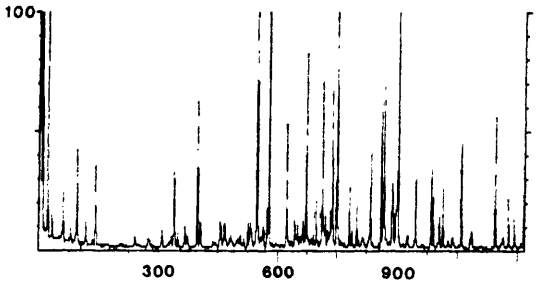

Figure 7

Total ion chromatogram of volatiles from hair of a chronic schizophrenic patient. Volatiles were purged from the hair with 15 ml min^{-1} of palladium purified hydrogen at 50°C for 30 min and accumulated by dynamic solvent effect using *n*-hexane as solvent. Column 25 m × 0.3 mm × 0.4 μm SE-30, carrier gas helium at a linear flow rate of 30 cm s^{-1}. Temperature program 40°C for 8 min then 4°/min^{-1} to 220°C. Inlet ballistically heated to 240°C after 6.5 min solvent evaporation. Open split interface, 70 eV ionization energy.

3.5 Sampling Rate

The factors determining sampling rate are treated in detail elsewhere [8,9]. In practice the gas flow rate is set so that the liquid film just reaches the top of the porous bed. Gas flows of 15-20 ml min^{-1} are typical with hexane as a solvent at 25°C and 20-30 ml min^{-1} for heptane at 30°C. For best quantitative reproducibility the sampling rate must be carefully standardized. Reproducibility is inversely dependent on sampling rate and for qualitative work the sampling rate may be substantially increased [8,9].

4 Results and Applications

In order to test for peak distortion due to adsorptive activity in the concentrators or the inlet our test mix for solvent effect inlets [13] was run. **Figure 5** shows clearly that peak distortion is negligible even at the nanogram level.

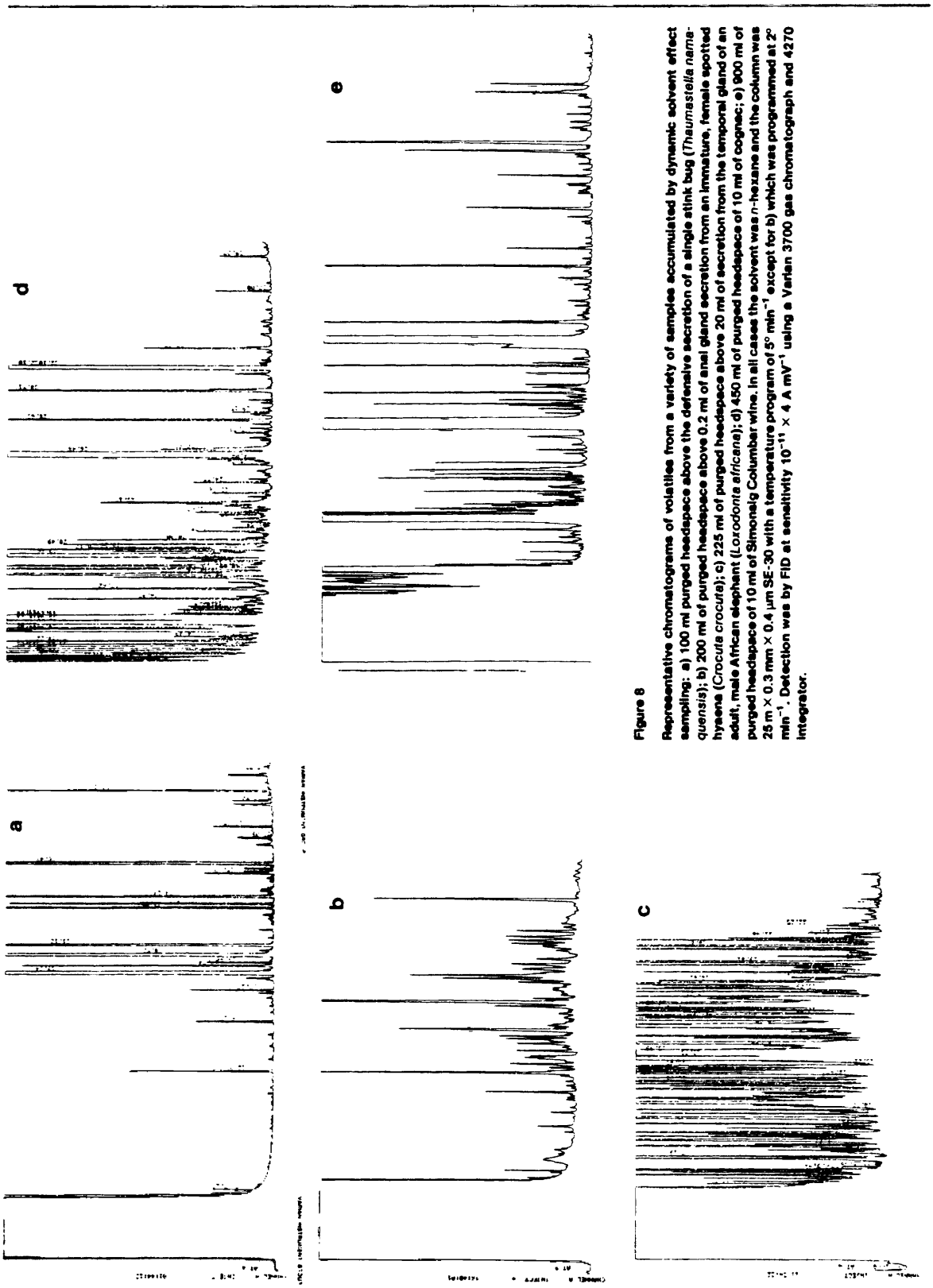

Figure 8

Representative chromatograms of volatiles from a variety of samples accumulated by dynamic solvent effect sampling: a) 100 ml purged headspace above the defensive secretion of a single stink bug (*Thaumastella namaquensis*); b) 200 ml of purged headspace above 0.2 ml of anal gland secretion from an immature, female spotted hyaena (*Crocuta crocuta*); c) 225 ml of purged headspace above 20 ml of secretion from the temporal gland of an adult, male African elephant (*Loxodonta africana*); d) 450 ml of purged headspace of 10 ml of cognac; e) 900 ml of purged headspace of 10 ml of Simonsig Columbar wine. In all cases the solvent was *n*-hexane and the column was 25 m × 0.3 mm × 0.4 µm SE-30 with a temperature program of 5° min^{-1} except for b) which was programmed at 2° min^{-1}. Detection was by FID at sensitivity 10^{-11} × 4 A mV^{-1} using a Varian 3700 gas chromatograph and 4270 integrator.

The necessity for, and effectiveness of, solvent purification by gas-solid chromatography is illustrated in **Figure 6** which shows a comparison between the impurities in 200 µl of purified solvent and in one tenth of the volume (20 µl) of a commercial "distilled in glass" solvent.

Dynamic solvent effect techniques are compatible with GC-MS (Fig. 7) provided that some means of solvent venting is employed at the outlet of the separating column to prevent overloading of the ion source with large volumes of saturated solvent vapor.

A detailed investigation of the quantitative performance of dynamic solvent effect sampling is to be presented separately [9]. Briefly, representative experimental variation as percentages of means for a selection of sample types may be given as follows: orange juice volatiles 1-15% for peak % areas; "fingerprinting" of commercial apple drinks 0.1-10% for peak % areas; decane as a test impurity at 10 ppb in hexane 11% for peak areas; volatiles sampled directly from anogenital region of sheep 30% for peak area representing less than 10 ng of *p*-cresol; liquid test mix [13] at a concentration of $2{:}10^7$ 0.6-2.1% for % areas of alkanes and 1.1-4.7% for % areas of polar compounds.

Chromatographic separations of volatiles from some of the more interesting and challenging samples to which we have applied dynamic solvent effect sampling are shown in **Figure 8**.

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We would like to thank *Mrs. A. de Klerk* and *Mr. D. Masemula* for making the columns.

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Volatile components of the anal gland secretion of the striped polecat *Ictonyx striatus*

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The volatile components of the anal gland secretion of an adult, male, *Ictonyx striatus* were separated and identified by dynamic solvent effect sampling, capillary gas-liquid chromatography, and mass spectrometry. The major component was 2-ethylthiacyclobutane. Nine other sulphur compounds, a ketone, and a hydrocarbon were also identified. Some of these occur in the anal gland secretions of other mustelids.

Die vlugtige komponente van die anale klierafskeiding van die volwasse, manlike *Ictonyx striatus* is geskei en geïdentifiseer deur dinamiese oplosmiddeleffek-monsterneming, gaschromatografie, en massaspectrometrie. Die hoofkomponent was 2-etieltiasiklobutaan. Nege ander swaelverbindings, 'n ketoon, en 'n koolwaterstof is ook geïdentifiseer. Van hierdie verbindings kom ook in die anale klierafskeiding van ander mustelidae voor.

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The striped polecat *Ictonyx striatus* is a small, African mustelid which in appearance, habits and ecology resembles the skunks of North America. Its biology has been authoritatively reviewed by Smithers (1983). Like the North American skunks *Mephitis mephitis* and *Spilogale interrupta*, *Ictonyx* is equipped with a well developed anal gland whose foul-smelling secretion is used for defence. The chemistry of this secretion has not previously been reported. We deal here with the identification of the major volatile components which give the secretion its defensive properties.

Anal gland secretion was taken from an adult male *Ictonyx* obtained as a fresh road casualty in the northern Cape Province, South Africa and kept deep-frozen until analysis. The anal gland was excised and its secretion expelled into a 10 ml glass tube. Volatiles were accumulated from 10 ml of the head space above the secretion by dynamic, off-line, solvent effect sampling (Apps, Pretorius, Lawson, Rohwer, Centner, Viljoen & Hulse 1987) using n-hexane as solvent at 25°C.

Table 1 Volatile components of the anal gland secretion of a male *Ictonyx striatus*

Peak[1]	Identification	Criterion[2]
1	2-methylthiacyclobutane	L
2	2,2-dimethylthiacyclobutane	P a
3	3-methyl-1-butanethiol	L
4	1-pentanethiol	L
5	2-ethylthiacyclobutane	P b,d
6	2-ethyl-3-methylthiacyclobutane	P c
7	4-methyl-3-heptanone	P e
8	propylthiacyclobutane isomer	I
9	methyldithiacyclopentane isomer	I
10	a ketone	I
11	1,2-dithiacycloheptane	P b
12	2-methyl-1-decene	L
13	ethyldithiacyclopentane isomer	I
14	a ketone	I
15	a ketone	I

[1]Peak numbers correspond to Figure 1.
[2]L = found in mass spectra library, P = published spectrum: a, Brinck *et al.* 1983; b, Brinck *et al.* 1978; c, Crump & Moors 1985; d, Sokolov *et al.* 1980; e, Stenhagen *et al.* 1974, I = mass spectrum interpretation.

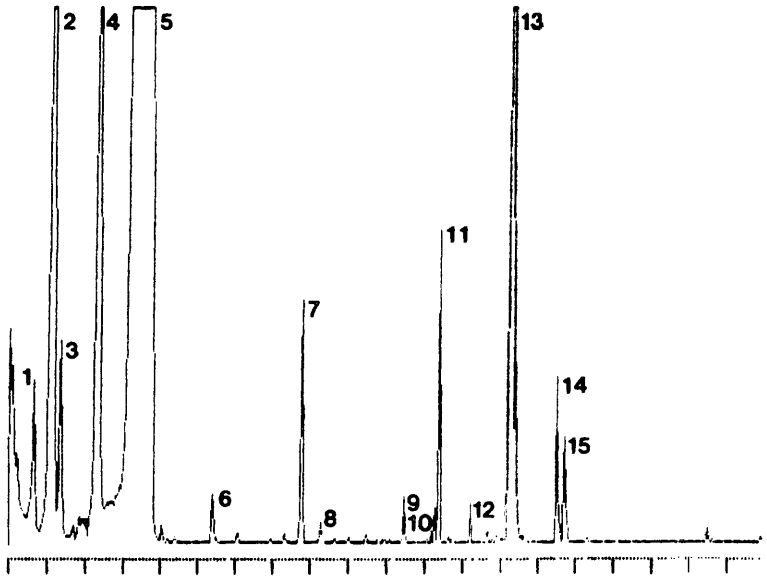

Figure 1 Total ion chromatogram of volatiles from 10 ml of headspace above the anal gland secretion of a male *Ictonyx striatus* sampled by dynamic solvent effect. For analytical conditions see text.

Table 2 Occurrence among Mustelidae of compounds found in anal gland secretion of *Ictonyx striatus*

	Mustela erminea[a]	*Mustela nivalis*[a]	*Mustela putorius*[a]	*Mustela putorius furo*[a]	*Mustela vison*[a]	*Mephitis mephitis*[b]
1. 2-methylthiacyclobutane	-	-	-	-	-	-
2. 2,2-dimethylthiacyclobutane	o	o	o	o	x	-
3. 3-methyl-1-butanethiol	-	-	-	-	-	x
4. 1-pentanethiol	-	-	-	-	-	-
5. 2-ethylthiacyclobutane	x	-	-	-	x	-
6. 2-ethyl-3-methylthiacyclobutane	-	-	-	o	-	-
7. 4-methyl-3-heptanone	-	-	-	-	-	-
8. propyldithiacyclobutane isomer	o	-	o	o	-	-
9. methyldithiacyclopentane isomer	-	-	-	-	-	-
11. 1,2-dithiacycloheptane	-	-	-	-	x	-
12. 2-methyl-1-decene	-	-	-	-	-	-
13. ethyldithiacyclopentane	-	o	-	-	o	-

x = same isomer; o = different isomer (based on published mass spectral); [a]Brink *et al.* (1983); [b]Andersen & Bernstein 1975.

Accumulated volatiles were separated and identified by high resolution gas-liquid chromatography — mass spectrometry. A dynamic solvent effect inlet (Apps *et al.* 1987) was used to transfer the volatiles to a 25 m × 0,3 mm × 0,4 μm methylpolysiloxane capillary column in a Varian 1400 gas chromatograph. The carrier gas was helium with a linear flow rate of 30 cm/s. The injector was held at 40°C for 7 min then heated ballistically to 220°C. The column was held at 40°C for 10 min then programmed at 4°C / min to 180°C. The column was coupled by an open-split interface to a Micromass 16 F mass spectrometer operating in the electron impact mode with an electron energy of 70 eV.

Components were identified by comparison of their mass spectra with library material or with published spectra, or by mass spectral interpretation. Where possible chromatographic retention data were used for confirmation.

The anal gland secretion of the male *Ictonyx striatus* consisted of a light, mobile, yellow oil and a colourless, translucent aqueous phase. The secretion emitted a strong, penetrating, metallic odour.

Fifteen clearly resolved peaks appeared on the total ion chromatogram (Figure 1), of which 12 were identified (Table 1).

The occurrence in other mustelids of the compounds identified in *Ictonyx striatus* is summarized in Table 2.

The presence of sulphur compounds conforms to expectation since these are usually strong-smelling and are important components of the anal gland secretions of other mustelids. The major, volatile component of the *Ictonyx* secretion; 2-ethylthiacyclobutane, made up 90% of the headspace volatiles. This compound was found as a minor component of the anal gland secretion of mink *(Mustela vison)* by Sokolov *et al.* (1980 compound V) but was then incorrectly identified as a dimethylthiacyclobutane isomer (compare Brinck, Erlinge & Sandell 1983 compound 5). The offensive, odorous compounds of the anal gland of the striped skunk are trans-2-butene-1-thiol, 3-methyl-1-butanethiol and trans-2-butenyl-methyldisulphide (Andersen & Bernstein 1975). Of these only 3-methyl-1-butanethiol was found in the striped polecat where it was a minor component.

The presence of ketones is unusual among the mustelidae (Brinck *et al.* 1983) although they are important components of fox *(Vulpes vulpes)* urine (Jorgenson *et al.* 1978).

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