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Identification of pheromones in mouse urine by head-space solid phase microextraction followed by gas chromatography–mass spectrometry

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Abstract

Given the key role of pheromones in animal communication and behaviour, there is need to identify the different classes of these molecules under varying physiological conditions. However, the highly volatile nature of pheromones and the fact that they occur at very low concentrations in urine makes this task all the more difficult. Herein, we present a method of detecting and identifying the five main pheromones known: 2-*sec*-butyl-4,5-dihydrothiazole, geraniol, indole, *trans*-beta farnesene and *trans*-alpha farnesene in individual urine microsamples taken from male mice. Urine volumes as small as 20 μl were subjected to solid phase microextraction (SPME) followed by gas chromatography–mass spectrometry (GC–MS). This selective analytical method permits the rapid detection of these pheromones free from cross-contaminants as a clearly distinguishable spectral signals. Highest recovery rates of natural pheromones were achieved by extraction on a carboxen/polydimethylsiloxane (CAR/PDMS) fibre of 85 μm film thickness. This selective, sensitive and accurate method will help address the question of possible links between certain pheromone classes, and social and reproductive behaviour in mice.

Keywords: Pheromones

1. Introduction

Communication among animals of the same gender occurs in many ways but the most widespread is probably that mediated by olfactory cues. In mammals, chemical signals excreted through the faeces, urine, genital organs, saliva or skin play a key role in sexual and social behaviour [1]. These chemical signals, or pheromones, are generally detected by a secondary olfactory system called the vomeronasal system (VNS), which is highly sensitive to non-volatile molecules [2]. It has been recently shown that the VNS can also detect odorants [3] and certain small volatile compounds [4]. High levels of major urinary proteins

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(MUPs) have been detected in mice urine. These proteins are members of the lipocalin family and show a beta-barrel structure enclosing a ligand binding domain [5,6]. MUPs bind compounds with prospective pheromonal effects such as thiazole, heptanone, brevicomin and farnesene [7,8], which may be detected by the VNS as volatile molecules. It has also been possible to demonstrate a very high level of polymorphism in MUPs in samples of urine from the wild house mouse even in individual animals of the same population [9].

To date, four main detection systems coupled to gas chromatography (GC) have been used to accurately determine pheromones: mass spectrometry (MS) [10], Fourier transform infrared spectroscopy (FTIR) [11], flame ionisation detection (FID) [12] and electroantennographic detection (EAD) [13].

Pheromones are commonly extracted from urine by size-exclusion chromatography or liquid–liquid extraction. Nevertheless, the small urine sample that can be obtained from an individual mouse and its low pheromone concentration have meant the need to pool individual samples for their determination. About 20 ml of urine from male mice is the volume generally used for reliable pheromone determination by Novotny [14]. Such a large volume cannot be obtained from a single mouse by a non-invasive procedure.

A new extraction technique for determining low concentrations of volatile and semi-volatile organic compounds has recently been developed [15]. This method, solid phase microextraction (SPME), is based on the use of a syringe-mounted fused-silica rod (fibre) coated with an absorptive organic phase (e.g. polydimethylsiloxane (PDMS)), or an adsorptive one (e.g. carboxen (CAR), carbowax (CW)); film thicknesses ranging from 7 to 100 μm. Analytes are sorbed from water or from head-space samples. After SPME extraction, the analytes can be thermally desorbed into the heated gas chromatograph injection port and analysed directly by gas chromatography [8,16]. SPME completely eliminates the use of organic solvents and substantially reduces the amount of sample needed to run an analysis from millilitres to microlitres, thus improving analytical conditions when using biological samples from small animals. The method here described has been applied to the five more common pheromones well studied in mice research. Research on pheromones in other species has demonstrated a high variety of them whose distribution is different among species (see Keverne [17] and Novotny [18] for more information about pheromones and their biological role). At present, understanding of their biological function is the subject of the most active research on this field which is requiring more efficient analytical tools.

To satisfy the requirement of accurate pheromone class detection in microsamples of urine from individual male mice, we developed a new, rapid method based on head-space solid phase microextraction and gas chromatography–mass spectrometry.

2. Experimental

2.1. Chemicals

All solvents for GC–MS were HPLC grade (Scharlau, Barcelona, Spain). Ultra-pure water was prepared using a Milli-Q system (Millipore; Milford, MA).

2.2. Samples

Urine samples were taken from single-housed male mice of inbred strain CBA/Ca (at the animal house of the University of Santiago de Compostela, Registry No. 15003AE).

2.3. Material

Sample collection required the use of Petri dishes (20 cm ∅), 20 ml head-space vials, Teflon-coated butyl septa and a hand crimper for 20 mm seals (Supelco, Bellefonte, PA, USA). Solid phase microextraction was performed on carboxen/polydimethylsiloxane, carbowax/divinylbenzene (CW/DVB) and polydimethylsiloxane fibres of 85, 65 and 100 μm film thickness, respectively (all supplied by Supelco).

2.4. Equipment and conditions

A double-port NURKA 390 fibre cleaner was used for cleaning and conditioning the fibres. The head-space temperature of the vial containing the urine sample for SPME was controlled by a NURKA 390 thermostat.

Analysis of the extracted components in the fibre was performed with a Hewlett-Packard 5989A Engine GC–MS equipped with a 5890 Series II gas chromatograph equipped with a split/splitless injection port, and a quadrupole mass spectrometer. A 30 m VA-5 column (0.25 mm i.d., 0.25 μm film thickness) was used with helium as the carrier gas. The mass spectrometer was operated in the full-scan mode between 20 and 800 amu. Ionisation was achieved by electron impact (70 eV). The transfer line temperature was maintained at 280 °C, the ion source temperature was 250 °C, and the quadrupole temperature was 100 °C.

2.5. Urine sample collection

Urine was collected by gently massaging the bladder area of a male mouse over a clean Petri dish. Care was taken to avoid faecal contamination of the urine. Each urine sample (20–80 μl) was immediately transferred to a 20 ml vial by micropipetting, and stored at −20 °C until analysis. Disposable pipette tips were used to avoid cross-contamination. All samples were extracted within 72 h of collection and immediately subjected to GC–MS. Controls with water were processed by the same procedure and always ran in parallel with the analysis of urine samples.

2.6. Solid phase microextraction

Optimum SPME results were obtained using the carboxen/polydimethylsiloxane fibre of 85 μm film thickness housed in its manual holder. Prior to extraction, the fibre was cleaned at 280 °C for 1 h under helium flow using the fibre cleaner. A 60 min exposure of the fibre to the head-space of the urine sample was performed. The vial was kept at 130 °C using the NURKA 390 thermostat. Once extraction was complete, the fibre was exposed inside the hot split/splitless injection port at 260 °C for 10 min and chromatographic separation was performed. The column temperature program was 35 °C for 15 min, ramped at 3 °C/min to 60 °C and held at 60 °C for 5 min, ramped at 10 °C/min to 150 °C and held at 150 °C for 1 min, ramped at 25 °C/min to 290 °C and held at 290 °C for 18 min.

3. Results and discussion

3.1. Optimising urine sample collection

The protocol developed was designed to analyse urine from a single male mouse and was thus based on the SPME technique, which only requires a few microlitres of sample. First, we tested several methods of collecting urine samples.

The use of metabolism cages was soon rejected because of extensive cross-contamination with other odorants in addition to a high cost. We then tried to directly collect urine onto filter paper, followed by cutting to the size of the sample and placing in an extraction vial. This collection method was also rejected due to cross-contamination with substances bound to the paper as detected by GC–MS due to the high extracting capacity of the SPME fibres. Finally, we found that holding the mouse over a Petri dish and transferring the deposited urine to a vial using a micropipette provided the best results. The background signal of blanks (water placed in contact with the Petri dish and micropipette) was negligible and the method was very reliable, quick, simple and economic. Nevertheless, it must be stressed that urine obtained by this method, or any similar procedure, has been in contact with the preputial glands, which could add some components to the normal male mouse urine [19].

3.2. Optimising the experimental conditions for SPME

An evaluation was made of the main variables affecting the SPME–GC signal. These results are outlined in Table 1.

The number and amount of target pheromones retained by the fibre were highest when the CAR/PDMS fibre was used. The chromatogram and mass spectra were sharper using this fibre. The volatility of these compounds seems to be sufficient for detection in the head-space. Their retention on mixed fibres containing carboxen and polydimethylsiloxane can be explained by their polarity. Probably, the large surface area provided by carboxen helps to improve retention [20].

Since the SPME method requires that equilibrium be reached between the fibre and head-space, the time of extraction conditions the method's sensitivity and is crucial for good reproducibility [21]. Based of the

Table 1
Optimisation of SPME experimental conditions

Studied variables	Range	Selected
Film thickness and fibre phase	85 μm (CAR/PDMS), 65 μm (CW/DVB), 100 μm (PDMS)	85 μm (CAR/PDMS)
Extraction time (min)	15, 30 and 60	60
Extraction temperature (°C)	40, 80, 130 and 150	130
Desorption time (min)	2, 5 and 10	10

CAR: carboxen, PDMS: polydimethylsiloxane, CW: carbowax, DVB: divinylbenzene.

saturation curves (peak area as a function of time) we were able to compromise sensitivity and analysis time, and selected an extraction time of 60 min as reasonable for detecting the target pheromones. However, this variable must be strictly controlled when conducting large analytical series.

A further significant factor was the extraction temperature of the vial containing the urine sample and the fibre. The CARD/PDMS fibre was chosen because best overall performance. Sensitivity was observed to increase with temperature from 40 to 130 °C; only to fall again at 150 °C. This phenomenon can be explained by a competitive adsorption effect of the less volatile compounds that are retained more strongly by the non-polar fibre. No pheromones were detected in fibres subjected to temperatures below 40 °C and interfering compounds were detected at temperatures higher than 130 °C.

In ideal conditions, a flash followed by complete desorption should occur for the GC–MS analysis. Under the optimal conditions determined for the pheromones adsorption to the CAR/PDMS fibre, best results were obtained by subjecting the fibre to a temperature of 260 °C for 10 min.

Table 2
Chromatographic validation of detecting pheromones

Analyte	Retention time (min)	R.S.D. (%)[a]	r^2[b]
2-*sec*-Butyl-4,5-dihydrothiazole	26.23	0.20	56
Geraniol	34.00	0.11	78
Indole	34.84	0.29	86
Trans-beta farnesene	37.46	0.04	98
Trans-alpha farnesene	38.24	0.17	70

[a] R.S.D. (%): the relative standard deviation within the series of replicate analyses ($n = 4$) of the retention time (t_R).
[b] r: library quality factor of compound identification.

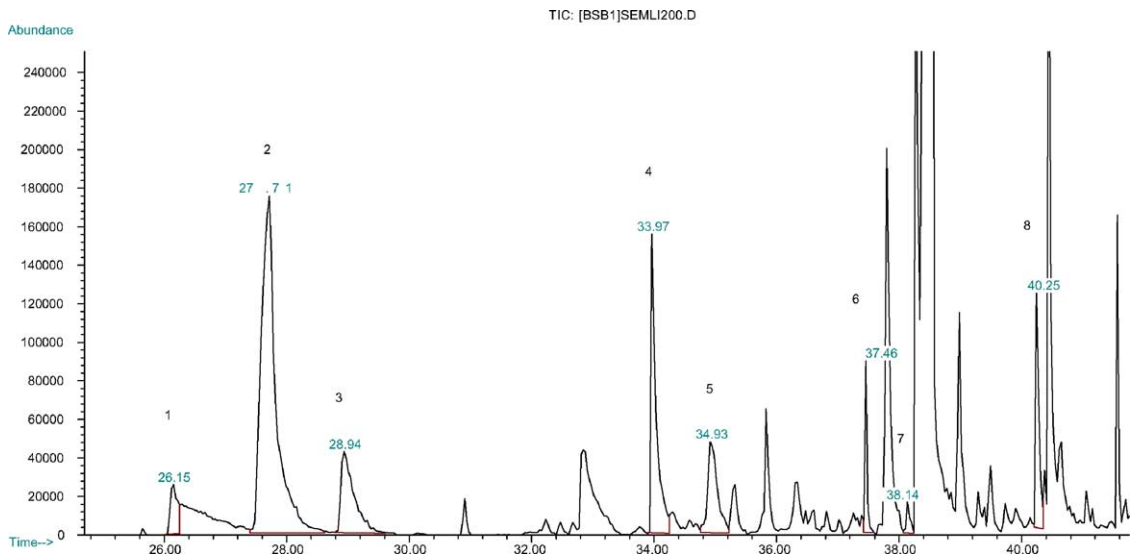

Fig. 1. Total ion chromatogram, obtained by HS–SPME–GC–MS of an urine sample from a single male mouse. Labelled chromatographic peaks were identified by mass spectrometry as follow: (1) 2-*sec*-butyl-4,5-dihydrothiazole; (2) methyl methylthiomethyl disulphide; (3) *N*-ethyl benzenamine; (4) geraniol; (5) indole; (6) *trans*-beta farnesene; (7) *trans*-alpha farnesene and (8) 1-tetradecanol.

3.3. Identifying the class of pheromone molecules

The proposed method was used to identify pheromones in several male mouse urine samples. Fig. 1 shows a total ion chromatogram obtained by HS–SPME–GC–MS according to our optimised procedure. Many volatile compounds, such as methyl methylthiomethyl disulphide, *N*-ethyl benzenamine

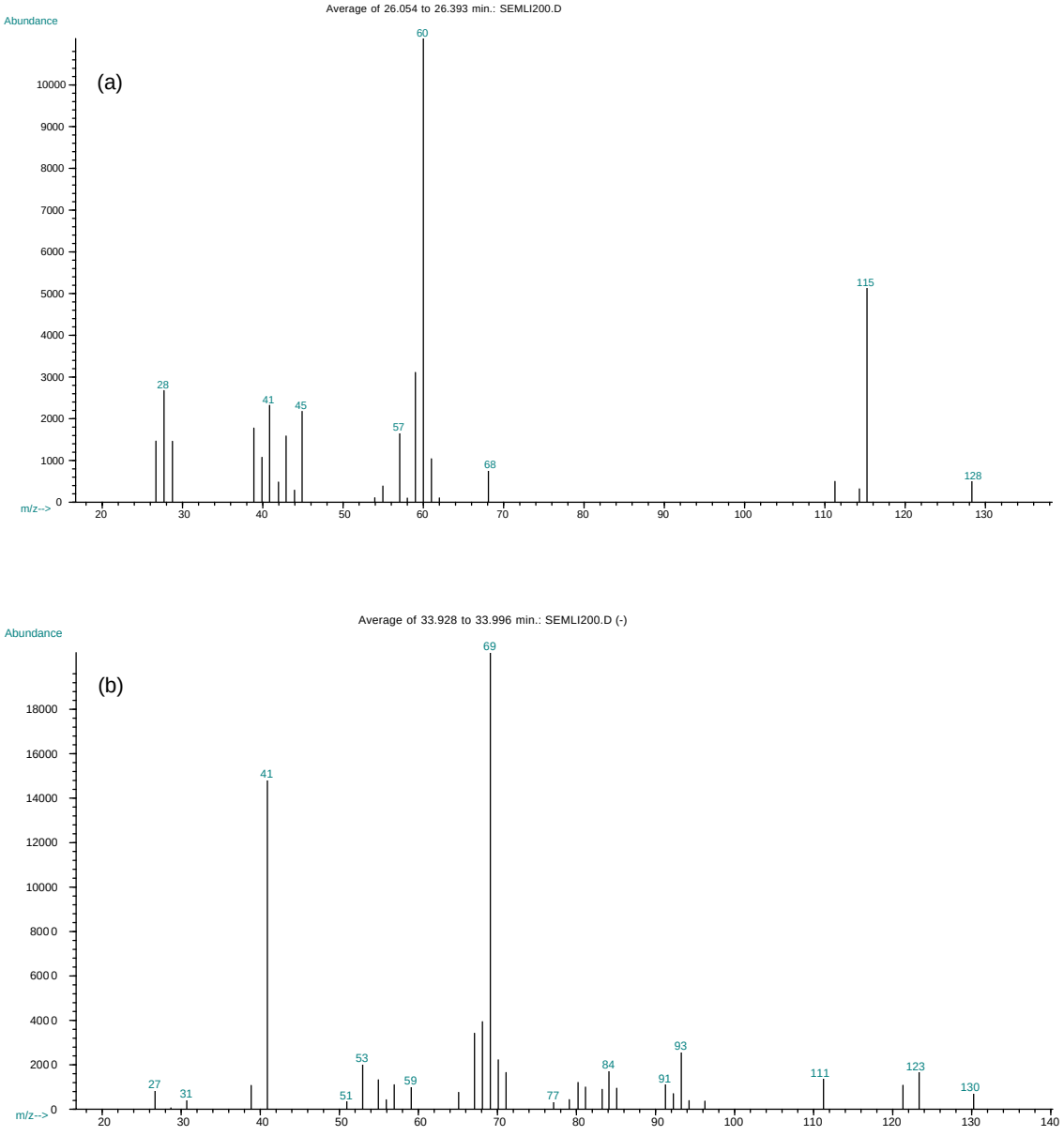

Fig. 2. Mass spectra identifying five different pheromones in male mouse urine indicating M^+, and other diagnostic fragments ions with their relative abundance in parentheses. (a) 2-*sec*-Butyl-4,5-dihydrothiazole: M^+ 143 (0), 128 (8), 115 (45) and 60 (100); (b) geraniol M^+ 154 (0), 123 (6), 69 (100) and 41 (68); (c) indole M^+ 117 (100), 90 (49), 89 (100) and 58 (24); (d) *trans*-beta farnesene M^+ 204 (0), 161 (6); 69 (100) and 41 (68); and (e) *trans*-alpha farnesene M^+ 204 (9), 107 (41), 93 (100) and 69 (96).

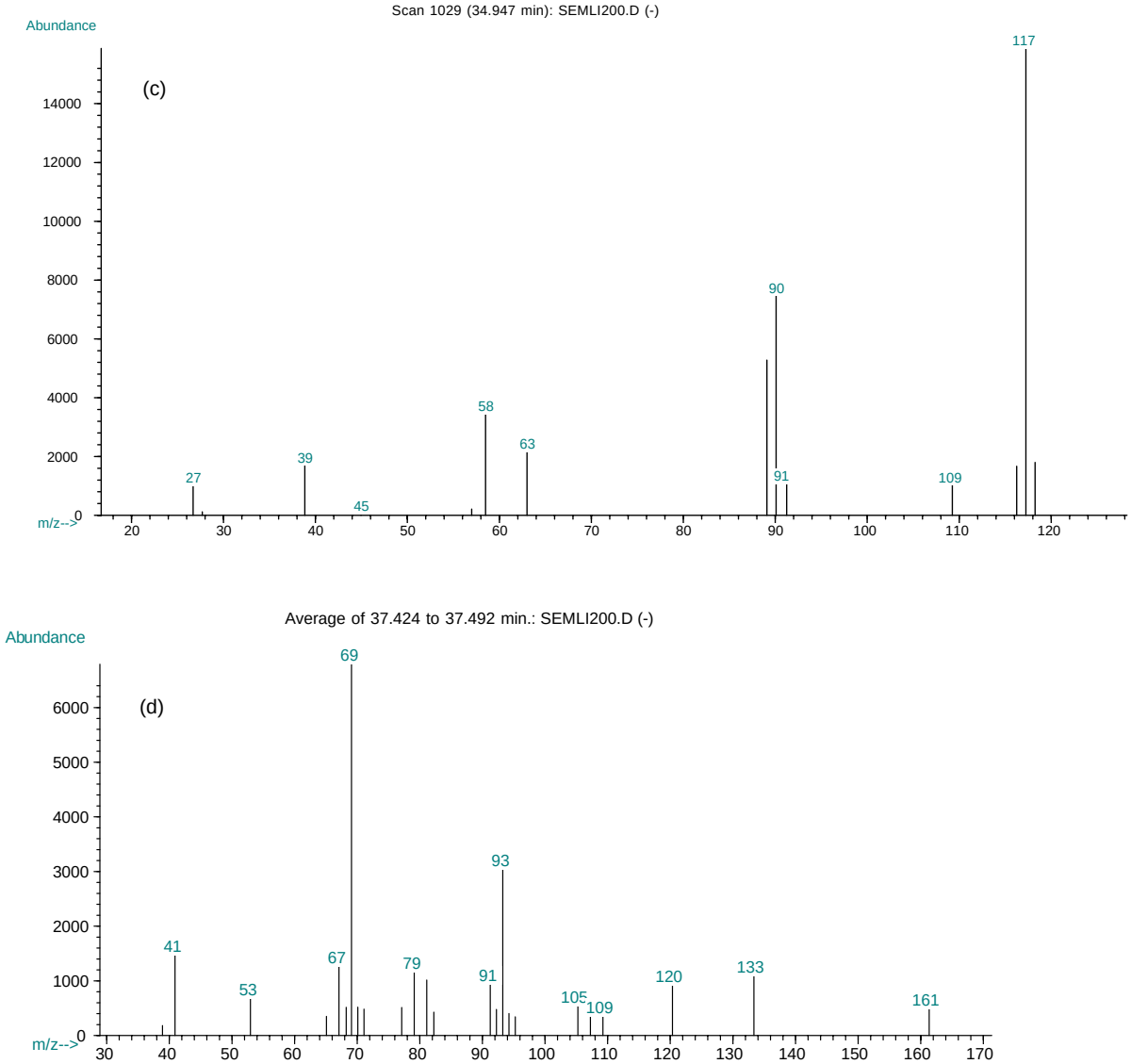

Fig. 2. (*Continued*).

and 1-tetradecanol, as well as the target pheromones 2-*sec*-butyl-4,5-dihydrothiazole, geraniol, indole, *trans*-beta farnesene, *trans*-alpha farnesene were identified. Fig. 2 (a–e, respectively) shows the mass spectra of these pheromones. The retention time (t_R), its relative standard deviation and library quality factor of pheromones identification are specified in Table 2. Relative amounts of the pheromones in the urine samples was determined with standards of geraniol and indole The values obtained were in the range of μg/ml (200–400 and 60–80 μg/ml for geraniol and indole respectively, in male mice urine).

In the mouse, these compounds are usually associated with and stabilised by binding to MUPs [22,23]. Thus, their detection by our procedure might indicate

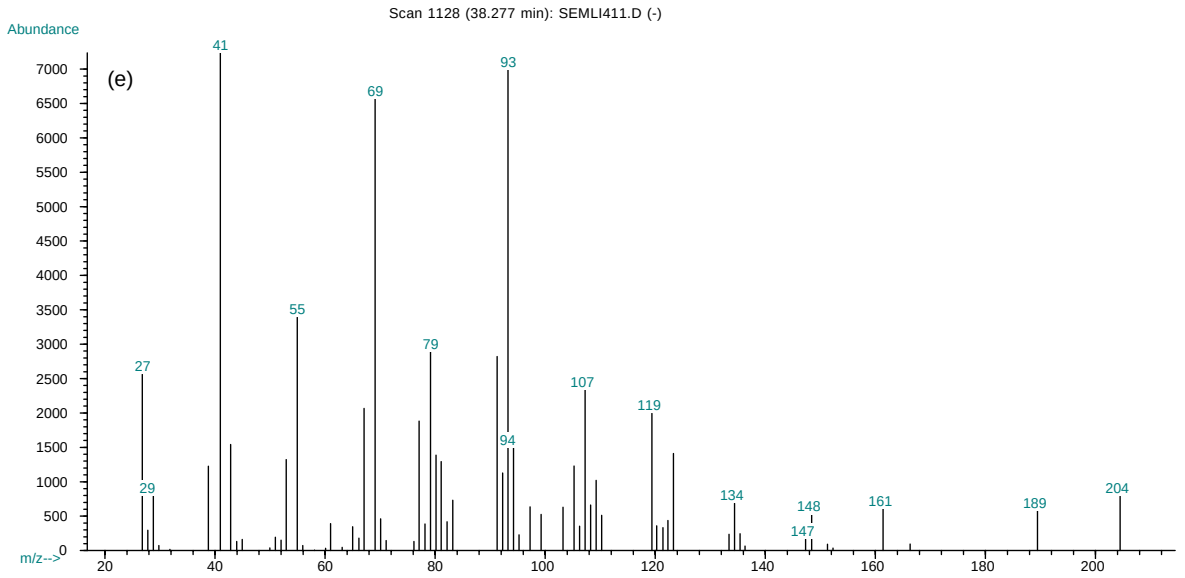

Fig. 2. (*Continued*).

a high affinity of the fibre employed for microextraction of pheromones from MUPs. The high level of polymorphism shown by MUPs [24], which are excreted in high concentrations, could give rise to the different patterns of pheromone binding shown by each urine sample. Indeed, it has been established that an animal's MUP profile plays a major role in urine mark identity [9,25]. This "fingerprint" in turn determines the individuality of urine marks related to the territorial behaviour of mice [9]. Animal behavioural studies require detailed analytical descriptions of the molecules that interact with the VNO sensory recognition system at the species, populational, familial and individual levels [26]. It is in the context of individual behaviour patterns that the SPME method could prove to be of most use, as suggested by its high sensitivity towards the small volumes tested in this pilot study. This minimally-invasive urine collection and pheromone extraction procedure could have applications in relating individual behaviour patterns, such as aggressiveness and jealousy, to pheromone secretion during the life cycle of an animal. It may even prove to be a useful tool for the rapid and reliable phenotypic analysis of transgenic (knock-out and knock-in) mice engineered to have a modified pheromone metabolism.

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