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Red deer gender and populations analysis using scats' volatile profile

Perfil volátil de excrementos de veado: distinção de populações e sexo

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Dedico este trabalho à minha avó Alice e ao meu avô David.

o júri

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palavras-chave

Cervus elaphus, compostos voláteis, dieta, distinção de géneros, excrementos, herança genética, populações, comunicação química.

resumo

Os recentes programas de reintrodução de veados (*Cervus elaphus*) e os movimentos naturais de dispersão de indivíduos provenientes de Espanha resumem a história das actuais populações deste mamífero em Portugal. À medida que estas se expandem e que populações com diferentes origens se reproduzem, aumenta a necessidade de metodologias mais sofisticadas capazes de as diferenciar independentemente da origem, e de determinar o rácio sexual, dois parâmetros usados na gestão de populações.

A microextracção em fase sólida e em modo espaço de cabeça e cromatografia abrangente bidimensional em fase gasosa acoplada à espectrometria de massa por tempo de voo (HS-SPME/GC×GC– TOFMS) surge como possível solução para a determinação destes parâmetros. Assim, este trabalho tem como objetivos diferenciar populações de veados, usando animais em cativeiro como modelo, bem como efectuar a distinção entre sexos. Os compostos voláteis obtidos são uma tentativa de determinar marcadores (compostos ou famílias químicas) para machos, fêmeas e para cada população, visando o uso na gestão de vida selvagem. De facto, os resultados deste estudo mostram que a diferenciação bem sucedida de populações e sexos foi baseada num sub-conjunto de compostos possivelmente derivados da dieta e comunicação química. O mecanismo que poderá estar na base destas distinções é a interação da genética e de fatores ambientais que conduziram a alterações metabólicas e fisiológicas nos animais, expressas com base nos metabolitos voláteis detetados nos seus excrementos. A ausência de literatura que relacione os compostos identificados (principalmente cetonas e hidrocarbonetos alifáticos) com a comunicação química entre veados leva à necessidade de bioensaios futuros onde se testem os compostos identificados neste estudo e a resposta dos animais. Considera-se que os resultados e conclusões deste trabalho poderão ter uma aplicação ecológica ao nível de populações selvagens de grande escala, permitindo a determinação de parâmetros ecológicos que de outro modo seriam dispendiosos e morosos.

abstract   Current populations of red deer (*Cervus elaphus*) in Portugal derive from recent reintroduction processes and natural dispersion movements from Spain. These movements promote the expansion of the species and reproduction among different populations, increasing the need for more sophisticated methodologies able to differentiate populations and determine sex-ratio, two demographic parameters used in the management of populations. Headspace solid phase microextraction and comprehensive two-dimensional chromatography coupled to mass spectrometry for time of flight gas phase mode (HS-SPME/GC×GC–ToFMS) arises as a possible solution to the determination of these parameters. Thus, this work aims to test for differences between red deer populations, using scats from natural fenced red deer populations as a model to distinguish between sexes. The obtained volatiles are an attempt to determine specific sets of compounds or chemical families' markers for males, females and for each population, targeting the use in wildlife approaches. In fact, results showed that populations and gender successful differentiation was based on a sub-set of probably, diet and chemical communication compounds, respectively. The mechanism underling this differentiation is probably the interaction of genetics and environment leading to changes in animals' physiology, based in the expressed volatile metabolites detected in the scats. The absence of literature data that relates the tentatively identified compounds (mainly aliphatic ketones and hydrocarbons) to chemical signals between red deer requires future bioassays testing the achieved compounds to pre-determined behaviours. It is considered that the results and conclusions of this work may be applied to big scale wild populations, allowing the assessment of ecological parameters that otherwise would be expansive and time consuming.

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1. General Introduction

The declining of red deer (*Cervus elaphus* Linnaeus, 1758) populations in Portugal, mainly due to hunting reasons, habitat losses and bad management (Fonseca et al., 2005), has been a study subject for biologists in the last forty decades. Such decrease led to small nuclei and fragmented populations, which was overcome by reintroduction programs with animals arising from several locations in the country. In the majority of the study cases, these reintroduction programs allowed recovering red deer population densities in Portugal, a species with a superior ecological interest, because it is an important prey of the endangered iberian-wolf (*Canis lupus signatus*) (Barja, 2009) as well as economic importance, because it is an hunting species.

Between 1995 and 1999 the first reintroduction program in Central Portugal occurred in Lousã mountains, which turned out to be the first reserve created in Portugal for *Cervus elpahus* (Ferreira et al., 1995) with a total of about 120 individuals successfully introduced (Fonseca et al., 2005). Besides reintroduction programs, free range animals from neighbouring Spain crossed the border and established themselves in the centre of Portugal, specifically in Tejo International Natural Park; in Alentejo, in the south (Salazar, 2009); and in the north, in Montesinho Natural Park, whose individuals arose from Serra de la Culebra populations (Santos, 2009). Thus, those population dispersion movements from Spain combined with reintroductions, resulted in a demographic growth and territory expansion of the species, making it difficult to access its distribution range.

To accomplish a sustainable growth, biologists need to assess some population parameters, such as distributional limits, population size (Santos, 2009) and sex-ratio

(Coulson et al., 2004). The most common methodologies used to assess such demographic parameters are radio-tracking surveys (Ferreira et al., 1995), fecal pellet group counts (Torres et al., 2012) and line transect sampling (Buckland et al., 1993), but these require numerous human resources, time and costs which may vary depending on the methodology applied. Moreover, achieving distributional limits in a population in expansion may turn out to be a difficult labour due social and organisational behavior, *e.g.* a male may roam from 5.000 to 20.000 ha each year depending on the habitat quality and human presence (Martinez, 1989) and in this process it might intercept and reproduce with females from other populations, biasing the results. Genetic approaches might also be suggested, but the ancestry background of Portuguese deer population may reveal some barriers.

Sex-ratio determination allows to understand the equilibrium of deer populations (Coulson et al., 2004). Faecal pellet dimension analysis is the most common non-invasive methodology used to distinguish sexes in deer. However, morphological changes between year and season within a class could constraint the use of this method (Tolleson et al., 2005). Regarding those problems, there is a need for reliable methods that allow biologists to assess such population parameters.

Near infrared reflectance spectroscopy (NIRS) was recently applied to determine fallow and red deer sex and species using faecal NIR spectra from animals in captivity. Tolleson et al., (2005) achieved good results in distinguishing red deer from fallow deer and males from females with scats from one campaign based in discriminant analysis. However, when combining two campaigns, discriminant equations were not able to predict accurately gender and species distinction. Although the referred distinction, it is very

unlikely that NIRS methodology would work in free-ranging populations, where animal's conditions are different from the studied by Tolleson et al., (2005). The individuals (males and females) where kept in a farmland environment, characterised by grassland of ryegrass (*Lolium perenne* L.); diet was similar between groups; males were all the same age (>1.5 years) and in a low point in the yearly cycle of nutritional needs (during the growth of velvet antlers); and females were all pregnant of about 90 days. Due to evident differences in male and female hormonal and reproductive state, it is very likely for an influence in scats NIR spectra to occur driven by the observed gender distinction.

Molecular advances in determining the degree of relationship among red deer populations were already achieved through microsatellite polymorphism extracted from small pieces of muscle in dead animals (Poetsch et al., 2001). In order for this to be applied in wild populations to determine population parameters in demographic studies, non-invasive methodologies are required.

Chromatographic techniques have also been used to study the origin of populations of ungulates. At least one study has been done regarding this subject (Lawson et al., 2001), using gas chromatographic techniques to analyse glands segregations, instead of scats. One dimension (1D) gas chromatography coupled with dynamic headspace sampling GC-MS/FID was performed with the aim of studying volatiles and their potential to code for attributes, such as population of origin in red deer, sika deer (*Cervus nippon*), chinese muntjac (*Muntiacus reevesi*) and fallow deer (*Dama dama*). Discrimination of population, based in discriminant analysis, was only possible in sika deer metatarsal and muntjac preorbital gland while discrimination in population of origin of red and sika deer in preorbital glands was weak. Muntjac samples were 93.8% correctly discriminated to

source population, while 22 sika samples were correctly classified to population of origin from the two tested populations. Although good results were observed, the number of volatiles that 1D gas chromatography allowed to elute from the samples (minimum of 6 and maximum of 72 compounds) is very low comparing to what more advanced chromatographic techniques enable to perform (Kalinová et al., 2006).

In order to use Gas Chromatography to analyse natural matrices, such as glands secretions or scats, organic compounds must be obtained with a previous extraction step. A method to extract volatile organic compounds (VOCs) is through headspace solid-phase microextraction HS-SPME (Figure 1– A). This technique developed by Janusz Pawliszyn in 1990 is used in natural matrices as food, wine, atmospheric air and biological samples. It consists in a fast way to extract VOCs with very simple devices. A retractable fibre coating with polymeric sorbent inside of a chirurgical needle is used to collect volatiles from the sample inside a vial and then, to transfer these volatiles into an analytical instrument for analysis. The vial containing the sample is placed in a thermostatic bath for a pre-determined time and temperature (according to the nature of the sample) (Figure 1– B I). The volatiles are then released from the sample to the headspace of the vial. In the second step, the fibre is introduced into the headspace and the organic volatiles are concentrated and extracted in the fibre coating (Figure 1– A II), being then transferred to the injector port of the chromatographic machine (Figure 1– B III) (Zhang and Pawliszyn, 1993).
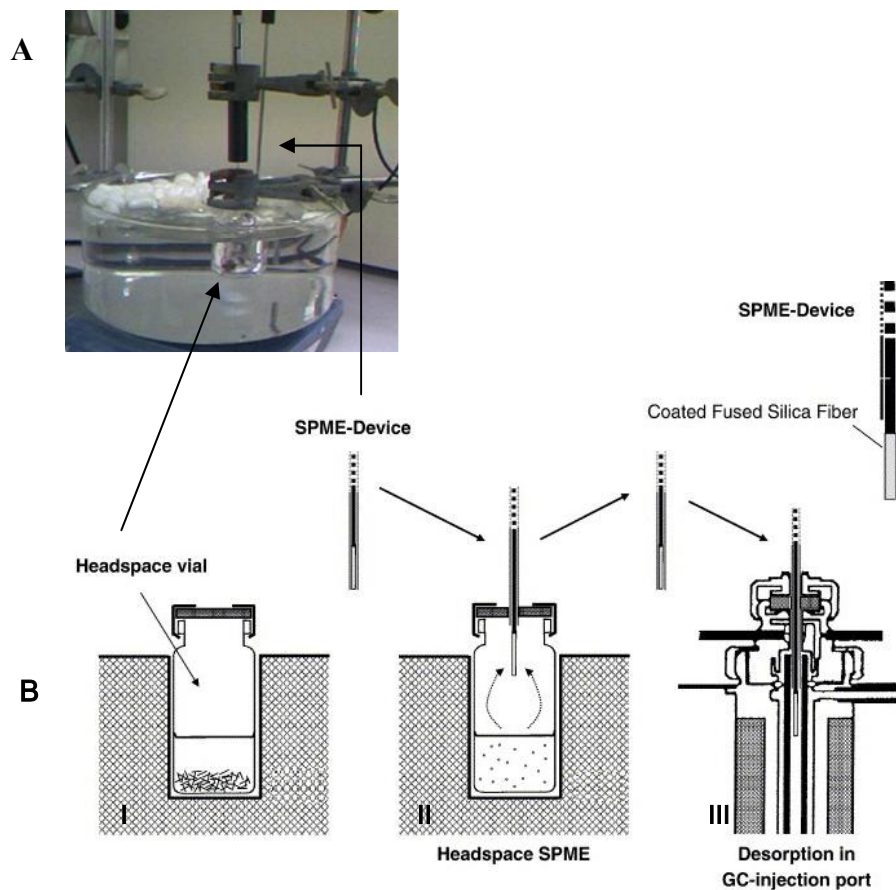
Figure 1 - A) HS-SPME apparatus; B) HS-SPME extraction representation and devices. Adapted from (Pragst and Balikova, 2006).

The advantages of HS-PME are diverse. It allows a faster analytes extraction; it does not require the previous treatment of samples as it is a direct analysis; it avoids losses of compounds with low volatility and the formation of artefacts formation and prevents losses of analytes to the sorbent, which were normally co-eluted in it (Zhang and Pawliszyn, 1993).

A

7. Hexane
8. Ethyl Acetate
9. Chloroform

1st Dimension (s)

B

2nd Dimension (s)
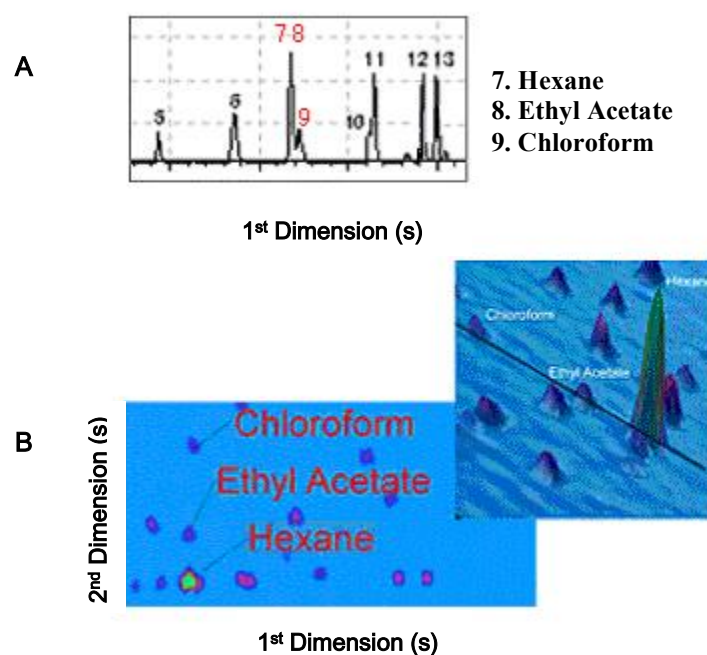
1st Dimension (s)

Figure 2- A) Co-eluted peaks obtained by one dimensional gas chromatography. B) GC×GC-ToFMS chromatogram contour plot showing enhanced hexane, ethyl acetate and chloroform separation. Adapted from Shimadzu Comprehensive GC×GC system on-line.

Separate compounds of a complex matrix (as scats) or nearby co-eluted peaks, is a very common limitation of the conventional one dimensional gas chromatography system, as the above announced technique, GC-FID (Kalinová et al., 2006). An important part of the information can be lost during the process due to this co-elution problem. It consists in a peak overlap in chromatographic separations (Figure 2-A), especially for highly complex samples, resulting in wrong similarity matches in the MS library and in the wrong compounds identification.

Comprehensive two-dimensional gas chromatography (GC×GC) was developed to solve this co-elution problem, providing a technique with a high resolution power. GC×GC chromatographic systems consists in two GC columns serially connected, coated with

different stationary phases, attached by an interface with a cryogenic modulator (Figure 3). After eluted from the first column, compounds are constantly condensed by a cryogenic modulator which releases them, quantitatively segmented, in short pulses into the second column (Kalinová et al., 2006). In fact, the peaks produced by those columns are so narrow that a very sensitive - high acquisition rate detector - is needed, as the Time-of-Flight Mass spectrometer (ToFMS). This detector receives the signal and then realigns it to form a three dimensional structured chromatogram: a visual information of each one of the separated peaks (Figure 2- B) that brings the possibility to relate chemical structures with their chromatographic position, facilitating the identification process. This way, GC×GC– ToFMS offers an excellent resolving power, with a high peak detectability, sensitivity and analysis speed (Dimandja, 2003).
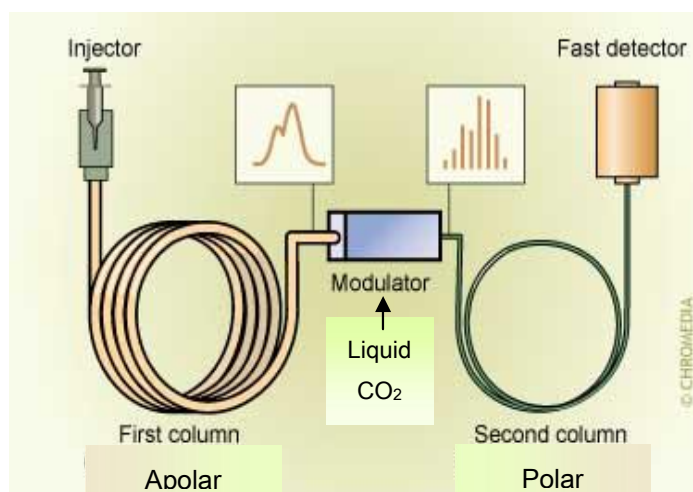
Figure 3 - Schematic representation of GC×GC system with two columns, the modulator and the ToFMS detector. Adapted from (Sindona and Taverna, 2012).

Thousands of compounds can be detected and identified from scat samples (Saraiva et al., 2013). Scats are easier to access due to their abundance, frequently being used in

censuses, and other ecological approaches, to estimate and track multiple variables such as population size (Kohn et al., 1999; Webbon et al., 2004), distributional patterns, species richness, diets, stress levels or even in environmental impact assessments. Saraiva et al. (2013), used scats to study the red deer and fallow deer using GC×GC– ToFMS. They obtained an average of 300 compounds in each sample, mainly hydrocarbons, ketones, aromatic hydrocarbons, monoterpenes, sesquiterpenic compounds (hydrocarbons and oxygen containing forms) and diterpenes. The results suggest that red deer volatile profile is characterised by terpens compounds, presumably related with diet, while fallow deer also achieved chemical communication markers: 2-propanone and hexane (aliphatic ketone and hydrocarbon, respectively) (Saraiva et al., 2013).

It is now clear that GC×GC– ToFMS offers a great separation potential, sensitivity and limits of detection comparing with 1D Chromatography (Dalluge et al., 2003; Marriott and Shellie, 2002; Zrostlikova et al., 2003), especially if the samples are as complex as scats.

Thus, considering the potential of this combined technique and the need for reliable methodologies to evaluate red deer demographic parameters, this work aims to study the feasibility to distinguish different red deer populations and sexes using HS-SPME combined with GC×GC– ToFMS. Population and gender related volatile markers are expected to be achieved for future applications in studies that aim to establish population distribution range and sex-ratio in wild animals.

2. Red deer population and gender distinction through the analysis of the volatile organic compounds released by scats

2.1.   Abstract

Current populations of red deer (*Cervus elaphus*) in Portugal derive from recent reintroduction process and natural dispersion movements from Spain. These movements promote the expansion of the species and reproduction among different populations, increasing the need for more sophisticated methodologies able to differentiate populations and to determine other parameters (such as sex-ratio) used in population management.

In order to study the feasibility to identify different populations and sexes, scats of seven red deer males and twelve females (n=19) from four different populations of natural fenced areas were analysed. Headspace solid phase microextraction (HS-SPME) combined with comprehensive two-dimensional gas chromatography coupled to mass spectrometry with time of flight (GC×GC- TOFMS) were used to extract and analyse the volatile organic compounds (VOCs) of scats. Principal Component Analysis (PCA) was performed to all 571 tentatively identified compounds. Results allowed to differentiate Tapada Nacional de Mafra (TNM), Parque Biológico da Serra da Lousã (PBSL), Parque Biológico de Vinhais (PBV) and Parque Ecológico de Gouveia (PEG) red deer populations based on a subset of 10 compounds. Partial Least Squares Discriminant Analysis (PLS-DA) generated a very good model of gender distinction in which its robustness was validated through Monte Carlo Cross Validation. The presence of aliphatic ketones and hydrocarbons may be related to chemical signalling in social behaviour while terpenes may arise from an herbivorous diet. In this regard, dietary components have contributed

more to the distinction between populations while possible chemical communication factors favoured gender differentiation. Additionally, metabolic variations resulting from the interaction of genetics and environment leads to changes in animal physiology, an aspect which may help to explain populations distinction.

Key words: *Cervus elaphus*, chemical communication, diet, gender distinction, genetic background, populations, scats, volatile compounds

2.2.   Introduction

Red deer (*Cervus elaphus* Linnaeus, 1758) situation in Portugal as suffered different stages, it has been declining in the last 50 decades ago and increased in the last 10 years due to human reintroduction programs (Fonseca et al., 2005) and natural expansion from Spanish border areas (Salazar, 2009). Such demographic increments exalts the need of management measures and a sustainable growth, supported by the role that this species assumes in the Portuguese wildlife trophic chain, since it is an important prey of the endangered iberian-wolf (*Canis lupus signatus*) (Barja, 2009).

To accomplish a sustainable growth, biologists need to assess population parameters, such as distributional limits, populations size (Santos, 2009) and sex-ratio (Coulson et al., 2004). The determination of these parameters might present some barriers since current Portuguese red deer wild populations are a mixture of different backgrounds, a result of different animal sources and population inbreeding. Up to the present, the methodologies used in demographic surveys have been traditionally based on radio-tracking (Ferreira et al., 1995), faecal pellet group counts (Torres et al., 2012)

and line transect distance sampling (Buckland et al., 1993), but all of them require a great number of human resources, time and costs which vary depending on the methodology applied.

Saraiva et al. (2013), applied more recently comprehensive two-dimensional gas chromatography (GC×GC) with time-of-flight mass spectrometry (ToFMS) detector, to scats of red deer, fallow deer (*Dama dama*) and Eurasian otter (*Lutra lutra*), obtaining a full separation of groups (carnivores from ungulates). These results were achieved combining GC×GC– ToFMS analysis with headspace solid-phase microextraction HS-SPME, proving to be a fast way to extract volatile organic compounds (VOCs) from scats with very simple devices. Furthermore, the combination of all these steps, revealed an effective way of distinguishing ungulates, red deer and fallow deer, having obtained an average of 300 compounds per sample and showing great sensitivity and enhanced limits of detection when compared with other applied methods. In fact, besides Saraiva et al. (2013), work, HS-SPME/GC×GC– ToFMS was already used in several fields of analysis, revealing benefits especially for analysis of complex samples and detection of trace components (Rocha et al., 2012; Rocha et al., 2013).

GC×GC– ToFMS represents a successful combination, a result of investigations made in the last twenty years aiming the strengthening resolving power of previous 1D-GC (Kidwell and Riggs, 2004; Tranchida et al., 2004). GC×GC employs two orthogonal mechanisms to separate the constituents of samples within a single analysis, a technique based on the application of two GC columns (instead of just one column) coated with different stationary phases. Hence, sensitivity, separation potential and limits of detection are greatly enhanced when compared to 1D-GC (Dalluge et al., 2003; Marriott and

Shellie, 2002; Zrostlikova et al., 2003) Attending the effective species differentiation observed in Saraiva et al., 2013, inferring great potential of HS-SPME/GC×GC– ToFMS on biological study applications, we hypothesise that the use of these multi-step technique could represent a great help in wildlife demographic surveys. Through a correct distinction between populations and sexes, using this non-invasive and fast technique applied in abundant natural matrices like scats, researchers could easily access to range determination and sex-ratio.

Thus, the aim of this study is to test for differences between natural fenced red deer populations and sexes through HS-SPME/GC×GC– ToFMS, attempting to obtain specific sets of compound or chemical family markers for males, females and for each population, targeting the use in wildlife approaches.

2.3.   Materials and Methods

2.3.1. Study area

Scat collection was performed in four Portuguese captivity areas: Parque Biológico de Vinhais (PBV) in the north, Parque Ecológico de Gouveia (PEG) and Parque Biológico da Serra da Lousã (PBSL) in the centre of Portugal and Tapada Nacional de Mafra (TNM) in the Lisbon and Tejo valley region, near the coast (Figure 4). Tapada Nacional de Mafra is a 16 km walled natural hunting area, with no natural predators that uses hunting to keeps the balance of the different animal populations and simultaneously limit the pressure of animals on vegetation. PBSL, PEG and PVP natural fenced parks display to visitors the main species that inhabit the Portuguese territory in an environment that

simulates their habitat in nature. In all areas veterinary cares such as vaccinations against major diseases and internal and external deworming is practiced.

2.3.2. Samples

A total of 19 samples (scats samples from 19 *Cervus elaphus* individuals) were collected from males, females, young and adults in four natural fenced areas. Parque Ecológico de Gouveia (PEG) contributed with one male (M) animal (n=1; M2), Parque Biológico de Vinhais (PBV) with one male and one female (F) (n=2; M3,F1), Parque Biológico da Serra da Lousã (PBSL) with six animals (n=6; F9, F10, F11, F12; M1 and M7) and Tapada Nacional de Mafra (TNM) contributed with ten animals (n=10; F2-8; M4-6). Samples from TNM were collected in two different fenced areas, the upper (M6 and F8) and lower fence but, for statistical analysis, they were considered one single population. Individuals from the upper area were separated from the others due to their appearance: the steams of the male appeared to be damage and were not in the best conditions to be displayed to the public. All fragments were collected immediately after animal defecation to avoid re-collections.

Samples were collected in different years and months (Table 1). Scats samples coming from PBV (F1 and M3), PEG (M2) and one from PBSL (M1) were collected in February 2011 and the last two in November 2010, respectively. Three samples (M4, F2, and F3) were collected in May 2011 while all the other samples were collected in July 2012.

After collection, scats were wrapped in aluminium foil and then stored in one glass vial for each species at -20 ºC until analysis.

Table 1 - Year, month and gender description of the collected samples.

Year	Month	Sample
2010	November	M1
2011	November	M2
2011	February	F1, M3
2011	May	M4, F2, F3
2012	July	M5-M7, F4-F12

F – Female (n=12), M – Male (n=7)

2.3.3. Population ancestry background

An ancestry background of the populations included in this study was achieved, in order to find relations between individuals. Figure 4 represents the translocation direction of the animals from the original place to the final natural fenced areas in study and is a summary of Figure 5.
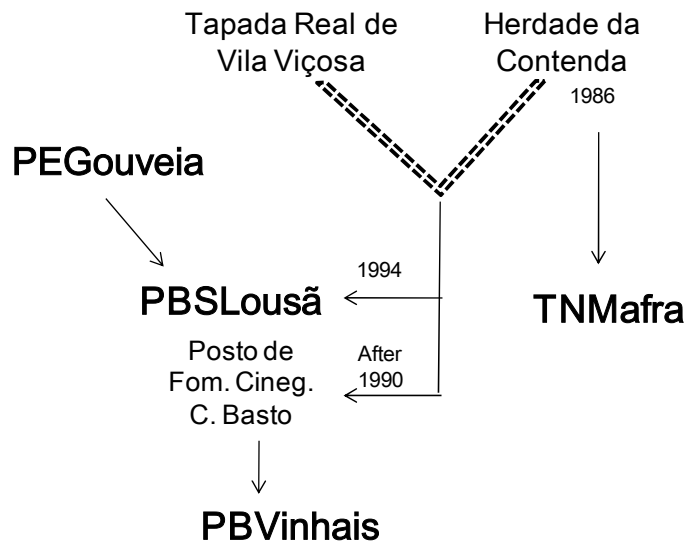
Figure 4- Summary of the translocation of the animals from the original place to the final natural fenced areas in study located in Portugal.

Figure 5 shows, for some cases, the number of individuals that were transferred in the last decades (in blue) from the original place to the final natural fenced areas. The population from the down fence in Tapada Nacional de Mafra (TNM) had its origin in Quinta da Torre Bela (around 1920), to which was added an individual from Herdade da Contenda (in 1986) (Cabral and Guerreiro, 1986). TNM animals from Herdade da Contenda reproduced successfully and were the main ancestor of animals sampled in this study. In regards to the Parque Biológico da Serrra da Lousã (PBSL), two female founders were donated in 1999, whereas the male founder was brought from Serra da Lousã surroundings, whose primary origin was Herdade da Contenda or Tapada Real de Vila Viçosa, as a result of the reintroduction program in 1995. Thus, TNM and PBSL populations share some genetic background due to Herdade da Contenda ancestry, and PBSL has genetic bounds with Parque Ecológico de Gouveia PEG due to the offered female. Also Parque Biológico de Vinhais (PBV) individuals share some genetic background with both TNM and PBSL. Both animals were brought from a game promoting reserve (Posto de fomento cinegético de Cabeceiras de Basto), which in turn were donated by Tapada Real de Vila Viçosa and by Herdade da Contenda, Álvaro Gonçalves, personal communication.
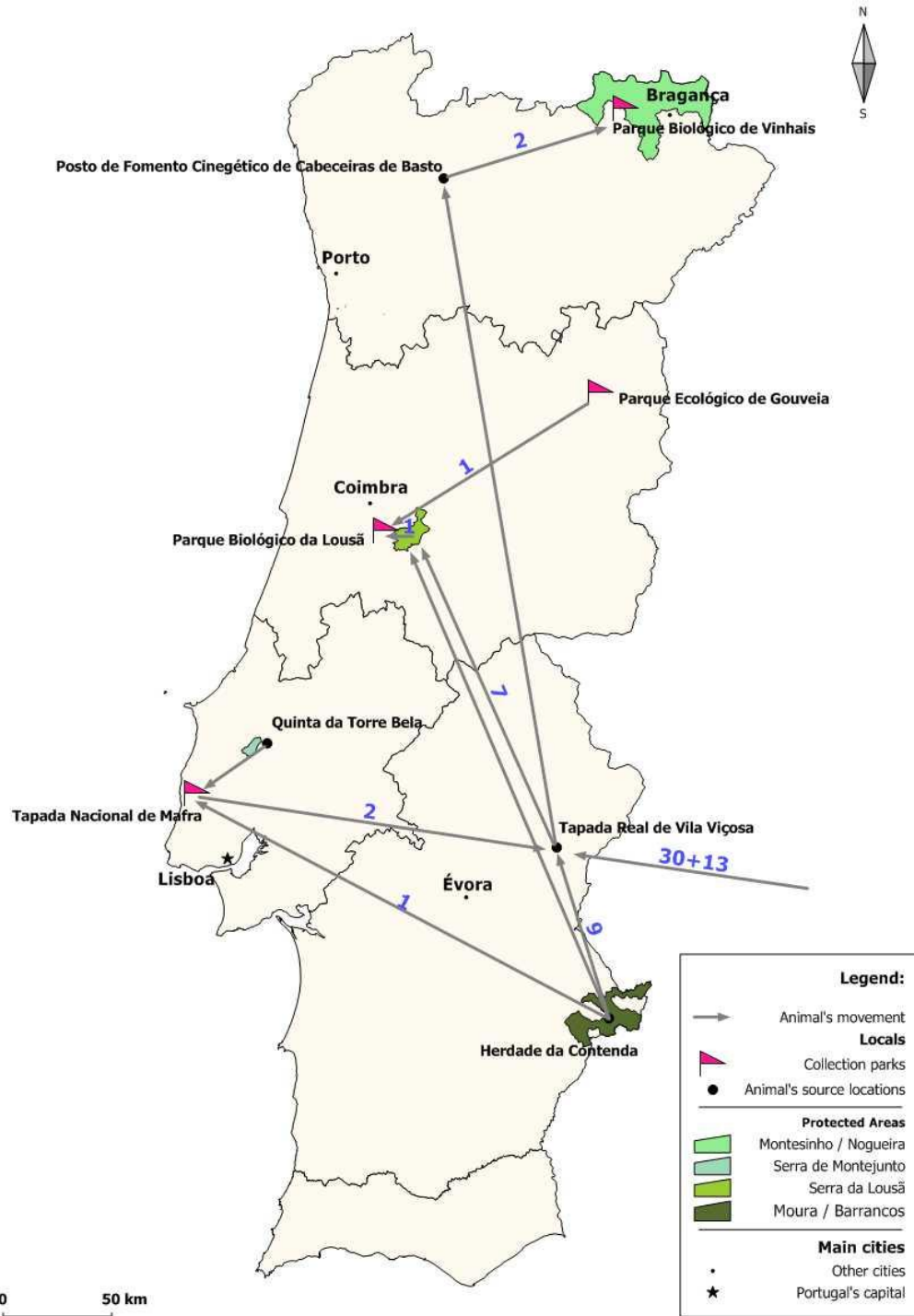
Figure 5-Translocation direction of the animals from the original place to the final natural fenced areas in study located in Portugal: Parque Biológico de Vinhais, Parque Ecológico de Gouveia, Parque Biológico da Serra da Lousã and Tapada Nacional de Mafra.

2.3.4. HS-SPME Methodology

The SPME holder for manual sampling and fibre were purchased from Supelco (Aldrich, Bellefonte, PA, USA). The SPME device included a fused silica fibre coating partially cross-linked with 50/30µ m divinylbenzene-carboxen-poly(dimethylsiloxane), PDMS/DVB/CAR. Prior to use, the SPME fibre was conditioned at 270 °C for 60 min in the GC injector, according to the recommendations of the manufacturers. Then, the fibre was daily conditioned for 10 min at 250 ºC.

The HS-SPME experimental parameters were based on a previous study (Saraiva et al., 2013). For HS-SPME assay, aliquots of 0.5 g of scat were placed into a 25 mL glass vial. The vial was capped with a PTFE septum and an aluminum cap (Chromacol, Hertfordshire, UK), and was placed in a thermostatted bath adjusted to 30.0±0.1 ºC. Then, the SPME fibre was inserted in the headspace for 10 min. Two aliquots of each animal scat were analysed (Figure 6). Blanks corresponding to the analysis of the coating fibre were not submitted to any extraction procedure, and were run between sets of two analysis.

2.3.5. GC×GC – ToFMS Analysis

After the extraction/concentration step, the SPME fibre coating was manually introduced into the GC×GC– ToFMS injection port (Figure 6) at 250 °C for desorption. The injection port was lined with a 0.75 mm I.D. splitless glass liner. Splitless injections were used (30s). LECO Pegasus 4D (LECO, St. Joseph, MI, USA) GC×GC– ToFMS system consisted of an Agilent GC 7890A gas chromatograph, with a dual stage jet

cryogenic modulator (licensed from Zoex) and a secondary oven. The detector was a high-speed ToF mass spectrometer. An HP-5 column (30 m x 0.32 mm I.D., 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA, USA) was used as a first-dimension column (1D) and a DB-FFAP (0.79 m x 0.25 mm I.D., 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA, USA) was used as a second-dimension column (2D). The carrier gas was helium at a constant flow rate of 2.50 mL min-1. The injection port was at 250 °C. The primary oven temperature program was: initial temperature 35 ºC (held for 2 min), rose to 100 ºC (6 ºC min− 1) and then rose to 220 ºC (10 ºC min− 1, hold 2 min). The secondary oven temperature program was 15 ºC offset above the primary oven. The MS transfer line and the MS source temperature operated at 250 ºC. The modulation time was 5s; the modulator temperature was kept at 20 ºC offset (above primary oven). The ToFMS was operated at a spectrum storage rate of 125 spectra s-1. The mass spectrometer was operated in the EI mode at 70 eV using a range of m/z 33-350 and the detector voltage was -1695 V. Total ion chromatograms (TIC) were processed using the automated data processing software ChromaTOF (LECO) at signal-to-noise threshold of 10. Contour plots were used to evaluate the general quality of the separation and for manual peak identification; a signal-to-noise threshold of 50 was used. For identification purposes, the mass spectrum of each compound detected was compared to those in mass spectral libraries which included an in-house library of standards, and two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0 - Mainlib and Replib). The identification was also supported by experimentally determined retention index (RI) values that were compared, when available, with values reported in the bibliography for chromatographic columns similar to those on the present work as the first

dimension column (5%-phenyl-95%-dimethylpolysiloxane) (Adams, 1995; Carrapiso et al., 2002; Högnadóttir and Rouseff, 2003; Leffingwell and Alford, 2005; Rembold et al., 1989; Rychlik et al., 1998; Valim et al., 2003; Venkateshwarlu et al., 2004; Wang et al., 2006; Xu et al., 2003; Xu et al., 2007). For determination of RI values a C8-C20 n-alkanes series was used, calculated according to the van den Dool and Kratz equation (van Den Dool and Kratz, 1963). The majority (90%) of the identified compounds presented similarity matches >800. The GC×GC area data were used as an approach to estimate the relative content of each volatile component of scats. The RI parameter supported the identification, since the calculated retention index differed 0 to 5.5% when compared to literature data for the 1D column or equivalents (Adams, 1995; Berdague et al., 1991; Choi, 2003; El-Sayed et al., 2005; Engel et al., 2002; Gomez et al., 1993; Hierro et al., 2004; Machiels et al., 2003; Rembold et al., 1989; Venkateshwarlu et al., 2004; Zehentbauer and Reineccius, 2002).
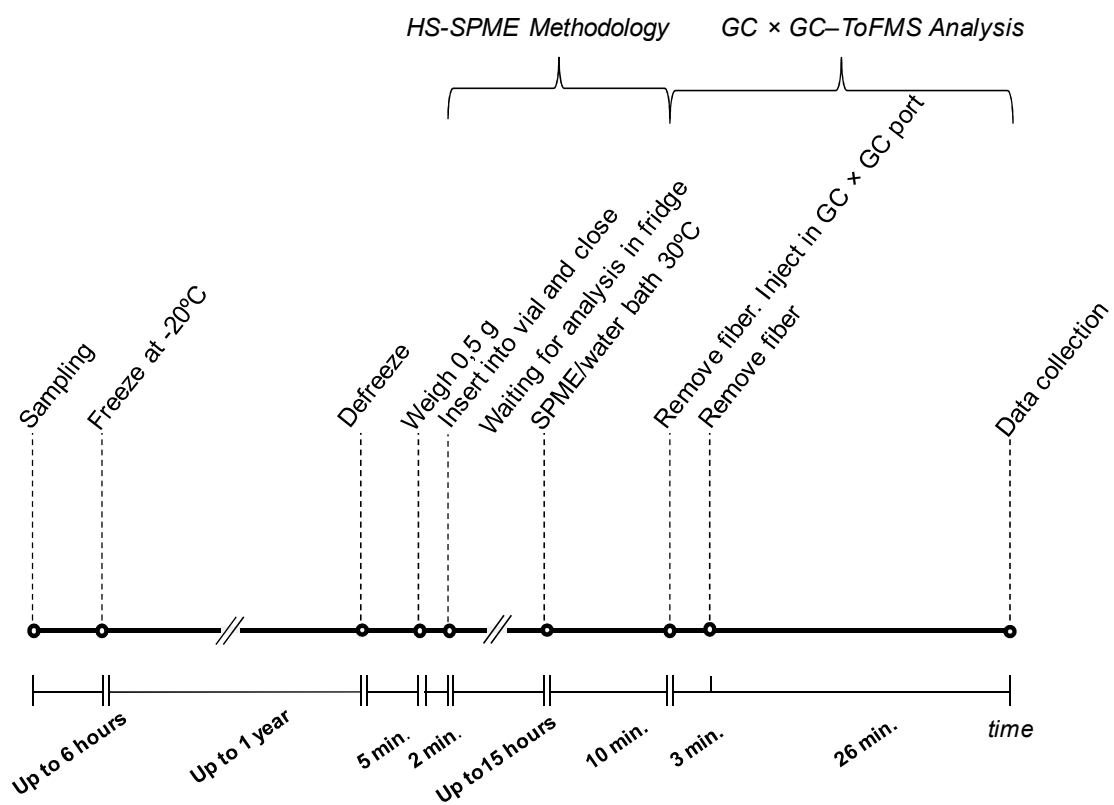
Figure 6 - Methodology resume from sampling to data collection.

2.3.6. Multivariate analysis

The Principal Component Analysis was applied to the normalised areas (mean normalisation) of a matrix consisting of 571 compounds (variables) and 38 replicates (2 replicates analysis x 19 individual scats samples). PCA establishes a relationship between objects and variables (Jolliffe, 2002) and extracts the most important information from the data set, expressing this information into new variables called principal components (PC1 and PC2). This characteristic is major because in this type of technique, variables can reach the hundreds of compounds and PCA reduces it to a set of the ones with the strongest pattern of similarity (Abdi and Williams, 2010). The scores scatter plot obtained plotting PC1 against PC2, shows similarities between the different samples and groups. The loading plot shows the trends in the data through the positioning of the compounds correspondent to the groups originated in the score scatter plot.

Aiming for the gender distinction, the previous matrix formed by 571 compounds, was also used for the comparison of sample groups (seven males and twelve females). After normalisation to total area, classification analysis in Partial Least Squares Discriminant Analysis (PLS-DA) was performed in SIMCA-P11.5 (Umetrics, Umeå, Sweden). Additionally, a selection of a sub-set (Table 2) comprising aliphatic ketones and hydrocarbons, mono and sesquiterpens and norisoprenoids was used to unveil the possible effects of diet or chemical communication in the distinction of samples. Scores scatter plots were used for visualising the variability between samples, and VIP (variable importance in the projection) values, were inspected for underlining the main compounds of the differentiation between the compared groups. Model robustness was assessed

using ROCCET, generated by Monte Carlo Cross Validation (MCCV). The sensitivity and the specificity of the model were extracted to assess the significance of the results.

2.4. Results

2.4.1. Contour plot analysis

Figure 7 shows the GC×GC-ToFMS chromatogram contour plot: a structured chromatogram that displays all analytes separated according to their volatility in the first dimension (^{1}D) and polarity (^{2}D) in the second dimension, allowing to depicture the major volatile chemical families present in *C. elaphus* scats. Chromatogram contour plot is represented with clusters and bands to elucidate the relevant chemical families: aromatic and aliphatic hydrocarbons, aliphatc ketones, monoterpenes, sesquiterpenic compounds (hydrocarbons and oxygen containing forms) and diterpenes.
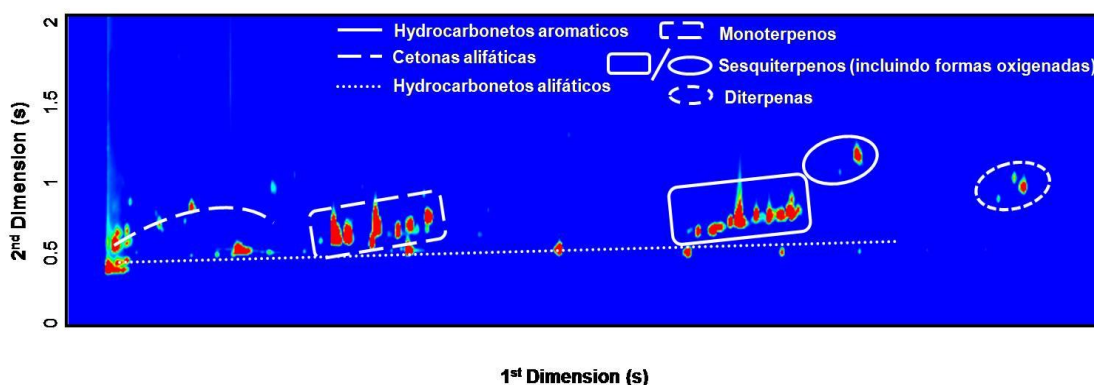
Figure 7- GC×GC chromatogram contour plots of *Cervus elaphus*. Bands and clusters formed by structurally related compounds are indicated.

For multivariate analysis and comparisons between sample groups (males and females), peak areas of the 571 compounds were manually extracted from the

chromatograms and used to create the data matrix. Besides the above mentioned chemical families, alcohols, aldehydes, anisoles, carboxylic acids, esters, ethers, furans, halocarbons, aromatic hydrocarbons and ketones, ketals, norisoprenoids, sulfur and nitrogen derived compounds were also detected on samples.

A list containing a selection of specific compounds and chemical families identified in chromatogram contour plot reported to be related with diet (mono and sesquiterpens) and with chemical communication (aliphatic hydrocarbons and ketones) is provided in Table 2, with the corresponding retention times t_R, retention index obtained ($RI_{calc.}$) through the modulate chromatogram and the RI reported in the literature (($RI_{lit.}$) for one dimensional GC with a 5%-phenyl-95%-dimethylpolysiloxane GC column or equivalent.

Table 2- Sub-set of 305 volatile compounds of scats selected for Partial Least Squares Discriminant Analysis (PLS-DA) analysis

$^1t_R, {}^2t_R$	Compound	Formula	CAS	RI_{calc}	RI_{lit}
	Aliphatic hydrocarbons				
90 , 0,384	C5 isomer	-	-	588	-
95 , 0,424	C5 isomer	-	-	594	-
95 , 0,432	C5 isomer	-	-	594	-
100 , 0,376	Hexane	C6H14	110-54-3	600	600
105 , 0.400	3-Methyl-2-pentene, isomer	C6H12	922-62-3	607	613
110 , 0,360	C6 isomer	-	-	613	-
110 , 0,368	C6 isomer	-	-	613	-
115 , 0,448	2,4-Hexadiene, isomer	C6H10	5194-51-4	620	631
125 , 0,354	1-Hexene	C6H12	592-41-6	633	584
125 , 0,368	2-Methyl-1-pentene	C6H12	763-29-1	633	663
125 , 0,400	3,3-Dimethyl-pentane	C7H16	562-49-2	633	652
125 , 0,528	C7 isomer	-	-	633	-
130 , 0,400	2-Methyl-hexane	C7H16	591-76-4	639	664
135 , 0,392	3-Methyl-hexane	C7H16	589-34-4	646	649
140 , 0,360	C7 isomer	-	-	652	-
145 , 0,376	1-Heptene	C7H14	592-76-7	659	-
145 , 0,392	C7 isomer	-	-	659	-

$^1t_R, {}^2t_R$	Compound	Formula	CAS	RI_{calc}	RI_{lit}
150 , 0,398	Heptane	C7H16	142-82-5	665	700
155 , 0,376	3-Ethyl-2-pentene	C7H14	816-79-5	671	701
180 , 0,384	C7 isomer	-	-	704	-
180 , 0,416	C7 isomer	-	-	704	-
180 , 0,416	C7 isomer	-	-	704	-
190 , 0,448	2,3-Dimethyl-1,3-pentadiene	C7H12	1113-56-0	717	733
195 , 0,416	2,3,4-Trimethyl-pentane	C8H18	565-75-3	723	760
200 , 0,400	2,3-Dimethyl-1,4-hexadiene	C8H14	18669-52-8	730	748
205 , 0,376	3-Ethyl-2-methyl-pentane	C8H18	609-26-7	736	760
205 , 0,392	C8 isomer	-	-	736	-
210 , 0,376	C8 isomer	-	-	742	-
215 , 0,376	C8 isomer	-	-	749	-
215 , 0.424	2-Methyl-heptane	C8H18	592-27-8	749	773
225 , 0,400	2-Octene, isomer	C8H16	08/04/7642	762	809
225 , 0,432	3-Methyl-heptane	C8H18	589-81-1	762	775
230 , 0,376	C8 isomer	-	-	768	-
240 , 0,400	1-Octene	C8H16	111-66-0	781	791
245 , 0,456	2-Octene	C8H16	111-67-1	788	792
255 , 0,408	4-Methyl-3-heptene	C8H16	4485-16-9	800	800
255 , 0,424	Octane	C8H18	111-65-9	800	798
265 , 0,400	3-Octene	C8H16	14919-01-8	804	798
265 , 0,408	3-Octene, isomer	C8H16	14850-22-7	807	-
275 , 0,408	2-Octene, isomer	C8H16	13389-42-9	814	817
280 , 0,448	C8 isomer	-	-	818	-
285 , 0,384	2,4-Dimethyl-heptane	C9H20	2213-23-2	821	821
285 , 0,432	2,3,4-Trimethyl-hexane	C9H20	921-47-1	821	-
290 , 0,472	1,3-Octadiene	C8H14	1002-33-1	824	830
300 , 0,384	3,5-Dimethyl-heptane	C9H20	926-82-9	831	-
310 , 0,472	2,5,5-Trimethyl-1-hexen-3-yne	C9H14	37439-53-5	838	-
330 , 0,416	C9 isomer	-	-	852	-
340 , 0,400	*mz*(41,55,67)	-	-	859	-
345 , 0,384	2,3-Dimethyl-heptane	C9H20	3074-71-3	862	-
345 , 0,392	2-Methyl-octane	C9H20	3221-61-2	862	864
355 , 0,392	2,5-Dimethyl-heptane	C9H20	2216-30-0	869	-
355 , 0,416	7-Methyl-3-octene	C9H18	-	869	-
365 , 0,376	C9 isomer	-	-	876	-
375 , 0,392	C9 isomer	-	-	883	-
385 , 0,432	C9 isomer	-	-	890	-
390 , 0,488	1-Nonene	C9H18	124-11-8	893	889
395 , 0,424	4-Nonene	C9H18	2198-23-4	897	-
400 , 0,408	C9 isomer	-	-	900	-
400 , 0.448	Nonane	C9H20	111-84-2	900	899
425 , 0,400	2,4,6-Trimethyl-heptane	C10H22	2613-61-8	916	875

$^1t_R, ^2t_R$	Compound	Formula	CAS	RI$_{calc}$	RI$_{lit}$
425 , 0,440	3-Ethyl-4,5-dimethyl-1,4-hexadiene	C10H18	-	916	-
435 , 0,392	3,5-Dimethyl-octane	C10H22	15869-93-9	923	928
435 , 0,400	2,3,5-trimethyl-heptane	C10H22	20278-85-7	923	-
450 , 0,400	2,6-Dimethyloctane	C10H22	2051-30-1	933	933
455 , 0,400	3-Methyl-nonane	C10H22	06-04-5911	936	968
455 , 0,480	C10 isomer	-	-	936	-
460 , 0,400	C10 isomer	-	-	939	-
460 , 0,408	3-Ethyl-2-methyl-heptane	C10H22	14676-29-0	939	942
465 , 0,424	C10 isomer	-	-	942	-
475 , 0,424	C10 isomer	-	-	949	-
480 , 0,400	C10 isomer	-	-	952	-
485 , 0,440	C10 isomer	-	-	955	-
490 , 0,392	2,2-Dimethyl-octane	C10H22	15869-87-1	958	917
490 , 0,440	C10 isomer	-	-	926	-
495 , 0,400	C10 isomer	-	-	962	-
495 , 0,416	C10 isomer	-	-	955	-
505 , 0,456	2-Methyl-nonane	C10H22	871-83-0	968	964
505 , 0,488	3,7-Dimethyl-2-octene	C10H20	13827-59-3	968	-
510 , 0,408	C10 isomer	-	-	971	-
510 , 0,416	C10 isomer	-	-	971	971
520 , 0,416	C10 isomer	-	-	978	-
525 , 0,488	C10 isomer	-	-	981	-
535 , 0,440	C10 isomer	-	-	987	-
535 , 0,544	2,6-Dimethyl-2,6-octadiene	C10H18	2492-22-0	987	-
540 , 0,408	3-Methyl-4-nonene	C10H20	63830-69-3	991	-
540 , 0.448	C10 isomer	-	-	991	-
540 , 0,952	2,7-Dimethyl-3,5-octadiene	C10H18	55682-64-9	991	-
545 , 0,440	4-Decene	C10H20	19398-89-1	994	994
555 , 0,464	Decane	C10H22	124-18-5	1000	999
560 , 0,492	2,6-Dimethyl-2,6-octadiene	C10H18	2609-23-6	1003	1004
565 , 0,456	2-Decene	C10H20	20348-51-0	1007	1013
570 , 0,500	3-Ethyl-2-methyl-1,3-heptadiene	C10H18	61142-35-6	981	-
570 , 0,516	4,6-Decadiene	C10H18	55682-65-0	1010	-
575 , 0,400	C10 isomer	-	-	1013	-
575 , 0,408	C10 isomer	-	-	1013	-
575 , 0,440	C10 isomer	-	-	1013	-
590 , 0,408	C10 isomer	-	-	1023	-
590 , 0,424	2-Methyl-3-ethyl-2-heptene	C10H20	19780-61-1	1023	-
595 , 0,456	C10 isomer	-	-	1026	-
600 , 0,416	C10 isomer	-	-	1029	-
605 , 0,400	C10 isomer	-	-	1033	-
610 , 0,512	C10 isomer	-	-	1036	-
635 , 0,456	C10 isomer	-	-	1052	-

$^1t_R, ^2t_R$	Compound	Formula	CAS	RI$_{calc}$	RI$_{lit}$
655 , 0,480	C10 isomer	-	-	1065	-
670 , 0,432	C10 isomer	-	-	1075	-
670 , 0,440	C10 isomer	-	-	1075	-
675 , 0,544	C10 isomer	-	-	1078	-
695 , 0,448	3-Undecene	C11H22	821-97-6	1091	-
695 , 0,512	1-Undecene	C11H22	821-95-4	1091	1092
705 , 0,440	C11 isomer	-	-	1097	-
710 , 0,432	C11 isomer	-	-	1100	-
710 , 0,480	Undecane	C11H24	1120-21-4	1100	1099
715 , 0,448	5-Methyl-4-undecene	C12H24	41851-94-9	1104	-
735 , 0,496	C12 isomer	-	-	1118	-
780 , 0,488	7-Methyl-3-(1-methylethyl)-1,5-octadiene	C12H22	74630-12-9	1150	-
800 , 0,416	2,9-Dimethyl-decane	C12H26	1002-17-1	1165	-
810 , 0,424	3-Methyl-undecane	C12H26	1002-43-3	1172	1169
835 , 0,448	4-Dodecene	C12H24	7206-15-7	1190	-
840 , 0,448	C12 isomer	-	-	1193	-
840 , 0,456	1-Dodecene	C11H22	112-41-4	1193	1192
840 , 0,480	2,9-Dimethyl-3,7-decadiene	C12H22	74630-13-0	1197	-
845 , 0,416	C12 isomer	-	-	1197	-
850 , 0,488	Dodecane	C12H26	112-40-3	1200	1200
855 , 0,448	C12 isomer	-	-	1205	-
920 , 0,416	C13 isomer	-	-	1264	-
920 , 0,424	C13 isomer	-	-	1264	-
930 , 0,416	C13 isomer	-	-	1273	-
945 , 0,440	5-Tridecene	C13H26	23051-84-5	1287	-
950 , 0,440	6-Tridecene	C13H26	6508-77-6	1291	-
960 , 0,496	Tridecane	C13H28	629-50-5	1301	1299
960 , 0,624	C13 isomer	-	-	1301	-
980 , 0,408	C13 isomer	-	-	1323	-
985 , 0,464	C13 isomer	-	-	1328	-
1030 , 0,416	C14 isomer	-	-	1378	-
1030 , 0,416	2,6,10-Trimethyl-dodecane	C15H32	3891-98-3	1378	1378
1045 , 0,440	1-Tetradecene	C14H28	1120-36-1	1395	1393
1045 , 0,448	C13 isomer	-	-	1395	-
1050 , 0,432	C14 isomer	-	-	1401	-
1055 , 0,608	2,6,10-Trimethyl-1,5,9-undecatriene	C14H24	62951-96-6	1407	-
1095 , 0,496	C15 isomer	-	-	1457	-
1100 , 0,632	C15 isomer	-	-	1463	-
1125 , 0,440	C15 isomer	-	-	1494	-
1125 , 0,448	1-Pentadecene	C15H30	13360-61-7	1494	1490
1130 , 0,432	C15 isomer	-	-	1501	-
1130 , 0,640	Pentadecane	C15H32	629-62-9	1501	1500
1205 , 0,440	C16 isomer	-	-	1601	-

^1t_R, ^2t_R	Compound	Formula	CAS	RI$_{calc}$	RI$_{lit}$
1205 , 0.568	Hexadecane	C16H34	544-76-3	1601	1600
1275 , 0,432	2,6,10,14-tetramethyl-hexadecane	C20H42	638-36-8	1701	-
1275 , 0.640	Heptadecane	C17H36	629-78-7	1701	1700
1330 , 0,440	1-Heptadecene	C17H34	6765-39-5	1792	1700
1330 , 0,448	2-Methyl-7-octadecene	C19H38	35354-39-3	1792	-
1345 , 0,432	2-Methyl-nonadecane	C20H42	1560-86-7	1817	-
1370 , 0,448	C19 isomer	-	-	1859	-
	Aliphatic ketones				
80 , 0,456	2-Propanone	C3H6O	67-64-1	575	545
95 , 0,560	Butanone	C4H6O	78-94-4	594	581
95 , 0,688	2,3-Butanedione	C4H6O2	431-03-8	594	595
100 , 0,544	2-Butanone	C4H8O	78-93-3	601	601
110 , 0,648	1-Acetyloxy- 2-propanone	C5H8O3	592-20-1	614	622
130 , 0.592	3-Methyl-2-butanone	C5H10O	563-80-4	639	663
135 , 0,720	3-Methyl-3-buten-2-one	C5H8O	814-78-8	646	671
145 , 0,672	2-Pentanone	C5H10O	107-87-9	659	-
145 , 0,800	1-Penten-3-one	C5H8O	1629-58-9	659	-
150 , 0,912	2,3-Pentanedione	C5H8O2	600-14-6	666	684
150 , 0.664	3-Pentanone	C5H10O	96-22-0	665	696
165 , 0,472	C5 isomer	C5H10O	115-22-0	684	-
190 , 0.696	C5 isomer	-	-	717	-
190 , 1,128	3-Penten-2-one	C5H8O	3102-33-8	718	735
200 , 0,584	2-Methyl-3-pentanone	C6H12O	565-69-5	730	752
200 , 0,616	3-Methyl-2-pentanone	C6H12O	565-61-7	730	-
220 , 0,832	2-Methyl-1-penten-3-one	C6H10O	25044-01-3	756	-
235 , 0,904	C6 isomer	-	-	775	-
240 , 0,544	C6 isomer	-	-	781	-
240 , 0,672	3-Hexanone	C6H12O	589-38-8	782	786
240 , 0,912	4-Methyl-2,3-pentanedione	C6H10O2	7493-58-5	782	-
245 , 0,824	2-Hexanone	C6H12O	591-78-6	788	787
250 , 0,616	C6 isomer	-	-	794	-
255 , 0,880	4-Methyl-3-penten-2-one	C6H10O	141-79-7	801	801
265 , 2,264	3-Hydroxy-2-pentanone	C5H10O2	3142-66-3	808	803
305 , 0,648	5-Methyl-3-hexanone	C7H14O	623-56-3	835	865
305 , 0,944	Heptane-2,3-dione	C7H12O2	96-04-8	835	-
320 , 0,720	4-Methyl-2-hexanone	C7H14O	105-42-0	845	846
325 , 0,728	C7 isomer	-	-	849	-
330 , 0,616	C7 isomer	-	-	852	-
340 , 0,760	5-Methyl-2-hexanone	C7H14O	110-12-3	859	884
360 , 0,696	4-Heptanone	C7H14O	123-19-3	873	860
380 , 0,688	C7 isomer	-	-	887	-
380 , 0,728	3-Heptanone	C7H14O	106-35-4	887	887
385 , 0,880	2-Heptanone	C7H14O	110-43-0	890	882

^1t_R, ^2t_R	Compound	Formula	CAS	RI$_{calc}$	RI$_{lit}$
390 , 0,968	C8 isomer	-	-	894	-
390 , 2,200	5-Hydroxy-4-octanone	C8H16O2	496-77-5	895	-
400 , 0,664	3,4-Dimethyl-2-hexanone	C8H16O	19550-10-8	900	-
440 , 0,632	3-Methyl-4-heptanone	C8H16O	15726-15-5	926	932
505 , 0,968	2-Methyl-1-hepten-6-one	C8H14O	-	968	966
520 , 0,864	4-Octen-3-one	C8H14O	14129-48-7	978	-
530 , 0,928	2,3-Octanedione	C8H14O2	585-25-1	984	980
535 , 0.824	3-Octanone	C8H16O	106-68-3	988	984
535 , 1,080	Sulcatone	C8H14O	110-93-0	988	985
540 , 0,800	2-Octanone	C8H16O	111-13-7	991	991
565 , 0,760	C9 isomer	-	-	1007	-
605 , 0,704	C9 isomer	-	-	1033	-
630 , 0,792	5-Ethyl-2-heptanone	C9H18O	-	1049	-
665 , 0,696	4-Nonanone	C9H18O	4485-09-0	1071	1030
695 , 0,904	2-Nonanone	C9H18O	821-55-6	1091	1091
750 , 0,640	5-Ethyl-4-methyl-3-heptanone	C10H20O	27607-63-2	1126	-
800 , 0,744	C10 isomer	-	-	1165	-
840 , 0,840	2-Decanone	C10H20O	693-54-9	1194	1188
950 , 0,848	2-Undecanone	C11H22O	112-12-9	1292	1291
1135 , 0,624	2-Dodecanone	C12H24O	6175-49-1	1508	-
1135 , 0,728	C15 isomer	-	-	1508	-
	Monoterpens				
405 , 0,504	Bornylene	C10H16	464-17-5	904	-
410 , 0,488	Santolinatriene	C10H16	2153-66-4	907	908
435 , 0,464	α-Thujene	C10H16	02-05-2867	923	937
445 , 0,520	α-Pinene	C10H16	80-56-8	929	933
450 , 0,464	α-Pinene, isomer	C10H16	7785-26-4	933	939
470 , 0,520	β-Citronellene	C10H18	10281-55-7	946	-
470 , 0,544	Camphene	C10H16	79-92-5	946	953
480 , 0,496	α-Pyronene	C10H16	514-94-3	952	-
480 , 0,568	Verbenene	C10H14	-	952	967
495 , 0,440	C10 isomer	C10H16	-	952	-
510 , 0,544	Sabinene	C10H16	3387-41-5	971	973
510 , 0,576	β-Pinene	C10H16	127-91-3	971	980
540 , 0,568	Myrcene	C10H16	123-35-3	991	991
550 , 0,552	2-Menthene	C10H18	5256-65-5	997	1009
560 , 0,560	α-Phellandrene	C10H16	99-83-2	1004	1011
565 , 0,592	Δ-Carene	C10H16	13466-78-9	1007	1004
595 , 0,576	Limonene	C10H16	138-86-3	1026	1028
595 , 0,592	β-Phellandrene	C10H16	555-10-2	1026	1031
595 , 0,600	α-Pinene, isomer	C10H16	2437-95-8	1026	-
595 , 0,640	l-Limonene	C10H16	5989-54-8	1026	1030
615 , 0,496	β-Ocimene	C10H16	13877-91-3	1039	1050

$^1t_R, ^2t_R$	Compound	Formula	CAS	RI$_{calc}$	RI$_{lit}$
625 , 0,400	β-Ocimene, isomer	C10H16	3779-61-1	1045	1050
630 , 0,616	β-Ocimene, isomer	C10H16	3338-55-4	1049	1040
645 , 0,608	γ-Terpinene	C10H16	99-85-4	1059	1062
690 , 0,616	α-Terpinolene	C10H16	586-62-9	1088	1084
1005 , 0,720	L-Camphene	C10H16	5794-04-07	1351	-
Oxygenated monoterpens					
550 , 0,696	Epoxy-ocimene	C10H16O	-	997	-
560 , 1,088	Yomogialkohol	C10H18O	-	1004	998
600 , 0.656	1,8-Cineole	C10H18O	470-82-6	1030	1031
610 , 0,968	Santolina alcohol	C10H18O	21149-19-9	1036	1038
635 , 0,728	Santolina epoxide	C10H16O	60485-45-2	1052	-
645 , 1,352	Hotrienol	C10H6O	29957-43-5	1059	1101
685 , 0,952	Fenchone	C10H16O	126-21-6	1085	1083
685 , 1,144	Artemisia alcohol	C10H18O	27644-04-8	1085	-
690 , 1,008	Linalool	C10H18O2	5989-33-3	1088	1072
710 , 0,544	Metoxycitronellal	C11H22O2	3613-30-7	1100	-
710 , 1,592	L-Pinocarveol	C10H16O	547-61-5	1101	1141
715 , 1,368	Thujenol	C10H16O	36431-72-8	1105	-
770 , 1,136	Camphor	C10H16O	464-48-2	1144	1145
775 , 1,640	Verbenol	C10H16O	473-67-6	1148	1140
785 , 0,808	ρ-Menthone	C10H18O	89-80-5	1154	1146
795 , 1,072	Pinocarvone	C10H14O	30460-92-5	1162	-
800 , 0,840	Isomenthone	C10H18O	491-07-6	1165	1164
800 , 1,552	Borneol	C10H18O	464-45-9	1165	-
810 , 1,088	Isocamphopinone	C10H16O	15358-88-0	1172	1173
815 , 1,168	Menthol	C10H20O	2216-51-5	1176	-
820 , 1,040	Terpinen-4-ol	C10H18O	562-74-3	1179	1177
870 , 0,936	β-Cyclocitral	C10H16O	432-25-7	1219	-
895 , 0,912	Pulegone	C10H16O	89-82-7	1242	1241
915 , 0,672	Linalyl acetate	C12H20O2	115-95-7	1260	-
935 , 0,624	Menthyl acetate	C12H22O2	16409-45-3	1278	1290
945 , 0,688	Endobornyl acetate	C12H20O2	76-49-3	1287	1285
1105 , 0,728	Geranyl acetone	C13H22O	3796-70-1	1470	1436
Norisoprenoids					
605 , 0,880	2,2,6-Trimethyl-cyclohexanone	C9H16O	2408-37-9	1033	1036
620 , 1,080	Dihydroisophorone	C9H16O	873-94-9	1043	-
740 , 1,072	Isophorone	C9H14O	78-59-1	1126	1121
770 , 2,048	Ketoisophorone	C9H12O2	1125-21-9	1144	1142
Sesquiterpens					
985 , 0,488	Neoclovene	C15H24	4545-68-0	1328	-
1000 , 0,504	γ-Gurjunene	C15H24	22567-17-5	1345	-
1010 , 0,536	α-Gurjunene	C15H24	489-40-7	1356	-
1010 , 0,560	α-Cubebene	C15H24	17699-14-8	1356	1351

| $^1t_R, {}^2t_R$	Compound	Formula	CAS	RI$_{calc}$	RI$_{lit}$
1015 , 0,544	Vatirenene	C15H22	-	1362	-
1030 , 0,572	Copaene	C15H24	3856-25-5	1378	1391
1030 , 0,576	Longicylene	C15H24	1137-12-8	1378	1373
1040 , 0,520	Viridiflorene	C15H24	21747-46-6	1390	-
1040 , 0,592	β-Bourbonene	C15H24	5208-59-3	1390	1384
1045 , 0,552	β-Elemene	C15H24	515-13-9	1395	1375
1060 , 0,616	Longifolene	C15H24	475-20-7	1413	1416
1070 , 0,640	Caryophyllene	C15H24	87-44-5	1426	1428
1075 , 0,624	Aromadendrene	C15H24	109119-91-7	1432	1439
1090 , 0,632	Allo-aromadendrene	C15H24	25246-27-9	1451	1455
1095 , 0,664	β-Farnesene	C15H24	28973-97-9	1457	1443
1100 , 0,664	α-Humulene	C15H24	6753-98-6	1463	1454
1115 , 0,656	γ-Muurolene	C15H24	30021-74-0	1482	1477
1120 , 0,560	α-Amorphene	C15H24	483-75-0	1488	1485
1120 , 0,576	Germacrene D	C15H24	23986-74-5	1488	1480
1125 , 0,736	α-Selinene	C15H24	473-13-2	1495	1494
1130 , 0,568	Valencene	C15H24	4630-07-03	1501	1495
1135 , 0,584	Germacrene B	C15H24	15423-57-1	1507	1561
1135 , 0,672	α-Muurolene	C15H24	31983-22-9	1508	1499
1140 , 0,600	β-Elemene	C15H24	33880-83-0	1514	-
1145 , 0,712	γ-Cadinene	C15H24	39029-41-9	1521	1513
1155 , 0,576	β-Farnesene	C15H24	77129-48-7	1534	1458
1155 , 0,680	ë-Cadinene	C15H24	483-76-1	1534	1530
1155 , 0,816	Calamenene	C15H22	483-77-2	1534	1532
1165 , 0,776	α-Bisabolene	C15H24	29837-07-8	1548	1522
1170 , 0,968	α-Calacorene	C15H20	21391-99-1	1555	1540
1255 , 1,272	Dihydro-neoclovene	C15H26	-	1673	-
1265 , 0,920	Cadalene	C15H18	483-78-3	1687	1668
	Oxygenated sesquiterpens				
1015 , 0,464	Longipinene epoxide	C15H24O	-	1362	
1200 , 0,848	Globulol	C15H26O	489-41-8	1595	-
1225 , 0,776	Viridiflorol	C15H26O	552-02-3	1630	-
1250 , 1,152	β-Eudesmol	C15H26O	473-15-4	1666	1649
	Diterpens				
1475 , 0,640	Sclarene	C20H32	511-02-4	2024	1981
1515 , 1,216	Kaur-16-ene	C20H32	562-28-7	2087	2043
1500 , 0,744	Abietatriene	C20H30	19407-28-4	2063	2051

Retention times of first (^1t_R) and second (^2t_R) dimensions.

[b] RI: retention index obtained through the modulated chromatogram.

[c] RI: retention index reported in the literature for one dimensional GC with 5%-Phenyl-methylpolysiloxane GC column or equivalent.

2.4.2. Heatmap

The heatmap (Figure 8) was performed with the normalised GC peak areas (logarithmic normalisation) to all individuals sampled at the four natural fenced areas. Heatmap shows differences in GC areas according to natural fenced areas: while PBSL is represented by almost all chemical families (low number of blanks in blue), TNM reveals the absence of compounds from several chemical families (high blank areas in blue), namely mono and sequiterpenoids and some hydrocarbons. Terpens are the chemical family with the highest GC area (1.1×10^{10} arbitrary units), appearing in the majority of analysed samples, followed by ketones (7.9×10^{9}), alcohols (4.37×10^{9}) and hydrocarbons (3.74×10^{9}), while esthers and halocarbons are the families with less GC areas. Of all the chemical families with the highest GC area, a sub-set of ketones are the chemical family more consistent across all samples (red line from M2 to M6). PBSL is, in general, the natural fenced area where there are more compounds with the largest areas (1.4×10^{13}), being mono and sesquiterpenoids the chemical family that most contributes to this fact. Another information that the heatmap allows us to obtain is that the male 2 (M2) and male 1 (M1) are the samples with the highest GC peaks areas (3.9×10^{9} and 3.76×10^{9}, respectively) corresponding to the sole samples being collected during rut season (November).
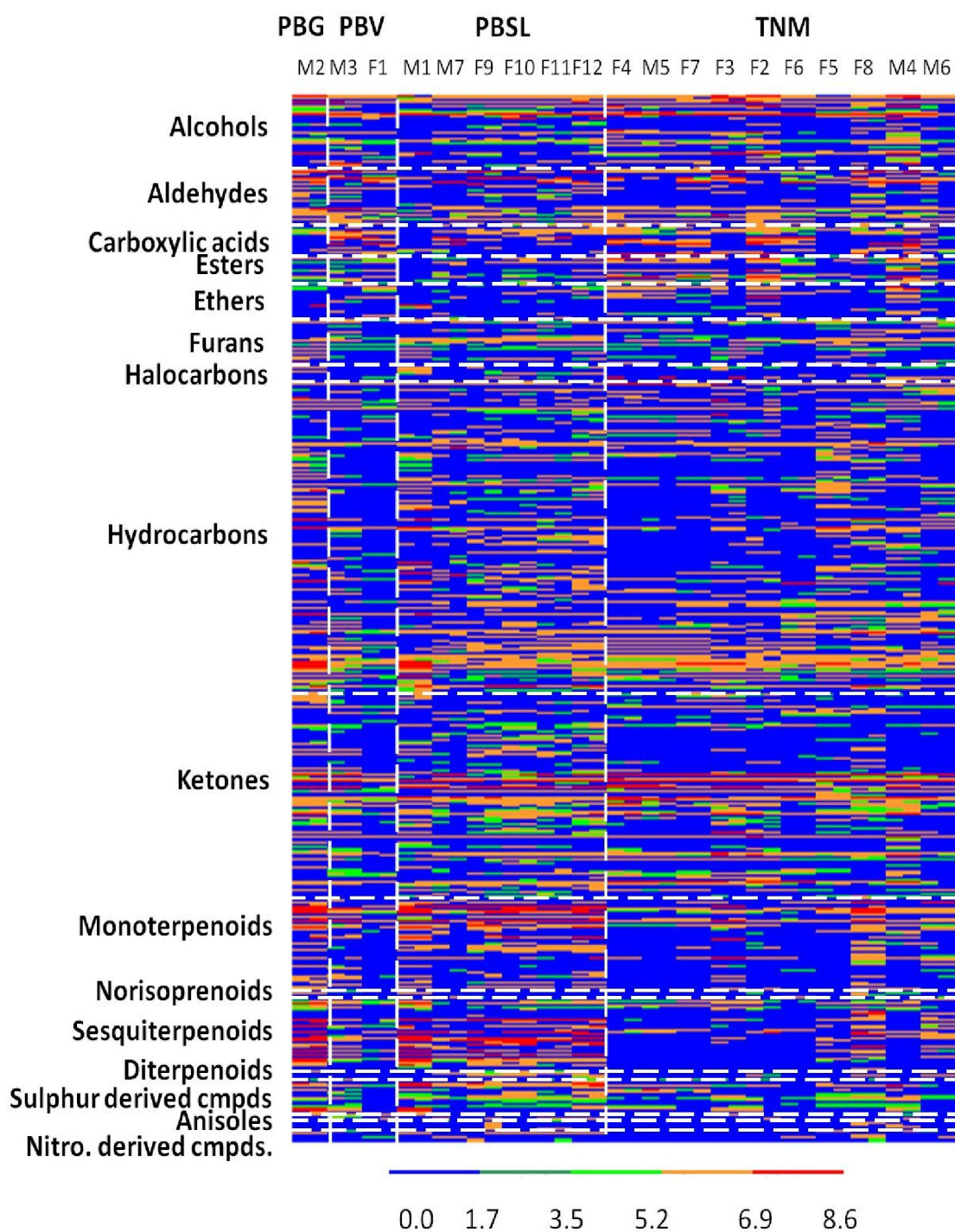
Figure 8 - Heatmap of the logarithmic normalised GC peak areas of the identified compounds on Parque Biológico de Gouveia, Parque Biológico de Vinhais, Parque Biológico da Serra da Lousã and Tapada Nacional de Mafra.

2.4.3. PCA analysis of all natural fenced area populations

In order to study the main sources of variability between samples, PCA was performed to GC×GC peak areas of 571 compounds. Figure 9-A shows the scores scatter plot results (explaining a total of 45% of variability), in which is seen a clear separation of the individuals from TMN and PBSL. PBSL samples are mainly distributed across PC1 and PC2 negative. PBSL differentiation from the remaining natural fenced areas was based on terpen compounds (Figure 8-B) because its samples are mostly characterised by the monoterpens α-pinene (444) and β-pinene (452) and the sesquiterpen aromadendrene (513).

TNM samples are located on PC1 and PC2 positive and its differentiation can be explained by the alcohol 3-methyl-1-butanol (11), ketones 2-propanone (372), 2-pentanone (379) and acetophenone (430) and by ester ethyl acetate (87). Regarding PBV and PEG samples, the distinctions were not so clear, probably due to the low number of individuals from each area. PBV samples are dispersed diagonally across PC2 positive and PEG in PC1 negative axis. The aldehyde 3-methylbutanal (46) is related to PEG, PBV or TNM and the ketone 2-butanone (375) to TNM, PBV or PBSL. Regarding PEG and PBV analysis, no more relevant information can be extracted, regarding the number of individuals sampled. For a better visual understanding, different coloured bands were delineated for each population of origin in the following PCAs score plots.

An association can be made between the space/distance of samples in scatter plot and the genetic background: PBSL, PEG samples very close positioning agreed with the most recent link between samples (dating back to the nineties) and PBSL and PBV close

positioning may also be explained because they share H. Contenda and/or T. N. V. Viçosa locals as the origin of the animals.

Other information that can be perceived by the visual analysis of the scores plot is the existence of two TNM individuals that deviate from the main TNM individuals group, corresponding to M6 and F8 individuals living in the upper TNM fence, in PC1 positive and PC2 negative. Although those results were not explored in this study, PCA shows a separation between individuals from upper and lower fenced areas, which it occurs in TNM, where morphological differences led to the segregation of the animals in two fences.
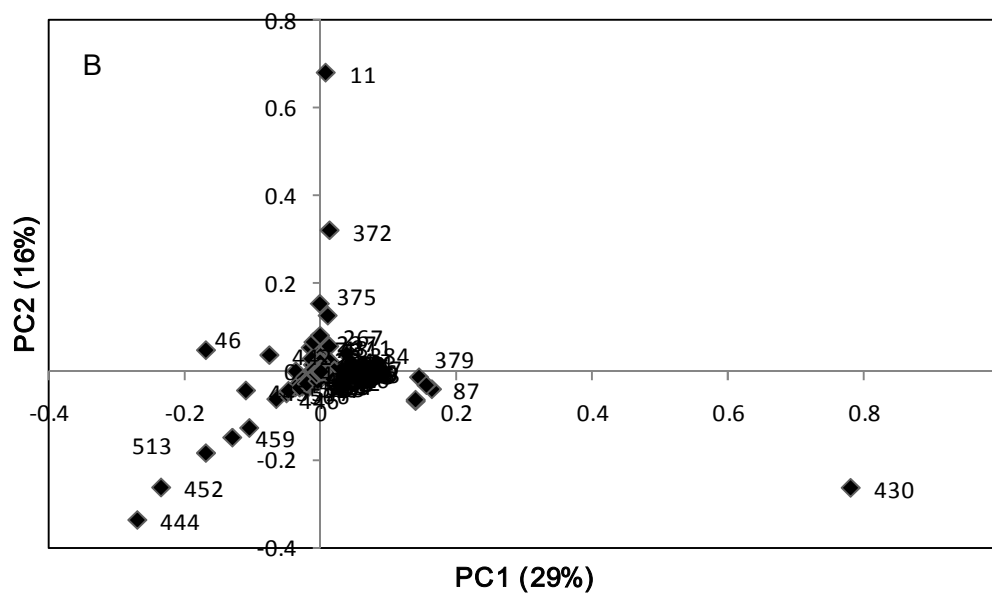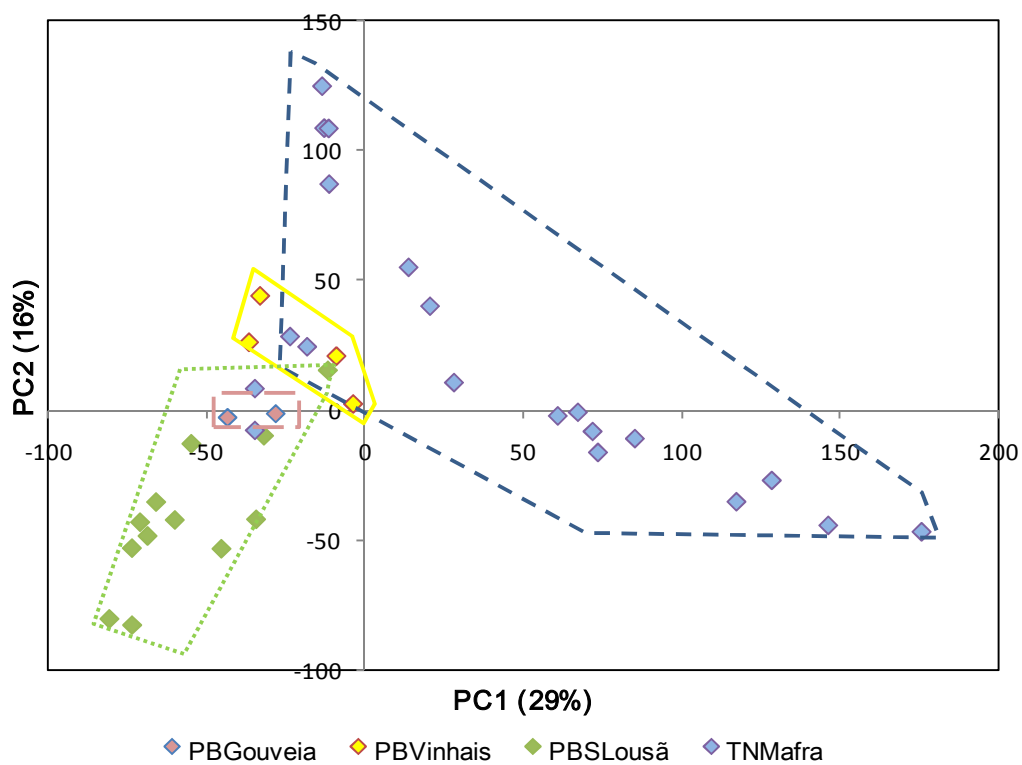
Figure 9-PC1×PC2 scores (A) and loadings (B) scatter plots of the selected 571 scats VOCs from PBSL, TNM and PBV/PEG. The highlighted peak numbers correspond to the compounds that may explain the samples distinction (see peak number attribution in Table 2)

Loadings plot of the PCA (Figure 9-B) allows perceiving which compounds are characterising TNM, PBSL and PEG or PBV populations that correspond to a sub-set of 10 compounds. Table 3 summarises the compounds that seem to characterise each one of the populations of origin (compound numbers below correspond to those presented in Figure 8). For each identified compound the respective retention time for the first (^1t_R) and second dimension (^2t_R), the peak number, the RI, the respective place of samples origin and the respective reference are described below.

Table 3 - Sub-set of 10 volatile compounds that explains the PCA distinction (Figure 9-A).

$^1tR, {}^2tR$ [a]	Peak no.	Local	Compound	RI$_{calc.}$ [b]	RI$_{lit.}$ [c]
445 , 0,520	444	PBSL	α -Pinene	929	933
510 , 0,576	452	PBSL	β -Pinene	971	964
1075 , 0,624	513	PBSL	Aromadendrene	1432	1439
80 , 0,456	372	TNM	2-Propanone	574	545
105 , 0,528	87	TNM	Ethyl acetate	607	608
145 , 0,672	379	TNM	2-Pentanone	659	685
185 , 1,712	11	TNM	3-Methyl-1-butanol	712	734
650 , 2,632	430	TNM	Acetophenone	1053	1065
125 , 0,576	46	PEG/PBV/TNM	3-Methylbutanal	633	646
100 , 0,544	375	TNM/PBV/PBSL	2-Butanone	659	601

Retention times of first (^1tR) and second (^2tR) dimensions.

[b] RI: retention index obtained through the modulated chromatogram.

[c] RI: retention index reported in the literature for one dimensional GC with 5%-Phenyl-methylpolysiloxane GC column or equivalent (Adams, 1995; Choi, 2003; El-Sayed et al., 2005; Engel et al., 2002; Machiels et al., 2003; Venkateshwarlu et al., 2004; Zehentbauer and Reineccius, 2002)

2.4.4. PLS-DA comparison of male and female groups

In order to explore the influence of other factors in the distinction of samples, such as gender, PLS-DA was applied to the GC×GC chromatographic areas to establish the relationship between the compounds and the samples under study. Figure 10-A shows the scores scatter plot regarding the distinction of female and male deer. Is observed a good separation between sample classes (males and females) along LV1 (Latent Variable): males are dispersed in LV1 positive values whereas females are linked to LV1 negative, which explains 7,3% of the variance. M5 sample displayed in LV2 axis is relative to a 1 year old fawn. Considering that in natural fenced areas adult males need to establish their social dominance towards fawns, a behaviour that is mostly performed by adults than by young deer (Barroso and Rosa, 1999), it is possible that this behaviour is separating this fawn from adult males through metabolic or physiological differences which are reflected is scats volatiles.

Considering the VIP values (variable importance in the projection) (Figure 10-B), they establish the contribution of each compound to the distinction among groups. One aldehyde (pentadecanal), one monoterpen (γ-terpinene) and one ketone (4-methyl-2,3-pentanedione) were attributed to females, whereas males are linked to two anisoles (1-methoxy-4-methyl-benzene, anisole), four aliphatic hydrocarbons (2,3,5-trimethyl-heptane, 3-methyl-undecane, decane and 2-octene), one aromatic hydrocarbon (4-ethyl-1,2-dimethyl-benzene), one ether (hexyl-oxirane) one undetermined compound (mz(41,55,67)), one norisoprenoid (isophorone) and two aliphatic ketones (5-ethyl-4-methyl-3-heptanone and 3,4-dimethyl-2-hexanone).
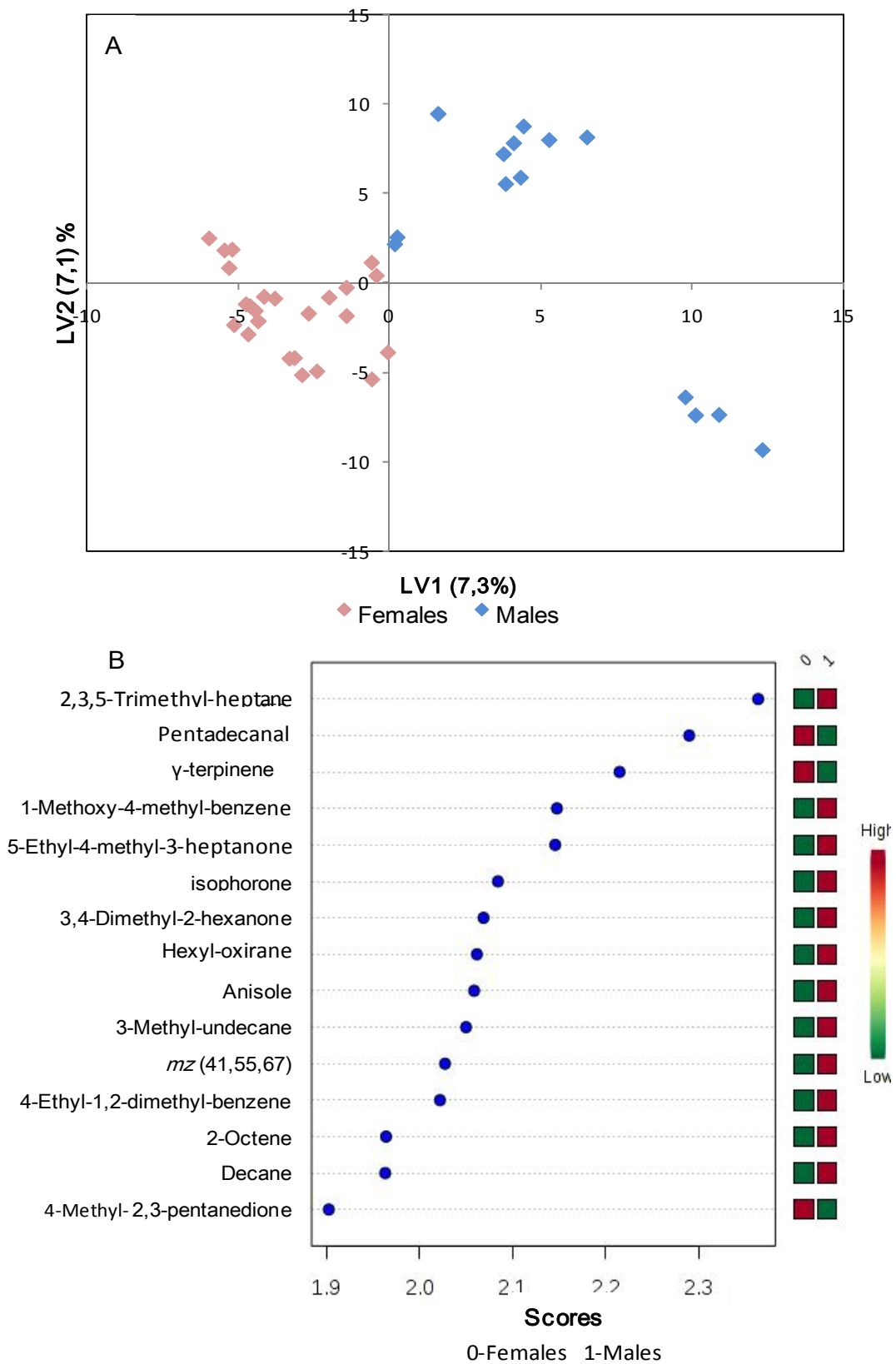
Figure 10 - A) PLS-DA scores scatter plot (LV1 vs. LV2), R^{2}X 0.72, R^{2}Y 0.87, Q^2=0.37. B) VIP plot explaining the PLS-DA scores plot.

This classification analysis provided a model with explained variance of R^2X 0.72 and R^2Y 0.87, and predictive capability of Q^2 0.37. To better assess the predictive ability of the males and females PLS-DA model a MCCV was applied, revealing a classification rate of 78.9%, 87.5% of sensitivity and 64.3% of specificity. Attending to the amount of uncontrollable factors: the number of females and males not being the same; various age-classes; different physiological states; season and diet, this can be considered a good model.

In an attempt to explain the main factor (diet or chemical communication) underlying sample class (males and females) distinction, several exploratory classification analysis (PLS-DA) were performed using target families combinations (chemical family associated with diet or chemical communication). Scores, loadings and VIP values plots are shown for both exploratory analysis. The first PLS-DA analysis was performed to a matrix comprising chemical families (alcohols, aldehydes, furans and terpenoids) which have been related in literature to diet (forage) (Cramer et al., 2005; Matsui, 2006; Saraiva et al., 2013) and to chemical communication (aliphatic ketones and aliphatic hydrocarbons) (Martín et al., 2010; Rasmussen and Krishnamurthy, 2000; Saraiva et al., 2013). Scores scatter plot (Figure 11-A) show a clear distinction between males and females along LV1 (6.2% variance) and (Figure 11-B) shows terpens chemical family widely dispersed across all loadings plot.
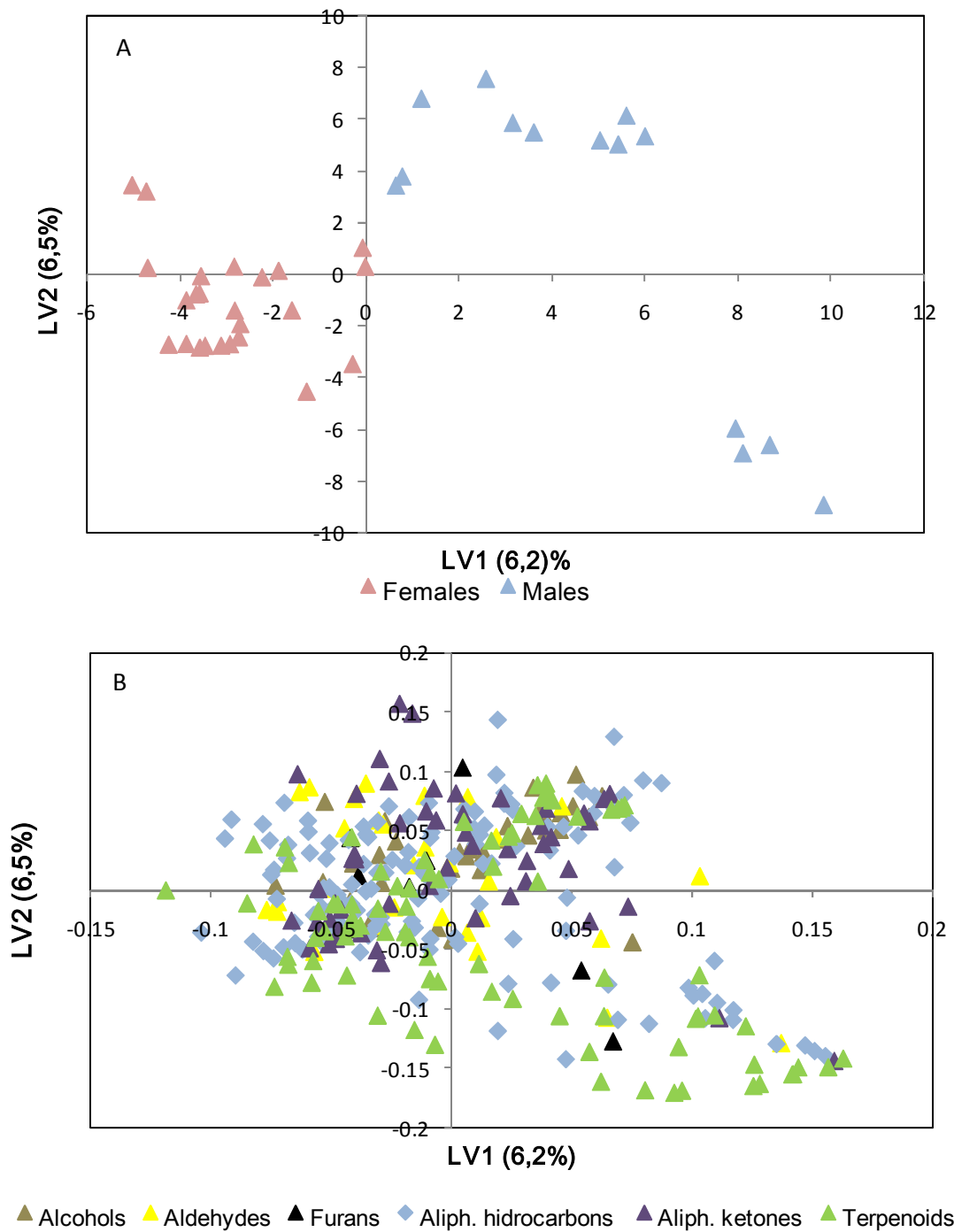
Figure 11- A) PLS-DA (LV1 vs. LV2) scores scatter plot regarding the distinction of female and male deer performed to alcohols, aldehydes, aliphatic hydrocarbons and ketones, furans and terpens chemical families, B) Loadings scores. R2X=0.75, R2Y=0.81, Q2=0.4.
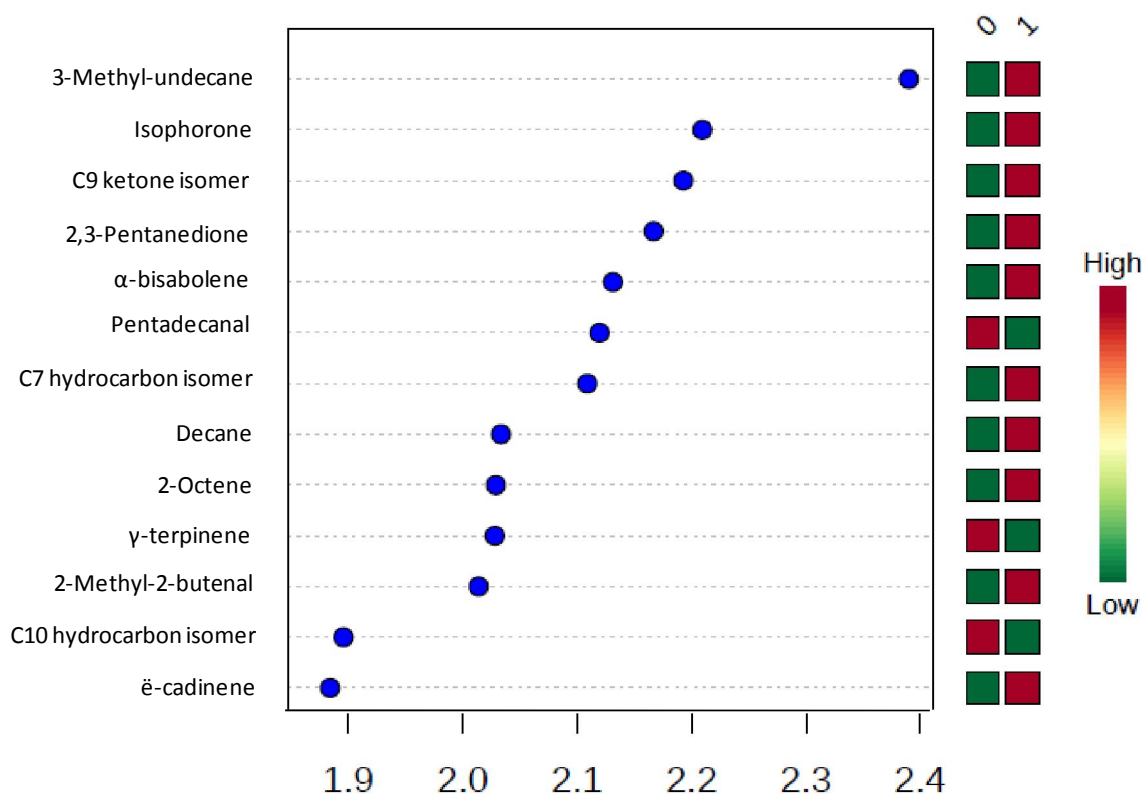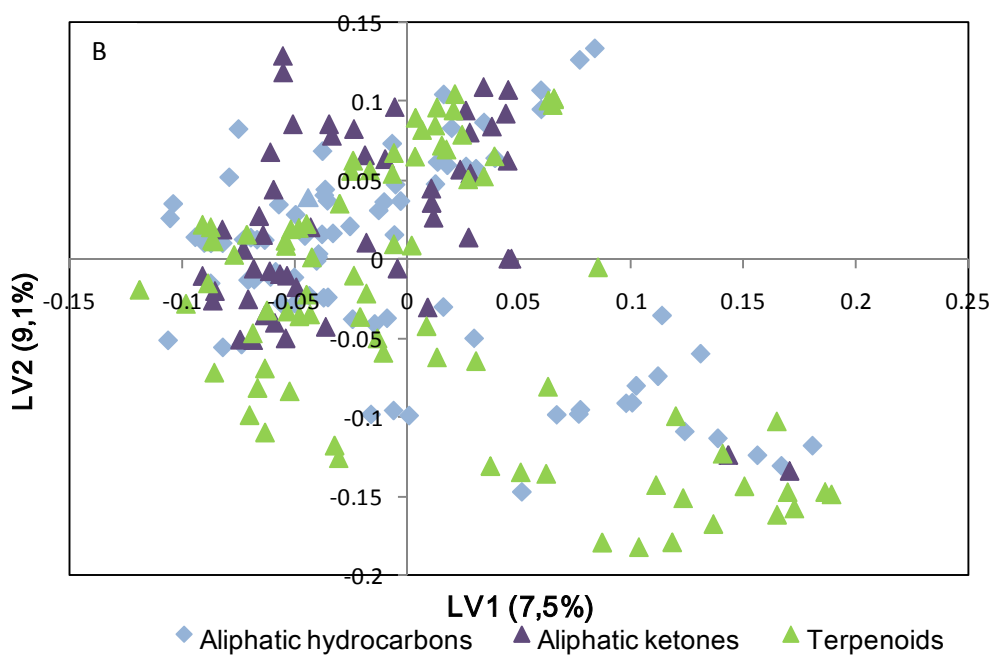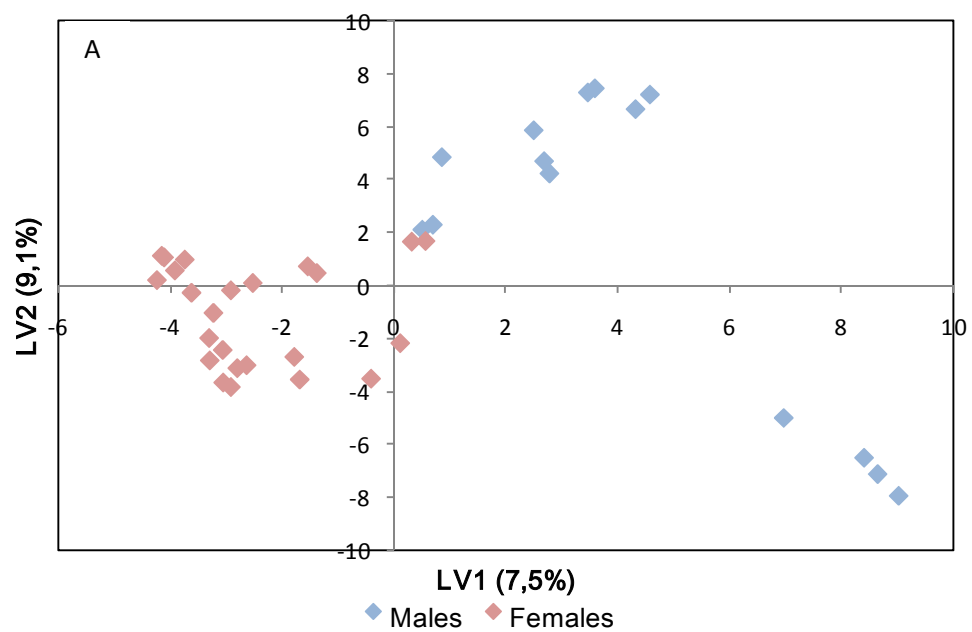
Figure 12- VIP scores of the PLS-DA (LV1 vs. LV2) analysis regarding the distinction of female and male deer performed to alcohols, aldehydes, aliphatic hydrocarbons and ketones, furans and terpens chemical families.

VIP values (Figure 12) descendant chemical families contributions: aliphatic hydrocarbons>aliphatic ketones and aldehydes>terpens, suggest that aliphatic ketones and hydrocarbon were the major contributor to class distinction. This model showed $R^2X=0.75$, $R^2Y=0.81$ of explained variance and $Q^2=0.4$ of predictive capability.

A second exploratory analysis was performed reducing the amount of chemical families to three in the matrix, excluding alcohols, aldehydes and furans, due to their ambiguous link to diet and/or chemical communication factors. Terpens and aliphatic ketones and hydrocarbons were maintained because they were the main chemical families in VIP scores (Figure 10) achieved for female and male distinction and also

because they were reported as diet or chemical communication volatiles in deer study (Saraiva et al., 2013). Scores scatter plot (Figure 13-A) and loadings (Figure 13-B) show again a group sample distinction along LV1 (7.5 variance) and terpens displayed across LV1 and LV2 plot, showing affinity to both male and female samples.

Figure 13- A) PLS-DA (LV1 vs. LV2) scores scatter plot regarding the distinction of female and male deer performed to aliphatic hydrocarbons and ketones and terpens chemical families. B) Loadings scores. R2X=0.80, R2Y=0.84, Q2=56.

VIP scores values (Figure 14) allow us to perceive that hydrocarbons are the major contributors to group samples distinction.
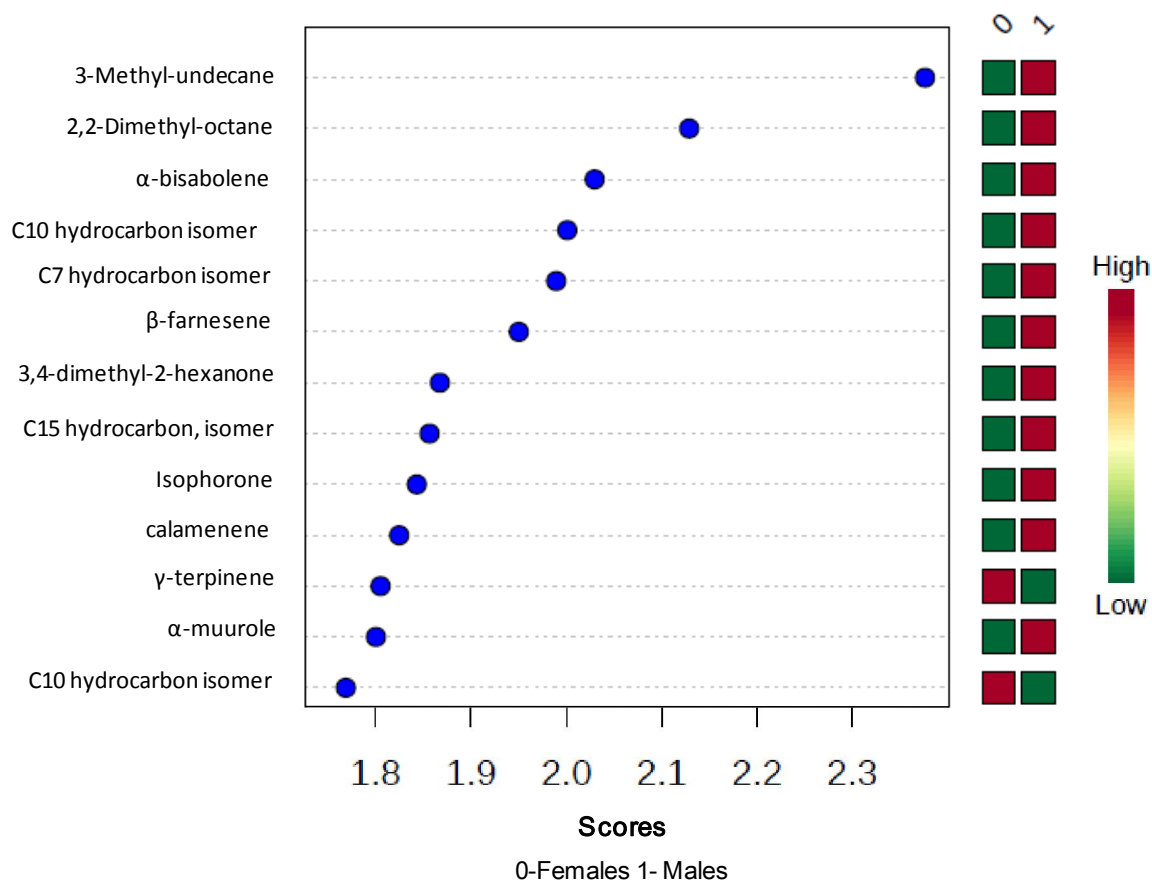
Figure 14-VIP scores of the PLS-DA (LV1 vs. LV2) analysis regarding the distinction of female and male deer performed to aliphatic hydrocarbons and ketones and terpens chemical families.

The PLS-DA results for male and female distinction shows that pentadecanal and γ - terpinene occur repeatedly as female compounds while 3-methyl-undecane, decane, 2-octene and 3,4-dimethyl-2-hexanone appear as male compounds. The mentioned compounds need to be further studied in order to confirm their applicability as female and male markers. This second exploratory model also provided a well explained variance ($R^2X=0.80$, $R^2Y=0.84$, $Q^2=56$).

2.5.   Discussion

2.5.1. Contour plot analysis

GC×GC-ToFMS originates a chromatogram contour plot which allows to perceive the global volatile profile of a red deer scat. This representation was only possible due to the orthogonal system with a non-polar/polar thick-film column combination that provides a separation according to analytes volatility on the 1D, and on the 2D, separating them according to their polarity. This separation originates a pattern visible in the GC×GC chromatogram contour plot, also called chromatogram of total ions. In it, compounds with similar mass spectrum appear as one spot and with a colour-code according to its GC area. If the display showing total ion current (TIC) is selected, a spot pattern is formed, allowing comparisons between different chromatograms, and to search between certain differences or resemblances (Kalinová et al., 2006). Although being a paramount advantage on the identification process and relationship assessment through spatial positioning on the 2D chromatograms allowing an easy and rapid snapshot of the species, the chromatogram contour plot did not allow a visual distinction between samples.

2.5.2. Heatmap

Heatmap (Figure 8) allowed to obtain the relative proportions of each chemical family from the tentatively identified compounds on the different samples. While terpens areas are larger in PBSL samples, ketones and hydrocarbons areas are more intense in TNM. Distinct chemical families areas proportions according to natural fenced areas might be explained by different constraints affecting differently each population, namely stress

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caused by predators, different food needs, climate, the stress caused for being closed in a natural fenced area and the need to define new social status or physiological status may be some factors affecting and differentiating populations.

2.5.3. PCA analysis of all natural fenced area populations

Different chemical families contributed to the distinction of red deer populations. Terpens contributed most to PBSL samples while aliphatic ketones, one ether and one aliphatic alcohol are more related to TNM samples. Terpens derive from the secondary metabolites of plants and their biosynthesis is carried out by plants and some microorganisms (Belitz et al., 2004), so it is very likely that these results may be explained by the diet of red deer. Additionally, mono and sesquiterpens, have been described in steams and leaves of wheat straw, a major component in the daily diet of PBSL (Butter et al., 1985). Given the herbivorous diet of red deer, green leaf volatiles (GLV) are the most probable origin of some aldehydes, alcohols and their esters (Matsui, 2006). When green leaves are crushed or injured (which happens when red deer eat them) GLV are formed through oxidation reactions of linoleic and linolenic acids. Lipoxygenase (LOX) is one of several enzymes that catalyse this reaction, whose product is then metabolised by a hydroperoxide lyase, an enzyme family found in alfalfa (Matsui, 2006). During the leaf metabolisation process, which occurs in red deer rumen, linoleic acid forms n-hexanal and then C6 aldehydes which are metabolised into its corresponding C6-alcohol. Those results are also in accordance with the main chemical families described in literature regarding their daily diet. Besides hydroperoxide lyase, an enzyme found in alfalfa, animals at TNM were also fed with a blend of dry feed composed by broad beans, barley,

corn and carob residues. Volatiles described in literature for these forage/grains belong mainly to aldehydes, furans, terpens and alcohols chemical families. The main chemical families of barley are aldehydes, alcohols and furan contents (Cramer et al., 2005). Aldehydes such as 3-methylbutanal are usually associated to corn (Maga, 1978) while the major described compounds for wheat straw are (Z)-3-hexenyl-acetate, (Z)-2-hexanol, (E)-2-hexenal and caryophilene (Butter et al., 1985). Ketones 2-propanone (372), 2-butanone (375), 2-pentanone (379) and acetophenone (430) were also described in literature, this time regarding chemical communication through chemical signals in animals. Ketones result from the oxidation of fatty acids thus, are metabolic byproducts of plants and animals. They occur in nature, but also in mammalian bodies in low quantities, as part of ketone bodies in the blood stream (Enig, 2000). Despite the lack of literature that relate these ketones with the chemical communication between deer, such compounds have been described in other species. 2-Butanone and 2-propanone were reported in samples of temporal gland and urinary exudates of elephants at early mid musth and mid musth, respectively (Rasmussen and Krishnamurthy, 2000) and in the volatile scats of *Dama dama* individuals (Saraiva et al., 2013). 2-Pentanone was detected in vaginal mucus and urine of female white-tailed deer during estrus (Jemiolo et al., 1995) and as scent-marker of non-breeding white tailed deer male (Miller et al., 1998). Acetophenone was identified in scats of adult Iberian wolves with a probable scent marking function (Martín et al., 2010). Thus, it is expectable that these compounds tentatively identified on TNM samples, are related to chemical communication in this population. Additionally, the aldehyde 3-methylbutanal corresponding to PBV or PEG or TNM samples was also detected in the nonbreeding season of former dominant white-

tailed deer (Miller et al., 1998). Taking into consideration that samples of PBV were collected in February (nonbreading season) and that 3-methylbutanal was detected in white-tailed deer males in nonbreading season, it is reasonable to attribute this compound to PBV instead of PEG samples if we assume it as a chemical signal and not a dietary component. Little is known concerning the chemical communication of ungulates, but at least three mechanisms have been suggested to transmit information through olfactory signals, where each individual has an identity signal that attributes for age or sex; secondly the signal itself codes for a particular attribute (sex or age) or state (reproductive or dominance) and thirdly, no attributes information within the signal, but the message is simply limited to resident vs. non-resident, familiar vs. non-familiar (Gosling, 1990; Lawson et al., 2000). The absence of literature data that relates the tentatively identified ketones with the chemical communication among red deer leads to uncertainties in the attribution of this explanation to the occurrence of these ketones in TNM samples. However, a methylated ketone (2-tridecanone) has been reported as a pheromone thus, related to chemical communication, for two different species: in an ant setting off an alarm and in one termite as a defence mechanism. This may support the fact that despite ketones 2-butanone, 2-propanone, 2-butanone and acetophenone have been reported in other species as pheromones with a particular function (Wyatt, 2003), it does not mean that they cannot be associated with a specific deer population with a different function of the one described previously in other species. Those results suggest that terpens influenced most PBSL samples, probably related with diet, while ketones compounds are possibly arising from chemical signals in chemical communication between deer.

2.5.4. PLS-DA comparison between male and female groups

The achieved VIP scores were reported in other studies as pheromones used in chemical communication in insects, in scent-marking in wolves and in white-tailed deer. Anisole has been described as a pheromone precursor in insects (Ward et al., 2002), pentadecanal in insect pheromones (McDaniel et al., 1992) and in scats of wild adults Iberian wolves (Martín et al., 2010). Isophorene is an insect pheromone (Schulz et al., 1993) while the aliphatic hydrocarbon decane was found in the interdigital gland of dominant white-tailed male deer (Gassett et al., 1996) and in the urine volatiles of male mices (Achiraman and Archunan, 2005). 3-Methyl-undecane, an aliphatic hydrocarbon, was also detected and extracted from urine of male mice (Achiraman and Archunan, 2005). Furthermore, aliphatic hydrocarbons and ketones were detected in volatiles extracted from the scats of the ungulates *Cervus elaphus* and *Dama dama*, also with a putative chemical communication function (Saraiva et al., 2013). From what was possible to obtain in literature data, no studies have discussed gender distinction by the analysis of volatiles thus, one can do no more that hypothesise about the compounds also being considered male markers with a chemical signalling function. Regarding these results, several species seem to use the reported VIPs in their chemical communication system (anisole, pentadecanal, isophorone, decane, 3-methyl-undecane and probably 3,4-dimethyl-2-hexanone) what may suggest that the scats profile of males and females is not directly influenced by diet-derived compounds (mainly terpens), but by chemical-communication related compounds. This conclusion was expected, because gender is not

determined by what individuals eat, but by intrinsic factors, such as genetics, hormonal and physiological differences (Rinn and Snyder, 2005).

The exploratory analysis using two PLS-DA matrices also allows us to conclude that diet related compounds/chemical families were not the major factor contributing to the distinction of sex-samples. Although, the presence of some terpens and norisoprenoid compounds (terpenes γ -terpinene, α -bisabolene, β -farnesene and the norisoprenoid isophorone) attributed in VIP scores may be indicative of differences/preferences of female and male for certain diets. According to some authors (Beier, 1987; Staines et al., 1982), females have a tendency to consume more nutritious forage. Therefore, although the compounds derived from the diet (terpens) are not the most decisive regarding the distinction of sex, the presence of these compounds may be explained by differences between male and female preferences for certain types of diet.

An integrative explanation is needed to understand how genetic background and physiology combined can influence populations and gender distinction. In this study, animals arising from different locations were introduced and bred in different reserves, making them more susceptible to stress induced by new climate, environment and by the need to define new social status.

Crews et al. (2012) found that a single exposure to environmental factors (i.g. environment toxicant), three generations prior, alters the physiology, behaviour, metabolic activity, and transcriptome in discrete brain development in descendant males, causing them to respond differently to chronic stress. This alteration of baseline brain promotes a change in neural genomic activity that correlates with changes in physiology and

behaviour, revealing the interaction of genetics, environment, and epigenetic transgenerational inheritance in the shaping of the descendant adult phenotype. In fact, each population is characterised by a particular constraint and can be more affected by the stress caused for being closed in a natural fenced area, by near predators, the need to define new social status, differences in climate and food scarcity might be the environmental factors or the chronic stress that may be playing part in ancestral environment exposure or chronic stress that lead to changes in physiology and behaviour of the descendants causes changes in physiology. Exploring the differences in climate, natural fenced areas in the north of the country (PBV), centre (PEG and PBSL) and coastland-centre (TNM) are affected differently by climate. In Portugal, atmospheric features and asymmetry in relief are responsible for decreasing rainfall from north to south. Inversely, temperature increase from north to south and high amplitudes are sensed in the hinterland (Ferreira, 2000). Such characterisation is in accordance with the average-3 year temperature recorded in weather stations nearby our natural fenced areas. Lisbon (near TNM), registered the highest average temperatures, with 17.8ºC; Bragança in the north had the lowest temperatures with 12.8ºC and highest fluctuations (negative winter temperatures). Finally, the Castelo Branco weather station, which was the nearest to PBG and PBSL parks (64km) recorded 16,1ºC of average temperature (PORDATA, 2009). Additionally, Brown et al. (2004) showed with their Metabolic Theory of Ecology how the metabolic rate varies with temperature: organisms that operate at warm temperatures by i.e., living in warm environments tend towards higher metabolic rates than organisms that operate at colder temperatures. High metabolic rates produce more free radicals as a by-product of energy production. In turn, the increment of

metabolic rate produces different by-products at different concentrations that may be excreted in urine or scats which can be traceable in released volatiles (Howe and Schilmiller, 2002; Obata et al., 2000).

3. General conclusions and perspectives to go further

This study reinforces the successful application of HS-SPME/GC×GC– ToFMS in the study of complex matrices such as scats and in wild life management studies that aim to assess the distributional limits and sex-ratio of populations. Saraiva et al. (2013) had previously used this technique to identify and distinguish between Eurasian otter, red deer and fallow deer. At this time, this technique was applied for the first time to distinguish among *Cervus elaphus* populations, and between male and female individuals, equally obtaining good results.

Being able to distinguish between different populations may give us the opportunity to explore different constraints. Stress caused by predators, food scarcity, climate, the stress caused for being closed in a natural fenced area and the need to define new social status, may be some factors affecting and differentiating populations, which in the future may be related to a specific pattern of potential markers. In fact, TNM and PBSL samples were distinguished based on different classes of volatile compounds, even having a similar genetic background, which might be an indicative of some physiologic state derived from that particular constraint. Natural fenced areas distinction was based on a sub-set of ten tentatively identified compounds, mainly aliphatic ketones and terpens, obtained by Principal Component Analysis (PCA). Chemical signalling in chemical

communication and diet derived compounds may be related with occurrence of aliphatic ketones and terpenes, respectively. Additionally, the interaction of genetics and environment leading to changes in animal physiology which may be detected trough metabolic variations might help to explain distinction between populations.

Gender distinction was achieved through a classification analysis (PLS-DA) and validation (MCCV) tool. Aldehydes, anisoles, aliphatic hydrocarbons and ketones, were the main compounds achieved to distinguish between samples. Literature data (Achiraman and Archunan, 2005; Gassett et al., 1996; McDaniel et al., 1992; Ward et al., 2002) relates some of these compounds to insect pheromone precursors and chemical signals found in glands or in scats of wolves, white tailed deer or red deer. Further studies should be done in order to confirm the link between these compounds (aliphatic ketones and hydrocarbons and anisoles) and chemical communication. The most common way to assess such information is by performing bioassays, exposing the animals to that particular chemical (isolated) and then assessing if the animals respond or not, through a certain behaviour (a set of expected behaviours is required, based on the species pre-knowledge ecology/behaviour). For example, frontalin is a chemical that was found to be increased during a specific musth state of Asian Elephants, right before mating. To link this chemical to intra-specific communication, bioassays were done exposing young and adult males and females in different oestrus states to frontalin to observe their responses: avoidance (circling motions around samples with no contact), repulsion (backing up or moving away from the sample after chemosensory responses such as main olfactory), vocalisations or mating-related behaviours. Females in the follicular phase (ready to mate) were the most responsive and often demonstrated mating-related behaviors (spent

extended periods at frontalin samples, and performed penile and temporal gland checks on male elephants), suggesting that frontalin acts as a pheromone, which was statistically proven (Rasmussen and Greenwood, 2003). Thus, a vast knowledge of red deer social behaviour responses, characteristics and physiological parameters, chemical origin and metabolic pathways are needed in order to interpret, complement and confirm the results achieved in this study.

HS-SPME/GC×GC– ToFMS combined methodology can possibly be applied to big scale populations, allowing the assessment of populations and ecological parameters that would otherwise be expensive and time consuming, once is an accurate, fast and non-invasive technique.

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