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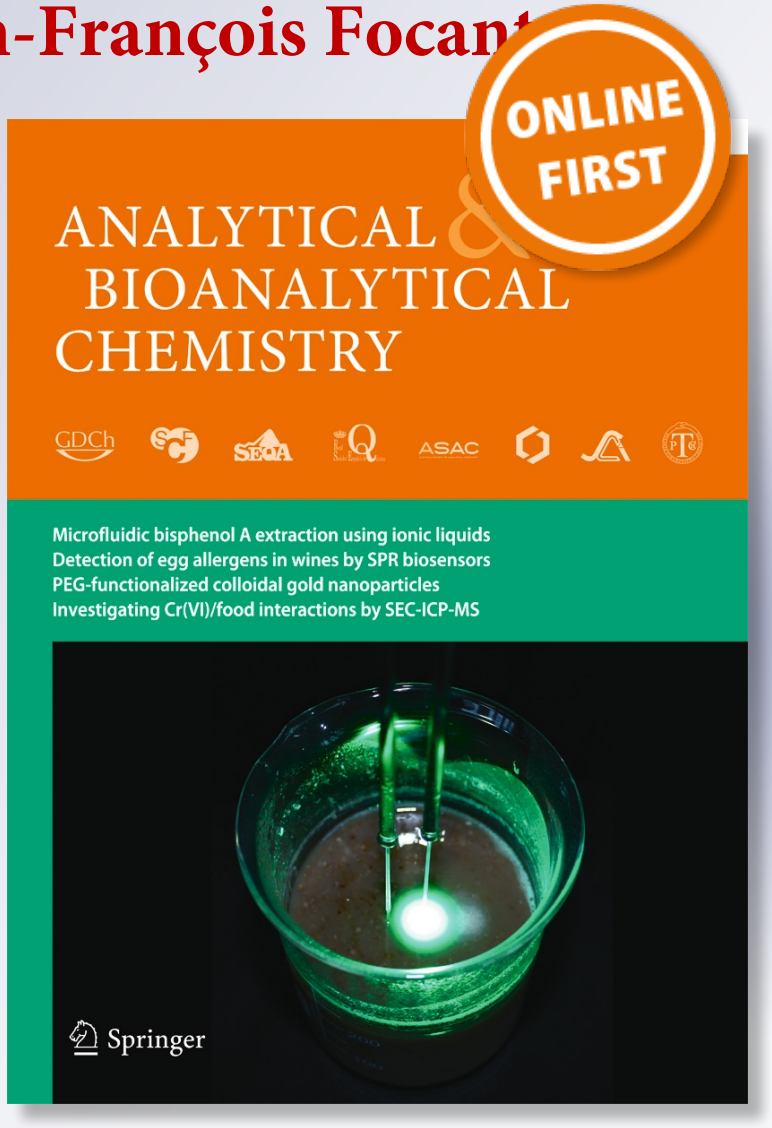

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RESEARCH PAPER

GC×GC–TOFMS and supervised multivariate approaches to study human cadaveric decomposition olfactive signatures

Pierre-Hugues Stefanuto[1] · Katelynn A. Perrault[2] · Sonja Stadler[3] · Romain Pesesse[1] · Helene N. LeBlanc[3] · Shari L. Forbes[2] · Jean-François Focant[1]

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Abstract In forensic thanato-chemistry, the understanding of the process of soft tissue decomposition is still limited. A better understanding of the decomposition process and the characterization of the associated volatile organic compounds (VOC) can help to improve the training of victim recovery (VR) canines, which are used to search for trapped victims in natural disasters or to locate corpses during criminal investigations. The complexity of matrices and the dynamic nature of this process require the use of comprehensive analytical methods for investigation. Moreover, the variability of the environment and between individuals creates additional difficulties in terms of normalization. The resolution of the complex mixture of VOCs emitted by a decaying corpse can be improved using comprehensive two-dimensional gas chromatography (GC×GC), compared to classical single-dimensional gas chromatography (1DGC). This study combines the analytical advantages of GC×GC coupled to time-of-flight mass spectrometry (TOFMS) with the data handling robustness of supervised multivariate statistics to investigate the VOC profile of human remains during early stages of decomposition. Various supervised multivariate approaches are compared to interpret the large data set. Moreover, early decomposition stages of pig carcasses (typically used as human surrogates in field studies) are also monitored to obtain a direct comparison of the two VOC profiles and estimate the robustness of this human decomposition analog model. In this research, we demonstrate that pig and human decomposition processes can be described by the same trends for the major compounds produced during the early stages of soft tissue decomposition.

Keywords Human decomposition · Victim recovery canines · Forensic thanato-chemistry · GC×GC–TOFMS · Volatile organic compounds · Multivariate statistics

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Introduction

In the past decade, numerous studies have investigated the volatile organic compounds (VOCs) released from deceased individuals. However, comprehensive profiling of "the scent of death" still faces several challenges. Limitations surrounding the use of human remains for decomposition studies are numerous, including both ethical and legal restrictions that differ between regions. When these restrictions are accounted for, obtaining multiple cadavers simultaneously is logistically challenging due to the nature of established body donation procedures. A lack of replicates used in previous studies [1–5] does not allow for a powerful comparison between the odor produced by human cadavers and other potential surrogates. In order to deal with these challenges, many of the previous studies on decomposition VOCs have been

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conducted using pig carcasses (*Sus scrofa domesticus*) as human analogs [1, 6–8]. Existing studies utilize either human or pig remains to investigate the decomposition VOC profile. Although the majority of the decomposition VOCs are thought to be similar between species [5, 9], a comparison of decomposition VOCs produced by human remains and pig carcasses has never been performed in the same environment and under the same conditions. A direct comparison of this nature is imperative in order to validate the use of pig carcasses as suitable human decomposition odor analogs and identify decomposition VOCs that may be specific to each species. Differences in the decomposition VOC profiles on a species level will improve understanding regarding the ability of victim recovery (VR) canines to differentiate between animal and human remains.

Isolating, separating, and identifying decomposition VOCs still remain an analytical challenge. The complexity of the VOC mixture, the large dynamic range, and the dynamic nature of the decomposition process seriously challenge classical single-dimensional gas chromatography (1DGC), even when coupled to mass spectrometry (MS), for proper separation and identification of decomposition VOCs. The recent use of comprehensive two-dimensional gas chromatography (GC×GC) has considerably enhanced the chromatographic resolution of decomposition VOCs to provide an improved description of the profile originally reported using 1DGC [7]. The detection of trace levels of VOCs in early decomposition has improved by the increased sensitivity, allowing for a wider array of compounds to be identified. In addition to the group of 400 compounds listed by means of 1DGC separation in the decomposition odor database [10], GC×GC opened the list to much more specific molecules that showed that this list was not yet exhaustive [1, 8]. A full profile of decomposition VOCs covers such a large range of polarities, concentrations, and molecular families [1, 4, 6–8, 10–13] that only the implementation of powerful separation science tools will allow further exploration.

In GC×GC, the use of two column phases with a certain level of orthogonality allows bi-dimensional chromatographic separation and offers a significant improvement in terms of peak capacity. Moreover, the modulation process focuses the peaks and improves the sensitivity by means of cryogenic zone compression [14]. All 1DGC detectors can be hyphenated to a GC×GC system, with the only requirement being that a fast acquisition rate be used to properly reconstruct narrow cryo-focused chromatographic signals. Fast acquisition time-of-flight mass spectrometers (TOFMS) are often the preferred choice of detectors as their intrinsic design allows them to generate unskewed mass spectra that permit efficient mass spectral deconvolution for the production of high-quality spectra, further easily compared with library databases such as NIST and Wiley for identification. A complete description of the GC×GC–TOFMS system used in this study can be found in previous papers [7, 8].

In non-targeted analysis, the major challenge associated with GC×GC is the complexity of the generated data. As the multidimensional data include several slices of modulation, two retention times (^1t_r and ^2t_r), and deconvoluted mass spectral information, powerful software is required to efficiently perform data processing and facilitate the treatment of the information and comparisons between different chromatograms. Proper comparison greatly relies on the correct alignment of the chromatograms, based on matching both dimension retention times and mass spectral data. Statistical comparison tools have been designed for this purpose and are featured in some commercially available software [15]. They further allow direct statistical investigation. For example, classes of samples can be created to categorize replicate analyses and increase statistical power. The data treatment can then be founded on Fisher ratio (FR) calculations that allow identification of significant differences between classes for each of the analytes identified after classical peak finding algorithms are applied. The FR is the mathematical ratio of class-to-class variance to intra-class variance and helps the user to quickly identify analytes that are present at significantly different levels between classes [7]. The level of specificity to be applied can be tuned by setting a FR threshold value. Such specific data can then be exported and processed more rigorously in any statistical software package. Such a supervised approach has the advantage of reducing the influence of biological variation between replicate samples and is often used in metabolomics when GC×GC is employed [16–18]. Because blanks are generally quite complex and the dynamic range of the different compounds is large, chromatogram alignment followed by various statistical treatments has been the preferred solution to deal with this issue and avoid false identification of biomarkers associated with complex biological processes. Alternatively, another approach for GC×GC data analysis is the use of unsupervised multivariate statistics, which drastically increase the size of the matrix of data to be processed. Multivariate approaches capable of reducing data dimensionality are then used to allow a clear visualization of the main clustering behavior. Principal component analysis (PCA) is one type of multivariate approach that has successfully been used in previous decomposition VOC studies [1, 4, 6–8, 11–13, 15].

This study represents the first GC×GC–TOFMS evaluation of decomposition VOCs from human remains. The purpose of this study was to investigate multivariate analysis approaches as tools to aid in the differentiation of decomposition VOCs from human cadavers and pig remains as studies of this nature compile in the field. There was also an interest in evaluating whether the profile of human remains analyzed by GC×GC–TOFMS could be differentiated under different conditions (i.e., in the presence and absence of insect activity). The lack of replicates available in studies of this nature was the driving force for the development of statistical methods to

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increase the objectivity when developing complex lists of decomposition VOCs by GC×GC–TOFMS. The current study involved the collection of decomposition VOCs for 6 days post-mortem during two trials at the same geographic location. This period is particularly important for the early investigation of natural and mass disasters, as VR canines are regularly tasked with locating victims during the early stages of decomposition in these scenarios. Since early decomposition produces VOCs at lower concentrations compared with more advanced stages, developing an extensive VOC profile for this period is challenging but necessary in order to increase the success of disaster victim recovery [19, 20]. FRs and PCA were used to develop a list of decomposition VOCs of interest during the early post-mortem period. A third approach was also investigated combining the FR and PCA approaches in order to identify the most appropriate method of identifying decomposition VOCs of forensic significance.

Material and methods

Sampling

Field design

In order to compare the VOC profile of pig carcasses and human remains within the same decomposition environment, research trials were conducted at the Forensic Anthropology Research Facility (FARF) at Texas State University (San Marcos, TX) in collaboration with the recipients of a US Department of Justice grant (No. 2010-DN-BX-K243). Their study involved two experimental treatment groups for the human cadavers, insect inclusion and insect exclusion (Table 1). The insect inclusion treatment was allowed to be colonized by insects, whereas the insect exclusion treatment prevented arthropod access. This is an important variable of interest from a taphonomic perspective because when temperature is accounted for, insect activity has the largest impact on the rate of decomposition of a human body [21]. The insect-exclusion cadaver, insect-inclusion cadaver, and a pig carcass were each placed unclothed on the ground separated by a minimum distance of 10 m. The site was located in an open grassy field surrounded by trees. NexSens DS1923 μ-T temperature loggers (Fondriest Environmental Inc., Alpha, OH, USA) were placed within 1 m of each set of remains, and the temperature (°C) was recorded at 0.25-h intervals. A control site was designated for each trial, containing the same soil and vegetation but no decomposing remains. The insect-inclusion remains were placed beneath anti-scavenging cages with dimensions 5.0 m×5.0 m×3.0 m, whereas pig remains were placed beneath anti-scavenging cages with dimensions 0.9 m×0.6 m×0.6 m. The insect-exclusion cadavers were placed beneath a 3.7 m×1.8 m×1.8 m Lumite® screen (18×14 mesh size) (BioQuip Products, Rancho Dominguez, CA, USA) inside the anti-scavenging cage in order to inhibit arthropod access to the remains.

A VOC sample was collected daily for 6 days post-mortem from each of the four sites (insect-included cadaver, insect-excluded cadaver, pig carcass, and control site). This was performed for two replicate field trials that occurred in May 2012 and November 2012 (i.e., four samples per day for 6 days in each trial). This yielded 48 VOC samples for the whole study. Due to the nature of the body donation program, transport and storage conditions varied between each specimen. The pre-deposition post-mortem interval ranged between trials from 3 to 9 days for the human cadavers and 0 to 3 days for pig carcasses. Transport of cadavers to the site was conducted at room temperature or on dry ice but was not specified on delivery. Upon arrival, cadavers were stored in human remain coolers at approximately 4.5 °C until deposition.

VOC sampling

A rectangular stainless steel collection hood was used for the accumulation of VOCs from the remains. Due to the size of the remains, the hood was transported in two pieces, each with the dimensions 120 cm×76 cm×76 cm (l×w×h). At the time of collection, the hood was assembled by aligning the two pieces to overlap at the midpoint. A 1/4″ Swagelok® bulkhead connector was used as a sampling port for connection of a dual sorbent tube containing Tenax® GR and Carbopack™ B (Markes International Ltd, UK). The hood was placed over

Table 1 Summary of the different samples collected in the spring and summer trials

	Species	Condition	Figure 6 label	Day 1	Day 2	Day 3	Day 4	Day 5
Spring (May)	Pig	Insect included	Pig	Fresh	Fresh	Fresh	Bloat	Bloat
	Human	Insect included	I-Incl	Fresh	Fresh	Fresh	Bloat	Bloat
	Human	Insect excluded	I-Excl	Fresh	Fresh	Fresh	Fresh	Fresh
Fall (November)	Pig	Insect included	–	Fresh	Fresh	Fresh	Fresh	Fresh
	Human	Insect included	–	Fresh	Fresh	Fresh	Fresh	Fresh
	Human	Insect excluded	–	Fresh	Fresh	Fresh	Fresh	Fresh

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each set of remains for 30 min to allow for headspace accumulation. A BD constant-flow air sampling pump (LaMotte Company, Chestertown, MD, USA) was used to draw the VOC sample onto the sorbent tube for 5 min at a rate of 200 mL/min. Samples were capped using brass long-term storage caps and were transported to the laboratory for analysis.

Chemicals

Each set of samples was analyzed in conjunction with a set of *n*-alkane standards from C_8 to C_{20} (Sigma-Aldrich®, Bellefonte, USA) in order to calculate retention indices. The *n*-alkane mix was injected separately as an external standard. The retention indices were used to check the consistency of each relevant compound's retention times between the different trials.

Sample analysis

Samples were analyzed by thermal desorption (TD) coupled to GC×GC–TOFMS. A LECO Pegasus 4D system was equipped with a secondary oven, a quad-jet dual-stage modulator, and a unit mass TOFMS (LECO, St Joseph, MI, USA). The first dimension (1D) column was a low-polarity Restek Rxi-5Sil (5 % phenyl–95 % dimethyl polysiloxane) (Bellefonte, PA, USA) (30 m×0.25 mm id×0.25 μm df), and the second dimension (2D) column was a mid-polarity Restek Rxi-17 (50 % phenyl–50 % dimethyl polysiloxane) (Bellefonte, PA, USA) (1.0 m×0.15 mm id×0.15 μm df). This column combination provides two complementary mechanisms of separation allowing an efficient separation, ordered chromatograms, and good flexibility in terms of temperature stability [22]. Moreover, this column set was successfully used in previous decomposition VOC studies investigating pig carcass decomposition [1, 7, 8].

Thermal desorption and injection of samples were carried out using a Unity 2 series thermal desorber (Markes International Ltd.). Each sample tube was desorbed at a temperature of 300 °C for 5 min. Samples were recollected on a cold trap with a Tenax TA/Carbograph 1TD sorbent bed at a temperature of −10 °C. Desorption of the cold trap occurred at 300 °C for 3 min. The split flow during desorption and injection was fixed at 20 mL/min and kept constant for all the injections. The split flow was recollected on an additional tube. The GC oven temperature was initially set at 35 °C and held for 5 min, before being increased at 5 °C/min to 240 °C where it was held for another 5 min. The temperature offset for the secondary oven was 5 °C. The modulation period (P_M) was 4 s, with a hot pulse time of 0.70 s and a temperature offset of 10 °C. The transfer line was held at 250 °C. The TOFMS was operated in electron ionization (EI) mode at 70 eV, with a mass range of 29–450 amu, an acquisition rate of 100 Hz, and a detector voltage of 1500 V. More details on the analytical procedure and comparison to 1D-GC analysis can be found in previous reports [7, 22, 23].

Data processing

GC×GC–TOFMS data were first acquired and processed with the ChromaTOF® 4.5 software (LECO Corp.). This software was used for peak finding, mass deconvolution, integration, and library searching. Wiley (2011) and NIST (2011) databases were used for mass spectral identifications with a match factor threshold >700. A detailed explanation of the raw data processing can be found in previous work [1, 7, 8]. After initial processing, the chromatograms were aligned using the statistical compare (SC) option of the ChromaTOF® 4.5 software (LECO) to obtain a proper sample comparison [15, 24, 25]. The criteria used for class-to-class comparison and alignment were a minimum of five samples that contain analytes and a minimum of 50 % of the samples in a class that contains the analytes. All of the following processes were conducted on unique mass in order to facilitate the use of SC. During this alignment process, sample classes were created. A class represents a group of replicate samples that need to be compared with one or more other groups of samples (other classes). An individual comparison between the control samples and experimental samples was made for each sample type in SC. Therefore, the control samples were processed and aligned to the insect-included cadaver samples, insect-excluded cadaver samples, and pig carcass samples independently.

Following alignment of data processing tables, the basic statistical information from various peak calculations was compared in order to identify the compounds showing the highest between-class variance. FRs were calculated from the compound table for each analyte in order to identify the compounds showing the highest variance [24–26]. Results were exported as .csv files to apply statistical methods with external software. First, data sorting and cleaning were conducted in Excel®. During this step, non-specific compounds, such as siloxane compounds, were manually removed. Multivariate statistical analyses were conducted using The Unscrambler X® software from CAMO.

To follow and compare the decomposition process over the experimental days, various supervised multivariate approaches were compared. The aim of this comparison was to select the best way to obtain valuable information from a complex and dynamic mixture. All the approaches were based on principal component analysis (PCA). PCA was used to give a visual representation of the distinction between the decomposition samples and control samples. This separation between the two groups was based on the compound intensity as variables. The scores and loadings plots were the main tools of this visual investigation. First, PCA was performed using all exported compounds following the alignment in SC. In the

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second approach, compounds in the PCA were only input where the FR was undefined. This was hypothesized to be valuable based on the theory that an undefined FR should indicate that the compound was specific to one class. Finally, a FR threshold was tested whereby compounds above a certain FR were input into the PCA model. The threshold approach would incorporate some compounds that were specific to a class, in addition to the ones that are highly variable in levels between classes.

Results and discussion

General approaches

Previous studies describe the general VOC profile produced during decomposition of pig carcasses used as human analogs by GC×GC [7, 8]. Based on literature comparisons with other studies, similarities in the major compounds between the odor profiles of pig and human remains have been identified [4, 7, 8, 10–13]. The GC×GC chromatograms in Fig. 1 show the direct comparison of the VOC profile produced by the two cadavers (insect included and insect excluded) and the pig carcass on day 5 of the May 2012 trial. Upon visual inspection of these chromatograms, a difference in the VOC profile is apparent. During early-stage decomposition, an increase in VOC production can be associated with further progression through the decomposition process. Figure 1 shows that the pig carcass has apparently reached a more advanced stage of decomposition compared to the insect-excluded cadaver. Additionally, the insect-included and insect-excluded cadavers can be differentiated. The exclusion of insects during decomposition is known to result in a reduced rate of decomposition, which is reflected in the VOC profiles in Fig. 1 [19, 27, 28]. The field observations support this, as on day 5, the pig carcass was reported to be in the bloat stage, the insect-included cadaver was exhibiting early signs of putrefaction but could not be classified as having entered the bloat stage yet, and the insect-excluded cadaver was still in the fresh stage.

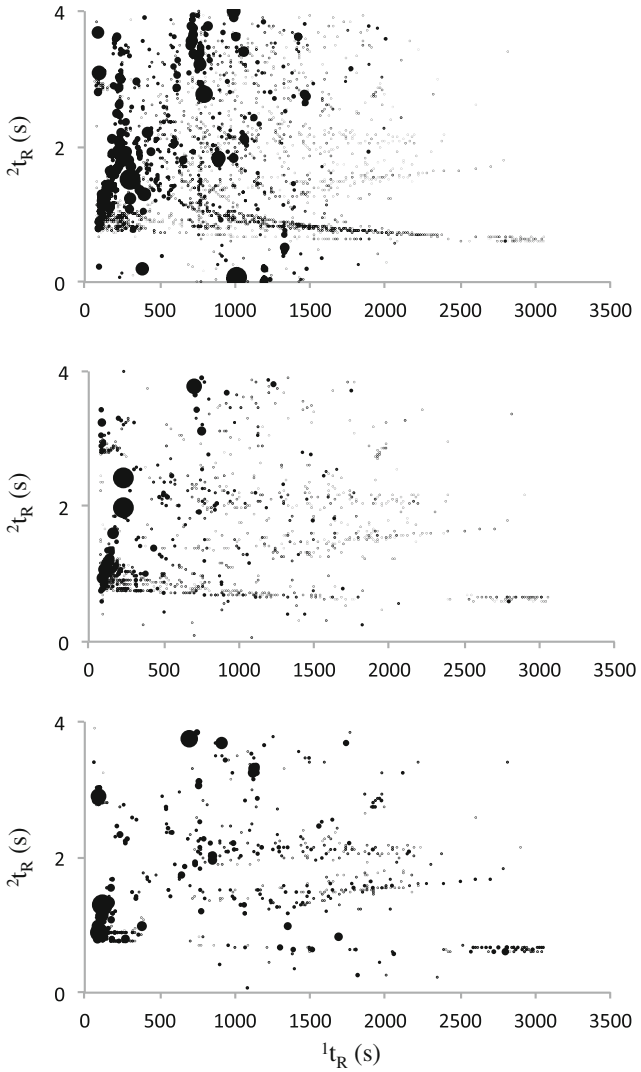

Fig. 1 Comparison of the three GC×GC profile bubble plots (^1t_R and ^2t_R are retention times for the 1D and 2D, respectively) for VOCs sampled on experimental day 5 (*top* is pig carcass, *middle* is insect-included carcass, and *bottom* is insect-excluded carcass). After 5 days, a shift in the decomposition profile is observed in the different sample types, whereby the pig was the most advanced and the insect-excluded cadaver was the least decomposed

After an initial comparison, the chemical composition of the VOC profile from each of the samples was studied. Based on SC alignment and comparison of remains headspace vs. controls, decomposition-specific VOCs were isolated. These compounds facilitated the evaluation of both the seasonal variation as well as any inter-species differences of the decomposition odor. Prior studies have provided information on the decomposition VOC profile from pig carcasses [6–8, 11]; thus, an inter-seasonal comparison could be performed during this study between the May and November 2012 trials. Figure 2 shows that the relative percentage of each chemical family is comparable between the pig carcasses in both trials on day 2. For day 4 and day 5, there is a higher degree of difference displayed between seasons. In Texas, temperatures in May are much warmer than in November. Since higher temperatures are known to increase the rate of decomposition due to the acceleration of biochemical processes, it follows that the decomposition process was faster in May. This is evidenced by the importance of sulfide compounds during pig decomposition. These sulfide compounds were mainly dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS). They are the major products of mammalian decomposition. Despite the fact that similarities are identified between the two trials, comparison beyond early stages is complicated by differences in decomposition rate. The importance of replicate field studies is demonstrated here due to the variance in the

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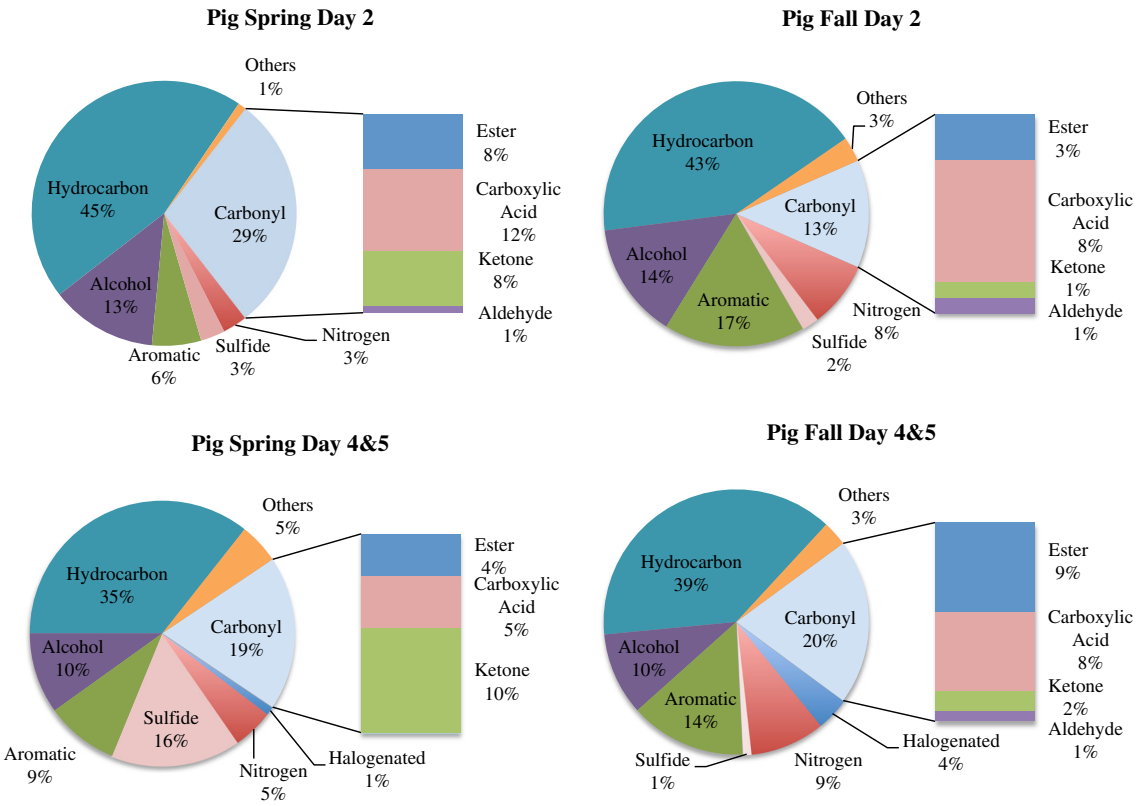

Fig. 2 Seasonal variation of relative values for major chemical families present during the early stage of decomposition for the pig carcass in spring (May 2012) and fall (November 2012)

VOC profile produced between individuals and between conditions.

Furthermore, the decomposition VOC profile from the pig carcass in the May 2012 trial is compared to that of a previous study using pig carcasses in Fig. 3 [7]. For this comparison, the data from this trial were processed with the same method used by Stadler et al. [7]. This allowed for a comparison between geographical locations (Southern Ontario, Canada [7] vs. San Marcos, USA) for the same season (spring). Figure 3 shows that the resulting VOC profile from both studies was similar based on the identified chemical families. The overwhelming presence of nitrogen and sulfide compounds in the profile was seen in both studies, and similarities can be noted in the amounts of aromatics, halogenated compounds, and carboxylic acids. This demonstrates the robust nature of the decomposition VOC profile produced during the bloat stage regardless of season and geographical location.

After studying each species separately, the cadaver with insect-included and the pig carcass decomposition VOC profiles from the May 2012 trial were compared to provide information regarding the use of pig carcasses as human odor analogs. Only the insect-included cadaver is displayed since it was closest in decomposition stage to the pig carcass. Figure 3 shows that the VOC profile based on chemical families differs between the pig carcasses (Southern Ontario, Canada [7] vs. San Marcos, USA) and the human cadaver during the same time frame. For the human sample, the presence of nitrogen and sulfide compounds was less pronounced and the aromatics were a major contributor to the VOC profile. The major compounds from decomposition, i.e., DMDS and DMTS, were less prominent in the human decomposition VOC profile. However, some similarities (e.g., hydrocarbons) were exhibited between pig and human decomposition VOC profiles at this time. These data are of interest in the quest for human decomposition VOC biomarker identification. Nevertheless, it still remains unclear whether differences in the decomposition VOC profile can be attributed to the use of different species (pigs vs. humans) or whether a shift in the decomposition rate was responsible.

In order to identify VOCs from human decomposition, an attempt was made to monitor individual VOC production throughout the decomposition process for control sites, pig carcasses, and human cadaver samples. However, upon initial investigation, the fluctuations of each compound observed for the pig carcass and human cadaver samples, as well as in the control sites, made interpretation challenging. Due to the fact that neither trends nor biomarkers could be identified with this general approach, the power of multivariate statistic approaches was necessary for further interpretation.

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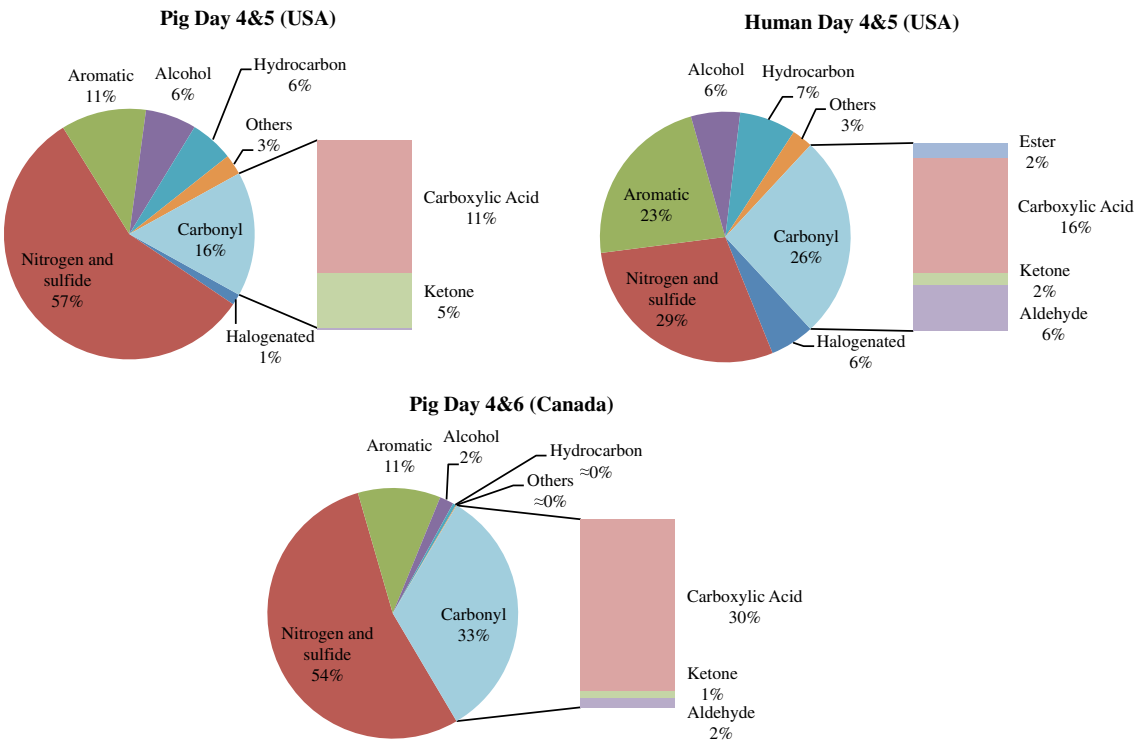

Fig. 3 Comparison of decomposition VOC profiles recorded during two separate studies on pig carcasses (USA and Canada) and one study using a human cadaver (insect included)

Supervised multivariate approaches

The next step for developing a comprehensive decomposition VOC profile was the establishment of a method for identifying potential decomposition VOC biomarkers. To this end, three different approaches were compared for decomposition VOC identification as illustrated in Fig. 4. To assess which approach was the most valuable, the pig carcass and human cadavers were compared during the first 6 days of decomposition in May 2012. The results presented for the insect-included human cadaver are representative of the results observed for the other samples.

For the first of the multivariate approaches, processing was based on PCA of matrices of data containing all individual compounds found above a certain *S/N* value (i.e., *S/N* of 50) during peak finding (PCA on a full data set). No valuable information was obtained in terms of identification of specific biomarkers for early decomposition (Fig. 5a). The principal components (PCs) only accounted for 21 % (PC-1) and 15 % (PC-2) of the overall variability, and most samples congregated in the same region of the PCA score plot. As it appears in loading and correlation loading plots (Fig. 5a), most of the analytes of the large data matrix (1200 entries) were not specific to cadaveric decomposition. One of the major reasons was that levels of analytes were very low at the early stage of decomposition and therefore can easily fluctuate in levels and exhibit "noise-type" fluctuations, which caused the PCA to become over fitted, thereby reducing the value of the information obtained using this approach. This clearly directed us towards a more controlled or supervised approach where some level of data filtration should be performed to minimize this noise fluctuation effect and concentrate the multivariate treatment on more prominent analytes in order to visualize the discriminatory information in the data set.

In the second approach, with the intent of exclusively isolating compounds that were specific to cadaveric decomposition, Fisher ratio (FR) calculations were performed between two classes for each sample type, one class of decomposition VOC samples and another class of control samples. After FR calculations, analytes were only selected when classified with an "undefined" FR value. This classification resulted from the fact that such analytes are present in the decomposition sample class and not in the control class (i.e., "exclusive" to decomposition). A data matrix was built with these specific analytes. Despite the expectation that such an approach would maximize the impact of highly specific biomarkers (e.g., sulfides) compared to less specific analytes, the resulting PCA did not produce a clear separation between the classes (Fig. 5b). Compared to the PCA performed on the full data set, PC-1 and PC-2 axis loadings increased slightly (33 and 17 %, respectively) but the discrimination of sample groupings over the PC axes was still unclear. It was noted that, although each class was comprised of samples only from decomposition or controls, the decomposition process is

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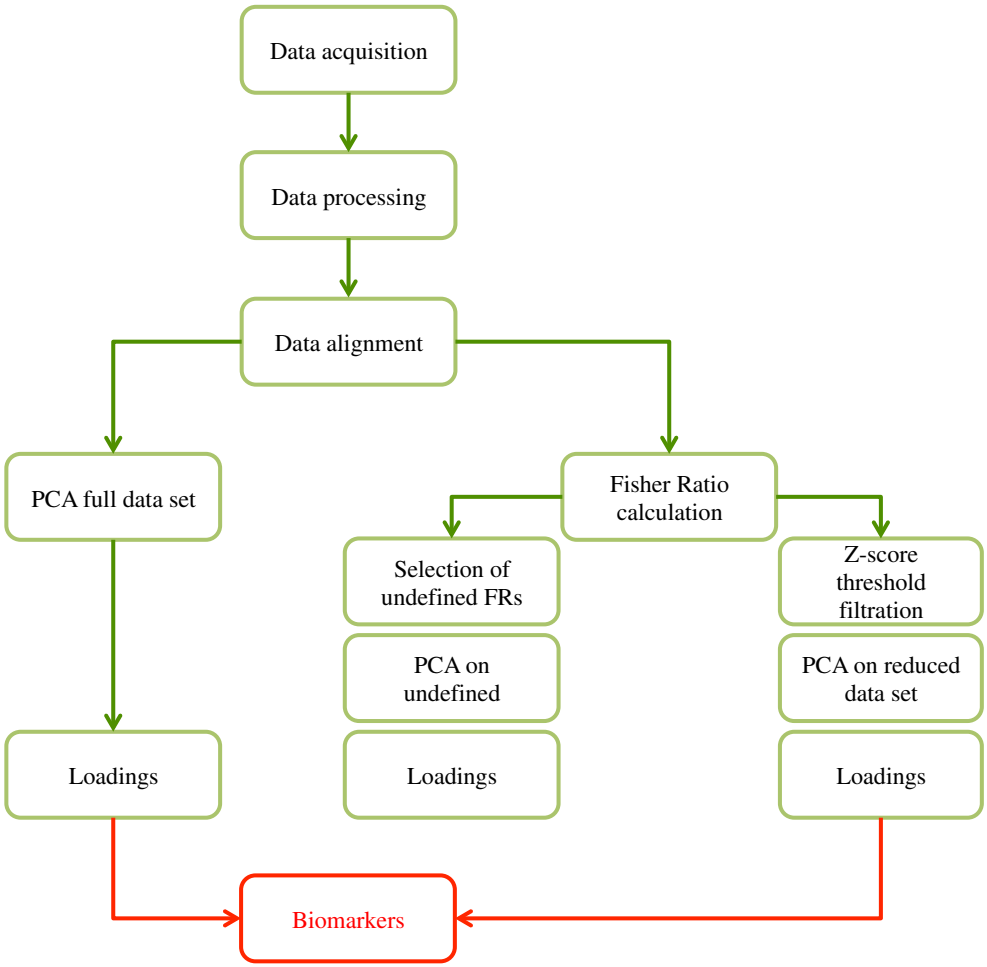

Fig. 4 Scheme of the three different multivariate supervised and non-supervised methods used for decomposition VOC identification

dynamic which would significantly increase the variance in the decomposition class. Loadings and correlation loadings plots started to provide useful information using this approach. Contrary to the first approach, it was possible to identify compounds responsible for the PCA separation from these plots (Fig. 5b). The compounds with no impact on the PCA from the previous unfiltered approach (i.e., the ones close to the center of the plot in Fig. 5b) are removed, making visualization clearer. However, the PCA performed on this approach using undefined FRs was still inconclusive for determining biomarkers of decomposition. It is nevertheless suggested that the investigation of undefined FRs can be valuable for screening of samples prior to statistical analyses. This data can contain important information or an initial indication of sample type.

The proposed third approach was also supervised as it included pre-filtering of compounds prior to PCA. This was done by means of FR calculations for the two classes and the use of numerical FR values. Previous studies have indicated that the use of FRs is valuable in deconvoluting biological variation. Indeed, Pierce et al. [18, 26] demonstrate the advantage of using FRs for PCA to identify biomarkers when the biological diversity was high [26]. However, the analysis of all FR values (e.g., for each analyte) is time consuming and often provides no additional information from low FR values. Therefore, a decision to apply a FR threshold was made, in order to fix the limit between compounds of interest (high FR values) and background compounds (low FR values), prior to the subsequent use of PCA. Because of the fact that the range of FR numerical values varies from one study to another depending on class-to-class variance and intra-class variance, it was not possible to select a single threshold numerical value to be used as a reference to differentiate compounds of interest in all studies. The challenge was to find a standardized way to determine a versatile threshold value that could be efficiently applied to studies independently of the FR numerical range. The calculation of *z*-score is often used to determine outliers in analytical data sets. In our supervised approach, compounds that are responsible for the differentiation between the two classes (high FR values) can, to some extent, be considered as the FR outliers. Therefore, the establishment of a threshold value based on *z*-score calculation could be a way to normalize the threshold approach for various class comparisons in any study. In this way, the FR threshold value above which

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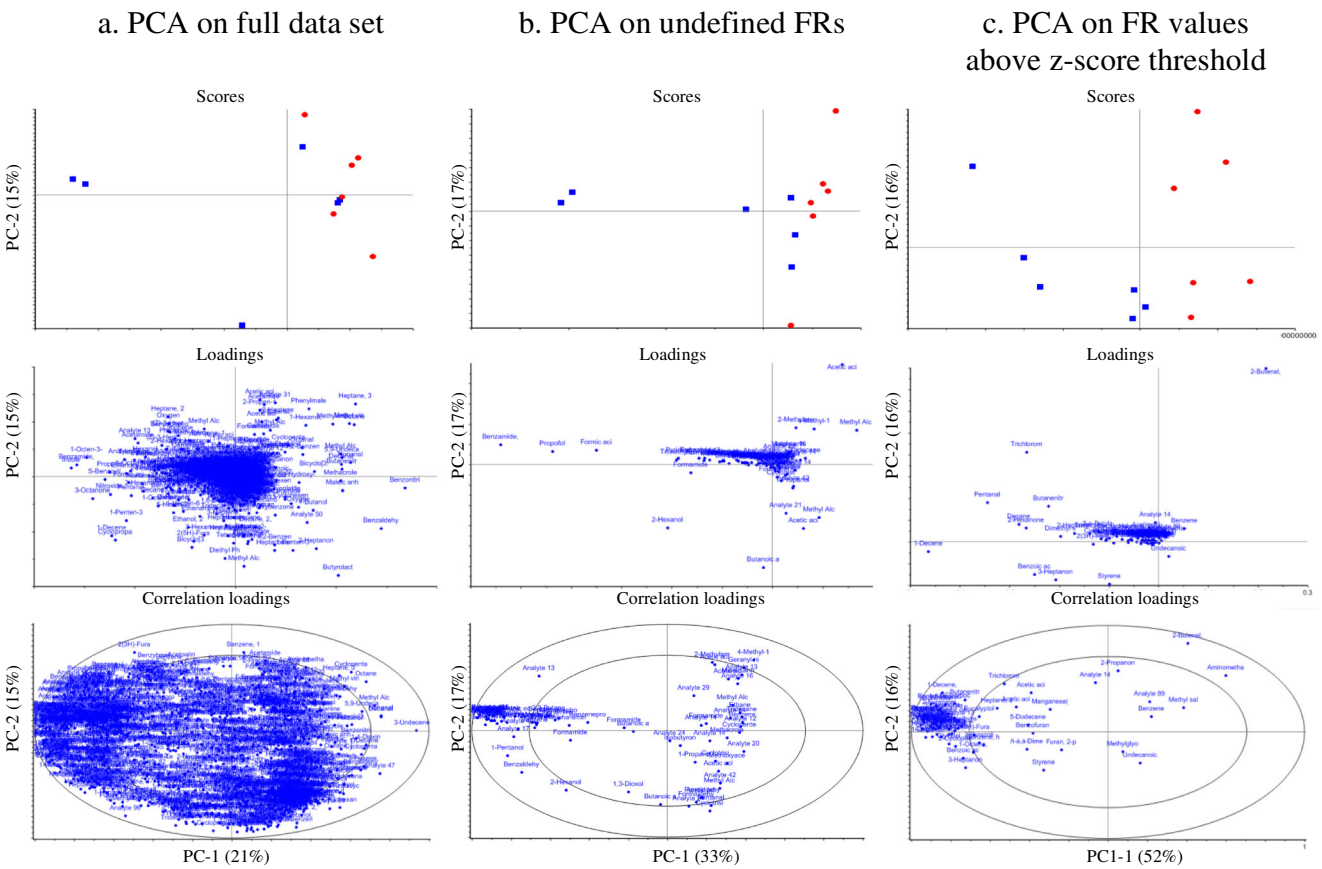

Fig. 5 PCA scores and loadings for the three different multivariate supervised and non-supervised methods used for decomposition VOC identification on the insect-included human sample from the spring trial. *Blue squares* represent human samples and *red circles* represent control samples. **a** PCA on all the data from the headspace; **b** PCA on the undefined FR only present in body headspace; **c** PCA on compounds with a minimum FR value

compounds are included as biomarkers would be different for each class comparison. A specific *z*-score value must be determined in order to select the level of stringency applied. Moreover, only the positive values of the *z*-score were kept for the PCA. Indeed, a negative *z*-score indicated a low FR value under the mean. A weak FR corresponds to non-specific compounds. The mean and the standard deviation of the FR numerical values were calculated, and *z*-score values were calculated for each analyte. As illustrated in Table 2, several *z*-score threshold values were applied to demonstrate the effect of these thresholds on the number of selected analytes for three different class comparisons. A compromise between the severity of the *z*-score threshold and the number of selected analytes was necessary. Between extreme situations where a *z*-score threshold of 5 selected only 3 analytes and a *z*-score threshold of 0.25 selected 101 analytes, a threshold of 0.5 was selected as it produced a reasonable number of analytes in terms of further statistical treatment using PCA. In practice, from the 1500 analytes typically present and for which FR values are automatically calculated, the *z*-score threshold approach reduced the list to less than 75 compounds, representing less than 5 % of the original data set. Such a reduction not only permitted a focus on major analytes, but it also greatly simplified the data handling that still requires manual input. A comprehensive fingerprinting approach was not desired whereby every compound, including those in trace quantities, was identified and characterized. Rather, a focus was placed on identifying important decomposition VOCs that can provide information of practical purpose. By focusing on compounds above a defined threshold, the most important compounds of interest can be targeted. This will be most beneficial when translating complex GC×GC data into manageable information for understanding VR dog mechanisms and improving their training. Setting the threshold based on *z*-scores, however, might suffer in robustness because the distribution of the FR values did not follow a normal distribution. As shown in Table 2, a *z*-score threshold of 2 and 3 did not result in isolating 2.3 and 0.15 % of the FR values above the threshold, respectively. Long-term testing of this approach in various studies, where classes of analytes will be considered, is necessary to determine if another approach is required. Nevertheless, the combined approach of FR *z*-score threshold and PCA produced the best result during the statistical analysis. As shown in Fig. 5c, a much clearer separation was

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Table 2 Effect of applying various FR z-score threshold values for three different comparisons of sample classes

z-score value	5		4		3		2		1.5		1		0.5		0.25	
Analytes above threshold	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
Pig vs. control	2	0.2	4	0.4	9	0.9	18	1.7	24	2.3	43	4.2	69	6.7	103	9.9
Human (I-Incl.) vs. control	4	0.4	5	0.5	7	0.6	15	1.7	29	2.6	41	3.7	70	6.3	104	9.4
Human (I-Excl.) vs. control	3	0.3	6	0.5	9	0.8	15	1.3	23	2	34	3	73	6.4	96	8.4
Average number of analytes above threshold	3		5		8		16		25		39		71		101	
Average percentage of analytes above threshold		0.3		0.5		0.8		1.6		2.3		3.6		6.5		9.2

distinguished between decomposition and control samples, with more than half of the variance explained on PC-1. In order to find the compounds responsible for the separation, the loading and correlation loading plots were investigated. Analytes located inside the region defined by the two ellipses indicate how much variance is taken into account by the model (100 and 50 % for the outer and the inner ellipse, respectively) and are listed in Table S1 (see Electronic Supplementary Material, ESM). These compounds appeared to be similar to the typical chemical families reported in previous decomposition VOC studies [6–8, 10, 11, 29]. Nevertheless, the compounds displayed in this table still have to be validated in further studies.

Inter-species comparison

Elaborating on the use of this approach, PCA was used to compare the VOC profile of the pig carcass, the two human cadavers, and the control simultaneously (Fig. 6). PCA demonstrated that the pig carcass and the human cadavers followed similar trends. Days 4 and 5 for the pig carcass and cadaver form a distinct group away from the cluster of controls and the first few experimental days. This separation can be mostly attributed to the difference in sulfides and alcohol compounds. The axis loading of the first principal component (PC-1) suggests that the major compounds in the decomposition VOC profile were similar between pig and human remains. This is shown by the separation on the PC-1 axis, with the control group on the left and the pig and human remains group on the right. On the right side of the loadings plot, ethanol and 2-methyl-1-propanol were responsible for differentiating the human samples upwards from pig samples. The pig samples separated downwards due to 1-propanol, 1-butanol, 2-bromo-1-propanol, and DMDS. These compounds were responsible for the positioning on PC-2, and therefore, they represent the separation between the human and the pig VOC profile. Looking for human-specific biomarkers, further investigation of the GC×GC data is necessary. Indeed, the general profile is the same for both species [30], which was demonstrated in this study along PC-1 (Fig. 6). However, pig and human samples for D4 and D5 are separated along PC-2. One of the main interests of the investigation of human decomposition VOCs is to isolate which compounds are human specific. Using the third approach, it was possible to go further in this direction. Nevertheless, several repetitions of the present study will be necessary to validate the compounds responsible for this difference.

Further, the effect of insect colonization on early decomposition is also displayed in Fig. 6. The insect-excluded human cadaver is shown to be most similar to the first days of decomposition from the other pig samples. As expected from the general approaches, decomposition progressed at a reduced rate when insect access was restricted. This information demonstrated that exhaustive analysis of cadaveric VOC profiles has the ability to clearly reflect the dynamic aspect of the decomposition process under various conditions. The PCA visualization is extremely valuable for this type of interpretation.

Determination of human-specific biomarkers could aid police services and rescue teams to improve cadaver-detection dog training, especially in early-stage victim recovery. Specific biomarkers could be targeted in training regimes or by instrumentation in order to improve sensitivity and efficacy towards human remains. The sensitivity of the canine olfactory system is sufficient for recognizing these compounds; however, for instrumental analysis, it is proposed that the sensitivity of GC×GC may be required to detect volatile biomarkers of human decomposition [31].

Conclusions

This study was conducted to provide information regarding the use of pig carcasses as human odor analogs in decomposition studies. This is the first study using TD–GC×GC–TOFMS to investigate the human decomposition VOC profile. Monitoring the first 6 days of decomposition allowed the identification of early-decomposition VOCs. Use of GC×GC–TOFMS was able to efficiently resolve the complexity of the decomposition VOC profile. Unfortunately, the copious information generated and the data handling can prove to be

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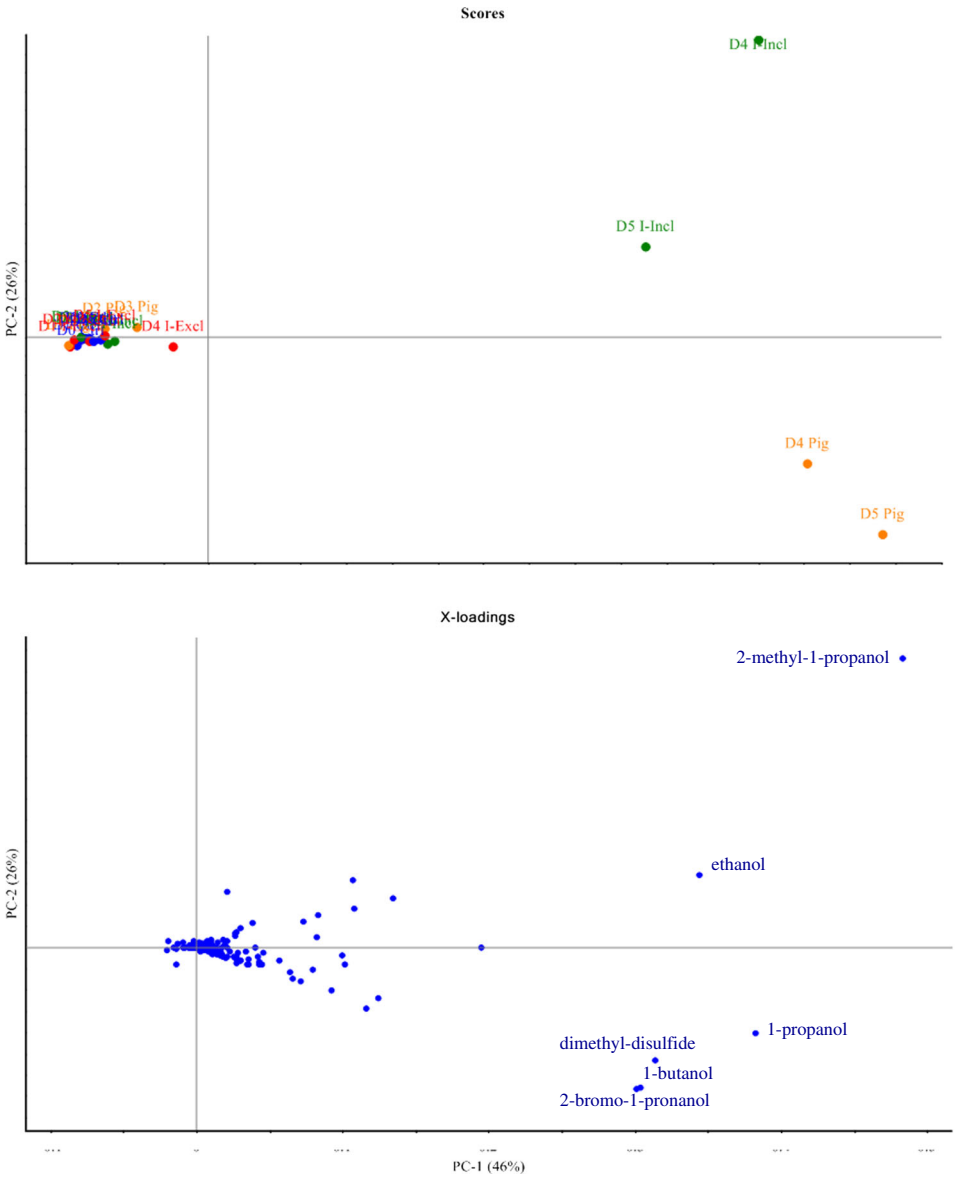

Fig. 6 Supervised FR *z*-score threshold PCA (z=0.5) of the control (*blue*), the pig (*orange*) carcass, and the two human cadaveric samples (insect inclusion (*green*); insect exclusion (*red*)). The global mammalian signature is defined along PC-1, and the species-specific signature is defined along PC-2

difficult. In this paper, three methods were used to identify potential decomposition VOCs of interest. The most efficient method was to determine a FR threshold using a *z*-score-based approach, followed by PCA visualization. Although the results are promising, the mathematical meaning of the *z*-score threshold, especially in terms of the normality of the *z*-score distribution, must be further investigated and may be beneficial for future work investigating human-specific biomarkers. Indeed, the applications of other threshold normalization methods are under investigation [32, 33]. However, applying this pre-filtering of the raw data allowed clear statistical grouping for the comparison between species and for two treatments (insect-included and insect-excluded cadavers). Nevertheless, the validation of the list of decomposition VOCs will require further study on human cadavers in subsequent field studies. A compiled list in the future will be valuable to improve VR dog training methods for locating recently deceased individuals and trapped victims. Moreover, the approach could also be used for long-term research studies to identify other advanced decomposition biomarkers significant for the location of individuals following extended post-mortem intervals of months or years.

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