Optimisation of dynamic headspace sampling conditions for the identification of Palaeolithic adhesives

Anika Lokker[1], Pierre-Hugues Stefanuto[1], Dries Cnuts[2], Veerle Rots[2,3*], Jean-Francois Focant[1*]

1 – University of Liège, Organic and Biological Analytical Chemistry Group, MolSys Research Unit, Allée du 6 Août 11, Quartier Agora, Sart-Tilman, Liège 4000, Belgium

2 – University of Liège, TraceoLab/Prehistory, Quai Roosevelt 1B, Liège 4000, Belgium

3 – F.R.S-FNRS, Brussels, Belgium

Corresponding author: alokker@uliege.be (A. Lokker)

*co-last

Abstract

The characterisation of Palaeolithic adhesives holds great potential for understanding human behaviour and its evolution. GC-MS is the most accurate identification method, unfortunately, it is destructive and requires a large sample size. Hence, most Palaeolithic adhesives are not characterised with GC-MS. Here, a new non-destructive identification method is introduced; dynamic headspace with two-dimensional GC coupled to a time-of-flight MS. The dynamic headspace extraction is optimised with a design of experiment approach. Four parameters were selected, and the optimised values were: incubation temperature: 50°C, incubation time: 20 min, purge volume: 450 mL and purge flow: 22.5 $mL.min^{-1}$, pine resin was chosen as a proxy for Palaeolithic adhesives. Subsequently, dynamic headspace was also tested on hide glue, which has less volatiles than pine resin and the universality of the extraction was tested. With untargeted techniques a distinction between hide glue and pine resin could be made based on their chromatographic profile. Lastly, dynamic headspace was tested against an existing headspace – solid-phase microextraction method. Dynamic headspace showed a higher response in the total area of the chemical groups of interest. Thus, dynamic headspace has a higher sensitivity for prehistoric adhesives than solid phase micro extraction, which is desired for minimal samples.

Key words

Prehistory, natural substances, optimisation, headspace, GCxGC-MS

Abbreviations

CIS	cool injector system
DHS	dynamic headspace
DoE	design of experiment
F_{purge}	purge flow
FFD	full factorial design
FR	Fischer ratio
HCMT	hydrocarbon monoterpenoids
HCST	hydrocarbon sesquiterpenoids
HS-SPME	headspace solid phase microextraction
OVAT	One-variable-at-time
OXMT	oxygenated monoterpenoids
OXST	oxygenated sesquiterpenoids
PCA	principal component analysis
T_{incu}	incubation temperature
TDU	thermal desorption unit
$time_{incu}$	incubation time
V_{purge}	purge volume
VOCs	volatile organic compounds

1. Introduction

Stone tools are the main cultural remnants of the Palaeolithic period, this makes them the objects of prime importance for the study of prehistorical behaviours. The scope of investigation is however limited because of the absence of organic remains that rarely preserve on stone tools. Many stone tools were not used as such but were mounted in or on organic handles thereby testifying to human's creativity and capacity for planning. The practise is referred to as hafting and the first evidence of hafting is dated to about 250,000 years ago in Europe [1] and about 200,000 years ago in Africa [2], but none of these yet include evidence for the use of adhesives. Adhesives haven been demonstrated to been used at the site of Campitello, Italy [2] where it possibly dates to around 200,000 years ago. All other evidences are more recent, and it is only after about 80,000 years ago that adhesive remains are more regularly discovered [3–7]. While Palaeolithic adhesives remain rare and are systematically of vegetal origin (e.g., resin, tar), other adhesives may have been used (e.g., protein glue). The

identification and characterisation of adhesives is important as, they contain a wealth of information and permit to reveal the technical expertise – and perhaps cognitive abilities – of their makers [8,9].

Natural materials are chemically complex. In addition, prehistoric adhesives could have been mixed with a plasticiser (e.g., beeswax) and been subject to significant degradation over time. The intensity of the latter process, moreover, depends on the archaeological context in which the stone tools were preserved. This makes the characterisation of prehistoric adhesives challenging. Optical microscopy, Fourier transformation infrared (microscopy) (FTIR(M)), Raman spectroscopy, scanning electron microscope with energy-dispersive X-ray spectroscopy (SEM-EDS) and gas chromatography coupled to mass spectrometry (GC-MS) are major analytical instruments used for the identification and characterisation of adhesives [3,4,6,10–13]. With the exception of GC-MS, these instrumental techniques are minimally to non-invasive and easy to implement, but only tentative identifications are possible [6,11]. Most of the time, a combination of several of these complimentary techniques is employed to characterise prehistoric adhesives [3–6,14].

GC-MS allows a better chemical description of adhesives but is a destructive approach [6,11,15,16]. Traditional GC-MS measurements of adhesives, involves extraction of the adhesive with a solvent followed by chemical derivatisation of non-volatile thermally sensitive molecules [6,17]. The identification of the adhesive is currently done following of the so-called 'biomarker' method that relies on the measurement of a set of specific chemicals that have been reported as unique for the different types of adhesives [16]. This practise is however destructive for the adhesive and involves complex sample preparation procedures. Alternative more direct sample introduction methods such as pyrolysis [15,18] and headspace (HS) trapping [17,19,20] have therefore also been reported.

Amongst HS techniques, HS solid phase microextration (HS-SPME) is commonly used because of its high level of automation and ease of operation. In HS-SPME, a fibre with a polymer coating is inserted in the vial, the VOCs present in the HS are adsorbed onto the fibre. After a certain extraction time, the fibre is removed from the vial and inserted into the GC inlet. Then the VOCs are desorbed and released into the column. The extraction is not exhaustive but allows a certain accumulation of analytes onto the fibre until a new equilibrium between the HS and the fibre is formed [21]. The sensitivity as well as the capacity of the SPME fibre to trap more chemicals is limited by the low volume of the polymer coating on the fibre [21–23]. The first reported results for stone tool adhesives were promising. However, the necessary sensitivity was only achievable when the incubation temperature was raised at values that degraded the adhesive [19].

Because of its non-destructive nature and suitability for highly degraded samples, HS-SPME continued to create interest in both archaeology and cultural heritage research [24]. It is supported by more

recent studies reporting on the successful usage of HS-SPME for blind-testing on a large range of adhesives [17,25,26]. The routine use of HS-SPME is still not really established for the identification of prehistoric adhesive [26]. As the well-known biomarkers of prehistoric adhesives are not present in the VOCs profiles and a new set of specific VOC chemicals has yet to be proposed for identifying the adhesives.

To use HS techniques in an identification method for prehistoric adhesives, a better characterisation of their VOCs is pivotal. HS-SPME might not be sensitive enough for the initial exhaustive screening of the HS profile of the adhesives. Compared to SPME, dynamic headspace (DHS) is an active sampling technique involving a continuous displacement of the HS equilibrium, in favour of a larger VOC release that enhance the sensitivity. Moreover, the volume of trapping sorbent is larger in comparison with SPME, resulting in a higher amount of VOCs adsorbed [21]. The increase of trapping efficiency of DHS well counterbalance the increased complexity to perform DHS over HS-SPME. [22,23,27–29]. When exhaustive screening of complex HS profiles is required, GC-MS using quadrupole MS can be replaced by comprehensive two-dimensional GC – time-of-flight MS (GC×GC-TOFMS). It offers higher chromatographic peak capacity, sensitivity enhancement by zone compression, and mass spectral deconvolution for improved analyte identification [25,30–33].

In GC×GC, two GC columns are used in series and connected to each other by the modulator responsible for subsampling eluent from the first dimension and reinjecting narrow bands of signals into the second dimension. This modulation process is the basis of the separation principle in the 2-D space [30,34,35]. Additionally, the 2-D chromatograms are chemically structured and compounds with similar chemical properties elute in the same space of the chromatogram, which supports the identification of ‘unknowns’ [30]. A HS-SPME-GC×GC-TOFMS instrument was used for the blind test investigation of Palaeolithic adhesives mentioned earlier [17,25,26]. DHS is also one of the sample introduction techniques that can be coupled to GC×GC-TOFMS and provides a powerful tool for biomarker discovery [31].

The aim of the present work is to investigate a new non-destructive identification method of Palaeolithic adhesives by mean of VOC profile measurements using DHS-GC×GC-TOFMS. For the optimisation of the DHS parameters a design of experiment (DoE) approach is used. Pine resin is selected as a test sample for its representation of Palaeolithic adhesives earlier identified on archaeological stone tools [6,13]. Subsequently, an untargeted identification approach is investigated by measuring pine resin and hide glue with DHS-GC×GC-TOFMS. Hide glue is selected as an example of protein glues, which are notoriously more difficult to detect with HS techniques than resins [17]. Lastly, DHS is compared with HS-SPME in the context of archaeological artefact analysis.

2. Materials & Methods

2.1. Samples

The pine resin (Pinus *nigra* subsp. laricio (Corsican pine)) was collected in Corsica in September 2009, stored in a plastic container until the measurements in October 2022. The hide glue was produced in-house from a deer hide by an experienced experimenter (C. Lepers, TraceoLab), see SI1 for more information.

All samples (5 mg of pine resin and 10 mg of hide glue) were placed in 20 mL screw cap headspace vials with a 1.3 mm PFTE septa (Gerstel®, Kortrijk, Belgium). Any instrumental variances were checked by measuring a Cannabis terpenes standard (#1 and #2, 2500 ppm) (Restek® Corporation, Bellefonte, PA, USA) for DHS and the grobmix. The standard was diluted to 25 ppm in methanol (≥ 99.8%, HPLC grade, Fisher scientific, Loughborough, UK) and 40 µL was pipetted in a 20 mL screw cap headspace vial. No internal standard was added, due to the solid nature of the samples. A n-alkane standard solution (C7-C30, Sigma Aldrich, Saint louis, USA, 1000 ppm) was diluted in MeOH to 20 ppm and 2 µL was pipetted inside a 20 mL vial.

2.2. Headspace techniques

DHS and HS-SPME extractions were automated using a MultiPurposeSampler autosampler (MPS, Gerstel K.K, Mülheim an der Ruhr, Germany), connected to the GC system.

2.2.1. HS-SPME extraction

The HS-SPME extractions were performed with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm Stableflex 24 Ga fibre (Supelco®, Bellefonte, PA, USA) [19]. The incubation time was 10 min at 50°C. The adsorption time was 25 min at a penetration depth of 43 mm. There was no agitation during the incubation and adsorption. After the adsorption, the fibre was desorbed in the inlet of the GC system, with a penetration depth of 54 mm at 270°C for 3 min. The injection was performed with a split ratio of 5:1. After each injection, the fibre was reconditioned in the bake-out port at 270°C for 20 min with a penetration depth of 54 mm.

2.2.3. DHS extraction – design of experiment

For the DHS extraction, the parameters were first optimised via a full factorial design (FFD) approach. Four parameters were selected for the optimisation: incubation time ($time_{incu}$, min), incubation temperature (T_{incu}, °C), purge flow (F_{purge}, $mL.min^{-1}$) and purge volume (V_{purge}, mL), see Table 1. The centre points were measured in triplicated, and the runs were executed in random order. The optimal DHS parameters were determined with a Pareto chart and main effect plot and were chosen to be:

141 $time_{incu}$: 20 min, T_{incu}: 50°C, F_{purge}: 22.5 mL.min^{-1} and V_{purge}: 450 mL. These parameters were used for all
142 other DHS measurements conducted in this research.

143 VOCs were trapped in a thermal desorption tube filled with Tenax TA. Tenax TA was chosen as it can
144 absorb a wide range of VOCs and it was found, in a previous study, that it had the best reproducibility
145 among the tested TD sorbents [36]. During the trapping, the temperature of the tubes was 20°C for
146 the T_{incu} 40°C and 30°C (minimal temperature) and 30°C for T_{incu} 50°C. The needle temperature was set
147 at 120°C for all extractions [22].

148 After the DHS extraction, VOCs were desorbed in the thermal desorption unit (TDU, Gerstel K.K.), the
149 TDU was heated from 40°C to 280°C at 300°C.min^{-1}, and held isothermal for 3 min. The desorption was
150 conducted in splitless mode, and VOCs were trapped on a liner filled with Tenax TA at -20°C in a cooled
151 injector system (CIS, CIS4 Gerstel K.K.). Subsequently, VOCs were released into the GC by heating of
152 the CIS to 250°C at 12°C.s^{-1} and held for 3 min. The CIS was operated in split mode (5:1). Before the
153 sequence starts and after each run, the tube was reconditioned for 15 min in the TDU at 300°C.

154 ## 2.3. GC×GC-TOFMS analysis

155 All samples were analysed on a Pegasus GC-4D (LECO® Corporation, St. Joseph, MI, USA) GC×GC-
156 TOFMS equipped with a secondary oven and a quad-jet, dual stage thermal modulator. The column
157 set was chosen from an earlier study conducted on similar compounds [17], and consisted of a semi-
158 polar Rxi-624silMS (Restek® Corporation) (30 m x 0.25 mm i.d 1.4 µm df) in 1-D and a polar Stabilwax
159 (Restek® Corporation) (2 m x 0.25 mm i.d 0.5 µm df) in 2-D. The connection between the two columns
160 was made with a SilTite µ-union (Trajan Scientific and Medical®, Australia). The oven program started
161 at 40°C (hold time 3 min) and increased to 240°C at 5°C.min^{-1} (hold time 2 min). The modulator
162 temperature offset was +15°C in relation to the primary oven and the modulator period was 4 s, with
163 a 0.60 s hot pulse time. For the carrier gas, high purity helium (Alphagaz 2, Air Liquide, Liège, Belgium)
164 was used. The flow rate was kept constant at 1 mL.min^{-1}. After every five samples, a blank was
165 measured and a terpenoid standard was injected at the beginning, middle and end of the sequence.

166 The temperatures of the MS transfer line and the ion source were 250°C and 230°C, respectively. The
167 mass range was 35-550 amu, the acquisition rate was 200 spectra.s^{-1}, the electron ionisation was 70
168 eV, and an acquisition delay of 300 s was used. Before each sequence, mass calibration and tuning
169 were performed with perfluorotributylamine (PFTBA).

170 ## 2.4. Data analysis

171 The data acquisition and processing of all samples were done with ChromaTOF® (LECO Corp., v 4.72).
172 For the data processing the S/N threshold ratio was 50, the baseline offset was 1 (just above the noise),
173 1-D and 2-D peak widths reference values were 12 s and 0.125 s, respectively. The detected peaks were

tentatively identified by a forward search using the NIST 2017 database, with a minimum similarity of 700 and a linear retention index window of 20 [33].

For the FFD calculations, the response was calculated as the total area of four different classes, using a quant mass of 93 m/z or 91 m/z:

- Hydrocarbon monoterpenoids (HCMT, $C_{10}H_{14}$ / $C_{10}H_{16}$ / $C_{10}H_{18}$)
- Oxygenated monoterpenoids (OXMT, $C_{10}H_{14}O$ / $C_{10}H_{16}O$ / $C_{10}H_{18}O$)
- Hydrocarbon sesquiterpenoids (HCST, $C_{15}H_{22}$ /$C_{15}H_{24}$/ $C_{15}H_{26}$)
- Oxygenated sesquiterpenoids (OXST, $C_{15}H_{22}O$ / $C_{15}H_{24}O$ / $C_{15}H_{26}O$)

The response was then imported in Minitab LLC (State College, PA, U.S.A) and further processed for the generation of the Pareto charts and the main effect plots.

The samples (pine resin and hide glue) measured with the optimised DHS method were imported in Statistical Compare (LECO Corp.) and divided in two classes: samples and blank. Then, the chromatograms were processed, and peak alignment was performed. For the data processing the S/N ratio was 50, baseline offset was 1 (just above the noise), 1-D and 2-D peak width reference values were 20 s and 0.25 s, respectively. Area calculations were performed using unique masses and peaks were aligned if the spectra match was 650 or higher and if the peaks were not more than 2 modulation periods apart. If peaks were not found with the first peak finding, the S/N ratio was reduced to 20. Subsequently, the Fischer ratio (FR) was calculated, all analytes with a FR > FR_{crit} (5.59) or which were undefined, were kept and used for generating the peak table. This approach was already found successful in defining which analytes were only present in the samples and are thus discriminatory and which were not [17]. The aligned peak table was exported to Excel and principal component analysis (PCA) was calculated based on the unique masses of the peaks present in the table. The PCA was performed in MetaboAnalyst6.0 [37].

Some visual post-processing has been used to change the colours in order to enhance the readability of the Pareto charts, main effect plots and to highlight the chemical classes in the pine resin chromatogram.

3. Results and discussion

3.1. Optimisation - full factorial design

The DHS was optimised using a FFD approach for four parameters, selected based on existing literature [27]: incubation temperature (T_{incu}, °C), incubation time ($time_{incu}$, min), purge volume (V_{purge}, mL) and purge flowrate (F_{purge}, $mL.min^{-1}$). The total number of FFD runs was nineteen, 2^4 plus three centre

points. For the minimal and maximum values of the four parameters see Table 1. The FFD was conducted on pine resin and the response was the total area of the four selected chemical classes: HCMT, OXMT, HCST and OXST. Because the method needs to be versatile and resilient to sample and concentration diversity, it has been decided to optimise the response parameters for four groups with different concentration and volatility range. The RSD of the centre points for each group was 17% (HCMT), 32% (OXMT), 37 (HCST), 22% (OXST), which is in the expected range for DHS. A closer look to the RSD of individual analytes shows a higher variation (see peak table of pine resin in Support Information 2 (SI2)). Working with chemical family instead of individual markers had many advantages. In addition to minimise the RSD, it also makes the optimisation more versatile prior to identifying potential marker of interest at a later stage. Finally, the focus on different type of terpenoids makes this work easily transferable to the characterization of the headspace from other natural substances.

Table 1. The minimum and maximum values of the parameters investigated during the FFD runs.

Parameter	Min value	Max value
$\mathbf{T_{inc}}$	30°C	50°C
$\mathbf{time_{inc}}$	10 min	30 min
$\mathbf{V_{purge}}$	300 mL	600 mL
$\mathbf{F_{purge}}$	15 mL.min^{-1}	30 mL.min^{-1}

The values of the parameters and the responses for each experimental run are displayed in Table S1 (see SI1). In Fig. 1, a Pareto chart is depicted for each chemical group, the pink line represents the 95% confidence interval, every value that crosses this line has a significant influence on the response. For HCMT and HCST, none of the parameters have a significant influence on the response. On the other hand, the temperature has a major influence for OXMT and for OXST almost all parameters have a significant influence on the extraction. In Fig. 2, the main effect plots of each parameter on the total response per group are shown. Combining Fig. 1 and Fig. 2, the parameters which have a large impact on the total area can been understood. T_{incu} of 50°C is selected, this gives the highest response for all except HCMT. However, T_{incu} has an non-significant influence on the total response for HCMT, therefore the decrease in response is found minimal between 40°C and 50°C. Moreover, OXMT and OXST might even benefit from a higher T_{incu}, as the plateau of the response has not been reached. However, to keep the integrity of the resin, the temperature cannot be raised above 50°C [26]. For the other three parameters, the highest response is found with the same values for the HCMT, OXMT and HCST classes, namely: $time_{incu}$ of 20 min, the V_{purge} of 450 mL and F_{purge} of 22.5 mL.min^{-1}.

The selected values might not be optimal for the OXST class, but the response for this class was minimal and therefore even a slight change in total area resulted in a larger relative response change. It is not surprising that the response for OXST is very minimal, as these compounds are the least volatile and are more difficult to extract with DHS. They would benefit from a higher temperature, longer incubation and extraction time and a faster purge flow.

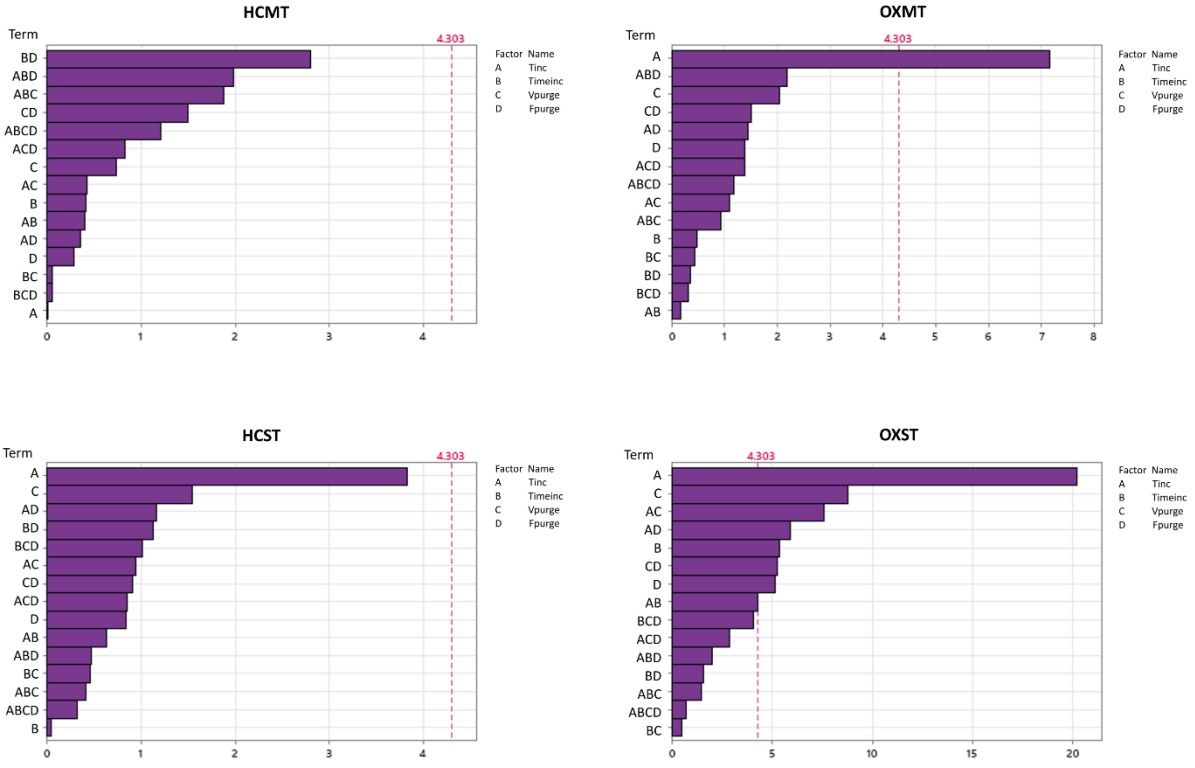


Fig. 1. The pareto charts depicting the effect of the extraction parameters have on the response, each chemical group has its own chart: HCMT, OXMT, HCST and OXST. If a standardised effect crosses the pink line, this effect has a significant influence on the response. The alpha is 0.05.

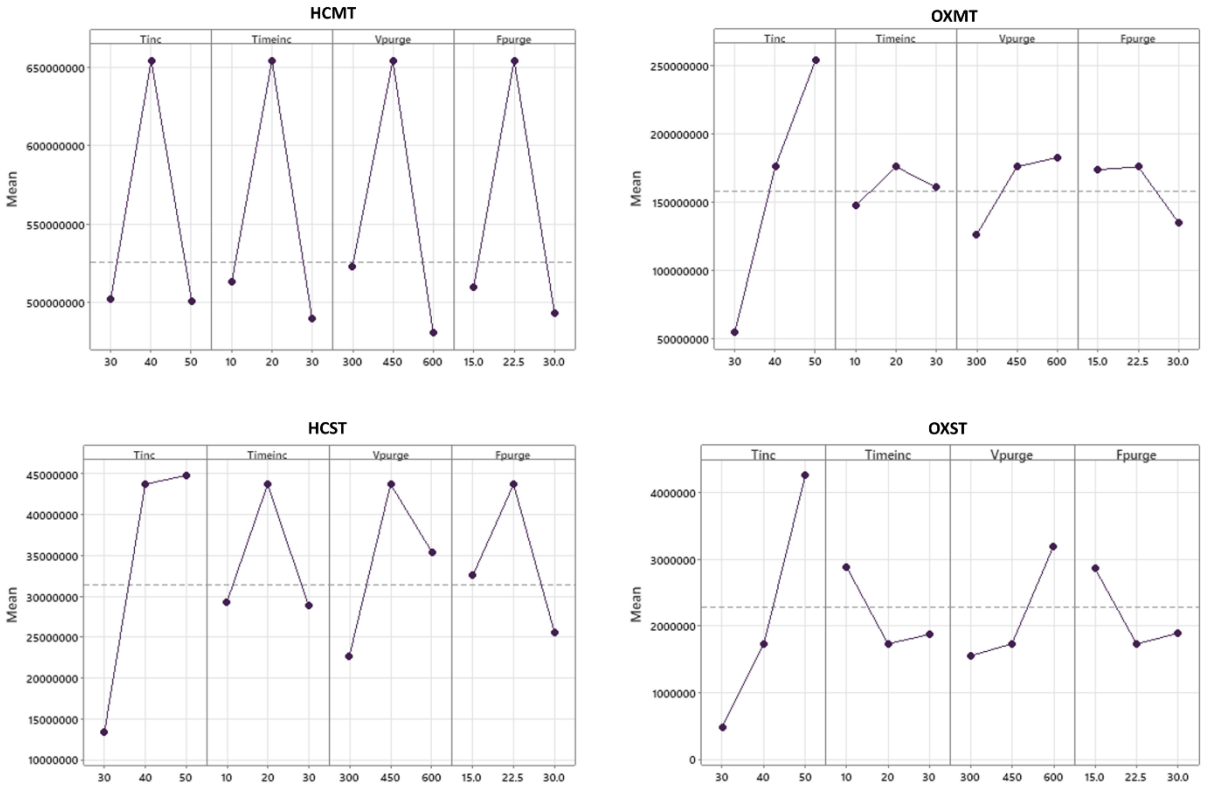
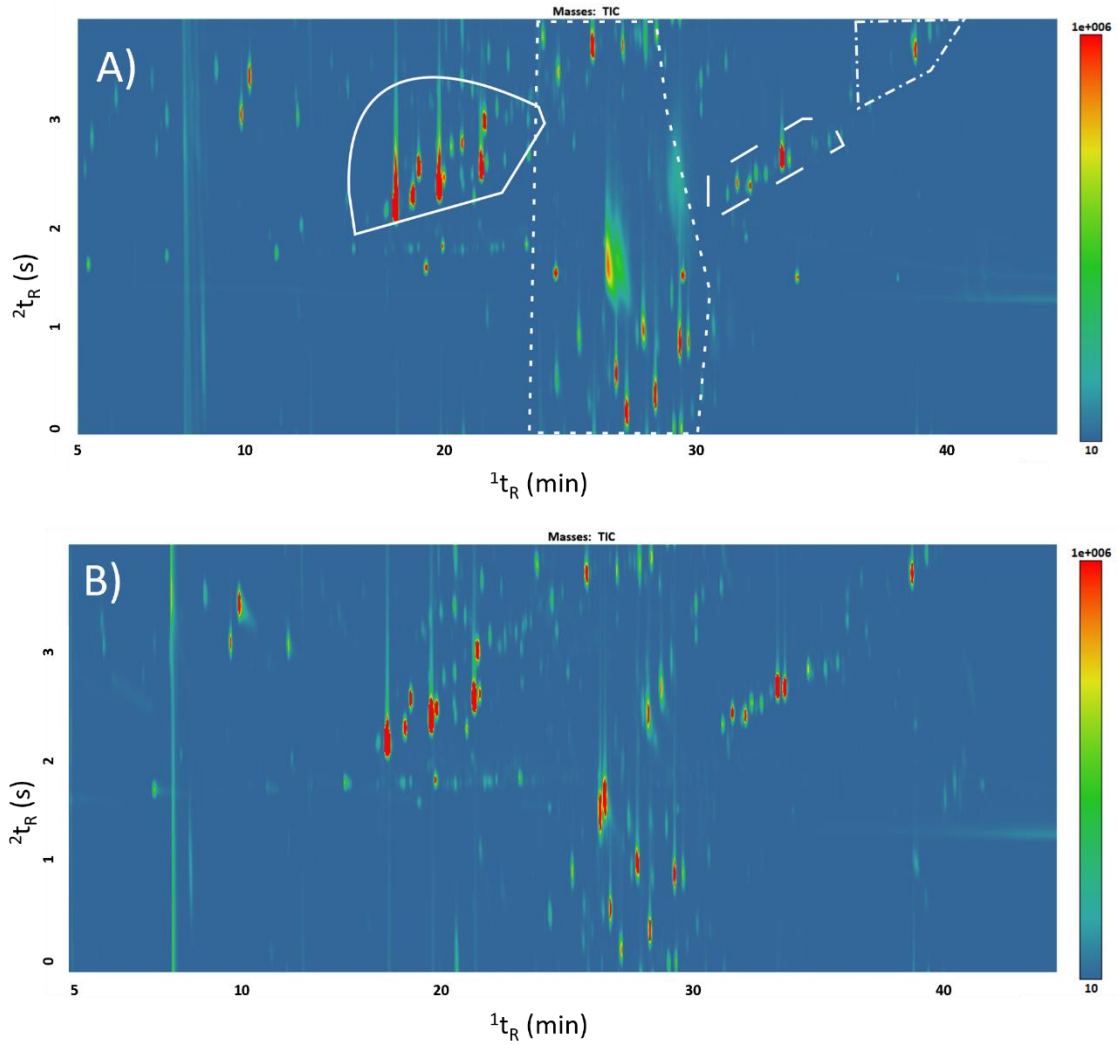


Fig. 2. The main effect plots show the mean response for each value of each parameter per class: HCMT, OXMT, HCST, OXST.

3.2. Adhesives measurement

After optimisation of the DHS parameters, a sample of pine resin and hide glue were extracted with DHS (Fig. 3A and 4). The adhesives were selected to cover a broad range of possible prehistoric adhesives and to evaluate if the DHS method was universal. For this reason, the analytes were divided in four different chemical classes; Terpenoids (HCMT, OXMT, HCST, OXST), Hydrocarbons, Aromatics, Others.

Some compounds could be classified in two classes and in such cases, one class was selected as the main class (e.g., p-cymene was put in terpenoid class as it elutes closer to the terpenoids than the other aromatics). Moreover, upon further processing of the data, terpenoids with two oxygen containing functional groups were also placed in the terpenoid class, even though they were kept out of it during the optimisation response calculations. Due to the difficulties of assigning a unique name to all peaks, the recommendation of the Metabolomic Standards Initiative was followed [38]. Since there are no LRI in the NIST library for a semi-polar column as used in this research, only the chemical group was assigned. The only names assigned were terpenoids matching with the used standard. See the final peak tables in Support Information 2 (SI2), they are also uploaded in the open-source database of Harvard Dataverse.



261

262 *Fig. 3. A) Chromatogram of pine resin, HS extracted with DHS, four classes highlighted, straight line: HCMT, dash line: OXMT,*
263 *long dash line: HCST, dash dot line: OXST. B) Chromatogram of pine resin, HS extracted with HS-SPME. Note the higher intensity*
264 *of the upper chromatogram and the increased number of peaks, especially in the HCMT and OXMT groups.*

265

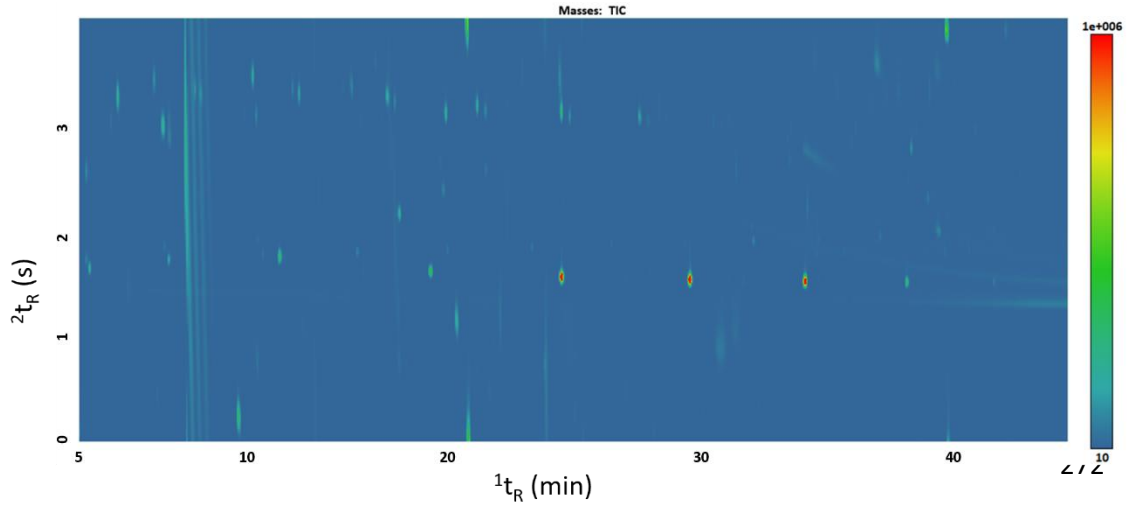


Fig. 4. Chromatogram of hide glue, HS extracted with DHS.

Additionally, for some analytes the RSD on the triplicates measurements was very high (>75%), this issue was also addressed in section 3.1. during the optimisation of the DHS parameters. This might be resulting from the intrinsic variability of the sample itself. Pine resin and hide glue are natural substances which are not homogeneous. Homogenising the samples is difficult as the samples have to be melted and stirred, this means a loss of volatiles and it is difficult, if not impossible, to predict the change of the VOCs profile due to heat treatment. Hence, it was decided not to homogenise the samples. Measuring a range of different natural adhesives will provide more knowledge about the general composition of the adhesives. This issue may also be overcome by identifying the adhesive not based on individual compounds but on the composition of chemical classes. Another advantage of identifying by chemical classes is that less knowledge about the preservation of certain molecules is required. This knowledge is still not known for most adhesives and research to the degradation pathways of prehistoric adhesives is limited.

Furthermore, natural occurring compounds are often present in a variety of species but in different concentrations. This can pose problems when trying to identify a species purely based on the presences of biomarkers. As proposed in earlier work an untargeted approach based on statistical calculations of the intensities of the compounds found in the adhesives might better [25]. Especially for artefacts of which the formation and degradation history is not known. Instead of looking of a few known compounds, the overall fingerprint is taken into account. Differences in intensity of the present compounds are visualised with statistical calculations such as PCA. This approach was also used in this

296 work, a PCA was calculated based on the generated peak table for pine resin and hide glue (Fig. 5). In
297 Fig. 5, both adhesives are nicely separated, and the triplicates are in proximity of each other, indicating
298 that the overall standard deviation is acceptable. The PCA shows that untargeted alignment of the
299 peak tables might be a good start for the identification of the adhesives. Evidently, this comparison
300 only stands for modern adhesives, in future research the change of the VOC profile during ageing has
301 to be investigated.

302

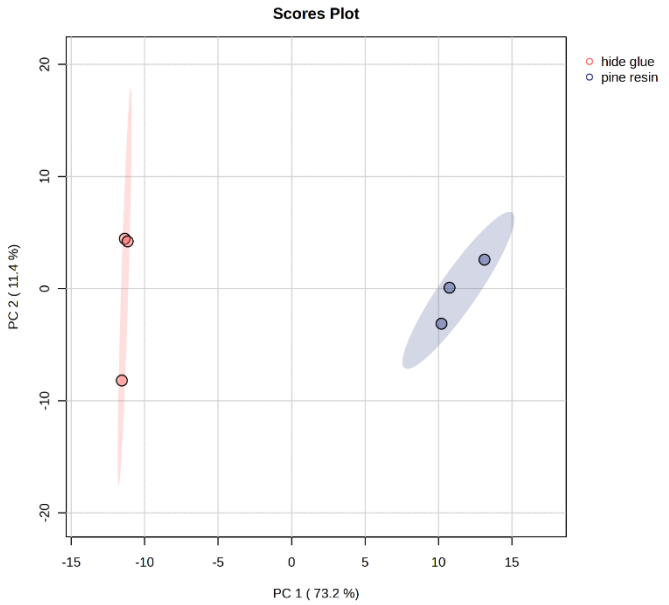


Fig. 5. PCA of the principal components 1 and 2 of pine resin and hide glue, the 95% interval is represented by the oval around the dots.

3.3. HS-SPME

3.3.1. Optimisation HS-SPME

The HS-SPME parameters were based on earlier research [17,25]. Nonetheless, a few injections with a different incubation temperature and extraction time were conducted to see if the chosen parameters were indeed optimal for the extraction. The tested incubation temperatures were 30°C, 40°C and 50°C. Similarly with the DHS extraction, an incubation temperature of 50°C was deemed to give the best results. For the extraction time, 20 and 25 minutes were assessed, the shorter extraction resulted in a significant decrease of the signal and therefore 25 minutes was chosen. 25 minutes is longer than the extraction time used in previous researched, but it gave the optimal results for the current measurements. For all the parameters see section 2.2.1.

3.3.2. Comparison DHS vs. HS-SPME

A 5 mg pine resin sample was analysed using HS-SPME and DHS for extraction, in triplicate for both techniques, see Fig. 3 and Table 2. The terpenes standard was used to calculate the RSD of both techniques, see Table 2. The RSD show the same trend for both HS-SPME and DHS, HCMT has the lowest RSD while HCST has the highest RSD. The other two classes have a similar RSD. Moreover, the

RSD for DHS is more or less three times higher than for HS-SPME, this is in accordance with literature [29,36]. In order to compare the two methods better, the total area was calculated based on the four terpenoids groups identified earlier and with a quantitative mass of 91 or 93 m/z. The main difference between these extractions is the higher sensitivity of DHS over HS-SPME, especially for the oxygenated, and less volatile, classes the total area almost doubled for DHS. This is important, as prehistoric adhesives have lost most of their very volatile fraction and only semi-volatiles are still present in the sample.

Table 2. The average total area and RSD (in percentage) per chemical group for pine resin detected with DHS and HS-SPME. The RSD is calculated with the terpenes standard.

	DHS		**HS-SPME**	
	Total area	**RSD**	**Total area**	**RSD**
HCMT	7.13E+08	11%	5.51E+08	5%
OXMT	4.57E+08	36%	2.46E+08	10%
HCST	1.52E+08	43%	9.99E+07	16%
OXST	2.15E+07	37%	9.84E+06	9%

Another important advantage of DHS over HS-SPME is the robustness of DHS. The SPME fibre is fragile and there is a risk of losing the sorbent during sampling, and hence, a loss of the sample. For most applications this is not a major issue as the sample can be remeasured, but when it comes to unique prehistoric artefacts, the loss of sample is of much higher concern. Moreover, if the fibre coating is accidentally lost while inside the vial, this might contaminate the artefact. DHS does not have a high risk of losing the sorbent and no risk of contaminating the artefact as the sorbent is inside a tube that stands apart of the sample vial. A downside of DHS is that the RSDs are higher, even if acceptable, on the present samples, and it is a more complex extraction technique.

The extraction time was comparable for both methods. For HS-SPME, the total extraction time was 35 min (10 min incubation and 25 min extraction), while for DHS the total time was 40 min (20 min incubation and 20 min trapping). For DHS, the TDU injection time comes on top of that, which was around 7 minutes. This results in an extra time of 12 minutes for DHS compared to HS-SPME. Moreover, both HS-SPME and DHS are fully automated, and the extraction can take place during the measuring time of the GC instrument. The extraction time is fast compared to the traditional liquid extractions, which takes several hours to complete, and it is not possible to fully automate the extraction.

4. Conclusions

A new identification method for Palaeolithic adhesives was investigated, DHS-GC×GC-TOFMS. DHS is a sampling technique which traps VOCs present in the HS of a sample and has great potential for archaeological applications. In the case of hafted stone tools, the adhesive can be characterised without the need to remove the adhesive from the stone tool and no complicated sample preparation is required. The preservation of the adhesive on the stone tool creates opportunities for future generations to investigate the adhesive with better instrumentations.

The DHS extraction is optimised via a FFD approach with pine resin as a proxy for prehistoric adhesives. Four parameters were selected (T_{inc}, $time_{incu}$, V_{purge}, F_{purge}) and four chemical classes were identified for the response ((HCMT), (OXMT), (HCST), (OXST)). The selected parameters were as follows: T_{inc}: 50°C, $time_{incu}$: 20 min, V_{purge}: 450 mL, F_{purge}: 22.5 $mL.min^{-1}$.

Furthermore, the DHS method was compared against an HS-SPME-GC×GC-TOFMS method, which was partly based on previous research and partly optimised with a OVAT approach [17,25]. Indeed, DHS had a higher sensitivity than HS-SPME, when measuring a pine resin sample. This is promising for the characterisation of prehistoric artefacts with DHS-GC×GC-TOFMS.

A downside of using HS methods is that the traditional biomarker approach cannot be applied when identifying prehistoric adhesives as the established biomarkers are not volatile enough to be present in the HS. Therefore, a new way to identify the adhesives needs to be investigated. In this research, an untargeted approach to generate a peak table combined with a statistical analysis (PCA) was employed to test the identification abilities of VOCs present in the HS between pine resin and hide glue. The resulting peak tables are stored in an open access database, in the future more peak tables of adhesives will be added including artificially aged adhesives [26]. Although every archaeological artefact is unique and has a different degradation pathway [39], artificially degraded adhesives might help to understand the differences in the VOCs profile of a prehistoric adhesive. This database will

serve as a library for VOCs detected in natural adhesives and can be used for comparison of 'unknowns' peak tables, for example, a peak table resulted from an archaeological artefact.

Acknowledgments

We would like to thank Christian Lepers (Traceolab) for providing the pine resin and hide glue samples. We would also like to thank Restek® Corp. for consumables support this research received.

Conflict of Interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding statement

This research was funded by the ARC-project GLUE (PI: V. Rots) funded by the University of Liège through the collaborative research actions (Actions de Recherche Concertée). V. Rots is also indebted to the F.R.S-FNRS.

References

[1] Rots, V., Insights into early Middle Palaeolithic tool use and hafting in Western Europe. The functional analysis of level IIa of the early Middle Palaeolithic site of Biache-Saint-Vaast (France). *J. Archaeol. Sci.* 2013, 40, 497–506.

[2] Rots, V., Peer, P. V., Early evidence of complexity in lithic economy: core-axe production, hafting and use at Late Middle Pleistocene site 8-B-11, Sai Island (Sudan). *J. Archaeol. Sci.* 2006, 33, 360–371.

[3] Mazza, P. P. A., Martini, F., Sala, B., Magi, M., Colombini, M. P., Giachi, G., Landucci, F., Lemorini, C., Modugno, F., Ribechini, E., A new Palaeolithic discovery: tar-hafted stone tools in a European Mid-Pleistocene bone-bearing bed. *J. Archaeol. Sci.* 2006, 33, 1310–1318.

[4] Pawlik, A. F., Thissen, J. P., Hafted armatures and multi-component tool design at the Micoquian site of Inden-Altdorf, Germany. *J. Archaeol. Sci.* 2011, 38, 1699–1708.

[5] Boëda, É., Bonilauri, S., Connan, J., Jarvie, D., Mercier, N., Tobey, M., Valladas, H., Sakhel, H. A., New Evidence for Significant Use of Bitumen in Middle Palaeolithic Technical Systems at Umm el Tlel (Syria) around 70,000 BP. *Paléorient* 2008, 34, 67–83.

[6] Degano, I., Soriano, S., Villa, P., Pollarolo, L., Lucejko, J. J., Jacobs, Z., Douka, K., Vitagliano, S., Tozzi, C., Hafting of Middle Paleolithic tools in Latium (central Italy): New data from Fossellone and Sant'Agostino caves. *PLoS ONE* 2019, 14, 1–29.

[7] Charrié-Duhaut, A., Porraz, G., Cartwright, C. R., Igreja, M., Connan, J., Poggenpoel, C., Texier, P. J., First molecular identification of a hafting adhesive in the Late Howiesons Poort at Diepkloof Rock Shelter (Western Cape, South Africa). *J. Archaeol. Sci.* 2013, 40, 3506–3518.

[8] Wragg Sykes, R. M., in: Coward, F., Hosfield, R., Pope, M., Wenban-Smith, F. (Eds.), Settlement, Society and Cognition in Human Evolution. Cambridge University Press 2015, pp. 117–137.

[9] Wadley, L., Hodgskiss, T., Grant, M., Implications for complex cognition from the hafting of tools with compound adhesives in the Middle Stone Age, South Africa. *PNAS* 2009, 106, 9590–9594.

[10] Chen, S., Vahur, S., Teearu, A., Juus, T., Zhilin, M., Savchenko, S., Oshibkina, S., Asheichyk, V., Vashanau, A., Lychagina, E., Kashina, E., German, K., Dubovtseva, E., Kriiska, A., Leito, I., Oras, E.,

425 Classification of archaeological adhesives from Eastern Europe and Urals by ATR-FT-IR spectroscopy
426 and chemometric analysis. *Archaeometry* 2021, 1–18.

427 [11] Bradtmöller, M., Sarmiento, A., Perales, U., Zuluaga, M. C., Investigation of Upper Palaeolithic
428 adhesive residues from Cueva Morín, Northern Spain. *J. Archaeol. Sci. Rep.* 2016, 7, 1–13.

429 [12] Villa, P., Soriano, S., Tsanova, T., Degano, I., Higham, T. F. G., D'Errico, F., Backwell, L., Lucejko, J.
430 J., Colombini, M. P., Beaumont, P. B., Border Cave and the beginning of the Later Stone Age in South
431 Africa. *PNAS* 2012, 109, 13208–13213.

432 [13] Regert, M., Investigating the history of prehistoric glues by gas chromatography-mass
433 spectrometry. *J. Sep. Sci.* 2004, 27, 244–254.

434 [14] Cârciumaru, M., Ion, R. M., Niţu, E. C., Ştefânescu, R., New evidence of adhesive as hafting
435 material on Middle and Upper Palaeolithic artefacts from Gura Cheii-Râşnov Cave (Romania). *J.*
436 *Archaeol. Sci.* 2012, 39, 1942–1950.

437 [15] Nardella, F., Duce, C., Ribechini, E., Analytical pyrolysis and thermal analysis to chemically
438 characterise bitumen from Italian geological deposits and Neolithic stone tools. *J. Anal. Appl. Pyrol.*
439 2021, 158, 105262.

440 [16] Evershed, R. P., Organic residue analysis in archaeology: The archaeological biomarker
441 revolution. *Archaeometry* 2008, 50, 895–924.

442 [17] Perrault, K. A., Stefanuto, P. H., Dubois, L., Cnuts, D., Rots, V., Focant, J. F., A new approach for
443 the characterization of organic residues from stone tools using GC×GC-TOFMS. *Separations* 2016, 3,
444 8–10.

445 [18] Niekus, M. J. L. T., Kozowyk, P. R. B., Langejans, G. H. J., Ngan-Tillard, D., van Keulen, H.,
446 van der Plicht, J., Cohen, K. M., van Wingerden, W., van Os, B., Smit, B. I., Amkreutz, L. W. S. W.,
447 Johansen, L., Verbaas, A., Dusseldorp, G. L., Middle paleolithic complex technology and a Neandertal
448 tar-backed tool from the Dutch North Sea. *PNAS* 2019, 116, 22081–22087.

449 [19] Regert, M., Alexandre, V., Thomas, N., Lattuati-Derieux, A., Molecular characterisation of birch
450 bark tar by headspace solid-phase microextraction gas chromatography-mass spectrometry: A new
451 way for identifying archaeological glues. *J. Chromatogr. A* 2006, 1101, 245–253.

[20] Hamm, S., Bleton, J., Tchapla, A., Headspace solid phase microextraction for screening for the presence of resins in Egyptian archaeological samples. *J. Sep. Sci.* 2004, 27, 235–243.

[21] Sithersingh, M. J., Snow, N. H., Headspace gas chromatography. *Gas Chromatography* 2021, 251–265.

[22] Manzini, S., Durante, C., Baschieri, C., Cocchi, M., Sighinolfi, S., Totaro, S., Marchetti, A., Optimization of a Dynamic Headspace - Thermal Desorption - Gas Chromatography/Mass Spectrometry procedure for the determination of furfurals in vinegars. *Talanta* 2011, 85, 863–869.

[23] Stefanuto, P. H., Perrault, K. A., Dubois, L. M., L'Homme, B., Allen, C., Loughnane, C., Ochiai, N., Focant, J. F., Advanced method optimization for volatile aroma profiling of beer using two-dimensional gas chromatography time-of-flight mass spectrometry. *J. Chromatogr. A* 2017, 1507, 45–52.

[24] Bonaduce, I., Ribechini, E., Modugno, F., Colombini, M. P., Analytical approaches based on gas chromatography mass spectrometry (GC/MS) to study organic materials in artworks and archaeological objects. *Top. Curr. Chem.* 2016, 374, 1–37.

[25] Perrault, K. A., Dubois, L. M., Cnuts, D., Rots, V., Focant, J.-F., Stefanuto, P.-H., Characterization of hafting adhesives using comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry. *Sep. Sci. plus* 2018, 1, 726–737.

[26] Cnuts, D., Perrault, K. A., Stefanuto, P. H., Dubois, L. M., Focant, J. F., Rots, V., Fingerprinting Glues Using HS-SPME GC×GC–HRTOFMS: a New Powerful Method Allows Tracking Glues Back in Time. *Archaeometry* 2018, 60, 1361–1376.

[27] Vyviurska, O., Hanobiková, M., Gomes, A. A., Špánik, I., Multivariate optimization of dual-sorbent dynamic headspace extraction of volatiles in wine analysis. *Food Chem.* 2021, 365, 130449.

[28] Zhang, P., Carlin, S., Lotti, C., Mattivi, F., Vrhovsek, U., On sample preparation methods for fermented beverage VOCs profiling by GCxGC-TOFMS. *Metabolomics* 2020, 16, 102.

[29] Diez-Simon, C., Ammerlaan, B., Berg, M. van den, Duynhoven, J. van, Jacobs, D., Mumm, R., Hall, R. D., Comparison of volatile trapping techniques for the comprehensive analysis of food flavourings by Gas Chromatography-Mass Spectrometry. *J. Chromatogr. A* 2020, 1624, 461191.

[30] Han, B., Lob, S., Sablier, M., Benefit of the Use of GCxGC/MS Profiles for 1D GC/MS Data Treatment Illustrated by the Analysis of Pyrolysis Products from East Asian Handmade Papers. *J. Am. Soc. Mass Spectrom.* 2018, 29, 1582–1593.

[31] Franchina, F. A., Zanella, D., Dubois, L. M., Focant, J., The role of sample preparation in multidimensional gas chromatographic separations for non-targeted analysis with the focus on recent biomedical, food, and plant applications. *J. Sep. Sci.* 2021, 44, 188–210.

[32] Patterson, D. G., Welch, S. M., Turner, W. E., Sjödin, A., Focant, J.-F., Cryogenic zone compression for the measurement of dioxins in human serum by isotope dilution at the attogram level using modulated gas chromatography coupled to high resolution magnetic sector mass spectrometry. *J. Chromatogr. A* 2011, 1218, 3274–3281.

[33] Stefanuto, P.-H., Smolinska, A., Focant, J.-F., Advanced chemometric and data handling tools for GC×GC-TOF-MS Application of chemometrics and related advanced data handling in chemical separations. *TrAC - Trends Anal. Chem.* 2021, 139, 116251.

[34] Mommers, J., Wal, S. van der, Column Selection and Optimization for Comprehensive Two-Dimensional Gas Chromatography: A Review. *Crit. Rev. Anal. Chem.* 2021, 51, 183–202.

[35] Mondello, L., Tranchida, P. Q., Dugo, P., Dugo, G., Comprehensive two-dimensional gas chromatography-mass spectrometry: A review. *Mass. Spec. Rev.* 2008, 27, 101–124.

[36] Franchina, F. A., Zanella, D., Dejong, T., Focant, J. F., Impact of the adsorbent material on volatile metabolites during in vitro and in vivo bio-sampling. *Talanta* 2021, 222, DOI: 10.1016/j.talanta.2020.121569.

[37] Xia, J., Psychogios, N., Young, N., Wishart, D. S., MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Res.* 2009, 37, W652–W660.

[38] Salek, R. M., Steinbeck, C., Viant, M. R., Goodacre, R., Dunn, W. B., The role of reporting standards for metabolite annotation and identification in metabolomic studies. *Gigascience* 2013, 2, 1–3.

[39] Nigra, B. T., Faull, K. F., Barnard, H., Analytical chemistry in archaeological research. *Anal. Chem.* 2015, 87, 3–18.

Support information

Additional supporting information can be found online in the Supporting Information section at the end of this article.