# Title
Breaking the secret of prehistoric stone tools design using multidimensional chromatography

# Key words

# Abstract

Hafting relies on adding a handle to a stone tool, often with the use of an organic glue (plant or animal based).[1] Understanding the hafting processes used during prehistory gives us an indication of the human technical capacity during those times, as it requires abstract thinking and some control of fire.[2] Here dynamic headspace with two-dimensional gas chromatography coupled to a time-of-flight mass spectrometer (DHS-GCxGC-TOFMS) is applied for the characterization of pine resin and hide glue. The DHS parameters are optimized using a design of experiment approach. Several figures of merits were evaluated for quality assessment and the DHS method was challenged against an existing headspace-solid phase micro extraction (HS-SPME) – GCxGC-TOFMS approach.[3]

Generally, DHS was found more sensitive than HS-SPME, but the relative standard deviation was sensibly higher. With this method, a first step in a new, minimal invasive chemical identification method for prehistoric glues and resins is established.

# Experimental

*Samples*

A 5 mg pine resin sample was weighted in a 20 mL vial for each run. For the hide glue measurements, a 10 mg piece of dried hide glue was inserted in a 20 mL vial and 5 µL of MilliQ water was added, after a few hours this formed a gluey substance. The headspace measurements were performed on this gluey substance. Pine resin was chosen as a good representative of the tree resins available in the prehistory, hide glue was chosen as a good representative for the animal glues.[4]

*Headspace techniques*

Both the DHS and the SPME techniques were performed with a MuliPurposeSampler autosampler (MPS, Gerstel K.K), connected to the gas chromatography system.

The DHS parameters selected for optimization were incubation time ($t_{incu}$), incubation temperature ($T_{incu}$), purge flow ($F_{purge}$) and purge volume ($V_{purge}$), see Table 1. The optimized parameters were $t_{incu}$: 20 min, $T_{incu}$: 50°C, $F_{purge}$: 22.5 mL.min$^{-1}$ and $V_{purge}$: 450 mL. A TD tube filled with Tenax TA was used to trap the volatiles, the trap temperature was 20°C below the $T_{incu}$, expect for $T_{incu}$ 30°C, then the trap temperature was 20°C. The needle temperature was set at 120°C. The parameters were optimized via a full factorial design (FFD) with center point measurement in triplicate (a total of 19 runs). The samples were desorbed in the inlet by thermal desorption united (TDU) with a cooled injector system (CIS) The initial temperature of the TDU was set at 40°C and heated up to 280°C with 300°C.min$^{-1}$ and hold for 3 min. The CIS started at -20°C, hold time of 0.02 s, and was heated up to 250°C at 12°C.min$^{-1}$ and hold for 3 min. The TDU was operated in splitless mode, and the analytes were trapped on a liner filled with Tenax TA at -20°C. The CIS was operated in solvent vent mode.

For SPME a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco®, Bellefonte, PA, USA) fibre was used. The incubation time was 10 min at 50°C, the fibre was exposed for 25 min with a penetration depth of 43 mm, there was no agitation during the incubation and absorption. Subsequently, the fibre was desorbed in the inlet with a penetration depth of 54 mm at 270°C for 180 s, split ratio was 5:1. The gas chromatography and mass spectrometer method where the same for both techniques, see below.

*GCxGC-TOFMS analyses*

All samples were measured with a Pegasus GC-4D (LECO® Corporation, St. Joseph, MI, USA) GCxGC-TOFMS equipped with a secondary oven and a quad-jet, dual stage thermal modulator. The first dimension ($^1$D) column is a semi-polar Rxi-624silMS (30 m x 0.25 mm i.d 1.4 µm df) and the second dimension ($^2$D) column is a polar Stabilwax (2 m x 0.25 mm i.d 0.5 µm df). The connection between the columns was made with a SilTite µ-union (Trajan Scientific and Medical®, Australia). The carrier gas was high purity helium (Alphagaz 2, Air Liquide®,Liège, Belgium), and the flow rate was 1 mL min$^{-1}$. The temperature program was 40°C - 240°C with 5°C min$^{-1}$, the $^2$D oven followed the $^1$D temperature program. The modulator off-set was 15°C and the modulator period was 4 s, with a 0.60 s hot pulse time. The MS transfer line was 250°C and the MS ion source was kept at 230°C. The mass range was 35-550 m/z, the acquisition rate was 200 Hz, the electron ionisation was 70 eV and an acquisition delay of 300 s was used.

*Data analysis and statistical calculations.*

Data acquisition, alignment and processing were done with ChromaTOF (LECO Corp., v 4.72). For the data processing the signal-to-noise (S/N) ratio was set at 50. The detected peaks were tentatively identified by a forward search using the NIST 2017 database (minimum similarity 700).
The statistical analysis for the DoE were performed with Minitab LLC (State College, PA, U.S.A). The following chemical classes were used to establish the DoE: Hydrocarbon monoterpenoids (HCMT, $C_{10}H_{16}$ / $C_{10}H_{14}$ / $C_{10}H_{18}$), Oxygenated monoterpenoids (OXMT, $C_{10}H_{16}O$ / $C_{10}H_{14}O$ / $C_{10}H_{18}O$), 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 quantity mass selected was 93 m/z or 91 m/z for all the analytes. The selection of variety of chemical markers is critical for optimization of untargeted methods. They have to be representative of the expected markers.

Table 1. The parameters used for DoE with the minimum and maximum values

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

# Results and Discussion

*Design of Experiment*

The DHS method was optimized by a design of experiment, in Fig. 1 the pareto chart of the four classes is depicted. The red line gives the 95% confidence interval, all values left of the line are not significant while all values at the right are significant. The pareto charts of HCMT and HCST shows that none of the parameters crosses the 95% confidence interval line, thus none of the investigated parameters and their interactions have significant influence on the response. While, for OXMT and OXST the incubation temperature has the most significant influence on the extraction (see Fig. 1.).
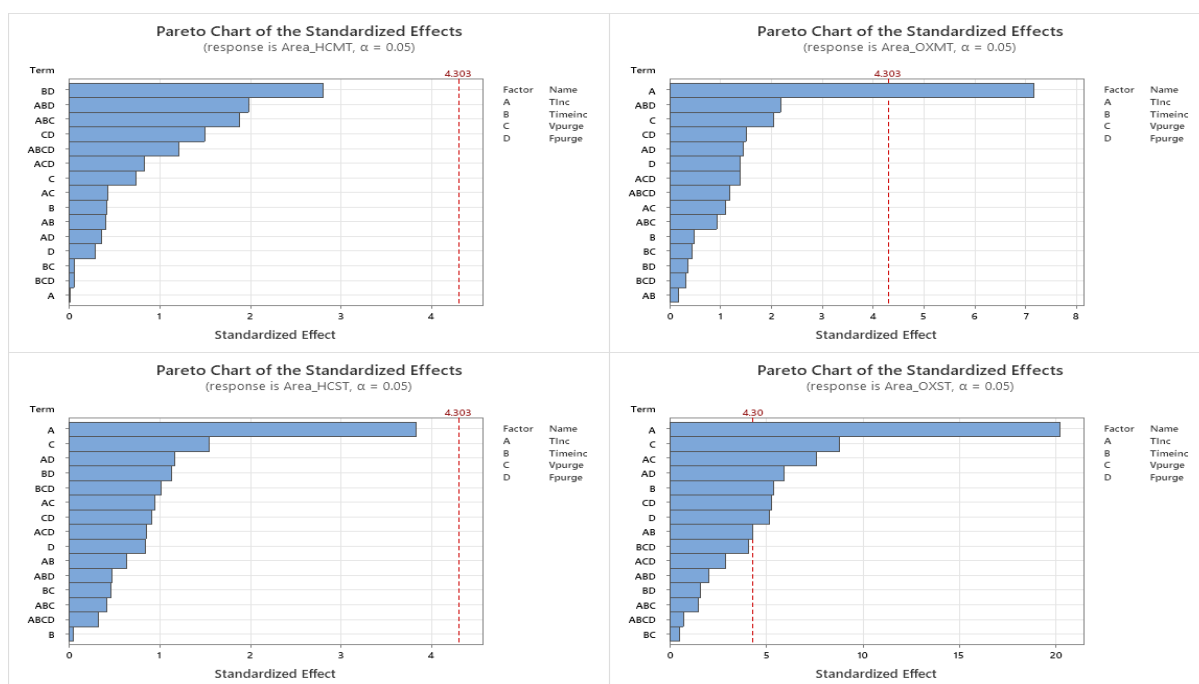


Fig. 1. The pareto charts of the four chemical classes selected for analysis.

Fig. 2 depicts the response (total area) per class for all the parameters assessed in the nineteen runs. An incubation temperature of 50°C gives the best results for all classes expect for HCMT. OXMT and OXST might even benefit from a higher temperature, but in order to preserve the integrity of the resin a higher temperature is not possible (melting point of resins lies around 60°C). An incubation time of 20 min, a purge volume of 450 mL and a purge flow of 22.5 mL.min$^{-1}$ gives the best results for all except for the OXST. However, the optimal parameters for OXST result in less-than-optimal extraction of the other four classes, therefore, the parameters which are sub-optimal for only OXST were chosen.
In conclusion, the selected parameters for the optimized DHS method are: $T_{inc}$:50°C, $t_{incu}$: 20 min, $V_{purge}$: 450 mL, $F_{purge}$: 22.5 mL.min$^{-1}$.
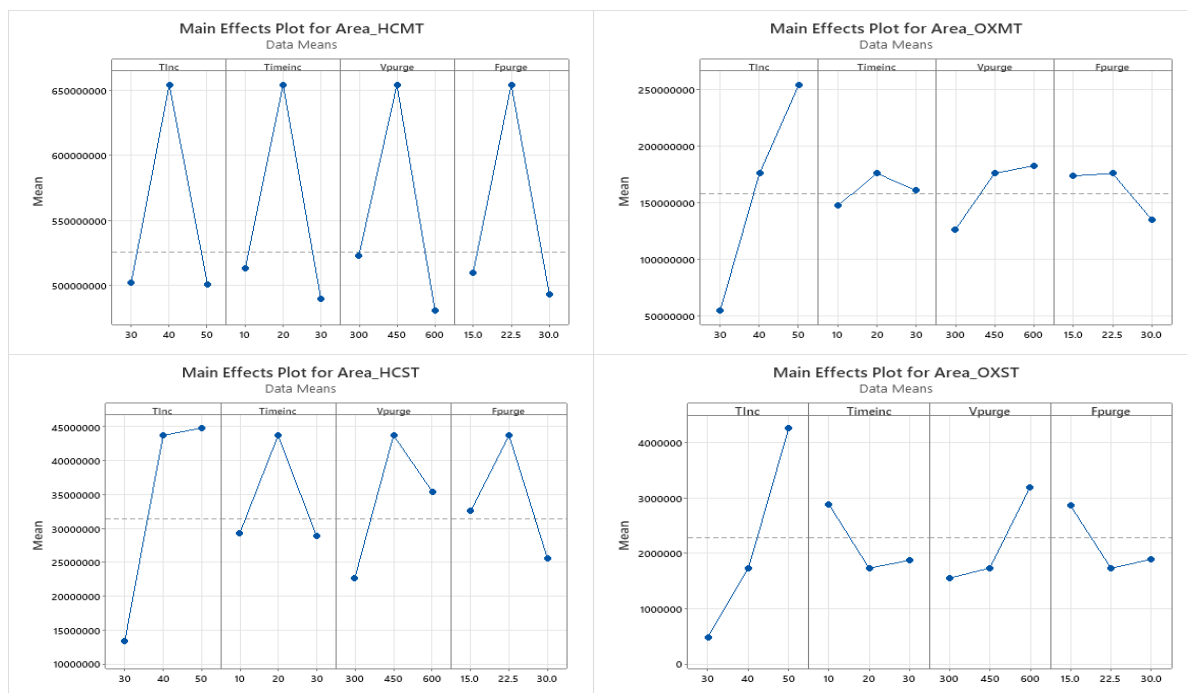
Fig. 2. The main effect plots showing the responses for each parameter evaluated per class.

With the optimized DHS method, a sample of pine resin was measure in triplicate and the RSD for the four classes where established, which are shown in Table 2. The values are between 12 – 21% which is acceptable for headspace measurements.

In Fig. 3a. a chromatogram of pine resin is shown with the four chemical classes highlighted. In Fig. 3b. a chromatogram of hide glue is depicted. For hide glue mostly aldehydes and ketones are detected. A noticeable group is the 2-hexanone until 2-hexadecanone and the hexanal until hexadecanal (see Fig. 3b, highlighted in the red box).

*Comparison DHS vs. SPME*

In Table 2, the average, standard deviation, and the relative standard deviation of both DHS and SPME measurements on pine resin are given. Generally, the total average area is higher for DHS than for SPME. Although, the DHS parameters for OXST were sub-optimal, the total average area is still higher than when the extraction is performed with SPME. This implies that even in sub-optimal conditions, the response for the OXST is better with DHS.

On the other hand, the RSD values for DHS are a bit higher, but still in the accepted range. However, DHS is more robust than SPME and has less risk of losing the absorbent during sampling, and thus the sample.

Table 2. The differences between the DHS and SPME techniques in terms of average total area, standard deviation and relative standard deviation (RSD%) are stated.

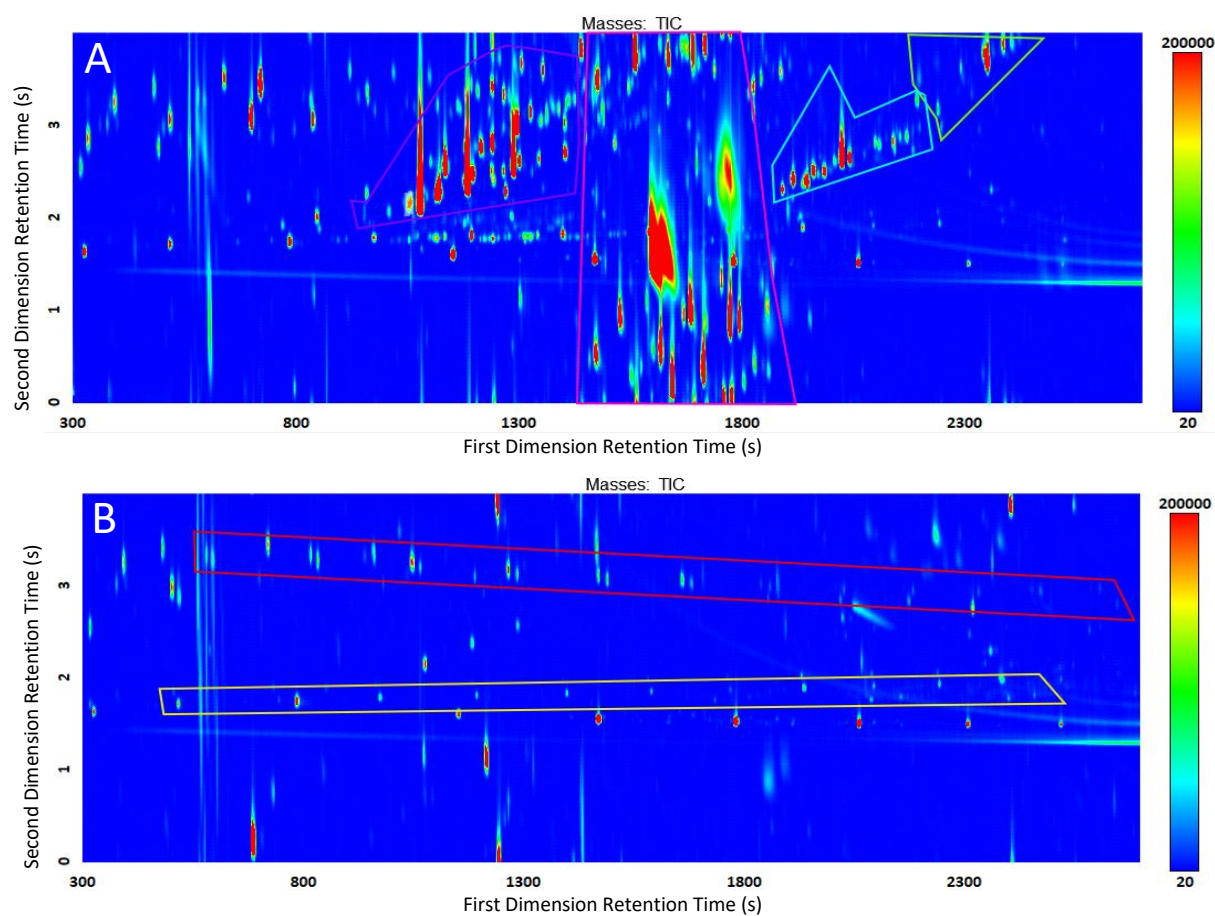| | | DHS | SPME |
|---|---|---|---|
| HCMT | average | 2.14E+09 | 5.40E+08 |
| | standard deviation | 2.66E+08 | 4.77E+07 |
| | RSD% | 12 | 9 |
| OXMT | average | 1.37E+09 | 2.55E+08 |
| | standard deviation | 2.53E+08 | 3.16E+07 |
| | RSD% | 18 | 12 |
| HCST | average | 4.54E+08 | 9.97E+07 |
| | standard deviation | 9.55E+07 | 3.01E+07 |
| | RSD | 21 | 30 |
| OXST | average | 6.47E+07 | 9.86E+06 |
| | standard deviation | 1.29E+07 | 1.43E+06 |
| | RSD% | 20 | 14 |



Fig. 3 A) pine resin, colour code: purple = HCMT, pink = OXMT, light blue = HCST, light green = OXST. B) hide glue, red = ketones and aldehydes, yellow = n-alkanes

## Conclusion

In this application, a new characterisation method of the volatile components in pine resin and hide glue is presented. Pine resin and hide glue are both good representatives of possible hafting adhesives.[4]

The method involves DHS sampling followed by GCxGC-TOFMS; the DHS parameters were optimized by design of experiment. For the response, the total area of four classes (hydrocarbon monoterpenoids, oxygenated monoterpenoids, hydrocarbons sesquiterpenoids, oxygenated sesquiterpenoids) was calculated. The pareto chart showed no significant parameters for the hydrocarbons monoterpenoids and sesquiterpenoids, for the oxygenated terpenoids temperature was the most significant parameter. The optimized parameters were $t_{incu}$: 20 min, $T_{incu}$: 50°C, $F_{purge}$: 22.5 mL.min$^{-1}$ and $V_{purge}$: 450 mL. The RSD was between 12-21% for all classes. These values are comparable with SPME, but DHS has a higher sensitivity and is more robust than SPME. Therefore, DHS is preferred over SPME.

However, the limit of detection for the DHS method has yet to be investigated. Moreover, as the method does not rely on the established biomarkers approach for the identification of the prehistoric glues[5] a library of the volatile profile of each glue has to be made. The library should include different resins, animal glues, gums, tars and mixtures as well as artificially aged resins and glues in order for it to be useful for archaeological identification.

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