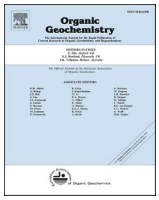
Organic Geochemistry 197 (2024) 104863

Contents lists available at ScienceDirect

Organic Geochemistry

journal homepage: www.elsevier.com/locate/orggeochem



A matter of extraction – Extraction yields and ratios of faecal lipid biomarker from archaeological soils using Soxhlet, microwave-assisted and accelerated-solvent extraction

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ARTICLE INFO

Associate Editor — Joe Werne

Keywords:
Faecal lipid biomarker
Archaeological soils
Extraction method
Lipid yields
Replicate analysis

ABSTRACT

The performance of Soxhlet (SOX), microwave-assisted (MAE) and accelerated-solvent extraction (ASE) in the analysis of faecal lipid biomarkers (FLB, Δ^5-sterols, stanols, stanones) from archaeological soils was investigated to assess effectiveness and reproducibility of the extraction methods. Results from two Anthrosols that were analysed in six replicates show that SOX achieves significantly higher extraction yields for individual substances and steroid sums than MAE, while ASE produces the lowest lipid yields. Regarding the FLB ratios, which are used for sourcing of faeces, the three extraction methods show comparable values with three out of five ratios differing significantly between different soil samples. The reproducibility of extraction yields decreases with SOX > ASE > MAE, as well as for concentrations <100 ng g^{-1} sediment. Analyses of six different soils indicate a weak influence of soil properties (pH, texture, total organic carbon and cation exchange capacity) on the effectiveness of extraction methods. From our study we conclude that the classical SOX is still the preferred extraction approach when reliably higher FLB yields are of foremost interest or low concentrations are expected, as it is most effective and reproducible. However, considering the drawbacks of SOX (high extraction times and high solvent consumption), MAE and ASE appear to be comparably attractive for extracting FLBs in archaeological contexts. In addition to comparable FLB ratios, MAE and ASE are economically more efficient, as they reach a higher sample throughput and waste lower amounts of extractant.

1. Introduction

The analysis of faecal lipid biomarkers (FLB, Δ^5-sterols, stanols, stanones) from archaeological soils gives insights into past human-environment interaction, such as livestock farming, the use of manure as well as dietary habits (Bemmann et al., 2014; Harrault et al., 2019; Vázquez et al., 2021). This is of particular interest when archaeological evidence (e.g., bones) is degraded due to weathering processes and microbial turnover, or when the periphery of ancient settlements is investigated (Lerch et al., 2022; Scherer et al., 2021a). In both cases, FLBs can provide additional information on past subsistence strategies (Birk et al., 2011; Gea et al., 2017; Scherer et al., 2021b).

Lipid biomarkers are organic molecules that have sufficient structural integrity to be traced back to their sources and are hydrophobic in nature, showing strong binding behaviour to the mineral phase in soils as well as to soil organic matter (Bull et al., 2002; Islam et al., 2023). Biomarkers such as Δ^5-sterols, stanols, stanones are derivates of the sterane hydrocarbon and are structurally assigned to the class of tetracyclic triterpenoids. These biomarkers are endogenous substances that enter the environment via excretion (Brueggemeier and Li, 2021; Lednicer, 2013).

Although FLBs have been frequently analysed to reconstruct past human-environment interaction for more than two decades (Bull et al., 2002; Prost et al., 2017; Simpson et al., 1999), the type of extraction is still under discussion (Andaluri et al., 2017; Manley et al., 2020; Parera et al., 2004; Shen and Shao, 2005). The extraction, which is the first step of the analysis, transfers biomolecules from a solid phase (soil sample) to a liquid phase (organic solvent), ready for further clean-up before gas chromatography – mass spectroscopy (GC–MS) analysis. Thus, the effectiveness of extraction controls the overall biomarker yield. There are various types of extraction used to analyse FLBs in archaeological contexts, of which Soxhlet (SOX), microwave-assisted (MAE) and

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https://doi.org/10.1016/j.orggeochem.2024.104863
Received 5 April 2024; Received in revised form 17 June 2024; Accepted 22 August 2024
Available online 23 August 2024

accelerated-solvent extraction (ASE) are most commonly applied (Birk et al., 2012; Lauer et al., 2014; Vallejo et al., 2022).

SOX can be seen as the 'classical' standard procedure of lipid extraction from solid materials (von Soxhlet, 1879). Numerous adaptations to the Soxhlet laboratory extractor have been proposed (López-Bascón and Luque De Castro, 2020). The foremost advantage of SOX is that sample material is repeatedly rinsed with fresh portions of extractant, which facilitates displacement of the transfer equilibrium. Furthermore, SOX is associated with low basic equipment costs and is reported to extract higher yields of lipids compared to alternative approaches (Jansen et al., 2006; Luque De Castro and Priego-Capote, 2010). The most serious drawback of SOX is its high extraction time (>24 h), which limits the capacity for laboratory replicates. Additionally, the large amount of extractant required increases costs and is environmentally problematic to dispose of (Jansen et al., 2006; Luque De Castro and Priego-Capote, 2010).

MAE uses microwave energy to give rotation to molecules with permanent dipole and disordered motion, resulting in rapid heating of the target compounds and extractants. This facilitates the leaching process due to enhanced desorption and diffusion of analytes from the soil matrix (Ganzler et al., 1986). In contrast to SOX, MAE can be fully automated, has extraction times of less than one hour, wastes smaller volumes of extractants and reaches higher sample throughputs (Barriada-Pereira et al., 2003; Medina et al., 2015; Pastor et al., 1997).

During ASE, samples are leached under elevated temperature and pressure, the latter ensuring that volatile extractants and target compounds remain liquid (Richter et al., 1996). Similar to MAE, the ASE method requires only low to moderate extraction times and smaller volumes of solvents (Giergielewicz-Możajska et al., 2001; Jansen et al., 2006; Wiesenberg et al., 2004). However, the acquisition of basic laboratory equipment for both the MAE and ASE method is comparatively cost-intensive (Jansen et al., 2006).

This study aims to investigate the extraction of FLBs (Δ^5-sterols, stanols, stanones) from archaeological soils using SOX, MAE, and ASE in order to assess the effectiveness and reproducibility of these extraction methods. Beyond absolute FLB concentrations, a qualitative interpretation of biomarkers regarding sourcing of faeces is of particular interest in archaeological contexts (Prost et al., 2017; Vázquez et al., 2021). Therefore, this study further evaluates if FLB ratios vary by extraction method. Considering physiochemical soil properties to assess the interaction between soil solution and solid matter (mineral and organic phase), it is assumed that the soil milieu, the soil organic carbon content and the binding capacity of soils influence the mobility and persistence of biomarkers in soils (Islam et al., 2023; Lloyd et al., 2012). So, this study will additionally investigate whether SOX, MAE and ASE achieve different biomarker yields over varying physiochemical soil properties (soil texture, soil organic carbon content, pH, cation exchange capacity).

We hypothesize that

(i) the faecal lipid biomarker concentrations are significantly different for the three extraction methods (SOX, MAE, ASE).
(ii) the faecal lipid biomarker ratios are comparable between the three extraction methods for the same soil sample.
(iii) the three extraction methods are inherently reproducible with low coefficients of variation for six replicates per extraction.
(iv) the extraction methods produce different biomarker yields over varying physiochemical soil properties (texture, soil organic carbon content, pH, cation exchange capacity).

2. Material and methods

2.1. Sample origin

The samples were chosen based on FLB yields from unpublished and already published literature (Birk et al., 2012; Glaser et al., 2000; Scherer et al., 2021b) as well as based on different soil properties (Table 1). All samples are related to different contexts of past human-environment interactions from Amazonia, Central Europe, Southeast Europe and the Middle East. In these cultural-historical contexts, anthropogenic soils such as Anthrosols and Colluvisols (colluvic Regosols according to WRB 2015) developed as a result of land use.

2.2. Experimental design

The experimental design comprised the extraction of Δ^5-sterols, stanols, stanones from eight different samples via SOX, MAE, and ASE. Herein, samples A and B (Table 1) referred to hypotheses (i-iii) and samples C-H (Table 1) to hypothesis (iv). For the samples A and B, two extractions with three replicates for each extraction method were performed. For the samples C-H, a single extraction without replicates was performed using SOX, ASE and MAE (Fig. 1).

2.3. Sample preparation and acquisition of total lipid extracts (TLE)

Soil samples were dried at 40 °C, sieved < 2 mm and finely ground in a ball mill. All laboratory consumables (glass ware, quartz sand,

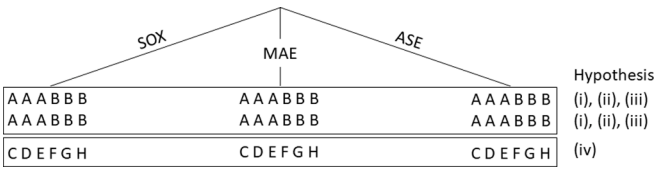

Fig. 1. Experimental design of the study. SOX: Soxhlet extraction; MAE: Microwave-assisted extraction; ASE: Accelerated-solvent extraction; A-H: Arbitrary sample designation (see Table 1).

Table 1
Physiochemical characteristics of soil samples. BY: Bavaria, RP: Rhineland-Palatinate, BW: Baden-Württemberg. TOC: Total organic carbon, TN: Total nitrogen, CEC: Cation exchange capacity.

Sample	Location	Soil type[a]	Depth	Sand, silt clay[b]	TOC[c]	TN[c]	pH	CEC (eff)[d]
			[cm]	[%]	[%]	[%]	($CaCl_2$)	[$cmol_{c+}$ kg^{-1}]
A	Brazil	Anthrosol	10–20	29, 47, 24	8.1	0.6	5.7	20
B	Germany (BY)	Anthrosol	30–40	7, 59, 34	2.3	0.2	5.7	39
C	French Guiana	Anthrosol	30–40	93, 4, 3	0.9	0.1	4.4	1
D	Jordan	Cambisol	0–15	3, 73, 24	1.6	0.1	7.2	58
E	Serbia	Anthrosol	0–10	59, 33, 8	2.0	0.1	6.6	27
F	Germany (RP)	Anthrosol	55–58	35, 51, 14	2.0	0.1	4.2	2
G	Germany (BW)	Colluvic Regosol	155–160	39, 34, 27	2.0	0.1	6.9	34
H	Germany (BW)	Colluvic Regosol	295–300	27, 43, 30	1.0	0.1	7.1	26

[a] Reference soil group of the WRB (IUSS Working Group, 2014), World Reference Base for Soil Resources, FAO, 2015.
[b] Textural analysis according to Köhn (1928).
[c] Carbon and nitrogen analysis via dry combustion at 950 °C (Vario ELcube, Elementar).
[d] Effective cation exchange capacity analysed according to Trüby and Aldinger (1989).

fibreglass thimbles, silverware, etc.) were pretreated with organic solvents and heated at 300 °C for 8 h prior to use.

For SOX, 5 g of soil were weighed into 23 × 100 mm fibreglass thimbles (Macherey-Nagel, Düren, Germany) and covered by a thin layer of quartz sand. Extraction was conducted in extractors (100 mL) with 210 mL of dichloromethane/methanol (DCM:MeOH, 2:1, *v/v*) for 36 h. To avoid boiling delay, up to ten boiling chips were added to the round-bottom flasks.

For MAE, 5 g of soil were weighed into 100 mL PTFE-TFM tubes. To each tube, 40 mL of DCM:MeOH (2:1, *v/v*) and a stirring bar were added. Extraction was performed with a Multiwave Pro (Microwave Reaction System Solv, Anton Paar GmbH, Austria) using one cycle of 10 min heating time to 100 °C, 20 min static time and 10 min cooling time.

For ASE, 5 g of soil were filled into 22 mL extraction cells together with diatomaceous earth. Extraction was performed with Dionex (350, Thermo Fischer Scientific, USA) using DCM:MeOH (2:1, *v/v*) and three extraction cycles. Maximal temperature was set to 100 °C (5 min heating and static time, respectively), rinse volume to 50% and purging time to 300 sec.

2.4. Sample purification

After extraction, internal standards (IS1) pregnanone, cholesterol-d_7 and cholestanol-d_5 were added to the TLE (Table 2). Solutions were concentrated under reduced pressure and completely dried under a gentle stream of nitrogen. Samples were saponified at room temperature for 12–14 h using 3.5 mL of 0.7 M potassium hydroxide (KOH) in MeOH. A liquid–liquid extraction was performed to further separate Δ^5-sterols, stanols, stanones (SSS-fraction) using 10 mL of water and three times 15 mL of chloroform ($CHCl_3$). The SSS-fraction was purified by solid-phase extraction using PE-columns (1 mL) packed with 5% deactivated silica gel (50 mm, mesh: 70–230, pore size: 100 Å, Polygoprep, Macherey Nagel, Germany). The SSS-fraction was eluted with 3 mL of DCM and 2 mL of DCM:acetone (2:1, *v/v*) and was thereafter silylated with a mixture of *N,O*-Bis(trimethylsilyl)trifluoroacetamide (containing 1% trimethylsilyl chloride, 99:1, *v/v*) and pyridine (3:1, *v/v*) at 90 °C for one hour. Cholestanol-d_4 was added as IS2 to all samples before analysis.

2.5. GC–MS analysis

All substances were analysed using a 7890B/7000D GC-QQQ-MS system, equipped with a DB5-ms Ultra Inert column (30 m × 0.25 mm × 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA/USA). Data was acquired in selected ion monitoring (SIM) mode for quantification and in scan mode to verify peak identification (e.g., Fig. 2). He (99.9990% purity) was used as carrier gas (flow rate 1 mL min^{-1}). The injection port (split/splitless) was set to 250 °C and 1 μL sample was injected in splitless mode (1 min splitless time). Oven temperature programme was 80 °C (held 1.5 min) to 260 °C at 12 °C min^{-1}, to 274 °C at 0.5 °C min^{-1} and to 300 °C (held 10 min) at 10 °C min^{-1}. Transfer line temperature was set to 250 °C, ion source temperature to 230 °C, and MS1/MS2 quadrupole temperature to 150 °C. Scanned masses of the characteristic ion fragments (*m/z*) are listed in Table 2. Workup efficiency was 89 ± 13% (n: 216) for all analysed substances.

2.6. Statistical analysis

All statistical computations were performed using *R* (version 4.2.2). Arithmetic means and standard deviation were calculated for all substances based on three repetitions from two extraction runs. The coefficient of variation (CV, relative size of standard deviation compared to the mean, given in %) was used to identify the degree of variance for different biomarker concentrations within the experimental design. Further, a multiple regression and a subsequent analysis of variance (ANOVA) was performed using different variables (extraction method, extraction run, concentration and steroidal compound). Paired Welch's *t*-test for unequal variances (level of significance $p < 0.01$) was used to determine differences in biomarker yields regarding the SOX, MAE and ASE extractions.

3. Results and discussion

In this study Δ^5-sterols, stanols, stanones were extracted using SOX, MAE, ASE in order to compare overall biomarker yields, degrees of variance and qualitative significance (FLB ratios) to enhance the economical and analytical efficiency in laboratory workflows (higher sample throughput, maximal and reproducible lipid yields, reduced costs). Based on the experimental design (Fig. 1) and the laboratory equipment used in this study (Table 3), extraction time could be reduced by > 4 days and extractant volume by > 2 L when soil samples were extracted using MAE or ASE compared to SOX.

Table 2
Steroid compounds and used internal standards, their trivial names, retention times (RT) and selected characteristic ion fragments. a, b, d: internal standard 1, c: internal standard 2.

Biomarker group	Substance	Trivial name	RT (min)	Characteristic ion fragments (*m/z*)
Δ^5-sterols	cholest-5-en-3β-ol	Cholesterol	31.3	329.0
	cholest-5-en-3β-ol-d_7[a]	Cholesterol-d_7	31.1	465.0
	stigmasta-5,22-dien-3β-ol	Stigmasterin	36.0	394.0
	stigmast-5-en-3β-ol	Sitosterol	38.4	396.0
Stanols	5α-pregnan-3β-ol	Pregnanol	18.6	361.0
	5β-cholestan-3β-ol	Coprostanol	28.6	215.0
	5β-cholestan-3α-ol	Epi-coprostanol	29.4	355.0
	5α-cholestan-3β-ol	Cholestanol	31.7	445.0
	5α-cholestan-3α-ol	Epicholestanol	29.1	355.0
	5α-cholestan-3β-ol-d_5[b]	Cholestanol-d_5	31.6	465.0
	5α-cholestan-3β-ol-d_4[c]	Cholestanol-d_4	24.0	376.5
	5β-stigmastan-3β-ol	5β-Stigmastanol	34.8	398.0
	5β-stigmastan-3α-ol	Epi-5β-Stigmastanol	35.8	398.0
	5α-stigmastan-3β-ol	Stigmastanol	35.4	215.0
	5α-stigmastan-3α-ol	Epistigmastanol	35.4	383.0
Stanones	5α-cholestan-3-one	α-Cholestanone	32.0	231.0
	5β-cholestan-3-one	β-Cholestanone	30.6	316.0
	5α-pregnan-3-one[d]	Pregnanone	19.3	302.5

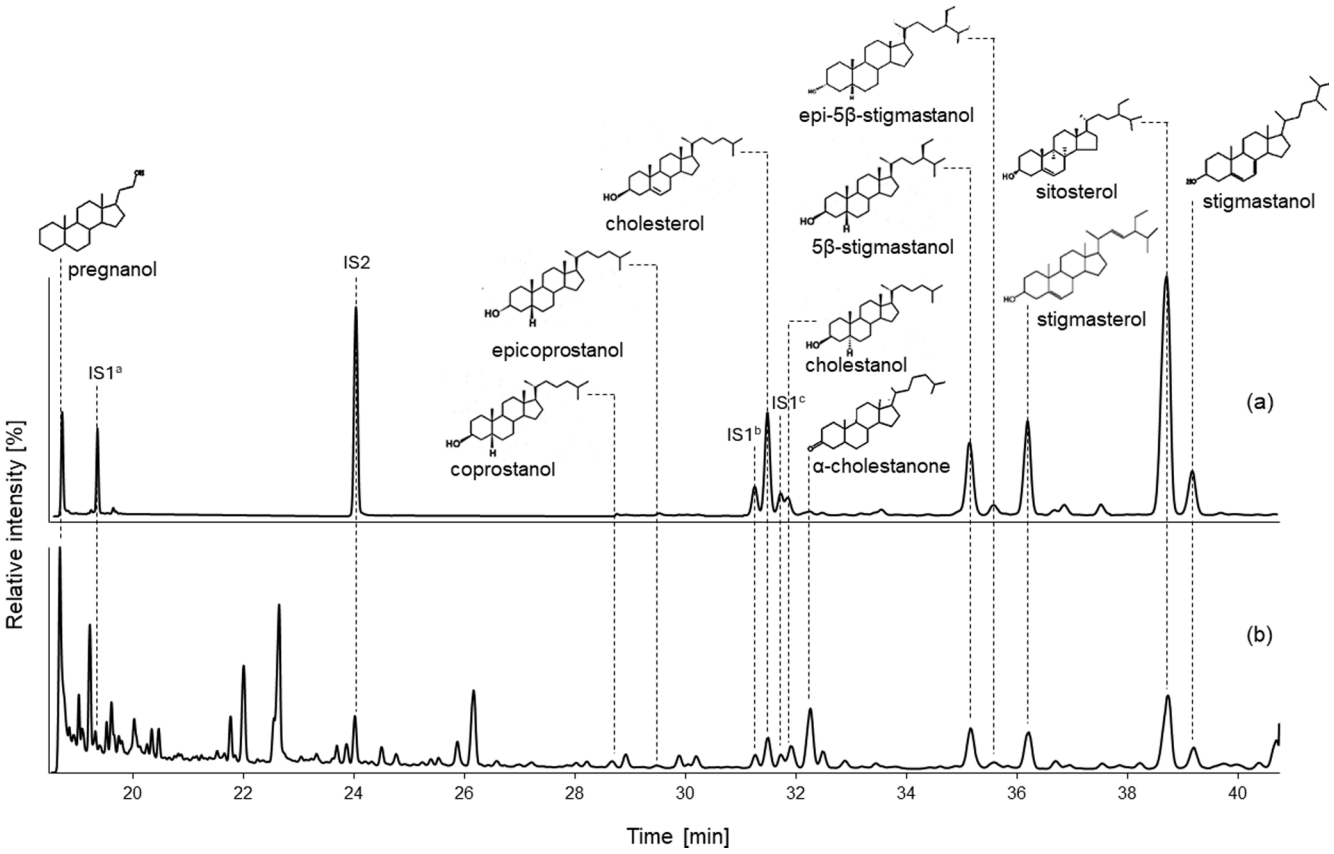

Fig. 2. Gas-chromatograms of the Δ^5-sterols, stanols, stanones profiles for the sample A (run 1, extracted by accelerated-solvent extraction). (a): SIM-mode, (b) SCAN-mode. IS1[a]: pregnanone, IS1[b]: cholesterol-d_7, IS1[c]: cholestanol-d_5, IS2: cholestanol-d_4.

Table 3
Comparison of Soxhlet extraction (SOX), microwave-assisted extraction (MAE) and accelerated-solvent extraction (ASE) in regard to extraction time, number of samples per run, volume of organic solvent per sample and equipment costs (based on laboratory equipment used in this study).

Method	Extraction time [h]	Samples/ run	Solvent volume/ sample [mL]	Equipment costs [€]
SOX	>24	6	210	~2.500
MAE	1	15	90	~40.000
ASE	10	20	60	~55.000

3.1. Faecal lipid biomarker yields and ratios

Summarized extraction yields for individual Δ^5-sterols, stanols, stanones are provided in Fig. 3; raw data for all identified compounds are given in Table S1. The steroid profiles show a similar pattern for the analysed Anthrosols (A, B) and the three extraction methods (SOX, MAE, ASE): Plant and plant-derived steroids such as stigmasterol (stigmasta-5,22-dien-3β-ol), sitosterol (stigmast-5-en-3β-ol) and stigmastanol (5α-stigmastan-3β-ol) reach higher lipid concentrations than faecal-derived 5β-stanols. The steroid sums for SOX, MAE, ASE are 4535 ± 75, 3847 ± 116 and 3117 ± 414 ng g^{-1} sediment for the Brazilian Anthrosol (A) and 1703 ± 23, 1465 ± 189 and 1323 ± 105 ng g^{-1} sediment for the Bavarian Anthrosol (B), respectively. Both for the steroid sums and for individual substances, a partially significant ($p < 0.01$) pattern is evident in which SOX achieves higher yields than MAE, and ASE mostly the lowest extraction performance. Our results are consistent with a study of Shen and Shao (2005) who extracted sterols and other lipids from tobacco and found that SOX was more effective than ASE. Manley et al. (2020) analysed steroids from soils and slurry and found no significant differences between SOX and ASE. For other molecular compounds such as polychlorinated biphenyls (PCB), organochlorine pesticides (OCP), polycyclic aromatic hydrocarbons (PAH), polybrominated diphenyl ethers (PBDE) and short-chain chlorinated paraffins (SCCP) from soil and vegetation biomass samples, comparisons of extraction methods have also been conducted. Most studies showed that MAE and ASE achieve comparable or even better yields of lipid biomarker than SOX. Therefore, these studies concluded that MAE and ASE are suitable for replacing the classic, time-consuming and solvent-intensive Soxhlet extraction (Barriada-Pereira et al., 2003; Parera et al., 2004; Sporring et al., 2005; Wang et al., 2010, 2007). This partially contrasts our data, as more than half of the steroid compounds achieve significantly higher yields when extracted with SOX compared to MAE and ASE. In addition to the extraction techniques, their specific characteristics (extraction time, solvent, temperature, pressure) are reported to influence overall lipid yields, too (Alcántara-Concepción et al., 2013; Shen and Shao, 2005; Wu et al., 1995). Since manipulation of these specifics is not the focus of the manuscript, we kept them constant for each extraction technique. However, considering the tremendous difference in extraction times (SOX to MAE/ASE) as well as the acknowledged influence of temperature on sterol extraction (Alcántara-Concepción et al., 2013; Barriada-Pereira et al., 2003; Quénéa et al., 2012), this might explain significant differences in FLB yields, while future research will be needed to comprehend and optimize MAE and ASE to extract FLBs from soil matrices.

Consistently lower biomarker yields may be accepted in certain cases e.g. when sourcing of faecal matter is of foremost interest. Especially in archaeological contexts, FLBs are used to reconstruct the origin of faeces when archaeological and zooarchaeological records are incomplete (Lauer et al., 2014; Scherer et al., 2021b). Then, FLB ratios are used to differentiate between omnivorous and herbivorous as well as between certain individual species (Birk et al., 2022; Harrault et al., 2019; Lerch

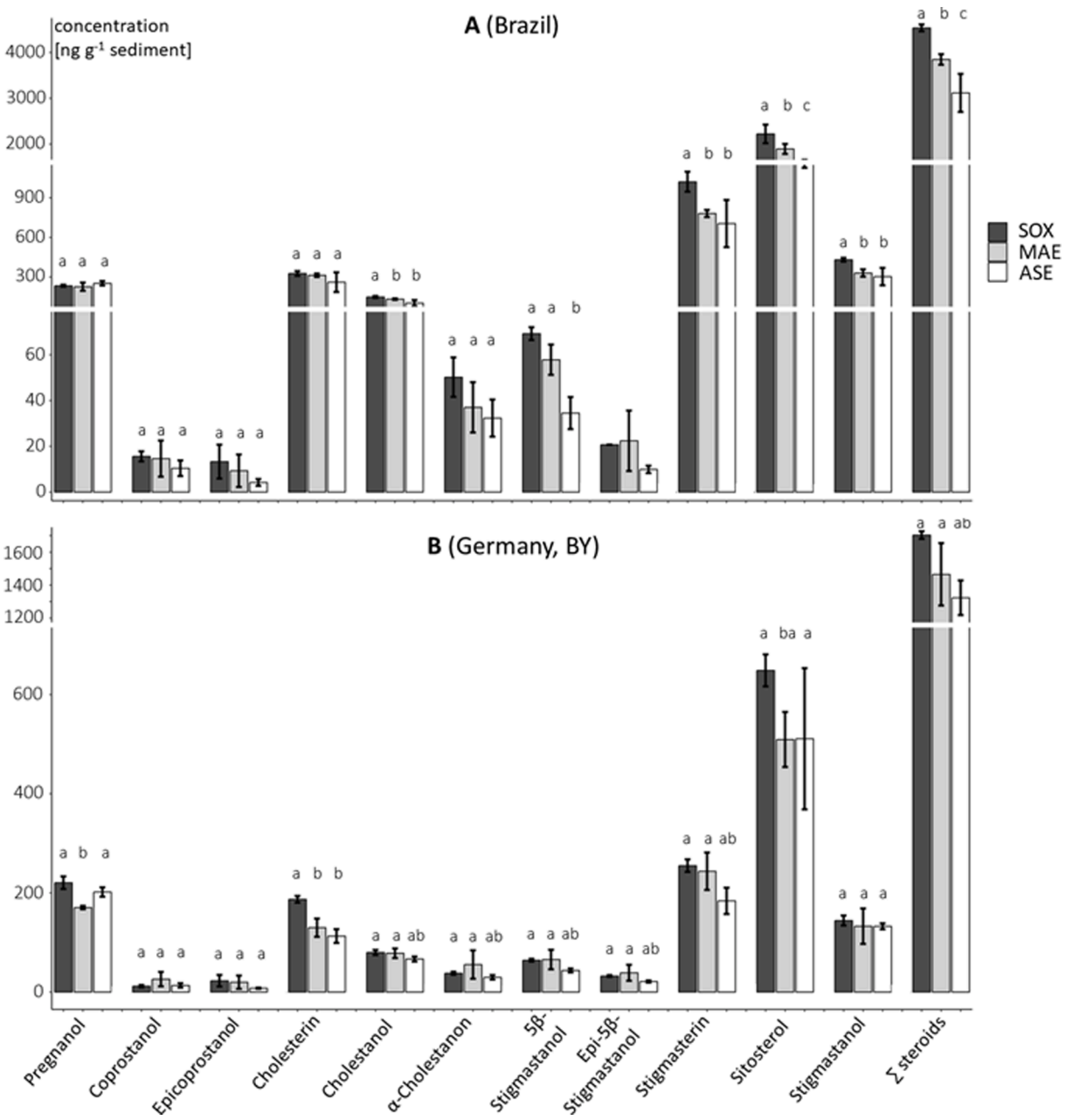

Fig. 3. Lipid biomarker yields (Δ^5-sterols, stanols, stanones) of the first and second sample run (samples A, B). Each bar has n = 6 (three repetitions, two sample runs). Small letters indicate significant differences between the extraction methods ($p < 0.01$). For better visualization, scale breaks are added to the y-axis.

et al., 2022). A comprehensive summary of FLB ratios is given in Vázquez et al. (2021) and Prost et al. (2017). Five ratios (R1-R5) were chosen to assess whether different concentrations of Δ^5-sterols, stanols, stanones obtained by different extraction methods lead to varying biomarker ratios (Fig. 4). R1 and R2 provide information on faecal inputs in general (Bull et al., 2001; Prost et al., 2017; Simpson et al., 1998), R3 allows for a differentiation between omnivorous and herbivorous faecal input (Bull et al., 2002) and R4 and R5 for an input of pig and horse faeces in specific (Jardé et al., 2007; Prost et al., 2017).

Comparable FLB ratios for the three extraction methods for the same soil samples, but significant differences between soil samples (A, B), indicate a robust source identification via biomarker ratios that is largely independent of the extraction method. This applies to R1, R2 and R4, which produced comparable FLB ratios for SOX, MAE and ASE and revealed significant differences of FLB ratios between the soil samples. A similar tendency, but not statistically significant ($p > 0.05$), can be observed for R3 and R5. It is also shown that ASE produces consistently lower FLB ratios than SOX and MAE. We refrain from including an interpretation of FLB ratios along specific threshold values, as this does not meet the aim of the study. Moreover, it should be considered that some of these thresholds are based on the investigation of fresh faecal matter and that lower threshold values are to be expected for archaeological soil samples due to the disproportionate degradation of steroid compounds over time (Bull et al., 2001; Prost et al., 2017).

In summary, our data show that the highest yield of Δ^5-sterols, stanols, stanones is achieved with SOX, while MAE is more effective than ASE. The comparison of FLB ratios reveals similar ratios obtained by SOX, MAE and ASE for individual soil samples. For the two analysed Anthrosols (A, B), this means that despite different biomarker concentrations, the extraction methods show comparable FLB ratios that are used for sourcing of ancient faecal matter.

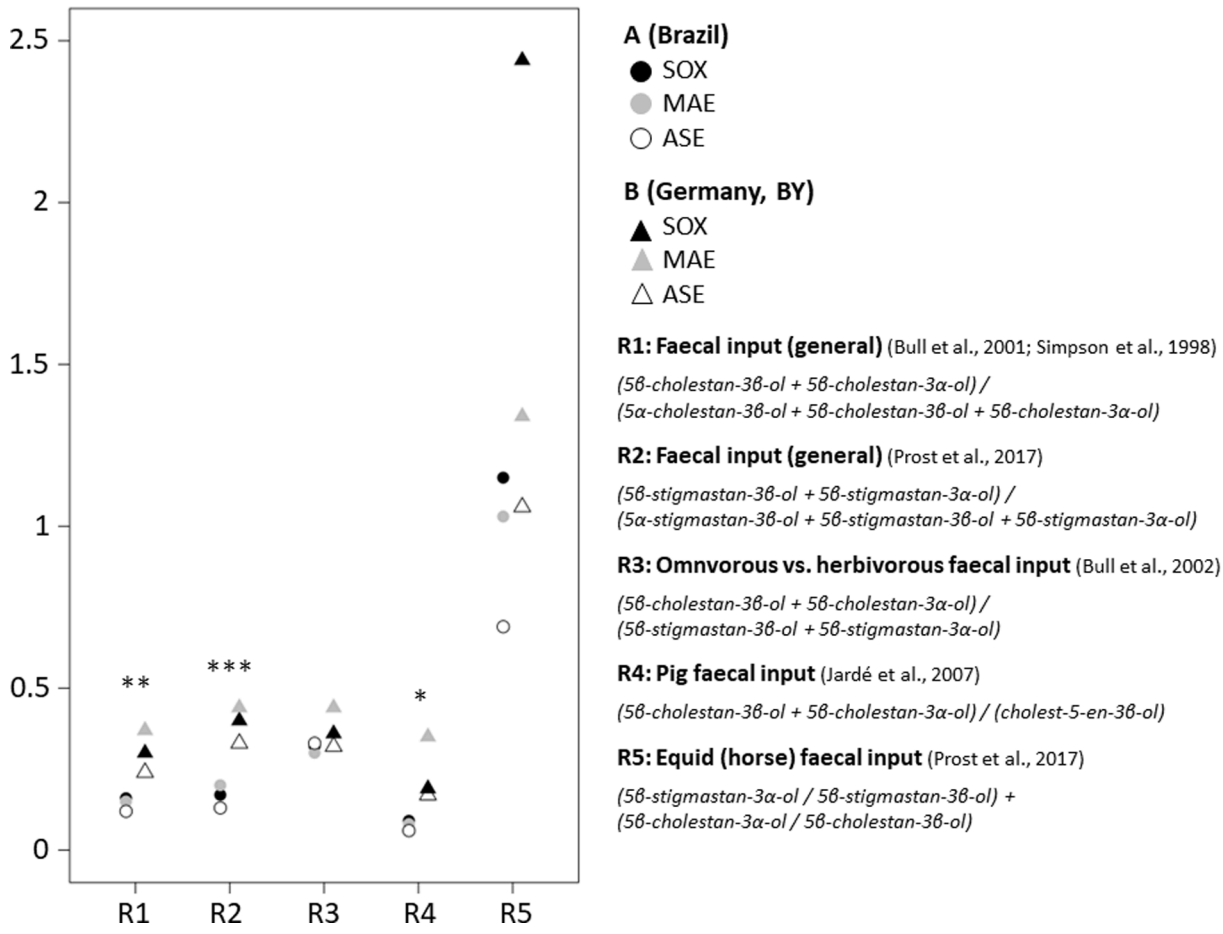

Fig. 4. Lipid biomarker ratios (Δ^5-sterols, stanols, stanones) of the 1st experimental level. * $p < 0.1$; ** $p < 0.5$; *** $p < 0.01$.

3.2. Reproducibility of extraction methods

The analysis of Δ^5-sterols, stanols, stanones from archaeological soil samples commonly produces lower concentrations (in the range of ng g^{-1} sediment) compared to samples from river sediments, slurry, fresh biomass and faecal matter (µg g^{-1} sediment) (Cordeiro et al., 2008; Kemp et al., 2022; Manley et al., 2020). To increase data reliability, lower FLB yields must be verified by replicates. Our data reveal average coefficients of variation (CV) for SOX, MAE and ASE of 8.2 ± 4.9%, 23.3 ± 24.9% and 23.1 ± 8.0% for sample A and 7.6 ± 4.7%, 28.2 ± 19.1% and 14.1 ± 9.1% for sample B, respectively (Table 4). These are higher CVs compared to other studies (Isobe et al., 2002), whereas our biomarker concentrations are 100–1000-fold lower. For both samples SOX shows a higher reproducibility than ASE, while MAE achieves highest average CVs. This tendency remains, even if relative instead of absolute extraction yields are considered. Relating all substances to cholesterol, average CVs for SOX, MAE and ASE are 8.8 ± 7.0%, 25.2 ± 23.6% and 12.5 ± 5.4% for sample A and 7.8 ± 5.3%, 25.6 ± 18.8% and 12.5 ± 8.1% for sample B, respectively (Tab. S2). This strengthens the quality of reproducibility for the analysed samples in the order SOX > ASE > MAE and contrasts a study of Shen and Shao (2005) who showed a higher reproducibility in biomarker yields extracted with ASE compared to SOX. The commonly observed relationship between variance and concentration (Shen and Shao, 2005) is also given for our data. Distinguishing between lower (< 100 ng g^{-1} sediment) and higher FLB concentrations (> 100 ng g^{-1} sediment), a distinct trend is evident: Lower concentrations result in higher (mean: 26.1 ± 8.2%) as well as in a wider range of average CVs (13.3 ± 10.1% – 35.2 ± 26.2%) compared to higher concentrated substances (mean: 10.6 ± 2.5%, range: 6.0 ± 4.1% – 12.9 ± 8.9%). This trend is particular evident for coprostanol (5β-cholestan-3β-ol) and epicoprostanol (5β-cholestan-3α-ol) with lipid yields < 20 ng g^{-1} sediment and average CVs > 30. Coprostanol and epicoprostanol are 5β-stanols that originate mainly from microbial reduction of Δ^5-sterols in the gut of mammals and are predominantly present in omnivorous faeces (Birk et al., 2011; Prost et al., 2017). Therefore, they are commonly used for deducing an input of human and pig faecal matter and have a high significance in reconstructing past human-environment interaction (Bull et al., 2003; Cordeiro et al., 2008).

However, in most studies dealing with the analysis of Δ^5-sterols, stanols, stanones in archaeological contexts, the number of replicates is

Table 4
Coefficients of variation given in % for sample A (Brazil) and B (Germany, BY), for Soxhlet extraction (SOX), microwave-assissted extraction (MAE) and accelerated-solvent extraction (ASE) as well as for first and second sample run; n.d.: not detectable.

	Sample A (Brazil)			**Sample B** (Germany, BY)		
	SOX	**MAE**	**ASE**	**SOX**	**MAE**	**ASE**
Pregnanol	3.3	13.6	6.7	5.8	2.0	4.7
Coprostanol	14.0	40.2	32.9	19.7	43.1	33.2
Epicoprostanol	12.8	72.4	35.9	12.7	61.2	16.3
Cholesterin	5.4	4.1	28.5	3.6	14.2	12.2
Cholestanol	5.1	4.1	22.3	6.4	12.5	7.5
α-Cholestanon	17.1	29.7	25.2	8.2	51.6	14.8
5β-Stigmastanol	4.0	11.4	20.4	4.6	30.1	9.3
Epi-5ß-stigmastanol	n.d.	62.9	16.2	5.7	41.7	9.9
Stigmasterin	7.4	3.5	25.3	5.0	15.5	14.4
Sitosterol	9.1	5.7	19.0	5.0	10.9	27.9
Stigmastanol	3.3	8.5	21.8	7.0	27.0	4.6
mean	8.2	23.3	23.1	7.6	28.2	14.1
standard deviation	4.9	24.9	8.0	4.7	19.1	9.1

limited, or no replicates are considered at all, especially when high biomarker concentrations are reached, or source identification is of foremost interest (Bull et al., 2003; Lauer et al., 2014). This makes an evaluation of the reproducibility of different extraction methods difficult to compare.

In summary, our data reveal two main observations regarding the reproducibility of extraction methods in the analysis FLBs on the example of the two analysed Anthrosols (A, B): The reproducibility increases for higher biomarker concentrations, and SOX is better reproducible and produce distinctly lower CVs compared to MAE and ASE.

3.3. Faecal lipid biomarker yields over varying soil characteristics

The fate of lipids in soils and sediments is influenced by the physiochemical characteristics of the environment. Among a variety of soil properties, pH, texture, total organic carbon (TOC) and cation exchange capacity (CEC) are reported to have a significant impact on the mobility and persistence of lipids in soils (Lloyd et al., 2012; Thomas et al., 2021; Wei et al., 2021). For example, the soil milieu regulates the charge of the –OH functional groups of Δ^5-sterols and stanols (Bull et al., 2000). At low pH-value, these functional groups are positively charged and are attracted to negatively charged soil surfaces (Klitzke et al., 2011). Both SOM and clay minerals have an increased number of negatively charged exchange sites for cationic molecules and ions due to isomorphic substitution of Si^{4+} and their large specific surface area (Higgins and Luthy, 2006; Oukali-Haouchine et al., 2013; Tang et al., 2009; Wu et al., 2015). Therefore, low pH-values and high contents of soil organic matter and clay minerals contribute to a high persistence and low mobility of Δ^5-sterols and stanols in soils. However, especially in tropical soils, acidic soil milieus contribute negatively to the persistence of cationic molecules and ions in soils, as the variable charge of oxides and SOM is reduced and available clay minerals tend to permanent positive charges (Gallez et al., 1976). To what extent a higher persistence of lipids in soils correlates with biomarker yields from different extraction types has not yet been investigated.

The physiochemical soil properties of the samples C-H (Table 1) vary significantly with high contents of silt (up to 73%) and clay (up to 30%) for the Jordanian Cambisol (D) and the German colluvic Regosols (G, H). The sand fraction dominates in the Serbian and French Guinian Anthrosols (C, E) with contents between 59 and 93%. The CEC varies between 1 and 58 $cmol_{c+}\ kg^{-1}$ with the highest values for silty and clay-rich soils and the lowest values at acidic soil milieus with high contents of sand. TOC contents are highest for the soils from Serbia (E) and Germany (F, G) with values up to 2.0%. The pH ($CaCl_2$) ranges from acidic (4.2 to 4.4; C, F) to neutral (6.6–7.2; D, E, G, H) soil milieus.

For samples with steroid sums > 2000 ng g^{-1} sediment (D, E), the MAE extracted more effectively than SOX (Fig. 5). For steroid sums < 2000 ng g^{-1} sediment, SOX produces slightly higher lipid yields than MAE, while ASE achieves the lowest FLB sums for all analysed samples. With the exception of samples D and E, the physiochemical soil properties do not appear to distinctly influence the FLB yield, as the differences of biomarker yields are comparable for the three extraction methods. Nevertheless, some trends are cautiously indicated, bearing in mind that the relationship between physiochemical soil properties and extraction efficiency requires further research attention. Our data indicate that SOX extraction achieves higher FLB yields in clay-rich matrices and in predominantly acidic milieus. The reason could be that SOX uses extraction times > 24 h with more time to break down intermolecular H-bonds that are more abundant in clay-rich soils (higher CEC) and at low pH-values (Islam et al., 2023). However, it cannot be ruled out that the overall biomarker concentration in the analysed soils mainly dominates the effectiveness of the different extraction methods, with MAE performing the best at medium to high and SOX at (very) low to medium concentrations.

In summary, our data show that the physiochemical soil properties do not appear to influence the FLB yields for most of the samples, as the differences between extraction methods are small. However, as cautiously indicated, SOX seems to achieve higher biomarker outputs from clay-rich matrices and at acidic soil milieus.

4. Conclusion

The extraction of FLBs (Δ^5-sterols, stanols, stanones) from archaeological soils using SOX, MAE and ASE was performed to assess the effectiveness and reproducibility of the extraction methods. Based on our results, we could show that the three extraction methods produce significantly different FLB yields for at least six replicates in the order SOX > MAE > ASE. Despite of varying biomarker concentrations the FLB ratios are comparable for the three extraction methods with larger and partially significant differences between soil samples. From the replicate analysis we conclude that high FLB concentrations as well as SOX reveal a better reproducibility compared to low concentrations and MAE and

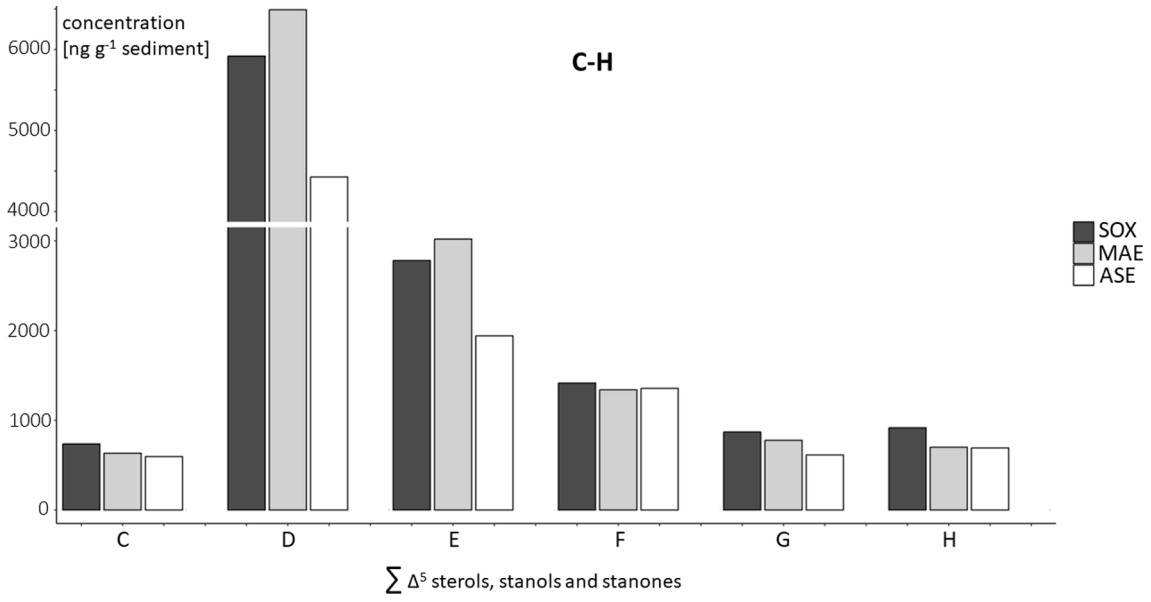

Fig. 5. Sums of Δ^5-sterols, stanols, stanones of the third sample run (samples C–H). For better visualization, scale breaks are added to the y-axis. C: Anthrosol (French Guiana), D: Cambisol (Jordan), E: Anthrosol (Serbia), F: Anthrosol (Germany, Rhineland-Palatinate), G, H: Colluvic Regosols (Germany, Baden-Württemberg).

ASE. We recommend using replicates when FLB concentrations < 100 ng g^{-1} sediment for individual substances are determined. Considering varying physiochemical soil properties, the three extraction methods appear to produce comparable FLB yields for most of the soil samples with SOX extracting slightly better at acidic soil milieus and from clay-rich matrices. However, the overall lipid concentration seems to be the key driver in extraction effectiveness with SOX performing the best at (very) low to medium concentrations and MAE at medium to high concentrations.

Based on our study, we recommend SOX when reliably higher lipid biomarker concentrations are of foremost interest or low concentrations are expected. However, SOX is known to be economically inefficient due to long extraction times and high solvent consumption. MAE and ASE have proven to be comparably attractive to extract Δ^5-sterols, stanols, stanones from archaeological soils for economic and analytical reasons, especially when the focus is source identification of faecal matter. In addition, the use of replicates, enabled by a higher sample throughput when extraction is performed with MAE or ASE, provides more reliable data on FLBs.

CRediT authorship contribution statement

Sascha Scherer: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jago Jonathan Birk:** Writing – review & editing, Methodology, Conceptualization. **Stefanie Klassen:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Sabine Fiedler:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

Acknowledgements

We would like to thank the following people for carrying out the soil sampling: B. Glaser supported by W.G. Teixeira, G. Schroth and Embrapa Amazônia Ocidental sampled the Anthrosol in Brazil. B. Lucke supported by G. Schörner, N. Voß, H. al Sababha, S. al Karaimeh collected soil samples in Jordan. M. van den Bel, S. Jérémie, D. McKey, S. Rostain and Inrap Base archéologique de Guyane, supported soil sampling in French Guiana. Collection of soil samples in Serbia (Caričin Grad) was supported by V. Ivanišević, I.Bugarski, R. Schreg, Römisch-Germanisches Zentralmuseum Mainz and Institute of Archaeology Belgrade. The Anthrosol in Bavaria was sampled by M. Dippold. E. Dietze generously allowed J. Doyle to perform the ASE extractions in the laboratory of Landscape Geoscience in the Department Physical Geography at the University of Göttingen. We thank G. Borngässer for performing the soil texture analysis and George Janzen for critically reviewing the manuscript (both University of Mainz) and we are grateful to J. Hutchings and an anonymous reviewer who provided constructive comments and helped to improve the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.orggeochem.2024.104863.

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