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Green Chemistry and Archaeological Biomarkers: a new and safe DES-based approach for the extraction of absorbed lipid residues from archaeological samples of ceramic potsherds

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***Abstract*–This work aimed at comprehensively evaluate the potential and effectiveness of natural Deep Eutectic Solvents (DESs) for the extraction of different natural compounds from archaeological samples. We compared the procedure by DESs, which are now emerging as green and sustainable solvents, with the more conventional solvent extraction protocols, which gave measurable yields of lipid extract. The different techniques were applied on the absorbed residues of small samples of a replica pottery vessel after cooking experiments of animal fats. Direct extraction by DES and derivatization proved efficient to obtain enough fatty acids for quantification analysis of absorbed lipid residues by GC-MS. The next step was the application of DES extraction procedure on some archaeological samples previously submitted to conventional extraction methods. GC-MS analyses gave comparable results as regards the amounts and relative proportions of fatty acids identified in the archaeological samples, thus encouraging to further refine in the future the analytical protocol by DES.**

INTRODUCTION

Lipid residue analysis has become a common technique for the identification of the organic residues deriving from resources processed in archaeological ceramic vessels, integrating traditional archaeological investigation for the determination of vessel use and contributing to the reconstruction of food habits, cultural and economic practices in antiquity [1,2].

Lipids are the best preserved biomolecules and are often used as archaeological biomarkers. With this approach, it has been possible to identify a wide range of commodities, such as terrestrial and marine animal fats, beeswax, vegetable oils, plant waxes, plant resins, etc. [3].

Lipid components absorbed into the archaeological material are extracted using different techniques and analysed by the employment of a range of different analytical instruments suitable for their separation, identification and quantification, e.g. High Temperature-Gas Chromatography (HT-GC), Gas Chromatography-Mass Spectrometry (GC-MS), High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS), in order to link the lipid residues to their original biological and environmental source.

The commonly employed extraction procedures use a solvent wash with chloroform/methanol and the extracted compounds are subsequently silylated before molecular analyses [4, 5, 6]. Nevertheless, it has been demonstrated that some lipids can establish stronger interactions with the ceramic matrix and their recovery is possible only through alkaline hydrolysis using strong base extraction [7], which is a long and laborious procedure.

Many scientists are now developing more efficient and rapid extraction methods [8, 9], with the aim to process increasingly large numbers of potsherds in a short time and to deal more efficiently and systematically with important archaeological questions.

However, despite the significant results of organic residues analysis on archaeological pottery, the recovery of lipids from ancient vessels still requires expensive and toxic organic solvents for each step of the sample preparation procedure.

Here we present an alternative extraction technique using natural Deep Eutectic Solvents (DESs), which are

emerging as green and sustainable solvents for efficient extraction of natural compounds.

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Deep eutectic solvents (DESs) represent a promising class of alternative solvents that have become of growing interest both at academic and industrial levels [10-15]. Compared to conventional volatile organic solvents (VOCs), DESs show high thermal stability, non-flammability and practically no vapour pressure, therefore low volatility. Formation of DESs can be easily accomplished by simply mixing and warming at least two safe, cheap, renewable and biodegradable components, generally a hydrogen bond donor and a hydrogen bond acceptor, which are capable of forming a eutectic mixture having a melting point markedly, lower than either of the individual components (fig. 1).

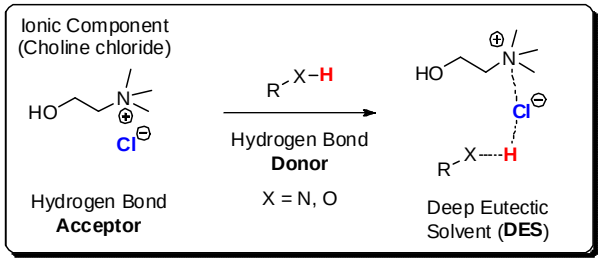

Fig. 1 Schematic representation of a choline chloride based Deep Eutectic Solvent.

Deep Eutectic Solvents (DES) can be formed between a variety of ammonium salts (e.g., choline chloride, betaine, glycine, proline, etc.), and a very large range of hydrogen bond donors such as urea, glycerol, carboxylic acids, amino acids and carbohydrates, polyalcohols. All these components come from renewable sources; thus, their biodegradability is extraordinarily high, and their toxicity is non-existent or very low if compared with classical VOCs deriving from petroleum.

Moreover, it is noteworthy that the extraction capability of DESs may be finely tuned: by choosing the optimal combination between the ionic part and the hydrogen bond donor, it is possible to design the best solvent with the optimal physical−chemical properties for the selective extraction of a peculiar biological marker.

DES are made of two components, the ionic part and the hydrogen-donor that is often a molecule with higher lipophilicity compared to the other constituent. Such double nature gives to DES a wide polar range and consequently a high degree of solubilisation strength for many classes of compounds characterized by dissimilar molecular structures. Therefore, the extractions performed with DES, instead then classical VOCs, can be accomplished without employing other additives such as strong acids or bases, often responsible of chemical alteration of the original sample (e.g. hydrolysis of tri-, di- and monoglycerides into glycerol and fatty acids).

[AS]

1. MATERIALS AND METHODS

A. *Samples*

In order to validate the protocols, two pottery samples were taken from a replica ceramic vessel after cooking experiments of animal fats (beef).

One sample was submitted to conventional extraction by chloroform/methanol followed by alkaline treatment of the insoluble residue of previously solvent-extracted potsherd.

Another sample was treated with DES, following the procedure described in the following paragraph.

The next step was the application of the extraction procedure on some archaeological pottery samples that had been already submitted to conventional extraction methods [16, 17]. The samples were taken from two cooking pots retrieved during the excavations in the indigenous settlement of Castello di Alceste (S.Vito dei Normanni-BR). These thin-walled containers have narrow mouth, rounded and everted rim, globular body and they begin to appear during the archaic age in many indigenous settlements in southern Italy. Most of them are Greek imports that, together with many other artefacts of Greek provenance, reflect important innovations in food preparation and consumption among the archaic indigenous societies. The sampled pots, datable to the VIth century B.C., presented burnt traces on the exterior surfaces and no visible residues adhering on the interior surfaces.

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B. *Lipid extraction of powdered ceramic samples*

A typical extraction procedure with DES can be described in three simple steps:

1. The suspension of the ground potsherd in the proper deep eutectic solvent. 3 ml of a glycerol/choline (2:1) mixture was used for the extraction of the powdered sherd samples (ca 2 g), after the addition of 1 ml of a standard solution of nonadecane.

2. The extraction by mixing, stirring and heating the ingredients. Ultrasounds or microwaves can also assist the extraction process, in order to optimize the yield of extraction. Extraction was performed by ultrasonication for 1 hour at 70°C.

3. After cooling and centrifugation the liquid fraction is ready to be analysed by liquid chromatography techniques (HPLC, LC-HPLC, etc.), but for chromatographic analysis by GC-MS or HT-GC, a second extraction to remove the analytes from the DES is required. This can be easily done using a common organic solvent such as ethyl acetate, diethyl ether, hexane, etc. and adding some water in order to reduce the typical high viscosity of DES and the solubility of analites in the polar phase (water/DES). In this experiment, hexane (2x4ml) was used as extraction

solvent. The solvent was then evaporated under a gentle stream of nitrogen, for subsequent derivatization.
The dried extracts were derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) at 80°C for 1 hour. Prior to analyses by GC-MS, the BSTFA was evaporated under a slow stream of nitrogen and the samples redissolved in an appropriate volume of hexane.

D. Gas chromatography – mass spectrometry
Samples were analysed using an Agilent Technologies 6850 II series gas chromatograph (5% phenyl-polymethylsiloxane capillary column, 30 m, internal diameter 0.25 mm, 0.25 µm film thickness), with a split/splitless injection system used in the split mode and maintained at 300°C, coupled to an Agilent 5973 Network mass spectrometer operated in the EI mode (70 eV). The mass spectrometer was set to scan in the range of m/z 50 to 600 in a total cycle time of 1 s. The GC oven temperature was programmed from 100°C to 280 °C at 10°C/min, and held at 280°C for 15 min. Helium was used as the carrier gas at a constant flow rate of 1 ml/min.
Compounds were identified partially by their retention time within the GC, based on comparisons with analysed reference compounds, but mainly by their mass spectra, interpreted manually with the aid of the NIST Mass Spectral Library.

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2. RESULTS

Replica pottery vessel
The sample submitted to extraction with chloroform/methanol gave very high lipid concentrations. The major fatty acids identified (fig. 2a) are palmitic (C16:0), oleic (C18:1) and stearic (C18:0) acids, together with tetradecanoic acid (C14:0). Cholesterol, branched chain and odd-numbered straight chain fatty acids (C15:0 and C17:0) were also detected, confirming the presence of fats from ruminant animals [18].
The sample treated by DES shows equivalent lipid concentrations as evidenced by the enhanced peak areas compared to the internal standard (fig. 2b), which was added at the same volume (1ml) in both extractions.
We observed also a higher percentage of nonanoic and decanoic acids (C9:0 and C10:0) but a lower abundance of stearic acid. The differences observed in the absolute amounts of lipids are probably due to variations in the yield of lipids absorbed by different points of the vessel profile.
The highest peak is represented by glycerol, deriving from the solvent used in the extraction mixture. Phthalate plasticizers were observed in lipid extracts and derive from plastic bottles the solvents have been stored in. Despite this, none of the samples was excluded from the study, as these contaminations are quite easy to detect, but we decided to store the solvents in glass bottles, in order to avoid modern contaminations in future experiments.
However, the distribution of fatty acids and their relative concentrations were extremely consistent in all extractions.

Archaeological samples
The data obtained from the experimental vessel was then compared with lipid concentration data for archaeological pottery sherds sampled from two cooking pots, which had been analysed previously following the extraction methods published in literature [4, 5, 6, 7]. GC-MS analyses of the total lipid extract showed the presence of fats from ruminant animals [16, 17], due to predominance of palmitic and stearic acid and the detection of odd numbered and branched chains fatty acids. Cholesterol was also detected.
GC-MS results of the samples treated with the extraction procedure by DES showed a similar fatty acids distribution (fig. 3). The percentage of the total lipid extracts was close to that obtained from the samples treated with chloroform/methanol, although the peak of cholesterol was recognizable only by its retention time and not by the mass spectra, due to low concentration. The samples analysed didn't gave higher concentrations of lipid residues compared to conventional extraction protocols, but the alternative method proved efficient to liberate enough fatty acids from the pottery matrix of archaeological samples.
These preliminary results show the potential of this new protocol, but they also suggest the necessity to optimize it through further experiments, in order to achieve better results on archaeological samples.

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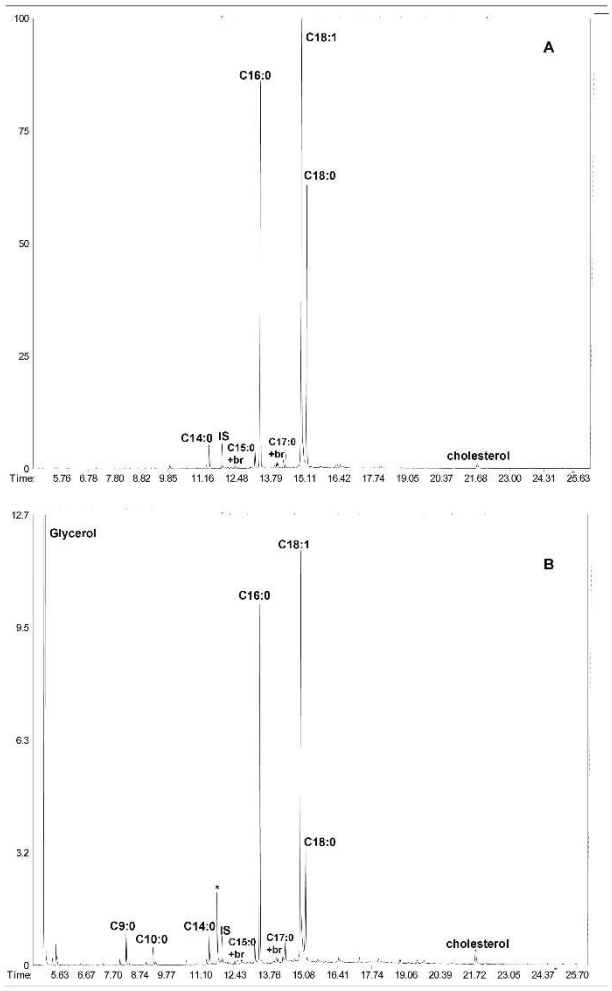

*Figure 2. Chromatograms of the total lipid extract of samples of replica pottery vessel treated with chloroform/methanol (a) and DES (b), showing the major fatty acids (as TMS derivatives). IS=Internal Standard; *=phthalate (plasticizer).*

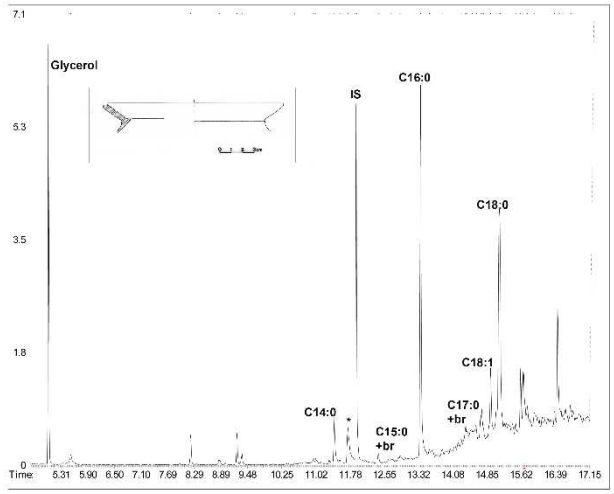

*Figure 3. Chromatogram of the total lipid extract of the archaeological sample (SV05 505/6) treated with DES, showing the profile of fats from ruminant animals. IS=Internal Standard; *=phthalate (plasticizer).*

CONCLUSIONS

The analyses by GC-MS of the pottery samples submitted to DES extraction gave comparable results with the analyses of the samples treated with chloroform/methanol and alkaline hydrolysis of the insoluble residue, as regards the amounts and relative proportions of fatty acids identified. For archaeological samples, this alternative method proved efficient to liberate enough fatty acids from the pottery matrix for quantification analysis of absorbed lipid residues. Nevertheless, the procedure still requires further investigation in order to improve and enhance the extraction potential of DESs and to develop its application on the chemical characterization of different classes of organic compounds. Future experiments on this new approach will be aimed also at the evaluation of the results by the application of different analytical techniques, in particular HPLC-MS, that would allow direct sample analysis after the extraction by DES, thus greatly reducing the sample preparation procedure.

The extraction technique proposed here could offer in fact an efficient, rapid and low cost analytical protocol for extracting lipid residues from archaeological pottery, making the processing of large numbers of potsherds feasible within the period of most research projects. In addition, also archaeologists could carry out this simple procedure without necessarily using chemical laboratories, because the solvents have the advantages of biodegradability and ease of handling with very low toxicity.

In conclusion, the development of this protocol intends to offer an alternative method for absorbed lipid residue analysis, aimed at saving time and laboratory consumables in the preparation of the extracts for the analyses by the common employed analytical instruments: gas chromatography-mass spectrometry (GC-MS), High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS), etc.

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REFERENCES

[1] R. P. Evershed, “Organic residue analysis in archaeology: the archaeological biomarker revolution”, Archaeometry, 50, 2008, pp. 895-924.

[2] R.P. Evershed, S.N. Dudd, M.J. Lockheart, S. Jim, “Lipids in archaeology”, in D.R. Brothwell, A.M. Pollard (edd.), Handbook of Archaeological Sciences, John Wiley & Sons, West Sussex 2001, p. 331.

[3] M. Roffet-Salque, J. Dunne, D.T. Altoft, E. Casanova, L.J.E. Cramp, J. Smyth, H. Whelton, R.P. Evershed, “From the inside out: Upscaling organic residue analyses of archaeological ceramics”, Journal of Archaeological Science: Reports (2016) (http://dx.doi.org/10.1016/j.jasrep.2016.04.005).

[4] M. S. Copley, H. A. Bland, P. Rose, M. Horton, R. P. Evershed, “Gas chromatographic, mass spectrometric and stable carbon isotopic investigations of organic residues of plant oils and animal fats employed as illuminants in archaeological lamps from Egypt”, The Analyst, 2005, 130, pp. 860-871.

[5] S. Charters, R. P. Evershed, L. J. Goad, P. W. Blinkhorn, V. Denham, “Quantification and distribution of lipid in archaeological ceramics: implications for sampling potsherds for organic residue analysis”, Archaeometry, 35, 1993, pp. 211-223.

[6] S. N. Dudd, R. P. Evershed, “Evidence for Varying Patterns of Exploitation of Animal Products in Different Prehistoric Pottery Traditions Based on Lipids Preserved in Surface and Absorbed Residues”, Journal of Archaeological Science, 26, 1999, pp. 1473-1482

[7] O.E. Craig, G.D. Love, S. Isaksson, G. Taylor, C.E. Snape, “Stable carbon isotopic characterisation of free and bound lipid constituents of archaeological ceramic vessels released by solvent extraction, alkaline hydrolysis and catalytic hydropyrolysis”, J. Anal. Appl. Pyrolysis, 71, 2004, pp. 613–634.

[8] M. Correa-Ascencio, R. P. Evershed, “High throughput screening of organic residues in archaeological potsherds using direct acidified methanol extraction”, Anal. Methods, 2014, 6, pp. 1330–1340.

[9] V. Papakosta, R. H. Smittenberg, K. Gibbs, P. Jordan, S. Isaksson, “Extraction and derivatization of absorbed lipid residues from very small and very old samples of ceramic potsherds for molecular analysis by gas chromatography–mass spectrometry (GC–MS) and single compound stable carbon isotope analysis by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS)”, Microchemical Journal, 123, 2015, pp. 196–200.

[10] A.P. Abbott, G. Capper, D.L. Davies, R.K. Rasheed, V. Tambyrajah, “Novel solvent properties of choline chloride/urea mixtures”, Chem. Commun. 2003, pp. 70–71.

[11] A.P. Abbott, J.C. Barron, K.S. Ryder, D. Wilson, “Eutectic-based ionic liquids with metal-containing anions and cations”, Chem. Eur. J. 2007, 13, pp. 6495–6501.

[12] M. Francisco, A. van den Bruinhorst, M.C. Kroon, “Low-Transition-Temperature Mixtures (LTTMs): A New Generation of Designer Solvents”, Angew. Chem. Int. Ed. 2013, 52, pp. 3074–3085.

[13] K. De Oliveira Vigier, G. Chatel, F. Jérôme, “Contribution of Deep Eutectic Solvents for Biomass Processing: Opportunities, Challenges, and Limitations”, ChemCatChem, 2015, 7, pp. 1250–1260.

[14] E.L. Smith, A.P. Abbott, K.S. Ryder, “Deep Eutectic Solvents (DESs) and Their Applications”, Chem. Rev. 2014, 114, pp. 11060–11082.

[15] D.A. Alonso, A. Baeza, R. Chinchilla, G. Guillena, I.M. Pastor, D.J. Ramón, “Deep Eutectic Solvents: The Organic Reaction Medium of the Century”, Eur. J. Org. Chem., 2016, 4, pp. 612–632.

[16] F. Notarstefano, “Ceramica e alimentazione. L’analisi chimica dei residui organici nelle ceramiche applicata ai contesti archeologici”, BACT 10, Bari 2012.

[17] F. Notarstefano, M. Lettieri, G. Semeraro, L. Troisi, “Food Habits and Social Identity during the Archaic Age: Chemical Analyses of Organic Residues Found on Pottery Vessels from the Messapian Settlement of San Vito dei Normanni (Southern Italy)”, in I. Turbanti-Memmi (ed.), Proceedings of the 37th International Symposium on Archaeometry, Berlin Heidelberg, 2011, pp. 465-471.

[18] H.R. Mottram, S.N. Dudd, G.J. Lawrence, A.W. Stott, R.P. Evershed, “New chromatographic, mass spectrometric and stable isotope approaches to the classification of degraded animal fats preserved in archaeological pottery”, Journal of Chromatography A, 833, 1999, pp. 209–221.