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Separation of molecular constituents from a humic acid by solid-phase extraction following a transesterification reaction

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Abstract

A selective removal of humic constituents involved in ester groups was conducted by a boron trifluoride–methanol transesterification reaction after removal of structurally unbound lipids. An analytical separation of subfractions containing specific classes of compound liberated from the humic matrix simplifies their identification by gas chromatography–mass spectrometry. We compared the traditional liquid–liquid separation into phenolic and aliphatic fractions with the modern and versatile fractionation technique using solid-phase extraction (SPE) on aminopropyl-bonded phases. Our results showed that both methods ensured separation of the same compounds, such as di- and tri-hydroxyalkanoic acids, α-, β- and ω-hydroxy fatty acids, alkanoic acids, α,ω-alkanedioic acids, *n*-alkanols, phenolic acids and sterols. Moreover, the SPE method not only provided a larger recovery of compounds, but involved smaller sample and solvent requirements, and larger ease and rapidity of sample handling than the traditional liquid–liquid separation. The SPE method should be thus recommended in structural studies of natural organic matter.

Keywords: Humic acids; Natural organic matter; Transesterification; Liquid–liquid extraction; Solid-phase extraction; Lipids; Hydroxyalkanoic acids

1. Introduction

Natural organic matter (NOM) occurs widely in soils, waters and sediments, and plays a key role within several natural and anthropogenic processes, such as the global carbon geochemical cycle and the transport of organic and inorganic pollutants across the environmental compartments. Humic substances (HS), being the main component of NOM, largely affect these processes. The molecular properties of humic and fulvic acids, and humin have been recognized to influence the binding and transport of pesticides and other apolar organic compounds [1–5]. Moreover, as they are the most persistent pool of soil organic carbon (SOC) with mean residence times of several hundreds of years, HS are involved in the control of CO_2 emission to the atmosphere, being a sink of soil OC [6]. Therefore, an improved molecular characterisation of HS is of large importance for understanding their role in environmental dynamics.

Different methods have been used for HS characterisation, including elemental analysis [7], oxidative and reductive degradation [8], UV-spectroscopy [9,10], ^{13}C NMR-spectroscopy [11], tangential membrane filtration [12], high performance size exclusion chromatography (HPSEC) [13], pyrolysis-GC/MS [14,15] and ESI-MS [16]. Based on the body of these recent results on HS chemistry, Piccolo [17] proposed that HS, rather than being macropolymers as traditionally believed, should be better described as supramolecular associations of relatively small (<1000 Da) heterogeneous molecules held together by weak dispersive forces and hydrogen bonds, in only apparently large molecular dimensions. However, the specific molecular composition of HS varies according with the nature and spatial variability of soils.

Recent investigations indicated the contribution of aliphatic constituents in the progressive accumulation of SOM and the relevance of esterification as a mode of binding and cross-linking for organic bioesters during the humifi-

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cation process [18]. Alkyl compounds, derived from both plant residues decomposition and microbial re-synthesis, are progressively incorporated into more stable SOM fractions and bound in forms extremely resistant to chemical degradation [19–21]. Furthermore, hydrophilic components released from biodegrading plant tissues are entrapped in the hydrophobic domains of humic matter and, thus, protected from further degradation [22]. Different sources of aliphatic compounds can be distinguished in soils, such as free extractable lipids, biopolyesters like cutin and suberin, which are present in both aerial and underground parts of plants, and non-hydrolyzable biopolymers like cutan and suberan. However, the majority of ester-bound moieties in soil seems to be related to cutin and suberin [23–26].

Boron trifluoride–methanol transesterification, that cleaves ester-linkages, has been successfully applied to structural studies of plant biopolyesters [27] and heterogeneous materials such as humic acids and humin [23,28]. The advantage of this technique relies in the selective removal of humic constituents and identification of labile diagnostic molecules without the profound structural alteration of more drastic degradation methods. Commonly, after liquid–liquid extraction of transesterified digests, the chloroform-soluble material is divided into aliphatic and aromatic subfractions according to the traditional procedure proposed by Kolattukudy et al. [27]. However, it is conceivable that a similar fractionation may be achieved by applying solid-phase extraction with aminopropyl cartridges [29] with considerable savings in time and materials. The SPE technique has been extensively used for lipid component separation, in both biological and food matrices [30,31] and is considered one of the most powerful techniques currently available for rapid and selective sample preparation and purification. In comparison to the conventional liquid–liquid extraction method, advantages of SPE include smaller sample and solvent requirements as well as simplicity and ease of handling. Therefore, SPE can be considered an environment-friendly method because of the reduced usage of toxic solvents.

The objective of this study was to compare two different techniques used to isolate the molecules involved into ester bonds in HS following a BF_3–CH_3OH transesterification reaction: the conventional liquid–liquid separation into aromatic and aliphatic fractions used by Kolattukudy et al. [27] and the rapid and versatile fractionation with SPE cartridges.

2. Experimental

2.1. Humic samples

Humic matter was obtained from a volcanic soil (Allic Fulvudand) from Vico, near Rome (Italy). Humic acids (HA) were isolated and purified as by standard procedures [17]. Briefly, the soil was shaken over night in a solution of 1 M NaOH and 0.1 M $Na_4P_2O_7$ under N_2 atmosphere. The HA were separated from the alkaline extracts by lowering the pH to 1 with 6 M HCl and extensively purified by three cycles of dissolution in 1 M NaOH solution and subsequent precipitation in 6 M HCl. The HA were then treated with a 0.5% (v/v) HCl–HF solution for 48 h, dialyzed (Spectrapore dialysis tubes, 3500 MW cut-off) against distilled water until chloride-free and freeze-dried. The humic samples were characterized for their elemental content using a Fison EA 1108 Elemental Analyzer. The resulting ash content was less than 3%.

2.2. Solvents and reagents

Methanol and all the other solvents were of reagent grade and used without further purification. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Macherey-Nagel, Germany. Acetyl chloride was from Fluka, whereas internal (tridecanoic acid, 98%) and external standards (docosanedioic acid, 85%; 16-hydroxyhexadecanoic acid, 98%; and β-sitosterol, 40%) were from Aldrich. The SPE cartridges used in this study had a bonded aminopropyl solid-phase (Strata NH_2 500 mg/3 ml, Phenomenex).

2.3. Gas chromatography–mass spectrometry (GC/MS)

GC/MS analyses were conducted on a Perkin-Elmer Autosystem XL gas chromatograph, equipped with a Perkin-Elmer Turbomass Gold mass spectrometer. The injector was held at a constant temperature of 250 °C and a fused-silica capillary column (Restek Rtx©-5MS, 30 m length × 0.25 mm i.d. × 0.25 μm film thickness) was used for analytical separation. Helium was the carrier gas at a flow rate of 1.6 ml/min. The oven was temperature-programmed from 100 to 300 °C, at a rate of 4 °C/min, and held there for 20 min. The mass spectrometer operated in full scan mode in the range of m/z 50–600 and by an electron impact ionisation energy of 70 eV with a cycle time of 1.0 s. Compounds identification was based on comparing the mass spectra of the chromatographic peaks to those reported in the NIST-MS library.

2.4. Sample treatment and transesterification

The main steps of sample treatment are shown in Fig. 1 and were conducted on triplicate samples. Typically, 500 mg of HA were initially oven dried at 40 °C and the free lipids extracted by shaking for 2 h at room temperature with 100 ml of a (2:1, v/v) solution of dichloromethane (DCM) and methanol (MeOH). This solvent combination was adopted to increase the polarity of the removing solution of structurally unbound lipids as compared to a sequential water and chloroform extraction [32]. The extract was separated from residue through centrifugation (25 min, 7000 rpm) and the supernatant removed. The residue was further extracted

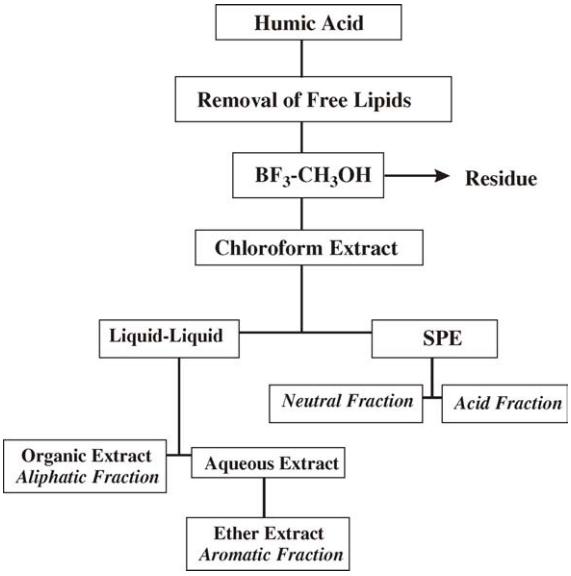

Fig. 1. Scheme of chemical treatments undergone by the HA sample.

with 100 ml of the DCM/MeOH (2:1, v/v) solution over night at room temperature, and again separated from the supernatant by centrifugation. The residue remaining from the lipids extraction was air-dried, added with 15 ml of 12% BF_3–CH_3OH solution and heated at 90 °C over night. This treatment was repeated twice. The supernatants were recovered by centrifugation (15 min, 7000 rpm), combined, treated with an excess of water in order to destroy the remaining BF_3, and then liquid–liquid extracted with chloroform. The total extract was dehydrated with anhydrous Na_2SO_4 and then divided into two aliquots,which underwent different fractionation procedures.

2.5. Liquid–liquid separation

The first aliquot of the chloroform organic phase was treated with 1 M KOH in a separation funnel and the remaining traces of alkali neutralized with 3 N HCl. The aliphatic components partitioned into the organic phase, which was separated from the aqueous phase and dehydrated with anhydrous Na_2SO_4. The aromatic components were recovered from the acidified aqueous phase by liquid–liquid extraction with diethylether [23,27,28]. Both aliphatic and aromatic subfractions were dried by rotoevaporation, derivatised and analysed by GC/MS.

2.6. SPE fractionation

The second aliquot was rotoevaporated to dryness, dissolved again in DCM/isopropanol (2:1, v/v) and loaded into a SPE aminopropyl cartridge column, previously conditioned with hexane (4 ml). The column was first eluted with DCM/isopropanol (2:1, v/v; 8 ml) in order to obtain the neutral subfraction, and then flushed with 2% v/v acetic acid in diethylether (8 ml) to elute the acid subfraction [29]. Both neutral and acid subfractions were derivatised and analysed by GC/MS.

2.7. Derivatisation

All fractions were derivatised before GC/MS analyses. Following the addition of a known quantity of tridecanoic acid as internal standard, each sample was firstly methylated, by refluxing for 30 min at 60 °C with an excess of MeOH and acetyl chloride. The solvent was evaporated to dryness under a gentle stream of nitrogen, and the resulting residue was silylated with 100 μl of BSTFA containing 1% TMCS, for 1 h at 70 °C, added with 400 μl of hexane, and analysed by GC/MS. Preliminary tests showed that the amount of each class of compounds was underestimated by the internal standard method. Therefore, the quantitative analysis was conducted using calibration curves of different external standards. The standard deviation of the quantitative evaluation of each compound for triplicate samples varied from 5 to 15% for the liquid–liquid separation and from 4 to 12% for the SPE method.

3. Results and discussion

The GC/MS chromatograms of the aliphatic and aromatic subfractions, from the conventional liquid–liquid separation, and the neutral and acid subfractions, from the SPE fractionation, are shown in Fig. 2. Both procedures, following the BF_3–CH_3OH transesterification of HA, gave rise to the separation of a large number of organic compounds. The main compounds detected in the fractions comprised di- and tri-hydroxyalkanoic acids (C_{16}, C_{18}), α- and β-hydroxyalkanoic acids (C_{10}–C_{26}), ω-hydroxyalkanoic acids (C_{14}–C_{26}), alkanoic acids (C_9–C_{32}), α,ω-alkanedioic acids (C_{16}–C_{24}) and minor amounts of *n*-alkanols (C_7–C_{30}), phenolic compounds and sterols. The distribution of the identified compounds is comparable to that previously reported for the monomeric constituents of biopolymers, studied with different methods, such as base hydrolysis [29], high performance liquid chromatography [33] or thermochemolysis with tetramethylammonium hydroxide [34,35].

Table 1 summarizes all identified molecules and their relative content with reference to the net weight of compounds extracted by the two treatments. The total organic carbon (TOC) in the dried humic acid sample was 51%. The total yield obtained after the BF_3–CH_3OH transesterification, through the conventional liquid–liquid separation, constituted 2.15% of the TOC in the humic acid sample, whereas 3.56% was obtained through the SPE fractionation. Additionally, Table 2 reports the total net weight extraction for each class of identified compounds and their distribution in the aliphatic and aromatic fractions, and in the neutral and acid fractions, for the liquid–liquid and SEP method, respectively.

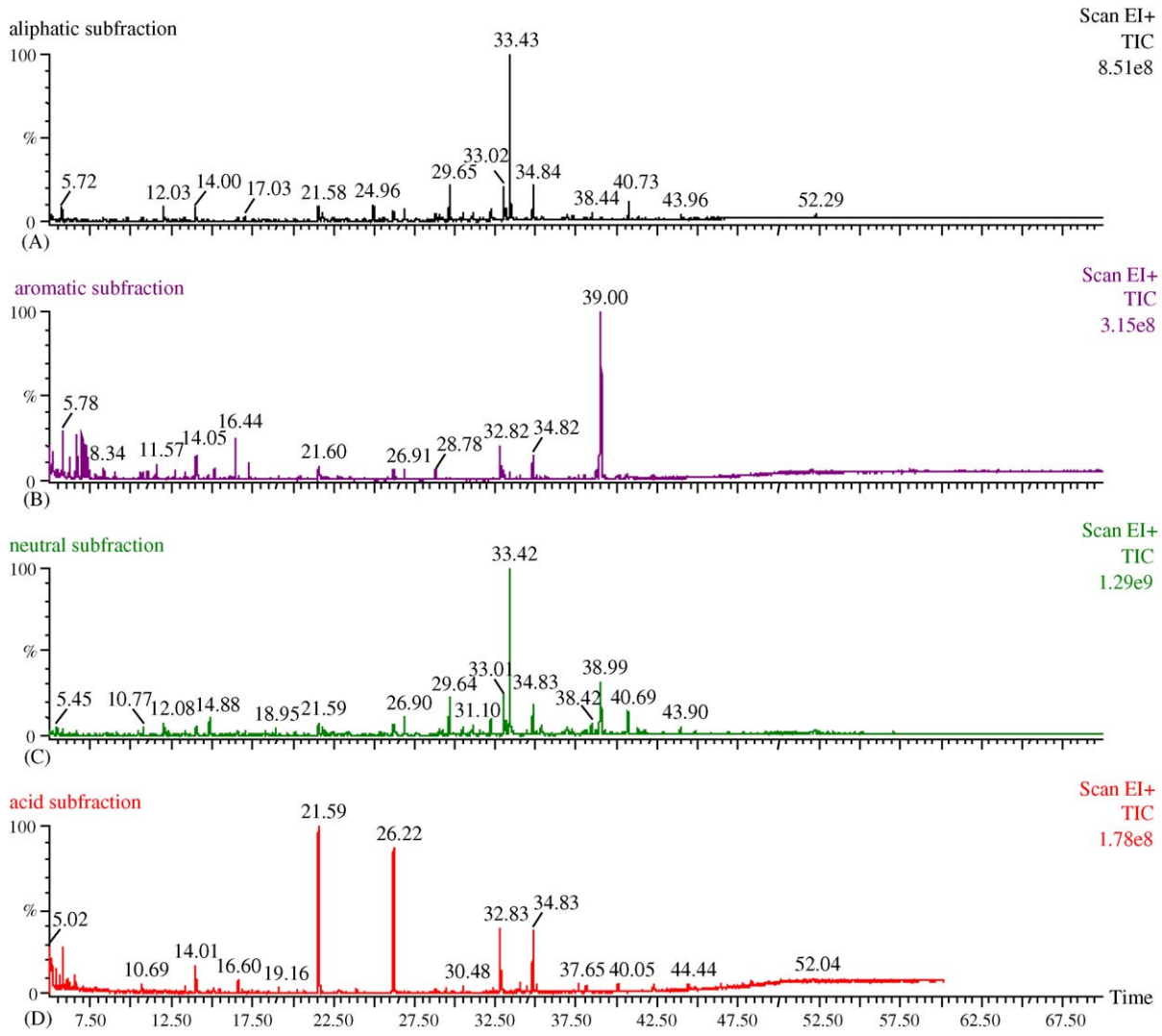

Fig. 2. GC/MS total ion chromatograms of HA, after the BF_3–CH_3OH treatment: (A) and (B) represent aliphatic and aromatic extracts, respectively, obtained after conventional liquid–liquid extraction; (C) and (D) represent neutral and acid fractions, respectively, obtained after SPE fractionation.

3.1. Di- and tri-hydroxy acids

This class of compounds represented more than a quarter of the total detected compounds. Results by either liquid–liquid separation or SPE fractionation were comparable, although the latter was quantitatively more efficient (Fig. 3). In fact, the quantitative analysis based on C_{16} ω-hydroxy acid external standard and a calibration curve ranging from 20 to 150 μg, showed that 10.81 and 8.10 mg g^{-1}_{TOC} of C_{16} and C_{18} di- and tri-hydroxy acids were recovered, respectively, by SPE and liquid–liquid separation. The most abundant components in all analysed subfractions were 9,10- and 10,18-dihydroxyoctadecanoic acids, though smaller amounts of 9,16-, 9,10- and 10,16-dihydroxyhexadecanoic acid isomers were also detected. As already reported by Naafs and van Bergen [24], all identified C_{16} dihydroxy acids were substituted in the ω-position and near the centre of the aliphatic chain, at positions 9

Table 1
Total quantity (μg) and μg g^{-1}_{TOC} of class of compounds in humic matter extracted by the BF_3–CH_3OH transesterification reaction and measured by GC/MS for both liquid–liquid and SPE methods

Compounds	Liquid–liquid		SPE	
	μg	μg g^{-1}_{TOC}	μg	μg g^{-1}_{TOC}
Total	6109	24544	9068	36432
Di-, tri-hydroxy C_{16}, C_{18} acids	2017	8103	2697	10810
α-, β-Hydroxy acids	1551	6233	2516	10110
ω-Hydroxy acids	1305	5243	1805	7251
Saturated and unsaturated fatty acids	338	1360	539	2166
Dicarboxylic acids	401	1612	564	2265
Linear alcohols	301	1208	438	1761
Phenolic acids	118	474	437	1755
Terpenoids	78	312	78	313

Table 2
Quantity (μg) and percentage of classes of compound as total and in separated fractions by both liquid–liquid and SPE methods following extraction by the BF_3–CH_3OH transesterification reaction and measurement by GC/MS

Compounds	Liquid–liquid[a]		SPE[b]	
	μg	(%)	μg	(%)
Di-, tri-hydroxy C_{16}, C_{18} acids	2017	33.01	2697	29.67
Fraction A	1319	21.59	2594	28.61
Fraction B	698	11.42	97	1.06
α-, β-Hydroxy acids	1551	25.40	2516	27.75
Fraction A	1322	21.65	2451	27.03
Fraction B	229	3.75	65	0.72
ω-Hydroxy acids	1305	21.36	1805	19.90
Fraction A	1072	17.55	1805	19.90
Fraction B	233	3.81	0.00	0.00
Saturated and unsaturated fatty acids	338	5.54	540	5.95
Fraction A	281	4.60	358	3.94
Fraction B	57	0.94	182	2.00
Dicarboxylic acids	401	6.57	564	6.22
Fraction A	341	5.58	524	5.78
Fraction B	61	0.99	40	0.43
Linear alcohols	301	4.92	438	4.83
Fraction A	267	4.35	426	4.70
Fraction B	35	0.57	12	0.13
Phenolic acids	118	1.93	437	4.82
Fraction A	61	1.00	377	4.16
Fraction B	57	0.93	59	0.65
Terpenoids	78	1.27	78	0.86
Fraction A	72	1.18	78	0.86
Fraction B	6	0.09	0	0.00

[a] Fraction A = aliphatic fraction; Fraction B = aromatic fraction.
[b] Fraction A = neutral fraction; Fraction B = acid fraction.

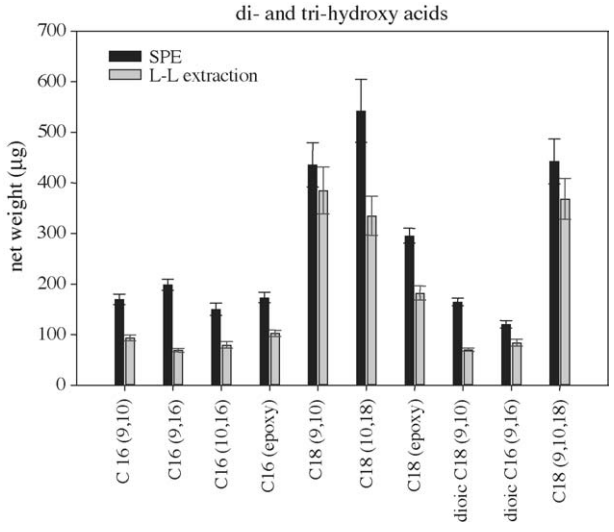

Fig. 3. Weight (μg) of C_{16} and C_{18} di- and tri-hydroxy acids identified in chromatograms in relation to external standards for either liquid–liquid extraction or SPE fractionation. Numbers in parentheses refer to position of hydroxy groups.

or 10, but differently from Naafs and van Bergen [24] C_{14} dihydroxy acids were not detected. The 10,16- and 9,16-dihydroxyhexadecanoic acids are thought to be the major compounds deriving from plant polymers, such as cutin and suberin [36–39]. The C_{18} dihydroxy acids are usually less common, but the range of positional isomers found here was narrower than that reported in these early works since only 9,10- and 10,18-isomers were detected in the samples of this study.

The 16-hydroxy-9,10-epoxyhexadecanoic acid and 18-hydroxy-9,10-epoxyoctadecanoic acid were also identified. Since epoxy compounds are the first intermediates in the oxidation of double bonds, the epoxy acids detected here may be formed during the intermediate steps in the conversion of unsaturated fatty acids to the corresponding tri-hydroxymonobasic acids [23]. Therefore, the presence of considerable amounts of 9,10,18-trihydroxyoctadecanoic acid may have been partially due to the C_{18} trihydroxy acids originally present in suberized tissues [25,35] and partially to those derived from C_{18} epoxide, which may have been hydrolysed during the transesterification reaction [37].

3.2. Monohydroxy acids

This class of compounds includes α-, β- and ω-hydroxy acids, with the latter present in lesser amounts. The compounds obtained by liquid–liquid extraction were similar to those by SPE (Fig. 4), though the SPE method extracted larger quantities of each compound, except for the C_8 ω-hydroxy acid, that was not present in the SPE extracts. The quantitative analysis was performed by using C_{16} ω-hydroxy acid as external standard and the relative calibration curve ranging from 20 to 150 μg. Based on this, the liquid–liquid separation extracted 6.23 mg g^{-1}_{TOC} of α- and β-hydroxy acids and 5.24 mg g^{-1}_{TOC} of ω-hydroxy acids, whereas the SPE

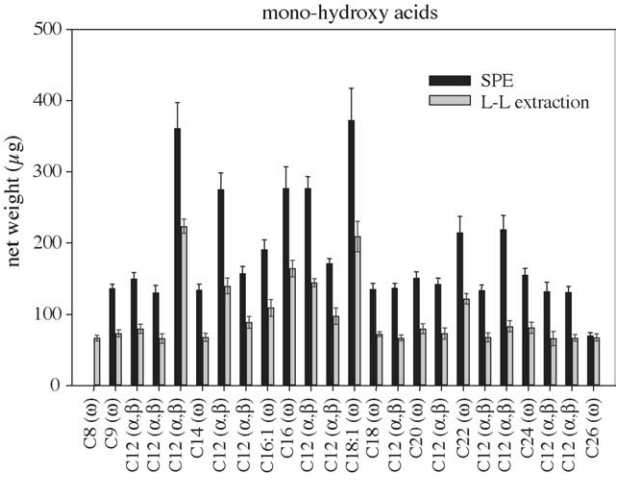

Fig. 4. Weight (μg) of monohydroxy acids identified in chromatograms in relation to external standards for either liquid–liquid extraction or SPE fractionation. Letters in parentheses refer to position of the hydroxy groups (α or β, and ω).

method gave 10.11 mg g^{-1}_{TOC} of α- and β-hydroxy acids and 7.25 mg g^{-1}_{TOC} of ω-hydroxy acids. The distribution of α- and β-hydroxy acids is uniform, ranging from C_{12} to C_{26}, with a maximum for the C_{14} compound. β-Hydroxy acids have already been detected in humus extracts by other authors [23] and are assumed to be indicators of bacterial metabolism. In particular, they are considered molecular markers derived from lipopolysaccharides of Gram-negative bacteria [40,41]. The distribution of ω-hydroxy acids is characterised by the occurrence of long chain (C_{14}–C_{26}) members showing a strong predominance of even over odd carbon number, $C_{18:1}$ and $C_{16:1}$ being the dominant molecules. Similar results have been reported by Naafs et al. [24] and Matzke and Riederer [41], but the dominance of $C_{18:1}$ was less pronounced. ω-Hydroxy acids are very common constituents of cutins [37], though the presence of a large content of long chain ($\geq C_{16}$) ω-hydroxy acids was attributed mainly to free extractable polyesters and suberin associated with overlying plant roots [36,43]. The relatively low concentration of C_{22} ω-hydroxy acid, in comparison with $C_{18:1}$ and C_{16} ω-hydroxy acids, has been suggested to be a consequence of biotic degradation by micro-organisms, which preferentially degrade the C_{22} component [29]. Smaller amounts of C_9 ω-hydroxy acid have also been detected and assumed to be the result of microbial β-oxidation or auto-oxidation of a double bond in 18-hydroxyoctadec-9-enoic acid [44] and subsequently preserved through chemical bonding, most likely via ester linkages, into insoluble polymeric matrices [45]. However, according to Naafs and van Bergen [24], this compound is likely to be indirectly derived from plant suberins, without ruling out a contribution from cutins.

3.3. Fatty acids

Both fractionation methods showed a similar distribution of *n*-alkanoic acids, derived from cutins ($<C_{20}$) [37] and from suberins ($\geq C_{20}$) [36,43]. On a quantitative basis, the SPE fractionation was more effective than the liquid–liquid separation in the extraction of fatty acids (Fig. 5). The quantitative analysis was conducted by using a C_{13} linear fatty acid as external standard and a calibration curve ranging from 10 to 150 μg. This showed that 1.36 mg g^{-1}_{TOC} of fatty acids, only 2.6% of which was represented by unsaturated C_{18}, have been extracted by liquid–liquid separation, whilst the SPE method produced 2.17 mg g^{-1}_{TOC} of fatty acids, 1.8% of which was the unsaturated C_{18} acid. Qualitatively, there was not a significant difference between the two methods, since fatty acids ranged from C_9 to C_{32} by liquid–liquid separation and from C_9 to C_{28} by SPE, being the C_{16} and C_{18} molecules the largest in both methods. Similar patterns have been observed previously in fractions deriving from alkaline hydrolysis of plant [29] and soil residue [18]. A minor contribution came from unsaturated C_{18} acids since mono and di-unsaturated C_{18} fatty acids have been identified, although the position of double bonds in the chain was still undefined. The dominance of long-chain members, in particular fatty acids with

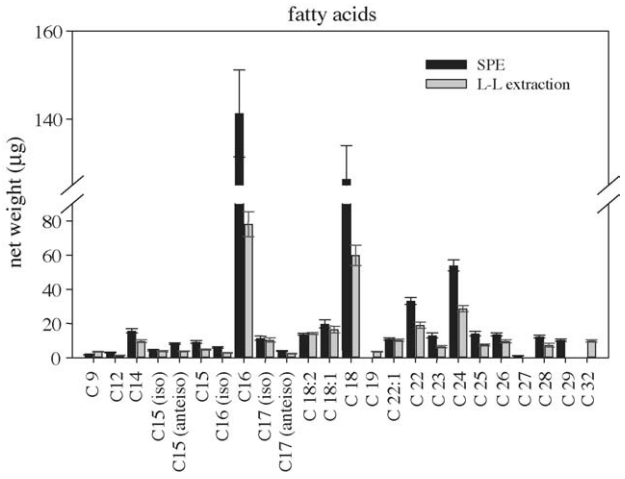

Fig. 5. Weight (μg) of saturated and unsaturated fatty acids identified in chromatograms in relation to external standards for either liquid–liquid extraction or SPE fractionation.

even carbon number, was pronounced, indicating an origin from higher plants [46,47]. Nevertheless, the concentration of unsaturated C_{18} fatty acids, which are commonly dominant in higher plant tissues [48], was very low with respect to saturated C_{18} acid. An explanation may reside in the oxidation of double bonds due to bacterial degradation. Evidence for such process could be represented by the occurrence in all the analysed subfractions of *iso* and *anteiso* branched C_{15} and C_{17} alkanoic acids, which reflect a bacterial activity [18,46].

3.4. Dicarboxylic acids

Both fractionation methods were characterised by the detection of short chain α,ω-diacids, ranging from C_8 to C_{10}, and of long chain α,ω-diacids, ranging from C_{16} to C_{24}. Fig. 6 shows that analogous qualitative and quantitative results were obtained by using both treatments, although a slightly larger effectiveness of the SPE method was observed in the extrac-

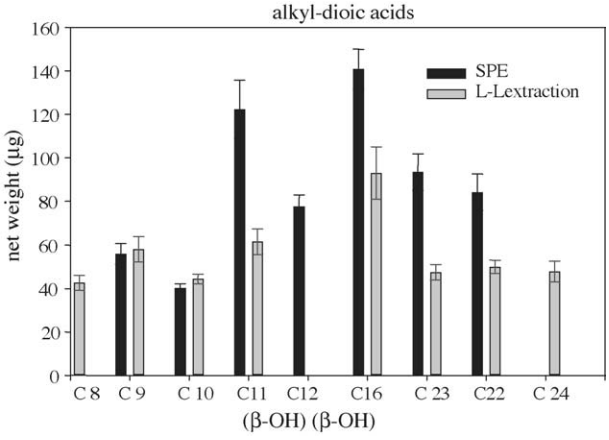

Fig. 6. Weight (μg) of alkyl dioic acids identified in chromatograms in relation to external standards for either liquid–liquid extraction or SPE fractionation.

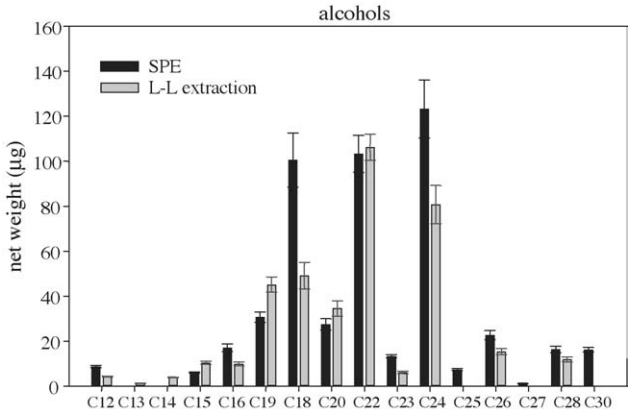

Fig. 7. Weight (μg) of alcohols identified in chromatograms in relation to external standards for either liquid–liquid extraction or SPE fractionation.

tion of long chain α,ω-diacids. The quantitative analysis was done by using a C_{22} dioic acid as external standard and a calibration curve ranging from 20 to 100 μg. The dicarboxylic acids extracted by liquid–liquid separation amounted to 1.61 mg g^{-1}_{TOC}, while they were up to 2.26 mg g^{-1}_{TOC} by the SPE method. The most abundant member was the C_{16} dicarboxilic acid, that has been reported in plant cutins [37], although is commonly associated with suberins [35,36,43]. A relevant presence of C_9 diacids was also found and it confirmed previous findings [18,24]. The origin of this diacid appears to be associated with the microbial oxidation of Δ^9-unsaturated acids such as oleic acid [18,45], as in the case of C_9 ω-hydroxy acid.

3.5. Linear alcohols

The *n*-alkanols were present in all the analysed subfractions, reaching about 5% of the identified compounds in both fractionation methods. The quantitative analysis was conducted by using a C_{13} linear fatty acid as external standard and a calibration curve ranging from 10 to 150 μg. It was found that 1.21 and 1.76 mg g^{-1}_{TOC} of alcohols were recovered by liquid–liquid separation and solid-phase extraction, respectively. As shown in Fig. 7, no significant difference was observed between the two analytical methods. The distribution of *n*-alkanols ranged from C_{12} to C_{30}, exhibiting a large predominance of even over odd carbon number in the chain, being the C_{18}, C_{22} and C_{24} the most abundant chains. The *n*-alkanols are biopolyester-derived compounds. In general, homologues with more than 18 carbon atoms distinguish suberin from cutin [36,42]. A wide range characterised by the predominance of C_{20} alcohol was previously reported in hydrolysis products of suberin [43] and of humin [18], although C_{22} and C_{24} members were also commonly found as dominant suberin derived products [43,25]. Grasset and Amblès [18] related the detection of *iso* C_{15} alkanol components to a relevant bacterial activity. However, this compound was not identified in the humic extracts of this study.

3.6. Phenolic acids

A great variety of phenolic acids were found in all the analysed subfractions. Di- and tri-methoxy benzencarboxylic acids, para, meta and ortho-methoxy cinnamic and mandelic acids, mono and di-methoxy benzoic acids were all identified on the basis of their mass spectra. The yields of these compounds were larger in SPE fractions than in the aliphatic and aromatic fractions obtained by the liquid–liquid separation. The amount was 1.75 mg g^{-1}_{TOC} by the SPE procedure and only 0.47 mg g^{-1}_{TOC} by the conventional liquid–liquid separation. The quantitative analysis made use of β-sitosterol as external standard, due to its structural similarity, and of a calibration curve ranging from 4 to 80 μg. Phenolic acids, also reported by Almendros and Sanz [23,28] as the second major group of transesterification products, are known to be part of the aromatic domain of suberins, usually linked as esters [36], but they can also derive from lignins [28].

3.7. Terpenoids

Small quantities of tetracyclic sterols and pentacyclic triterpenes were detected, amounting to about 0.31 mg g^{-1}_{TOC} in both studied methods. The quantitative analysis related to β-sitosterol as external standard and to a calibration curve ranging from 4 to 80 μg. The β-sitosterol has been identified in depolymerisates from soils [48–50] and from suberins [43]. Other sterols and triterpenes are usually found in waxes, but their identification and the exact nature of ester-bound moieties from which they derive are still unclear.

4. Conclusions

Our findings showed that the SPE procedure appears to represent a valid alternative to the traditional liquid–liquid separation of ester-bound components of HS released after a BF_3–CH_3OH transesterification reaction. The results by the SPE technique were comparable to those obtained using the conventional solvent extraction technique for all the classes of compounds identified. While the SPE method was found to be as efficient as the liquid–liquid extraction method on both qualitative and quantitative bases, the former should be recommended due to the smaller sample and solvent requirements and simpler handling. Moreover, the reproducibility of results suggests that the SPE method can be efficiently used to compare HS of different origin.

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