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Impact of lipid extraction on the dearomatisation of an *Eisenia foetida* protein powder

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ABSTRACT

Delipidation was studied as a way to dearomatise a non-conventional protein powder obtained from *Eisenia foetida* earthworms. In the first step, we studied the impact of several factors such as solvent type, extraction method and particle size on the yield of extracted lipids. Lipid extraction from samples was considerably improved using an ultrasound method with a chloroform/methanol mixture and small particle size. In the second step, the volatile compounds were extracted from the delipidated protein powder by the SAFE extraction method and by the HS-SPME method, and were further analysed by GC–MS. The chloroform/methanol mixture and the ultrasound method extracted a wide range of volatile compounds very efficiently. Moreover, whatever method was used to extract the lipids, the volatile compounds detected in the headspace of the delipidated powder represented less than ¼ of the volatile compounds detected in the headspace of the regular powder.

1. Introduction

Some authors have pointed out the great potential of *Eisenia foetida* as a non-conventional protein source (Vielma-Rondón, Ovalles-Durán, León-Leal, & Medina, 2003). They determined that earthworms had a high protein content (62% dry weight; Medina et al., 2003) and that the content of essential amino acids, except for tyrosine (Vielma-Rondón et al., 2003), was higher than that recommended by the FAO. Earthworms could then have many different nutritional applications as animal food and as an ingredient in food products for humans. They have already been used in an experimental diet for rainbow trout (Bastardo, Medina, & Sofia, 2005) and as chicken feed (Taboga, 1980).

For practical reasons, earthworms are often dried and used as a powder in the different applications. One of the main problems of using this earthworm protein powder in food products is related to its organoleptic properties. Off-flavours, especially those due to the fishy odour of this earthworm protein powder can make it unacceptable to humans.

It is well known from the literature that relationships between lipids and aroma compounds are important (Druaux, Le Thanh, Seuvre, & Voilley, 1998). Firstly, as aroma compounds are mainly hydrophobic molecules, they often interact with the lipid fraction. Secondly, phenomena such as oxidation and enzymatic degradation of lipids may be responsible for the appearance of off-flavours and have been reported for various products. For example, many volatile flavour compounds in commercial oil-free soybean lecithin are attributed to the autooxidative decomposition products of unsaturated fatty acids of phospholipids (Kim, Ho, & Chang, 1984). In meat, Wood et al. (2008) reported that fatty acid composition determines the firmness/oiliness of adipose tissue and the oxidative stability of muscle, which in turn affects flavour and muscle colour in vertebrates.

In fish flesh, Sérot, Regost, and Arzel (2002) reported that aliphatic aldehydes commonly derive from lipid oxidation. They underlined that autooxidation of unsaturated fatty acids can be initiated by a physical catalyst such as light, or by enzymes or microorganisms present in fish flesh. In an extensive study on volatile aldehydes in smoked fish, Varlet, Prost, and Serot (2007) reported that the large quantities of *n*-alkanals found in smoked fish flesh (heptanal and hexadecanal, for example) could be related to the large amounts of their lipidic precursors found in unsmoked fish flesh. In the same study, the aroma descriptor "fishy" was found for heptanal and E-2-nonenal.

For these reasons, in the present study, we tried to remove lipids from dried earthworms to dearomatise them as has been done for soybean products. Eldridge, Friedrich, Warner, and Kwolek (1986) reported that flavour scores of defatted soybean meals were considerably better than full-fat soybean flakes with the usual

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grassy-beany and bitter flavours only minimally detectable. Samoto, Miyazaki, Kanamori, Akasaka, and Kawamura (1998) reduced the off-flavour of soy protein isolate by removing lipid-linked proteins and polar lipids.

There are many methodologies for removal of lipids from food matrices. The nature of these methods can be very different. Some methods involve the use of enzymes (Snabe, Neves-Petersen, & Petersen, 2005), other methods use membranes (Bergqvist, Strandberg, & Rappe, 1999), or supercritical fluids (Nagy & Simándi, 2008), but the most popular method for lipid extraction is Soxhlet where lipids are extracted using solvents. In the last ten years, the extraction by ultrasounds has also proved to be a rapid and efficient tool for lipid extraction especially in food chemistry applications (Vilkhu, Mawson, Simons, & Bates, 2008).

Apart from the extraction method, other parameters may affect extraction efficiency. These parameters include the particle size of the sample and the nature of the extracting solvent. The particle size controls the mass transfer kinetics and access of the solvent to the soluble components. Higher extraction efficiencies can be achieved by applying smaller particle sizes, resulting in an increase in mass transfer, and in the quantity of the accessible soluble fraction (Nagy & Simándi, 2008). Regarding the solvent, it is possible to extract lipids of different nature depending on their polarity (Zarnowski & Suzuki, 2004).

The aim of the present work was to study to what extent the delipidation of the earthworm powder had on the elimination of volatile compounds. For this purpose, an experiment was designed to map the effects of three qualitative parameters (method of extraction, solvent type, particle size) on the extraction yield of lipids from the regular earthworm protein powder and to determine the optimal conditions for lipid extraction. In the second step, volatile compounds were extracted from the delipidated protein powder both by the SAFE extraction method and by the headspace solid phase microextraction method, and were further analysed by GC–MS. This allowed us to estimate the dearomatisation induced by delipidation.

2. Material and methods

2.1. Samples and reagents

Earthworm protein powder obtained from *E. foetida* was kindly donated by Professor Ana Luisa Medina of *Los Andes* University, Mérida (Venezuela). Samples were obtained from the species *E. foetida* at an adult stage of development (3 months), with an average length and weight of 8.5 cm and 0.45 g, respectively (earthworm cultures from "Luis Ruiz Terán" Herbarium at the Faculty of Pharmacy, University of the Andes, Merida-Venezuela). The earthworms were fed a diet of organic waste compost, obtained from a university canteen in the region. In order to guarantee optimum growth conditions, the temperature, moisture and pH of the compost were controlled. The earthworms were thoroughly washed and were subsequently stored for 12 h in an air insufflated water container. The previously washed earthworms were put in boiling water for 1 min to kill them. Excess water was then removed and the earthworms were dried in an oven at 60 °C for four hours and finally ground to a homogeneous flour. The dried earthworm protein powder obtained (regular protein powder = RPP) was placed in closed plastic bags and stored at 4 °C until further analyses. Its protein content was determined by the Kjeldahl method and was found to be 65.5 ± 0.4% using a conversion factor of 6.25.

Hexane (⩾96.5%) was purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France), chloroform (>99%) from Aldrich, and methanol (⩾99.9%) from Carlo Erba Reagents. Dichloromethane (⩾99.8%) from Carlo Erba Reagents was distilled before use. Deionised water obtained from a MilliQ RG, Millipore apparatus was employed. The chromatographic standard (2-methylpropyl 3-methylbutanoate) with a purity ⩾ 98.0% was purchased from Sigma–Aldrich.

2.2. Particle size reduction

The particle size of the RPP was determined by sifting in a sifting device (AS200 basic, Retsch, Haan, Germany) comprising of six sieves from 3150 to 200 μm pore size. The particles obtained were between 200 and 500 μm for 46% of the particles, between 500 and 800 μm for 16%, larger than 800 μm for 13%, and smaller than 200 μm for 25% for the rest. When required, the regular earthworm protein powder was ground in an IKA – WERKE GmBH & CO apparatus after cooling in liquid nitrogen. The ground particles were sifted through a set of sieves of different sizes (250, 100 and 50 μm). The remaining particles in the 50 μm sieve were collected and called the 'reduced particle size' fraction.

2.3. Soxhlet extraction

Soxhlet extraction was carried out following the official method (Horwitz & AOAC, 1980). Thirty gram of RPP was placed into an extraction thimble bought from Schleicher and Schuell, MicroScience. The RPP was covered with cotton to avoid sample loss and placed in a Soxhlet device which was filled with 225 ml of solvent (hexane or chloroform/methanol mixture in a ratio 9:1). The Soxhlet was run for 8 h. After this time, the delipidated earthworm protein powder (DPP) was put into a watch glass and the solvent was evaporated overnight at room temperature. The standard deviation was determined for this method and was 0.28.

2.4. Ultrasound extraction

Ultrasound extraction experiments were performed using a US bath Bransonic Mod 3210 (Branson Europe B.V.) with an ultrasound fixed-frequency of 47 kHz ± 6%. The extraction time and the number of extraction steps were optimised.

2.4.1. Optimisation of extraction time

For the optimisation of the extraction time, the following kinetic experiments were done: six flasks containing 5 g of RPP and 25 ml of hexane each were placed into the ultrasound bath for a given time (1, 2, 3, 4, 5 and 10 min). Immediately afterwards, the suspensions were removed from the ultrasound bath and filtered off. The solvent fraction was evaporated at 45 °C under reduced pressure, using a rotary evaporator, in order to collect extracted lipids. The amount of extracted lipids was weighed for each different time.

2.4.2. Optimisation of the number of extraction steps

In order to optimise the number of extraction steps, 30 g of RPP were extracted with 150 ml of a mixture of solvents (chloroform/methanol in a ratio 87:13) for 3 min in an ultrasound bath. After the first extraction step, the delipidated earthworm protein powder (DPP) was filtered off under vacuum and the solvent was evaporated at 45 °C under reduced pressure. This process was repeated several times using the DPP obtained from the previous extraction step.

2.5. Extraction of volatile compounds

2.5.1. SAFE extraction method

The extraction of volatile compounds was done using the SAFE (solvent assisted flavour evaporation) extraction method (Engel, Bahr, & Schieberle, 1999). For this extraction technique, 20 g of

sample (RPP or DPP) were mixed with 150 ml of deionised water. The suspension was placed in the SAFE apparatus and vacuum distillation (10^{-2} Pa) was performed for two hours at 30 °C. The water phase containing the volatile compounds was stirred three times for 15 min with 15 ml of distilled dichloromethane. After liquid–liquid separation, the organic phase was collected, dried over anhydrous sodium sulphate and filtered off through glass wool. The extract was concentrated to 222 μl using a Kuderna–Danish apparatus in a 70 °C water bath.

2-Methylpropyl 3-methylbutanoate was dissolved in dichloromethane and was used as the internal standard at a concentration of 23.3 ng/μl. One microlitre of the internal standard was added to 100 μl of the extract immediately before GC–MS analysis. The standard deviation of this method was equal to five.

2.5.2. Headspace solid phase extraction method (HS-SPME)

For the HS-SPME analysis, 5 g of each protein powder was transferred into a 20 ml vial and the vial was immediately sealed with a Teflon-lined septum and screw cap. The equilibration time was 24 h at room temperature. Incubation at 30 °C during 90 min with an agitation of 250 rpm at intervals of 20 s was then done using an autosampler (Gerstel MPS2). After incubation, the headspace volatiles were extracted using an SPME fiber (2 cm–50/30 μm DVB/Carboxen/PDMS/StableFlex, Supelco, USA) for 60 min at 30 °C with agitation. Finally, for the injection, the fiber was inserted into the GC injection port and desorbed for 3 min. The fiber was cleaned at 240 °C for 12 min after each injection. The standard deviation of this method was equal to 1.00.

2.6. Gas chromatography–mass spectrometry analysis (GC–MS)

Volatile compounds were analysed on a 5973 gas chromatograph (Hewlett–Packard, Palo Alto, CA, USA) equipped with a fused-silica capillary column (30 m × 0.32 mm ID, 0.5 μm film thickness) coated with a DB-Wax stationary phase (J and W Scientific, USA). The instrument was equipped with a split/splitless injection port operating at 240 °C in the splitless mode. Helium was used as the carrier gas and the chromatographic temperature was programmed from 40 to 240 °C at a rate of 4 °C/min, with a final isotherm of 10 min. Mass spectrometry was taken in the electron ionisation mode at 70 eV and the scan range between 29 and 350 amu. The ion source was set at 230 °C and the transfer line at 240 °C. Compounds were identified by comparison with mass spectra libraries (WILLEY138, NIST, and INRA database) and by the calculation and comparison of the GC retention index of a series of alkanes (C8–C30) with the retention index from published data calculated under the same conditions. The quantitative data were obtained by electronic integration of the TIC peak areas with the ChemStation program.

2.7. Experimental design for the optimisation of delipidation and for the data analysis of dearomatisation

A 2^3 factorial experiment design (Table 1) was carried out to map the effects of three qualitative factors on the extraction yield of lipids in the regular earthworm protein powder and to determine the optimal conditions for the lipid extraction. The selected factors were considered at two levels: solvent (hexane or chloroform/methanol), extraction type (soxhlet or ultrasound) and particle size (normal: >250 μm or reduced: between 50 and 100 μm).

Observed variables were quantitative variables: the percentage of extracted lipids, the percentage of dearomatisation calculated from results obtained after SAFE extraction, and the percentage of dearomatisation calculated from results obtained after HS-SPME extraction. Experiment DPP1 (solvent = hexane, extraction method = Soxhlet, particle size = normal) was repeated four times to obtain an estimate of the experimental error. The main effects and interactions were calculated on variables as described by Box, Hunter, and Hunter (1978).

Additionally, the volatile compounds extracted by HS-SPME (qualitative variables) were identified for each experiment. On the basis of the simultaneous absence (the aim was elimination) or mismatch of the volatile compounds in each sample, Sokal and Sneath two similarity coefficients (Sokal & Sneath, 1963) were calculated and the corresponding dissimilarity matrix was computed. Cluster analysis following Ward's aggregation method was

Table 1
Coded levels and corresponding physical values of the 2^3 factorial experiment designs.

Factors	Label	Levels	
		−1	+1
Solvent	SOL	Hexane	$CHCl_3$/MetOH
Extraction method	EXT	Soxhlet	Ultrasound
Particle size	PAR	Normal[a]	Reduced[b]

[a] Particle size > 250 μm.
[b] 50 μm < particle size < 100 μm.

Table 2
Experimental conditions and response values of the eight experiments used to study the percentage of delipidation and the consequent reduction in volatile compounds.

Test labels	Factors			Observed variables		
	SOL	EXT	PAR	Extracted lipids	Volatile compounds	
				% (w/w of RPP)	% of dearomatisation using SAFE	% of dearomatisation using HS-SPME
DPP1	−(Hexane)	−(Soxhlet)	−(Normal)	9.95	77	83
DPP2	−(Hexane)	−(Soxhlet)	+(Reduced)	10.27	88	89
DPP3	−(Hexane)	+(Ultrasound)	−(Normal)	9.1	78	78
DPP4	−(Hexane)	+(Ultrasound)	+(Reduced)	11.5	73	79
DPP5	+($CHCl_3$/MetOH)	−(Soxhlet)	−(Normal)	8.63	47	92
DPP6	+($CHCl_3$/MetOH)	−(Soxhlet)	+(Reduced)	7.8	26	76
DPP7	+($CHCl_3$/MetOH)	+(Ultrasound)	−(Normal)	20.24	93	89
DPP8	+($CHCl_3$/MetOH)	+(Ultrasound)	+(Reduced)	24.5	87	83

NB: Dearomatisation refers to the comparison of the sum of the peak areas obtained after GC–MS analyses for each sample with the sum of the peak areas obtained for the regular earthworm protein powder.
The percentage of dearomatisation using SAFE was calculated using the following equation: % of dearomatisation = 100–100 ($\sum$ relative peak areas of DPPi/$\sum$ relative peak areas of RPP).
Relative peak areas were calculated from the chromatogram of a SAFE extract by dividing absolute area of each peak by the peak area of the internal standard. In this case, $\sum$ relative peak areas of RPP and DPP7 were found to be 91 and 6.6, respectively.
The percentage of dearomatisation using HS-SPME was calculated using the following equation: % of dearomatisation = 100–100 ($\sum$ absolute peak areas of DPPi/$\sum$ absolute peak areas of RPP).
In this case the absolute peak areas, obtained from a headspace chromatogram, were used to calculate the percentage of deodourisation. $\sum$ absolute peak areas of RPP was found to be 60411740.

used to identify homogeneous subgroups of cases among the different experiments.

3. Results and discussion

3.1. Optimisation of ultrasound extraction

Different extraction times ranging from 1 to 10 min were tested in the extraction of the RPP with hexane. The amount of extracted lipids was quite constant whatever the extraction time and was 0.32 g ± 0.02, representing 6.4% (w/w) of RPP. Although there were no significant differences between the six extraction times chosen, the extraction time was established at $t = 3$ min as a compromise between duration of the experiment and efficiency of the extraction. The effect of the number of extraction steps was checked by successive extractions on the same sample using a mixture of chloroform/methanol solvents. After the first extraction, the amount of extracted lipids accounted for 12.83% (w/w) of the RPP, which is far more than was obtained with hexane. During the following extraction steps, the amount of extracted lipids decreased considerably at each successive extraction: 3.07% for the second extraction, 2.10% for the third and 1.27% for the fourth. After the fifth extraction, the mass of extracted lipids accounted for less than 1% (w/w) of the RPP. For this reason, the number of extractions was set at five. The total amount of extracted lipids with five steps of 3 min was 20.2% (w/w) of the RPP powder.

3.2. Impact of the extraction of lipids on dearomatisation

Factors of the 2^3 factorial experiment and corresponding percentages of extracted lipids are reported in Table 2. The amount of extracted lipids by using both methods (ultrasound and Soxhlet) ranged from 7.8% to 24.5% with a mean value at 12.75%.

Dearomatisation was quantified by two complementary methods. For all samples, extraction of the volatile fraction was done by SAFE extraction and by HS-SPME extraction. The SAFE method is known to give a relatively complete extraction of the volatile compounds without heating the sample (Engel et al., 1999; Pozo-Bayón, Guichard, & Cayot, 2006), but the concentration of the sample using this method can lead to the loss of the most volatile compounds. The HS-SPME method is complementary since it allows the determination of the most volatile compounds. We can consider that the SPME fiber gave a representative analysis of the headspace because the DVB/carboxen/PDMS fiber we used was able to trap a wide range of volatile compounds. The analysis of the collected volatile compounds was done by GC–MS. The dearomatisation was expressed as a percentage of dearomatisation.

Table 3
Calculated effects and standard errors for the 2^3 factorial experimental design used to study delipidation and dearomatisation of an earthworm powder.

Effect	Extracted lipids Estimate ± standard error	% of dearomatisation using SAFE Estimate ± standard error	% of dearomatisation using HS-SPME Estimate ± standard error
Average	12.75 ± 0.28	71 ± 5	83.63 ± 1.00
Main effects			
SOL	+2.54 ± 0.55	−8 ± 10	+1.38 ± 1.96
EXT	+3.59 ± 0.55	+12 ± 10	−1.38 ± 1.96
PAR	+0.77 ± 0.55	−3 ± 10	−1.88 ± 1.96
Two-factor interactions			
SOL × EXT	+3.49 ± 0.55	+15 ± 10	+2.38 ± 1.96
SOL × PAR	+0.09 ± 0.55	−4 ± 10	3.63 ± 1.96
EXT × PAR	+0.90 ± 0.55	0 ± 10	+0.63 ± 1.96
Three-factor interactions			
SOL × EXT × PAR	+0.38 ± 0.55	+4 ± 10	+1.88 ± 1.96

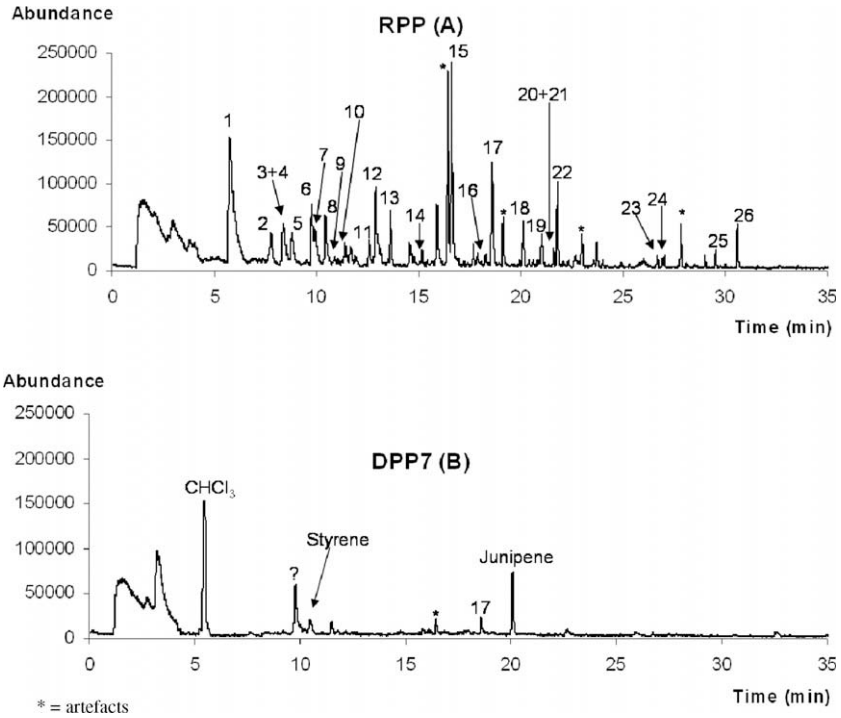

Fig. 1. Volatile compound analysis done by GC–MS after HS-SPME extraction. (A) Regular earthworm protein powder (RPP) chromatogram. (B) Delipidated earthworm protein powder (DPP7) chromatogram.

The volatile fraction of the regular earthworm protein powder (RPP) was analysed and the HS-SPME chromatogram obtained is shown in Fig. 1A. The same procedure was applied to DPP samples corresponding to each experiment of the factorial design. An example of a HS-SPME chromatogram obtained for DPP7 is given in Fig. 1B.

As it can be seen on Fig. 1, there was a considerable difference between the peak areas for all volatile compounds when comparing DPP chromatograms to the RPP chromatogram. The peak areas of all volatile compounds were lower in the case of the delipidated earthworm protein powder.

Using the SAFE method, between 90 and 150 volatile compounds were detected depending on the samples. The percentage of dearomatisation using SAFE varied from 26% to 93% (Table 2). The lowest dearomatisation effect was found for DPP6, which was also the experiment that gave the lowest percentage of extracted lipids. In contrast, the highest dearomatisation effect was measured in DPP7, which corresponded to one of the highest amounts of extracted lipids, but not the highest. For the SAFE method, the percentage of dearomatisation increases with the percentage of extracted lipids to reach a maximum at about 10% of extracted lipids. Beyond this amount of extracted lipids, the percentage of dearomatisation is quite constant. It can be assumed that the extracted lipids are of two types: (i) lipids that participate to the formation of the flavour or that interact with the volatile compounds. (ii) Lipids that do not take part in the formation of the flavour and whose extraction increases the percentage of extracted lipids but not the percentage of dearomatisation.

Using the HS-SPME method, between 2 and 38 volatile compounds were detected depending on the samples. The percentages of dearomatisation using HS-SPME were higher than those calculated for the SAFE results and varied from 76% to 92% (Table 2). Nevertheless, the relationships between the percentage of dearomatisation and the percentage of extracted lipids are not clearly established.

3.3. Main effects and interactions of the different factors on the delipidation and dearomatisation of the earthworm powder

The main effects and interactions of the different factors of the experimental design on delipidation and dearomatisation were calculated and given in Table 3.

The main effects of the three factors on the percentage of extracted lipids were all significant because contrasts were higher than the experimental error and they were positive for the three factors.

Table 4
Volatile compounds identified by GC–MS in an HS-SPME extract from regular protein powder and from delipidated protein powders.

Identification number	RT	Calculated RI[e]	Reference RI	Compounds	LogP[f]	CAS	DPP1	DPP2	DPP3	DPP4	DPP5	DPP6	DPP7	DPP8
1	5.75	1082	1080[a]	Hexanal	1.8	66-25-1	√	√	√	√	√	√		
2	7.77	1164	1169[c]	1-Penten-3-ol	1.12	616-25-1			√					
3	8.39	1187	1186[c]	2-Heptanone	1.73	110-43-0								
4	8.41	1189	1189[c]	Heptanal	2.29	111-71-7			√	√	√			
5	8.79	1202	1200[c]	Dipentene	4.83	7705-14-8								
6	9.77	1235	1237[c]	2-Pentyl-furan	3.87	3777-69-3	√		√	√	√	√		
			1240[a]											
7	9.95	1241	1221[b]	6-Methyl-2-heptanone	2.15	928-68-7			√					
8	10.42	1257	1256[a]	1-Pentanol	1.33	71-41-0			√	√				
9	10.90	1273	1264[a]	1-Methyl-3-isopropylbenzene	4	535-77-3								
10	11.40	1289	1280[a]	2-Octanone	2.22	111-13-7			√	√				
			1264[d]											
11	12.59	1328	1376[d]	2,3-Octanedione	0.63	585-25-1	√		√	√		√		
12	13.00	1342	1340[a]	6-Methyl-5-hepten-2-one	2.06	110-93-0			√	√				
			1342[c]											
13	13.63	1362	1354[a]	1-Hexanol	1.82	111-27-3	√	√	√	√				
			1366[c]											
14	15.19	1412	1411[c]	3-Octen-2-one	2.29	1669-44-9			√	√	√			
15	16.63	1459	1456[a]	1-Octen-3-ol	2.60	3391-86-4	√	√	√	√	√	√		
			1463[c]											
16	17.87	1499	1498[c]	2-Decanone	3.2	693-54-9								
17	18.60	1522	1528[a]	Benzaldehyde	1.71	100-52-7	√	√	√	√	√	√	√	√
			1520.9[c]											
18	20.12	1573	1539[b]	E,E-3,5-octadien-2-one	2.08	30086-02-3	√	√	√	√	√	√		
19	21.01	1604	1606[a]	2-Undecanone	3.69	112-12-9			√	√	√	√		
			1603[c]											
20	21.60	1624	1587[b]	E-2-octen-1-ol	2.59	18409-17-1			√	√				
21	21.61	1625	1640[d]	Dihydro-2(3H)-furanone	−0.31	96-48-0	√			√		√		
22	21.78	1630	1628[d]	Butanoic acid	1.07	107-92-6	√		√	√		√		
23	26.69	1805	1800[d]	4-Methyl pentanoic acid	1.98	646-07-1								
24	26.93	1815	1815[c]	2-Tridecanone	4.68	593-08-8				√	√			
25	29.53	1914	1918[c]	Benzene ethanol	1.57	60-12-8		√	√	√	√	√		
26	30.58	1955	1954 or 1956[d]	Heptanoic acid	2.54	111-14-8	√	√	√	√	√	√	√	

√: means that the volatile compound is present in the sample.
[a] Retention index published from a Supelcowax-10 stationary phase (Bianchi, Careri, Mangia, & Musci, 2007).
[b] Retention index published from a CW20 M stationary phase (Kondjoyan & Berdagué, 1996).
[c] Retention index published using a DB-Wax stationary phase (Farkas, Le Quéré, Maarse, & Kovác, 1994).
[d] Retention index published from a DB-Wax stationary phase or a similar polarity phase (The pherobase: database of insect pheromones and semiochemicals, <http://www.pherobase.net>, El-Sayed, 2008).
[e] Retention index calculated with a DB-Wax stationary phase using a series of alkanes between C8 and C30.
[f] Expressed by the estimated logarithm of the *n*-octanol/water partition coefficient (from KOWWIN v. 1.67).

The factor with the strongest influence was the extraction method: the ultrasound method gave a higher percentage of extracted lipids than the Soxhlet method did.

Some authors (Luque-Garcia & Luque de Castro, 2003; Perez-Serradilla, Priego-Capote, & Luque de Castro, 2007) demonstrated that ultrasound facilitates the extraction due to the cavitation process. During this process, vapour bubbles form in the liquid, grow and undergo implosive collapse. This leads to an increase in temperature and pressure in local hot spots. The increased temperature enhances the solubility of the analytes (lipids) in the leachant (solvent) and facilitates their diffusion from the sample matrix to the outer region; on the other hand, the increased pressure facilitates penetration of the leachant into the sample matrix and transfer from the matrix to liquid phase at the interface. It can also be assumed that the cavitation process leads to the liberation of lipids covalently bound to proteins and carbohydrates, which can explain the better extraction efficiency obtained with the ultrasound extraction than with the Soxhlet extraction.

The solvent also played a significant role in the extraction. The polarity of the chloroform/methanol mixture is higher than that of hexane, and for this reason, this mixture is able to extract a higher range of lipids.

Finally, the factor with the weakest influence was the particle size of the sample. In fact, the use of small particle size samples only slightly increased the amount of extracted lipids.

It is noticeable that two two-factor interactions of the three were significant and positive: SOL × EXT, and EXT × PAR to a lesser extent. Extraction by ultrasound was significantly improved by using the chloroform/methanol mixture, and was slightly improved by using the sample with reduced particle size. The three-factor effect was not significant.

For the dearomatisation measured by SAFE, the method of lipid extraction was the only factor to influence the percentage of dearomatisation. Dearomatisation was significantly improved by the use of ultrasound. Additionally, the effect of the SOL × EXT interaction was significant and positive, showing that dearomatisation was even better using chloroform/methanol. As assumed for the yield of extracted lipids, the high polarity of the chloroform/methanol mixture allowed the extraction of a wide range of aroma compounds and not only non polar compounds as was the case when hexane was used.

The three-factor effect was not significant.

In the case of dearomatisation measured by HS-SPME, none of the main effects was significant. Two two-factor interactions of the three were significant: SOL × EXT had a positive effect; i.e. a synergistic effect was observed using ultrasound with chloroform/methanol, and SOL × PAR had a negative effect; i.e. an antagonistic effect was observed using chloroform/methanol with reduced particle size. It can be hypothesised that the reduced particle size may have led to caking and a lower surface of exchange. The three-factor effect was not significant.

3.4. Hierarchical cluster analysis (HCA)

The global dearomatisation parameter (surfaces of all detected compounds) determined using SAFE extraction showed a clear relationship between the amount of extracted lipids and the percentage of dearomatisation. On the opposite, the global parameter determined using HS-SPME gave no clear tendency. A more "qualitative" analysis of the chromatograms was conducted considering the number of volatile compounds detected using HS-SPME for the different DPP. Among the volatile compounds extracted by HS-SPME, only the compounds identified both in DPP samples and in the RPP sample were kept for the hierarchical cluster analysis. Moreover some acids were not taken into account because they were found to be artifacts. The resulting volatile compounds identified in RPP and the eight samples of DPP were reported in Table 4. Among these volatile compounds, some might come from the raw material (i.e. the earthworm itself, its food and its environment such as earth, water, ...), others might be due to microbiological modifications or lipid peroxidation. Additionally, drying of the protein powder may have concentrated off-flavours and/or created new odorant compounds. Many chemical groups were found in the volatile fraction among which ketones (6-methyl-5-hepten-2one), alcohols (1-hexanol), aldehydes (hexanal), and acids (heptanoic acid). Some of the aldehydes and acids found in the volatile

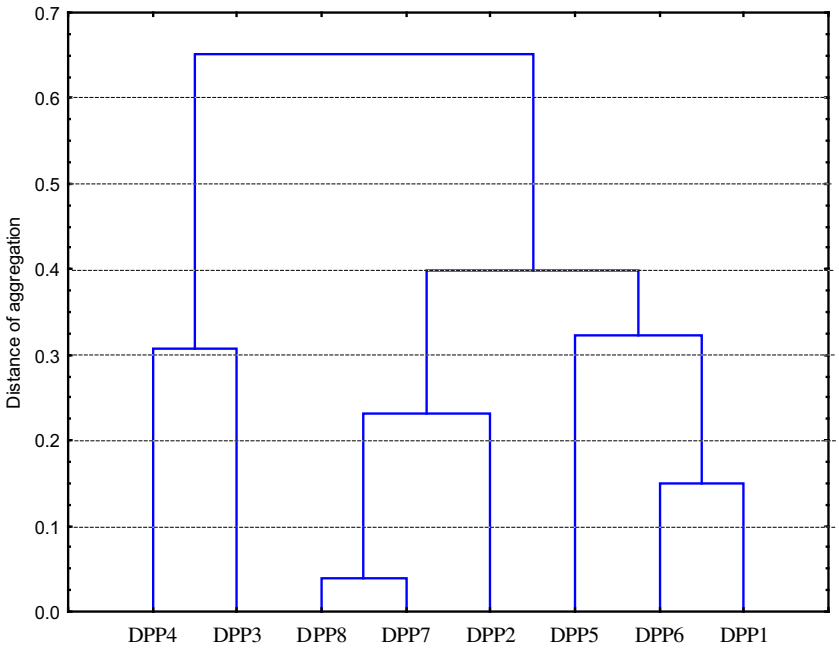

Fig. 2. Dendrogram obtained from the hierarchical cluster analysis (Method of Ward. Dissimilarity matrix of Sokal and Sneath $n°2$) of the identified volatile compounds analysed by HS-SPME in DPP samples.

fraction may come from the degradation of lipids. In fact, in the first step, lipid oxidation generates hydroperoxides, the decomposition of which leads to the formation of aldehydes, acids, esters and hydrocarbons. For example, model experiments of autooxidation of oleic, linoleic and linolenic acids gave pentanal, hexanal, heptanal, E-hex-2-enal (Belitz, Grosch, & Schieberle, 2004).

For hierarchical cluster analysis, the dissimilarity matrix was computed as explained in the Section 2 and the dendrogram (graphical representation on the hierarchical cluster analysis) was reported in Fig. 2. Experiments DPP8 and DPP7 were very close with a distance of aggregation below 0.05. They formed a group with DPP2 (distance of aggregation about 0.25) and were characterised with few volatile compounds detected in the corresponding headspace samples: one, two and seven volatile compounds, respectively. For experiments DPP1, DPP5 and DPP6 (distance of aggregation about 0.35), 10 or 11 compounds were detected, six of which were found in the three samples. Finally, for DPP3 and DPP4 (distance of aggregation about 0.3), 19 volatile compounds were detected, among which 17 were found in both samples. Experiments DPP3 and DPP4 were clearly different from the other experiments (distance of aggregation about 0.65).

We hypothesised that hexane was not able to remove polar volatile compounds. In fact, as shown in Table 4, volatile compounds with low logP values (below 1.5) were identified only in samples DPP1, DPP3, DPP4 and DPP6, which, except for DPP6, were all obtained using hexane.

Among these samples, DPP1 and DPP6 were obtained using Soxhlet that is to say that they underwent an 8 h-delipidation process that would have increased the extraction of volatile compounds. As a consequence, DPP3 and DPP4 exhibited the highest number of remaining volatile compounds.

For samples DPP2, DPP7 and DPP8, for which a maximum of seven volatile compounds were detected in the headspace, it should be noticed that the percentages of dearomatisation calculated from HS-SPME quantitative results were high and ranged from 83% to 89%. The corresponding percentage of dearomatisation calculated after SAFE extractions were the highest of the experimental design.

4. Conclusions

Ultrasound extraction of 5×3 min was found to extract lipids from a dried earthworms powder very efficiently and rapidly. The 2^3 factorial experimental designs allowed us to conclude that the chloroform/methanol (9:1) mixture used together with the ultrasound method gave the highest extraction yield for lipids and led to an impressive decrease in the volatile fraction of the samples. It is noticeable that hexane, contrarily to the chloroform/methanol mixture, did not lead to the total elimination of the volatile compounds of low hydrophobicity and that the Soxhlet method permitted the elimination of the most volatile compounds as well as the ultrasound extraction method.

Delipidation resulted in a significant decrease in the peak areas of the volatile compounds extracted from the protein powders but also in the number of detectable compounds. Whatever the method used to extract the lipids, the volatile compounds detected in the headspace of delipidated protein powders using HS-SPME represented less than ¼ of the volatile compounds detected in the headspace of regular protein powder.

The main disadvantage of this study is the use of organic solvent in the delipidation step in comparison with solvent-free techniques such as the supercritical CO_2 extraction. Nevertheless, the main advantages of the ultrasound technique over the supercritical CO_2 extraction are as follows: (i) the equipment is much simpler, so the overall cost of the process is much lower. (ii) Ultrasound-assisted leaching allows the extraction of a wide variety of compounds, whatever their polarity, as it can be used with any solvent (Luque-Garcia & Luque de Castro, 2003).

Nevertheless, the residual amount of solvent must be checked before the efficiency of the dearomatisation can be validated by sensory analysis of the delipidated powder and before sensory identification of the remaining volatile compounds can be done using olfactometry.

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