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Stable isotope ratio analysis for the characterization of edible insects

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Abstract

Entomophagy, or the act of eating insects, has been practiced since ancient times, but it started to gain more popularity, especially in Western countries, only recently. As sustainability is one of the current emerging themes, the inclusion of insects in our diet is a valid alternative that might help reduce the amount of water and land used for livestock and the associated emissions of greenhouse gasses. Moreover, insects are a source of protein, fibres, vitamins, minerals and fats. Edible insects are considered a novel food, for which no isotopic reference values are yet available. In the present work, samples of farmed edible insects (n=40) belonging to different orders (namely, Coleoptera, Hemiptera, Hymenoptera, Lepidoptera, Odonata and Orthoptera) and insect-based food items (n=4) for human consumption were analysed. The following isotopes, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O$ and $\delta^{2}H$ of the defatted samples, together with the $\delta^{13}C$ of the fat, were investigated. The aim of the work was to provide the first reference isotopic ratios that can be used for future investigations in the food quality field. The variability of these parameters was dependent on the life stage and diet of insects, their geographical origin, and the addition of ingredients as seasoning.

Keywords *entomophagy, edible insects, stable isotope ratios, database, authenticity*

1. Introduction

Entomophagy, or the act of eating insects, has been part of the culinary tradition of many countries since ancient times, but started to increase in popularity only recently, gaining momentum especially in Western countries. Nowadays, more than 100 countries and about two billion people in the world practice entomophagy, mostly in some parts of Asia, Africa and Latin America (Barennes *et al.*, 2015). There are over 2000 species of edible insects in the world (Roos, 2018), but the most commonly consumed belong predominantly to the orders Coleoptera, Lepidoptera, Hymenoptera, Hemiptera, Orthoptera, Odonata, Isoptera, and Diptera (van Huis, 2013). Edible insects can be consumed at different life stages as larvae, pupae and adult insects (Stamer, 2015).

Despite the common prejudices that make people reject entomophagy, they are a valuable source of protein, fibres, fats, vitamins (i.e. ascorbic and folic acid, thiamine, riboflavin, niacin) and minerals (i.e. calcium, potassium, magnesium, phosphor, sodium, and iron). Most species of insects generally contain high levels of proteins (50-70% on dry basis), resulting in good digestibility (Sosa and Fogliano, 2017). As for fats, their content ranges from 10 to 50% on dry basis (Xiaoming *et al.*, 2010). The percentage can differ according to the life stage of the insect (the amount is usually higher in the larvae compared to the adults), while their composition in terms of fatty acids depends on factors such as the species, sex, stage of life, diet and environmental temperature (Oonincx *et al.*, 2015). The fats of the insects, which are generally liquid at room temperature, can derive from their diet or from endogenous synthesis. The essential fatty acids contained in their fat are linoleic (18:2n-6) and linolenic (18:3n-3) acids, which are more abundant in insects than in other animal and vegetal sources (da Silva Lucas, 2020).

Besides the nutritional value of edible insects, entomophagy provides also environmental advantages (Govorushko, 2019). As sustainability is one of the emerging themes of our times, the inclusion of insects in our diet can help reduce the amount of water and land used for livestock. As reported in other studies, indeed, the land area and the water needed to rear insects is less consistent than those needed for the production of chicken, pork, and beef (Nadeau *et al.*, 2015; Dennis G. A. B. Oonincx *et al.*, 2012). Rearing insects also entails a very high conversion of food into protein, giving solution to the growing demand by the humanity for food and nutrition, and especially for animal protein (Govorushko, 2019). Furthermore, the inclusion of insects into our diet would contribute to the decrease of greenhouse gases and ammonia emissions deriving from this practice (Nadeau *et al.*, 2015; Ooninck *et al*., 2010).

From a regulatory point of view, in Europe insects are considered as a "novel food" by Regulation 2015/2283EC. Meanwhile, only a limited amount of studies have been carried out to investigate the chemical and microbiological safety of edible insects (Poma *et al*., 2019; Poma *et al*., 2017; Baiano, 2020). The European Food Safety Authority (EFSA) mentioned *Musca domestica*, *Zophobas atratus*, *Galleria mellonella*, *Bombyx mori*, *Achroia grisella*, *Locusta migratoria migratorioides*, *Schistocerca Americana*, *Tenebrio molitor*, *Hermetia illucens*, *Acheta domesticus*, *Gryllodes sigillatus* and *Alphitobius diaperinus* as the species having the greatest potential to be used as food and feed in Europe (EFSA Journal, 2015).

Stable isotope ratio analysis of light elements (C, N, S, O and H) has been widely used to investigate food authenticity. Examples are reported for wine (Dordevic *et al*., 2013), vinegar (Camin *et al*., 2013; Perini *et al*., 2014), orange juice (Simpkins *et al*., 1999), coffee (Santato *et al*., 2012), olive oil (Camin *et al*., 2010), cheese (Camin *et al*., 2012; Pianezze *et al*., 2020), saffron (Perini *et al*., 2020), tomatoes (Bontempo *et al*., 2020; Bontempo *et al*., 2011), honey (Bontempo *et al*., 2017), cereals (Asfaha *et al*., 2011) and also food supplements (Perini *et al*., 2017). It has also been recognised, since the 90s by several standardisation organizations (CEN, AOAC, OIV, EU Reg.) as an official method to detect adulterations in wine, honey, fruit juice, vinegar, cheese and other food matrices (Rossmann, 2001). An authenticity or adulteration assessment encompasses the analysis of genuine samples to create reference datasets (Camin *et al*., 2017; Donarski *et al.*, 2019) which are then used for comparing the data of samples on the market. Isotopic values are therefore available for several categories of food, but to the best of our knowledge, no isotopic values for edible insects are yet reported in literature.

In this work, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O$ and $\delta^{2}H$ of 40 defatted farmed insect samples and 4 insect-based food items, as well as the $\delta^{13}C$ of their fats, have been measured using Isotope Ratio Mass Spectrometry. The aim of this work is to provide the first background reference stable isotope ratios that could be useful for future investigations on their authenticity. This first dataset, once confirmed by analysing a larger set of samples, can be used to characterise the different species of edible insects and then, based on data comparison, to verify their authenticity, i.e. the correspondence to what is reported on the label of insect based food products, in terms of insect composition and origin.

2. Materials and Methods

2.1 Sampling

The current dataset includes 44 samples, 40 of which were edible insects authorized for human consumption and 4 were insect-based food items, purchased between November 2015 and September 2018 from different European and Asian shops, e-shops, and supermarkets. A detailed description of the collected samples is reported elsewhere (Poma *et al.*, 2019; Poma *et al.*, 2017) and summarized in **Table 1**. Briefly, the selected species of edible insects belonged to six different Orders: Orthoptera (n=19), Coleoptera (n=14), Lepidoptera (n=4), Hemiptera (n=1), Odonata (n=1), Hymenoptera (n=1) and were purchased in their natural state (n=35, i.e. without addition of any ingredients) or seasoned (n=5, i.e. with added flavours and dressings, such as sugar, soy sauce and syrup). Insect-based food was plant-based food containing small percentages of insects (5-10% in weight) and were buffalo worm-based “bugballs”, cricket croquettes, and buffalo worm-based “bugburgers”. Multiple insects composing a sample were pooled for analysis, freeze-dried, homogenized, and stored in aluminium foil at −20°C pending analysis. The fat content of each sample was determined gravimetrically (Poma *et al.*, 2019; Poma *et al.*, 2017).

2.2 Sample preparation

To separate the fats from the defatted fraction, all samples were washed with three 10 mL aliquots of ethyl ether:petroleum ether (1:2, *v/v*). The washed samples were then dried, while the etheric aliquots were collected and evaporated to get the fatty fraction. The procedure was performed according to (Camin *et al.*, 2004). Finally, to exclude the presence of carbonates, all samples were treated with 10 µL of HCl (0.1 N) and dried in oven at 60 °C overnight.

2.3 Stable Isotope Ratios analysis

The defatted samples were weighed using a microbalance into tin (1 mg for $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$, of defatted insect and 0.5 mg of fat) or silver capsules (0.2 mg for $^{2}H/^{1}H$ and $^{18}O/^{16}O$). For the analysis of $^{2}H/^{1}H$, the comparative-equilibration method was used. Samples and standards were left at lab air moisture for at least 96 hours and then placed in a desiccator with P_2O_5 under nitrogen atmosphere. Each sample was weighted and analysed in duplicate.

The $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ ratios were measured simultaneously using an isotope ratio mass spectrometer (IsoPrime, Isoprime Limited, Germany) after total combustion in an elemental analyser (VARIO CUBE, Isoprime Limited, Germany). The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios were measured simultaneously using an isotope ratio mass spectrometer (Finnigan

DELTA XP, Thermo Scientific) coupled with a pyrolizer (Finningan DELTA TC/EA, high temperature conversion elemental analyser, Thermo Scientific).

According to the IUPAC protocol, the isotopic values are expressed in *delta* in relation to the international standard V-PDB (Vienna-Pee Dee Belemnite) for $\delta^{13}C$, V-SMOW (Vienna-Standard Mean Ocean Water) for $\delta^{2}H$ and $\delta^{18}O$, V-CDT (Vienna Canyon Diablo Troilite) for $\delta^{34}S$ and Air (atmospheric N_2) for $\delta^{15}N$, according to the following general equation:

$$\delta^{i} E = (i\ R_{SA} - i\ R_{REF})/i\ R_{REF}$$

where i is the mass number of the heavier isotope of element E (for instance ^{13}C), R_{SA} is the respective isotope ratio of the sample (for instance, number of ^{13}C atoms/ number of ^{12}C atoms or, as an approximation, $^{13}C/^{12}C$) and R_{REF} is the relevant internationally recognised reference material, such as VPDB for CO_2 (Galimov, 1985). The delta values are multiplied by 1000 and expressed commonly in units "per mil" (‰) or, according with the International System of Units (SI), in unit 'milliurey' (mUr) (Coplen, 2011).

The isotopic values were calculated against 2 standards through the creation of a linear equation. For $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$, two in-house working standards were used. They were calibrated against international reference materials, namely, for $^{13}C/^{12}C$: fuel oil NBS-22 ($\delta^{13}C$=−30.03‰), sucrose IAEA-CH-6 ($\delta^{13}C$=−10.45‰) (IAEA-International Atomic Energy Agency, Vienna, Austria), and L-glutamic acid USGS 40 ($\delta^{13}C$=−26.39‰ and $\delta^{15}N$=−4.52‰ (U.S. Geological Survey, Reston, VA, USA); for $^{15}N/^{14}N$: L-glutamic acid USGS 40 and potassium nitrate IAEA-NO3 ($\delta^{15}N$=+4.7‰); for $^{34}S/^{32}S$: Barium sulphates IAEA-SO-5 ($\delta^{34}S$=+0.5‰) and NBS 127 ($\delta^{34}S$=+20.3‰). Keratins CBS (Caribou Hoof Standard $\delta^{2}H$=−157 ± 2‰ and $\delta^{18}O$=+3.8 ± 0.1‰) and KHS (Kudu Horn Standard, $\delta^{2}H$=−35 ± 1‰ and $\delta^{18}O$=+20.3 ± 0.2‰) from U.S. Geological Survey were used to normalise $^{18}O/^{16}O$ and $^{2}H/^{1}H$ values. The maximum standard deviations of repeatability accepted were 0.3‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, of 0.5‰ for $\delta^{18}O$ and of 4‰ for $\delta^{2}H$.

2.4 Statistical analysis

Statistical analysis was carried out using RStudio version 1.2.5033 (2019). Non-parametric tests were applied due to the low number of samples. The Kolmogorov-Smirnov test was used to compare two independent groups and the Kruskal-Wallis test was used to compare more than two independent groups. Moreover, a dendrogram was performed in RStudio to display potential sample groupings in clusters formed according to similar features.

3. Results and discussion

SAMPLE	*ORDER*	PRODUCT	LIFE STAGE	ORIGIN CONTINENT	$\delta^{13}C$‰ defatted (vs V-PDB)	$\delta^{15}N$‰ defatted (vs Air)	$\delta^{34}S$‰ defatted (vs V-CDT)	$\delta^{2}H$‰ defatted (vs V-SMOW)	$\delta^{18}O$ defatted (vs V-SMOW)	$\delta^{13}C$ fat vs (V-PDB)
O1	*Orthoptera*	Natural	Adult	Europe	-23.5	3.3	4.2	-87.2	18.1	-27.3
O2	*Orthoptera*	Natural	Adult	Europe	-23.5	3.5	5.2	-83.3	17.1	-27.9
O3	*Orthoptera*	Seasoned	Adult	Asia	-19.9	4.3	4.5	-95.9	16.2	-23.8
O4	*Orthoptera*	Natural	Adult	Europe	-23.5	3.2	5.7	-84.9	18.1	-27.5
O5	*Orthoptera*	Natural	Adult	Europe	-21.3	4.1	6.4	-73.6	20.1	-24.2
O6	*Orthoptera*	Natural	Adult	Europe	-28.7	4.8	4.4	-88.5	19.8	-32.7
O7	*Orthoptera*	Natural	Adult	Europe	-28.4	6.1	4.7	-89.6	19.5	-32.5
O8	*Orthoptera*	Natural	Adult	Asia	-22.6	3.9	2.1	-90.9	19.6	-25.5
O9	*Orthoptera*	Natural	Adult	Europe	-28.9	4.1	5.7	-92.2	18.6	-33.0
O10	*Orthoptera*	Natural	Adult	Europe	-28.6	6.0	4.5	-86.3	18.2	-32.1
O11	*Orthoptera*	Natural	Adult	Asia	-20.3	2.0	2.9	-89.4	14.6	-22.1
O12	*Orthoptera*	Natural	Adult	Asia	-18.7	3.1	1.6	-85.4	17.0	-25.6
O13	*Orthoptera*	Seasoned	Adult	Asia	-17.9	4.7	2.3	-49.2	26.7	nd
O14	*Orthoptera*	Natural	Adult	Asia	-26.3	5.2	1.2	-101.1	17.7	nd
O15	*Orthoptera*	Natural	Adult	Asia	-12.2	9.9	-0.1	-67.5	22.3	-21.1
O16	*Orthoptera*	Natural	Adult	Asia	-18.9	2.5	-2.6	-98.7	15.7	-19.8
O17	*Orthoptera*	Natural	Adult	Asia	-25.2	10.5	7.8	-76.6	19.2	-31.2
O18	*Orthoptera*	Natural	Adult	Europe	-28.4	5.5	2.8	-83.1	19.4	-32.5
O19	*Orthoptera*	Natural	Adult	Europe	-27.9	4.7	1.7	-90.1	20.4	-32.5
C1	*Coleoptera*	Natural	Larva	Europe	-24.3	4.7	5.2	-75.6	19.9	-28.4
C2	*Coleoptera*	Natural	Larva	Europe	-23.9	5.5	4.6	-77.2	19.6	-28.3
C3	*Coleoptera*	Natural	Larva	Asia	-20.2	4.4	0.7	-77.5	18.9	-23.0
C4	*Coleoptera*	Natural	Larva	Europe	-24.5	5.4	5.2	-70.9	19.9	-28.6
C5	*Coleoptera*	Natural	Larva	Europe	-24.3	4.9	2.8	-71.2	19.9	-28.6
C6	*Coleoptera*	Natural	Larva	Asia	-26.9	1.8	7.7	-77.1	17.8	-32.3
C7	*Coleoptera*	Natural	Larva	Asia	-24.8	5.9	1.6	-59.7	20.3	-31.3
C8	*Coleoptera*	Natural	Larva	Asia	-24.5	11.5	0.8	-58.5	19.0	-29.8
C9	*Coleoptera*	Natural	Larva	Asia	-24.2	5.4	5.6	-64.4	22.6	-28.6
C10	*Coleoptera*	Natural	Larva	Asia	-24.5	6.0	2.9	-71.5	19.6	-29.1
C11	*Coleoptera*	Natural	Larva	Asia	-17.9	3.2	3.0	-100.7	15.0	-19.4
C12	*Coleoptera*	Natural	Larva	Asia	-27.3	2.3	4.4	-80.5	20.5	-32.2
C13	*Coleoptera*	Natural	Larva	Europe	-23.9	5	1.9	-77.8	19.9	-28.5
C14	*Coleoptera*	Natural	Larva	Europe	-24.1	6.5	3.2	-71.6	19.9	-29.2
L1	*Lepidoptera*	Natural	Larva	Asia	-28.9	6.4	-2.4	-99.6	13.5	-35.7
L2	*Lepidoptera*	Seasoned	Larva	Asia	-21.4	6.7	2.0	-55.5	26.0	-34
L3	*Lepidoptera*	Seasoned	Larva	Asia	-27.6	5.6	2.4	-87.6	18.2	-35.4
L4	*Lepidoptera*	Natural	Larva	Europe	-23.8	6.6	-2.6	-70.3	16.4	-27.9
He1	*Hemiptera*	Natural	Larva	Asia	-27.8	2.1	3.2	-82.6	14.9	-31.1
Od1	*Odonata*	Natural	Larva	Asia	-31.3	7.6	-0.6	-83.7	17.5	-37.6
Hy1	*Hymenoptera*	Seasoned	Larva	Asia	-19.6	1.9	2.8	-59.3	28.9	-30.0
IBFI1	*Commercial*	Insect-based Food Items	-	-	-26.2	3.9	2.9	-40.9	27.4	nd
IBFI2	*Commercial*	Insect-based Food Items	-	-	-25.1	4.3	4.1	-43.7	29.9	nd

IBFI3	*Commercial*	Insect-based Food Items	-	-	-26	4.1	2.4	-62.9	24.0	-29.9
IBFI4	*Commercial*	Insect-based Food Items	-	-	-23.7	2.1	3.8	-46.6	27.6	nd

Table 1. Dataset of $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{2}H$ and $\delta^{18}O$ of the defatted samples and $\delta^{13}C$ of the fats

3.1 $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ of the defatted insect samples

The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ dataset, grouped based on the order of each edible insect sample, is represented in Figure 1.

Values of $\delta^{13}C$ ranged from -12.2 to -31.3‰ (Table 1). The carbon stable isotope ratio is related to the diet of the insect. In particular, it is known that C4 plants have $\delta^{13}C$ values between -14 and -12‰, while C3 plants range between -30 and -23‰ (Knobbe *et al*. 2006; Molkentin and Giesemann, 2010). A few insect samples showed $\delta^{13}C$ higher than -23‰, up to -12.2‰ for an Asian grasshopper, likely attributable to ingestion of C4 plants. Anyway, most samples had $\delta^{13}C$ values fitting the range of a C3 plant, indicating this is a major component of insects' diet. Kruskal-Wallis test has been carried out, yielding no significant differences among orders (Figure 1A).

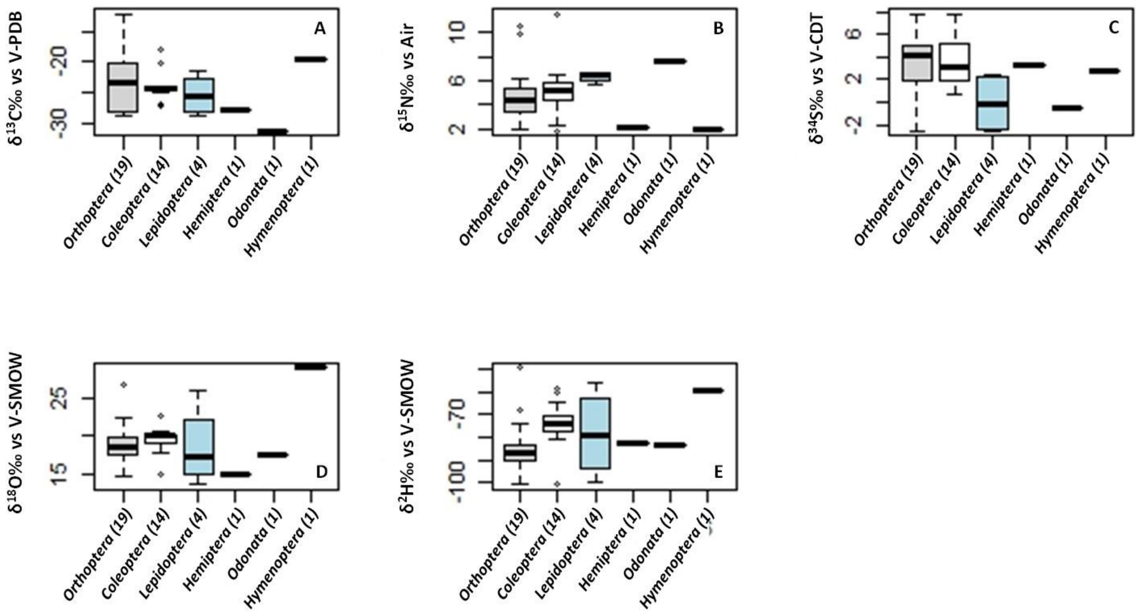

Figure 1. A, $\delta^{13}C$, B, $\delta^{15}N$, C, $\delta^{34}S$, D, $\delta^{18}O$ and E, $\delta^{2}H$ of the defatted samples grouped according to the order of the insects (number of samples belonging to each order is given in brackets)

Values of $\delta^{15}N$ in insects ranged from 1.8 to 11.5 ‰ (Table 1). The Kruskal-Wallis test was carried out but did not show any significant differences among the orders (Figure 1B). The nitrogen stable isotope ratio of the animals is closely related to their trophic level (O'Brien, 2015). Herbivorous species are expected to have a lower $\delta^{15}N$ than carnivorous ones, as ^{15}N

accumulates along the trophic chain (Camin *et al.*, 2016). The range of values found here for insects indicates an omnivorous diet, comprehensive of plants and animals. The $\delta^{15}N$ and $\delta^{13}C$ values reported in this work resemble the isotopic values described in an article on Asian longhorn beetle (Heinrich and Collins, 2017).

Finally, the values of $\delta^{34}S$ ranged from -2.6 to 7.8‰ (Table 1). As for $\delta^{13}C$, the $\delta^{34}S$ values of animals tend to reflect the values of the plants they feed on. Indeed, the sulphur trophic shift between animals and their diet was estimated in literature between 0‰ and 1‰. (McCutchan *et al.*, 2003; Tanz *et al.*, 2015; Harrison *et al.*, 2011). In turn, the $\delta^{34}S$ values of the plants are influenced by factors like the geology of the soil the plant grew on (e.g. presence of sulphides in the soil, type of underlying local bedrocks, etc.), aerobic or anaerobic growing conditions (Rubenstein and Hobson, 2004) of the plant itself, active microbial process into the soil, closeness to the sea (sea-spray effect), and the fertilization practices (Rubenstein and Hobson, 2004; Krouse *et al.*, 2000). To test the differences among the orders (Figure 1C), the Kruskal-Wallis test was used, giving no significant differences. Since the sulphur isotopic ratio is also influenced by the geology of the soil in which the insect dietary plants grow, the samples were divided in 2 groups: from Asia (n=23) and from Europe (n=17). The $\delta^{34}S$ of the European samples ($\delta^{34}S$ 3.9±3.1‰) showed statistically higher values that of the Asian ones ($\delta^{34}S$ 2.3±2.6‰) ($p<0.05$). For $\delta^{13}C$ and $\delta^{15}N$, we did not find significant differences between Asian and European insects.

3.2 δ^2H and $\delta^{18}O$ of the defatted samples

As reported in the literature, δ^2H and $\delta^{18}O$ of water are strictly related parameters (Araguas *et al.*, 1996) that depend on variables such as latitude, altitude, closeness to the sea (Bowen *et al.*, 2007). For plants, the correlation between δ^2H and $\delta^{18}O$ is still valid, but not that strict, as the only source of hydrogen is the water absorbed through the roots, while oxygen derives from both O_2 and CO_2 absorbed through the stomata (Barbour, 2007). As the sources of oxygen and hydrogen of the insects are both the water they drink and the plants they eat, their δ^2H and $\delta^{18}O$ is influenced by such factors. The correlation between the oxygen and hydrogen isotopic ratios (δ^2H = 3.41‰ $\delta^{18}O$ - 144‰; r^2 = 0.84), is represented in Figure 2. A similar correlation has already been reported in literature concerning isotopic ratios of oxygen and hydrogen in human scalp (δ^2H = 5.73‰ $\delta^{18}O$ - 166‰; r^2 = 0.873) (Ehleringer *et al.*, 2008). Furthermore, the slope of the correlation found in this work is similar to that described in another one concerning crickets and spiders, in which the slope was reported to range between 3.8 and 5.8 (McCluney and Sabo, 2010).

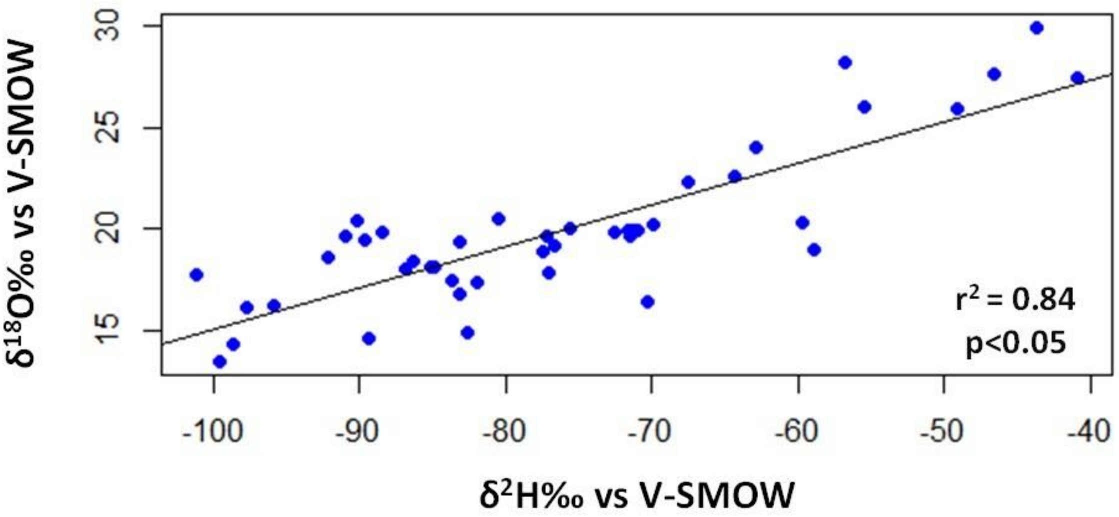

Figure 2. Linear correlation between $\delta^{18}O$ and $\delta^{2}H$

Our results ranged from -40.9 to -101.1‰ for $\delta^{2}H$ and from 13.5 to 29.9‰ for $\delta^{18}O$ (Table 1) and as for the other isotopic data, they were not significantly different from one insect order to another (Kruskal-Wallis test) (Figure 1D,E). These ranges of values resemble the isotopic ratios described in other studies on insects (Heinrich and Collins, 2017; Myers *et al.*, 2012). Our hydrogen and oxygen isotopic ratios seem to be higher in insects compared to animal muscle, probably because of the presence of chitin with higher $\delta^{2}H$ values compared to collagen (Soto *et al.*, 2017). As for $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and $\delta^{18}O$, we did not find significant differences between Asian and European insects. The only significant difference was between larvae (-74.9±12.1‰) and adults (-84.9±11.9‰), with significantly higher $\delta^{2}H$ values in larvae, probably due to the different tissue composition. Moreover, we found higher $\delta^{2}H$ and especially $\delta^{18}O$ values, even though they were not statistically significant, in seasoned samples (mean values -77.1±18.4‰ and 23.2±5.6‰, respectively) in comparison to natural samples (-76.6±14.9‰ and 18.6±2.1‰, respectively). A possible reason thereof can be the presence of sugar in the seasoning samples, as sugar shows high $\delta^{18}O$ values of around 30‰ (Houerou *et al.*, 1999; Spangenberg, 2006).

3.3 $\delta^{13}C$ of fats

The $\delta^{13}C$ of fats ranged from -19.4 to -37.6 ‰. $\delta^{13}C_{fat}$ and $\delta^{13}C_{defatted}$ are expected to be related, as the only source of carbon is the diet. The linear correlation resulting from our dataset is represented in Figure 3 ($\delta^{13}C_{fat}$ = 0.955 $\delta^{13}C_{defatted}$ - 5.89, r2 = 0.73). An average depletion of 4.9‰ in fat compared to the defatted samples was calculated, in line with the findings of other authors in other animal species (Camin *et al.*, 2018; Kiljunen *et al.*, 2006; Smet *et al.*, 2004). Due to the depletion in the lighter isotope (fractionation) during the synthesis of fats, the $\delta^{13}C_{defatted}$ (which is actually the carbon isotopic ratio of the insect proteins) is higher than $\delta^{13}C_{fat}$. (Piasentier *et al.*, 2003; Deniro and Epstein,1978).

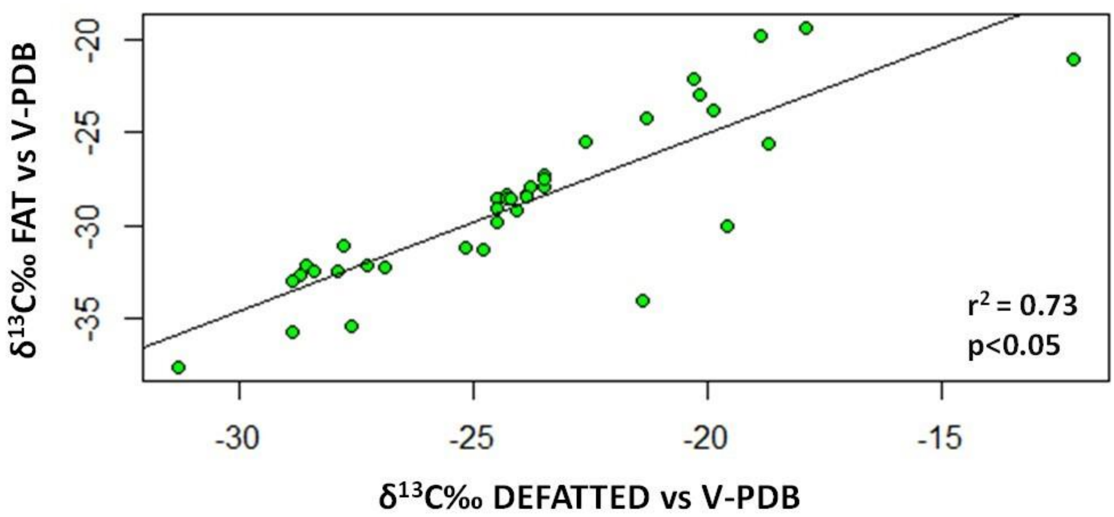

Figure 3. Linear correlation between $\delta^{13}C$ in defatted samples and $\delta^{13}C$ in fats

3.4 Insect-based food items

The isotopic values of the 4 insect-based food items are reported in Table 1. The insect-based food items IBFI1, IBFI2, IBFI3 and IBFI4 contained 7.4%, 15.8%, 8.9% and 10.0% of insects, respectively (Poma et al., 2017). Their mean isotopic values ($\delta^{13}C_{defatted}$ = -25.3±1.1‰, $\delta^{15}N_{defatted}$ = 3.6±1.0‰, $\delta^{34}S_{defatted}$ = 3.3±0.8‰) are comparable (not statistically different, as shown by the Kolmogorov-Smirnov test) to the defatted insect. As for $\delta^{13}C_{fats}$, it was possible to measure only one value among the 4 food items (-29.9‰) due to the low fat concentration of the samples. As for $\delta^{2}H_{defatted}$ (-48.5±9.9‰) and $\delta^{18}O_{defatted}$ (27.2±2.4‰), the insect-based food items present significantly higher values compared to the insects. If we divide the farmed insects in natural and seasoned ones, the insect-based products show values of $\delta^{2}H_{defatted}$ and $\delta^{18}O_{defatted}$ similar to those of the seasoned insects. (Figure 4).

Figure 4. δ^2H and $\delta^{18}O$ values vs type of product considered, whether farmed natural (FN) or seasoned (FN) insects or insect-based food items (IBFI). Significantly different mean values are identified by different letters ($p<0.05$)

This variation seems therefore imputable to the seasoning added to the insects. The results are represented in Figure 4, where F are the farmed insect samples (natural and seasoned) and IBFI are the insect-based food items. The Kolmogorov-Smirnov test revealed significant differences between the IBFI and FN in terms of both δ^2H ($p<0.05$) and $\delta^{18}O$ ($p<0.05$). Sugar, soy sauce, syrup, amino acids, vegetal oils, and flavours are just a few of the ingredients that can be added to insects and these additives can change their isotopic values. Higher $\delta^{18}O$ values may be explained with the addition of sugars to the insects. Indeed, as reported in several works, sugar usually has an oxygen isotopic ratio of around 30‰ (Houerou *et al.*, 1999; Spangenberg, 2006).

3.5 Dataset dendrogram

To check whether the samples of the dataset can be grouped in clusters according to similar features, a dendrogram was created (Figure 5). This representation makes it possible to display potential likenesses between groups of samples, to highlight a clustering feature, i.e. a specific characteristic which appears in different and apparently non connected samples.The number on the x axis corresponds to the code shown in Table 1, while the hierarchical level of aggregation, which represents the distance or dissimilarity between clusters, is shown on the y axis.

By considering a hierarchical level of 40, three groups can be distinguished (Group 1(20 samples) labelled in blue, Group 2 (6 samples) in yellow and Group 3 (18 samples) in red, (Figure 5). Group 1 and Group 3 are mainly composed of natural samples (90% and 94%, respectively), while Group 2 includes 3 insect-based food items and 3 seasoned insects. This confirms the ability of isotopic analysis, in particular $\delta^2H_{defatted}$ and $\delta^{18}O_{defatted}$, to differentiate seasoned from natural insects, as previously discussed. Focusing on the differences between Group 1 and Group 3, most (75%) of the first one is represented by natural adult insects, while Group 2 is mostly represented by natural insects in their larval stage (78%). Thus, the insect life stage seems to be the clustering feature that makes it possible to discriminate between the two groups, as discussed for hydrogen isotopic ratio.

Figure 5. Dendrogram of the dataset. Group 1 (n=20, blue); Group 2 (n=6, yellow); Group 3 (n=18, red). On the x axis is reported the same code as in Table 1, on the y axis the dissimilarity between clusters.

4. Conclusions

For the first time, data of $\delta^{13}C$, $\delta^{34}S$, $\delta^{15}N$, $\delta^{2}H$ and $\delta^{18}O$ of the defatted samples and the $\delta^{13}C$ of the fat have been provided for different edible insect species and some insect-based food items. These first data can be used to give a first isotopic characterization of edible insects and, once confirmed by the analysis of a larger dataset, can be used as reference of authenticity in order to verify the compliance of what is reported on the label of insect-based food products on the market. Parameters like $\delta^{2}H$ and $\delta^{18}O$ appear to allow the differentiation between natural and seasoned insects and insect-based food items. This is due to the addition of ingredients and flavours to the latter, which makes their isotopic values shift as against the values of natural insects. Furthermore, other parameters seem to be promising in the characterization of the insect diet ($\delta^{13}C$), geographical origin ($\delta^{34}S$) and the trophic level ($\delta^{2}H$) of edible insects.

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