Nutritional content of the Green Walnut (*Juglans regia* L.) Husk, the natural host of the Walnut Husk Fly *(Rhagoletis completa)*

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ABSTRACT

Walnut orchards face challenges from various pests that compromises fruit quality. The Walnut Husk Fly (WHF), *Rhagoletis completa* Cresson (Diptera: Tephritidae), which infests the Green Walnut Husk (GWH), is one of them. The dietary requirements of WHF larvae, as well as the nutritional informations regarding the GWH are scarce, affecting the ability to fully rear this species under laboratory conditions for research purposes. In this study, we analysed the content in macronutrients, inorganics, and vitamins, and the profiles of free sugars, amino acids, fatty acids, and sterols of GWH from *Juglans regia* L. (cv. 'Franquette'). These findings will serve as valuable guidance for the development of larval diets in WHF rearing.

Keywords: macronutrient, micronutrient, fruit, mesocarp, Juglandaceae, Tephritidae

INTRODUCTION

The Green Walnut Husk (GWH), the fleshy mesocarp of the walnut fruit, is a byproduct of walnut production that plays a crucial role in the fruit's development as any damage to its integrity can result in a decrease in the quality of the nut. By feeding on the GWH, the Walnut Husk fly (WHF) larvae, *Rhagoletis completa* Cresson (Diptera: Tephritidae), can cause up to 80% yield loss in infested orchards (Verheggen et al., 2017). Infested fruits present a stained shell, and a significant reduction in shell weight, kernel weight, kernel percentage (Solar et al., 2020), and can fall prematurely.

Native from North-America, this specialist insect was detected in Switzerland in 1986 and had since spread to 15 European countries (EPPO, 2024), most likely through adult flight and hitchhiking behavior (Verheggen et al., 2017). Boyce (1934) described extensively the life cycle of the WHF in the field. Adults emerge from the soil during summer (July-August). A female lays a single cluster of 15 eggs (on average) per fruit, below the skin of the GWH, where they hatch within 4-10 days. Larvae feed inside the husk for about 3-5 weeks before exiting the fruit and falling on the soil to burrow, pupate, and enter winter diapause.

Current control methods include synthetic and natural insecticides, such as acetamiprid (neocotinoid) and spinosad (bacteria-derived), and trapping with ammonium carbonate lure, coupled or not with an insecticide depending on the trap design (yellow sticky trap for monitoring or bowl for mass-trapping). Prophylactic methods are also used: kaolin spraying on fruits as a mechanical barrier to prevent oviposition; collection and burning of infested fallen fruits; and shallow tillage of the soil (Medic et al., 2022). However, these methods have limitations. Insecticides show toxicity to non-target organisms and are subject to frequent shifting in legislation, while large number of traps are required on the field to effectively mass-trap the flies (Medic et al., 2022), and thus can be costly and time consuming. Lastly, kaolin spraying requires 4-5 applications per year, and is inconvenient for taller trees which constitute the majority of walnut orchards (ANSES, 2014). Recent efforts have been made to characterize WHF pheromone and use it in the field to improve monitoring (Sarles et al., 2018), although an efficient pheromone-based strategy is yet to be deployed.

The development of safe control methods is hindered by the difficulty to fully rear *R. completa* under laboratory conditions, primarily due to its specialised behavior towards *Juglans* spp. fruits and the limited understanding of larval nutritional requirements. Previous attempt for WHF larval rearing reached an unsatisfactory 48% of pupal recovery (Ciociola, 1982). Furthermore, existing literature has predominantly focused on the pharmacologically relevant compounds found in the GWH, such as phenolics (Jahanban-Esfahlan et al. 2019), while providing minimal information regarding its nutritional components. The objective of this study is to fill this research gap by characterizing the nutritional content of GWH, which will provide the missing reference to develop an efficient nutritional substrate for WHF larvae.

MATERIALS AND METHODS

Analysis of GWH content

Green walnuts of *Juglans regia* L. (cv. 'Franquette') were collected from organic orchards located in France (Chatte, 45°08'07.8"N 5°17'18.5"E) and Belgium (Grand-Leez, 50°34'53.3"N 4°45'06.2"E), between August 8th and August 19th, 2022. This cultivar is widely cultivated in France were the WHF is present, with a peak of larval infestation during august. Green walnuts were at their maximal caliber, closed, with a fully formed shell and a filled kernel, corresponding to stage 799 on the BBCH scale (Robin et al., 2024). Depending on the requirement for each analysis, 70-150 fruits on at least 3 different trees were collected for representative samples preparation. In total, 4 batches of green walnuts were collected: 3 in Belgium (for fresh and dried samples), and 1 in France (for fresh sample). Green walnuts were deprived of disease or pest presence (visual assessment for insect's punctures and fungal/bacterial stains). For analyses on Fresh Matter (FM), the green walnuts were either immediately brought to the analysis provider, or sent in dry ice on the same day. In the laboratory, the green walnuts mesocarps were dried at 50°C overnight after the removal of the skin (epicarp) and shell for analyses on Dry Matter (DM).

The analyses were conducted on peeled GWH, focusing on the mesocarp, as the darkened epicarp is a marker of WHF infestation because the larva mostly consume the pulp beneath (Boyce, 1934). All analyses were performed in Gembloux Agro-biotech (Belgium), except for vitamins and iodine which were quantified by SGS laboratory in Rouen (France), on the samples of GWH collected in Belgium and France respectively. In most cases, DM content was quantified, and then FM content calculated according to the reference humidity percentage of fresh mesocarp. In the results section, the number of measured samples drawn from the main representative sample is indicated with "n", and number of technical replicates for each sample with "N" (if more than one). All results are mean FM content. Values preceded by '<' denotes quantification limit for the nutrient.

pH was measured on fresh mesocarp with a pHmeter WTW 340i/SET. To assess DM and humidity percentage, fresh mesocarp was lyophilised using a freeze-dryer. Lyophilised mesocarp was used for all the analyses mentioned below. Total sugars were quantified according to the *Dubois* method, and total dietary fibers using the *AOAC Official Method 991.43 Total, Soluble and Insoluble Dietary Fibre in Foods* method. Proteins were quantified with *QSPA-026* (*AFNOR NF V 04-407*) method. Lipids were quantified by SOXTHERM.

Fresh mesocarp was mineralised with TMAH (*MLR-ME309* method), and iodine quantified by ICP-MS (*MLR-ME309* method). Dried mesocarps were used. Mineralisation was performed with Aqua regia for 2 hours under reflux (*NBN EN 16174* method). Metals (CaO, MgO, K_2O_T, Na_2O, Fe, Cu, Zn, Mn, Mo, Sn, Cr, V, Si, Se) were quantified by GF-AAS/ETAAS (*CWEAS S-II-2* method), sulfates (SO_4) by nephelometer (*8.9. EU regulation rule 2003/2003* method), fluorides (F^-) by potentiometry (ion selective electrode) after calcination and alkaline fusion, chlorides (Cl^-) by potentiometric titration ($AgNO_3$) after leaching, phosphorus (P_2O_5) by colorimetry (MoO_2P blue). For ions, DM contents of targeted elements were calculated using their atomic weights.

Using fresh mesocarp, the following (pro)vitamins were quantified by UV-HPLC: Beta-carotene (456nm), vitamin A (retinol) (325nm; *MLR-ME577* method), vitamin B7 (biotin)(204nm), vitamin B9 (folacin) (280nm; *MLR-ME348* method), vitamin B12 (cobalamin) (361nm; *SOP M844/SOP M577 microbiology/AOAC 952.20(AOAC 986.23) microbiology (single determination)* methods), vitamin C (ascorbic acid) (254nm; *MLR-ME186*). The following vitamins were quantified by fluorescence-HPLC: B1 (thiamine) (excitation 366nm –emission 435nm; *DIN EN14122/SOP M580/SOP M843* methods), B2 (riboflavin) (excitation 422nm –emission 522nm; *DIN EN14152/SOP M931/SOP M843*), B3/PP (nicotinic acid) (excitation 322nm –emission 380nm; *NFEN15652* method), B5 (pantothenic acid) (excitation 345nm –emission 455nm), B6 (pyridoxine) (excitation 290nm – emission 395nm; *MLR-ME99*), E (DL-α-Tocopherol) (excitation 295nm – emission 326nm; *MLR-ME577* method), K1 (phylloquinone) (excitation 366nm – emission 435nm; *DIN EN 14148/SOP M2986/SOP M548* methods). D2 (ergocalciferol) and D3 (cholecalciferol) were quantified by MS/MS-HPLC (*MLR-ME595* method).

Dried mesocarp was used for free sugars quantification by HPAED-PAD (Dionex) after 3 hours agitation in water at room temperature: glucose, fructose, sucrose, raffinose, maltose, ribose, fucose. It was also used for amino acids analysis. Total amino acids (glutamic acid, asparatic acid, leucine, threonine, lysine, valine, alanine, serine, glycine, phenylalanine, arginine, isoleucine, proline, tyrosine, histidine) were hydrolysed (HCl) for 24 hours and quantified according to the *Stein and Moor* method. The same process was used for sulfur-containing amino acids (methionine, cysteine), except that hydrolysis was preceded by a performic oxidation (CH_2O_3) of the sample. For tryptophane, sample was alkaline hydrolysed and quantified by UV-HPLC (280nm), with separation on a C18 column and 0.07M acetate buffer (pH 4.5) in acetonitrile as eluant. Dried mesocarp was finally used for further lipids analyses. Fatty acids were quantified according to the method described in Fina and al. (2022), with a standard F.A.M.E. mix of 21 fatty acids (C6-C20). Sterols were quantified by *COI/ T.20/ Doc. No 30/Rev. 2 2017* method, with injections of cholesterol and stigmasterol standards.

RESULTS

GWH DM is 10.84% (RH 89.16%) and pH 4.03 (n=3). Macronutrients (n=3) include total sugars 8.13% (total fibers 2.11%), proteins 0.48%, lipids 0.05% and ashes (n=2) 0.46%. The following inorganics (n=1) are quantified in mg/100g: potassium 250.59, calcium 47.50, phosphorus 18.59, chlorine 14.04, magnesium 8.47, sodium 7.38, sulfur 4.53, iron 1.86, zinc 0.16, manganese 0.10, copper 0.08, silicon <0.51. The following inorganics (n=1) are quantified in µg/100g: fluorine 93.00, vanadium 64.00, chromium 43.00, selenium 5.00, tin 4.00, molybdene 1.00, iodine (n=2, N=3) <5.00 (not detected).

Vitamins (n=1) include (in mg/100g): C (ascorbic acid) 146.70, E (DL-α-tocopherol) 1.10, B3/PP (nicotinic acid) 0.50, B2 (riboflavin) 0.12, B6 (pyridoxine) 0.07, B1 (thiamine) 0.01 and B5 (pantothenic acid) <50.00 (not detected), as well as (in µg/100g) β-Carotene 700.00, K1 (phylloquinone) 13.00, D2 (ergocalciferol) 7.01, B12 (cobalamin) 0.10, B7 (biotin) <2.00, B9 (folacin) <2.00, A (retinol) <0.80, D3 (cholecalciferol) <0.40.

Free sugars include glucose 3.12%, fructose 1.35%, sucrose 0.73%, raffinose 0.22% and likely maltose (close RT to standard).

After proteins hydrolysis, GWH total amino acids 0.467% (n=3), include glutamic acid 0.066%, aspartic acid 0.052%, leucine 0.035%, threonine 0.034%, lysine 0.032%, valine 0.031%, alanine 0.029%, serine 0.026%, glycine 0.022%, phenylalanine 0.022%, arginine 0.020%, cystine 0.019%, isoleucine 0.019%, proline 0.018%, tyrosine 0.013%, histidine 0.011%, tryptophan 0.010%, methionine 0.008%.

Fatty acids (n=3) relative content is mostly saturated (73.79%), including lauric acid 53.99%, tridecylic acid 6.72%, myristic acid 5.12%, palmitic acid 4.35%, caprylic acid 1.94%, capric acid 1.13%, stearic acid 0.32%, arachidic acid 0.23% ; polyunsaturated (11.59%) include linolelaidic acid 7.80% and α-linolenic acid 3.79%; monosaturated (1.02%) include oleic/elaidic acids 0.81% and palmitoleic acid 0.21% ; and 6 unidentified fatty acids accounting for 13.60%.

Sterols (n=3) include (in µg/100g): ß-sitosterol 5.27, clerosterol 2.50, Δ5-avenasterol 0.51, cholesterol 0.17, campesterol 0.15, stigmasterol 0.13, Δ7-campesterol, 0.08, and 4 unidentified accounting for 0.62. Total sterols is 9.43 µg/100g.

DISCUSSION

We enhanced our understanding of WHF larval nutrition by providing a description of the physicochemical properties of the GWH, its natural food source. In comparison to other fruit' pulps, GWH stands out for its high content in vitamins C and K1, calcium, iron and selenium (Fig. S1-3) and thus appears distant from other fruits' pulps nutritional composition (Fig. S4A). Interestingly, when removing these high values from the comparison (Fig. S4B), GWH nutrients composition becomes closer to the other pulps, including peach and orange (Fig. S5). Peach (Kasana & AliNiazee, 1995; Yokoyama & Miller, 1994) and, to a lesser extent, english hawthorn (Yee & Goughnour, 2008) and orange pulp (Boyce, 1934) are three others fruits known to support the full larval development of *R. completa*. This suggests that their nutritional composition is sufficiently similar to GWH's to meet the larval nutritional requirements.

Beyond the mains macronutrients categories (proteins, lipids, and sugars), the specific nutrients essentials or useful to almost all insects are: the amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine; sterols (cholesterol or phytosterols); polyunsaturated fatty acids; the (pro)vitamins A, B1, B2, B3, B5, B6, B7, B9, B12, C, E, β-carotene, inositol and choline; the inorganics calcium, chlorine, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, sulfur, and zinc (Behmer, 2008; Kraus et al., 2019). With the exception of inositol and choline, all nutrients mentioned above have been addressed in the present study on the GWH.

Based on these findings, two approaches can be considered for the development of artificial diets for WHF: (a) create a chemically defined diet, mimicking the nutrient content of GWH (holidic diet) or (b) develop chemically undefined (oligidic) or semi-defined (meridic) diets, incorporating nutritionally complex ingredients and supplementing refined nutrients as needed. The former (a) type of diet can be time-consuming and costly to implement, as well as overlook the importance of essential trace elements in the recipe, but is useful to assess the importance of each nutrient by dietary deletion experiments (Thompson & Simpson, 2009). The latter (b) is commonly used in the laboratory and mass rearing of insects, including other Tephritidae species (Dominguez Gordillo, 1999), and offer practical and cost-effective alternatives to chemically defined diets.

It should be pointed out that optimal nutrition of insects in nature relies on far more complex and intricated aspects than just nutrient concentrations. These notably include symbiotic relationship with micro-organisms, interactions with other non-nutritious compounds, and behavioral factors (Thompson & Simpson, 2009). Nonetheless, an artificial diet nutritionally close to the natural diet of the insect often gives good results (Thompson & Simpson, 2009). In this regard, understanding the nutritional needs of WHF larva is essential, and we think that characterization of its natural food is a step in the right direction.

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