A new green insecticide for stored wheat grains: Efficiency against Rhyzopertha dominica and risk assessment

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Abstract

This work evidenced the insecticidal activity of encapsulated *Mentha spicata* essential oil (EO) against *Rhyzopertha dominica* in presence of stored wheat grain and evaluated the residual EO amount in wheat grains after treatment. EO distribution in the different fractions recovered after grain milling was also determined. The mortality rate against *R. dominica* reached more than 90% and was dependent on the amount of EO released from the encapsulation matrix. The quantification of the two major compounds of EO, (*R)*-limonene and (*R)*-carvone, showed that wheat grains contained different amount depending on the storage conditions. When wheat grains were stored in open jars, the EO residual amount in grains was especially low (1.98mg/kg) compared to amount in closed jar (51.6 mg/kg). The flour resulting from milling contained lower amount of EO compounds in comparison with the bran fractions. The contact of wheat grains with EO clearly modified the native aromatic profile of the wheat flour as the most represented compounds were those belonging to the EO. In the native flour, ten volatile compounds, mainly alcohols and aldehydes due to oxidation of lipids were identified, which represented only 14% of the aromatic profile when the wheat grains were treated by EO. However, when the storage condition of grains mimics the silos conditions, the level of (*R)*-carvone, the major component of spearmint EO is found around 3 mg/kg of flour which was far below its currently use in bakery products.

Key words: D-carvone, essential oil, insect, *Mentha spicata*, solid phase micro-extraction

1. Introduction

The infestation of grain stocks after harvest by harmful insects constitutes a "permanent" risk during the storage period, and represents the major cause of deterioration of the quality of grains and seeds in post-harvest situations. Thus, in the international business and, to a lesser extent for the supplying of agri-food industries, the presence of insects in unprocessed cereals is not tolerated because it is the matter of severe depreciations when they are detected. The economic losses resulting from a detected infestation represent between 2 and 5% of the commercial value. This percentage is in relation to the reduction of the price if some insects persisted or additional costs of sanitation and cleaning measures to make infested batches in conformity with the commercial standards (Campbell & Arbogast, 2004). To moderate these damages liquid insecticides are usually pulverised on the stored grains such as organophosphorus compounds (pirimiphos methyl, chloropyrifos etc.) or pyrethrinoides (such as deltamethrin or cypermethrin, etc.). However, some of these treatments induce residues with long persistence activity (Leblanc et al 2014; Skerritt et al , 1992). It was demonstrated that the residues present in cereal foods are generated almost exclusively by the treatments of grain bulks after harvest and that numerous grains contain harmful residues. The long persistence activities of these residues have conducted to the tightening of regulations and to impose maximum residue limits for each active insecticide molecule and/or to prohibit some of them such as chloropyrifos and chlorpyrifos-methyl (European Commission, 2020).

To replace liquid treatment, phosphine a highly toxic gas was proposed because it has the advantage to not generate residues in the treated product (Nayak et al 2020). This fumigant is now widely used as curative treatment to control stored grain pests due to its low price and its proven effectiveness against various targets. However, even if its application is adapted to the different storage structures, the treatment needs to guarantee the air tightness of the involved silos which is not always easy to obtain with old storage grain facilities. Furthermore, the emergence of phosphine resistance among some insects calls into question its widespread use (Nayak et al., 2020). Moreover, the new green approach promoted by consumers, but also by farmers, has opened many discussions about the safety of substances used in agriculture and of their impact on health and on the environment and push to develop new alternatives. Now the question is how to replace usual insecticides and by which products without the same defect?

During the last two decades, essential oils (EOs) and their constituents were strongly investigated as potential insecticides to replace synthetic products and their efficacy was clearly demonstrated (Isman, 2006; Regnault-Roger et al, 2012). Essential oils are aromatic oily liquids obtained from different parts of the plant (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). Major compounds can constitute up to 85% of the total EO's content, whereas other compounds are presented only as traces. They are usually used as flavouring in food or beverages, as a compound of fragrances and aftershaves and as medicines in the pharmaceutical industry. Besides, some essential oils or certain aromatic plants have been traditionally used to preserve food, to repel and kill insects at home or in granaries, to inhibit microorganisms and increase foodstuff shelf-life (Pavela, 2016; Regnault-Roger et al., 2012). The positive insecticidal effect of EOs and their major compounds was demonstrated under fumigation against the different insects known to infest food grains (Isman, 2006; Kedia et al, 2014). Coleoptera such as *Sitophilus* species (*granarium*, *zeamais* and *oryzae*), *Rhyzopertha dominica* (*Fabricius, 1792*) known as the lesser grain borer, is primary pest of grains which can strongly damage them, rendering it susceptible to attack by secondary pests. *R. dominica* is also highly resistant to phosphine treatment (Nayak et al., 2020) and essential oils appeared as an efficient but challenging alternative (Kumar et al, 2011; De Souza et al, 2016).

Among essential oils, the insecticidal potential of *Mentha spicata* (L.) is clearly established against various stored pests and specifically against weevil of stored grains (Kedia et al., 2014, Kumar et al, 2011). The essential oil of *M. spicata*, is rich in (*R)*-carvone and has a characteristic smell of spearmint.

Among the challenges associated with the use of EOs for pest management, the control of their impact on transformed products (*e.g.* flour, brans…) in term of residual concentration and sensory properties is determinant for their acceptation as an alternative solution. Indeed, the potential adsorption of EOs into the grains during storage can provoke the reject due to odorant grains. Moreover, in absence of perceptible odour, the presence of residual traces into the fractions obtained by milling (flour and brans) could be damageable because the consumption of some compounds could be limited and regulated.

To avoid strong adsorption into grains during storage, the use of encapsulated EO is an alternative way to better control the released amount and the risk of contamination (Werdin et al, 2014). Encapsulation allows a better adjustment of the amounts and a

reduction of the quantities of active substances required for pest species control. The formulation of active ingredients in matrices with delayed release is usually used in the pharmaceutical, textile, agri-food and plant health fields, *e.g.* formulation of some fertilizers or pesticides in agriculture (Chevillard et al, 2012). In addition, since the compounds of EO are volatile, the contact with insects and grains is indirect and occur via the atmosphere. It can be expected that this mode of action should limit the EO residues.
To get a representative result of the aromatic profile of a foodstuff the extraction of these compounds is an essential step. To choose the best method, the knowledge of aroma compounds present in the commodity is important. Direct extraction by solvent of cereal flour provides efficient recovery of aroma compounds if mild conditions are applied to avoid the potential destruction of the desired compounds (Murat et al, 2012). The use of solid phase micro-extraction (SPME) is on its rise thanks to its simplicity, rapidity, the absence of solvent, high sensitivity, small sample volume and lower costs. It is known that with this method ,the quantification of compounds can be complex due to the difference of aroma compounds affinity for the used fiber but an overall profile can be obtained (Murat et al, 2012).
The aims of this study are:

- to evaluate the insecticidal activity of encapsulated spearmint (*M. spicata*) EO against relevant granary pest, *R. dominica*;
- to determine the impact of spearmint EO treatment on the quality of wheat grains and its milling products.

2. Materials and methods

2.1. Materials

Common wheat (*Triticum aestivum*) grown in organic conditions was purchased from *Salvagnac Agribio Union – RD 999 81630* (Salvagnac, France). Spearmint (*Mentha spicata*) EO from India was purchased from Golgemma (Esperaza, France). Hexane (>99% purity for analysis) was used as a solvent for the extraction of EO and 2-heptanol (≥99%) was used as internal standard. *(R)*-limonene, *(R)*-carvone and other chemical compounds were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Badineb® composed of pyrethrin and piperonyl butoxide (used as synergist) was purchased from Lodi Group (Grand-Fougeray, France).

142 The insects (pest beetle species *R. dominica)* were purchased by Sitona AgroExpert (Saint
143 Médard en Jalles, France). They were grown on wheat grains to reach their adult stage (at
144 least 2 weeks) in favourable conditions of temperature and humidity determined by
145 ARVALIS (Institut du Végétal in Boigneville, France).
146
147 **2.2. Methods**
148 *2.2.1. Encapsulation of spearmint EO*
149 For the encapsulation of the spearmint EO, organic matrices used as carriers were
150 formulated by a repeatable process that consisted in four formulation phases: 1) carrier
151 elaboration, 2) shaping of the carrier, 3) drying of the carrier and 4) EO addition. Each
152 carrier can be pictured as a similar size of a wheat grain, due to confidentiality purposes
153 the process cannot be detailed any further. The carriers contained mainly starch, proteins
154 and a low percentage of lipids. After production, the carriers were stored in hermetic glass
155 jars at refrigeration temperature until analysis.
156 *2.2.2. Insecticidal assays*
157 Common wheat grains (*Triticum aestivum*) of the harvest 2016 and 2017 from the organic
158 wheat plots at ARVALIS were used for the insecticide experiments. An analysis of
159 phytosanitary residues was performed over the wheat grain before the insecticide assays
160 in order to ensure the absence of any fortuitous contamination. After its reception, the
161 wheat grain sample was cleaned using a MINI-PETKUS 200 separator. Next, phosphine-
162 based fumigation was performed to assure the absence of any insect. Finally, the wheat
163 grain was stored in a cold room at 10 °C and before the insecticide analysis the wheat
164 grain was humidified in order to achieve a moisture content of 14 % (humid basis) taking
165 into-account its initial water content. Before each assay, the needed quantity of wheat
166 grain was equilibrated at 25 °C during two days.
167 The experiments were carried out with one kilogram of common wheat grain introduced
168 in 1.5 L jar. The wheat grains were infested with 50 targeted insects (*R. dominica*) and
169 closed by a metallic grid to avoid the insects escape.
170 The jars were maintained under infestation risk conditions (25±4 °C and 70±5% RH). The
171 stored conditions were monitored using a KIMO® probe in the controlled chamber and
172 another one inside the grain mass stored in a jar.
173 Two specific amounts of carriers containing spearmint EO named EEO 1 and EEO 2, for
174 which EO content was previously determined were put in contact with grains. In parallel,

175 a negative control without carrier nor EO (NC1), a negative control with carrier but
176 without EO (NC2) and a positive control (PC) were assessed. For the positive control, the
177 common wheat grain was treated with Badineb Bio® as described in the point 2.1 of the
178 CEB 106 (AFPP, 1994) protocol. The approved dose of 10 % was nebulized over the wheat
179 grain (0.165 L/t) in order to compensate the loss occurring during the application. Once
180 given the density of the product (0.845 g/mL), the Badineb Bio ® mass nebulized was of
181 8.5 g. The nebulization of the product was done using an atomizer nozzle coupled with a
182 9700 Boxer® peristaltic pump. The duration of the nebulization process took around 33
183 s. This quantity of wheat grain was stored in a cold room at 10 °C inside a closed plastic
184 barrel until assay.
185 The insecticide effect of the different treatments was determined by counting the number
186 of adult insects alive or dead after 1, 2 and 14 days (3 repetitions) and the mortality was
187 measured using the following formula:

$$Mortality\ (\%) = \frac{Number\ of\ dead\ insects}{Total\ of\ insects\ recovered} \times 100$$

188 To count the insects after the assays, a double sieving of the wheat grain of each
189 experimental unit was performed using a 2 mm mesh.
190 At the end of each block, the wheat grains and the carriers were recovered and the EO
191 amount was estimated after solvent extraction and analysis by GC-FID (see below).
192 The percentage of mortality of *R. dominica* was analyzed by an ANOVA statistical analysis
193 followed by a Tukey test ($p<0.05$) with a multiple mean comparison objective.
194 *2.2.3 Contact of wheat grains with encapsulated spearmint EO*
195 To determine the magnitude of EO absorption into the wheat grains but also in milling
196 products, other assays than insecticidal assays were performed. The assays consisted in
197 putting wheat grains in contact with the carriers (containing encapsulated EO). Four 1L
198 glass jars containing 200 g of wheat grain were prepared and a determined amount of
199 carrier containing EO was added on the surface of the grains. The rate of EO in the carrier
200 matrix was previously determined. Two jars were remained closed (CJ) and two remained
201 open (OJ). Then, the four jars were incubated during three weeks in an oven with a
202 headspace of 253 L with weak ventilation <0.3m/s under controlled conditions of RH (72
203 ± 1%) and temperature (25 ± 1°C). Finally, the grains were removed and a sample of 10g
204 were drawn to be submitted to solvent extraction and to quantify the adsorbed EO ad
205 posteriori. The remaining part of the treated grains was submitted to the milling process.

206 *2.2.4. Milling process of wheat grains*

207 Wheat grains (without or after contact with EO) were used for milling. First, the wheat
208 grains were tempered to reach 16.5 % (w/w) of moisture content for 17h. Moisture
209 content was verified using a *Precisa XM 50* infrared moisture analyser.

210 A micro-mill already was used to simulate the industrial milling process which is divided
211 in four steps including two breaking stages, one sizing and one reduction stage leading to
212 4 fractions; flour, coarse bran, fine bran and shorts. Each step consisted of a size reduction
213 phase and a sieving phase. During the sieving process, each fraction was collected,
214 weighted, and proceed again if needed. For the last two phases, the flour obtained after
215 milling was processed with a bran finisher (CHOPIN S.A) for 1.5 min. Then, the product
216 was sieved to achieve a high yield of flour.

217 *2.2.5. Proximal characterization of wheat grain and milled fractions*

218 *Moisture content* (MC): was determined using standardized international method (ISO
219 712:2009) on ground grains and milling fractions.

220 *Starch:* Total starch concentration was determined in duplicate on ground grains or on
221 milling fractions using a Megazyme kit "Amyloglucosidase/alpha-Amylase method"
222 according to AACCI 76-13-01 method (K-TSTA assay kit, Megazyme International Int.,
223 Ireland). Before analysis coarse bran fractions were ground with a ball grinder (MM400,
224 Retsch, Haan, Germany) after being frozen in liquid nitrogen.

225 *Ash:* ash content was determined according to AACC Method 08-12.01

226 *Fat:* lipid content was determined using a semi continuous solvent extraction method
227 using a Soxhlet (AOAC Method 934.01). Ten g of dried sample were inserted in a pre-dried
228 extraction thimble. The sample was covered with glass wool and extraction was
229 performed during 5 h at a rate of six drops per second using hexane as solvent.

230 Except for fat (duplicate), all the experiments were performed in triplicate and statistical
231 significance was determined by one-way analysis of variance (ANOVA) followed by a
232 Tukey test at $p<0.05$.

233 *2.2.6. Liquid extraction of volatile compounds from the carrier, wheat grain, flour and*
234 *brans.*

235 A common solvent extraction method was used to determine the quantity of volatile
236 compounds in the carrier before and after contact with the wheat grain, or in the grain
237 and in the milling fractions obtained from the EO treated grains. A sample of 0.3 g of
238 carrier or 1 g of grains or fractions (bran and flour) was put in a 25ml vial with 9.9 ml of

hexane and □□□□□l of internal standard solution (3 g/l of 2-heptanol in hexane). Then, the sample was stirred during 18 h at 350 rpm. Finally, the organic phase was removed and filtered using a PFTE membrane (pore size 0.2 µm) (*Acrodisc* syringe filters, Sigma-Aldrich) and injected directly into the GC-FID or GC-MS. The quantification of the two major compounds ((*R)*-carvone and (*R)*-limonene) of spearmint EO was done from all samples considering their response coefficients in comparison with 2-heptanol previously determined by calibration of GC-FID. For carrier or grains, the extractions were done in triplicate. For fractions, the extractions were performed using two samples obtained by a milling process (independent replicate) and repeated 3 times (technical repeat). Significant differences between the fractions were carried out by ANOVA and Tukey test ($p < 0.05$).

2.2.7. Extraction of volatile compounds in the flour by SPME

Solid Phase Micro-Extraction (SPME) was used as an extraction technique to analyse the different volatiles compounds in the flour. The volatile compounds in the headspace were extracted when the equilibriums between the different phases (sample/headspace and headspace/fiber) were reached. For this, the samples, 0.1 g or 0.5g of flour from EEO-treated grains or non-treated grains respectively, were placed inside a 20 mL vial in presence of the most adapted volume (5 or100 µL) of internal standard solution (2 g/L of 2-heptanol in distilled water) and were incubated for 5 min at 50 °C. Next, the extraction process was carried out during 25 min at 50 °C using a SPME-fiber of 2 cm (30/50 □m DVB/CAR/PDMS, stableflex).

After extraction, the desorption was carried out for 5 min at 250°C in the GC-MS injector. Between each measurement the fibre was heated (baked-out) for 30 min at 270°C.

2.2.8. Analysis of spearmint EO by GC-FID and GC-MS

A GC-MS ISQ (Thermo-Scientific, Austin, Texas, USA) equipped with a DB-WAX polar capillary column (30 m, 0.25 mm i.d. x 0.25 µm of thickness) and a quadrupole detector was used for the identification of volatile compounds present in the spearmint EO but also in wheat grains, milled fractions obtained from wheat grains treated or not with encapsulated spearmint EO. Helium was used as the carrier gas with a flow rate of 1.2 ml/min. The GC-MS oven temperature was kept at 40°C for 5 min and programmed to 250 °C at a rate of 2°C/min. Spectra were obtained in the electron impact mode with 70 eV of ionization energy. The full scan mode was used, and the range of scans was between 40-

500 amu. Compounds were identified by using different libraries (INRA, NIST and Wiley) and confirmed by Kovat's index determination.

For quantification of (*R)*-limonene and (*R)*-carvone in the carrier, grains and fractions, the GC-FID analysis was performed thought a Varian-3800 GC (Les Ulis, France) equipped with a DB-5 capillary column (30 m x 0.32 mm i.d. x 0.25 µm of thickness, J&W scientific) and a flame ionization detector (FID) using the following conditions (H_2 30 ml/min, air 300ml/min, nitrogen 30 ml/min). Hydrogen was used as the carrier gas with a flow rate of 1 ml/min and analysis was carried out in split mode with a ratio of 20. Operating conditions of oven were: initial temperature of 40 °C (5 min), then raised to 110 °C at a rate of 2 °C/min with a final temperature raise to 250 °C at a rate of 10 °C/min with a detector temperature of 300 °C.

3. Results and discussion

3.1 Insecticidal assays

The spearmint EO used in this study was characterized by 25 compounds mainly monoterpenes and sesquiterpenes. The two major identified compounds were *(R)*-limonene and *(R)*-carvone which represented around 75% of the essential oil, the latter being 2 times (49.4%) more represented than the former (25.6%). The other compounds were present in weak amount remaining inferior or equal to 3%. This composition is in agreement with species coming from India and Asian regions (Kedia et al., 2014).

3.1.1 Insecticidal activity

As described in the Material and Methods, two different carriers amount containing spearmint EO were put in contact with grains and insects in parallel with negative and positive controls. The insect mortality was reported in Table 1.

The amount of EO contained in the carrier and used to act against insects were 894 mg/kg for EEO1 and 1788 mg/kg of wet grains for EEO2. After 14 days of treatment, the EO was not totally released since 37 and 25% of EO was retrieved in the carrier respectively for EEO1 and EEO2, *i.e.* 563 mg/kg and 1341 mg/kg of grains respectively. This showed that the release rate appeared to depend both on the initial amount and to the set-up of a dynamic equilibrium. By consequence the EO amount acting against the insects differed and it was clear that in the case of EEO1, the released amount was insufficient to display a strong insecticidal effect since only around 30% of mortality was observed after 14 days. The higher released amount in the case of EEO2 was clearly correlated to the stronger

mortality. Moreover, a strong lethal effect was already reached after 2 days of EEO2 treatment, and the quantification of EO released showed that only 650 mg/kg of grain was needed to provoke this insecticidal effect (81±9 %, Table 1). These results are difficult to compare with reported literature values due to a wide variation on their described experimental conditions. A lethal concentration of 86 µl/L of air (around 85mg/L of air) leading 100% of mortality was determined by fumigation of *M. spicata* EO against 10 *R. dominica* adults confined in 1L chamber (De Souza, 2016). But, the spearmint EO composition used in this study was different, mainly comprising menthol (35.20%), isomenthone (18.71%), and menthyl acetate (6.22%). Moreover, grains were not present during the fumigation experiment.

3.1.2 Residual EO amount in wheat grains

In parallel to the insecticidal activity the volatile compounds of spearmint EO adsorbed inside the wheat grain due to the treatment were quantified. As all the other compounds of EO were equal or inferior to 3%, we decided to focus our quantification on *(R)*-limonene and *(R)*-carvone which represent the major part of EO, *i.e.*75%.

Depending on the initial dose of spearmint EO in the carrier, *i.e.* EEO1 and EEO2, its quantity varied between 93±15 and 136±9 mg/kg in wet grains, *i.e.* 10.4 and 7.6 % of the initial EO dose respectively. It was clear that the EO absorbed by the wheat grains were not negligible and did not appear correlated with the initial EO amount. In regards to the EO released during the 14 days of experiment, 63% for EEO1 and 75% for EEO2, the EO absorbed inside grains corresponded to 16.5 and 10.1 % of the released amount of EO, respectively. It means, as already stated, that a dynamic equilibrium was established in relation to the initial amount and that the residual amount in grains could be controlled depending on the EO dose. According to the insect mortality (>80%) obtained with a release of 650 mg/kg, we decided to use an EO dose around 700 mg/kg for the second part of this study.

3.2 Impact of EO treatment on *(R)*-limonene and *(R)*-carvone amount in wheat grain and milling fractions

Wheat grains were put in contact with 700mg/kg of EO encapsulated in the carrier and maintained in open and/or closed jars. The contact time between the EO and the grain was increased to 21 days to allow a higher amount of EO to be released. These assays

allowed the comparison between two "extreme" conditions and EO repartition. When the jars are closed the headspace is limited and the EO accumulation in the grains is forced. When open jars were used, the headspace was larger and the EO repartition is modified with a major part of EO losses in the oven as this can occur in open and ventilated silos. This last condition can better mimic the real condition of grain storage in a silo.

3.2.1 Residual amount of spearmint EO into the carrier

The amount of EO into the carrier was characterised and quantified before and after contact with the grains. The total amount of both compounds (*(R)*-limonene and *(R)*-carvone) brought from the carrier in 1 kg of grains reached 697±39 mg where *(R)*-limonene only represented 24% (167±9 mg) of the sum of the two compounds against 34% in the original EO extract. *(R)*-carvone was present up to 76% against 66% in the native EO considering only the two major compounds. Indeed, the EO profile was modified during the encapsulation process because limonene is largely more volatile than *(R)*-carvone. (P_{vap}= 263.9 Pa and 15.33 Pa at 25°C respectively, the Good Scents Company Information system, 2021).

The residual amounts of spearmint EO inside the carrier, after contact with the wheat grain and along 21 days, were also estimated depending on the experimental conditions. In open jars, the total amount decreased from 697 to 6.7±1.4 mg (4.8±1.3 mg of *(R)*-carvone and 1.9±0.1 mg of *(R)*-limonene) but remained relatively high in closed jars with 69±1 mg (56.6±9.3 mg of *(R)*-carvone and 12.3±0.8 mg of *(R)*-limonene).

The difference between the residual EO in the carrier with the two types of jars, closed or open, was due to the contrasting headspace volume. In the case of closed jars, a thermodynamic equilibrium was created with a distribution of volatile compounds between the headspace of the jar (available volume around 0.9 L), the carrier and the grain (200g). In the open jar placed in the oven at controlled conditions (volume ≈ 250L), the equilibrium was never reached due to the large headspace volume of the oven and EO compounds were partially removed by the weak ventilation. Therefore, in the open jars, the majority of *(R)*-limonene and *(R)*-carvone trapped in the carrier was released (up to 99%) being volatilised in the headspace or adsorbed by the grain. Moreover, the major residual compound was *(R)*-carvone due to the preferential volatilisation of *(R)*-limonene. As expected in the closed jars, a great part of volatile compounds (10%) remained trapped

368 in the carrier and it could be hypothesized that a high quantity can also be adsorbed by
369 wheat grains.
370 *3.2.2 Residual volatile compounds amount in grains and milling fractions*
371 The amount of absorbed aroma compounds was estimated in grains before milling and
372 their distribution in each fraction; flour, fine bran, coarse bran and shorts were evaluated
373 after milling. Flour corresponds mainly to the starchy endosperm as attested by its starch
374 content (Table 2) whereas the coarse bran fractions are known to mainly contain the most
375 peripheral grain tissues (Hemery et al, 2007). Fine bran and shorts contain both part of
376 the endosperm and of the peripheral tissues. In addition, coarse and fine brans
377 representing 20.5 % of the issues were richer in lipids in comparison with the flour.
378 Values reported in Table 2 show that all these fractions display a percentage of yield and
379 ash content similar to those reported by (Greffeuille et al, 2006) on similar common
380 wheat grains and micro-mill (Table 2).

381 Before milling, for the grains in the closed jars, the two major compounds of spearmint
382 EO were found strongly absorbed with a total concentration of 257 mg/kg, *i.e*. around
383 37% of the EO initial amount in the carrier (Table 3). Moreover, *(R)*-limonene was well
384 represented (around 27.7%) because closing the jars avoided its volatilization. In
385 addition, limonene being a highly hydrophobic compound, its absorption in grains was
386 slightly favoured in comparison with *(R)*-carvone. As expected, in the open jars, a very low
387 compounds amount was absorbed, not exceeding 10 mg/kg which corresponds to 1.4%
388 of the EO initial amount. The most represented compound was*(R)*-carvone (93%).
389 Limonene was strongly volatilized during the storage due to its volatility that is much
390 higher than the one of *(R)*-carvone. As expected, the amount of EO absorbed in grains from
391 open jars (93 mg/kg) was inferior to the amount obtained in the previous experiments
392 (see above 3.1.2.) with carriers containing high value of EO (894 mg/kg) and in contact
393 with grains during a shorter time (14 days). This result confirms that the EO trapped in
394 grains was correlated to the initial dose but also to the release rate and time of storage.

395 In Table 3, the amount of *(R)*-limonene and *(R)*-carvone were expressed in mg/kg of dry
396 mass basis for each fraction allowing evaluating their specific EO content. Moreover, in
397 the objective to make a balance-sheet and to estimate the specific aroma compounds
398 absorption in each fraction, the amount of both aroma compounds was calculated
399 considering the fraction yield. Comparing the total amount found in the grains and in the

400 different fractions, it was observed that a high amount of *(R)*-carvone was recovered in
401 the fractions after milling since the losses did not exceed 30% and was limited to 14% in
402 the closed jars. In contrast, when grains from closed jars were milled, more than 50% of
403 limonene was lost during the milling steps. These loses were less pronounced using the
404 grain from the open jars (13%). As already highlighted, the strong volatility of limonene
405 favoured its loss during storage (open jars) but also during milling (closed jars). However,
406 the final amounts were always superior in the fractions obtained with grains from closed
407 jars. Indeed, it can be suggested that the absorption strength in the fraction was
408 dependent on the available amount but also on the presence of other compounds which
409 can compete. The difference in behaviour between *(R)*-carvone and *(R)*-limonene
410 influenced the total recovery which was clearly lower for the fractions obtained from the
411 open jars. Concerning the aroma distribution in milling fractions, a preferential recovery
412 was observed in the coarse bran (50%) from grains in closed jars, followed by flour
413 (25%), fine bran (17%), and the shorts (7%). With grains from the open jars, the major
414 part of EO was also found mainly in the coarse bran (36%) but unexpectedly increased in
415 the flour (33%). However, the concentrations found in the open jars were very low and
416 could approach the limit of quantification of the analytical method. Therefore, the
417 comparison of the amounts of compound absorbed in the different fraction may be
418 approximate.

419 In both cases *(R)*-carvone and *(R)*-limonene were preferentially accumulated in the whole
420 bran fractions with values between 54 to 68% (Table 3). The retention of those two aroma
421 compounds in the outer layers and thus in brans was expected as the peripheral tissues
422 play a barrier role to the EO diffusion into the other tissues such as endosperm. Indeed,
423 wheat grain is an indehiscent dry fruit (caryopsis) consisting of a single seed intimately
424 welded to the envelopes that contains it. The bran fraction is mainly composed of the
425 pericarp, testa and aleurone layer which are rich in polyosides such as cellulose, complex
426 xylans esterified with ferulic acid and that contributes to polymer crosslinking. The
427 polyosides were embedded by lignin, a phenolic polymer, in pericarp and testa, this latter
428 being considered as a hydrophobic layer in regard to its composition in lipids and lignin
429 (Antoine et al, 2003; Hemery et al, 2007). The richness of bran in lipids, around 5%
430 (against only 1.64% for the flour) was clearly established and it relates to the preferential
431 hydrophobic interactions between the aroma compounds and the brans (Table 2).

However, part of the outer layers can break along the milling process and part of the EO can also penetrate the grain which explains its non-negligible recovery in other fractions. Moreover, starch is also known to trap aroma compounds (Delarue & Giampaoli, 2000). In the flour where starch dominates, the capacity of absorption remained high but still inferior to the total brans fraction because the aroma compounds were first preferentially adsorbed by the external layers of the grains. Therefore, the flour which is the fraction the most often used to make cereal products is also the one that contains the lower amount of EO or aroma compounds. However, bran fractions can be used to obtain fibre rich food products or to produce animal feed. In these specific cases, the presence of aroma compounds could be an issue, depending on its concentration.

The flour obtained using grains from the open jars had a concentration of *(R)*-carvone equal to 3 mg/kg. Two enantiomers of carvone occur naturally in plants: the *(R)*-carvone which is levorotatory enantiomer (named also l-carvone) which is found in spearmint plant *Mentha spicata L.* and *(S)*-carvone, the dextrorotary enantiomer (named also d-carvone) which is found in caraway or dill seeds (*Carum-carvi L*, *Anethum graveolens L*).

Their use has been regulated in the European Union, and an Acceptable Daily Intake (ADI) of 0-1mg/kg of Body Weight (bw) per day for the two enantiomers was previously adopted (Joint FAO/WHO Expert Committee on Food Additives, 2004) (WHO, 2000). Recently, the ADI for *(S)*-carvone was evaluated to 0.6 mg/kg of body weight per day and the ADI for D-carvone was not increased due to insufficient toxicology data and the commission recommended to generated additional data to refine the current risk assessment (EFSA, 2014). Nevertheless, it was calculated that the level of aggregated exposure to *(R)*-carvone should be 1.8mg/kg of body weight per day, i.e. 3 times higher than that for *(S)*-carvone since exposure to carvone also occurs from non-food sources such as pesticides, feed additives, veterinary products, personal care products and herbal medicinal products (EFSA, 2014). Besides, in foodstuffs, *(R)*-carvone is added as a flavouring agent with level varying between 0.2 and 7170 mg/kg depending on product. The average amount of D-carvone in bakery is estimated to 4mg/kg but other references address an average value of 94 mg/kg with a maximum value of 116 mg/kg (The Good Scents Company Information System, 2021).

462 In brief, the weak residual amount of *(R)*-carvone found in the flour obtained from the
463 open jars wheat grain, which simulated open silos, should not impact the total exposure
464 expected for this compound. However, if the stored grain is not well ventilated, the
465 adsorption of the EO by the grain and the residual amount in flour could increase. In this
466 case, the consumption of flour could contribute to the total exposure (flavouring,
467 pesticides, personal care products *etc.*). For the other compounds present in the EO, there
468 is not a specific regulation and up to date there is not any toxicological problems related
469 to these compounds.

470 Regarding the sensorial impact, the perception threshold of *(R)*-carvone varied between
471 0.027 and 0.6 ppm (mg/kg), indicating that the residual amount of *(R)*-carvone could have
472 an impact on the flour's odour obtained with wheat grains from open as well as from
473 closed jars. In the same way, the perception threshold of *(R)*-limonene varied between
474 0.01 and 0.2 ppm which means it can be perceived in the flour. However the given values
475 for both compounds were obtained in water and the perception values can be largely
476 higher depending on the matrix and specific interactions (Plotto et al, 2004). Nonetheless,
477 we decided to analyse the total profile of the flour to evaluate the importance of
478 compounds in relation to EO absorption in comparison with the volatile compounds
479 naturally found in the flour.

480 **3.3 Comparative analysis of aromatic profile of flours obtained from native grains**
481 **or EO-treated grains**

482 First, the different fractions of milling without contact with the spearmint EO (native
483 fractions) were extracted using a SPME method adapted from Pico et al (2018) and the
484 aromatic profiles were characterised by GC-MS. This first identification allowed
485 determining the compounds that are part of the native flour aroma profile. All identified
486 compounds either from native flour or in presence of spearmint EO were semi-quantified
487 using an internal standard.

488 In the native flour, ten compounds, mainly alcohols and aldehydes due to oxidation of
489 lipids were identified (Table 4). The most represented compounds were 1-hexanol and
490 limonene followed by nonanal. Acetic acid and hexanal were previously reported as major
491 compounds in wheat flour (Czerny & Schieberle, 2002) but the extraction method used
492 by the authors was different (Soxlhet extraction using dichloromethane and
493 concentration by SAFE solvent-assisted flavour evaporation). It is also probable that the

494 respective flours were not stored for similar duration. In general, the main contributors
495 to the wheat flour aroma are the compounds derived from lipid peroxidation, such as 1-
496 pentanol, 1-hexanol, 1-octen-3-ol, hexanal and nonanal (Xu et al 2017), and the amount
497 of these compounds changed with the progress of reaction. These authors showed that
498 the longer the time before analysis, the greater the risk of oxidation causing volatile
499 profile modification. In their study they found that 1-hexanol was the major compound
500 followed by hexanal, naphthalene and nonanal of the flour produced from common wheat
501 grains.

502 Hexanal is the main volatile product from the autoxidation of linolenic acid and its high
503 presence is an indicator of a high extend of lipid oxidation. The low amount of this
504 compound in the flour agreed with its analysis just after production. It may also be the
505 result from the low amount of its corresponding precursor into the flour since the
506 presence of linoleic acid was not clearly evidenced by SPME.

507 Surprisingly, limonene was found in a significant amount in the native flour. The presence
508 of limonene was also evidenced in other studies (Kim et al 2017; Xu et al., 2017) and in
509 derived products of flour such as sourdough and bread (Pico et al, 2015). The
510 identification of limonene and/or other terpene compounds in the flour was explained by
511 the presence of other plants and flowers rich in these compounds that can be
512 concomitantly harvest with wheat grains. Another explanation is linked to the use of
513 limonene as insecticide in organic farming against pest insects to eradicate powdery
514 mildew in *vitis*, or for vegetable culture and potential wheat grain contamination. The
515 presence of 2-ethylhexan-ol has been already detected in flours. However, its origin
516 requires further investigations. Indeed, it is known as an indoor air pollutant with human
517 toxicity and its detection could be problematic (Wakayama et al. 2019).
518 The majority of the compounds present in all of the fractions were characterized by
519 having a green, vegetal aroma and are rather pleasant with the exception of acetic acid, 1-
520 pentanol and 1-hexanol that are perceive as pungent.

521 For the grains in contact with the spearmint EO, the identification and quantification were
522 performed in the fractions obtained from the grains in open jars. The amount of EO in the
523 closed jars was too high and therefore, the quality of the analysis could have been affected.
524 The semi-quantification with internal standard showed that flour contained 5.85 mg/kg

of aroma compounds belonging to spearmint EO for a total of 6.80 mg/kg, meaning that aroma compounds of EO represented 86 % of the aroma profiles. As expected, *(R)*-carvone was the major recovered compound.

The other compounds found in the flour from grains treated with spearmint EO represented between 1 and 3 % of the EO. Their relative quantification showed that they were in the same range of concentrations as 2-heptanone and 1-hexanol. It is also important to note that the distribution of the native compounds was modified: as an example, 2-heptanone becoming the most represented component while the total amount of native compounds was unchanged compared to flour obtained with grains without EO addition. This result confirms that the aromatic profile of flour was constantly changing and that this alteration depends on the storage conditions and the milling process.

In regards to the relative concentration of the EO compounds in the flour, those related to lipid oxidation, which display a strong and disagreeable odour, could be positively masked by the presence of terpene compounds due to the EO presence. Indeed, *(R)*-carvone and other compounds have specific odour of mint (Table 4) that may modify the flour's flavour. However, the sensorial impact of flavour can depend on the residual concentration of the compounds and on the perception threshold of the consumers. Indeed, the use of spearmint EO as an anti-oxidant was recently promoted in bread with a slight impact on its organoleptic quality when a limited quantity (2.5%) is used (Shori et al, 2020).

4.Conclusion

The spearmint EO encapsulated in a carrier was efficient against *R. dominica*, where the mortality rate reached up to 90% after 14 days for a total release of 700 mg/kg of grains. The EO components were detectable in wheat grains after contact with the encapsulated spearmint EO. Both major compounds (*(R)*-carvone and *(R)*-limonene) were retrieved in the milling fractions with higher amount in the issues. The residual EO amount was dependent on the storage conditions where the use of hermetic environments to store wheat grain (closed jars) favours the sorption of essential oils in grains and in milling fractions. By contrast, the use of an open system (open jars) showed lower amounts of EO in wheat grains and corresponding flour after the treatment. In addition, the use of open jars better mimics the common conditions used to store wheat grains, *i.e.* the use of non-hermetic silos coupled with a ventilation system. The consequence of treating wheat grain

with an encapsulated EO led to a modification of the corresponding flour aromatic profile. The majority of the volatile compounds identified in the native flour were lipid oxidation products. After EO treatment, these compounds represented only 14% of the flour aromatic profile which was described by the presence of *(R)*-carvone and *(R)*-limonene and others compounds related to spearmint EO. However, as the presence of limonene in native grains and their corresponding flour was also observed, it can be suggested that this compound could have limited impact in the flour sensorial profile. Whilst (*R*)-carvone has a pronounced mint odour, its controlled presence in flour could be favourable in regard to its anti-oxidative properties. For now, insufficient data about (*R*)-carvone toxicology are available to determine its limits for use. Until further clarification from the legislation on the use of (*R*)-carvone, the amounts of spearmint EO to be used as a green insecticide should be carefully adapted to the infestation rate. Hopefully, a desired amount of EO can be controlled and efficiently released by the use of a carrier. As shown in this study, the residual amount of (*R*)-carvone in a finished product was low in comparison with its intentional addition in bakery products. In addition, ventilation of wheat grain during storage and cleaning before commercialization should reduce to minimum the residual quantity of EO in the grain and consequently in the flour.

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Table 1. Mortality rate of *R. dominica* after treatment by encapsulated spearmint EO

Treatment/time	Mortality of *Rhyzopertha dominica* (%)		
	1 day	2 days	14 days
PC	99±1[a]	99±1[a]	100±1[a]
NC1	1±1[b]	1±1[b]	2±2[b]
NC2	0±0[b]	2±2[b]	5±4[b]
EEO1	17±1[c]	18±5[c]	29±7[c]
EEO2	70±15[a]	81±9[a]	93±6[a]

690 The experiments were performed in triplicate. Means followed by the same letter are not significantly
691 different at 5% level by one way ANOVA and Tukey comparison test.
692

693

694 Table 2. Moisture content, starch and ash composition of milled fractions

Composition	Yield (%)	Moisture content (g/g)	Starch (%)	Ash (%)	Lipids (%)
Flour	69.8± 0.9	0.869±0.002 [b]	75.4± 3.9 [c]	0.60±0.03 [a]	1.64
Coarse Bran	13.5±1.9	0.848±0.002 [a]	26.4±0.3 [a]	5.27±0.12 [c]	4.68
Fine Bran	7.0±1.4	0.880±0.004 [c]	28.2± 0.5 [a]	6.12±0.05 [c]	5.31
Shorts	9.6±0.3	0.861± 0.003 [b]	53.8±0.8 [b]	2.85±0.29 [b]	nd

695 nd: not determined. The experiments were performed in triplicate except for lipids (duplicate). Different
696 letters in the same column (a, b, c) indicate significant difference between samples at the $p < 0.05$ level
697 determined with Tukey's test. No statistical analysis was performed for lipids, the results reported is the
698 means of the duplicate.
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Table 3. Amount of *(R)*-limonene and *(R)*-carvone absorbed by wheat grains and its milling fractions when in contact with encapsulated spearmint EO in Closed (C Jars) or Open (O Jars) Jars.

Assay	Aroma compound		Concentration of aroma compound by fraction (expressed in dry weight)					
			Wheat grains	**Flour**	**Fine Bran**	**Coarse Bran**	**Shorts**	**Total (milled fractions)**
C Jars	*(R)*-limonene	mg/kg	71±12	6.12±1.0^{a}	74±3.0^{b}	105±2.2^{c}	6.95±0.7^{a}	
		mg/assay	14.2±2.5	0.84±0.14	0.91±0.02	3.19±0.06	0.13±0.013	5.07±0.39
	(R)-carvone	mg/kg	186±34	60.7±2.0 ^{a}	418±2.3^{c}	515±5.2 ^{d}	131.9±6^{b}	
		mg/assay	37.2±6.8	8.34±0.0	5.27±0.04	15.30±0.16	2.48±0.11	31.93±1.78
	Total	mg/assay	51.6±9.3	9.2±0.20^{c}	6.2±0.06^{b}	18.5±0.22^{d}	2.60±0.12^{a}	36.5±2.1*
		(%)		(25.2)	(16.9)	(50.7)	(7.1)	(100)
O Jars	*(R)*-limonene	mg/kg	0.71±0.54	0.44±0.15^{a}	0.77±0.35^{a} `	1.35±0.09^{b}	1.06±0.7^{b}	
		mg/assay	0.142±0.11	0.061±0.02	0.010±0.005	0.036±0.025	0.02±0.014	0.127±0.064
	(R)-carvone	mg/kg	9.22±0.58	3.00±0.85^{a}	17.7±1.3^{c}	17.9±0.8^{c}	8.5±1.41^{b}	
		mg/assay	1.84±0.16	0.41±0.11	0.24±0.018	0.48±0.022	0.16±0.027	1.30±0.18
	Total	mg/assay	1.98	0.475±0.13^{a}	0.25±0.02^{a}	0.52±0.05^{a}	0.18±0.04^{a}	1.43±0.19**
		(%)		(33.2)	(17.4)	(36.3)	(12.5)	(100)

The experiments were performed in duplicate and the extraction repeated 2 times. Different letters in the same line (a, b, c, d) indicate significant difference between fractions at the $p < 0.05$ level determined with Tukey's test. Statistical analysis was also performed to compare total amount in 0 and C jars (last column) and significant difference is indicated by * or **.

709
710 Table 4. Identification and semi-quantification of volatile compounds by SPME in native
711 flour and flour obtained from wheat grains after contact with spearmint encapsulated
712 EO.

Compound	Linear Retention Index (LRI)	Amount of volatile compounds (µg/kg) (SPME, GC-MS)		Odour qualification
		Native Flour	Flour obtained from grains in contact with EEO	
1-hexanal	1106	62	42	Fresh green fatty aldehydic grass leafy
2-heptanone	1220	41	255	Cheese, fruity, ketonic
(R)-limonene	1227	180	133	Sweet, citrus and peely
1-pentanol	1289	54	32	Pungent, fermented, bready, yeasty, and solvent-like
1-hexanol	1388	335	167	Pungent, sweet with a green top note
1-nonanal	1417	169	ND	Waxy, aldehydic, citrus,
Acetic acid	1481	42	114	Pungent, sour, fruit, overripe fruit and acetic
1-octen-3-ol	1487	31	36	Earthy, green, oily, vegetative and fungal
β-bourbonene	1524	ND	64	Herbal and woody
2-ethyl-1-hexanol	1525	68	traces	Citrus, fresh floral, oily sweet
β-caryophyllene	1601	ND	83	Sweet, woody, spice, clove and dry
Dihydrocarvone	1615	ND	40	Herbal, minty, mentholic
1-terpinen-4-ol	1630	ND	60	Pine,citrus, woody, floral
Menthol	1677	ND	104	Cooling mentholic, minty
(R)-carvone	1759	ND	5075	Minty, licorice
cis-dihydrocarveol	1765	ND	71	Minty, herbal
trans-dihydrocarveol	1788	ND	91	Minty, herbal
cis-carveol	1878	ND	197	Caraway
trans-carveol	1910	ND	71	Caraway, spearmint
Benzyl alcohol	1919	81	145	Sweet, floral, fruity with chemical nuances
Total aroma compounds		1009	6801	

713