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1 **A new green insecticide for stored wheat grains: efficiency against *Rhyzopertha***
2 ***dominica* and risk assessment**

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11

12 **Abstract**

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

31
32 Key words: D-carvone, essential oil, insect, *Mentha spicata*, solid phase micro-extraction

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45 **1. Introduction**

46 The infestation of grain stocks after harvest by harmful insects constitutes a “permanent”
47 risk during the storage period, and represents the major cause of deterioration of the
48 quality of grains and seeds in post-harvest situations. Thus, in the international business
49 and, to a lesser extent for the supplying of agri-food industries, the presence of insects in
50 unprocessed cereals is not tolerated because it is the matter of severe depreciations when
51 they are detected. The economic losses resulting from a detected infestation represent
52 between 2 and 5% of the commercial value. This percentage is in relation to the reduction
53 of the price if some insects persisted or additional costs of sanitation and cleaning
54 measures to make infested batches in conformity with the commercial standards
55 (Campbell & Arbogast, 2004). To moderate these damages liquid insecticides are usually
56 pulverised on the stored grains such as organophosphorus compounds (pirimiphos
57 methyl, chloropyrifos etc.) or pyrethrinoides (such as deltamethrin or cypermethrin, etc.).
58 However, some of these treatments induce residues with long persistence activity
59 (Leblanc et al 2014; Skerritt et al , 1992). It was demonstrated that the residues present
60 in cereal foods are generated almost exclusively by the treatments of grain bulks after
61 harvest and that numerous grains contain harmful residues. The long persistence
62 activities of these residues have conducted to the tightening of regulations and to impose
63 maximum residue limits for each active insecticide molecule and/or to prohibit some of
64 them such as chloropyrifos and chlorpyrifos-methyl (European Commission, 2020).
65 To replace liquid treatment, phosphine a highly toxic gas was proposed because it has the
66 advantage to not generate residues in the treated product (Nayak et al 2020). This
67 fumigant is now widely used as curative treatment to control stored grain pests due to its
68 low price and its proven effectiveness against various targets. However, even if its
69 application is adapted to the different storage structures, the treatment needs to
70 guarantee the air tightness of the involved silos which is not always easy to obtain with
71 old storage grain facilities. Furthermore, the emergence of phosphine resistance among
72 some insects calls into question its widespread use (Nayak et al., 2020). Moreover, the
73 new green approach promoted by consumers, but also by farmers, has opened many
74 discussions about the safety of substances used in agriculture and of their impact on
75 health and on the environment and push to develop new alternatives. Now the question
76 is how to replace usual insecticides and by which products without the same defect?

77 During the last two decades, essential oils (EOs) and their constituents were strongly
78 investigated as potential insecticides to replace synthetic products and their efficacy was
79 clearly demonstrated (Isman, 2006; Regnault-Roger et al, 2012). Essential oils are
80 aromatic oily liquids obtained from different parts of the plant (flowers, buds, seeds,
81 leaves, twigs, bark, herbs, wood, fruits and roots). Major compounds can constitute up to
82 85% of the total EO's content, whereas other compounds are presented only as traces.
83 They are usually used as flavouring in food or beverages, as a compound of fragrances and
84 aftershaves and as medicines in the pharmaceutical industry. Besides, some essential oils
85 or certain aromatic plants have been traditionally used to preserve food, to repel and kill
86 insects at home or in granaries, to inhibit microorganisms and increase foodstuff shelf-
87 life (Pavela, 2016; Regnault-Roger et al., 2012). The positive insecticidal effect of EOs and
88 their major compounds was demonstrated under fumigation against the different insects
89 known to infest food grains (Isman, 2006; Kedia et al, 2014). Coleoptera such as *Sitophilus*
90 species (*granarium*, *zeamais* and *oryzae*), *Rhyzopertha dominica* (Fabricius, 1792) known
91 as the lesser grain borer, is primary pest of grains which can strongly damage them,
92 rendering it susceptible to attack by secondary pests. *R. dominica* is also highly resistant
93 to phosphine treatment (Nayak et al., 2020) and essential oils appeared as an efficient but
94 challenging alternative (Kumar et al, 2011; De Souza et al, 2016).

95 Among essential oils, the insecticidal potential of *Mentha spicata* (L.) is clearly established
96 against various stored pests and specifically against weevil of stored grains (Kedia et al.,
97 2014, Kumar et al, 2011). The essential oil of *M. spicata*, is rich in (*R*)-carvone and has a
98 characteristic smell of spearmint.

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

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

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

126 The aims of this study are:

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

131

132 **2. Materials and methods**

133 **2.1. Materials**

134 Common wheat (*Triticum aestivum*) grown in organic conditions was purchased from
135 *Salvagnac Agribio Union – RD 999 81630* (Salvagnac, France). Spearmint (*Mentha spicata*)
136 EO from India was purchased from Golgemma (Esperaza, France). Hexane (>99% purity
137 for analysis) was used as a solvent for the extraction of EO and 2-heptanol (≥99%) was
138 used as internal standard. (*R*)-limonene, (*R*)-carvone and other chemical compounds
139 were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Badineb® composed
140 of pyrethrin and piperonyl butoxide (used as synergist) was purchased from Lodi Group
141 (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:

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

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 $< 0.3 \text{ m/s}$ under controlled conditions of RH (72
203 $\pm 1\%$) and temperature ($25 \pm 1^\circ\text{C}$). 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

239 hexane and 2222l of internal standard solution (3 g/l of 2-heptanol in hexane). Then,
240 the sample was stirred during 18 h at 350 rpm. Finally, the organic phase was removed
241 and filtered using a PTFE membrane (pore size 0.2 µm) (*Acrodisc* syringe filters, Sigma-
242 Aldrich) and injected directly into the GC-FID or GC-MS. The quantification of the two
243 major compounds ((*R*)-carvone and (*R*)-limonene) of spearmint EO was done from all
244 samples considering their response coefficients in comparison with 2-heptanol
245 previously determined by calibration of GC-FID. For carrier or grains, the extractions
246 were done in triplicate. For fractions, the extractions were performed using two samples
247 obtained by a milling process (independent replicate) and repeated 3 times (technical
248 repeat). Significant differences between the fractions were carried out by ANOVA and
249 Tukey test ($p < 0.05$).

250 2.2.7. Extraction of volatile compounds in the flour by SPME

251 Solid Phase Micro-Extraction (SPME) was used as an extraction technique to analyse the
252 different volatiles compounds in the flour. The volatile compounds in the headspace were
253 extracted when the equilibriums between the different phases (sample/headspace and
254 headspace/fiber) were reached. For this, the samples, 0.1 g or 0.5g of flour from EEO-
255 treated grains or non-treated grains respectively, were placed inside a 20 mL vial in
256 presence of the most adapted volume (5 or 100 µL) of internal standard solution (2 g/L of
257 2-heptanol in distilled water) and were incubated for 5 min at 50 °C. Next, the extraction
258 process was carried out during 25 min at 50 °C using a SPME-fiber of 2 cm (30/50 µm
259 DVB/CAR/PDMS, stableflex).

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

262 2.2.8. Analysis of spearmint EO by GC-FID and GC-MS

263 A GC-MS ISQ (Thermo-Scientific, Austin, Texas, USA) equipped with a DB-WAX polar
264 capillary column (30 m, 0.25 mm i.d. x 0.25 µm of thickness) and a quadrupole detector
265 was used for the identification of volatile compounds present in the spearmint EO but also
266 in wheat grains, milled fractions obtained from wheat grains treated or not with
267 encapsulated spearmint EO. Helium was used as the carrier gas with a flow rate of 1.2
268 ml/min. The GC-MS oven temperature was kept at 40°C for 5 min and programmed to 250
269 °C at a rate of 2°C/min. Spectra were obtained in the electron impact mode with 70 eV of
270 ionization energy. The full scan mode was used, and the range of scans was between 40-

271 500 amu. Compounds were identified by using different libraries (INRA, NIST and Wiley)
272 and confirmed by Kovat's index determination.

273 For quantification of (*R*)-limonene and (*R*)-carvone in the carrier, grains and fractions, the
274 GC-FID analysis was performed through a Varian-3800 GC (Les Ulis, France) equipped
275 with a DB-5 capillary column (30 m x 0.32 mm i.d. x 0.25 µm of thickness, J&W scientific)
276 and a flame ionization detector (FID) using the following conditions (H₂ 30 ml/min, air
277 300ml/min, nitrogen 30 ml/min). Hydrogen was used as the carrier gas with a flow rate
278 of 1 ml/min and analysis was carried out in split mode with a ratio of 20. Operating
279 conditions of oven were: initial temperature of 40 °C (5 min), then raised to 110 °C at a
280 rate of 2 °C/min with a final temperature raise to 250 °C at a rate of 10 °C/min with a
281 detector temperature of 300 °C.

282

283 **3. Results and discussion**

284 **3.1 Insecticidal assays**

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

291 *3.1.1 Insecticidal activity*

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

295 The amount of EO contained in the carrier and used to act against insects were 894 mg/kg
296 for EE01 and 1788 mg/kg of wet grains for EE02. After 14 days of treatment, the EO was
297 not totally released since 37 and 25% of EO was retrieved in the carrier respectively for
298 EE01 and EE02, *i.e.* 563 mg/kg and 1341 mg/kg of grains respectively. This showed that
299 the release rate appeared to depend both on the initial amount and to the set-up of a
300 dynamic equilibrium. By consequence the EO amount acting against the insects differed
301 and it was clear that in the case of EE01, the released amount was insufficient to display
302 a strong insecticidal effect since only around 30% of mortality was observed after 14 days.
303 The higher released amount in the case of EE02 was clearly correlated to the stronger

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

314

315 3.1.2 Residual EO amount in wheat grains

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

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

331

332 3.2 Impact of EO treatment on (*R*)-limonene and (*R*)-carvone amount in wheat 333 grain and milling fractions

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

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

342 *3.2.1 Residual amount of spearmint EO into the carrier*

343 The amount of EO into the carrier was characterised and quantified before and after
344 contact with the grains. The total amount of both compounds (*(R)*-limonene and *(R)*-
345 carvone) brought from the carrier in 1 kg of grains reached 697 ± 39 mg where *(R)*-
346 limonene only represented 24% (167 ± 9 mg) of the sum of the two compounds against
347 34% in the original EO extract. *(R)*-carvone was present up to 76% against 66% in the
348 native EO considering only the two major compounds. Indeed, the EO profile was
349 modified during the encapsulation process because limonene is largely more volatile than
350 *(R)*-carvone. ($P_{\text{vap}} = 263.9$ Pa and 15.33 Pa at 25°C respectively, the Good Scents Company
351 Information system, 2021).

352 The residual amounts of spearmint EO inside the carrier, after contact with the wheat
353 grain and along 21 days, were also estimated depending on the experimental conditions.
354 In open jars, the total amount decreased from 697 to 6.7 ± 1.4 mg (4.8 ± 1.3 mg of *(R)*-
355 carvone and 1.9 ± 0.1 mg of *(R)*-limonene) but remained relatively high in closed jars with
356 69 ± 1 mg (56.6 ± 9.3 mg of *(R)*-carvone and 12.3 ± 0.8 mg of *(R)*-limonene).

357 The difference between the residual EO in the carrier with the two types of jars, closed or
358 open, was due to the contrasting headspace volume. In the case of closed jars, a
359 thermodynamic equilibrium was created with a distribution of volatile compounds
360 between the headspace of the jar (available volume around 0.9 L), the carrier and the
361 grain (200g). In the open jar placed in the oven at controlled conditions (volume $\approx 250\text{L}$),
362 the equilibrium was never reached due to the large headspace volume of the oven and EO
363 compounds were partially removed by the weak ventilation. Therefore, in the open jars,
364 the majority of *(R)*-limonene and *(R)*-carvone trapped in the carrier was released (up to
365 99%) being volatilised in the headspace or adsorbed by the grain. Moreover, the major
366 residual compound was *(R)*-carvone due to the preferential volatilisation of *(R)*-limonene.
367 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 losses 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).

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

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

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

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

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

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

545

546 **4.Conclusion**

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

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

575

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582

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687
688 Table 1. Mortality rate of *R. dominica* after treatment by encapsulated spearmint EO
689

Treatment/time	Mortality of <i>Rhyzopertha dominica</i> (%)		
	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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702

703 Table 3. Amount of (*R*)-limonene and (*R*)-carvone absorbed by wheat grains and its milling fractions when in contact with encapsulated
 704 spearmint EO in Closed (C Jars) or Open (O Jars) Jars.

		Concentration of aroma compound by fraction (expressed in dry weight)						
Assay	Aroma compound		Wheat grains	Flour	Fine Bran	Coarse Bran	Shorts	Total (milled fractions)
C Jars	<i>(R)</i> -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
	<i>(R)</i> -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	<i>(R)</i> -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
	<i>(R)</i> -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)

705 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
 706 between fractions at the p < 0.05 level determined with Tukey's test. Statistical analysis was also performed to compare total amount in O and C jars (last column)
 707 and significant difference is indicated by * or **.

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

Compound	Linear Retention Index (LRI)	Amount of volatile compounds ($\mu\text{g}/\text{kg}$) (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	

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