1 **CAN OCCURRENCE OF PESTICIDE METABOLITES DETECTED IN CROPS PROVIDE THE**

2 **EVIDENCE ON ILLEGAL PRACTICES IN ORGANIC FARMING?**

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11 **Abstract**

12 Modern pesticides rapidly degrade after their application due to both physicochemical

13 factors and through biotransformation. Consequently, pesticide residues in samples might

14 be either undetectable or detected at low concentrations (≤ 10 µg/kg). Under such

15 conditions, a monitoring of pesticide metabolites in samples might be a conceivable solution

16 enabling the documentation of earlier pesticide use. Analysis of metabolites might pose

17 analytical challenges, because pesticide degradation leads to the production of a number of

18 metabolites, differing somewhat in their structure and polarity. This study was focused on

19 the determination of pesticide residues and their metabolites in samples of grapevine and

20 wine, using ultra-high-performance liquid chromatography coupled with high-resolution

21 mass spectrometry, with the objective of supporting the possibility of the verification of the

22 method of farming. It documents the identification of pesticide metabolites commonly used

23 in conventional farming and provides a characterization of pesticide degradation during

24 grapevine growth, maturation and during the wine-making process.

27

1. INTRODUCTION

The market of organic products is continuously growing in all EU member states, including the Czech Republic. Besides the concern about the impact of conventional farming practices on the environment, consumers´ purchase of organic foods is motivated by various beliefs including that they are safer, healthier and taste better. Although it is rather difficult to provide clear evidence of the last two features, for some consumers the major expectation is the absence of synthetic pesticides, which are perceived as a serious health risk. It is worth noting that the incidence of pesticide residues in organic food is generally low. For instance, the recent European Food Safety Authority (EFSA) report shows that 83.1 % of organic food produced in 2016 in EU countries was free of quantifiable residues, while it was only 53.3 % in the case of conventional foods.[1] Only a few substances (listed in Annex II of the Commission Regulation (EC) No 889/2008)[2] are permitted, but the use of synthetic pesticides is one of the restrictions applied in organic farming (Council Regulation (EC) No 834/2007).[3] Nevertheless, in some cases, contamination can be detected by current highly sensitive methods. In some cases, when applied in a conventional field, pesticides can drift through the air to a neighboring organic farm, leaving traces on the food crops. Similarly, some contamination may occur during transportation or storage of organic foods, if they are not carefully separated from conventional food items. The common practice of food inspection authorities or certification bodies is to tolerate residues at or below the level 0.01 mg/kg. However, when a rapidly degrading pesticide is employed in the pre-harvest period, no or very low residues can be detected at harvest time. Therefore, the unambiguous identification of such an illegal practice is fairly complicated. Laboratory analysis aimed at the control of compliance with legislation typically targets only compounds that are included in the definitions of maximum residue level (MRL)[4]. These mostly involve

merely the parent pesticides and, only in limited cases, certain toxicologically relevant metabolites. However, dissipation of modern pesticides after application leaves a number of various (nontoxic) metabolites in treated plants. These metabolites can be considered, in some respect, as ´markers´ of earlier unauthorized pesticide usage. Therefore, documentation of their presence at higher concentrations in a product labeled as ´organic´ may support identification of fraud even in the case when a parent pesticide is not detectable. (Bio)transformation of pesticides in plants has been a subject of many studies and has to be described in documentation submitted on the occasion of pesticide preparation registration.[5] In general terms, in Phase I, oxidation and hydrolysis and sometimes also reduction of the parent molecules take place. In Phase II, these products undergo conjugation with polar molecules such as sugars, amino acids or glutathione. In some cases, such conjugates occur directly with parent compounds. In Phase III, conversion of Phase II metabolites into (nontoxic) 2^0 conjugates takes place; metabolites move to the vacuole for storage or are incorporated into the cell wall.[6]

Analysis of pesticide metabolites is fairly challenging for several reasons: (i) their concentration might be very low, thus difficult to detect; as several metabolites originate from a parent pesticide; (ii) the dynamics of the origin of individual metabolites in specific crops are unknown, thus multiple metabolites should be always targeted; (iii) most of these metabolites are somewhat more polar compared to the parent compound, thus cannot be directly incorporated into common multiresidue methods, so new analytical procedures have to be developed and implemented; (iv) analytical standards are commercially available for only a few metabolites, thus accurate quantification of most of them is practically impossible.

75   As mentioned above, the popularity of organic products is rapidly growing. This also applies

76   in the case of ´organic wines´ offered on the Czech market. Like other organic farm products,

77   these wines are also regularly controlled for quality and authenticity. While the procedures

78   for the identification of a geographic origin, based on a combined analysis of elemental and

79   isotopic composition, are available in control laboratories, the methodology is still missing

80   for the reliable authentication of farming practice. To investigate the possibility of using

81   pesticide metabolites as markers of unauthorized pesticide usage, we performed a pilot

82   study within which a number of samples was collected from an experimental vineyard where

83   grapevine plants were treated with common fungicides according to an agreed treatment

84   plan. Using ultra-high-performance liquid chromatography coupled with high-resolution

85   mass spectrometry (UHPLC-HRMS(/MS)), the dynamics of parent compounds' dissipation

86   and the origin of the relevant metabolites were monitored. The transfer of these compounds

87   into wine was also studied. Several studies realized earlier by other authors documented the

88   fate of pesticides during wine making process, the impact of various procedures such as

89   filtration, clarification and maceration was described in detail. [7-8] Optimization of the

90   analytical strategy for the extraction of metabolites formed an integral part of this study.

91   **2.   MATERIALS AND METHODS**

92   **2.1.   Materials**

93   Certified standards of pesticides (dimethomorph, fenhexamid, iprovalicarb, metrafenone,

94   pyraclostrobin, quinoxyfen, spiroxamine, tebuconazole and triadimenol) were purchased

95   from Dr. Ehrenstorfer GmbH (Augsburg, Germany), Honeywell Fluka[TM] or Honeywell Riedel-

96   de Haen[TM] (both Seelze, Germany). The purity of standards was in the range of 98-99.9 %.

97   The internal standards for triphenyl phosphate (TPP) and nicarbazin were obtained from

98   Sigma-Aldrich (St. Louis, Missouri, USA). Individual pesticides' stock solutions were prepared

99   in pure methanol, acetonitrile or acetone containing 1 % formic acid (*v/v*), depending on the

100   solubility of the specific pesticide. A composite stock standard in acetonitrile was prepared

101   at 50 000 ng·mL^{-1} from stock solutions and was stored at −18 °C. The working standard

102   mixtures (20-2000 ng·mL^{-1}) used for matrix-matched calibration were prepared from stock

103   solution by further dilution with acetonitrile.

104   HPLC-grade acetonitrile, LC–MS-grade formic acid, ammonium formate and ammonium

105   acetate were obtained from Sigma-Aldrich. Methanol was obtained from Merck (Darmstadt,

106   Germany). Acetone and sodium chloride were obtained from Penta (Chrudim, Czech

107   Republic). Anhydrous magnesium sulfate was obtained from Honeywell Fluka^{TM}. Deionised

108   water (18 MΩ) was produced, using a Millipore Milli-Q system (Bedford, USA).

109   **2.2.   Samples**

110   All samples tested in this study were collected from the vineyard located at the Oblekovice

111   experimental station in the Czech Republic (South Moravian Region). The Ryzlink rynsky

112   (Riesling) grapevine cultivar, treated with various fungicides was used for the experiments.

113   The schedule of treatment and a list of applied Plant Protection Products (PPP) and other

114   preparations, together with the sampling intervals of tested materials, are documented in

115   **Table 1**. Treatments were performed at the doses recommended by the manufacturers.

116   Vine leaves were sampled from May to October (20-week period), wine grapes were

117   sampled during the ripening period from September to October (5 weeks). Both types of

118   samples were collected every 6-8 days (see **Table 1**).

119   Grapes were harvested after the pre-harvest interval (given on a label of PPP) of the

120   particular pesticide had elapsed. Thereafter, the grapes were processed into a white wine.

121   The wine-making process involved the following steps: (*i*) crushing and pressing of grapes

122   into a juice; (*ii*) clarification of juice (separation of lees); (*iii*) fermentation of must;

123   (*iv*) racking; (*v*) fining of young wine; (*vi*) filtration and (*vii*) bottling of wine. During the wine

124   production, sampling was done after crushing and pressing of the grapes (juice), after

125   racking (clarified juice), during the alcoholic fermentation (musts), after racking (young wine

126   and lees), and after bottling (white wine). During the alcoholic fermentation, samples of

127   musts were collected at 6- to 14-day intervals. The amount of an individual sample was 100-

128   200 g and 200-250 mL respectively. All samples were stored at -18 °C in polyethylene bags or

129   in plastic bottles. The list of samples is shown in **Table 1**.

130   **2.3.   Sample preparation**

131   Prior to the analysis, solid samples (vine leaves and wine grapes) were homogenized, using a

132   laboratory blender. Liquid samples (musts and wines) were mixed thoroughly. Parent

133   pesticides were determined, using an ISO 17025 accredited method routinely used in our

134   laboratory. A new extraction/detection method had to be implemented for the analysis of

135   metabolites.

136   **2.3.1. Extraction of parent pesticide residues**

137   The extraction procedure was based on the QuEChERS method. 2.5 g of homogenized vine

138   leaves were weighed into a 50-mL centrifugation tube, followed by the addition of 10 mL of

139   water containing 1 % (*v/v*) of formic acid. The matrix was allowed to soak for 20 min. In the

140   case of wine grapes/wine, 10 g of previously homogenized samples was weighed into a 50-

141   mL plastic centrifuge tube, without water addition. Subsequently, 10 mL of acetonitrile was

142   added and the tube was vigorously shaken for 2 min. In the next step, 1 g of NaCl and 4 g of

143    $MgSO_4$ were added and the shaking process was repeated for 1 min. Then 100 μL of mixture

144   of TPP and nicarbazin (5 μg·mL^{-1}) as an internal standard was added and the tubes were

145   centrifuged for 5 min at 11 200 rcf. An aliquot of the supernatant was transferred into a vial.

146   In the case of wine samples, the volume of the extract (top organic layer) was affected by

147   the ethanol naturally present in wines (11-15 vol.%). To compensate for this effect, an

148   addition of internal standards was used.

149   **2.3.2. Extraction of pesticide metabolites**

150   Four alternative sample preparation procedures were tested for the extraction of pesticide

151   metabolites from the experimental samples.

152   *Procedure A*

153   The same extraction procedure based on the modified QuEChERS method described above

154   for the extraction of parent pesticides (see Section **2.3.1**) was also tested for the extraction

155   of pesticide metabolites.

156   *Procedure B*

157   A sample of homogenized vine leaves (2.5 g) or wine grapes/wine (10 g) was weighed into

158   a 50-mL centrifugation tube, followed by the addition of 10 mL of a mixture of

159   methanol:water (80:20, *v/v*) containing 1 % (*v/v*) of formic acid. The tubes were closed and

160   shaken vigorously by a mechanical shaker for 20 min. The tubes were centrifuged for 5 min

161   at 11 200 rcf. An aliquot of the supernatant was filtered through a 0.22 μm PTFE filter and

162   transferred into a vial.

163   *Procedure C*

164   A sample of homogenized vine leaves (2.5 g) or wine grapes/wine (10 g) was weighed into

165   a 50-mL centrifugation tube, followed by the addition of 10 mL of mixture of

166   methanol:water (50:50, *v/v*) containing 1 % (*v/v*) of formic acid. The tubes were closed and

167   shaken vigorously by a mechanical shaker for 20 min. The tubes were centrifuged for 5 min

168   at 11 200 rcf. An aliquot of the supernatant was filtered through a 0.22 μm PTFE filter and

169   transferred into a vial.

170 *Procedure D*

171 10 g of wine grapes were weighed into a 50-mL centrifugation tube. Thereafter, the tubes

172 were centrifuged for 5 min at 11 200 rcf. An aliquot of the filtered juice was transferred into

173 a vial.

174 **2.4.   Identification and quantification of pesticide residues**

175 Identification of pesticide residues in the samples was based on a comparison of retention

176 time, accurate mass (*m/z*) of the (de)protonated molecule, isotopic pattern matching and

177 accurate mass of MS/MS fragments, to those obtained for pesticide reference standards.

178 The acceptable mass error of potential elemental composition for (de)protonated molecule

179 was ± 5 ppm. The identification criteria were in accordance with the requirements in the

180 European Commission's guideline SANTE/11813/2017.[9]

181 Quantification was performed by using a calibration curve based on matrix-matching

182 calibration standards. To obtain matrix-matched standards corresponding to concentration

183 level 1, 2, 5, 10, 20, 50, and 100 ng·mL^{-1}, 50 µL of a specific working standard mixture and

184 50 µL of internal standard (1 µg·mL^{-1}) were added to 900 µL of the blank extract (blank

185 extract diluted with acetonitrile in ratios of 1:9 and 1:99)

186 **2.5.   LC-MS parameters**

187 The LC–HRMS(/MS) analyses of fungicide residues and their metabolites were performed

188 using an Agilent Infinity 1290 LC system (Agilent Technologies, USA), equipped with an

189 Acquity UPLC HSS T3 analytical column (100 mm × 2.1 mm, 1.8 µm particle size, Waters,

190 USA). Mass spectrometry detection was performed using Quadrupole-Time of Flight

191 spectrometry (Agilent Ion-Mobility Q-TOF 6560, USA) in positive and negative ESI modes.

192 The column temperature was maintained at 40 °C. The injected sample volume was 4 µL.

193 The mobile phases were different for analyses in electrospray positive (ESI+) and negative

194　(ESI−) ionization modes. For compounds detected in the ESI+, mobile phases were (A) water

195　with 5 mM ammonium formate and 0.1 % (*v/v*) formic acid and (B) methanol, respectively.

196　For compounds detected in the ESI- , mobile phases were (A) water with 5 mM ammonium

197　acetate and (B) pure methanol. The gradient was the same in both polarities: the starting

198　mobile phase composition was 5 % of the organic phase (B) with flow 0.2 mL·min^{-1} and

199　linearly changed to 99 % (B) with flow 0.3 mL·min^{-1} in 10 min. This mobile phase composition

200　was held for 2 min simultaneously with the flow rate being changed from 0.3 to

201　0.4 mL·min^{-1}. The column was reconditioned for 2 min in the starting composition of 5 % (B)

202　(flow rate 0.4 mL·min^{-1}). The autosampler temperature was maintained at 5 °C.

203　The MS source conditions were as follows: capillary voltage (VCap) was 4 kV (-4kV in ESI-);

204　nozzle voltage was 1 kV; gas temperature and sheath gas temperature were 210 °C and

205　380 °C respectively; drying gas flow and sheath gas flow were 10 L·min^{-1} and 12 L·min^{-1}

206　respectively, and nebulizer pressure was 342.6 kPa (35 psig) in both acquisition modes.

207　Collision induced dissociation was performed, using nitrogen and the collision energy was

208　fixed (20 V). An Agilent MassHunter Workstation Software (version B.07.00; Agilent

209　Technologies, USA) was used for data acquisition and data analysis.

210　**2.6.　　Strategy for detection and identification of pesticide metabolites**

211　Detection and identification of pesticide metabolites in sample extracts (see Section **2.3.1**)

212　were based on calculated accurate mass (*m/z*), isotopic pattern matching and accurate mass

213　of MS/MS fragments. The acceptable mass error of potential elemental composition for

214　(de)protonated molecule was ± 5 ppm.

215　At first, HRMS mass spectra in a full-scan technique (without fragmentation – MS^{1}) were

216　acquired across the entire chromatographic run, using a mass range of *m/z* 100-1100. The

217　obtained data were searched against the database of elemental composition of metabolites

218 (*csv* format of file), created manually based on a survey in the available literature on

219 pesticide metabolism in plants (see **Table 2**), and consideration of common metabolic

220 reactions (*e.g.* oxidation, dealkylation).

221 In the next step, the identity confirmation of metabolites detected in MS^1 was based on data

222 acquired in the MS/MS run. 3 categories of fragments were searched:

223   (i)   diagnostic ions, known for some groups of fungicides [10];

224   (ii)   common fragments detected in the MS/MS spectrum of the parent pesticide

225   as well as its metabolite;

226   (iii) fragments characterizing a part of molecule with metabolic modification, not

227   detected in the MS/MS spectrum of the parent pesticide.

228 For identification of the conjugates of the parent pesticide and/or its metabolite, a search

229 for neutral losses (*e.g.* hexoses) in fragmentation mass spectra was performed.

230 **2.7.   Method validation**

231 Performance characteristics (recovery, repeatability, within-laboratory reproducibility and

232 limit of quantification) were determined for pesticide residues (parent compounds) in vine

233 leaves, grapes and wine. Validation studies were performed on spiked blank samples. Two

234 spiking levels (0.002 mg·kg^{-1} and 0.02 mg·kg^{-1} in grapes and wine or 0.008 mg·kg^{-1} and

235 0.08 mg·kg^{-1} in vine leaves) were used and analyzed in 6 replicates. Within-laboratory

236 reproducibility (RSD_R) was determined from on-going QC-data in routine analyses. Samples

237 were extracted by the extraction procedure described in Section **2.3.1**.

238 As standards of pesticide metabolites were not available, recovery experiments could not be

239 performed. The precision (repeatability) of the method was determined by an analysis of

240 samples containing incurred pesticide metabolites, extracted by *Procedure A* (see Section

241 **2.3.2**) in 6 replicates.

3. RESULTS AND DISCUSSION

Like other crops, grapevine can also be invaded by pests. In specific cases, fungal infections are common. In conventional vineyards, synthetic fungicides are commonly applied for prevention and treatment throughout the growing season, as well as after the harvest. However, these compounds are not permitted in organic grape production. The only chemicals conceivable for this purpose are *e.g.* sulfur, lime sulfur or some copper compounds.[2] The analysis of residues of synthetic pesticides might seem to be an efficient tool to control compliance with the restrictions in organic farming. Nevertheless, as mentioned in the Introduction, the absence of detectable pesticide residues, or the presence of their negligible traces, do not necessarily document that unauthorized use of banned plant protection products has occurred. To introduce some more reliable solution, we decided to investigate the possibility of screening the pesticide metabolites that are not involved in routine MRLs control (as they are not of toxicological concern), as treatment ´markers´. The implementation of the relevant screening method was the key task in the first phase of our research. In order to understand the dynamics of parent pesticides' dissipation and to have the relevant experimental matrices with incurred residues available, a number of fungicidal treatments were performed by our partners from the Central Institute for Supervising and Testing in Agriculture in their experimental vineyards. The outcome of our pilot study is described in the paragraphs below.

3.1. Development of method for pesticide metabolites

With the exception of pesticide metabolites that are involved in residue definition[11], in scientific literature, information on the occurrence of these compounds originated through plant metabolism and a description of their analysis is very limited. Since nontoxic pesticide metabolites are practically unavailable as analytical standards, the only applicable screening

266 method for them is high-resolution mass spectrometry, which allows for the detection of

267 their (de)protonated molecules, supposing that the presumable elemental formula of the

268 relevant compound is known. On this account, as described in the Section **2.6** and

269 summarized in **Table 2**, based on the available documentation on existing pesticide

270 metabolites, we established a database into which the exact masses of metabolites'

271 molecular ions calculated were inserted (see **Table 2**). To implement the method enabling

272 screening of these analytes, we had to perform simultaneous testing of an extraction

273 procedure and their HRMS detection in the respective plant extracts prepared from treated

274 grape wine samples.

275 ### 3.1.1. Optimization of sample preparation procedure

276 As pesticide metabolites are generally more polar than parent compounds (the increase of

277 polarity depends on the type of metabolic transformation), we were not sure whether a

278 common QuEChERS extraction method would provide the highest (apparent) recovery.

279 In total, four different procedures for the extraction of pesticide metabolites from pooled

280 wine grapes with incurred residues were tested (the grapes used for this purpose were not

281 treated by metrafenone). In addition to QuEChERS, also two methanol–water mixtures

282 (80:20 and 50:50, *v/v*) were used as more polar extraction solvents. The last sample

283 processing method, aimed at the illustration of metabolite transfer into juice, was realized

284 by a simple separation of solids by centrifugation of the grapes' homogenate.

285 With the exception of the QuEChERS extraction, in which phases partition results in

286 discrimination of the most polar matrix components (they are not transferred into an

287 acetonitrile layer), no clean-up step was employed in the above procedures, to prevent

288 losses of potentially occurring metabolites assumed to be more polar than parent pesticides.

289 **Figure 1** shows the apparent extraction efficiencies, expressed as signal intensities as

290 measured by the UHPLC-HRMS method. The ions of targeted metabolites were extracted

291 using their calculated masses (**Table 3**). Considering the QuEChERS extraction as a reference,

292 we can see that this sample preparation procedure provided, on average, the highest

293 analyte signals compared to the other procedures. It is worth noting that these results do

294 not necessarily mean that the highest concentrations of target analytes were contained in

295 QuEChERS extracts, since matrix effects (signal suppression) in samples prepared by other

296 procedures might be more severe. This assumption supports observation of more intensive

297 background signal in total ion chromatogram (TIC). It should be noted that, while the most

298 polar components such as sugars or hydroxy-carboxylic acids, remain in the aqueous phase

299 during the partition step in QuEChERS, these compounds elute in front part of the

300 chromatogram, where the polar metabolites also mostly elute. Considering these facts, the

301 QuEChERS method was employed in all follow-up experiments focused on pesticide

302 metabolite screening.

303   **3.1.2. Confirmation of metabolites' identity**

304 Altogether 18 metabolites originating from 7 pesticides were tentatively identified in the

305 experimental samples, with their quantity depending on the sampling time and processing

306 step. The list of parent compounds and detected metabolites, with exact mass of the

307 (de)protonated molecule and their fragments, ion type and retention time is shown in **Table**

308 **3**. The identity of pesticide metabolites tentatively identified by screening of their calculated

309 (de)protonated molecules (mass error tolerance ± 5 ppm) in both ionization polarity modes

310 and assessment of the isotopic pattern match were further confirmed by a critical analysis of

311 their fragmentation spectra. To illustrate this generic approach, an example of the

312 identification of three fenhexamid metabolites is given in the following paragraph.

313   **3.1.3. Identification of metabolites of fenhexamid**

314 Based on data from field trials[12], residues of fenhexamid were not extensively metabolized in

315 grapevine. Two metabolic pathways were described: (i) hydroxylation on the cyclohexyl

316 group of parent molecule at 2- or 4-position, followed by formation of sugar conjugates, and

317 (ii) glycosylation on the phenolic hydroxyl group of fenhexamid. The mass spectra acquired

318 during analysis were investigated to confirm the presence of these metabolites in the tested

319 samples.

320 A peak with a possible elemental composition $C_{14}H_{17}Cl_2NO_3$ (*m/z* 318.0658, [M+H]$^+$) that

321 might correspond to hydroxylated metabolite (Fen-OH) was detected in the chromatogram

322 at 8.56 min. Its final tentative identification was based on the presence of fragment ions

323 *m/z* 97.1006 and *m/z* 55.0527, which were the most abundant ions in the fragmentation

324 mass spectrum and were also detected in the MS/MS spectrum of the parent fenhexamid.

325 Fragment *m/z* 113.0960 in the spectrum of Fen-OH characterized the position of the

326 hydroxyl group cyclohexyl ring in the metabolite molecule. Finally, the fragment

327 *m/z* 300.0553 in the MS/MS spectrum corresponded to a loss of water molecule

328 (Δ*m/z* 18.0156 Da) from the metabolite Fen-OH (**Figure 2**).

329 A similar approach was used for the identification of two sugar conjugates, glycoside of

330 hydroxylated metabolite (Fen-OH-glycoside) and glycoside of fenhexamid (Fen-glycoside).

331 Peaks with possible elemental composition $C_{20}H_{27}Cl_2NO_8$ (*m/z* 480.1187, [M+H]$^+$) and

332 $C_{20}H_{27}Cl_2NO_7$ (*m/z* 464.1237, [M+H]$^+$) were detected in the chromatogram at 8.6 min and at

333 7.45 min, respectively. The identity of both conjugates was confirmed by matching of the

334 fragment ions with fenhexamid or Fen-OH respectively, and by a neutral loss of hexose ([M-

335 $C_6H_9O_5$]$^+$; Δ*m/z* 162.0535 Da) in the fragmentation mass spectra.

336 As expected, in reversed phase chromatography all the tentatively identified metabolites of

337 fenhexamid showed shorter retention times, compared to the parent pesticide, due to their

338 more polar nature. (**Figure 3**).

339 **3.2.   Validation**

340 Performance characteristics obtained within the validation method of parent pesticide

341 compound in various matrices are summarized in **Table 4**. Recoveries were in the range of

342 81-98 % and repeatabilities, expressed as relative standard deviation (RSD; %), were ≤16 % in

343 all tested matrices. Within-laboratory reproducibilities were in the range of 5-18 %. Limits of

344 quantification (LOQ) were in the range of 0.001-0.1 mg kg^{-1} for grapes and wine, and in the

345 range of 0.004-0.4 mg kg^{-1} for vine leaves.[9]

346 In the case of pesticide metabolites, the recovery values could not be determined, since, as

347 mentioned earlier, pure standards are not commercially available. Therefore, only

348 repeatabilities of measurements (repeatabilities of metabolites' signal intensities) were

349 calculated as relative standard deviations of 6 replicate injections of QuEChERS extracts

350 prepared from samples containing incurred metabolites. The obtained results of metabolites

351 in vine leaves and grapes are summarized in **Table 5**.

352 **3.3.   Changes in pesticide residues and levels of their metabolites**

353 The key objective of this study was to obtain further knowledge on the changes of pesticide

354 residues and the origin of their metabolite levels in various parts of grapevine, including

355 grapes, and during the wine-making process. With regard to the impossibility of quantifying

356 metabolites' concentration, ´Response´, the ratio between the area of detected metabolite

357 and area of internal standard (TPP in positive ionization mode and nicarbazin in negative

358 ionization mode) was used for illustration of the concentration trends.

359 **3.3.1. Vine leaves and wine grapes**

360   Following field treatment (a detailed description is in **Table 1**), 9 out of 13 LC-MS amenable

361   pesticide residues, together with their conceivable metabolites, were monitored in the

362   collected samples. Degradation of particular pesticide residues in vine leaves was monitored

363   during 8-18 weeks, based on their application in the vineyard. Sampling was performed

364   always on the 1st to 4th day after each pesticide treatment. The last five weeks of a

365   sampling period in the vineyard, and samples of wine grapes were collected, together with

366   samples of vine leaves. The complete results of pesticide residue analysis in vine leaves and

367   wine grapes are summarized in the **Supporting information – Figure S1**.

368   Overall, concentrations of pesticide residues in vine leaves significantly dropped in the first

369   week after specific treatment; the decrease of residues of dimethomorph, spiroxamin,

370   tebuconazole and triadimenol was in the range of 27-40 %. In the case of fenhexamid,

371   iprovalicarb, metrafenone, meptyldinocap, pyraclostrobin and quinoxyfen residues, it was

372   even higher, 60-69 %. In subsequent weeks, the decline was somewhat slower.

373   Nevertheless, with the exception of triadimenol, residues of applied fungicide were still

374   detectable in the vine leaves.

375   At the wine grape harvest, the last sampling was performed in the vineyard. In these

376   samples of vine leaves and wine grapes, all residues were detected except for triadimenol.

377   All residue levels decreased by 84-100 %.

378   Pesticide metabolites were already detectable in vine leaves collected within the first

379   sampling after the treatment. The spectrum of metabolites found in vine leaves and wine

380   grapes was similar in the case of fenhexamid, iprovalicarb and spiroxamine. Metabolites of

381   tebuconazole were detected in vine leaves only. In the case of dimethomorph, the pattern of

382   its metabolites differed between grapes and vine leaves. However, dimethomorph-demethyl

383   was found in both these matrices and, moreover, in vine leaves another Phase I metabolite,

dimethomorph-hydroxy, was also present. Regarding wine grapes, in addition to dimethomorph-demethyl, a Phase II metabolite, dimethomorph-demethyl glycoside, was also detected.

The obtained results for all tentatively identified metabolites are summarized in **Supporting information – Figures S3-S8**. To compare the changes in levels of metabolites and parent pesticides, the degradation dynamic of the parent pesticide is also displayed.

As an example, the data for metabolites of fenhexamid in both types of samples are shown in **Figure 4**. Fen-OH was the metabolite of fenhexamid with the highest signal intensity. Nevertheless, the response of fenhexamid was higher than the response of its metabolites in all tested samples. For instance, in samples collected at harvest time, the response of fenhexamid was 5 times (in wine grapes) and 10 times (in vine leaves) higher than the response of Fen-OH. In samples collected 8 weeks after the treatment, the response of Fen-OH and Fen-OH glycoside in vine leaves slightly increased. On the other hand, the response of the third metabolite, Fen-glycoside, decreased (it is noteworthy that the background of co-eluting compounds did not change, thus the analyte decrease is not due to stronger matrix effects). As the biotransformation of pesticides in plants is a complex of enzymatic reactions, Fen-glycoside might be an intermediate product of a metabolic pathway, resulting in the formation of the Phase III metabolites.

3.3.2. Wine-making process

We also attempted to find whether, and to what extent, pesticide residues and their metabolites are transferred from grapes into wine and could be used as markers of illegal practices in organic vineyards. For this purpose, samples were collected across the wine-making process. The obtained data are summarized in **Supporting information – Figure S2**. Residues of some pesticides, such as fenhexamid, iprovalicarb, pyraclostrobin in the juice

408   obtained after the crushing and pressing of grapes, did not significantly decrease when

409   compared with their levels in processed grapes. On the other hand, a remarkable reduction

410   of dimethomorph occurred. After clarification of must, concentrations of dimethomorph,

411   fenhexamid and iprovalicarb decreased by 59-67 %. No detectable residues of metrafenone,

412   pyraclostrobin, quinoxyfen, spiroxamine and tebuconazole were present in clarified must.

413   The elimination of residues corresponds to the physicochemical properties of the respective

414   pesticide. In general terms, less water soluble pesticides with a low octanol-water partition

415   coefficient (*Kow)* are not significantly transferred into wine, as they are mainly absorbed in

416   solid waste (seeds and skins).

417   During fermentation of must, the concentrations of three pesticide residues (dimethomorph,

418   fenhexamid and iprovalicarb) did not show any significant changes. In the final product –

419   bottled white wine – pesticide residues were detected in the same quantities as in

420   fermenting must. None of the processes following clarification resulted in considerable

421   changes in the residues of parent pesticides.

422   As described above, crushing, juicing of wine grapes and clarification of must led to reduced

423   concentrations of parent residues. These processes had a significant influence on the

424   metabolites of metrafenone, pyraclostrobin and spiroxamine; they were separated from

425   must as well as the parent compounds.

426   In samples collected during the wine-making process, metabolites of fenhexamid and

427   iprovalicarb were detected. Changes in the levels of parent compounds and their

428   metabolites are documented in **Figure 5**. In the case of fenhexamid, the response of Fen-OH

429   significantly increased and the response of glycosylated metabolites declined. These changes

430   were probably caused by hydrolysis of glycosidic bonds in the molecules of Fen-glycoside

431   and Fen-OH glycoside by yeast enzymes during the fermentation of must. Analysis of the

432   metabolites of iprovalicarb showed different results. Levels of both tentatively identified

433   metabolites showed no significant changes during the wine-making process. Based on the

434   results shown in **Figure *5*,** clarification of must resulted in a decrease of levels of parent

435   pesticides, but not their metabolites. All five metabolites were also detected in the final

436   product – white wine.

437   In conclusion, detection of pesticide metabolites in matrices with very low or non-detectable

438   residues of parent pesticides may indicate illegal practices in organic farming. Nevertheless,

439   for an unbiased conclusion whether contamination is not due to a drift of pesticides,

440   quantification of the respective metabolites might be helpful, as it would enable an

441   estimation of the earlier pesticide burden. However, for this purpose, the availability of pure

442   analytical standards of pesticide metabolites would be needed.

443   The strategy of pesticide metabolite screening could generically be employed for various

444   other plant matrices and products thereof, including *e.g.* baby food where high standards

445   regarding the quality of raw materials are required.

446   Also worthy of note is the fact that the knowledge on the occurrence of pesticide

447   metabolites in the human diet should be taken into consideration in biomonitoring studies,

448   where such compounds occurring in biological fluids are considered as exposure markers to

449   respective pesticides. The exposure to parent compounds might be overestimated,

450   supposing that the product of biotransformation of the parent compound in the human

451   body and that associated with (nontoxic) metabolites in the diet are identical.

452

453   **Abbreviations used:**

454   Fen-OH   fenhexamid-hydroxy

455   PPP   plant protection product

456 **Associated contents**

457 *Supporting information*

458 Complete results of pesticide residue analysis in vine leaves, wine grapes and musts/wines

459 (**Figure S1 – S2**) and changes in levels of specific pesticide metabolites in vine leaves and

460 wine grapes (**Figures S3 – S8**).

461

469

470 **References**

471 1. The 2016 European Union report on pesticide residues in food.

472 https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5348 (accessed

473 10/15/2018).

474 2. Commission Regulation (EC) No 889/2008 of 5 September 2008 laying down detailed

475 rules for the implementation of Council Regulation (EC) No 834/2007 on organic production

476 and labelling of organic products with regard to organic production, labelling and control

477 Official Journal of European Union, 2008; pp OJ L250, 1.

478 3. Council Regulation (EC) No 834/2007 of 28 June 2007 on organic production and

479 labelling of organic products and repealing Regulation (EEC) No 2092/91 Official Journal of

480 European Union, pp OJ L189, 1.

481 4. OECD Series on Testing and Assessment.

482 http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(20

483 09)30&doclanguage=en (accessed 10/20/2018).

484 5. GUIDELINE FOR THE TESTING OF CHEMICALS. https://www.oecd-

485 ilibrary.org/docserver/9789264061835-

486 en.pdf?expires=1531401580&id=id&accname=guest&checksum=EA54A5BDEC57271E3F277

487 02857981622 (accessed 10/10/2018).

488 6. Skidmore, M. W.; Benner, J. P.; Chun Lam, C. C.; Booth, J. D.; Clark, T.; Gledhill, A. J.;

489 Roberts, K. J., Bioavailability of Common Conjugates and Bound Residues. In *Pesticide*

490 *Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA: 2007; pp 383-393.

491 7. Fernández, M. J.; Oliva, J.; Barba, A.; Cámara, M. A., Effects of Clarification and

492 Filtration Processes on the Removal of Fungicide Residues in Red Wines (Var. Monastrell).

493 *Journal of Agricultural and Food Chemistry* **2005,** *53* (15), 6156-6161.

494 8. Fernández, M. J.; Oliva, J.; Barba, A.; Cámara, M. A., Fungicide Dissipation Curves in

495 Winemaking Processes with and without Maceration Step. *Journal of Agricultural and Food*

496 *Chemistry* **2005,** *53* (3), 804-811.

497 9. SANTE/11813/2017 - Guidance document on analytical quality control and method

498 validation procedures for pesticide residues and analysis in food and feed.

499 https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2

500 017-11813.pdf (accessed 10/10/2018).

501   10.   Lacina, O.; Urbanova, J.; Poustka, J.; Hajslova, J., Identification/quantification of

502   multiple pesticide residues in food plants by ultra-high-performance liquid chromatography-

503   time-of-flight mass spectrometry. *Journal of Chromatography A* **2010,** *1217* (5), 648-659.

504   11.   Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23

505   February 2005 on maximum residue levels of pesticides in or on food and feed of plant and

506   animal origin and amending Council Directive 91/414/EEC. Official Journal of European

507   Union, 2005; p OJ L 70.

508   12.   FAO   REPORT.

509   http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/JMP

510   R05report.pdf (accessed 10/20/2018).

511   13.   Modification of the existing MRLs for dimethomorph in various crops. *EFSA Journal*

512   **2010,** *8* (5).

513   14.   FAO   Evaluation.

514   http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Eval

515   uation06/Quinoxyfen06.pdf (accessed 11/20/2018).

516   15.   Conclusion on the peer review of the pesticide risk assessment of the active

517   substance spiroxamine. *EFSA Journal* **2010,** *8* (10).

518   16.   Conclusion on the peer review of the pesticide risk assessment of the active

519   substance iprovalicarb. *EFSA Journal* **2015,** *13* (4).

520   17.   Conclusion on the peer review of the pesticide risk assessment of the active

521   substance tebuconazole. *EFSA Journal* **2014,** *12* (1).

522   18.   Modification of the existing MRLs for metrafenone in table and wine grapes. *EFSA*

523   *Journal* **2011,** *9* (1).

524   19.   Conclusion regarding the peer review of the pesticide risk assessment of the active

525   substance triadimenol. *EFSA Journal* **2008,** *6* (10).

526   20.   FAO   Evaluation.

527   http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Eval

528   uation04/Pyraclostrobinaf.pdf (accessed 10/15/2018).

529

FIGURE CAPTIONS

531 **Figure 1** Comparison of HRMS signal intensities (peak area, mass window +/- 5ppm) for the

532 target pesticide metabolites in wine grape extracts prepared by various procedures

533 (reference: QuEChERS value = 100 %), n = 3. "Y error bars" represent repeatability of the

534 respective procedure in %.

535 **Figure 2** MS/MS fragmentation mass spectra of fenhexamid and its metabolites (collision

536 energy 20 V)

537 **Figure 3** Overlaid extracted ion chromatograms of fenhexamid (*m/z* 302.0709) and

538 metabolites Fen-OH (*m/z* 318.0658), Fen-glycoside (*m/z* 464.1237) and Fen-OH-glycoside

539 (*m/z* 480.1187)

540 **Figure 4** Changes in concentration of fenhexamid and levels of its metabolites in (A) vine

541 leaves and (B) wine grapes

542 **Figure 5** Changes in concentrations of fenhexamid, iprovalicarb and levels of their

543 metabolites during the wine-making process

544

546 **Table 1** Vineyard treatment by pesticide preparations and sampling of tested materials

Day of experiment	Type of action	Type of sample*	Characterization of pesticide treatment		Doses of applied a.i.
			Trade name of PPP	Common name of active ingredients (activity**)	
day 1 (4/16/2015)	pesticide treatment	-	Masai	tebufenpyrad (S)	0.20 kg·ha⁻¹
36	pesticide treatment	-	Kumulus WG	sulfur (C)	3.00 kg·ha⁻¹
			Polyram WG	metiram (C)	1.10 kg·ha⁻¹
36	sampling	VL	X		
43	sampling	VL			
48	pesticide treatment	-	Cabrio Top	metiram (C), pyraclostrobin (S)	1.00 kg·ha⁻¹
50	sampling	VL	X		
57	sampling	VL			
60	pesticide treatment	-	Melody Comby 65.3 WG	iprovalicarb (S), folpet (C)	2.00 kg·ha⁻¹
			Vivando	metrafenone (S)	0.32 L·ha⁻¹
64	sampling	VL	X		
69	pesticide treatment	-	Acrobat MZ	mancozeb (C), dimethomorph (QS)	2.50 kg·ha⁻¹
			Falcon 460 EC	tebuconazole (S), triadimenol (S), spiroxamine (S)	0.50 L·ha⁻¹
71	sampling	VL	X		
78	pesticide treatment	-	Karathane NEW	meptyldinocap (C)	0.50 L·ha⁻¹
			Melody Comby 65.3 WG	iprovalicarb (S), folpet (C)	2.00 kg·ha⁻¹
78	sampling	VL	X		
85	pesticide treatment	-	IQ-Crystal	quinoxyfen (S)	0.15 L·ha⁻¹
			Kumulus WG	sulfur (C)	3.00 kg·ha⁻¹
			Flowbrix	copper oxychloride (C)	3.00 L·ha⁻¹
85	sampling	VL	X		
92	sampling	VL			
97	pesticide treatment	-	Cabrio Top	metiram (C), pyraclostrobin (S)	2.00 kg·ha⁻¹
99	sampling	VL	X		
105	sampling	VL			
106	pesticide treatment	-	Karathane NEW	meptyldinocap (C)	0.50 L·ha⁻¹
			Melody Comby 65.3 WG	iprovalicarb (S), folpet (C)	2.00 kg·ha⁻¹
113	sampling	VL	X		
119	pesticide treatment	-	IQ-Crystal	quinoxyfen (S)	0.15 L·ha⁻¹
			Teldor 500 SC	fenhexamid (QS)	1.00 L·ha⁻¹
			Alliette Bordeaux	copper oxychloride (C), fosetyl-Al (S)	4.0 kg·ha⁻¹
120	sampling	VL	X		
127	sampling	VL			
134	sampling	VL			
141	sampling	VL			
148	sampling	VL+WG			
155	sampling	VL+WG			
162	sampling	VL+WG			
169	sampling	VL+WG			
176	sampling	VL+WG			
180	sampling	J			
187	sampling	M			
194	sampling	M			
201	sampling	M			
208	sampling	M			
216	sampling	M			
222	sampling	M			
236	sampling	M			
249	sampling	M			
292	sampling	YW			
412	sampling	W			

547   * VL – vine leaves; WG – wine grapes; J – grape juice; M – must; YW – young wine; W – wine

548   ** C – contact; S – systemic; QS – quasi-systemic; X – not relevant

549   **Table 2** Library of screened pesticide residues and their metabolites

#	analyte (parent pesticide and its metabolite)	elemental composition	ref.	#	analyte (parent pesticide and its metabolite)	elemental composition	ref.
1	**DIMETHOMORPH**	**$C_{21}H_{22}ClNO_4$**		**6**	**QUINOXYFEN**	**$C_{15}H_8Cl_2FNO$**	[14]
1a	dimethomorph-demethyl	$C_{20}H_{20}ClNO_4$		6a	3-hydroxy-quinoxyfen	$C_{15}H_8Cl_2FNO_2$	
1b	dimethomorph-demethyl glycoside	$C_{26}H_{30}ClNO_9$	[13]	6b	CFBPQ	$C_{15}H_7ClFNO$	
1c	dimethomorph-Z7	$C_{15}H_{13}ClNO_3$		**7**	**SPIROXAMINE**	**$C_{18}H_{35}NO_2$**	
1d	dimethomorph-Z37	$C_{21}H_{20}ClNO_5$		7a	spiroxamine-*N*-oxide	$C_{18}H_{35}NO_3$	
1e	dimethomorph-hydroxy	$C_{21}H_{22}ClNO_5$		7b	spiroxamine-*N*-desethyl	$C_{16}H_{31}NO_2$	
2	**FENHEXAMID**	**$C_{14}H_{17}Cl_2NO_2$**		7c	spiroxamine-*N*-despropyl	$C_{15}H_{29}NO_2$	[15]
2a	fenhexamid-glycoside	$C_{20}H_{27}Cl_2NO_7$		7d	spiroxamine-cyclohexanol	$C_{10}H_{20}O$	
2b	fenhexamid-hydroxy	$C_{14}H_{17}Cl_2NO_3$	[12]	7e	spiroxamine-cyclohexanol glycoside	$C_{16}H_{30}O_6$	
2c	fenhexamid-hydroxy glycoside	$C_{20}H_{27}Cl_2NO_8$		7f	spiroxamine-diol	$C_{10}H_{20}O$	
3	**IPROVALICARB**	**$C_{18}H_{28}N_2O_3$**		7g	spiroxamine-diol glycoside	$C_{16}H_{30}O_6$	
3a	iprovalicarb-hydroxy	$C_{18}H_{28}N_2O_4$	[16]	**8**	**TEBUCONAZOLE**	**$C_{16}H_{22}ClN_3O$**	[17]
3b	iprovalicarb-hydroxy glycoside	$C_{24}H_{38}N_2O_9$		8a	tebuconazole-hydroxy	$C_{16}H_{22}ClN_3O_2$	
4	**METRAFENONE**	**$C_{19}H_{21}BrO_5$**		8b	tebuconazole-hydroxy glycoside	$C_{22}H_{32}ClN_3O_7$	
4a	metrafenone CL 1500836	$C_{19}H_{20}O_6$		**9**	**TRIADIMENOL**	**$C_{14}H_{18}ClN_3O_2$**	
4b	metrafenone CL 3000402	$C_{19}H_{19}BrO_6$	[18]	9a	triadimenol glycoside	$C_{20}H_{28}ClN_3O_7$	[19]
4c	metrafenone CL 379395	$C_{19}H_{19}BrO_6$		9b	triadimenol-hydroxy	$C_{14}H_{18}ClN_3O_3$	
4d	metrafenone CL 197675	$C_{19}H_{19}BrO_7$		9c	triadimenol-hydroxy glycoside	$C_{20}H_{28}ClN_3O_8$	
5	**PYRACLOSTROBIN**	**$C_{19}H_{18}ClN_3O_4$**					
5a	pyraclostrobin-hydroxy	$C_{19}H_{18}ClN_3O_5$					
5b	pyraclostrobin-desmethoxy	$C_{18}H_{16}ClN_3O_3$	[20]				
5c	pyraclostrobin-hydroxy glycoside	$C_{25}H_{28}ClN_3O_{10}$					

550   **Table 3** List of targeted pesticide residues and their tentatively identified metabolites in

551   tested samples (* WG – wine grapes; VL – vine leaves; M – must; W – wine)

#	analyte (parent pesticide and its metabolite)	elemental composition	ion type	detected ion (MS¹)	measured *m/z* of fragments (MS²)	retention time [min]	sample type*
1	DIMETHOMORPH	$C_{21}H_{22}ClNO_4$	[M+H]$^+$	388.1310	301.0650 ($C_{17}H_{14}ClO_3$) 273.0655 ($C_{16}H_{14}ClO_2$) 165.0541 ($C_9H_9O_3$) 114.0546 ($C_5H_8NO_2$) 70.0271 (C_3H_4NO)	8.9; 9.1	WG; VL; M; W
1a	dimethomorph-demethyl	$C_{20}H_{20}ClNO_4$	[M+H]$^+$	374.1154	287.0480 ($C_{16}H_{12}ClO_3$) 151.0378 ($C_8H_7O_3$) 114.0546 ($C_5H_8NO_2$) 70.0271 (C_3H_4NO)	8.5; 8.7	WG; VL
1b	dimethomorph-demethyl glycoside	$C_{26}H_{30}ClNO_9$	[M+H]$^+$	536.1682	374.1154 ($C_{20}H_{21}ClNO_4$) 287.0480 ($C_{16}H_{12}ClO_3$) 151.0378 ($C_8H_7O_3$) 70.0271 (C_3H_4NO)	7.7	WG; VL
1e	dimethomorph-hydroxy	$C_{21}H_{22}ClNO_5$	[M+H]$^+$	404.1259	386.1135 ($C_{21}H_{21}ClNO_4$) 317.0558 ($C_{17}H_{14}ClO_4$) 289.0614 ($C_{16}H_{14}ClO_3$) 165.0541 ($C_9H_9O_3$) 114.0546 ($C_5H_8NO_2$) 70.0271 (C_3H_4NO)	8.1; 8.3; 8.5	VL
2	FENHEXAMID	$C_{14}H_{17}Cl_2NO_2$	[M+H]$^+$	302.0709	177.9817 ($C_6H_6Cl_2NO$) 143.0124 (C_6H_6ClNO) 97.1008 (C_7H_{13}) 55.0525 (C_4H_7)	9.4	WG; VL; M; W
			[M-H]$^-$	300.0564	264.0796 ($C_{14}H_{15}ClNO_2$) 249.0558 ($C_{13}H_{12}ClNO_2$) 221.0241 ($C_{11}H_8ClNO_2$)	9.3	

#	analyte (parent pesticide and its metabolite)	elemental composition	ion type	detected ion (MS^1)	measured m/z of fragments (MS^2)	retention time [min]	sample type*
2a	fenhexamid-glycoside	$C_{20}H_{27}Cl_2NO_7$	[M+H]$^+$	464.1237	302.0702 ($C_{14}H_{18}Cl_2NO_2$) 177.9817 ($C_6H_6Cl_2NO$) 143.0124 (C_6H_6ClNO) 97.1008 (C_7H_{13}) 55.0525 (C_4H_7)	8.7	WG; VL; M; W
2b	fenhexamid-hydroxy	$C_{14}H_{17}Cl_2NO_3$	[M+H]$^+$	318.0658	300.0549 ($C_{14}H_{16}Cl_2NO_2$) 175.9651 ($C_6H_4Cl_2NO$) 113.0961($C_7H_{13}O$) 97.1008 (C_7H_{13}) 55.0525 (C_4H_7)	8.6	WG; VL; M; W
			[M-H]$^-$	316.0513	280.0730 ($C_{14}H_{15}ClNO_3$) 237.0710 ($C_{11}H_8ClNO_3$)	8.5	
2c	fenhexamid-hydroxy glycoside	$C_{20}H_{27}Cl_2NO_8$	[M+H]$^+$	480.1187	318.0646 ($C_{14}H_{18}Cl_2NO_3$) 300.0549 ($C_{14}H_{16}Cl_2NO_2$) 175.9651 ($C_6H_4Cl_2NO$) 113.0961($C_7H_{13}O$) 97.1008 (C_7H_{13}) 55.0525 (C_4H_7)	7.6	WG; VL; M; W
3	IPROVALICARB	$C_{18}H_{28}N_2O_3$	[M+H]$^+$	321.2173	144.0644 ($C_6H_{10}NO_3$) 119.0852 (C_9H_{11}) 116.0700 ($C_5H_{10}NO_2$) 98.0591 (C_5H_8NO) 91.0533 (C_7H_7) 72.0797 ($C_4H_{10}N$)	9.4	WG; VL; M; W
			[M+CH$_3$COO]$^-$	319.2027	259.1470 ($C_{15}H_{19}N_2O_2$) 216.0911 ($C_{12}H_{12}N_2O_2$) 97.0040 ($C_3HN_2O_2$) 59.0128 ($C_2H_3O_2$)	9.3	
3a	iprovalicarb-hydroxy	$C_{18}H_{28}N_2O_4$	[M+H]$^+$	337.2122	319.1811 ($C_{18}H_{27}N_2O_3$) 144.0644 ($C_6H_{10}NO_3$) 135.0800 ($C_9H_{11}O$) 116.0696 ($C_5H_{10}NO_2$) 98.0591 (C_5H_8NO) 72.0797 ($C_4H_{10}N$)	7.9; 8.1	WG; VL; M; W
3b	iprovalicarb-hydroxy glycoside	$C_{24}H_{38}N_2O_9$	[M+H]$^+$	499.2650	337.2099 ($C_{18}H_{29}N_2O_4$) 319.1811 ($C_{18}H_{27}N_2O_3$) 144.0644 ($C_6H_{10}NO_3$) 135.0800 ($C_9H_{11}O$) 116.0696 ($C_5H_{10}NO_2$) 98.0591 (C_5H_8NO) 72.0797 ($C_4H_{10}N$)	7.3; 7.5	WG; VL; M; W
4	METRAFENONE	$C_{19}H_{21}BrO_5$	[M+H]$^+$	409.0645	226.9706 ($C_9H_8BrO_2$) 209.0808 ($C_{11}H_{13}O_4$) 194.0563 ($C_{10}H_{10}O_4$) 166.0626 ($C_6H_{10}O_3$)	10.1	WG; VL
4a	metrafenone CL 1500836	$C_{19}H_{20}O_6$	[M+H]$^+$	345.1333	253.0837 ($C_{16}H_{13}O_3$) 181.0849 ($C_{10}H_{13}O_3$) 165.0545 ($C_9H_9O_3$) 163.0387 ($C_9H_7O_3$)	8.5	VL
4b	metrafenone CL 3000402	$C_{19}H_{19}BrO_6$	[M+H]$^+$	423.0438	393.0310 ($C_{18}H_{18}BrO_5$) 268.1079 ($C_{17}H_{16}O_3$) 242.9640 ($C_9H_8BrO_3$) 240.9500 ($C_9H_6BrO_3$) 212.9530 ($C_8H_6BrO_2$)	9.7	WG; VL
4c	metrafenone CL 379395	$C_{19}H_{19}BrO_6$	[M+H]$^+$	423.0438	226.9674 ($C_9H_8BrO_2$) 225.0758 ($C_{11}H_{13}O_5$) 223.0596 ($C_{11}H_{11}O_5$) 212.9909 ($C_9H_{10}BrO$)	8.9	WG; VL

#	analyte (parent pesticide and its metabolite)	elemental composition	ion type	detected ion (MS^1)	measured m/z of fragments (MS^2)	retention time [min]	sample type*
					195.0648 (C$_{10}$H$_{11}$O$_4$)		
5	PYRACLOSTROBIN	C$_{19}$H$_{18}$ClN$_3$O$_4$	[M+H]$^+$	388.1059	324.0523 (C$_{17}$H$_{11}$ClN$_3$O$_2$) 296.0585 (C$_{16}$H$_{11}$ClN$_3$O) 194.0811 (C$_{10}$H$_{12}$NO$_3$) 163.0628 (C$_9$H$_9$NO$_2$) 149.0468 (C$_8$H$_7$NO$_2$) 133.0517 (C$_8$H$_7$NO)	9.9	WG; VL
5a	pyraclostrobin-hydroxy	C$_{19}$H$_{18}$ClN$_3$O$_5$	[M+H]$^+$	404.1008	312.0469 (C$_{16}$H$_{11}$ClN$_3$O$_2$) 194.0811 (C$_{10}$H$_{12}$NO$_3$) 163.0628 (C$_9$H$_9$NO$_2$) 149.0468 (C$_8$H$_7$NO$_2$) 133.0517 (C$_8$H$_7$NO)	9.9	WG; VL
			[M-H]$^-$	402.0862	208.0045 (C$_9$H$_5$ClN$_2$O$_2$) 164.0134 (C$_{11}$H$_{12}$NO) 157.0006 (C$_6$H$_4$ClNO$_2$)	9.9	
5b	pyraclostrobin-desmethoxy	C$_{18}$H$_{16}$ClN$_3$O$_3$	[M+H]$^+$	358.0953	326.0677 (C$_{17}$H$_{13}$ClN$_3$O$_2$) 298.0585 (C$_{16}$H$_{13}$ClN$_3$O) 164.0704 (C$_9$H$_{10}$NO$_2$) 132.0434 (C$_8$H$_6$NO)	9.9	WG; VL
5c	pyraclostrobin-hydroxy glycoside	C$_{25}$H$_{28}$ClN$_3$O$_{10}$	[M+H]$^+$	566.1536	404.1008 C$_{19}$H$_{19}$ClN$_3$O$_5$ 312.0469 (C$_{16}$H$_{11}$ClN$_3$O$_2$) 194.0811 (C$_{10}$H$_{12}$NO$_3$) 163.0628 (C$_9$H$_9$NO$_2$) 149.0468 (C$_8$H$_7$NO$_2$) 133.0517 (C$_8$H$_7$NO)	8.6	VL
6	SPIROXAMINE	C$_{18}$H$_{35}$NO$_2$	[M+H]$^+$	298.2741	144.1345 (C$_8$H$_{18}$NO) 100.1083 (C$_6$H$_{14}$N) 72.0795 (C$_4$H$_{10}$N)	8.9	WG; VL
6a	spiroxamine-N-oxide	C$_{18}$H$_{35}$NO$_3$	[M+H]$^+$	314.2690	160.1328 (C$_8$H$_{18}$NO$_2$) 130.1218 (C$_7$H$_{16}$NO) 100.1112 (C$_6$H$_{14}$N) 88.0750 (C$_4$H$_{10}$NO)	9.0; 9.2; 9.3	WG; VL
6b	spiroxamine-N-desethyl	C$_{16}$H$_{31}$NO$_2$	[M+H]$^+$	270.2428	116.1066 (C$_6$H$_{14}$NO) 72.0810 (C$_4$H$_{10}$N)	8.7	WG; VL
6c	spiroxamine-N-despropyl	C$_{15}$H$_{29}$NO$_2$	[M+H]$^+$	256.2271	102.0909 (C$_5$H$_{12}$NO) 84.0797 (C$_5$H$_{10}$N) 58.0639 (C$_3$H$_8$N)	8.4	WG; VL
7	TEBUCONAZOLE	C$_{16}$H$_{22}$ClN$_3$O	[M+H]$^+$	308.1524	151.0312 (C$_9$H$_8$Cl) 139.0285 (C$_8$H$_8$Cl) 125.0147 (C$_7$H$_6$Cl) 70.0390 (C$_2$H$_4$N$_3$) 57.0704 (C$_4$H$_9$)	9.8	WG; VL
			[M-H]$^-$	306.1379	223.0911 (C$_{13}$H$_{18}$ClO) 82.0407 (C$_3$H$_4$N$_3$) 68.0255 (C$_2$H$_2$N$_3$)	9.7	
7a	tebuconazole-hydroxy	C$_{16}$H$_{22}$ClN$_3$O$_2$	[M+H]$^+$	324.1473	141.0078 (C$_7$H$_6$ClO) 125.0147 (C$_7$H$_6$Cl) 70.0390 (C$_2$H$_4$N$_3$)	8.9, 9.4; 9.7	WG; VL
			[M-H]$^-$	322.1334	239.0838 (C$_{13}$H$_{18}$ClO$_2$) 223.0911 (C$_{13}$H$_{18}$ClO) 68.0255 (C$_2$H$_2$N$_3$)	9.2; 9.5	
7b	tebuconazole-hydroxy glycoside	C$_{22}$H$_{32}$ClN$_3$O$_7$	[M+H]$^+$	486.2002	324.1481 (C$_{16}$H$_{23}$ClN$_3$O$_2$) 141.0078 (C$_7$H$_6$ClO) 125.0147 (C$_7$H$_6$Cl) 70.0390 (C$_2$H$_4$N$_3$)	8.4	VL

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553 **Table 4** Recoveries (REC), LOQs, RSD_R and repeatabilities (n=6) in grapes, wine and vine
554 leaves

analyte	LOQ	GRAPES					
		0.002 mg kg^{-1}			0.02 mg kg^{-1}		
		REC	RSD	RSD_R	REC	RSD	RSD_R
	[mg kg^{-1}]	[%]	[%]	[%]	[%]	[%]	[%]
dimethomorph	0.001	93	2	7	92	1	6
fenhexamid	0.001	88	3	10	89	4	5
iprovalicarb	0.001	90	2	13	94	5	8
metrafenone	0.001	84	6	7	97	3	5
pyraclostrobin	0.001	90	2	5	89	1	7
quinoxyfen	0.001	83	4	11	85	2	9
spiroxamine	0.001	94	1	12	90	2	9
tebuconazole	0.001	87	3	9	92	2	5
triadimenol	0.01	<LOQ	-	-	92	4	16
analyte	LOQ	WINE					
		0.002 mg kg^{-1}			0.02 mg kg^{-1}		
		REC	RSD	RSD_R	REC	RSD	RSD_R
	[mg kg^{-1}]	[%]	[%]	[%]	[%]	[%]	[%]
dimethomorph	0.001	96	2	8	91	4	5
fenhexamid	0.001	98	3	9	94	2	5
iprovalicarb	0.001	94	16	11	93	2	9
metrafenone	0.001	94	2	8	95	1	7
pyraclostrobin	0.001	89	2	6	88	1	6
quinoxyfen	0.001	88	3	9	90	1	5
spiroxamine	0.001	91	1	10	84	9	7
tebuconazole	0.001	92	2	11	95	1	5
triadimenol	0.01	<LOQ	-	-	96	5	15
analyte	LOQ	VINE LEAVES					
		0.008 mg kg^{-1}			0.08 mg kg^{-1}		
		REC	RSD	RSD_R	REC	RSD	RSD_R
	[mg kg^{-1}]	[%]	[%]	[%]	[%]	[%]	[%]
dimethomorph	0.004	95	3	9	93	4	8
fenhexamid	0.008	89	3	11	88	2	10
iprovalicarb	0.004	90	11	17	90	2	15
metrafenone	0.004	88	2	9	93	2	9
pyraclostrobin	0.004	89	1	10	88	1	8
quinoxyfen	0.004	81	4	8	82	3	10
spiroxamine	0.004	92	2	12	93	3	10
tebuconazole	0.004	91	2	7	88	2	6
triadimenol	0.04	<LOQ	-	-	83	7	18

555 **Table 5** Method repeatability (RSD, n=6) for analysis of metabolites in vine leaves and grapes

| metabolite of pesticide	vine leaves RSD [%]	grapes RSD [%]
dimethomorph-demethyl	13	4
fenhexamid glycoside	8	4
fenhexamid-hydroxy	12	3
fenhexamid-hydroxy glycoside	5	2
iprovalicarb-hydroxy	14	8
iprovalicarb-hydroxy glycoside	13	3
metrafenone-CL 1500836	6	-
metrafenone-CL 379395	6	-
metrafenone-CL 3000402	4	-
pyraclostrobin-desmethoxy	6	4
pyraclostrobin-hydroxy	3	6
spiroxamine-N-desethyl	14	3
spiroxamine-N-despropyl	10	3
spiroxamine-N-oxide	2	5
tebuconazole-hydroxy	9	4
tebuconazole-hydroxy glycoside	8	4

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FIGURES & GRAPHICS
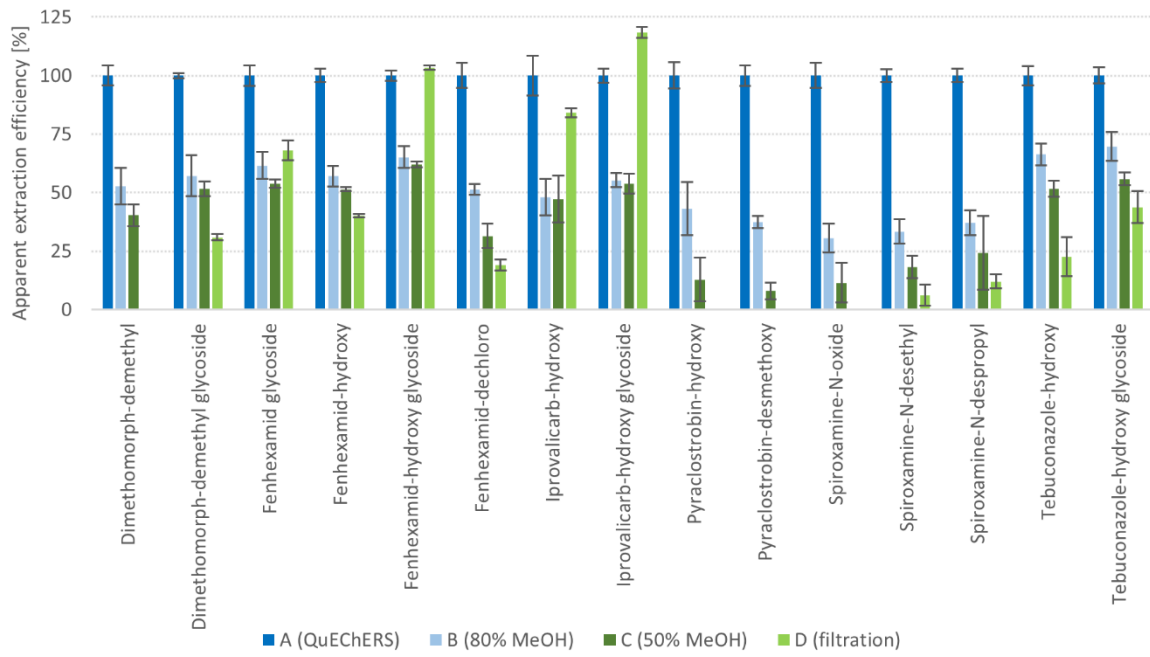
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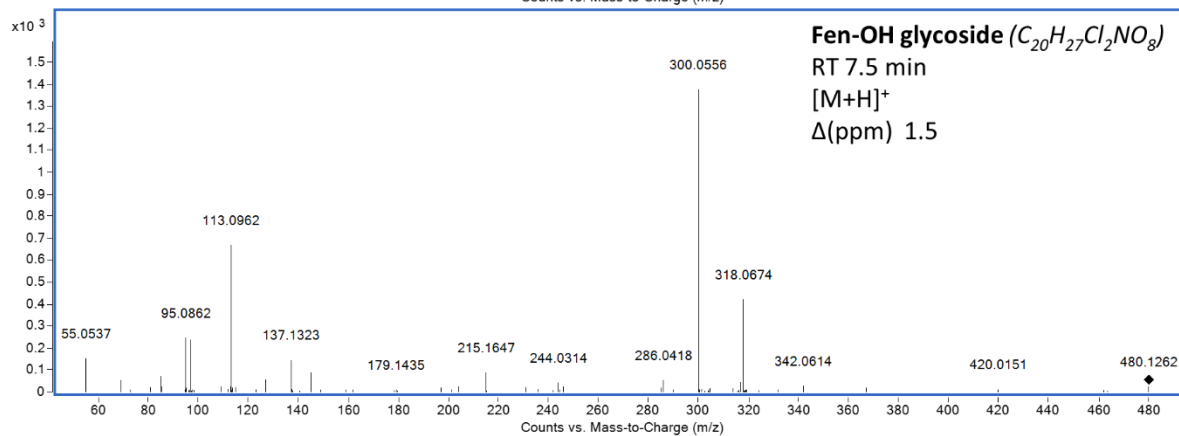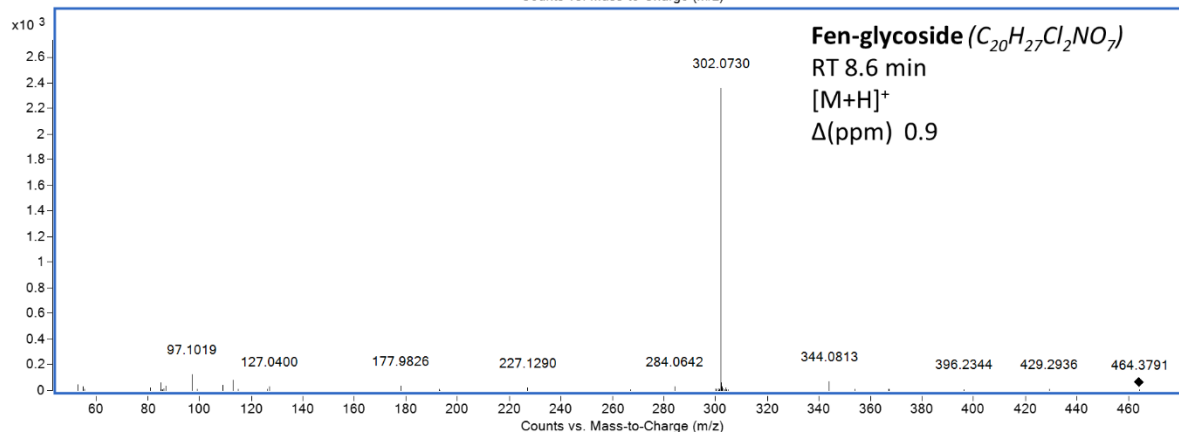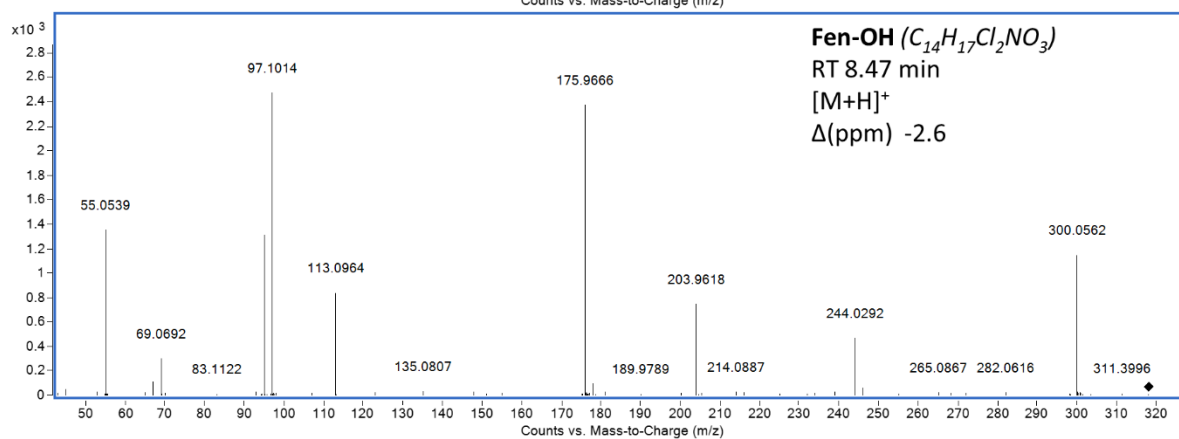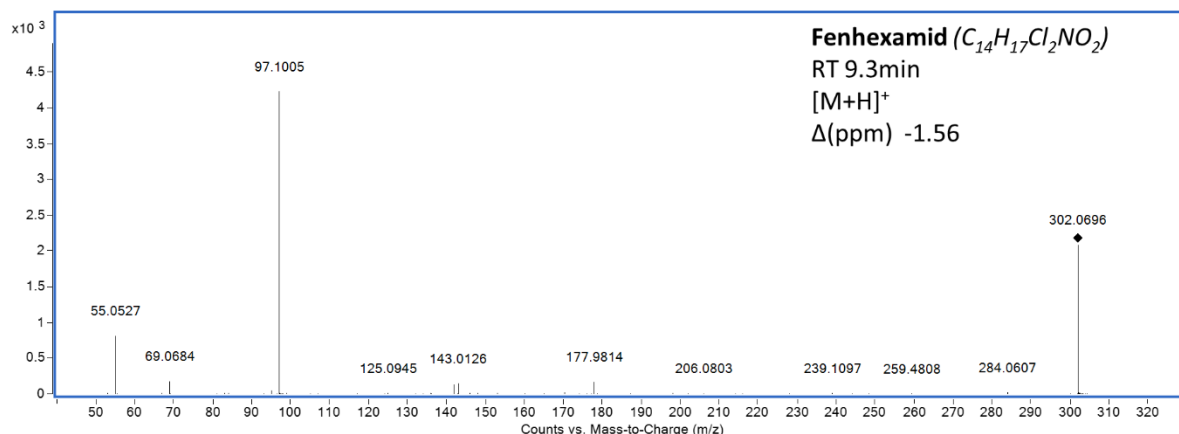
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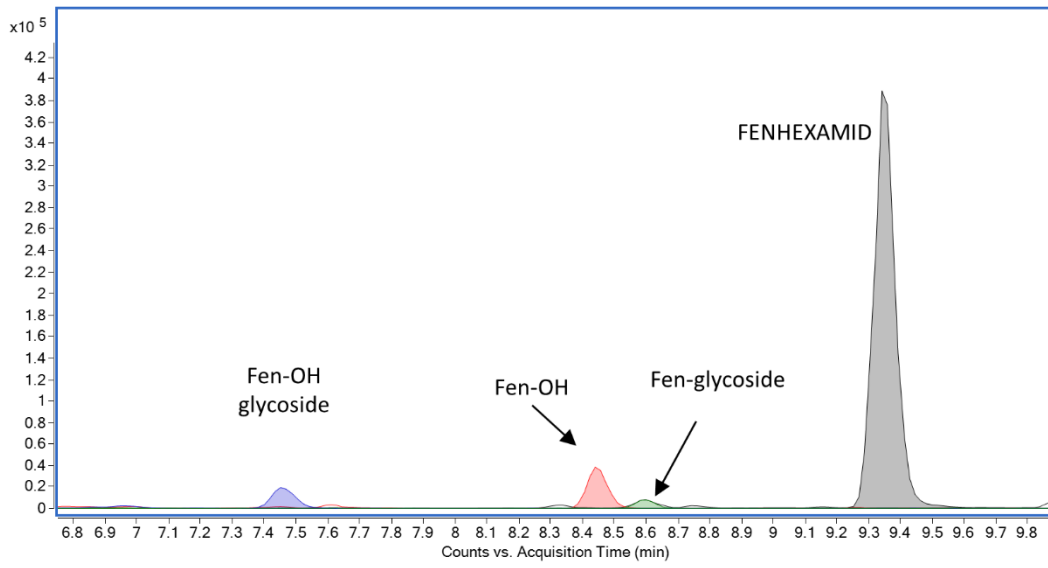
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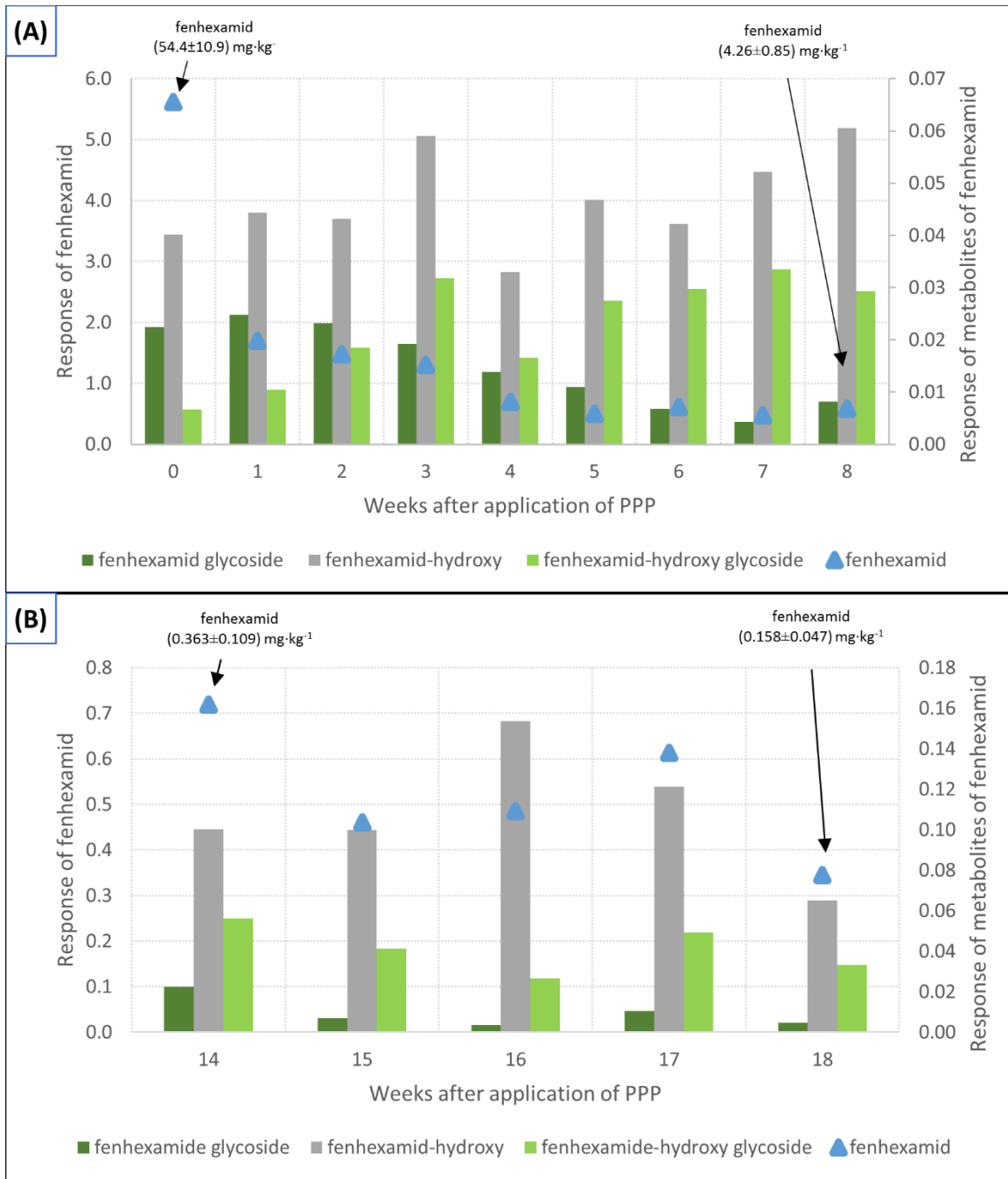
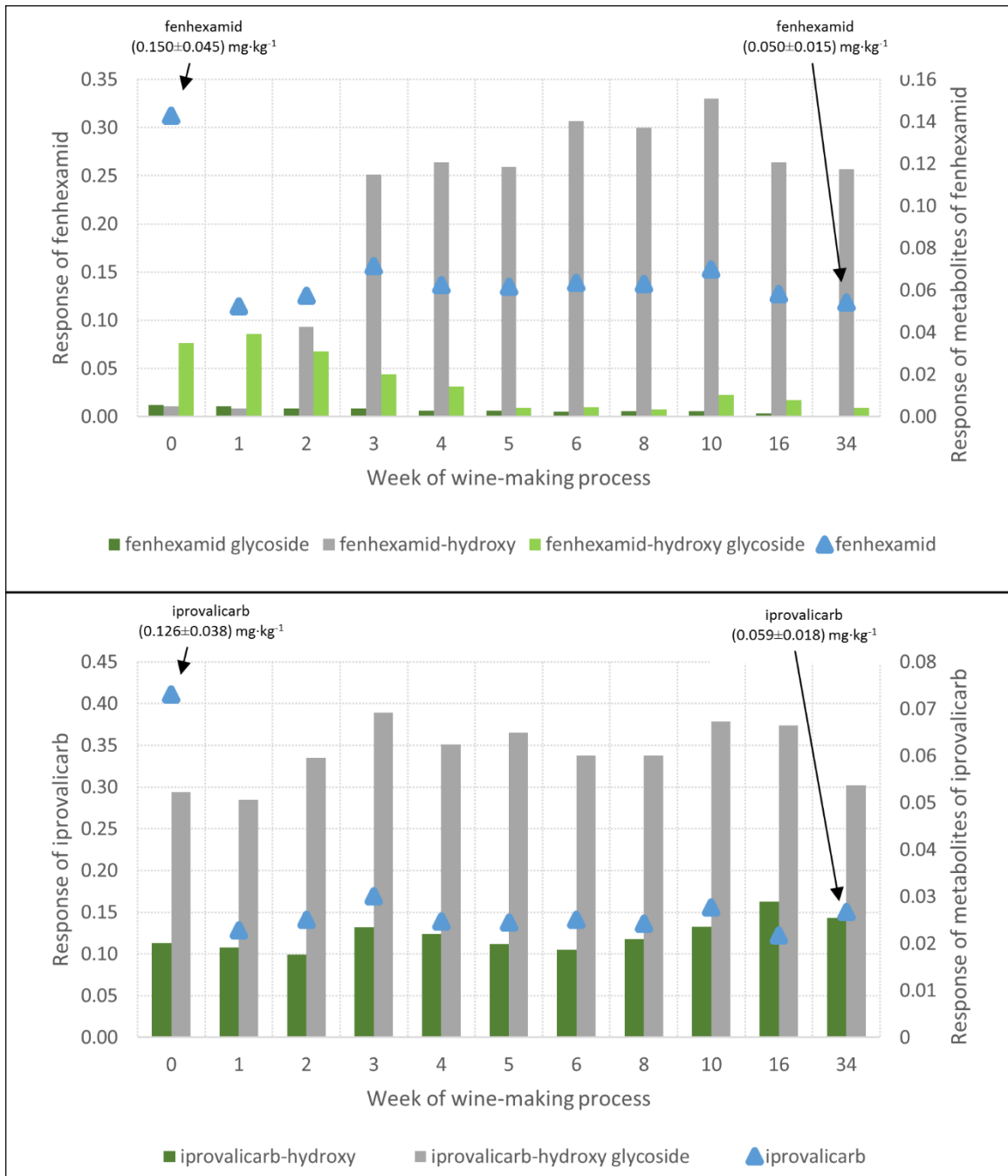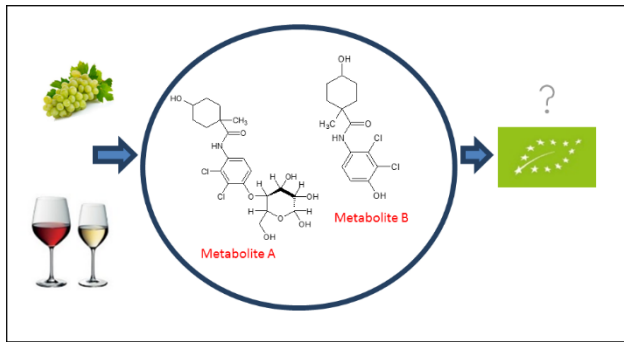564 **Figure 3**

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567 **Figure 4**

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569 **Figure 5**

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571 **For Table of Contents only**

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