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Title: Development and validation of a multi-residue method for pesticide determination in honey using on-column liquid-liquid extraction and liquid chromatography-tandem mass spectrometry

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PII: S0021-9673(07)00502-X
DOI: doi:10.1016/j.chroma.2007.03.035
Reference: CHROMA 347516

To appear in: *Journal of Chromatography A*

Received date: 25-10-2006
Revised date: 28-2-2007
Accepted date: 9-3-2007

Please cite this article as: C. Pirard, J. Widart, B.K. Nguyen, C. Deleuze, L. Heudt, E. Haubruge, E. De Pauw, J.-F. Focant, Development and validation of a multi-residue method for pesticide determination in honey using on-column liquid-liquid extraction and liquid chromatography-tandem mass spectrometry, *Journal of Chromatography A* (2007), doi:10.1016/j.chroma.2007.03.035

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Development and validation of a multi-residue method for pesticide determination in honey using on-column liquid-liquid extraction and liquid chromatography-tandem mass spectrometry

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Submitted for publication in a special issue of *Journal of Chromatography A*

ADV SAMPLE PREP 2006

Guest Editor: Dr L. Ramos

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45 **Abstract**

46 We report on the development and validation under ISO 17025 criteria of a multi-residue
47 confirmatory method to identify and quantify 17 widely chemically different pesticides
48 (insecticides: Carbofuran, Methiocarb, Pirimicarb, Dimethoate, Fipronil, Imidacloprid;
49 herbicides: Amidosulfuron, Rimsulfuron, Atrazine, Simazine, Chloroturon, Linuron,
50 Isoxaflutole, Metosulam; fungicides: Diethofencarb) and 2 metabolites (Methiocarb sulfoxide
51 and 2-Hydroxytertbutylazine) in honey. This method is based on an on-column liquid-liquid
52 extraction (OCLLE) using diatomaceous earth as inert solid support, and liquid
53 chromatography (LC) coupled to mass spectrometry (MS) operating in tandem mode
54 (MS/MS). Method specificity is ensured by checking retention time and theoretical ratio
55 between two transitions from a single precursor ion. Linearity is demonstrated all along the
56 range of concentration that was investigated, from 0.1 to 20 ng g^{-1} raw honey, with correlation
57 coefficients ranging from 0.921 to 0.999, depending on chemicals. Recovery rates obtained on
58 home-made quality control samples are between 71 and 90%, well above the range defined by
59 the EC/657/2002 document, but in the range we had fixed to ensure proper quantification, as
60 levels found in real samples could not be corrected for recovery rates. Reproducibility was
61 found to be between 8 and 27%. Calculated CCα and CCβ (0.0002-0.943 ng g^{-1} for CCα, and
62 0.0002-1.232 ng g^{-1} for CCβ) show the good sensitivity attained by this multi-residue
63 analytical method. The robustness of the method has been tested in analysing more than 100
64 raw honey samples collected in different areas in Belgium, as well as in some wax and bee
65 samples with a slightly adapted procedure.

66

67

68 **Key words:** Pesticides, liquid chromatography, tandem mass spectrometry, honey,
69 diatomaceous earth, on-column liquid-liquid extraction

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70 # 1. INTRODUCTION

71 During recent years, some beekeepers were confronted to hush of their beehives in Belgium.
72 As external observers did not find any classical illness in those hives, several hypotheses were
73 proposed to explain the withering: specific illnesses, pesticide contamination, poor beekeepers
74 practices, etc. Although, several projects were conducted in Europe for the study of separated
75 factors such as imidacloprid levels, no satisfactory explanation could however be given. A
76 multifactorial study has thus been initiated at the Belgian Walloon Region level. Specialized
77 observers have studied beekeeping practices, common illness in the hives and environmental
78 conditions around hives for selected Belgian locations. Consequently, a list of pesticides
79 including both product types used in apiculture and the surrounding agriculture has been
80 extracted. This list consists in a large number of compounds belonging to different chemical
81 classes of insecticides, herbicides and fungicides, which could be the source of the bee
82 decline. In order to verify this assumption, a wide range of pesticides has to be monitored in
83 different honey, wax and bee samples coming from different injured and safe areas.
84
85 In the past decades numerous publications have already reported analytical method for
86 pesticides determination in honey, and a review published four years ago has highlighted the
87 most relevant ones [1]. These studies however usually focused on the analysis very few
88 compounds, often belonging to one or two pesticide families at the most, such as
89 organochlorine or organophosphorous residues. As demands of pesticide analysis started to be
90 more motivated according similar agrarian uses rather than similar physico-chemical
91 properties of chemicals, multi-analyte determinations have appeared since the end of nineties,
92 covering several classes of pesticides [2-5]. The challenge of such strategy is to include in a
93 single procedure a broad range of compounds having widely different structures and
94 properties.

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95 Among the different reported analytical procedures, liquid-liquid extraction (LLE) is the most
96 popular technique for such difficult matrix as honey [1, 6-8]. However, LLE requires large
97 amounts of solvent, is time consuming, laborious and not well suited for automation [1, 6]. As
98 an alternative, solid phase extraction (SPE) or matrix solid phase dispersion (MSPD) have
99 been widely developed in the past decades. Their simplicity, robustness, rapidity and low
100 solvent consumption are attractive parameters for the analytical chemist. Whereas SPE is
101 based on the retention of selected analytes on cartridge sorbents and their elution with
102 appropriate solvent, MSPD consists in the dispersion of the matrix on a free-adsorbent and its
103 homogeneous packing on a column prior to elution of compounds with organic solvent
104 allowing the extraction of semi-solid and solid samples [9, 10]. The other side of the coin is
105 its poor capabilities for high sample input [11]. Solid-phase microextraction (SPME) has also
106 been studied for pesticide analysis in honey [2, 12] but showed sample input limitations and
107 relatively high limit of detection [11]. Supercritical fluid extraction (SFE) and stir-bar
108 sorptive extraction (SBSE) still remains quite marginal in this area until now [5, 13]. In this
109 study, an on-column liquid-liquid extraction (OCLLE) method has been tested as it seemed to
110 combine advantages of LLE, SPE and SPME.
111
112 Mass spectrometery represents the most selective detector for pesticides as it provides
113 structural information allowing unequivocal confirmation and its use in a multi-residue
114 screening context. Although GC is often reported as the most powerful separation tool, it
115 involves a derivatization step for thermally unstable compounds. This introduces additional
116 handling and reaction, thus potentially reducing reproducibility and recovery rates [1].
117 The goals of the present study were to develop and validate under ISO 17025 criteria a multi-
118 residue screening method to identify and quantify 17 widely chemically different insecticides
119 (Carbofuran, Methiocarb, Pirimicarb, Dimethoate, Fipronil, Imidacloprid), herbicides

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120 (Amidosulfuron, Rimsulfuron, Atrazine, Simazine, Chlorotoluron, Linuron, Isoxaflutole,
121 Metosulam), fungicides (Diethofencarb) and some metabolites (Methiocarb sulfoxide and 2-
122 Hydroxytertbutylazine) potentially present in honey. This method is based on on-column
123 liquid-liquid extraction (OCLLE) and liquid chromatography coupled to mass spectrometry
124 (LC-MS) operating in tandem mass spectrometry mode (MS/MS). This analytical procedure
125 was evaluated according to European Commission advice 2002/657/EC [14] in terms of
126 trueness, reproducibility, sensitivity, specificity and robustness. A slightly adapted procedure
127 was also developed and applied to wax and bee samples.

128

129 ## 2. EXPERIMENTAL

130 ### 2.1. Reagents and standards

131 Water was obtained from a Milli-Q® Ultrapure Water Purification Systems (Millipore,
132 Brussels, Belgium). Acetone, ethyl acetate, and acetonitrile were Pestanal® reagents
133 (Promochem, Molsheim, France) while NaCl was from Acros Organics (Geel, Belgium) and
134 acetic acid from JT Baker (Deventer, The Netherlands). Chem Elut cartridges (5 mL) were
135 purchased from Varian Inc. (Varian, Sint-Katelijne-Waver, Belgium). These disposable
136 cartridges contain cleaned diatomaceous earth packed in pure polypropylene housing and a
137 hydrophobic membrane at the base of the cartridge to ensure that moisture is excluded from
138 the extract. Liquid nitrogen was purchased at Air Liquide (Liege, Belgium). All pesticide
139 reference standards are produced by Dr. Ehrenstorfer (Augsburg, Germany), their
140 concentrations are listed in Table 1. Linuron D6 ($C_9H_4Cl_2N_2O_2D_6$) also produced by Dr.
141 Ehrenstorfer (100 µg mL^{-1}) was used as deuteriated surrogate standard to check extraction
142 step because of it easy commercial availability and its suitable retention time on the LC
143 column. PALL Bulk GHP Acrodic 13 mm syringe filters (pore diameter: 0.2 µm) were
144 purchased from VWR International Belgique (Leuven, Belgium)

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145 **[INSERT TABLE 1 ABOUT HERE]**

146 Honey, wax and bee samples were collected in 16 hives spread out in Wallonia (Belgium)
147 during the winter 2004-2005.

148 **2.2. Sample preparation**

149 Extraction of pesticides in honey was inspired by the procedure developed by Klein *et al.* [15]
150 for multi-residue determination in fruits and vegetables. It has been modified to extract the
151 selected chemicals from raw honey samples. Aliquots of 1 g of honey were spiked with 20 μL
152 of surrogate standard (see Table 1 for concentrations) before mechanical transversal agitation
153 with 1.25 ml of water and 2.5 mL of acetone for 1 hour. A 20% NaCl solution (1.25 mL) was
154 then added and the mix was loaded on the Chem Elut cartridge. After a waiting period of 15
155 min, analytes were eluted by gravity twice with 10 mL of ethyl acetate. Extracts were then
156 evaporated at 30°C until dryness under a gentle stream of nitrogen, and transferred with 200
157 μL of an acetonitrile-water solution (10:90) in vials suited for LC injection.

158 Classical LLE was performed as follow: 6.5 mL of acetonitrile were added to 1 g of honey
159 dissolved in 2 mL of water, and mechanically shaken for 30 min. Organic and aqueous phases
160 were separated by centrifugation (15 min at 2000 rpm). Organic layer was then evaporated
161 down to 100 μL, and added to 100 μL of water. This final extract is filtered before being
162 injected in LC-MS.

163 Bee samples were frozen with liquid nitrogen and crushed to obtain a fine homogeneous
164 powder. OCLL extraction was then performed on aliquots of 0.5 g with the same procedure
165 than described for honey.

166 Wax extraction was quite different. Samples were also frozen and ground in a fine powder.
167 0.5g was weighted, spiked with 20 μL of surrogate standard and agitated with 10 mL of
168 hexane and 10 mL of acetonitrile for 1 hour. Centrifugation was applied for 20 minutes and
169 the organic phase was re-extracted with 10 mL of acetonitrile. Both aqueous phases were

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170 evaporated at 30°C until dryness under a gentle stream of nitrogen. 200 µL of an acetonitrile-
171 water solution (10:90) were added, filtered and transferred in vials suited for LC injection.

172 **2.3. Instrumental analysis**

173 Analysis were carried out on a Quattro Ultima Platinum triple quadrupole mass spectrometer
174 coupled to an Alliance 2690 liquid chromatograph (Waters, Manchester, UK). The
175 chromatograph was equipped with a Polaris C18-A HPLC column (150 mm x 2.0 mm, 3 µm,
176 200 Å) from Varian Inc. (Varian, Sint-Katelijne-Waver, Belgium), kept at 40°C. The mobile
177 phase consisted of acetonitrile and water, both acidified with 0.1% of acetic acid. Gradient
178 was applied at a flow rate of 0.4 mL min^{-1} as follow: held the initial conditions of 10%
179 acetonitrile in water for 1 min, increased linearly to 80% in 14 min, increased linearly to
180 100% in 2 min, held at 100% during 1.9 min, returned to initial conditions in 0.1 min and
181 maintained for 4 min. The LC effluent was split using a T-splitter to produce a flow of 0.2 mL
182 min^{-1}. The quadrupole mass spectrometer was equipped with a Z-spray source for positive
183 electrospray ionization (ESI). Capillary and cone voltages were set respectively at 3 kV and
184 35 V, temperature source was kept at 125°C while desolvatation temperature was held at
185 250°C. Nitrogen was used as cone and desolvating gas at a flow rate of 100 and 680 L/h
186 respectively. Mass spectrometer operated in MS/MS mode using multiple reactions
187 monitoring (MRM). 99.8% pure argon from Air liquide (Liège, Belgium) was used as
188 collision gas at a constant pressure of $2x10^{-3}$ mbar. Table 2 summarises the acquisition
189 window definition, masses of parent and daughter ions that are monitored, and the optimized
190 collision induced dissociation (CID) voltages.

191 **[INSERT TABLE 2 ABOUT HERE]**

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192 # 3. RESULTS AND DISCUSSION

193 ## 3.1. Extraction assays

194 Pesticide extractions from raw honey were carried out by on column liquid-liquid extraction.
195 This technique is based on classical LLE principle, but assisted by inert solid support. This
196 inert matrix consisted in diatomaceous earth, well-known for its high porosity, high dispersing
197 capacities, and its high capacity for aqueous adsorption [16, 17]. It has been already used in
198 several chemical extraction applications [18-22]. In the present extraction step, diatomaceous
199 earth has been factory pre-packed in disposable cartridges commercially available under trade
200 name of Chem Elut, from Varian Inc. Comparison between OCLLE and classical LLE has
201 been carried out to check extraction efficiency and suitability of the procedure. Results are
202 shown in Figure 1. OCLLE seemed to provide similar or even higher extraction efficiency
203 and higher repeatability than LLE for some compounds. Moreover and above all, OCLLE
204 provides the real advantage over LLE to avoid emulsion formation in ensuring immiscibility
205 of organic solvents and aqueous matrix. This therefore significantly eases extraction
206 procedure [23]. Fidente *et al.* [24] have developed an extraction procedure based on identical
207 principles for insecticide analysis in honey but this study involved a single class of
208 insecticides and therefore included a limited number of chemically related compounds.
209 Moreover, cartridges that were used appeared to require a drying step using nitrogen flow,
210 increasing the analysis time and the procedure complexity. The strong points of Chem Elut
211 cartridges were their ease of use and the wide range of compounds that could be extracted
212 efficiently.

213 **[INSERT FIGURE 1 ABOUT HERE]**

214

215 ## 3.2. LC-MS/MS data

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216 Most of target pesticides are separated by LC prior to MS detection except Dimethoate and
217 Fipronil, which are reported to be analyzed by GC-MS or SPME-GC-MS. Avoid
218 derivatization step and allow a less rugged clean-up [6] were the reasons which led us to use
219 liquid instead of gas chromatography. The configuration of the Z-spray source designed at
220 first to prevent fragmentation during ionization [25] enhances this robustness in terms of
221 matrix related interferences, as only charged species enter in the detector. The use of tandem
222 mass spectrometry confers high specificity and reduces the risk of potential interferences
223 related to the complexity of the matrix. Each precursor ion was fragmented by Collision
224 Induced Dissociation (CID) and the two most abundant produced ions were monitored. In
225 addition to this gain of selectivity, the use of the MS/MS mode substantially increases
226 sensitivity by limiting the high background noise related to the honey matrix.
227 The Polaris C18-A column is normally dedicated to drug and drug metabolite discovery [26,
228 27]. The silica phase of this HPLC column is bonded to octadecyl chain with a polar group
229 maximizing polar retention and selectivity, and eliminating silanol residues. This allowed to
230 cover a broad range of chemically different compounds. LC gradient has been optimized to
231 distinguish the 17 pesticides keeping in mind that coeluted compounds showing different
232 masses could be separated by the mass spectrometer using multiple reaction monitoring
233 (MRM) mode. In order to achieve the best compromise between time analysis and sensitivity,
234 the number of transitions in a single window has been limited to 12. As for each precursor
235 compounds, two product ions have been recorded, this represented a maximum of 6 pesticides
236 monitored by acquisition window. An example of chromatogram is presented in Figure 2 for a
237 methanolic standard solution showing pesticide concentrations ranging between 0.4 and 20 ng
238 mL^{-1} depending on the congener (Tabe 1). Total LC cycling (separation and return to start
239 conditions) program was 23 min.
240 **[INSERT FIGURE 2 ABOUT HERE]**

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241

242 **3.3. Method validation**

243 Specific guidelines have been produced by the European Commission for the validation of
244 both vegetal and animal product analysis [14, 28]. As honey is product of animal origin such
245 as eggs or milk by-products, the analytical procedure has been validated in compliance with
246 the European Commission decision EC/657/2002 [14] dedicated to the measurement of
247 residues in living animals or their derived products. This group of contaminants includes
248 banned compounds or residues for which maximum levels (MRL) have been assigned
249 (compounds belonging to the group B from annex 1 of 96/23/EC [29]). Although pesticides
250 targeted in this work did not belong to this "blacklist", this guideline has been chosen because
251 of its particular rigor and precision. Validation will therefore involve determination of the
252 specificity, the calibration curves, the trueness, the accuracy (repeatability and
253 reproducibility), the sensitivity and the robustness.

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255 ***3.3.1. Specificity***

256 In order to prevent misidentification of analytes due to interferences, relative retention time
257 (RRT) has been checked for each pesticides and a maximum deviation of 2.5% from the
258 expected RRT accepted. Additionally, two transitions from a single precursor ion were
259 monitored to complete identification insurance. These transitions have been chosen for each
260 target on standard solution as both most abundant ions produced from precursor. Figure 3
261 shows an example for the determination of most intense fragments obtained by MS/MS for
262 Imidacloprid and optimisation of collision voltages. Identification of analytes was confirmed
263 if isotopic ratio bias from standard theoretical ratios were below 20%. Although two produced
264 ions have been recorded, quantification has been performed using only one mass because of
265 software limitation. These masses represent the most intense ions produced and are listed in
266 bold underlined in Table 2.

267 **[INSERT FIGURE 3 ABOUT HERE]**

268

269 ***3.3.2. Calibration curves***

270 Calibration curves have been produced for quantification. They were built using blank honey
271 matrix spiked after the extraction step at 5 different pesticide levels, the zero point included
272 (Table 3). This calibration procedure permits to avoid matrix effect in the electrospray source,
273 such as ion enhancement or suppression. Additionally to criteria required by the
274 2002/657/EC, a second calibration curve was run at the end of each sample series to check the
275 stability of the detector answer after unknown sample data aquisition. The requirement we set
276 was that the end curve had to show a bias lower than 20%, compared to initial calibration.

277 **[INSERT TABLE 3 ABOUT HERE]**

278 Linearity has been observed all along the area of concentration studied depending on the
279 chemicals. These ranges of concentrations were selected in function of the sensitivity of the

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280 mass spectrometer towards each pesticide. They are listed in Table 3, together with
281 correlation coefficient (R^2) of the linear regression. Very few compounds showed residual
282 level or background signal in the unfortified honey matrix. A chromatogram built with
283 specific masses of these compounds and the mass of deuteriated Linuron (D6) for comparison
284 is shown in Figure 2. This background noise was very low and usually non-significant. As
285 these traces were already taken into account during the calibration, no correction by mean of
286 subtracting blank matrix levels was necessary nor applied during the quantification process.
287 However, 10 blank matrices were run during the validation to ensure a minimal risk of
288 interferences and guarantee specificity of the method. Additionally, a blank matrix sample
289 was added to each unknown sample series in order to check for lab and solvent potential
290 contamination.

291

292 ***3.3.3. Recovery (Trueness)***

293 Other pools of blank honey have been fortified prior to the extraction step for home-made
294 quality control samples (QC) at 3 different levels, reported in Table 3. For each level, 6 QC
295 samples have been run. Recoveries have been calculated as the ratio between levels measured
296 in the QC and amounts really added to these blank samples. Particular care has to be taken for
297 the evaporation step. Most pesticides appeared to be really sensitive to dry evaporation, and
298 recoveries can be cut by more than a half if compounds remain to dryness a too long period
299 even at a maximum of 30°C.

300 Recoveries and relative standard deviation (RSD) are listed in Table 3. According to the
301 2002/657/EC document, these recoveries have to range between 50 and 120%. As already
302 mentioned, as levels found in real samples could not be corrected by the recovery rates, a
303 narrower range, between 70 and 110 %, was chosen to ensure at best proper quantification.
304 Although target analytes were characterized by significantly different physico-chemical

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305 properties (functional groups, polarity), recorded recoveries were constantly high, accounting
306 for the versatility and efficiency of the extraction and detection methods. One can also
307 mention that if one would use the less strict 50 to 120% acceptation range stated in the
308 European Directive, one could extend the list of pesticides adequately analyzed by this
309 procedure to other chemicals such as Tribenuron-methyl, Bitertanol, Difenoconazole,
310 Flusilazole, Difenoconazole, Metazachlor, Trifloxystrobin, Metconazole and, importantly, the
311 widely spread Rotenone.
312

313 ***3.3.4. Repeatability and reproducibility***

314 According to the 2002/657/EC document, 3 different QC levels have to be analyzed with six
315 replicate for each level, and these have to be performed on 3 distinct days in order to calculate
316 the method repeatability, as the standard deviation (SD) of the recovery mean.
317 Reproducibility has to be evaluated similarly with minor changes, such as with different
318 operators, different environment, with different solvent batches, etc. In this study, only 2
319 different QC levels (#1 and #3 in Table 3) were used to keep the validation cost in the budget,
320 resulting in a total of 36 QC measurements. Because different operators contributed to these
321 validation tests, only reproducibility was gathered in Table 3. RSD ranging between 8 and
322 27% were judged satisfactory regarding the low levels we dealt with in this work.
323

324 ***3.3.5. Decision limits and detection capacity (CCα and CCβ)***

325 Two different methods can be used to evaluate the decision limit (CCα) when there is no
326 maximal residue limits (MRL) applied for the target pesticides. The first one consists in the
327 analysis of 20 blank materials. The CCα is then equal to three times the signal-to-noise ratio
328 (S/N) in the chromatogram where the analyte is expected. As very few compounds have
329 shown background noise in the time window where they show up, this calculation approach

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330 appeared not to be appropriate to properly evaluate the decision limit. The other method is
331 based on the analysis of blank honey matrices spiked prior to extraction with decreasing
332 amounts of compounds, and the comparison between recorded MS signals and concentrations
333 added. CCα is then equal to the concentration corresponding to the sum of the intercept of the
334 linear regression and the reproducibility multiplied by 1.64 (α = 5% as stipulated for
335 compounds belonging to Group B from annex 1 of EU Directive 96/23/EC). Decision limit
336 values obtained for all investigated compounds are listed in Table 4. Detection capacities
337 (CCβ) have been calculated as the concentration corresponding to CCα added to the
338 reproducibility multiplied by a factor of 1.64 (β = 5%). Those values are also listed in Table 4.
339 **[INSERT TABLE 4 ABOUT HERE]**
340 Because very few authors have already reported such validation data on honey and, as
341 different criteria were applied when they did so, comparison with other reported methods is
342 difficult. Our procedure nevertheless demonstrates to offer very good sensitivity compared to
343 limits of detection (LODs) and quantification (LOQs) reported for some pesticides in honey
344 by Albero *et al.* [4] and Fidente *et al.* [24].
345
346 ***3.3.6. Robustness***
347 109 raw honey samples collected in different areas of Belgium have been analysed within the
348 scope of the multifactorial study described in the introduction. In practice, honey samples
349 appeared to be characterized by different appearances, depending on the area where they were
350 collected, with colour ranging from light yellow to dark brown, with different viscosity, etc.
351 Table 3 presents the number of samples in which target pesticides have been found
352 (considered as positive) and ranges of levels measured. Some pesticides which had not
353 successfully passed the validation criteria but had nevertheless showed recovery rates ranging
354 between 50 and 70% have been included in that Table (i.e. Rotenone, Bitertanol and

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355 Flusilasole). For those, CCα and CCβ have been calculated using the standard deviation
356 evaluated on 18 QCs instead of 36 QCs.
357
358 Additionally to honey samples, some bee (99) and wax (98) samples have also been analyzed
359 as a demand for those matrices appeared during the method development study. The slightly
360 modified procedure described in the sample preparation section was used. None of the
361 targeted pesticides was detected in all bee samples, whereas 26 wax samples showed
362 significant levels of Flusilazole, 17 presented positive results for Rotenone, Pirimicarb has
363 been found in 10 samples, 4 and 3 samples showed traces of Bitertanol and Atrazine,
364 respectively. Only one wax revealed levels in 2-Hydroxytertbutylazine. These results
365 demonstrated the flexibility of the procedure regarding matrix types. The range of chemicals
366 analyzed can be extended while keeping the same extraction method but combining LC-
367 MS/MS and GC-MS/MS. This approach has further been recently developed in our laboratory
368 and pesticides such as Coumaphos, Bromopropylate, Vinclozine, tau-Fluvalinate and
369 Lindane, among others, can now additionally be detected in honey, bee or wax samples (data
370 not shown).
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371 # 4. CONCLUSIONS

372 A rapid, reliable, time and resource saving analytical method is reported for the measurement
373 of a wide range of different chemicals used in apiculture or in the surrounding agriculture in
374 the context of a bee mortality study. The multi-residue analytical procedure developed in this
375 study was based on an on-column liquid-liquid extraction step using diatomaceous earth as
376 inert solid support. Extracts were analyzed without further purification by LC-MS/MS in ESI
377 mode. Extraction by OCLLE using the commercially available Chem Elut cartridges has
378 proven to be efficient for a wide range of pesticides, nearly independently of their polarity.
379 The use of LC-MS/MS permitted to avoid undesirable derivatization steps while lowering
380 sample clean-up requirements, compared to GC-MS. The extraction and purification have
381 then be considerably reduced and simplified. The MRM allowed to separate the 17 target
382 pesticides in less than 15 minutes with good specificity.
383 A complete validation following the European Commission decision 2002/657/EC dedicated
384 to some residues in living animals or their derived products has been performed for the 17
385 pesticides belonging to widely chemically different families, from organophosphorous to
386 triazines, including ureas, carbamates, pyrazoles, nicotinoids or pyrimidines. Specificity,
387 calibration curves, trueness, reproducibilty, sensitivity and robustness have been tested
388 successfully, demonstrating the suitability of this method for selected compounds. The list of
389 pesticides can easily be extended by adding a GC-MS/MS injection of the extracts. Other
390 matrices such as wax and bee have also been included in the study by slightly adapting the
391 extraction procedure.
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394 **Acknowledgements**

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395 The authors thank the Fonds Européen de Développement Régional (FEDER), the Fonds
396 Social Européen (FSE) and the Service Public Fédéral Santé Publique (Formerly Ministère
397 des Classes Moyennes et de l'Agriculture DG6) for their financial support.

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Table 1: List of the pesticide abbreviated names used all along this manuscript as well as method development concentration levels in the surrogate standard solution.

Pesticide name	Abbreviation used	State	Purity (%)	Level (ng mL^{-1})
Amidosulfuron	Am	solid	97.5	0.4
Atrazine	At	solution	99.5	0.4
Carbofuran	Ca	solid	99.5	0.4
Chlorotoluron	Ch	solution	99	20.0
Diethofencarb	De	solid	97.5	2.0
Dimethoate	Dm	solution	99	2.0
Fipronil	Fi	solid	96.5	10.0
Imidacloprid	Im	solution	97	2.0
Isoxaflutole	Is	solution	98.5	2.0
Linuron	Li	solution	99.5	2.0
Methiocarb	Mh	solution	98.5	10.0
Methiocarb sulfoxide	MhS	solution	96	20.0
Metosulam	Mo	solid	99.5	2.0
Pirimicarb	Pi	solution	98	0.4
Rimsulfuron	Ri	solid	99.5	0.4
Simazine	Si	solid	98	2.0
2-Hydroxyterbuthylazine	TOH	solution	98.5	1.0

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Table 2: List of acquisition parameters. Masses in bold underlined are those used for quantification.

Windows	Retention time (min)	Compounds	Precurseur ions (m/z)	Product ions (m/z)	Dwell times (s)	Collision voltages
1	5.84	Methiocarb sulfoxide	242	170	0.2	20
			242	**185**	0.2	10
	6.16	Imidacloprid	256	175	0.2	15
			256	**209**	0.2	15
	6.18	2-Hydroxytertbutylazine	212	114	0.1	20
			212	**156**	0.1	15
	6.38	Dimethoate	230	171	0.1	15
			230	**199**	0.1	10
2	8.26	Pirimicarb	239	**182**	0.2	15
			239	195	0.2	10
	8.68	Simazine	202	**124**	0.2	15
			202	132	0.2	15
3	9.57	Carbofuran	222	123	0.2	15
			222	**165**	0.2	10
	9.93	Amidosulfuron	370	218	0.2	20
			370	**261**	0.2	10
	10.09	Chlorotoluron	213	140	0.2	20
			213	**168**	0.2	15
	10.11	Rimsulfuron	432	**182**	0.2	20
			432	325	0.2	15
	10.27	Atrazine	216	146	0.2	20
			216	**174**	0.2	15
	10.61	Metosulam	419	**175**	0.1	20
			419	228	0.1	15
4	12.11	Methiocarb	226	121	0.2	15
			226	**169**	0.2	10
	12.25	Diethofencarb	268	180	0.1	15
			268	**226**	0.1	10
	12.29	Linuron	249	**160**	0.2	15
			249	182	0.2	15
	12.29	Linuron D6	256	**161**	0.1	15
	12.62	Isoxaflutole	360	**251**	0.2	10
			360	262	0.2	10
5	13.47	Flusilazole	316	165	0.2	25
			316	**247**	0.2	15
	13.68	Bitertanol	338	**99**	0.2	15
			338	269	0.2	5
	13.81	Rotenone	395	192	0.2	20
			395	**213**	0.2	20
6	14.55	Fipronil	437	290	0.2	25
			437	**368**	0.2	15

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Table 3: Validation process data showing the concentration range inside which the linearity was tested, levels (ng g^{-1} honey) of the 3 QC samples analyzed in 6 replicates, and RSD obtained for reproducibility test (QC level #1 and #3 analyzed in 6 replicates at 3 distinct days by 3 different operators).

	Linearity		QC levels			Trueness (n = 18)		Reproducibility
	Levels (ng g^{-1})	R^2	# 1 (ng g^{-1})	#2 (ng g^{-1})	#3 (ng g^{-1})	Recovery Mean %	RSD	RSD n = 36
Amidosulfuron	[0.1-0.4]	0.995	0.1	0.3	0.4	79	9	13
Atrazine	[0.1-0.4]	0.994	0.1	0.3	0.4	81	14	24
Carbofuran	[0.1-0.4]	0.977	0.1	0.3	0.4	89	12	15
Chlorotoluron	[5-20]	0.999	5.0	15	20	90	13	18
Diethofencarb	[0.5-2]	0.982	0.5	1.5	2.0	80	11	18
Dimethoate	[0.5-2]	0.999	0.5	1.5	2.0	89	11	13
Fipronil	[2.5-10]	0.979	2.5	7.5	10	72	12	20
Imidacloprid	[0.5-2]	0.999	0.5	1.5	2.0	86	10	14
Isoxaflutole	[0.5-2]	0.961	0.5	1.5	2.0	87	10	12
Linuron	[0.5-2]	0.961	0.5	1.5	2.0	79	10	27
Methiocarb	[2.5-10]	0.994	2.5	7.5	10	78	15	22
Methiocarb sulfoxide	[5-20]	0.999	5.0	15	20	89	11	11
Metosulam	[0.5-2]	0.986	0.5	1.5	2.0	83	16	19
Pirimicarb	[0.1-0.4]	0.994	0.1	0.3	0.4	88	7	21
Rimsulfuron	[0.1-0.4]	0.921	0.1	0.3	0.4	71	12	24
Simazine	[0.5-2]	0.985	0.5	1.5	2.0	82	9	18
2-Hydroxyterbuthylazine	[0.25-1]	0.996	0.25	0.75	1.0	78	9	8

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Table 4: Number of the real honey samples in which pesticides where detected and measured levels (ng g^{-1} honey). Decision limits and detection capacity (CCα and CCβ) are expressed in ng g^{-1} of raw honey. ND = non-detected.

Pesticides	Number of positive samples	Level range (ng g^{-1})		CCα	CCβ
		min	max	(ng g^{-1})	(ng g^{-1})
Rotenone	22	<0.5	> 2	0.057	0.069
Flusilazole	8	<0.05	<0.05	0.093	0.141
Methiocarb sulfoxide	8	0.09	0.31	0.0002	0.0002
Imidacloprid	5	<CCβ	<CCβ	0.069	0.084
Bitertanol	1	0.12	0.12	0.015	0.018
Carbofuran	1	> 0.6	> 0.6	0.253	0.317
Pirimicarb	1	<CCβ	<CCβ	0.053	0.071
Amidosulfuron	0	ND	ND	0.089	0.108
Atrazine	0	ND	ND	0.197	0.275
Chlorotoluron	0	ND	ND	0.003	0.004
Fipronil	0	ND	ND	0.011	0.015
Isoxaflutole	0	ND	ND	0.172	0.204
Linuron	0	ND	ND	0.321	0.462
Methiocarb	0	ND	ND	0.011	0.015
Rimsulfuron	0	ND	ND	0.670	0.930
Simazine	0	ND	ND	0.250	0.326
Diethofencarb	0	ND	ND	0.579	0.751
Dimethoate	0	ND	ND	0.060	0.073
Metosulam	0	ND	ND	0.943	1.232
2-Hydroxytertbutylazine	0	ND	ND	0.0003	0.0003

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Figure 1: Comparison betwween recoveries obtained by OCLLE and classical LLE

Figure 2: Typical reconstructed chromatogram based on specific masses (in bold underlined in Table 1) obtained for a methanolic standard solution using the Polaris C18-A column and the optimized LC conditions.

Figure 3: Collision voltage optimization for Imidacloprid. The retained voltage (15 V) produced the most intense signals for both transitions yielding to masses 175 and 209.

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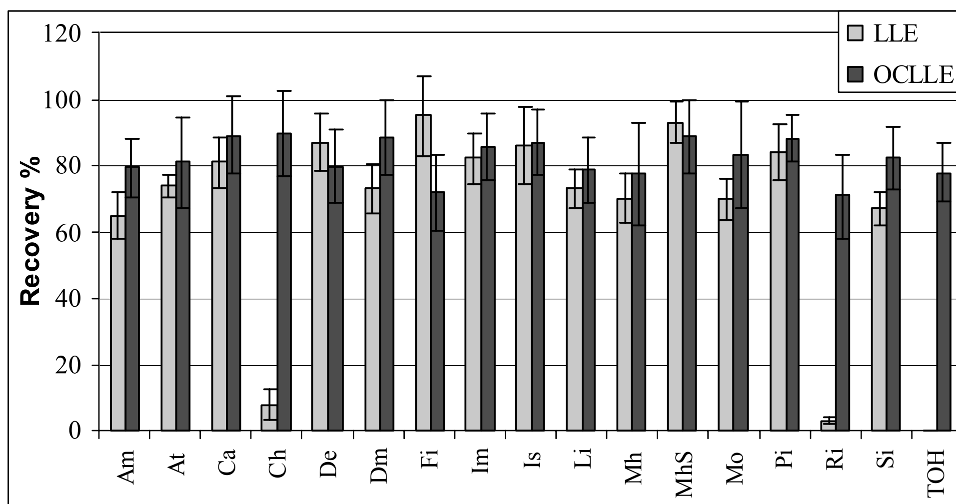

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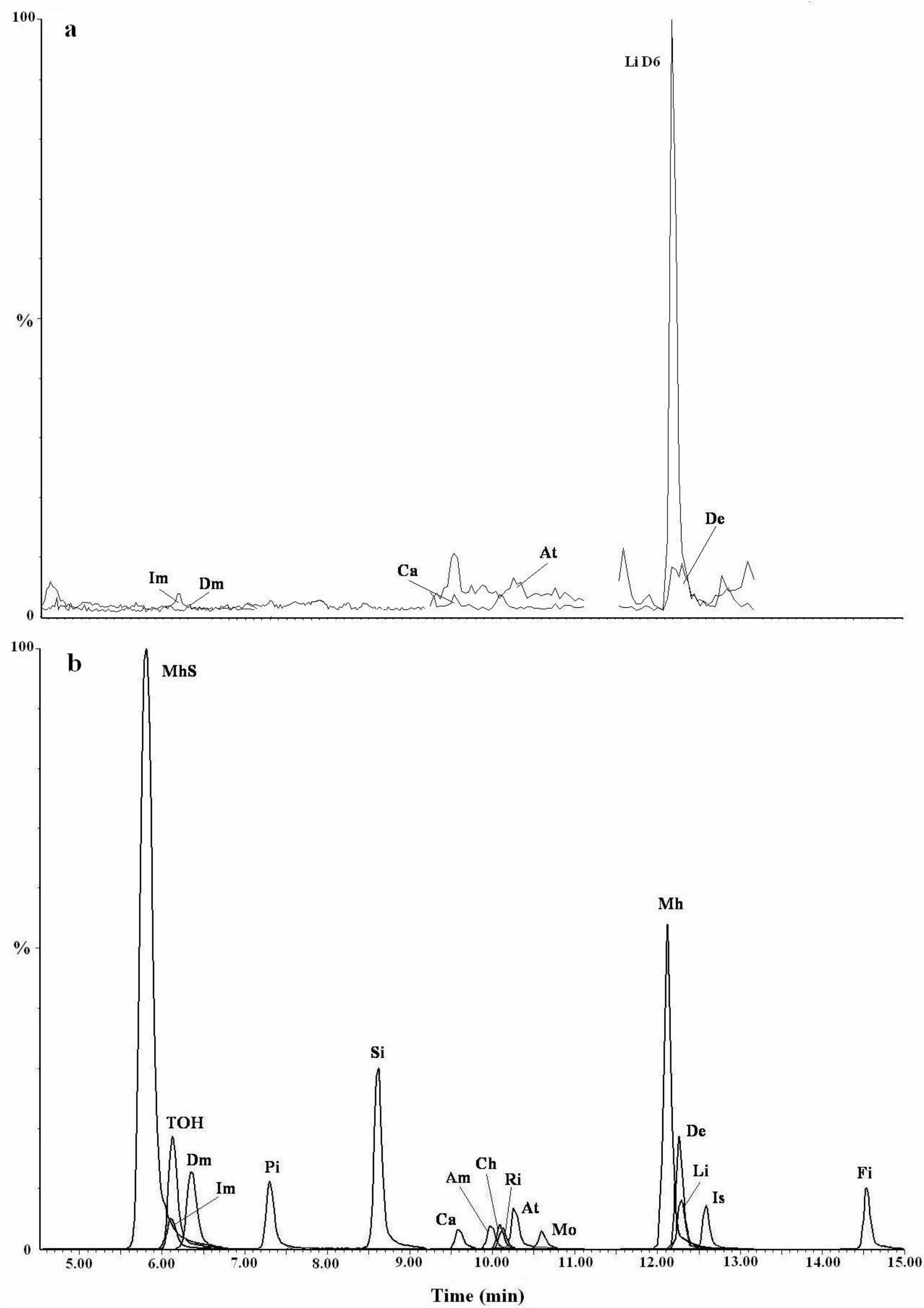

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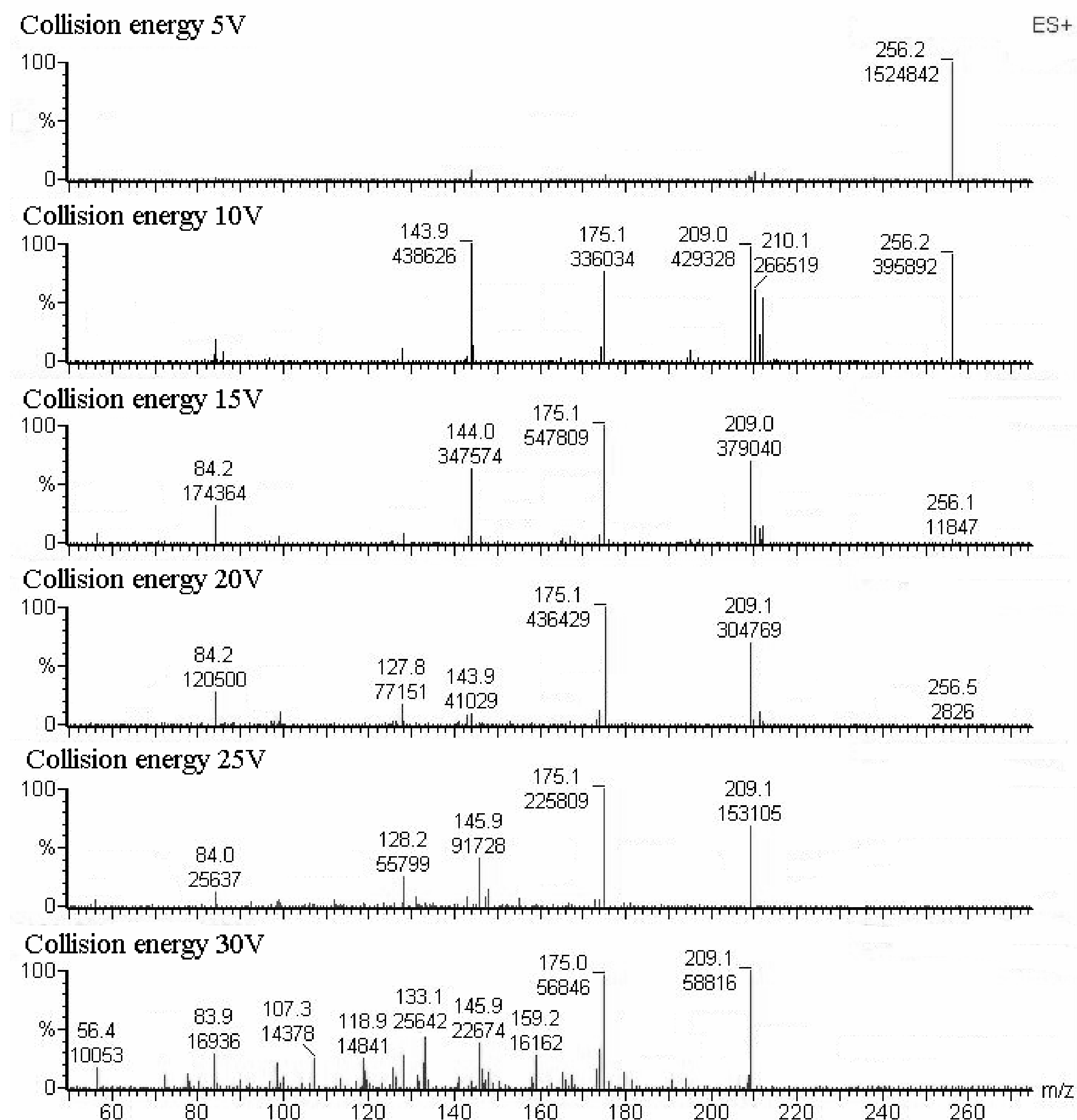

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