DEVELOPMENT AND COMPARISION OF ULTRA HIGH PERFORMANCE-LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY BASED METHODS FOR ANALYSIS OF NEONICOTINOID INSECTICIDES IN HONEY

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20 **ABSTRACT**

21 In this study, the feasibility of two different sample treatments (solid phase extraction
22 and QuEChERS), were investigated for the determination of seven neonicotinoid
23 insecticides (dinotefuran, nitenpyram, thiamethoxam, clothianidin, imidacloprid,
24 acetamiprid and thiacloprid) in honey from different botanical origins (multifloral,
25 rosemary and heather) using ultra-high performance liquid chromatography coupled to
26 tandem mass spectrometry (UHPLC-MS/MS). An efficient sample treatment involving
27 a solid phase extraction with a polymeric sorbent (Strata® X) was proposed for
28 analyzing dark honeys (heather); while a QuEChERS approach is recommend for
29 determining neonicotinoid insecticides in light honeys (multifloral and rosemary). In
30 both cases, the average analyte recoveries were between 80% and 109%.
31 Chromatographic analysis (6 min) was performed on a core-shell technology based
32 column (Kinetex® EVO C_{18}, 50 × 2.1 mm, 1.7 µm, 100 Å). The mobile phase consisted
33 of 0.1% formic acid in water and 0.1% of formic acid in acetonitrile, with a flow rate of
34 0.3 mL/min in gradient elution mode. The proposed methods were fully validated for
35 two different MS/MS detectors (quadrupole-time-of-flight-QTOF; triple quadrupole-
36 QqQ) The results showed that the best overall analytical performance was achieved
37 when using a QqQ detector mainly due to its better sensitivity and the reduced influence
38 of the matrix onto the analyte signals. Finally, the proposed methods were applied to
39 neonicotinoid analysis in commercial honey samples from different regions of Spain
40 and also from experimental apiaries.

41

42 **Keywords:** Honey; Mass spectrometry; Neonicotinoids; QuEChERS; Solid phase
43 extraction; Ultra-high performance liquid chromatography.

44 **1. Introduction**

45 Honey, which is one of the most used products of the hive, is a natural, unprocessed and
46 easily digested food that has been part of the human diet since ancient times [1]. It is a
47 highly valuable natural food product due to its characteristic flavor, nutritional value
48 and therapeutic applications; this has led to a significant increase in its consumption in
49 the last years [2]. However, food alerts caused by the detection of contaminants, like
50 pesticides, have recently affected its healthy image, as it could represent a potential risk
51 for consumers [1,3]. This contamination with pesticides may occur through direct
52 contamination from beekeeping practices as well as indirect contamination from
53 environmental sources [4,5]. Neonicotinoids are the most widely used insecticides in the
54 world due to their broad spectrum of efficacy, their systemic and translaminar action,
55 and their pronounced residual activity and a unique mode of action [6]. However,
56 concerns regarding the side effects on health and the environment of this family of
57 insecticides continue increasing, since they can be transferred to the environment and
58 the food chain, with potential adverse consequences for biodiversity, and for example
59 non-target organisms, such as honeybees. As a consequence of those negative effects
60 associated with the use of neonicotinoid insecticides, International legislations such as
61 the European Union has established stringent maximum residue levels (MRLs) for these
62 substances in honey (10-200 μg/kg; [7]) . Therefore, efficient, selective and sensitive
63 methods are needed for the simultaneous determination of these insecticides in honey.

64

65 In order to achieve an accurate and reliable analytical result, an efficient pre-
66 concentration/separation step is usually required prior to the determination of
67 neonicotinoid residues in honey (see Supplementary Information, Table S1), even when
68 such a sensitive detection method as tandem mass spectrometry (MS/MS) is used. From

69 an analytical point of view, honey can be considered as a highly concentrated sugar
70 solution (mostly fructose). Then, after a dilution with an aqueous solution it can be
71 extracted using protocols similar to those applied to water as solid phase extraction
72 (SPE) [4], as it has been done in several publications [7-10]. The SPE procedure usually
73 provides good results in terms of sensitivity, recovery and matrix effect, although it also
74 requires a significant cost in reagents and equipment, especially due to the SPE
75 sorbents. However, the current trend in sample preparation techniques is focused on the
76 simplification of those procedures to reduce costs, the amount of reagents and time
77 spent on this step, which are some of the principles of the green analytical chemistry
78 [4,11]. Thus, in recent years, the sample preparation known as quick, easy, cheap,
79 effective, rugged and safe (QuEChERS) has been predominately used for the extraction
80 of pesticides in food matrices, and in particular of neonicotinoids from honey [3,8,12-
81 16]. This method is usually based on liquid–liquid partitioning with acetonitrile
82 followed by a clean-up step via dispersive SPE (d-SPE) using primary secondary amine
83 (PSA) [5]. The simple steps involved and the relatively low cost of reagents and
84 equipment mean that it can be applied in most laboratories. Finally, it must be
85 mentioned that liquid-liquid microextraction (LLME), which overcomes some of the
86 problems of conventional liquid-liquid extraction (large volumes of organic solvents,
87 time and steps) [17], has been also successfully employed in several researches [10,18-
88 20]. Thus, in order to propose the most suitable sample treatment to perform this task, it
89 was decided to compare some relevant parameters (extraction efficiency, matrix effect,
90 organic solvent consumption, overall time, cost and number of steps) of two of the most
91 employed sample treatments (conventional-SPE; actual-QuEChERS) for determining
92 neonicotinoid insecticides in honey. It must be also mentioned that although in two of
93 the previous works related to the determination of neonicotinoids in honey [4,8], a

94 tentative comparison was made of the performance of SPE and QuEChERS, as the
95 conditions were not specifically developed, and more importantly, the methodologies
96 were not validated for honey samples different botanical origins, which could have a
97 strong influence of the insecticide determination (matrix effect) due to their different
98 chemical composition.
99
100 Due to their thermolability, low volatility and high polarity, neonicotinoid residues in
101 honey have usually been analyzed by high-performance liquid chromatography (HPLC)
102 in reverse phase mode with C_{18} based analytical columns (see Supplementary
103 Information, Table S1). In most of those studies the coupling with tandem mass
104 spectrometry (MS/MS) [3,4,7,9,10,13-15,16,20] has been predominately used as they
105 offer enough sensitivity and an unambiguous identification and quantification of the
106 insecticides. Although, a diode array detector (DAD), which is a simpler and cheaper
107 alternative to MS/MS especially when analyzing high concentrations, has been also
108 used in some cases [10,12,18,19]. In addition, it must be also stated that ultra-high
109 performance liquid chromatography (UHPLC), which usually provides better
110 resolutions and sensitivities in shorter running times, has been employed in some of
111 these works [3,7,14]. Then, it was decided that separation would be performed by
112 means of a UHPLC equipped with a C_{18} based stationary phase together; meanwhile,
113 the performance (sensitivity, linearity, matrix effect, precision) of two different MS/MS
114 detectors (quadrupole-time-of-flight-QTOF; triple quadrupole-QqQ), which has been
115 employed in previous publications (see Supplementary Information, Table S1), would
116 be also evaluated in order to select the most adequate to determine neonicotinoids in
117 honey.
118

119 Therefore, the aim of this study was to propose a specific analytical methodology to
120 quantify seven neonicotinoid insecticides (dinotefuran-DN, nitenpyram-NT,
121 thiamethoxam-TMX, clothianidin-CLO, imidacloprid-IMI, acetamiprid-ACET, and
122 thiacloprid-THIA; see proposed structures in Supplementary Information, Figure S1), in
123 honey using UHPLC–MS/MS. Although SPE, QuEChERS and UHPLC-MS/MS have
124 been previously used to determine those compounds in honey, we have optimized
125 specific and efficient extraction/determination procedures, and we have also compared
126 their performances in order to select the most adequate methodology. These new
127 conditions seek to ensure good recovery, sensitivity, and selectivity, as well as
128 minimizing the potential matrix effect and fulfilling as far as possible the principles of
129 green analytical chemistry. To the best of our knowledge, this is the first study in which
130 a simultaneous comparison was made of different combinations of sample treatment and
131 detector, which have been specifically developed and optimized in honeys from three
132 different botanical origins (multifloral, rosemary and heather). Further aims of the study
133 involved validating the proposed method for the three different botanical origins in
134 accordance with current European legislation [21], and analyzing samples from
135 different regions of Spain and also from experimental apiaries located close to cultivars
136 in which a TMX treatment had been applied.

137

138 **2. Materials and methods**

139 **2.1. Reagents and materials**

140 Fluka-Pestanal analytical standards of ACET (Det. Purity 99.9%), CLO (Det. Purity
141 99.9%), DN (Det. Purity 98.8%), IMI (Det. Purity 99.9%), NT (Det. Purity 99.8%),
142 THIA (Det. Purity 99.9%), TMX (Det. Purity 99.6%), and TMX-d3 (Det. Purity $\geq$ 98%)
143 were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). An

144 isotope-labeled standard (TMX-d3) was chosen as internal standard (IS), since it has the
145 same physical and chemical properties as the unlabeled analyte. Ethyl acetate, acetone,
146 methanol, ethanol and acetonitrile (HPLC grade) were supplied by Lab Scan Ltd.
147 (Dublin, Ireland). Formic acid (98-100% pure), ammonium acetate, ammonium
148 hydroxide, and magnesium sulfate anhydrous were obtained from Sigma–Aldrich
149 Chemie Gbmh (Steinheim, Germany). Sodium chloride, sodium acetate, trisodium
150 citrate dihydrate, and disodium hydrogen citrate sesquihydrate were supplied by
151 Panreac (Barcelona, Spain), while primary secondary amine (PSA) and C_{18} were
152 provided by Supelco (Bellefonte, PA, USA). Meanwhile, Strata® X (3 mL with 600 mg
153 of sorbent) SPE cartridges (Phenomenex, Torrance, CA, USA), and a 10-port Visiprep
154 vacuum manifold (Supelco, Bellefonte, PA, USA), were used in the SPE procedure. A
155 vibromatic mechanical shaker, a thermostated ultrasound system, and a drying oven,
156 both supplied by J.P. Selecta S.A. (Barcelona, Spain), a vortex mechanical mixer from
157 Heidolph (Schwabach, Germany), a 5810 R refrigerated bench-top Eppendorf
158 centrifuge (Hamburg, Germany), and an R-210/215 rotary evaporator from Buchi
159 (Flawil, Switzerland) were employed for all extractions. Nylon syringe filters (17 mm,
160 0.45 μm) were from Nalgene (Rochester, NY, USA), and ultrapure water was obtained
161 using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA, USA).
162
163 **2.2. Standards**
164 Standard stock solutions (~1000 mg/L) were prepared by dissolving approximately 10
165 mg of each neonicotinoid insecticide, accurately weighed, in 10 mL of methanol. These
166 solutions were further diluted with a water and methanol mixture (80:20, v/v) in order to
167 prepare the working solutions. Honey samples (5.0 g) were spiked before (BF samples)
168 or after (AF samples) sample treatment with different amounts of the neonicotinoid

169 insecticides and with 50 µg/kg of the IS to prepare the matrix-matched standards; this is
170 described in sub-section 2.3. The samples were employed for validation (quality control
171 (QC) samples and calibration curves), matrix effect, and treatment studies. Each QC
172 sample was prepared with 5.0 g of honey spiked with three concentrations of
173 neonicotinoids within the corresponding linear range for each MS/MS (QTOF and
174 QqQ). These were as follows: low QC- LOQ; medium QC- 10 µg/kg for QqQ and 50
175 µg/kg for QTOF; high QC- 50 µg/kg for QqQ and 300 µg/kg for QTOF. The stock
176 solution was stored in glass containers in darkness at -20ºC; working and matrix-
177 matched solutions were stored in glass containers and kept in the dark at +4ºC. All
178 solutions were stable for over two weeks (data not shown).
179

180 **2.3. Sample procurement and treatment**

181 **2.3.1. Samples**

182 Several honey types were selected according to their different color, composition and
183 botanical origin. Samples from different regions of Spain, in which a neonicotinoid
184 treatment has been employed in some crops, were kindly donated by the "Centro Apícola
185 Regional-CAR" at Marchamalo (Guadalajara, Spain). Their botanical origin, which was
186 confirmed by melissopalynological analysis, were: rosemary, *Rosmarinus officinalis* (n =
187 6); multifloral (n = 6); and heather, *Erica spp* (n = 6). In addition, multifloral honey
188 samples (n = 10) that were collected from controlled apiaries located close to experimental
189 crops, which had been previously treated with TMX dressed rapeseeds (1 L per 100 kg of
190 Cruiser 350 FS (Syngenta, Madrid, Spain) containing TMX-35%, w/v, were also supplied
191 by the CAR. In this study, all honey samples were examined in triplicate, and also
192 underwent a preliminary analysis by HPLC-MS/MS in order to check for the presence of
193 neonicotinoids. Once absence was confirmed is several samples, subsamples of the

194 corresponding honeys were used to prepare matrix-matched standards for validation and
195 sample treatment studies. The blank honey samples were stored in a fresh (4ºC) and dark
196 place before analysis.

197 **2.3.2. Sample treatment**

198 **2.3.2.1. QuEChERS protocol**

199 Briefly, 5.0 g of honey was weighed in a 50 mL centrifuge tube, diluted with 10 mL of
200 water, after which 10 mL of an acetonitrile and ethyl acetate (70:30, v/v) mixture were
201 added. The tube was then shaken for 30 s in a vortex device to dissolve until a homogenous
202 solution was obtained. Next, 2.0 g of $MgSO_4$, 0.5 g of sodium acetate, 1.5 g of trisodium
203 citrate dihydrate and 0.5 g of trisodium citrate sesquihydrate were added and the samples
204 were placed in an ultrasound device for 5 minutes at 30ºC. The mixture was then
205 centrifuged (5000 r.p.m, 5ºC) for 3 min. The supernatant was taken and evaporated to
206 dryness in a rotary evaporator (60ºC). The dry extract was reconstituted with 1 mL of a
207 methanol and water (80:20, v/v) mixture, and the resulting solution was passed through a
208 nylon filter (0.45 µm). After which, a 5 µL aliquot was injected into the UHPLC-MS/MS
209 system. Figure 1 outlines the QuEChERS procedure used during the present study.

210 **2.3.2.2. SPE protocol**

211 Briefly, 5.0 g of homogenized honey sample was diluted in 10 mL of ammonium formate
212 (10 mM) in water and the resulting solution was loaded onto a Strata® X cartridge
213 previously conditioned with 5 mL of methanol and 5 mL of water at about 1 mL/min by
214 means of a suction system. After 5 min of drying time, the analytes were eluted with 4 mL
215 of an acetonitrile and ethyl acetate (80:20, v/v) mixture. The resulting solution was
216 evaporated to dryness at 60ºC in a rotary evaporator; the dry residue was reconstituted with
217 1 mL of a methanol and water (80:20, v/v) mixture, filtered through a nylon 0.45-mm

218 filter, and injected (5 µL) into the UHPLC–MS/MS system. Figure 1 outlines the steps of
219 the SPE procedure used during the present study.
220

221 **2.4. UHPLC-MS/MS system**

222 **2.4.1. UHPLC conditions**

223 The chromatographic system consisted of an Acquity™ UHPLC system (Waters, Milford,
224 MA, USA) equipped with an online vacuum degasser, a binary solvent pump, an
225 autosampler, a thermostated column compartment. A Kinetex® EVO fused-core type
226 column (C_{18}, 50 × 2.1 mm, 1.7 µm, 100 Å) was employed for UHPLC analysis, and this
227 was protected by a Kinetex® EVO C_{18} guard column. Both were acquired from
228 Phenomenex (Torrance, CA, USA). After optimization studies, the mobile phase
229 composition and the flow rate, the injection volume and the column temperature were
230 selected; mobile phase was composed of 0.1% (v/v) formic acid in acetonitrile (solvent A)
231 and 0.1% (v/v) formic acid in water (solvent B) applied at a flow rate of 0.3 mL/min in the
232 following gradient mode: (**i**) 0.0-1.0 min (A–B, 10:90, v/v); (**ii**) 1.0-1.5 min (A–B, 60:40,
233 v/v); (**iii**) 1.5-2.5 min (A–B, 90:10, v/v); (i**v**) 2.5–3.5 min (A–B, 90:10, v/v); (**v**) 3.5–4.0
234 min (A–B, 60:40, v/v); (**vi**) 4.0–4.5 min (A–B, 10:90, v/v); (**vii**) 4.5–6.0 min (A–B, 10:90,
235 v/v). Injection volume and column temperature were set at 5 µL and 30ºC, respectively.

236 **2.4.2. QTOF conditions**

237 A QTOF mass spectrometer (maXis impact, Bruker Daltonik GmbH, Bremen, Germany)
238 were coupled through and electrospray (ESI) interface, which was operated in the positive
239 mode ionization mode, to the UHPLC system. Data was acquired and processed with
240 software Data Analysis 4.1 and Qualitative Analysis from Bruker Daltonik GmbH. The
241 optimal conditions were set as follows after several experiments (flow injection analysis in
242 infusion mode, 80 µL/min) were conducted: capillary voltage, 3500 V; drying gas

243 (nitrogen) flow, 12 L/min; drying gas (nitrogen) temperature, 220ºC; nebulizer pressure, 2
244 bar. Spectra were acquired in a mass range of mass/charge (*m/z*) 50–400. The *m/z* scale of
245 the mass spectra was calibrated daily by infusing a 0.01 mol/L sodium formate solution.
246 Compounds showed an intense $[M+H]^+$ (precursor ions) on their full-scan spectra, which
247 was selected as a precursor to obtain product ions for MS/MS analyses, which were carried
248 out by using an isolation width of 10 *m/z* and variable collision energies (10–30 eV; see
249 Table 1). A window of ±0.01 *m/z* for the extracted ion chromatograms (EIC) was used in
250 order to extract the exact mass.

251 **2.4.3. QqQ conditions**

252 A Xevo TQ-S-MS/MS (QqQ) mass spectrometer (Waters) equipped with an orthogonal Z-
253 spray ESI, which was operated in positive mode, coupled to the UHPLC. All data were
254 acquired and processed using Mass Lynx v 4.1software (Waters). Cone gas as well as
255 desolvation gas was nitrogen (Praxair, Valencia, Spain) setup 250 L/h and 1200L/h,
256 respectively. For operation in the MS/MS mode, collision gas was argon 99.995% (Praxair,
257 Madrid, Spain) with a pressure of 4×10^{-3} mbar in the collision cell (0.15 mL/ min). Other
258 parameters optimized were capillary voltages 3.5kV; source temperature 150ºC and
259 desolvation temperature 650ºC. Acquisition was performed in mode multiple monitoring
260 (MRM) mode, with the protonated molecular ion ($[M+H]^+$) of each compound chosen as
261 precursor ion. The most abundant product ion of each target neonicotinoid was used for
262 quantification and an additional product ion was used for confirmation. More specific
263 MS/MS parameters (MRM transitions, cone voltages and collision energies) are
264 summarized in Table 2. Finally, it must be commented that dwell times were automatically
265 selected in order to obtain enough points per peak and can be decreased down to 3 ms.

266

267 **2.5. Quality assurance/quality control (QA/QC)**

268 In order to compare the proposed method with other existing procedures (see
269 Supplementary Information, Table S1), validation was in line with the current European
270 legislation [21] as well as with recent studies [1,4]. Moreover, several of the main elements
271 of uncertainty [22] were taken into account when optimizing and validating this method,
272 such as the amount of sample used, the recovery value of the analytical procedure and
273 precision (RSD repeatability). The validation was performed with standard and matrix-
274 matched solutions, which were treated with the selected procedures for each botanical
275 origin (multifloral and rosemary-QuEChERS; heather-SPE), and for both detectors (QTOF
276 and QqQ) with the exception of the trueness (recoveries) that was only evaluated for
277 QTOF, as it was mentioned in subsection 3.1.2.

278 **2.5.1. Selectivity**

279 To determine the selectivity of the proposed method, a set of unspiked blank honey
280 samples (n=6) from the three different botanical origins was injected onto the
281 chromatographic system and the results were compared with those obtained for spiked
282 blank honey samples.

283 **2.5.2. Limits of detection and quantification**

284 The limits of detection (LODs) and quantification (LOQs) were experimentally
285 determined by injection of a number of blank honey samples (n=6), in which the
286 absence of insecticide residues was previously confirmed, and measurement of the
287 magnitude of background analytical response at the elution time of in each honey
288 sample for the different botanical origins investigated. The LODs and LOQs were
289 estimated to be three and ten times the signal-to-noise (S/N) ratio, respectively.

290 **2.5.3. Matrix effect**

291 To check how the matrix influenced ESI ionization, a comparison was made of the
292 results (analyte peak area/IS area) with standard working solutions and blank honey

293 samples of the different botanical origins spiked at three different concentrations (QC
294 levels) following sample treatment (AF samples).

295 **2.5.4. Linearity studies**

296 Matrix-matched standard calibration curves were used when using a QTOF detector to
297 quantify four (DN, NT, IMI and CLO) and one (ACET) neonicotinoid insecticides in
298 heather and multifloral honeys, respectively; while, only two insecticides (DN and NT)
299 in heather honeys must be quantified with matrix-matched standard calibration curves
300 when employing a QqQ detector. In contrast, neonicotinoid insecticides can be
301 quantified with standard calibration curves in all other cases: **i**) honey samples from
302 rosemary botanical origins for both MS/MS detectors; **ii**) honey samples from
303 multifloral botanical origins for QqQ; **iii**) all the analytes except ACET in honey
304 samples from multifloral botanical origins for QTOF; **iv**) three (TMX, THIA and
305 ACET; QTOF) and five (IMI, CLO, TMX, THIA and ACET; QqQ) compounds in
306 honey samples from heather botanical origins. Blank honey was treated accordingly
307 with the proposed procedure and spiked with variable amounts of the seven
308 neonicotinoids over an analytical range between LOQ and 300 µg/kg (calibration levels
309 of LOQ, 10, 25, 50, 100, 200, 300 µg/kg) and LOQ and 50 µg/kg (calibration levels of
310 LOQ, 1, 2, 5, 10, 20, 50 µg/kg) for matrix matched calibration curves using QTOF and
311 QqQ, respectively. The analytical ranges prepared for the standard calibration curves
312 were between LOQ and 1500 µg/L (calibration levels of LOQ, 50, 125, 250, 500, 1000,
313 1500 µg/L) for QTOF and LOQ and 250 µg/L (calibration levels of LOQ, 5, 10, 25, 50,
314 100, 250 µg/L) for QqQ. Neonicotinoid concentrations were the same in the standard
315 (µg/L) and matrix matched (µg/kg) solutions, in line with the proposed sample
316 treatment and unit conversion. Calibration curves (n = 6) were constructed by plotting

317 the signal on the *y*-axis (analyte peak area/IS area) against the analyte concentration on
318 the *x*-axis.

319 **2.5.5. Precision**

320 Intra-day precision experiments were performed concurrently by repeated sample
321 analysis using blank honey samples from the three different botanical origins spiked at
322 three different concentrations (low, medium and high QC levels) on the same day of
323 (n=6) (intra-day precision experiments), or over three consecutive days (n=6) (inter-day
324 precision).

325 **2.5.6. Trueness**

326 It was evaluated with the mean recoveries (as a measure of trueness), which were
327 calculated by comparing the results (analyte peak area/IS area) obtained from blank
328 honey samples from the different botanical origins spiked at three different
329 concentrations (low, medium and high QC levels), either prior to (BF samples) or
330 following (AF samples) sample treatment.

331

332 **3. Results and discussion**

333 **3.1. Optimization of sample treatment**

334 As it was previously mentioned in the Introduction, two different sample treatments
335 (QuEChERS and SPE) would be developed, optimized and compared in order to select
336 the most adequate to perform the determination of neonicotinoids in honeys from three
337 different botanical origins. The analysis of the different extracts obtained with both
338 sample treatments was conducted with the UHPLC-MS/MS equipped with a QTOF
339 detector.

340 **3.1.1. Optimization of the QuEChERS procedure**

341 Firstly, consideration was given to the amount of honey (1- 10 g) to be analyzed and the
342 most suitable volume of water (5-20 mL), which was chosen according to scientific
343 literature (see Supplementary Information, Table S1), to dissolve the honey. After
344 several tests (data not shown), 5.0 g of honey and 10 mL of water were selected as the
345 optimal amounts to be used. Recoveries were adequate with those values, and good
346 signal to noise (S/N) ratios were achieved in order to obtain the lowest possible limits of
347 detection (LODs) and quantification (LOQs). For the extraction step, some assays were
348 conducted with diverse volumes (5-15) of different solvent mixtures of acetonitrile with
349 water and ethyl acetate (100:0, 80:20, 50:50, v/v) which were chosen according to
350 preliminary experiments and the existing literature (see Supplementary Information,
351 Table S1). The best results in terms of recoveries were obtained with 10 mL of an
352 acetonitrile and ethyl acetate (80:20, v/v) mixture (data not shown). Afterwards, it was
353 optimized the amount of salts that should be employed in the partitioning step of the
354 QuEChERS procedure. Magnesium sulfate (2.0 g) was used in order to ensure the
355 dryness of the sample, leading to phase separation and extraction of the compounds by
356 the selected acetonitrile and ethyl acetate mixture [3]. Sodium acetate (1.0 g) served to
357 reduce the polar co-extractives, improved the insecticide stability, and increased the
358 extraction efficiency [3, 23]. Finally, trisodium citrate dihydrate (1.5 g), and disodium
359 hydrogen citrate sesquihydrate (0.5) were employed to buffer the liquid-liquid
360 extraction and provide an adequate media for the further extraction [23,24]. Once the
361 solvents and the salts were selected, the influence of certain extraction parameters, such
362 as the agitation source (vibromatic, vortex and ultrasound), extraction time (1-15 min),
363 and centrifugation time (1-10 min), was sequentially tested in order to obtain optimal
364 conditions. Optimal extraction (recovery percentages > 80%; see Figure 2) was
365 achieved with 5 min of agitation in the ultrasound, and 3 min of centrifuging at 5000

366 r.p.m and 5ºC. It must be also specified that different temperatures were tested when
367 performing the ultrasound agitation (20ºC-40ºC), and the highest recoveries were
368 obtained for 30ºC (data not shown). Afterwards, it was studied if a further clean-up step
369 would be necessary in order to reduce as much as possible the extraction of matrix-
370 components that could affect to analyte ionization, but without affecting the extraction
371 efficiency. Thus, the supernatant was collected and transferred to a centrifuge tube, in
372 which PSA (70 mg), C_{18} (70 mg), and a mixture of them (35 mg of each) were added in
373 different experiments with the aim of removing sugars and fatty acids (PSA) and non-
374 polar compounds. It was observed (see Supplementary Information, Figure S2) that the
375 clean-up step did not significantly reduce the matrix effect (see Supplementary
376 Information, Figure S2), which mainly affected the ionization of the insecticides in
377 heather honeys, but it had a marked negative effect onto the recovery percentages,
378 especially for DN and NT in heather honeys (< 60%) (see Supplementary Information,
379 Figure S3). Thus, it was decided that it was not required to perform a clean-up step, as it
380 would not have positive effects onto the neonicotinoid determination. Then, the
381 supernatant was directly transferred to a conical flask and gently evaporated to dryness
382 in a rotary evaporator at 60ºC. Different volumes (0.5-2.0 mL) of a methanol and water
383 (80:20,v/v) mixture, which were selected due to the good results obtained in previous
384 researches [7,23] were assayed in order to obtain the best results. Since it was observed
385 that amounts of solvent over 1 mL did not improve the recovery percentages, it was
386 decided that 1 mL of the mixture should be employed to reconstitute the dry residue.

387 **3.2. Optimization of the SPE procedure**

388 Firstly, the type of cartridge that would be used to perform the SPE procedure was
389 determined. As a result of the physicochemical properties of the neonicotinoids, our
390 research experience in honey [7] and previous works [9], we decided to check the

391 suitability of polymeric (Strata® X) SPE sorbents to extract the insecticides. Next, the
392 amount of honey (1-10 g), solvent (water; ammonium formate (10 mM) in water;
393 ammonium hydroxide 1% (v/v) in water; formic acid 1% (v/v) in water), and solvent
394 volume (5-15 mL) to dissolve the honey were selected. It must be specified that those
395 solvents were chosen based on preliminary experiments. After several tests, 5.0 g of honey
396 and 10 mL of ammonium formate (10 mM) in water were deemed the optimal values, as in
397 this way the highest S/N ratio for securing maximum sensitivity was obtained (data not
398 shown). Prior to loading the diluted sample onto the SPE cartridges, some parameters were
399 evaluated to optimize the extraction procedure. Firstly, different volumes of methanol and
400 water were tested in order to precondition the cartridge; 5 mL of both applied sequentially
401 was the most suitable. However, as honey contains sugars and substances such as pigments
402 and phenolic compounds, direct elution of the cartridges usually resulted in matrix
403 interference and unclean chromatograms. Consequently, it was normally required a
404 washing stage to avoid those problem. Several water and methanol mixtures (100:0, 90:10,
405 80:20, 70:30, 50:50, v/v) and volumes (5-15 mL) were tested for this purpose, as they have
406 provided good results in previous works [7,9]. It was found that in all cases two of the
407 neonicotinoids (DN and NT) were lost when performing the washing steps, and at the same
408 time, it was not observed a significant improvement in the matrix effect or in the removal
409 of the interferences (data not shown). Thus it was decided to eliminate the washing steps
410 from the SPE procedure. Optimal drying times for the cartridges were also determined and,
411 as no differences were observed between times of 5-20 min (data not shown), a 5-minute
412 drying period was chosen to avoid delays in the extraction procedure. Different mixtures of
413 acetonitrile with water or ethyl acetate (100:0, 80:20, 50:50, 20:80, 0:100; v/v) to elute
414 neonicotinoids from the cartridges were tested, as some of those mixtures have been
415 previously employed [7,9]. The best results in terms of extraction efficiency were obtained

416 when an acetonitrile and ethyl acetate (80:20,v/v) mixture was employed (data not shown).
417 Following testing of the elution volumes (ranging from 1.0-5.0 mL), it was also found that
418 4 mL of the selected mixture was appropriate for procuring satisfactory recoveries (>
419 75%). The solution obtained was transferred to a conical flask and gently evaporated to
420 dryness in a rotary evaporator at 60ºC. According to results obtained when optimizing the
421 QuEChERS procedure, 1 ml of a methanol and water (80:20,v/v) mixture was employed to
422 reconstitute the dry residue.

423 **3.3. Comparison of the proposed sample treatments**

424 In order to check the effectiveness of the proposed sample treatments, neonicotinoid
425 responses were compared as described in subsection 2.5.6: these were the peak areas
426 (analyte peak area/IS area) obtained from blank samples spiked at three different
427 neonicotinoid concentrations (QC levels), either prior to (BF samples) or following (AF
428 samples) sample treatment. Recovery values ranged from 80-108% when employing the
429 QuEChERS approach, while those values were quite slightly lower, except in some
430 cases for NT, ACET and THIA, when using SPE (see Figure 2). In relation to the
431 evaluation of the matrix effect, which was calculated as stated in subsection 2.5.3, no
432 significant differences were observed when comparing the responses for light honeys
433 (see Figure 3), with the exception of ACET in multifloral honeys. On the other hand, a
434 significant matrix effect (ion suppression) was observed for all the analytes in dark
435 honeys when using the QuEChERS approach; while, a lower signal suppression was
436 also observed for four of the neonicotinoids in the SPE treated samples, and in this case
437 three of the insecticides (TMX, ACET and THIA) were not affected by this effect (see
438 Figure 3). Thus, it can be concluded that the QuEChERS approach should be
439 employed when analyzing light honeys, as the results were comparable to those
440 obtained with SPE, but in rapid and cheaper way; whilst, the SPE procedure is the best

441 option when analyzing dark honeys. These results have demonstrated that the proposed
442 procedures are an efficient and green alternative to the existing procedures for analyzing
443 these insecticides in honeys. The recovery values are comparable with or better than the
444 reported values, and similar sample treatment times or volume/amount of reagents,
445 especially organic solvents, were required in previous studies (see Supplementary
446 Information, Table S1), but with the advantage that the matrix effect has been
447 minimized in such a way for multifloral and rosemary honey botanical origins, that
448 standard calibration curves could be used to quantify the neonicotinoid insectides. This
449 is particular relevant, if it is taken into account that matrix effect was not minimized in
450 most of previous publications, and it was necessary a much longer extraction time when
451 it was achieved (see Supplementary Information, Table S1).

452

453 **3.2. UHPLC-MS/MS optimization**

454 **3.2.1. UHPLC**

455 We recently published a paper concerning the analysis of the seven neonicotinoids in bee
456 pollen [23], we optimized the chromatographic conditions, selecting as mobile phase
457 components (0.1% (v/v) formic acid in ACN and 0.1% (v/v) formic acid in water and a
458 Kinetex® EVO (C_{18}, 50 x 2.1 mm, 1.7 µm, 100 Å) column due to the their good
459 performance. This core-shell column not only allows highly efficient separations with
460 relatively low back pressure, as happen with this type of columns, but also provides the
461 additional benefit of better peak shape for bases, wide pH 1 to 12 stability, and the
462 potential signal suppression caused by the presence of polar (basic compounds) is
463 decreased, as those compounds are more retained in those columns. We therefore decided
464 to optimize the separation with the Kinetex® EVO column and the same mobile phase
465 components. Several experiments were conducted in which standard and matrix matched

466 solutions were injected with diverse mobile phases and flow rates so as to elute
467 neonicotinoids rapidly whilst preventing co-elution. Tests were also carried out to study
468 the influence of the column temperature (between 20 and 50°C) and the injection volume
469 (between 2 and 10 μL) on the S/N ratio. Results showed an increase in the S/N when up to
470 5 μL was injected, and a loss of symmetry was observed at temperatures over 30ºC (data
471 not shown); consequently, 5 μL and 30ºC were selected as optimal values. The shortest
472 analysis time was obtained with the chromatographic conditions described in subsection
473 2.4.1. With such conditions the overall run time was 6.0 min, which, to our knowledge, is
474 the fastest proposal that has been published in relation to neonicotinoid analysis in honey
475 (see Supporting Information, Table 1S), eluting the last of the insecticides in less than 3.5
476 min with a gradient mobile phase composition at a flow rate of 0.3 mL/min (see Figures 4
477 and 5). It might be considered, after checking the MS/MS chromatograms of unspiked and
478 spiked (see Figures 4 and 5) blank honey samples, that the analysis times could be reduced
479 since no matrix peaks were observed at the shortest times. However, it should be
480 mentioned that although a baseline separation is not necessary for accurate quantification
481 when samples are analyzed by MS/MS (see Supplementary Information, Table S1), it is
482 recommendable to minimize as far as possible the potential effects on the analyte signal
483 (suppression or enhancement) caused by co-elution with matrix components [25]. In
484 addition, it is also interesting to mention that the proposed UHPLC method could be used
485 with more economical detectors (DAD or MS detectors) as the seven neonicotinoids were
486 baseline separated.

487 **3.2.2. MS/MS**

488 **3.2.2.1. QTOF**

489 Regarding optimization of the QTOF conditions, ESI in positive mode was chosen to
490 conduct the experiments as a result of our previous experience [7,23] and the existing

491 literature relating to HPLC-MS/MS analysis of neonicotinoids in honey (see
492 Supplementary Information, Table S1). To establish the optimal MS/MS conditions,
493 several experiments (flow injection analysis) were conducted in order to choose the
494 optimum parameters (see subsection 2.4.2 and Table 1) and achieve the maximum
495 sensitivity by the infusion mode of standard (250 µg/L) and matrix matched solutions (50
496 µg/kg). Neonicotinoids showed an intense $[M+H]^+$ (precursor ions) on their full-scan
497 spectra, which were were selected as a precursor ions to obtain product ions for MS/MS
498 analyses (see Table 1), and also as confirmation ions. The product ions with the highest
499 signals were used for quantification; meanwhile, the second products ions with the higher
500 signals were used for confirmation (see Table 1).

501 **3.2.2.2. QqQ**

502 The protonated molecules ($[M+H]^+$) were chosen as precursor ions (see Table 2). The two
503 most sensitive MRM transitions (in terms of signal-to-noise ratio) were selected for each
504 compound (see Table 2). The most abundant was used for quantification whereas the other
505 transitions were acquired for confirmation. Using this fast-acquisition QqQ mass analyzer,
506 dwell times as low as 3 ms per transition could be automatically set up allowing
507 satisfactory peak shape (at least 10 points-per peak) and sensitivity for the seven
508 compounds investigated.

509

510 **3.3. QA/QC**

511 **3.3.1. Selectivity**

512 No chromatographic interference was observed at analytes retention times in any of the
513 blank samples analysed of the three botanical origins for both detectors (see Figures 4 and
514 5). For the identification of neonicotinoid peaks in spiked samples, their mass spectra in
515 standard solutions and spiked samples were compared; the concentrations were similar and

516 the same conditions were employed for measurement. There was a considerable similarity
517 between both mass spectra. However, slight differences in the intensity of several ion were
518 observed and certain low intensity ions appeared in a few cases (data not shown).
519 Moreover, the relative intensities of the selected product ions/transitions in the matrix-
520 matched samples concurred with the corresponding standard solutions to within ± 10%
521 (data not shown); this is lower than the maximum rates permitted (± 30%; [21]). Therefore,
522 it can be concluded that the methods were selective for determing neonicotinoids in honeys
523 from different botanical origin.

524 **3.3.2. LODs and LOQs**

525 LODs and LOQs were determined experimentally in each botanical origin, as indicated in
526 Section (Tables 3-5). Low LODs and LOQs were obtained in all cases for both MS/MS
527 detectors, ranging the LODs values from 0.1 to 2.0 µg/kg (QTOF) or 0.01-0.20 µg/kg
528 (QqQ), and the LOQs from 0.30 to 6.70 µg/kg (QTOF) or 0.03-0.70 µg/kg (QqQ). As can
529 be seen, those values were ten times lower when using the QqQ, which is good agreement
530 with the existing literature (see Supplementary Information, Table S1). Moreover, the
531 LOQs we obtained with the QqQ detector are also lower than those in most of the previous
532 publications for different food matrices (see Supplementary Data, Table 1S), with only one
533 exceptions in which the values were quite similar, but in this work the sample treatment
534 was longer (SPE-DLLME), employed larger amount of solvents, and there was a
535 significant matrix-effect when performing the MS/MS detection [10]; while, the LOQs
536 obtained with the QTOF detectors are also comparable with most of the published data (see
537 Supplementary Information, Table S1). However, the sensitivity achieved with of both
538 MS/MS detectors is more than enough to fulfil the criteria of the European Commission in
539 relation to the maximum residue limits (MRL) established for some of this pesticides (not
540 for NT and DN) in honey and other apiculture products (10-200 µg/kg; [6]), that are much

541 higher than the LOQs obtained with our proposals. Thus, the excellent sensitivity achieved
542 with the proposed methods has been demonstrated.

543 **3.3.3. Matrix effect**

544 To ascertain how the matrix influenced ESI ionization for both detectors, a comparison
545 was made of the response obtained for each neonicotinoid insecticide as described in
546 subsection 2.5.3. Responses at the different concentrations (QC levels) assayed ranged
547 from 81% to 108% for multifloral and rosemary honey samples, with the exception of
548 ACET for QTOF that presented a much lower response (< 80%)(see Tables 6 and 7). As
549 can be observed, the values were slightly better in most cases when using a QqQ, but in
550 any case, there were not generally observed great differences in those values depending of
551 the MS/MS analyzer. On the other hand, significant differences between both detectors
552 were observed in the neonicotinoid responses when analyzing heather honey samples, as
553 four of the insecticides (DN, NT, CLO and IMI) presented responses lower than 70% in all
554 cases, while for QqQ detection only two of the analytes were significantly affected for the
555 matrix effect (DN and NT). In addition, it must be also commented that the responses of
556 the compounds were generally lower in honeys from heather botanical origin. To confirm
557 these findings the slopes of the standard and matrix-matched calibration curves were
558 contrasted (see Tables 3-5), and it was found that for multifloral and rosemary honeys
559 overlapping occurred at the confidence intervals, with the exception of ACET in
560 multifloral honeys for QTOF detection, but this was not the case for some of the
561 neonicotinoids (four-QTOF; two-QqQ) in heather honey (see Table 2). Therefore, it was
562 concluded that the matrix did not significantly affect ESI ionization of the analytes in
563 multifloral (with the exception of ACET for QTOF) and rosemary honey samples for both
564 detectors, results which complied with the criteria of the European Commission for
565 pesticide residue analysis (± 20% of the response from standard solutions; [21]).

566 Meanwhile, a significant matrix effect (signal suppression) was observed for some of the
567 compounds in heather honey samples. This is an important result not only because a
568 significant matrix effect have been reported in most of the existing literature dedicated to
569 analyze those compounds in honey (see Supporting Information, Table S1), but due to the
570 demonstration that there is a need of evaluating the matrix effect for different honey
571 botanical origins in order to avoid potential quantification errors.

572 **3.3.4. Linearity studies**

573 As mentioned in subsection 2.5.4, different calibration curves were used to quantify
574 neonicotinoid insecticides in accordance with the botanical origin of the honey and the
575 influence of the matrix effect onto the analyte ionization. Consequently, standard
576 calibration curves could be employed independently of the MS/MS detector when
577 determining neonicotinoids in multifloral and rosemary honey samples, with the exception
578 of the QTOF detection of ACET in multifloral honeys, as no significant matrix effect was
579 observed (see subsection 3.3.3). Meanwhile, standard or matrix-matched calibrations
580 curves should be employed in heather honeys depending on the neonicotinoids, and ACET
581 must be quantified with matrix-matched calibration curves when using a QTOF detector.
582 The graphs obtained in all the calibration curves were straight lines, with linearity across
583 the different concentration ranges studied, while the coefficient of the determination values
584 (R^2) was above 0.99 in all cases (see Tables 3-5). It must be also commented that the
585 linearity ranges were different according to the MS/MS detector and their corresponding
586 LOQ values for each insecticide. This is a relevant finding, as a dilution of the sample
587 would be necessary prior to their UHPLC-QqQ analysis for concentrations higher that 50
588 μg/kg in order to provide a correct quantification; while it would not required the dilution
589 until a highest concentration value (300 μg/kg) for QTOF detection. On the other hand,
590 QqQ is the best choice for determining the insecticides at the lowest concentrations.

591 **3.3.5. Precision**

592 Precision expressed as the percentage of relative standard deviation (%RSD), which was at
593 all times (intra and inter-day experiments) lower than 10% (see Supplementary
594 Information, Tables S2-S4). Moreover, there were not observed significant differences of
595 those values depending on the MS/MS detector. Those results indicate that the proposed
596 methods are precise according to existing normative (%RSD ≤ 20; [21]).

597 **3.3.6. Trueness**

598 Mean recoveries (as a measure of trueness), which were calculated as described in
599 subsections 2.5.6, ranged from 80 to 109% with %RSD values lower than 8% in all
600 cases (see Table 8). Those values, which are similar or better than the obtained in
601 previous works (see subsection 3.1.3), fulfilled the requirements established by the
602 European Commission [21] for pesticide residue analysis (recovery percentages
603 between 70% and 120% and %RSD ≤ 20).

604

605 **3.4 Application of the method**

606 The validated methodologies were applied to determine potential residues of the studied
607 neonicotinoids in eighteen commercial honey samples from three different botanical
608 origins, and ten multifloral honey samples collected from experimental apiaries (see
609 subsection 2.3.1). All of these were analyzed in triplicate, and the IS was added at the
610 same concentration (50 μg/kg) than in the matrix-matched samples. No residues of the
611 insecticides under study were detected in any of the commercial samples; while residues
612 of TMX and CLO were found in some of the honeys obtained from experimental
613 apiaries (see Table 9 and Figure 6). As can be observed, TMX was detected and
614 quantified in six samples (0.3-144 μg/kg) with QqQ detection, whilst it was detected in
615 the same samples when using a QTOF detector, but it can be only quantified in one of

616 them (141 μg/kg) due to the low concentrations observed (< 0.6 μg/kg). Meanwhile,
617 CLO was quantified in only one of the samples with both detectors (~ 45 μg/kg), and
618 the use of QqQ allowed its detection in another sample. Thus, it can be concluded, that
619 the use of QqQ is recommended for quantifying neonicotinoids in honey due to the
620 highest sensitivity provided that allowed the quantification at lowest concentrations.
621 Although, it must be also specified that for measuring the TMX amount in one of the
622 samples with a QqQ (#7), it was necessary to dilute the sample (1:3, v/v) with a
623 methanol and water mixture (80:20, v/v); while, it was directly analyzed by UHPLC-
624 QTOF.

625

626 **4. Conclusions**

627 Different analytical methods to simultaneously identify and quantify seven
628 neonicotinoids in honey samples from three very different botanical origins (multifloral,
629 rosemary and heather) have been developed, optimized and validated in order to
630 propose the most adequate methodology. The proposed extraction methods based on an
631 SPE (heather honeys) and QuEChERS (multifloral and rosemary honeys) procedures
632 have proven to be fast, efficient and to have a low consumption of organic solvents, as it
633 is recommended by the principles of green analytical chemistry. The QuEChERS
634 approach, which is fastest and more economical, is the best choice (efficiency of the
635 sample treatment and matrix effect) for analyzing light honeys (multifloral and
636 rosemary), but when determining neonicotinoids in dark honeys (heather), a significant
637 matrix effect was observed for the less retained analytes and the recovery percentages
638 were slightly lower in comparison with light honeys. Meanwhile, the SPE procedure
639 provided a good performance in all cases, although due to its highest cost in comparison
640 with the QuEChERS procedure, its use was recommended for dark honeys as the results

641 were clearly better than QuEChERS. The UHPLC separation of the insecticides was
642 achieved with a core-shell technology based column (Kinetex® EVO) in a shorter time
643 to that obtained in previous works in which those compounds were determined in
644 honey. In addition, the developed UHPLC method could be used with all types of
645 detectors, not only MS/MS, as the seven neonicotinoids were baseline separated. Once
646 the sample treatments were proposed for each of the honey botanical origins studied,
647 and the UHPLC conditions were selected, it was checked the performance of two
648 different MS/MS detectors (QqQ and QTOF). Thus, the main validation parameters
649 obtained for both detectors were compared: selectivity, LODs and LOQs, linearity,
650 matrix effect, trueness and precision. It can be concluded after examining the results
651 that the best overall analytical performance for determining neonicotinoids in honey
652 was achieved when using QqQ detection mainly due to its better sensitivity (LOQs ten
653 times lower) and the reduced influence of the matrix (only two compounds in heather
654 honeys) onto the analyte detection. Although, it would be required to dilute the sample
655 at lower concentrations that when using QTOF. It should be also mentioned that the
656 LOQs obtained with both detectors, especially for QqQ, are much lower than the MRLs
657 established by the European Commission in honey and than the values proposed in most
658 of the previous publications. Finally, commercial honey samples from the three
659 different botanical origins, and honey samples from experimental apiaries were
660 analyzed with the proposed sample treatments and both MS/MS detectors. No residues
661 of the insecticides under study were detected in any of the commercial samples; while
662 residues of TMX and CLO were found in some of the honeys obtained from
663 experimental apiaries. In conclusion, the best analytical performance for determining
664 neonicotinoids in honey was achieved when using a UHPLC-QqQ system after
665 performing a SPE (heather honeys) or QuEChERS (multifloral and rosemary honeys)

666 based treatments, which presented several advantages in relation to the existing
667 literature (extraction efficiency, matrix effect, sensitivity, analysis time, or the baseline
668 separation).

669

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765 **Figure captions**

766 **Figure 1.-** Analytical procedures work-up flow charts.

767 **Figure 2.-** Evaluation of the extraction efficiency (recoveries) obtained for blank honey
768 samples spiked at the medium QC (50 μg/kg) after performing the proposed SPE and
769 QuEChERS procedures. Data represent the mean of three replicates ± the standard
770 deviation of the mean (narrow bars).

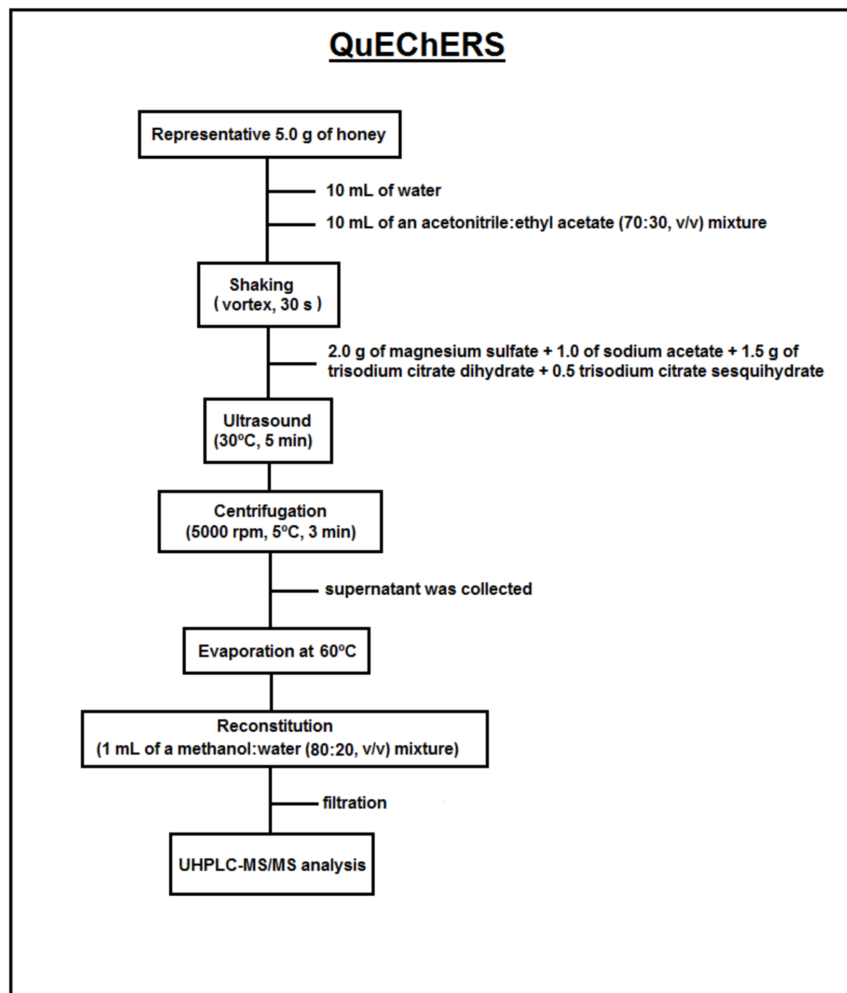
771 **Figure 3.-** Evaluation of the matrix effect (comparison of responses) obtained for blank
772 honey samples spiked at the medium QC (50 μg/kg) after performing the proposed SPE
773 and QuEChERS procedures with QTOF detection. Data represent the mean of three
774 replicates ± the standard deviation of the mean (narrow bars).

775 **Figure 4.-** Representative UHPLC-QTOF chromatograms (EIC in positive mode using
776 the quantification ions; see Table 1) obtained from: (**A**) non spiked rosemary honey
777 sample; (**B**) spiked (50 μg/kg) rosemary honey samples. The UHPLC-QTOF conditions
778 are summarized in subsection 2.4 and Table 1.

779 **Figure 5.-** Representative UHPLC-QqQ chromatograms (SRM or MRM in positive
780 mode using the quantification transitions; see Table 2) obtained from: (**A**) non spiked
781 rosemary honey sample; (**B**) spiked (50 μg/kg) rosemary honey samples. The UHPLC-
782 QTOF conditions are summarized in subsection 2.4 and Table 2.

783 **Figure 6.-** Representative UHPLC-MS/MS chromatograms (**A**-QTOF; **B**-QqQ)
784 obtained after analyzing a honey sample (#7; see Table 9) collected from experimental
785 apiaries. The UHPLC-QTOF conditions are summarized in subsection 2.4 and Tables 1
786 and 2.

Figure 1



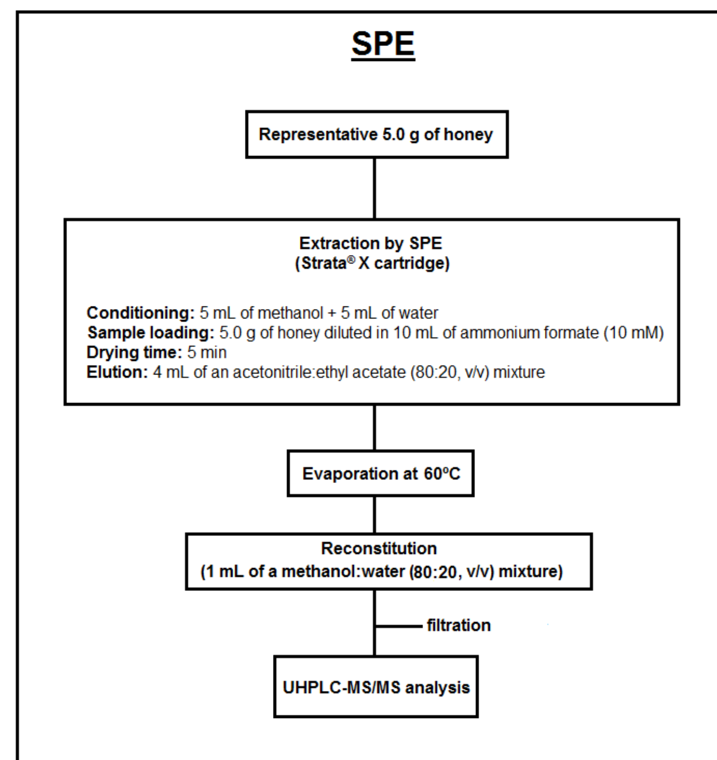


Figure 2

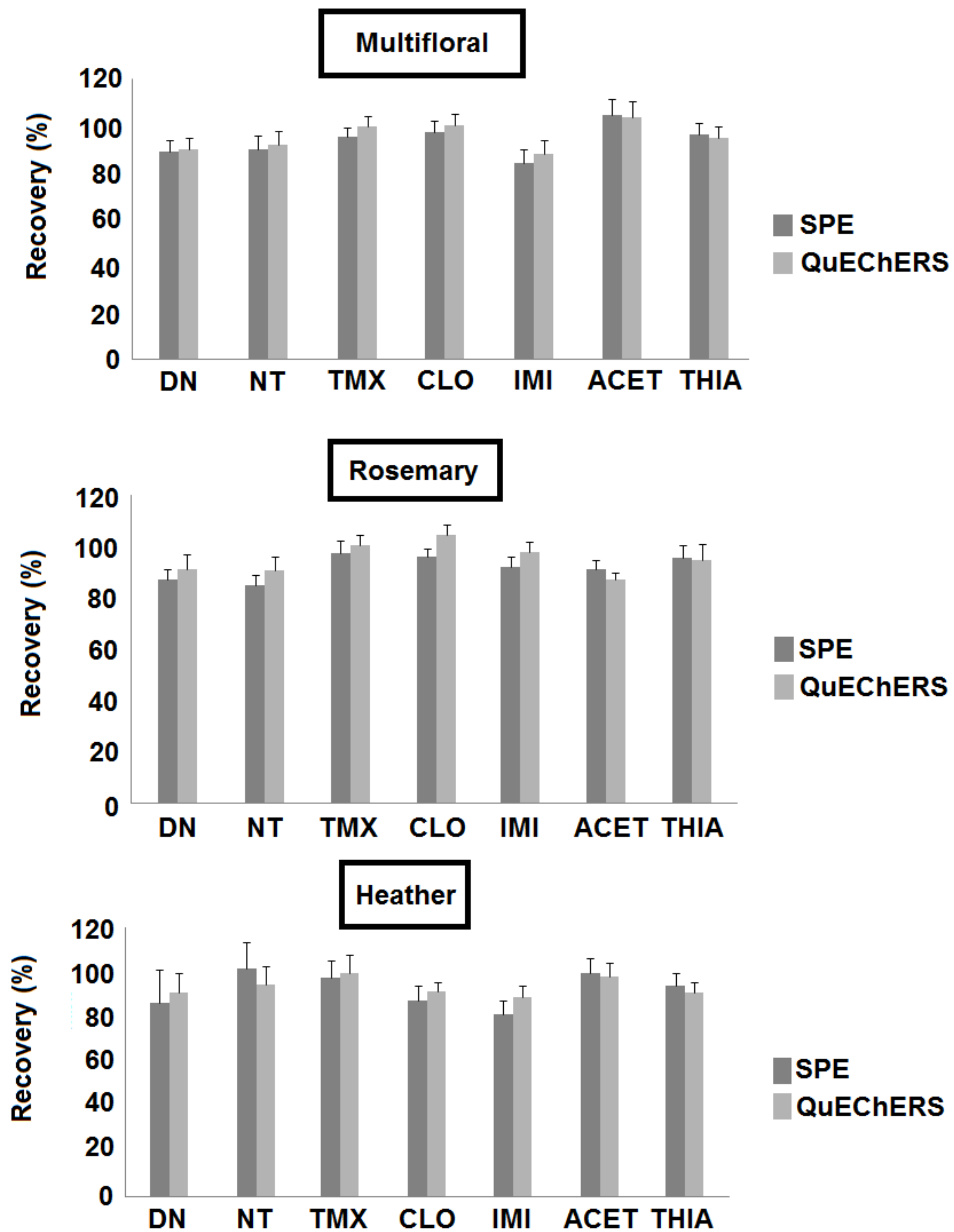


Figure 3

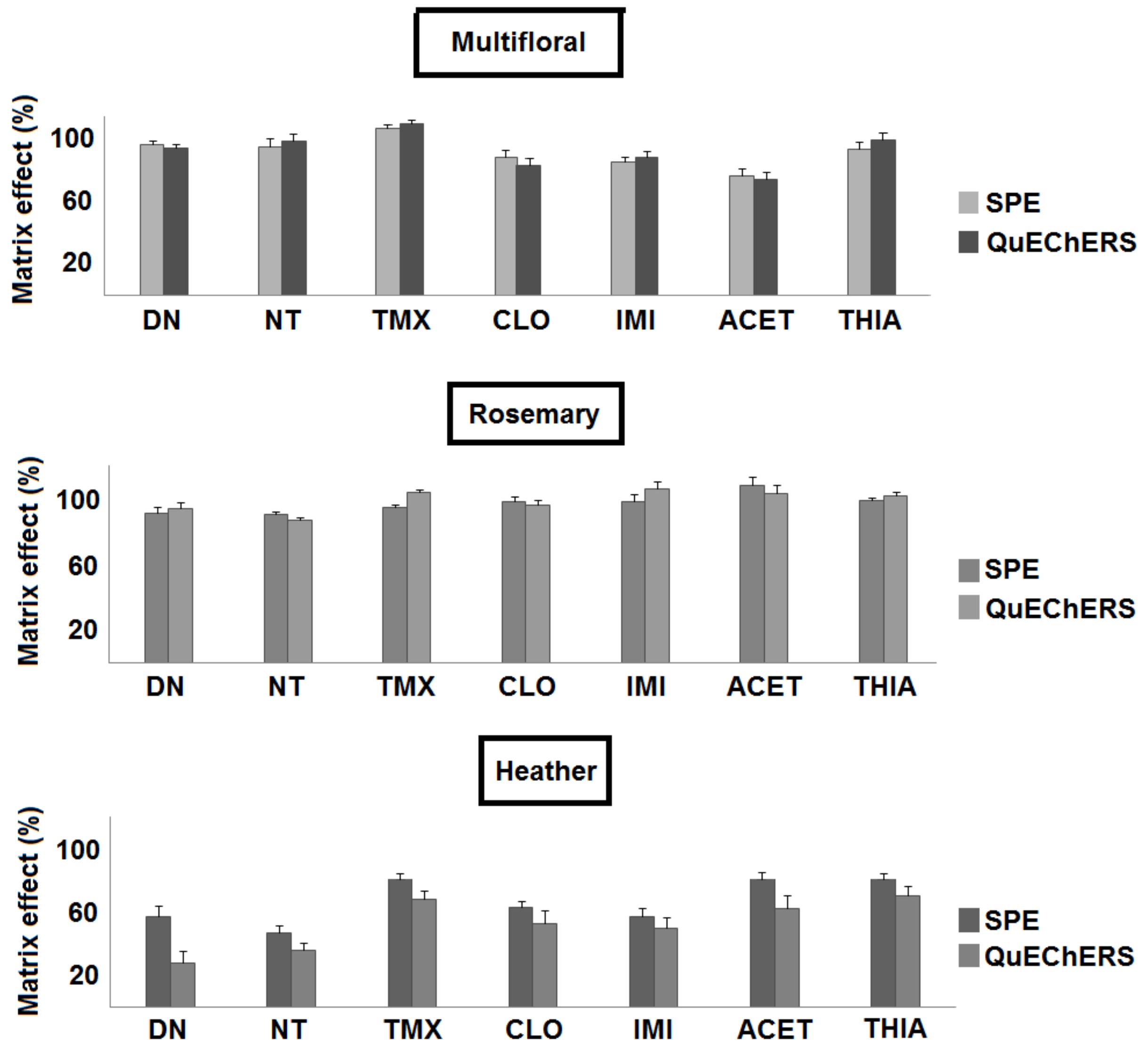


Figure 4

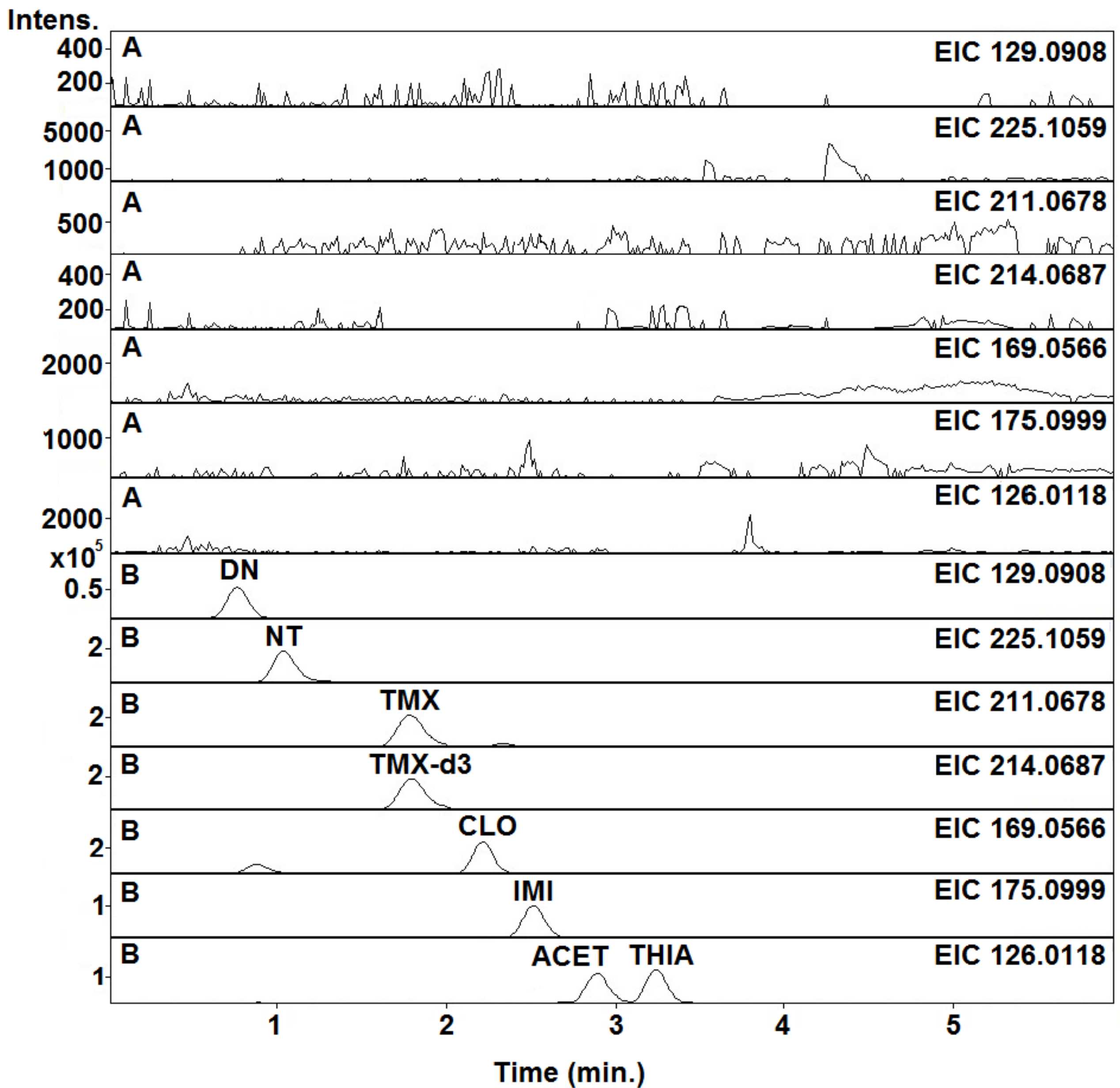


Figure 5

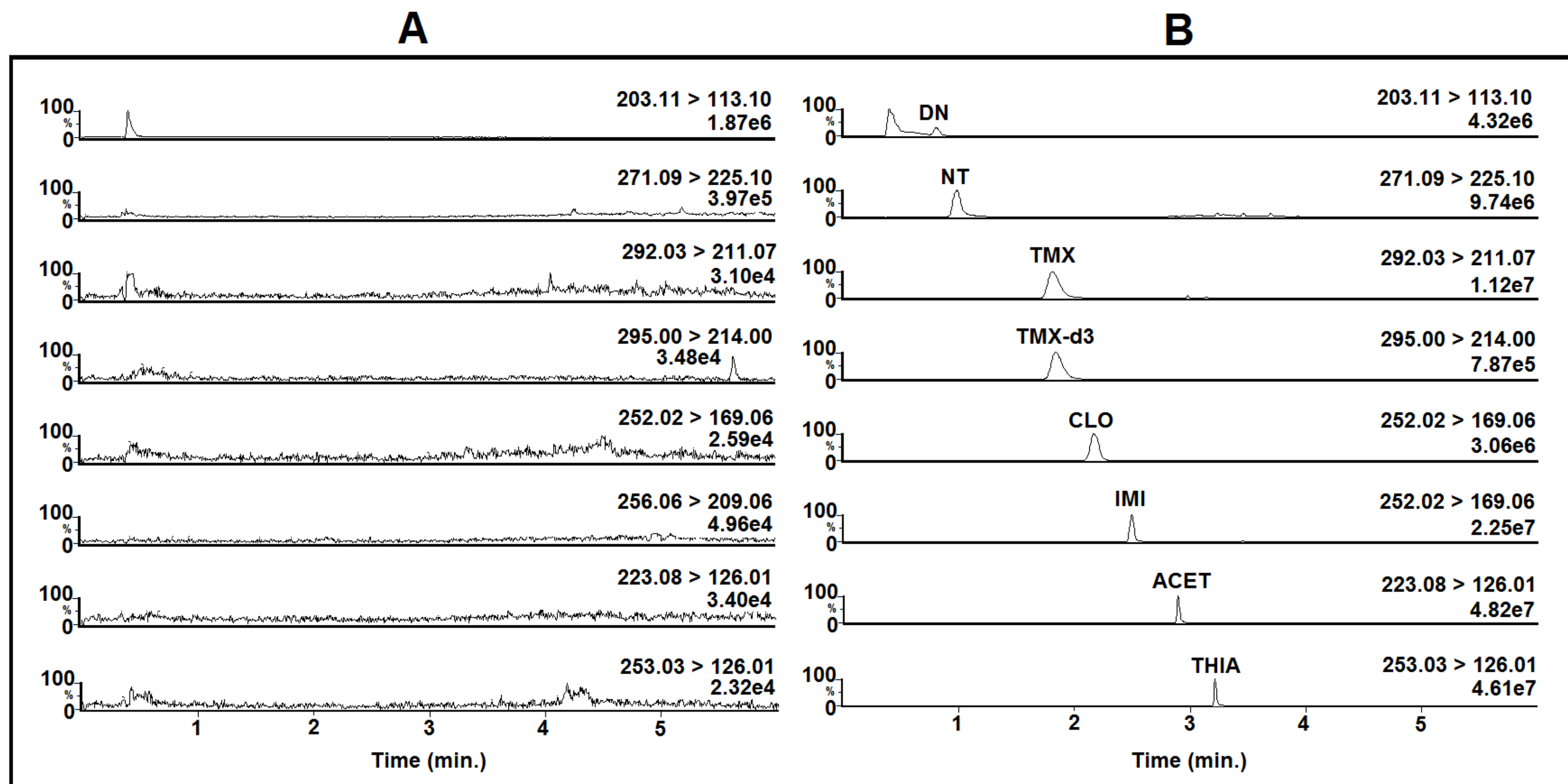
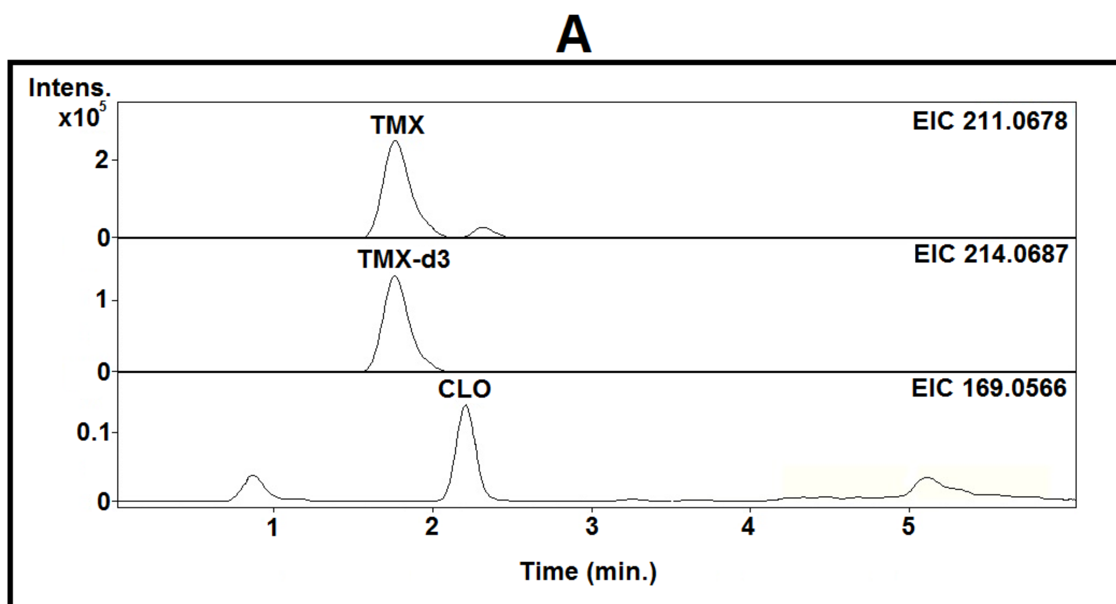


Figure 6



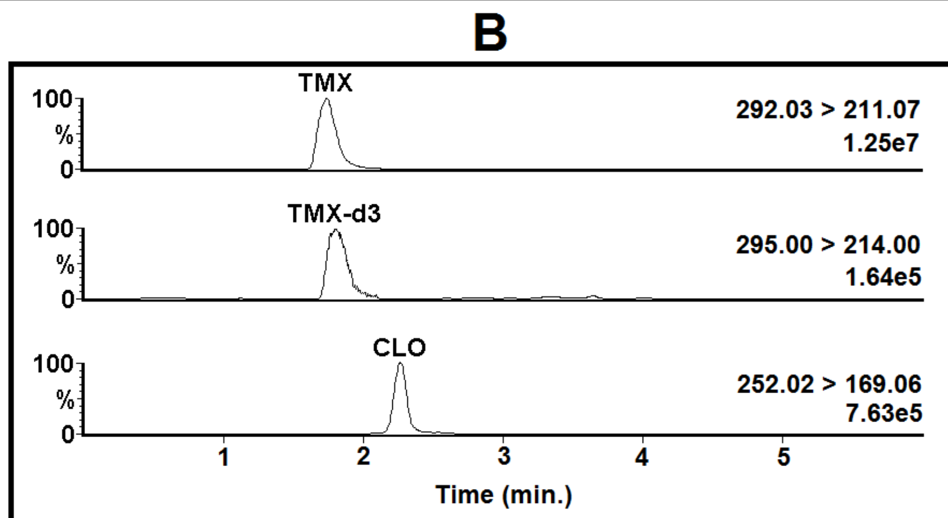


Table 1.-Specific QTOF parameters employed for each of the neonicotinoid insecticides.

Compound	Precursor ions (*m/z*)	Product ions (*m/z*)	CE (*eV*)
Dinotefuran	203.1163[A]	113.1039[A]	15
		129.0908[B]	15
Nitenpyram	271.0988[A]	99.0920[A]	15
		225.1059[B]	15
Thiamethoxam	292.0296[A]	131.9675[A]	15
		211.0678[B]	15
Thiamethoxam-d3 (IS)	295.0396[A]	131.9675[A]	15
		214.0687[B]	15
Clothianidin	250.0187[A]	134.9677[A]	15
		169.0566[B]	15
Imidacloprid	256.0623[A]	175.0999[B]	25
		209.0614[A]	25
Acetamiprid	223.0780[A]	56.1002[A]	30
		126.0117[B]	25
Thiacloprid	253.0342[A]	126.0118[B]	20
		186.0154[A]	20

[A]Confirmation ions; [B]Quantification ions; **CE**, collision energy

Table 2.-Specific QqQ parameters employed for each of the neonicotinoid insecticides.

Compound	CV (*V*)	Quantification ion transition	CE 1 (*eV*)	Confirmatory ion transition	CE 2 (*eV*)
Dinotefuran	20	203.11→ 113.10	15	203.11→ 129.09	15
Nitenpyram	20	271.09→225.10	15	271.09→99.09	20
Thiamethoxam	20	292.03→211.07	15	292.03→131.97	20
Thiamethoxam-d3 (IS)	20	295.00→214.00	15	295.00→134.00	20
Clothianidin	20	250.02→169.06	15	250.02→131.97	15
Imidacloprid	20	256.06→209.06	15	256.06→175.10	15
Acetamiprid	20	223.08→126.01	20	223.08→56.10	15
Thiacloprid	20	253.03→126.01	15	253.03→186.02	15

CV, cone voltages; **CE**, collision energy

Table 3.- Calibration curve data (n=6), LOD and LOQ values obtained for neonicotinoid insecticides in multifloral honeys.

		QTOF					QqQ				
Compound	**Calibration curve**	**Analytical range[A]**	**Slope confidence intervals**	**R^2**	**LOD[B]**	**LOQ[B]**	**Analytical range[A]**	**Slope confidence intervals**	**R^2**	**LOD[B]**	**LOQ[B]**
DN	Standard	1.0-300	$2.8\times10^3 \pm 0.9\times10^1$	0.993	0.3	1.0	0.4-50	$3.5\times10^2 \pm 1.2\times10^1$	0.993	0.10	0.40
	Matrix-matched		$2.7\times10^3 \pm 1.8\times10^2$	0.991				$3.5\times10^2 \pm 3.3\times10^1$	0.999		
NT	Standard	0.8-300	$1.7\times10^3 \pm 7.8\times10^1$	0.998	0.2	0.8	0.2-50	$1.4\times10^3 \pm 8.0\times10^1$	0.999	0.06	0.20
	Matrix-matched		$1.6\times10^3 \pm 1.2\times10^2$	0.992				$1.5\times10^3 \pm 1.9\times10^2$	0.999		
TMX	Standard	0.3-300	$3.5\times10^3 \pm 6.2\times10^1$	0.997	0.1	0.3	0.04-50	$5.8\times10^4 \pm 2.0\times10^2$	0.999	0.01	0.04
	Matrix-matched		$3.7\times10^3 \pm 9.5\times10^2$	0.995				$5.7\times10^4 \pm 9.7\times10^1$	0.997		
CLO	Standard	1.3-300	$1.5\times10^3 \pm 1.4\times10^2$	0.993	0.4	1.3	0.2-50	$1.7\times10^2 \pm 1.4\times10^2$	0.993	0.06	0.20
	Matrix-matched		$1.2\times10^3 \pm 2.6\times10^2$	0.991				$1.5\times10^2 \pm 9.0\times10^1$	0.992		
IMI	Standard	2.0-300	$1.6\times10^3 \pm 9.6\times10^1$	0.999	0.6	2.0	0.02-50	$6.1\times10^2 \pm 1.3\times10^2$	0.999	0.01	0.04
	Matrix-matched		$1.4\times10^3 \pm 1.9\times10^2$	0.992				$6.2\times10^2 \pm 5.5\times10^1$	0.996		
ACET	Standard	2.0-300	$3.1\times10^3 \pm 1.1\times10^2$	0.991	0.6	2.0	0.02-50	$1.5\times10^3 \pm 1.7\times10^2$	0.999	0.01	0.04
	Matrix-matched		$2.3\times10^3 \pm 8.5\times10^2$	0.999				$1.3\times10^3 \pm 1.1.\times10^2$	0.999		
THIA	Standard	1.5-300	$4.2\times10^3 \pm 1.6\times10^2$	0.993	0.5	1.5	0.01-50	$1.9\times10^3 \pm 6.0\times10^1$	0.999	0.01	0.03
	Matrix-matched		$4.0\times10^3 \pm 1.4\times10^2$	0.991				$2.0\times10^3 \pm 6.8\times10^1$	0.998		

[A]Neonicotinoid concentrations were same in the standard (µg/L) and matrix-matched (µg/kg) samples according to the proposed sample treatment and the unit conversion (1 mL of extract, 5.0 g of honey).

[B]LOD and LOQ values were calculated in matrix (honey, µg/kg).

Table 4.- Calibration curve data (n=6), LOD and LOQ values obtained for neonicotinoid insecticides in rosemary honeys.

		QTOF					QqQ				
Compound	**Calibration curve**	**Analytical range[A]**	**Slope confidence intervals**	**R^2**	**LOD[B]**	**LOQ[B]**	**Analytical range[A]**	**Slope confidence intervals**	**R^2**	**LOD[B]**	**LOQ[B]**
DN	Standard	2.6-300	$2.8\times10^3 \pm 1.5\times10^2$	0.998	0.8	2.6	0.5-50	$3.5\times10^2 \pm 0.2\times10^1$	0.993	0.20	0.50
	Matrix-matched		$2.5\times10^3 \pm 2.1\times10^2$	0.993				$3.5\times10^2 \pm 5.7\times10^1$	0.996		
NT	Standard	1.2-300	$1.7\times10^3 \pm 2.8\times10^2$	0.999	0.4	1.2	0.5-50	$1.4\times10^3 \pm 9.1\times10^1$	0.999	0.20	0.50
	Matrix-matched		$1.4\times10^3 \pm 1.9\times10^2$	0.991				$1.3\times10^3 \pm 2.0\times10^2$	0.991		
TMX	Standard	3.6-300	$2.5\times10^3 \pm 1.2\times10^2$	0.996	1.1	3.6	0.05-50	$5.8\times10^4 \pm 3.0\times10^2$	0.999	0.02	0.05
	Matrix-matched		$2.6\times10^3 \pm 9.8\times10^1$	0.995				$6.0\times10^4 \pm 1.4\times10^2$	0.992		
CLO	Standard	4.0-300	$1.3\times10^3 \pm 1.0\times10^2$	0.999	1.8	4.0	0.1-50	$1.7\times10^2 \pm 0.8\times10^1$	0.993	0.04	0.10
	Matrix-matched		$1.2\times10^3 \pm 9.2\times10^1$	0.992				$1.8\times10^2 \pm 1.1\times10^2$	0.992		
IMI	Standard	1.9-300	$1.6\times10^3 \pm 9.6\times10^1$	0.999	0.6	1.9	0.1-50	$6.3\times10^2 \pm 0.3\times10^1$	0.999	0.04	0.10
	Matrix-matched		$1.7\times10^3 \pm 7.5\times10^1$	0.991				$6.1\times10^2 \pm 2.9 \times10^2$	0.991		
ACET	Standard	2.6-300	$3.1\times10^3 \pm 1.1\times10^2$	0.999	0.8	2.6	0.05-50	$1.5\times10^3 \pm 7.0\times10^1$	0.999	0.02	0.05
	Matrix-matched		$3.1\times10^3 \pm 0.5\times10^2$	0.992				$1.6\times10^3 \pm 8.5\times10^1$	0.991		
THIA	Standard	2.0-300	$4.2\times10^3 \pm 1.6\times10^2$	0.999	0.6	2.0	0.02-50	$1.9\times10^3 \pm 6.0\times10^1$	0.999	0.01	0.03
	Matrix-matched		$4.4\times10^3 \pm 1.0\times10^2$	0.997				$2.1\times10^3 \pm 1.9\times10^2$	0.998		

[A]Neonicotinoid concentrations were same in the standard (μg/L) and matrix-matched (μg/kg) samples according to the proposed sample treatment and the unit conversion (1 mL of extract, 5.0 g of honey).

[B]LOD and LOQ values were calculated in matrix (honey, μg/kg).

Table 5.- Calibration curve data (n=6), LOD and LOQ values obtained for neonicotinoid insecticides in heather honeys.

		QTOF					QqQ				
Compound	**Calibration curve**	**Analytical range[A]**	**Slope confidence intervals**	**R^2**	**LOD[B]**	**LOQ[B]**	**Analytical range[A]**	**Slope confidence intervals**	**R^2**	**LOD[B]**	**LOQ[B]**
DN	Standard	5.2-300	$2.8\times10^3 \pm 4.0\times10^1$	0.991	1.5	5.2	0.20-50	$3.5\times10^2 \pm 0.2\times10^1$	0.993	0.20	0.70
	Matrix-matched		$1.5\times10^3 \pm 1.3\times10^1$	0.991				$2.1\times10^2 \pm 0.3\times10^1$	0.993		
NT	Standard	6.7-300	$1.7\times10^3 \pm 7.8\times10^1$	0.995	2.0	6.7	0.20-50	$1.4\times10^3 \pm 3.0\times10^1$	0.999	0.20	0.70
	Matrix-matched		$8.1\times10^2 \pm 3.4\times10^1$	0.993				$0.9\times10^3 \pm 3.0\times10^1$	0.998		
TMX	Standard	2.7-300	$2.5\times10^3 \pm 9.2\times10^1$	0.993	0.8	2.7	0.05-50	$5.8\times10^4 \pm 3.0\times10^2$	0.999	0.02	0.05
	Matrix-matched		$2.4\times10^2 \pm 1.7\times10^2$	0.995				$5.7\times10^4 \pm 2.0\times10^2$	0.996		
CLO	Standard	5.1-300	$1.3\times10^3 \pm 1.2\times10^1$	0.998	1.5	5.1	0.10-50	$1.7\times10^2 \pm 4.0\times10^1$	0.993	0.03	0.10
	Matrix-matched		$8.1\times10^2 \pm 4.5\times10^1$	0.991				$1.5\times10^2 \pm 3.0\times10^1$	0.999		
IMI	Standard	3.1-300	$1.6\times10^3 \pm 3.6\times10^1$	0.993	0.9	3.1	0.05-50	$6.3\times10^2 \pm 3.0\times10^1$	0.999	0.02	0.05
	Matrix-matched		$9.1\times10^2 \pm 4.3\times10^1$	0.991				$6.2\times10^2 \pm 6.0\times10^1$	0.995		
ACET	Standard	1.1-300	$3.1\times10^3 \pm 1.1\times10^2$	0.992	1.1	3.6	0.05-50	$1.5\times10^3 \pm 7.0\times10^1$	0.999	0.02	0.05
	Matrix-matched		$3.3\times10^3 \pm 1.4\times10^2$	0.999				$1.5\times10^3 \pm 6.9\times10^1$	0.993		
THIA	Standard	0.7-300	$4.2\times10^3 \pm 1.6\times10^2$	0.996	0.7	2.2	0.05-50	$1.9\times10^3 \pm 6.0\times10^1$	0.999	0.02	0.05
	Matrix-matched		$4.4\times10^3 \pm 2.7\times10^2$	0.996				$1.8\times10^3 \pm 7.0\times10^1$	0.998		

[A]Neonicotinoid concentrations were same in the standard (µg/L) and matrix-matched (µg/kg) samples according to the proposed sample treatment and the unit conversion (1 mL of extract, 5.0 g of honey).

[B]LOD and LOQ values were calculated in matrix (honey, µg/kg).

Table 6.- Evaluation of the matrix effect (comparison of responses) with the optimal sample treatment for each botanical origin (heather-SPE; multifloral and rosemary-QuEChERS) using a QTOF detector. Data obtained described in subsections 2.5.3 and 3.3.3 (n=6).

Quality control (QC) sample	Heather			Rosemary			Multifloral		
	Mean (%) ± RSD (%)			Mean (%) ± RSD (%)			Mean (%) ± RSD (%)		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
DN	55 ± 4	57 ± 7	50 ± 6	91 ± 2	93 ± 4	86 ± 3	99 ± 3	94 ± 3	102 ± 5
NT	45 ± 5	47 ± 5	45 ± 3	81 ± 3	86 ± 5	83 ± 3	94 ± 4	97 ± 5	96 ± 2
TMX	94 ± 7	97 ± 6	102 ± 4	103 ± 5	100 ± 3	102 ± 5	105 ± 3	107 ± 3	101 ± 6
CLO	60 ± 6	63 ± 4	65 ± 7	99 ± 2	95 ± 4	96 ± 3	80 ± 4	82 ± 5	85 ± 3
IMI	61 ± 5	57 ± 6	55 ± 5	103 ± 4	104 ± 2	107 ± 5	85 ± 5	87 ± 4	92 ± 3
ACET	102 ± 6	99 ± 7	105 ± 3	100 ± 6	102 ± 3	98 ± 4	71 ± 6	73 ± 5	75 ± 7
THIA	105 ± 8	102 ± 7	108 ± 4	102 ± 4	101 ± 3	105 ± 5	95 ± 6	98 ± 5	92 ± 5

Low QC1-LOQ (see Tables 3-5); **Medium QC**- 50 µg/kg; **High QC**-300 µg/kg.

Table 7.- Evaluation of the matrix effect (comparison of responses) with the optimal sample treatment for each botanical origin (heather-SPE; multifloral and rosemary-QuEChERS) using a QqQ detector. Data obtained described in subsections 2.5.3 and 3.3.3 (n=6).

Quality control (QC) sample	Heather			Rosemary			Multifloral		
	Mean (%) ± RSD (%)			Mean (%) ± RSD (%)			Mean (%) ± RSD (%)		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
DN	60 ± 4	62 ± 5	57 ± 7	101 ± 3	104 ± 2	98 ± 4	102 ± 2	98 ± 2	101 ± 2
NT	66 ± 6	68 ± 7	62 ± 6	95 ± 3	96 ± 3	93 ± 2	99 ± 4	103 ± 2	105 ± 5
TMX	98 ± 3	101 ± 3	102 ± 5	102 ± 2	101 ± 4	99 ± 3	99 ± 2	98 ± 2	97 ± 3
CLO	84 ± 5	86 ± 4	89 ± 5	105 ± 2	102 ± 2	105 ± 3	87 ± 5	92 ± 4	90 ± 5
IMI	93 ± 7	100 ± 6	104 ± 5	97 ± 3	99 ± 5	101 ± 5	98 ± 5	102 ± 3	104 ± 2
ACET	99 ± 4	100 ± 4	101 ± 6	105 ± 2	108 ± 2	102 ± 5	81 ± 5	84 ± 6	82 ± 5
THIA	95 ± 6	94 ± 3	97 ± 2	104 ± 3	107 ± 5	108 ± 6	101 ± 3	103 ± 5	99 ± 3

Low QC1-LOQ (see Tables 3-5); **Medium QC**- 10 µg/kg; **High QC**-50 µg/kg.

Table 8.- Evaluation of the efficiency (recoveries) of the optimized and selected sample treatment for each botanical origin (heather-SPE; multifloral and rosemary-QuEChERS). Data obtained as described in subsections 2.5.6 and 3.1 (n=6) using a QTOF detector.

Quality control (QC) sample	Heather			Rosemary			Multifloral		
	Mean (%) ± RSD (%)			Mean (%) ± RSD (%)			Mean (%) ± RSD (%)		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
DN	80 ± 5	85± 4	81 ± 4	102 ± 3	93 ± 5	95 ± 6	87 ± 4	90 ± 3	92 ± 5
NT	108 ± 7	102 ± 5	101 ± 4	94 ± 4	92 ± 2	88 ± 6	91 ± 5	94 ± 3	101 ± 3
TMX	104 ± 3	97 ± 4	92 ± 4	98 ± 2	102 ± 5	99 ± 4	96 ± 4	100 ± 5	103 ± 3
CLO	93 ± 5	87 ± 6	85 ± 4	109 ± 3	105 ± 2	98 ± 6	95 ± 6	101 ± 3	93 ± 4
IMI	87 ± 5	82 ± 4	83 ± 6	97 ± 3	100 ± 2	90 ± 4	94 ± 4	90 ± 5	92 ± 7
ACET	97 ± 4	100 ± 5	92 ± 3	90 ± 7	87 ± 5	85 ± 4	102 ± 4	107 ± 5	99 ± 3
THIA	95 ± 3	94 ± 4	91 ± 2	105 ± 4	95 ± 5	97 ± 6	100 ± 5	98 ± 4	97 ± 6

Low QC1-LOQ (see Tables 3-5); **Medium QC**- 50 µg/kg; **High QC**-300 µg/kg.

Table 9.- Results of the investigation of honey samples collected from experimental apiaries (means of triplicate analyses, µg/kg; %RSD < 10 in all cases)[A].

	QqQ		QTOF	
Sample	TMX	CLO	TMX	CLO
#1	<LOD	<LOD	<LOD	<LOD
#2	<LOD	<LOD	<LOD	<LOD
#3	0.5	<LOQ	<LOQ	<LOD
#4	0.5	<LOD	<LOQ	<LOD
#5	<LOD	<LOD	<LOD	<LOD
#6	0.5	<LOD	<LOQ	<LOD
#7	144	48	141	40
#8	0.5	<LOD	<LOQ	<LOD
#9	0.5	<LOD	<LOQ	<LOD
#10	<LOD	<LOD	<LOD	<LOD

[A]Other neonicotinoids were <LOD for all samples.

Supplementary Information

DEVELOPMENT AND COMPARISION OF ULTRA HIGH PERFORMANCE-LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY BASED METHODS FOR ANALYSIS OF NEONICOTINOID INSECTICIDES IN HONEY

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Table S1.- HPLC published methods for determining neonicotinoids in honey.

Analytes	Sample treatment (time)	Reagents (g, mL[T,O])	Matrix Effect[A]	Recoveries[A]	Baseline Separation[A]	LOQs (µg/Kg)[A]	Validation for different botanical origins	System (SP, time, MS/MS)	Ref.
6 and 7	QuEChERS (~25 min)	5.25 g, 20 mL (7 mL ACN)	Yes	92-104%	No	10-25	No	UHPLC-MS/MS (C_{18}, 13 min, QqQ)	**[3]**
3 and 5	[Q]QuEChERS (~15 min) [S]SPE (~20 min)	[Q]7.65 g, 17.5 mL (7 mL ACN) [S]55 mL (8 mL MeOH; 3 mL DCM)	NS	NS	NS	NS	NS	HPLC-MS/MS (C_{18}, 27.5 min, QqQ)	**[4]**
3-5	SPE (~30 min)	24 mL (6.4 mL MeOH; 0.9 mL ACN)	Yes	61-105%	Yes	4.1-50	Yes	UHPLC-MS/MS (C_{18}, 24 min, QTOF)	**[7]**
1-7	[Q]QuEChERS (~15 min) [S]SPE (~20 min)	[Q]7.2 g, 21 mL (10 mL ACN; 0.2 mL MeOH) [S]133 mL (17 mL MeOH; 40 mL CH)	Yes	[Q]60-107% [S]0-149%	No	[Q]2-10	No	HPLC-MS/MS (C_{18}, 17 min, QqQ)	**[8]**
1-7	SPE (~60 min)	28 mL (13 mL ACN; 3 mL MeOH)	No (< 10%)	88-110%	No	0.1-0.5	No	HPLC-MS/MS (C_{18}, 14 min, QqQ)	**[9]**
3-7	SPE-DLLME (~35 min)	36.5 mL (5 mL ACN)	Yes (IT) No (DAD)	90-104%	Yes	0.7-3.3 (DAD) 0.07-0.40 (IT)	Yes	HPLC-DAD-MS/MS (C_{18}, NS, IT)	**[10]**

[A]:data related only to neonicotinoids; [D]: DLLME; [O]: organic solvents; [Q]: QuEChERS [T]: total solvents.

1, dinotefuran; **2,** nitenpyram; **3,** thiamethoxam; **4,** clothianidin, **5,** imidacloprid; **6,** acetamiprid; **7,** thiacloprid; **ACN,** acetonitrile; **CH,** ciclohexane; **CP,** column partitioning; **DAD,** diode array detector; **DCM,** dichloromethane; **DLLME,** dispersive liquid-liquid microextraction; **HH,** heather honey; **IS,** in-syringe; **IT,** ion trap; **MH,** multifloral honey; **MS/MS**, tandem mass spectrometry; **NS**, not specified**; PDA,** photodiode array detector; **QqQ,** triple quadrupole; **QTOF:** quadrupole-time-of-flight; **QuEChERS**, quick, easy, cheap, effective, rugged and safe; **RTIL,** room-temperature ionic liquid; **SPE**, solid phase extraction.

Table S1.- Continued.

Analytes	Sample treatment (time)	Reagents (g, mL[T,O])	Matrix Effect[A]	Recoveries[A]	Baseline Separation[A]	LOQs (µg/Kg)[A]	Validation for different botanical origins	System (SP, time, MS/MS)	Ref.
1-7	[Q]QuEChERS (~20 min) [D]DLLME (~20 min)	[Q]7.55 g, 26 mL (10 mL ACN) [D]3 mL (0.55 mL ACN; 2 mL DCM)	Yes	[Q]73-98% [D]78-119%	Yes	[Q]5.0-10.0 [D]5.0-7.5	No	HPLC-DAD (C_8, 10 min)	**[12]**
3,5-7	QuEChERS (~10 min)	5.175 g, 20 mL (10mL ACN)	NS	NS	Yes	4.3-310	No	HPLC-MS/MS (C_{18}, 32 min, IT)	**[13]**
2-7	QuEChERS (< 10 min)	6.5 g, 20 mL (10 mL ACN)	NS	75-114%	No	0.1-4.0	No	UHPLC-MS/MS (C_{18}, 10 min, QqQ)	**[14]**
3 and 5	QuEChERS (~15 min)	4 g, 20 mL (10 mL ACN)	Yes	97-111%	Yes	0.1-0.5	No	HPLC-MS/MS (C_{18}, 15 min, QqQ)	**[15]**
2-7	QuEChERS (> 60 min)	7.55 g, 20 mL (10 mL ACN)	NS	NS	NS	0.1-2.0 (µg/L)	No	HPLC-MS/MS (C_{18}, 26 min, QqQ)	**[16]**
1-7	IS-DLLME (< 10 min)	NS, 100 µL octanol	Yes	96-108%	No	0.8-2.0 (µg/L)	No	HPLC-PDA (C_8, 18 min)	**[18]**
4-7	RTIL-DLLME (~15 min)	0.75 g, 250 µl (200 µL RTIL; 50 µl ACN)	Yes	84-100%	Yes	0.03 (µg/L)	No	HPLC-PDA (C_8, NS)	**[19]**

[A]:data related only to neonicotinoids; **[D]**: DLLME; **[O]**: organic solvents; **[Q]**: QuEChERS **[T]**: total solvents.

1, dinotefuran; **2,** nitenpyram; **3,** thiamethoxam; **4,** clothianidin, **5,** imidacloprid; **6,** acetamiprid; **7,** thiacloprid; **ACN,** acetonitrile; **CH,** ciclohexane; **CP,** column partitioning; **DAD,** diode array detector; **DCM,** dichloromethane; **DLLME,** dispersive liquid-liquid microextraction; **HH,** heather honey; **IS,** in-syringe; **IT,** ion trap; **MH,** multifloral honey; **MS/MS**, tandem mass spectrometry; **NS**, not specified**; PDA,** photodiode array detector; **QqQ,** triple quadrupole; **QTOF:** quadrupole-time-of-flight; **QuEChERS**, quick, easy, cheap, effective, rugged and safe; **RTIL,** room-temperature ionic liquid; **SPE**, solid phase extraction.

Table S1.- Continued.

Analytes	Sample treatment (time)	Reagents (g, mL^{T,O})	Matrix Effect^{A}	Recoveries^{A}	Baseline Separation^{A}	LOQs (µg/Kg)^{A}	Validation for different botanical origins	System (SP, time, MS/MS)	Ref.
1-7	DLLME (~ 25 min)	2.7 mL (0.5 mL ACN; 2.0 mL DCM)	Yes	74-114%	Yes	1.5-2.5	No	HPLC-MS/MS (C_{18}, 15 min, QqQ)	**[20]**
1-7	^{Q}QuEChERS (~15 min) ^{S}SPE (~20 min)	^{Q}4.5 g, 20 mL (7 mL ACN; 0.8 mL MeOH) ^{S}25 mL (5.8 mL MeOH; 3.2 mL ACN)	Yes (QTOF, HH-1,2,4,5; MH-6;QqQ, HH-1,2) No (Others)	^{Q}85-109% ^{S}80-108%	Yes	0.3-6.7 (QTOF) 0.03-0.70 (QqQ)	Yes	UHPLC-MS/MS (C_{18}, 17 min, QqQ and QTOF)	**Present study**

^{A}:data related only to neonicotinoids; ^{D}: DLLME; ^{O}: organic solvents; ^{Q}: QuEChERS ^{T}: total solvents.

1, dinotefuran; **2,** nitenpyram; **3,** thiamethoxam; **4,** clothianidin, **5,** imidacloprid; **6,** acetamiprid; **7,** thiacloprid; **ACN,** acetonitrile; **CH,** ciclohexane; **CP,** column partitioning; **DAD,** diode array detector; **DCM,** dichloromethane; **DLLME,** dispersive liquid-liquid microextraction; **HH,** heather honey; **IS,** in-syringe; **IT,** ion trap; **MH,** multifloral honey; **MS/MS**, tandem mass spectrometry; **NS**, not specified**; PDA,** photodiode array detector; **QqQ,** triple quadrupole; **QTOF:** quadrupole-time-of-flight; **QuEChERS**, quick, easy, cheap, effective, rugged and safe; **RTIL,** room-temperature ionic liquid; **SPE**, solid phase extraction.

Table S2.-Summary of precision and accuracy studies for the neonicotinoid determination in spiked blank multifloral honey samples (n=6).

	QTOF						**QqQ**					
	Intraday precision (%RSD)			**Interday precision (%RSD)**			**Intraday precision (%RSD)**			**Interday precision (%RSD)**		
Compound	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**
DN	8	9	8	9	9	7	7	8	9	6	7	7
NT	5	6	6	7	8	5	3	5	5	7	6	6
TMX	4	5	4	6	5	6	3	3	4	6	7	5
CLO	7	6	5	7	7	6	6	7	7	8	7	6
IMI	5	5	7	6	5	8	4	3	4	5	5	6
ACET	5	6	7	7	9	7	7	7	6	7	7	5
THIA	4	3	3	5	4	6	4	5	4	6	5	5

QTOF: Low QC1-LOQ (see Tables 3-5); **Medium QC**- 50 µg/kg; **High QC**-300 µg/kg.

QqQ: Low QC1-LOQ (see Tables 3-5); **Medium QC**- 10 µg/kg; **High QC**-50 µg/kg.

Table S3.-Summary of precision and accuracy studies for the neonicotinoid determination in spiked blank rosemary honey samples (n=6).

	QTOF						QqQ					
	Intraday precision (%RSD)			Interday precision (%RSD)			Intraday precision (%RSD)			Interday precision (%RSD)		
Compound	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**
DN	5	6	4	6	7	7	3	4	3	5	7	5
NT	6	7	6	8	7	8	5	6	5	7	8	8
TMX	4	3	6	5	5	8	3	2	2	5	4	3
CLO	6	4	5	7	7	9	5	6	5	6	6	8
IMI	5	5	6	7	8	8	4	3	5	4	4	6
ACET	7	7	5	6	5	8	4	3	7	5	5	7
THIA	4	5	6	7	8	6	5	3	7	5	5	8

QTOF: Low QC1-LOQ (see Tables 3-5); **Medium QC**- 50 µg/kg; **High QC**-300 µg/kg.

QqQ: Low QC1-LOQ (see Tables 3-5); **Medium QC**- 10 µg/kg; **High QC**-50 µg/kg.

Table S4.-Summary of precision and accuracy studies for the neonicotinoid determination in spiked blank heather honey samples (n=6).

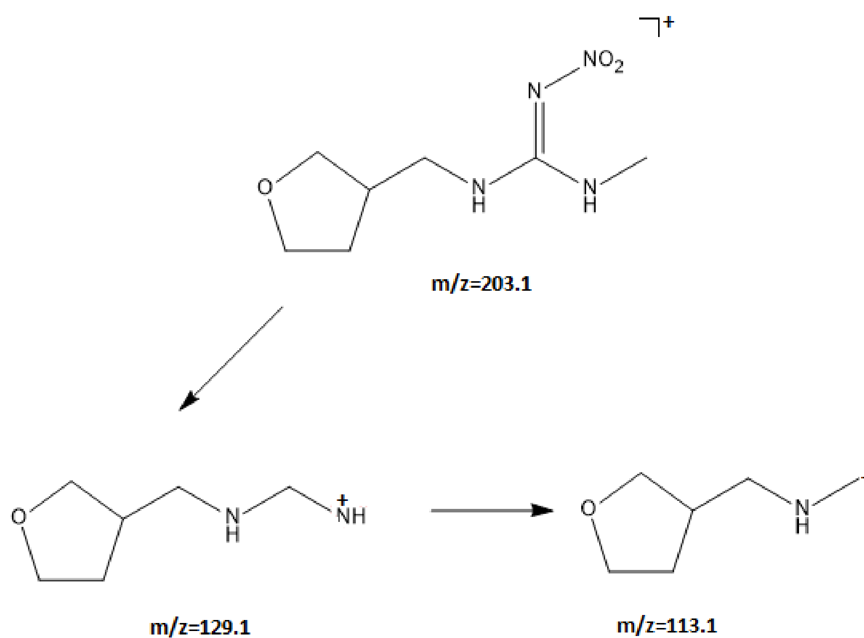
	QTOF						QqQ					
	Intraday precision (%RSD)			Interday precision (%RSD)			Intraday precision (%RSD)			Interday precision (%RSD)		
Compound	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**
DN	9	8	6	7	8	8	8	9	5	8	5	4
NT	9	9	5	8	8	4	7	5	3	7	8	6
TMX	8	6	2	8	6	2	6	4	4	6	5	3
CLO	9	8	5	7	7	4	9	7	7	5	6	5
IMI	9	8	4	8	7	5	9	9	3	6	5	4
ACET	8	8	7	8	8	6	7	6	5	7	6	5
THIA	8	19	7	9	7	5	7	9	8	5	6	4

QTOF: Low QC1-LOQ (see Tables 3-5); **Medium QC**- 50 µg/kg; **High QC**-300 µg/kg.

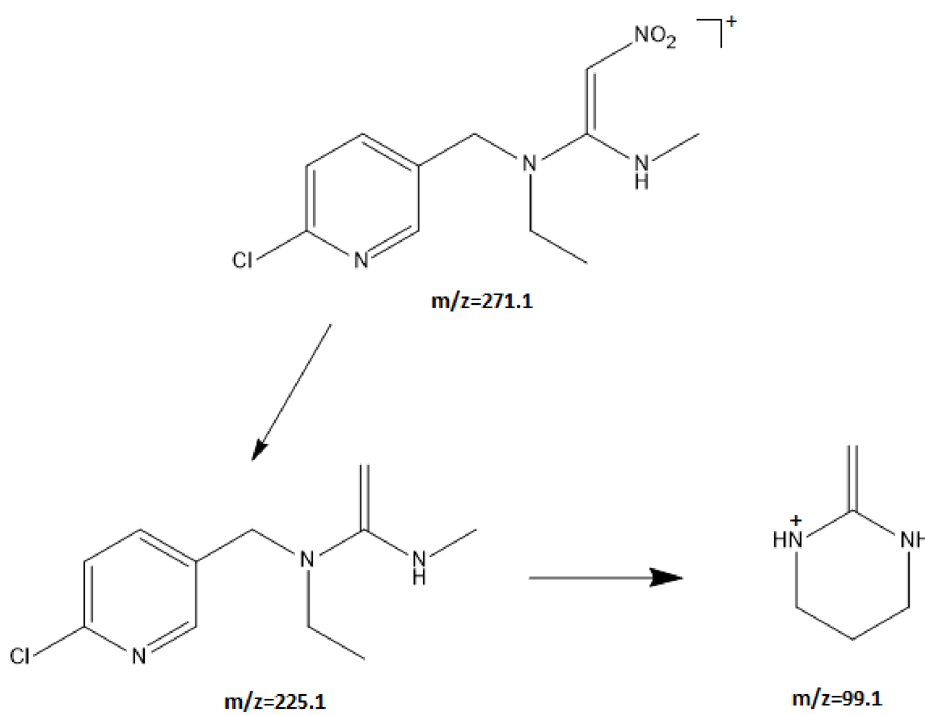
QqQ: Low QC1-LOQ (see Tables 3-5); **Medium QC**- 10 µg/kg; **High QC**-50 µg/kg.

Figure S1.- Proposed structures of the precursor and product ions for each neonicotinoid insecticide.

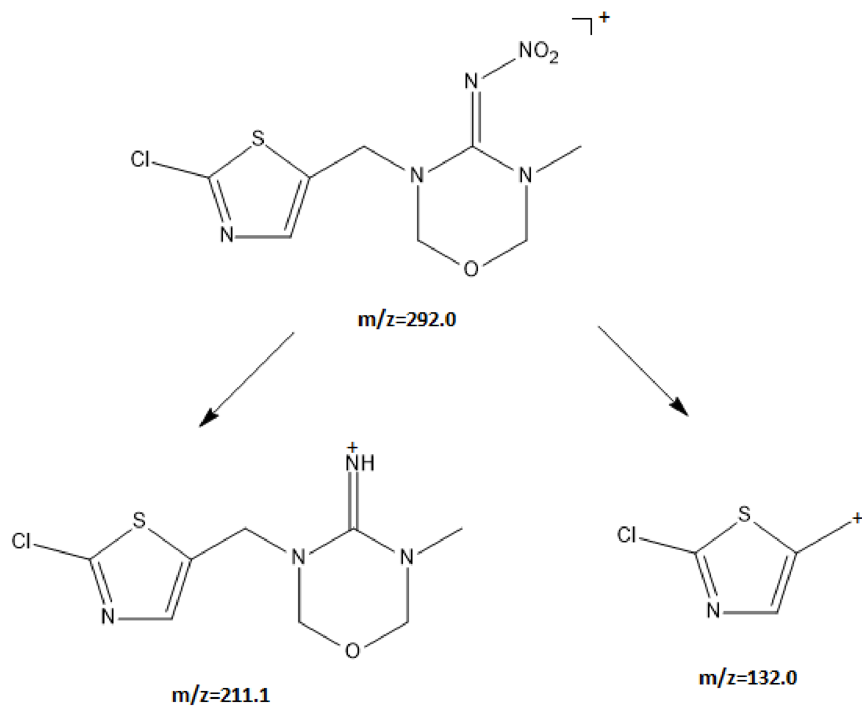
Dinotefuran (DN)



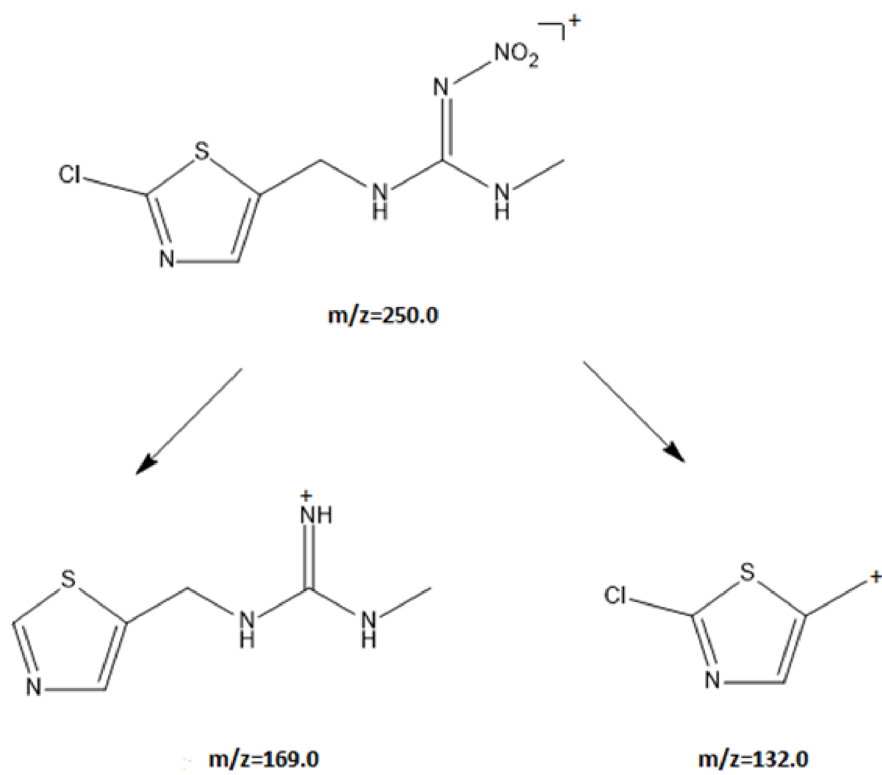
Nitenpyram (NT)



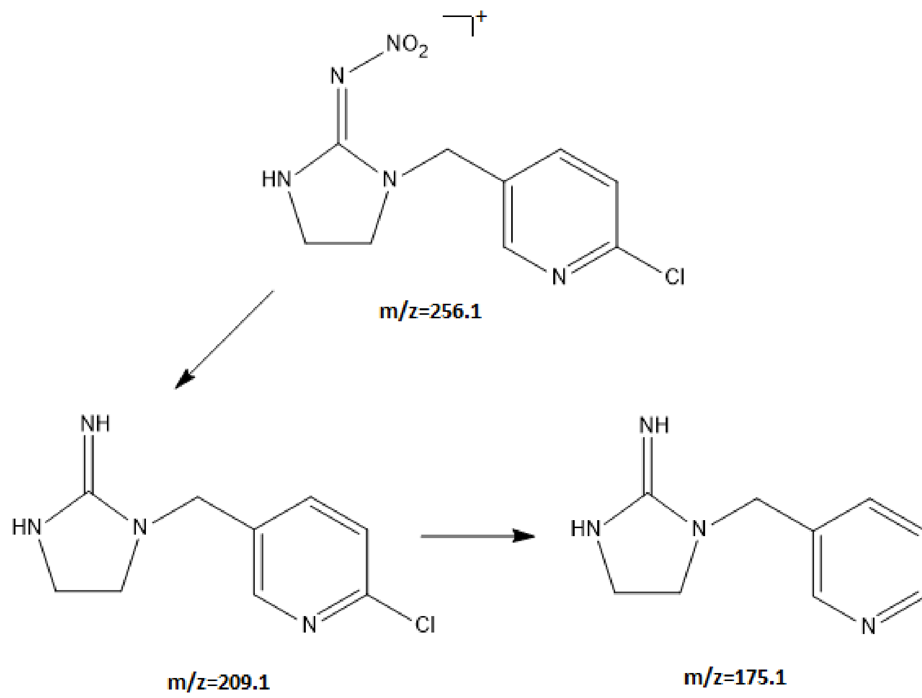
Thiamethoxam (TMX)



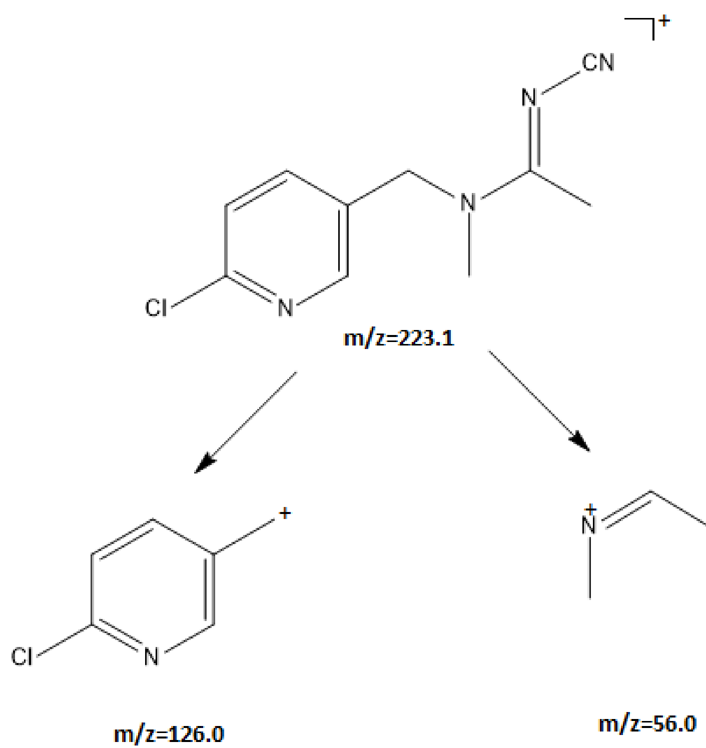
Clothianidin (CLO)



Imidacloprid (IMI)



Acetamiprid (ACET)



Thiacloprid (THIA)

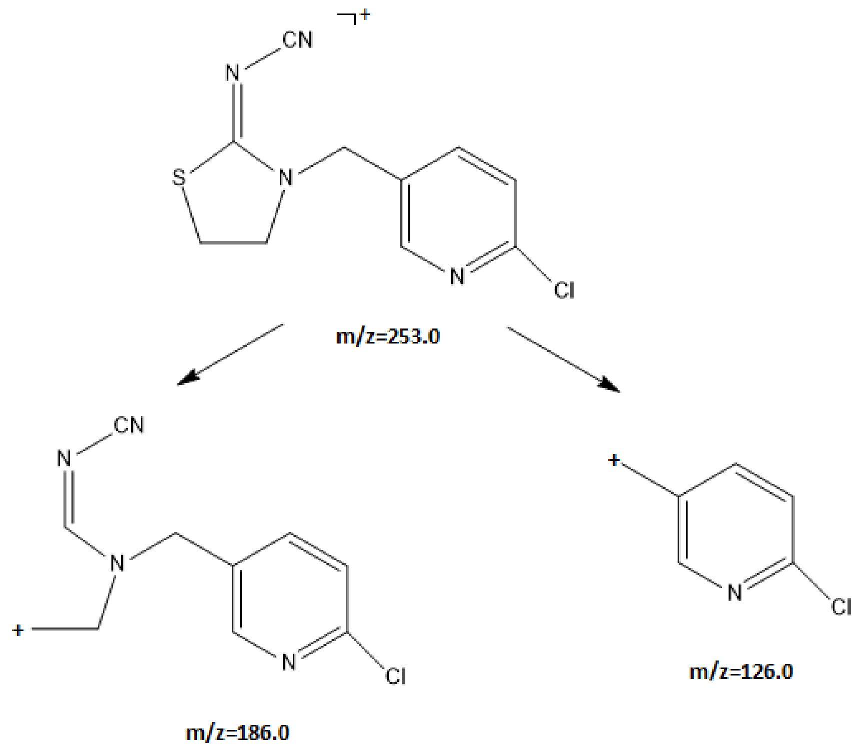


Figure S2.- Evaluation of the matrix effect (comparison of responses) obtained for heather blank honey samples spiked at the medium QC (50 µg/kg) after performing the proposed QuEChERS procedure with different clean-up strategies and using QTOF detection. Data represent the mean of three replicates ± the standard deviation of the mean (narrow bars).

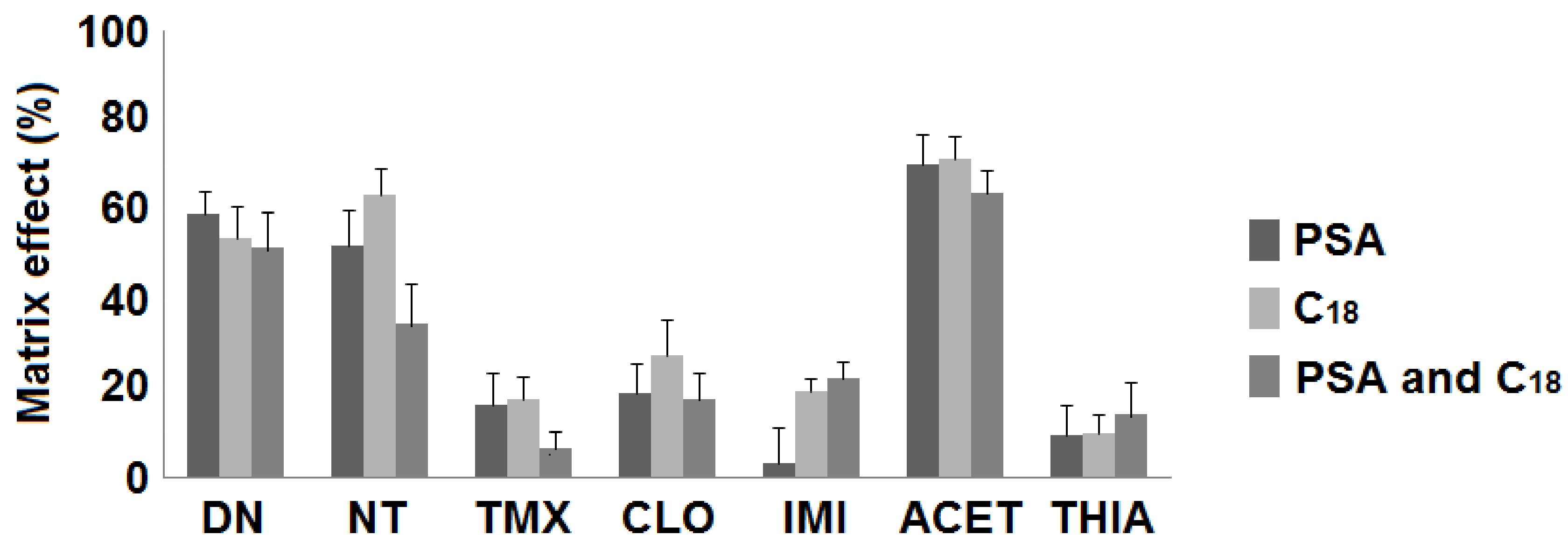


Figure S3.- Evaluation of the extraction efficiency (recoveries) obtained for heather blank honey samples spiked at the medium QC (50 µg/kg) after performing the proposed QuEChERS procedure with different clean-up strategies and using QTOF detection. Data represent the mean of three replicates ± the standard deviation of the mean (narrow bars).