**ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO QUADRUPOLE
ORBITRAP HIGH-RESOLUTION MASS SPECTROMETRY FOR MULTI-RESIDUE SCREENING OF
PESTICIDES, (VETERINARY) DRUGS AND MYCOTOXINS IN EDIBLE INSECTS**

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ABSTRACT

A generic extraction and UHPLC-Q-OrbitrapTM-HRMS method was developed for four insect species (mealworm, grasshopper, house cricket and black soldier fly) analyzing a large spectrum of organic chemical contaminants, including pesticides (n=25), (veterinary) drugs (n=29), and mycotoxins (n=23). To prove the method as 'fit-for-purpose', a successful validation was performed, both qualitatively, by determining the screening detection limit (SDL), selectivity and specificity, as well as semi-quantitatively, by assessing the within-day precision (relative standard deviation (RSD)) and recovery. For both the mealworm, grasshopper, house cricket and black soldier fly, 64, 61, 59 and 62 compounds were detected at the respective SDL levels (1 – 100 µg kg⁻¹), predominantly below existing maximum residue limits for other edible matrices. Mean recoveries ranged between 70% and 120% and RSD-values were in line with European regulations (CD 2002/657/EC; SANCO). Finally, the potential of the screening methodology was demonstrated on real insect samples, revealing minor to no contamination.

KEYWORDS

U-HPLC-Q-OrbitrapTM-HRMS, multi-residue, pesticides, (veterinary) drugs, mycotoxins, insects, food safety

1. INTRODUCTION

The continuously growing world population is considered a serious challenge to the integrity of our global food supply system, and therefore urges us to increase our production in order to anticipate to higher food requirements. However, these measures will inevitably have adverse effects on already limited resources, such as land, water, energy and fertilizers (Huis, Itterbeeck, Klunder, Mertens, Halloran, Muir, et al., 2013). From a historical point of view, especially in developed countries, only a restricted number of protein food sources are consumed, such as livestock and fish (Belluco, Losasso, Maggioletti, Alonzi, Paoletti, & Ricci, 2013). In combination with rising living standards, the demand for meat products is expected to more than double between 2000 and 2050 (Huis, et al., 2013), and as a consequence thereof, the livestock industry will increasingly exert an immense impact on the environment (deforestation, soil erosion, water pollution and greenhouse gas emissions). As such, the growing demand for food has led to an urgent need for alternative, safe supplies of protein from sustainable sources (Charlton, Dickinson, Wakefield, Fitches, Kenis, Han, et al., 2015).

Entomophagy, the consumption of edible insects by humans is already a common practice in several non-European countries (Belluco, Losasso, Maggioletti, Alonzi, Paoletti, & Ricci, 2013; Huis, et al., 2013) and entails great potential to compensate for the rising demand for meat and fish products, as they bring important environmental, economic, and food security benefits. Indeed, edible insects are known to have a nutritional value at least comparable with that of meat and are considered excellent sources of lipids, energy, vitamins and minerals (Rumpold & Schluter, 2013). However, as both the nutritional composition and the chemical profile of consumable insects are highly dependent on the feed composition, insects might accumulate hazardous undesired chemicals. Several insect species can be fed on agricultural co-products, by-products and waste streams that are currently not valorized, potentially accumulating contaminants such as heavy metals, (veterinary) drugs, mycotoxins and pesticides (Charlton, et al., 2015; Rumpold & Schluter, 2013). Insect species, stage of harvest, production methods, and especially the chosen substrate (source of nutrients) may all impact the occurrence and accumulation of contaminants in insect-based food (EFSA Scientific Committee, 2015).

The nutritional, economic and ecological benefits of insects as alternative protein source have led to a recent boost of interest in insect rearing in Europe. Insect species with a great potential for the production of feed and food for human consumption in the European market include the house cricket (*Acheta domesticus*), grasshopper (*Locusta migratoria*), yellow mealworm (*Tenebrio molitor*) and lesser mealworm (*Alphitobius diaperinus*), while the mealworms and the black soldier fly (*Hermetia illucens*) can be produced as animal food. However, according to regulation EC 999/2001, it is prohibited to use insects as a source of protein for animal feed, for animals intended for human consumption. With the implementation of this regulation, all processed animal protein (PAP), except hydrolyzed proteins and in some cases fishmeal, are excluded for use in animal feed. Recent amendments to this legislation (EU Regulation 56/2013 and EU Regulation 893/2017) allow the use of non-ruminant and insect-derived PAP in fish feed (European Commission, 2013).

73 Concerning the marketing of insects intended for human consumption, there were no specific regulations in Europe until
74 recently (FASFC, 2014). Since 2018, insects are considered a Novel Food in Europe for human consumption, and require
75 an application for authorization prior to market launch. Once a food is authorized and added to the Union list, it can be
76 placed in the European market (European Parliament and the Council, 2015). An authorization request includes several
77 aspects of which the risk assessment of the potential microbiological, chemical and physical hazards will be major issues
78 to investigate. Currently, scientific data on the latter topics are largely lacking, as especially in Europe very limited
79 research has been performed on the chemical food safety of insects (van der Spiegel, Noordam, & Fels-Klerx, 2013;
80 Vijver, Jager, Posthuma, & Peijnenburg, 2003). The food safety of non-European insects on the other hand has been
81 studied more widely, with reported food safety hazards including allergens, antinutritional factors, pesticides, heavy
82 metals and alkali, natural toxins and the presence of pathogenic or mycologic microorganisms (Saeed, Abudagga, &
83 Saraf, 1993; van der Spiegel, Noordam, & Fels-Klerx, 2013). These studies are still limited and the majority was
84 performed more than a decade ago. Therefore, improved analytical instrumentation and altered environmental conditions
85 may reveal different results. Recently, Belluco, Losasso, Maggioletti, Alonzi, Paoletti, and Ricci (2013) published a
86 review assessing the accumulation of a limited number of chemicals during the life cycle of European and non-European
87 insects, and demonstrated no additional hazards in comparison with generally consumed animal products. On the other
88 hand, metabolomic analysis, which is considered as the comprehensive quantitative and qualitative study of all small
89 molecules within a cell, tissue or organism (Mattoli, Cangi, Maidecchi, Ghiara, Ragazzi, Tubaro, et al., 2006), has already
90 been performed in order to analyze the reaction of insects on multiple pathogens (Aliferis, Copley, & Jabaji, 2012; Wang,
91 Carolan, Hao, Nicholson, Wilkinson, & Douglas, 2010) and to map insect development (Phalaraksh, Reynolds, Wilson,
92 Lenz, Nicholson, & Lindon, 2008). As such, metabolomics is considered a very powerful tool that could potentially offer
93 novel insight into the biological processes, including the insect's reaction to multiple organic chemicals or pathogens .

94 Taking into account the enormous possibilities of human and animal insect consumption, the development of a broad
95 range screening and monitoring method for the most relevant and common (organic) chemical contaminants and residues
96 is of crucial importance. Therefore, this study intended to develop and validate an appropriate extraction protocol and a
97 targeted ultra-high-performance liquid chromatography coupled to quadrupole Orbitrap high-resolution mass
98 spectrometry (UHPLC-Q-Orbitrap™-HRMS) methodology. Thanks to the high selectivity and sensitivity of both LC, as
99 well as ESI-MS, LC-ESI-MS is considered as a powerful tool for targeted, untargeted as well as suspect analysis in
100 metabolomics (Mattoli, et al., 2006). To the best of our knowledge, this is the first study to develop a targeted qualitative
101 and semi-quantitative screening methodology, allowing rapid and simultaneous analysis of pesticides (herbicides and
102 insecticides), (veterinary) drugs (coccidiostats, antimicrobial drugs, non-steroidal anti-inflammatory drugs (NSAIDs),
103 cardiovascular, anti-epileptic and antilipemic drugs) and mycotoxins (from different taxonomic origin i.e. *Aspergillus*,
104 *Alternaria*, *Fusarium*), with very diverse physico-chemical characteristics (log K_{ow} between -1.56 and 7.19) in multiple

105 insect species with a great potential for the production of feed and food (house cricket, grasshopper, mealworm and black
106 soldier fly). The robustness of the developed screening methodology was demonstrated by analyzing real insect samples
107 to assess the influence of different developmental stages and waste streams as rearing circumstances on the chemical food
108 safety. Additionally, the potential of our developed methodology for future suspect and untargeted analysis of insect
109 tissue was evaluated and the effectivity of the extraction protocol was assessed by means of light microscopy.

110 2. MATERIALS AND METHODS

111 2.1. Chemicals and reagents

112 The selection of 77 compounds was based on the prevalence of the most common (historical) residues and
113 contaminants in different matrices (e.g. water, food, feed) in Europe and their geographical, economical and physico-
114 chemical characteristics, such as persistence in the environment and potential bioaccumulation (Table S1 – S3) (Mol,
115 Plaza-Bolanos, Zomer, de Rijk, Stolker, & Mulder, 2008; Wiest, Bulete, Giroud, Fratta, Amic, Lambert, et al., 2011).
116 These 77 compounds included 25 pesticides, 29 (veterinary) drugs and 23 mycotoxins. Isoproturon-D6, atrazine-D5,
117 bezafibrate-D6, salicylic acid-D4, methyldiclazuril, nigericin, valinomycin and zearalanone were used as internal
118 standards. Analytical standards were purchased at Sigma Aldrich (Diegem, Belgium), the Federal Office of Consumer
119 Protection and Food Safety, EU and National Reference Laboratory (Berlin, Germany), Fermentek (Jerusalem, Israel),
120 Abcam (Cambridge, UK), Chiralix (Nijmegen, The Netherlands) and Toronto Research Chemicals (TRC) (Toronto,
121 Canada).

122 Primary stock solutions of each standard and internal standard were prepared in methanol (MeOH) at a concentration
123 of 1000 ng μL^{-1} , with the exception of sterigmatocystin, which was dissolved in MeOH/dimethylsulfide (80/20). Working
124 solutions (100 ng μL^{-1} , 10 ng μL^{-1} and 1 ng μL^{-1}) were generated by serial dilution of the stock solution in MeOH. When
125 necessary, sonication was applied to ensure complete dissolution of the analytical standards in MeOH. All solutions were
126 stored at -20 °C. Reagents were of analytical LC-MS grade. Formic acid (FA) (98-100 %) and ethyl acetate (99.5 %) were
127 supplied by Merck (Darmstadt, Germany), ammonium formate (99.99 %) was supplied by Sigma Aldrich (Diegem,
128 Belgium), MeOH (99.99 %) and acetonitrile (ACN) (99.99 %) were provided by Fisher Scientific (Loughborough, UK).
129 Ultra-pure water was produced in-house (Millipore, Brussels, Belgium).

131 2.2. Instrumentation

132 Chromatographic separation was achieved using reversed phase ultra-high-performance liquid chromatography
133 (UHPLC) (Dionex Ultimate 3000, Thermo Fisher Scientific), equipped with a degasser, autosampler, LC pump and
134 column oven. Separation was accomplished by gradient elution, using an HSS T3 column (2.1 x 100 mm, 1.8 μm)
135 (Waters, Milford, USA), with a column oven and tray temperature of 45 °C and 10 °C, respectively. The mobile phase
136 consisted of water (A) and MeOH (B), both containing 1 mM ammonium formate and 0.1 % FA, pumped at a flow rate
137 of 0.4 mL min^{-1} . The linear gradient was initiated with 90 % A and 10 % B, maintained for 0.5 min, followed by a slow
138 increase (1.5 min) of the percentage of B to 80 %. Over the next minute, the percentage of B was increased to 95 % and
139 to 99 % in the subsequent 3.5 min. At 7.5 min, the percentage of B reached 100 %, which was maintained for 0.5 min.

Equilibration at initial conditions was allowed the next 2 minutes, giving a total run time of 10 minutes. The sample injection volume was 10 μ L.

The UHPLC system was coupled to a Q-ExactiveTM benchtop mass spectrometer (Thermo Fisher Scientific, San Francisco, USA), equipped with a heated electrospray ionization (HESI-II) source, operating in polarity switching mode. Optimal ionization source parameters were spray voltage $\pm$ 2 kV, sheath gas flow 40 a.u. (arbitrary units), sweep gas flow 2 a.u., auxiliary gas flow 10 a.u., capillary temperature 250 $^{\circ}$ C, and vapour temperature 250 $^{\circ}$ C. The optimal MS parameters were S-lens Radio Frequency (RF)-level 50, operated with full-scan events. Detection was performed using a scan range between 100-1200 Da and a resolution of 140,000 FWHM (Full Width at Half Maximum) at 0.5 Hz. Furthermore, the scans were applied by targeting the automatic gain control (AGC) at ultimate mass accuracy ($1e^6$ ions) and maximum injection time of 50 ms. Instrument control and targeted data processing was carried out with Xcalibur 3.0 software (Thermo Fisher Scientific, San José, USA), whereby compounds were identified based on the m/z -value, isotope profile, and retention time relative to those of the internal standards. For untargeted and suspect data interpretation, the software package Compound Discoverer 3.0 (Thermo Fisher Scientific, San José, CA, USA) was used to achieve automated peak extraction, peak alignment, deconvolution, and noise removal. As main parameters, a minimum peak intensity of 200 000 a.u., retention time width of 0.2 min, and mass window of 5 ppm were employed for feature extraction, with retention time, m/z -value and signal intensity as main feature descriptors. For the suspect screening, ToxFinder 1.0 software (Thermo Fisher Scientific, San José, CA, USA) and an in-house database of hormones ($n = 74$) (Table S4) as well as additional pesticides and (veterinary) drugs ($n = 101$) (Table S5) allowed screening of the full scan HRMS spectra of the insect samples. Visualization of the suspect analysis was performed in MetaboAnalyst 3.0.

The effectivity of the homogenization step of the extraction method was microscopically evaluated by assessing multiple histological sections during yellow mealworm extraction. On each section, 3 micrographs (small (4x), medium (40x) and large (100x) magnification) were taken using a digital camera (Olympus DP50, Olympus NV, Aartselaar, Belgium), installed on a light microscope (Olympus BX61), and analyzed using the image analysis software Cell F (Olympus NV) (Figure S2 – S16).

164

2.3. Sample preparation and extraction

2.3.1 Insect material and sample preparation

Optimization and validation was performed on samples, including larvae of the yellow mealworm (*Tenebrio molitor*) and adults of the grasshopper (*Locusta migratoria*) and house cricket (*Acheta domesticus*), purchased at a local pet shop, while the larvae of the black soldier fly (*Hermetia illucens*) were obtained from research group RADIUS (Agro- and biotechnology of the Thomas More University of Applied Sciences, Belgium). Insects were placed in a refrigerator (4

171 °C) for a minimal of 24 hours to slow down their metabolism, and stored in the freezer awaiting analysis (-20 °C).
172 Subsequent to the validation, the newly developed method for routine analysis was employed on multiple screening
173 samples, received from the RADIUS group (Thomas More, Geel, Belgium) and intended for human or animal
174 consumption. Thereby the lesser mealworm (n = 6) and black soldier fly (n = 3) were analyzed at several time-points with
175 different processing methods, while for the house cricket (n = 6) and grasshopper (n = 1) different substrates were
176 evaluated. All samples were obtained from the Thomas More University of Applied Sciences and were blanched and/or
177 frozen (-20 °C) immediately after harvesting (Table S6; Figure S1). Blanching as pre-freezing thermal treatment is
178 designed to reduce residue levels and microbial load, and can therefore improve the shelf life and chemical safety of the
179 insect (EFSA Scientific Committee, 2015). Within one week after sample collection, the insects were freeze-dried (Alpha
180 1-4 LSC plus, Christ, Osterode am Harz, Germany) for 48 hours, physically homogenized using a pestle and mortar, and
181 stored at -20 °C awaiting analysis (Figure S2 – S10).

182 All histological sections were fixed in phosphate-buffered 4% formaldehyde solution (pH 7.4) for minimum 24h,
183 with the exception of the yellow mealworm samples following lyophilization, which were not fixed (Figure S5 – S7).
184 Following fixation, tissue samples were processed with an STP 420D Tissue Processor (Microm; Prosan, Merelbeke,
185 Belgium) and embedded (embedding center EC 350-1, Microm, Prosan). Specimens were sectioned with a rotary
186 microtome HM 360 (Microm, Prosan). Sections were stained with haematoxylin (Haematoxylin, C.I.75290, Merck
187 KGaA, Darmstadt, Germany) and eosin (Eosin yellow, C.I.45380, VWR International BVBA, Leuven, Belgium) (H&E),
188 using Sakura Linear Stainer II (Sakura, Berchem, Belgium).

189 190 2.3.2 Statistical experimental designs for optimizing the extraction protocol

191 Efficient optimization of the instrumental method and sample extraction relied on a sequential strategy of
192 experimental designs. To effectuate the chemometric optimization of both the instrumental method as well as the sample
193 extraction protocol, different variables with a possible significant effect (p-value < 0.05) on the extraction yield were
194 evaluated in a three-step statistical workflow, consisting of a screening phase, a fractional factorial resolution IV
195 experimental design (FFR) and a response surface modelling design (RSM). Optimization was performed on blank
196 insects, spiked with different amounts of (veterinary) drugs, pesticides and mycotoxins.

197 During the preliminary screening phase, 5 parameters that could affect the extraction efficiency were selected and
198 investigated for their effects (Table S7). In case of significance (p-value < 0.05), they were retained for further
199 optimization by means of the FFR design (n = 20), with the evaluation of 7 qualitative and quantitative parameters (Table
200 S8). The final step included the optimization of significant quantitative variables through an RSM, using a central
201 composited faced-centered (CCF) design (2 parameters, n = 16).

202 Hereby, statistical evaluation by Modde 5.0 (Umetrics, Umea, Sweden) was performed to select, evaluate and model
203 the appropriate experimental design. Taking into account the high number of analytes, optimization was based on the
204 summarized normalized area to ensure the optimal parameters for the combination of all compounds, according to
205 Huysman, Van Meulebroek, Vanryckeghem, Van Langenhove, Demeestere, and Vanhaecke (2017), as compound
206 specific optimization was not pursued. The models' suitability was evaluated through one-way variance analysis
207 (ANOVA) at a confidence interval of 95 % (p-value of 0.05).

208

209 2.3.3 Finalized extraction protocol

210 The extraction protocol was initiated by a chemical homogenization using 2 solid liquid extractions (SLE) (Figure
211 S11 – S16). For this purpose, 1 g of homogenized insect powder was weighted in a 50-mL falcon tube and spiked with a
212 mixture of internal standards (20 ng μL^{-1} or 50 ng μL^{-1}). The first step of the chemical homogenization was intended to
213 extract the polar fraction and was performed by a SLE in which 5.0 mL of ACN acidified with 1.0 % FA was added to
214 the physically homogenized insect powder. Subsequently, the samples were vigorously vortexed (10 seconds) and
215 manually shaken for 1 min, before placing the tubes in a hot water bath at 30 °C (30 min). Following this solid-liquid
216 extraction (SLE), the insect powder was precipitated through a centrifugation step (10 min at 14,127 g and 4 °C) (Rotanta
217 460 R, Hettisch Zentrifugen, Hamburg, Germany). The remaining supernatant was transferred to a 15-mL falcon tube,
218 after which it was evaporated to dryness with N_2 at a temperature of 55 °C. In the second step of the chemical
219 homogenization, the apolar fraction was obtained by repeating the above described SLE and centrifugation steps on
220 precipitated insect tissue using 5.0 mL of ethyl acetate as second extraction solvent. A second centrifugation (5 min at
221 13,765 g and 4 °C) preceded the addition of the supernatant to the 15-mL falcon tube with the previously evaporated,
222 polar fraction, which was then followed by evaporation (N_2 at 55 °C) of the combined fractions. However, the sample
223 could not be completely evaporated to dryness, leaving an oily droplet behind. This fraction was reconstituted in 5 mL of
224 ACN, 1 mL of ultra-pure water and 5 mL of *n*-hexane, after which it was vigorously vortexed (10 seconds) and manually
225 shaken for 1 min. Subsequent to centrifugation (2 min at 13,765 g at 4 °C), the upper *n*-hexane fraction was removed and
226 the sample was again evaporated to dryness, until 1 mL was left. A clean-up was executed by solid phase extraction (SPE)
227 (Oasis HLB SPE cartridges, 6cc, 500 mg, Waters, Asse, Belgium) to remove remaining lipids and other interfering matrix
228 effects. The cartridges were pre-conditioned with 6 mL of ACN/ H_2O (50/50), followed by sample-loading, after which
229 the cartridges were washed (3 mL of H_2O) and eluted with a mixture of 3 mL MeOH and 3 mL ACN, both acidified with
230 0.5 % FA. The eluents were evaporated to dryness (55 °C) with N_2 and finally reconstituted in 200 μL of mobile phase
231 (50/50). The obtained extract was transferred to an UHPLC-vial, after passing through a Chromafil AO 45/25 polyamide
232 filter (pore size 0.45 μm ; filter diameter 25 mm) (Marchery-Nagel, Düren, Germany) (Figure S17).

2.4. Quality assurance

Initial instrument calibration was established by infusing ready-to-use calibration mixtures for the positive and negative ion modes (Thermo Fisher Scientific, San Francisco, USA), using a Chemyx Fusion 100 syringe pump (Thermo Fisher Scientific, San Francisco, USA). Before and after sample analysis, a standard mixture of 25 pesticides, 29 (veterinary) drugs and 23 mycotoxins at a concentration of 0.1 ng μL^{-1} was injected to check the operational conditions of the device. Identification of the target compounds was based on their retention time relative to that of the internal standard, $^{12}\text{C}/^{13}\text{C}$ isotope profile, and the accurate mass of each compound, with a maximum mass deviation of 5 ppm allowed (Table S9). Appropriate (deuterated) internal standards were selected, capable of anticipating fluctuations in signal intensity during the extraction procedure. To this end, a mixture of the internal standards atrazine- D_5 (Atr), isoproturon- D_6 (Iso), bezafibrate- D_6 (Bez), salicylic acid- D_4 (Sal) and valinomycin (Val) at respective concentrations of 20.0 ng μL^{-1} and nigericin (Nig), methyldiclazuril (Met) and zearalanone (Zea) at respective concentrations of 50.0 ng μL^{-1} , was added to every sample, prior to extraction.

2.5. Method validation

The optimized extraction and instrumental method was validated on insect matrix in order to evaluate its fitness-for-purpose. Currently, no specific criteria for the validation of methods for analysis of organic chemical contaminants in insects are available. As this study was performed in Belgium, using insects intended for the European food market, validation was performed on the basis of the European analytical guidelines (European Commission, 2002; SANCO, 2007). This guideline states that the relative standard deviation (RSD) has to be calculated by the Horwitz Equitation (HE). However, for mass fractions lower than 100 $\mu\text{g kg}^{-1}$ application of the HE gives unacceptable high values. Therefore, the respective RSD-value can exceed 23 %, but has to be as low as possible.

Due to the broad range of physico-chemical characteristics of the compounds, it was the intent of this study to develop and validate a qualitative and semi-quantitative screening methodology. Qualitative validation was effectuated by determination of the screening detection limit (SDL), selectivity and specificity. The SDL was investigated as main validation parameter to estimate the threshold concentration at which detection of an analyte becomes reliable and is traditionally estimated by theoretical or empirical calculations based on signal-to-noise ratios (S/N-ratio). However, for HRMS this S/N-ratio is mainly of infinite magnitude, resulting in virtually low detection limits. Therefore, the SDL was established as the lowest concentration tested at which a compound was detected in all spiked samples under study. This was investigated by constructing an 11-point matrix-matched calibration curve (0, 0.5, 1.0, 1.5, 2.0, 5.0, 10.0, 15.0, 20.0, 40.0, 60.0 $\mu\text{g kg}^{-1}$), followed by theoretical calculations based on signal-to-noise ratios and additional spiking. To

264 guarantee the applicability of the SDL, an additional criterion was used, i.e. identity confirmation through the $^{13}\text{C}/^{12}\text{C}$ -
265 ratio of each target compound at the corresponding theoretical SDL (Huysman, Van Meulebroek, Vanryckeghem, Van
266 Langenhove, Demeestere, & Vanhaecke, 2017). As such, this validation comprised six different samples, spiked at their
267 SDL and analyzed together with their non-spiked counterparts (blanks). To provide a framework for achieving semi-
268 quantitative measurements, a more extensive validation was pursued by including additional validation parameters,
269 thereby generating data on the within-day precision and recovery of the method, examined by spiking reference insect
270 samples at 1 and 2 times the SDL in six-fold.

271

272 3. RESULTS AND DISCUSSION

273 3.1. Development of the UHPLC-Q-Orbitrap-HRMS methodology

274 Optimization of chromatographic conditions for multi-residue methods is always challenging because of the diversity
275 of physico-chemical properties ($\log K_{ow}$ between -1.56 and 7.19) that target analytes may possess (Table S9). Optimal
276 conditions, relating to the stationary phase, flow rate, column temperature, mobile phase composition and their additives,
277 were determined by studying the impact on inter-linked resolution, chromatographic peak shape, and interfering
278 background for the 77 target analytes. This resulted in the successful detection of 75 out of 77 compounds in at least one
279 of the insect matrices. Chromatographic separation of the target pesticides, (veterinary) drugs and mycotoxins was
280 evaluated and compared for five different UHPLC C_{18} columns, i.e. Acquity BEH (1.8 μm , 100 mm x 2.1 mm ID, Waters),
281 HSS T3 (1.8 μm , 100 mm x 2.1 mm ID, Waters), Hypersil Gold (1.8 μm , 100 mm x 2.1 mm ID, Thermo Scientific),
282 Nucleodur ISIS (1.8 μm , 100 mm x 2.1 mm ID, Marchery Nagel) and Nucleodur Pyramid (1.8 μm , 100 mm x 2.1 mm
283 ID, Marchery Nagel). Based on peak shape, baseline separation and retention time of the first and last eluting compounds,
284 the HSS T3 C_{18} column was retained as the most suitable column for the current application. Meticulous selection of the
285 mobile phase composition (solvents and buffering additives) ($n = 17$) and the multi-step gradient program ($n = 25$),
286 allowed detection of the analytes of interest, in a remarkable short total run time of 10 min. For the aqueous (water) as
287 well as the organic phase (MeOH or ACN), different buffer solutions were evaluated, including FA, acetic acid, related
288 ammonium salts (ammonium formate and acetate) and their combination at different concentration levels. Optimal
289 sensitivity and minor ion suppression were obtained using a mobile phase which consisted of ultra-pure water and MeOH,
290 both acidified with 0.1 % FA and 1.0 mM ammonium formate, as this resulted in good peak intensity and resolved
291 symmetrical peaks (Figure S18 – S20).

292 Optimization of the MS-parameters was based on the summarized normalized area, the number of recovered
293 compounds, the number of data points across the chromatographic peaks and their corresponding areas. More specifically,
294 the mass spectrometric resolution (70,000 and 140,000 FWHM) and AGC target (2e^4 , 5e^4 , 1e^5 , 2e^5 , 5e^5 , 1e^6 , 3e^6 and 5e^6)
295 were evaluated on both the analytical standard mixture as well as extracts of the black soldier fly. For the latter, the best

306 results were obtained with a mass resolution of 140,000 FWHM and AGC of $1e^6$, which included a 52.62 % increase in
307 peak area, > 8 data points across the chromatographic peak and amongst the highest number of recovered compounds
308 (68/75) (Figure S21). These results differed from the optimum parameters for the analytical standard, with a mass
309 resolution of 70,000 FWHM and an AGC of $2e^5$. Due to the complexity of insect matrix, the increased resolution improved
300 mass accuracy and selectivity, by superior exclusion of isobaric matrix interferences, and is therefore expected to benefit
301 mass spectrometric analysis (Huysman, Van Meulebroek, Vanryckeghem, Van Langenhove, Demeestere, & Vanhaecke,
302 2017). To guarantee the optimal HESI conditions, a serial statistical design of experiments was implemented, starting
303 with an FFR experimental design, followed by an RSM, using a CCF design. Factors included in the FFR were polarity
304 (positive/negative), spray voltage (2-4 kV), sheath gas flow (35-60 a.u.), sweep gas flow (0-10 a.u.), auxiliary gas flow
305 (10-20 a.u.), capillary temperature (250–450 °C), vaporizer temperature (250–500 °C) and the RF-level of the S-lens (0–
306 100). The parameters sweep gas, capillary temperature and S-lens displayed a significant effect (p-value < 0.05) on the
307 summarized normalized area of the contaminants (Figure S22). All quantitative factors were subsequently evaluated by
308 means of an RSM design, reaching the maximum summarized area for a spray voltage of ± 2 kV, sheath gas flow of 40
309 a.u. (arbitrary units), sweep gas flow of 2 a.u., auxiliary gas flow of 10 a.u., capillary temperature of 250 °C, and vapour
310 temperature of 250 °C. The optimal MS parameters were S-lens RF-level 50, operated with full-scan events (Figure 1).

311

312 **3.2. Optimization of the extraction method**

313 The generic extraction procedure was optimized on lyophilized insect matrices, spiked with a mixture of analytical
314 standards ($50 \mu\text{g kg}^{-1}$) (Table S10). Lyophilization was performed to prolong the storage and shelf-life of the samples and
315 to minimize any moisture in the sample, as the latter could reduce the recovery of analytes (Sun, Ge, Lv, & Wang, 2012).
316 Subsequent to the lyophilization, a physical homogenization using a pestle and a mortar was performed as a decreasing
317 particle size is considered to benefit the diffusion of analytes from the sample to the solvent extract (Sun, Ge, Lv, &
318 Wang, 2012). The extraction protocol was developed for four different insect species, with chemometric sample extraction
319 optimization first executed on the black soldier fly and consequently adapted to the three other insect species: i.e. yellow
320 mealworm, grasshopper and house cricket. Research has demonstrated that the diet rather than the developmental stage
321 of the insect determines its chemical composition (Oonincx & van der Poel, 2011). Therefore, no difficulties are expected
322 when analyzing different insect stages or species. The significance of a variety of extraction parameters on the detection
323 capability was evaluated through a sequential strategy of experimental designs, based on the summarized normalized
324 area.

325 The development of an extraction protocol for complex matrices, such as insects, is accompanied by several
326 analytical challenges. One of the major points of attention when analyzing these complex matrices is to avoid or minimize
327 matrix effects, in order to increase recoveries (Hajslova & Zrostlikova, 2003). Therefore, multiple parameters, such as

the extraction solvent, weight of the insects, freezing time and the type of sample clean-up have to be evaluated, to obtain extracts that are completely free of interfering lipids and proteins .

As such, the development of our screening methodology was initiated with a preliminary experiment, exploring the extraction efficiency on various amounts of freeze-dried insect powder (0.25, 0.5 and 1 g dry weight) to determine optimal sample amount. Based on the resulting peak intensity and signal-to-noise ratios, 1 g of freeze-dried insect powder was retained as optimal for efficient extraction of the target compounds.

Next to the weight of the insects, the choice of extraction solvent for the subsequent chemical homogenization is known to have a major impact, especially when extracting a broad range of analytes with different physico-chemical properties, mainly in terms of polarity ($\log_{\text{KOW}}$ between -1.56 and 7.19), pKa (between -9.10 and 17.79) and solubility (Table S9). ACN (Solvent Polarity index (SP_i) of 5.8) is known to be the most optimal choice for the majority of compounds due to its ability to extract analytes with a wide range of polarities (Wiest, et al., 2011). On the other hand, solvents such as ethyl acetate ($\text{SP}_i = 4.4$), *n*-hexane ($\text{SP}_i = 0.1$) or diethyl ether ($\text{SP}_i = 2.8$) were considered essential to dissolve the high lipid content of insects . Some analytes of interest were pH sensitive (e.g. chlorpyrifos, diazinon), as such, FA or acetic acid was added in order to maintain a low pH (< 3) and obtain higher extraction recoveries (> 70 %). In this study, the evaluation of multiple combinations of polar (MeOH and ACN) as well as non-polar extraction solvents (ethyl acetate and methyl-*tert*-butylether) for the chemical homogenization, resulted in a method with two consecutive SLEs using respectively ACN acidified with 1 % FA and ethyl acetate.

Both SLEs were followed by a centrifugation step, to clear the supernatant from all nuclear and cellular debris, thereby assuring no interference of cell debris (Barrera, Isaacson, Zhou, Bavro, Welch, Schaedler, et al., 2009). Subsequent to the double SLE, a lipid layer remained macroscopically noticeable, troubling further extraction and analysis. Indeed, the removal of lipids from extracts prior to chromatographic analysis is essential in order to avoid matrix effects, including strong ion suppression or enhancement, which could impede the analysis of contaminants at $\mu\text{g kg}^{-1}$ concentrations (Wiest, et al., 2011). To this end, an additional sample clean-up was effectuated by dissolving the lipid content of the insects through the addition of *n*-hexane, followed by centrifugation (10 min at 13,765 g and 4 °C) and removal of the *n*-hexane fraction. Additionally, three types of clean-up were tested: SPE, the QuEChERS method and accelerated solvent extraction (ASE), obtaining superior results for SPE clean-up. To optimize the SPE-protocol, different packing contents (200 and 500 mg) of Oasis HLB cartridges as well as Attract SPE LipRem cartridges (Affinisep, Petit Couronne, France) (6cc, 200 mg) were evaluated. The highest yield was obtained with Oasis HLB (6cc, 500 mg) cartridges, which is a reversed-phase polymer with a unique Hydrophilic-Lipophilic Balance, with a sorbent ideal for acidic, basic and neutral analytes as it is stable from pH 0 to 14 (Waters, 2008).

During the subsequent FFR design, the qualitative extraction parameters were tested, including the number of SLEs, the use of a hot water bath, the effect of shaking the samples, and the solvent for eluting the SPE. Tested quantitative

360 factors comprised the volume of solvent used for sample extraction, acidification of the SPE with FA and the temperature
361 of evaporation to dryness (Figure 2, Table S8). The only significant factor was the % of SPE acidification (% FA).

362 In the final RSM, optimal acidification of the eluent was observed at 0.5 % FA for adequate extraction of the target
363 compounds. At a low pH (< 3), acidic groups were protonated and in neutral state, while the basic groups were either
364 neutral or protonated, therefore ionic interaction between acidic and basic groups and the matrix was prevented (Mol,
365 Plaza-Bolanos, Zomer, de Rijk, Stolker, & Mulder, 2008).

366

367 3.3. Validation study

368 The goal of this work was to develop and validate an analytical methodology that allows the simultaneous, broad-
369 range detection of 77 different pesticides, mycotoxins and (veterinary) drugs in insect matrices. Therefore, validation of
370 the qualitative screening methodology included the determination of the screening detection limit (SDL), selectivity,
371 specificity. To provide a framework for achieving semi-quantitative measurements, a more extensive validation was
372 pursued by including additional validation parameters, thereby generating data on within-day precision and recovery.

373

374 3.3.1. Screening detection limit

375 Following insect matrix extraction, 75 out of the selected 77 compounds were successfully detected, with SDLs
376 ranging from 1 – 100 µg kg⁻¹ (Table S10), excluding fumonisin B1 and tenuazonic acid. Fumonisin is known to
377 covalently bind to polysaccharides and proteins via their two tricarballic acid side chains. As such, they form complexes
378 with matrix compounds, which are removed during extraction (Seefelder, Knecht, & Humpf, 2003). The chromatographic
379 separation of tenuazonic acid has been reported as very difficult, due to the formation of tautomers and rotamers.
380 Moreover, this compound is characterized by a poor peak shape in HPLC due to its high acidity at the prevailing pH (pKa
381 4.5) and metal chelation. This compound is normally not included in multi-residue methods as a pre-column derivatisation
382 has been shown as requisite to enhance its sensitivity (Man, Liang, Li, & Pan, 2017). For both compounds another sample
383 treatment and/or a more sensitive instrument might be required. However, as it was the purpose of this research to develop
384 a broad screening method, compound-specific optimization was not pursued.

385 To ascertain the applicability of our screening methodology, the obtained SDLs were compared to the range of
386 maximum residue levels (MRLs) in food or feed (Table S10), established by the European Commission to guarantee the
387 safety of the consumer. The MRL is the highest level of a contaminant or residue that is legally tolerated in food or feed
388 and can therefore serve as a basis for risk assessment. To the best of our knowledge, until now, no MRLs have been
389 established for residues in insect matrices. Therefore, the employed MRLs were based on CR 396/2005 for pesticides
390 (European Commission, 2005), CR 1881/2006 for mycotoxins (European Commission, 2006) and CR 37/2010 for

391 veterinary drugs (European Commission, 2010), while for pharmaceuticals used in human medicine, no maximum residue
392 levels have been determined yet. With regard to the chemical safety of the black soldier fly, Directive 2002/32/EC
393 regulates undesirable substances in animal feed, covering a range of contaminants and residues. However, only for
394 aflatoxin B1, an MRL between 5 and 20 $\mu\text{g kg}^{-1}$ has been determined (Commission, 2003). The SDLs obtained by our
395 developed methodology could be situated within the MRL-range for the majority of residues. For a minority of analytes
396 (Table S10), the SDL slightly exceeded the MRL. For these residues, no conclusions regarding their chemical safety
397 could be made and a more compound specific optimization might be necessary. For the yellow mealworm, grasshopper,
398 house cricket and black soldier fly, respectively, 64, 61, 59 and 62 out of 77 compounds were detected, while 4, 10, 10
399 and 7 compounds could be detected in a part of the samples at SDL. In total, 9, 6, 8 and 8 out of 77 compounds at SDL
400 could not be recovered for the yellow mealworm, grasshopper, house cricket and black soldier fly, respectively. The
401 majority of compounds that were not or only partially detected, were characterized by a log K_{ow} above 3, and thus
402 considered more hydrophobic. Difficulties in detection may thus be attributed to the inclusion of an *n*-hexane step, which
403 proved, however, essential since fatty clots formed, thereby impeding the analysis of the majority of compounds.

404

405 3.3.2. Specificity and selectivity

406 The power of discrimination between an analyte and closely related substances (isomers, metabolites, degradation
407 products, transformation products, endogenous substances, matrix constituents, etc.) is of crucial importance for reliable
408 analysis. As such, the specificity and selectivity of our developed method were evaluated by analyzing blank samples as
409 well as samples fortified with the analytes of interest at a concentration of 1 x SDL ($n = 6$), following SANCO-guidelines,
410 which requires a minimum of 5 replicates (SANCO, 2007). A significant increase in concentration was observed at the
411 accurate mass (signal-to-noise ≥ 3 ; mass deviation ≤ 5 ppm) and specific retention time when compounds were added to
412 blank samples, which confirmed that the optimized method was selective for the target residues in the presence of other
413 matrix constituents. Identification was based on accurate mass, $^{12}\text{C}/^{13}\text{C}$ isotope ratio and relative retention time, i.e. the
414 ratio between retention time of the analyte and its internal standard, which ensured the high selectivity of the method.
415 However, the isomeric compounds 3-acetyl deoxynivalenol and 15-acetyl deoxynivalenol only differ in the position of
416 the acetyl group (Figure S4), therefore, similar HPLC retention times impeded identification and quantification
417 (Yoshinari, Tanaka, Ishikuro, Horie, Nagayama, Nakajima, et al., 2013). However, due to reported co-occurrence of the
418 latter in food samples, it is not necessary to consider additional measures for their analysis, as measures with regard to
419 deoxynivalenol also protect the human population from unacceptable exposure to its acetylated derivatives (European
420 Commission, 2002).

421

3.3.3 Precision and recovery

Precision was evaluated based on the calculated RSD of a minimum of six replicates, at each spiking level. Only for those compounds for which all six samples were detected at the lowest concentration, RSD-values and recoveries were calculated (Table S11). As there are no guidelines for the qualitative and semi-quantitative screening of insect tissue, values of the CD 2002/657/EC, considering edible matrices, were implemented. As such, an RSD below 30 % was pursued, which was met for the majority of the analytes. In absence of certified reference material, trueness was investigated by calculating the recovery. A minimum of six replicates were used at both the SDL and at least one higher level, in this case 2 x SDL. For the majority of the compounds, the recovery ranged between 70 % and 120 %, with RSD-values calculated on the 2 x SDL below 30 %, in line with SANCO and CD 2002/657/EC (European Commission, 2002; SANCO, 2007) (Table 1, Figure 3 - 4).

The developed methodology provided acceptable RSD-values for pesticides and mycotoxins, which are considered important contaminants/toxins and as such a strong source of chemical residues in insect matrices, due to insect rearing on fruits or vegetables. However, the methodology proved less ideal for the determination of (veterinary) drugs, which are also potentially important residues as some insect species can be reared on manure or blood of animal origin (Table 1, Figure 3 – 4). The latter could be contributed to the higher presence of zwitterionic compounds within this group (Table S9). Indeed, mainly for the negatively charged zwitterionic compounds higher RSDs were obtained, in line with observations from Prat, Benito, Compano, Hernandez-Arteseros, and Granados (2004), who concluded that these compounds exhibit maximum retention at the pH value at which the zwitterionic species prevails. Moreover, the combined extraction of both acidic and zwitterionic compounds in the same extraction procedure is nearly impossible as the zwitterionic compounds are better extracted using basic solutions, while the anionic compounds prefer acidic conditions (Andreu, Blasco, & Pico, 2007). Additionally, it is well accepted that next to the log K_{ow} , the pKa as well is an essential parameter for determining the RSD and recovery (Pailler, Krein, Pfister, Hoffmann, & Guignard, 2009). Indeed, compounds with a log K_{ow} above 3 (apolar) and/or compounds with a high (> 7) pKa value also displayed higher RSD-values and recoveries. The latter is caused by the crucial role of the pH during solid phase extraction as it affects interaction with the polymeric sorbents of the Oasis HLB cartridge. Therefore, the pH should be adjusted to increase retention on the SPE cartridges. As the goal of this research was to develop one broad semi-quantitative screening methodology, this compound specific optimization was not pursued, especially as the majority of the compounds demonstrated an RSD below 30 %, which was considered acceptable according to the European guidelines (European Commission, 2002). Finally, it should be stressed that the SDL is the smallest concentration at which the compound could be detected, therefore prone to higher RSD-values compared to higher spiked levels as well as to recoveries below or above 70 – 120 % -range (European Commission, 2002).

3.4. Screening results in insect samples

The obtained samples were screened for the 75 compounds that could be detected with the newly developed screening methodology, as well as quality control samples, consisting of blank samples and samples spiked at SDL, used to support the performance of the screening method and to estimate the concentration of chemicals present in the samples. Several contaminants could be detected in the samples, including (veterinary) drugs (salicylic acid, metoprolol, and paracetamol), mycotoxins (alternariol, alternariol methyl ether, HT2-toxin, nicarbazin, nivalenol, roquefortine C, and zearalenone) and pesticides (isoproturon) (Figure S23 – S26). Salicylic acid could be detected in each sample, the majority at SDL or estimated concentration of 1 $\mu\text{g kg}^{-1}$ or 3 $\mu\text{g kg}^{-1}$ (type I and II error respectively 95.8 % and 0 %). Other compounds detected in a considerable number of samples below SDL-level were paracetamol (0, 0, 6 and 0) and zearalenone (0, 3, 0 and 0) for respectively the lesser mealworm, black soldier fly, house cricket and grasshopper, while metoprolol tartrate (6, 0, 6 and 0) and nicarbazin (6, 0, 0 and 1) were detected at levels above the SDL (Table 2). Similar observations could be made for HT2-toxin, which was detected above the SDL in several lesser mealworm samples. However, for those compounds which exceeded the SDL, no MRLs were determined and as such, they are considered safe.

Acetylsalicylic acid is an analgesic drug and exerts its pharmacological effect due to salicylic acid, a metabolite formed upon hydrolysis. Its presence in insect matrices might be explained by previous administration of sodium salicylate in animals, and the use of manure to rear insects. Besides this, it is also known to be found in several fruits and vegetables and aquatic environments (i.e. surface and groundwaters) and could therefore be present in feed ingredients. Furthermore, it has been previously detected in humans and animals not medicated by salicylic acid derived therapeutics (Nacher-Mestre, Ibanez, Serrano, Boix, Bijlsma, Lunestad, et al., 2016). Paracetamol is a widely used ‘over-the-counter’ analgesic and antipyretic drug with its pharmacological effects due to an inhibition of prostaglandin synthesis and cyclooxygenase. At therapeutic levels, it is considered to be safe for humans upon normal drug use. Paracetamol and structural analogues (i.e. 4'-hydroxyacetanilide, N-acetyl-p-aminophenol) are ubiquitously present and easily accumulate in aquatic environment, including surface waters, wastewater and drinking water throughout the world (Wu, Zhang, & Chen, 2012). Zearalenone is a natural contaminant of food of cereal origin, produced by *Fusarium* species infecting cereals such as corn, rice, and wheat. It has an immunosuppressive and estrogenic effect, and as such impairs fertility and fetal development in farm animals, especially pigs. Insects can be contaminated with this mycotoxin through growth on infested crops (Waskiewicz, Gromadzka, Bocianowski, Pluta, & Golinski, 2012). The MRL of zearalenone for animal consumption has not been established, therefore, no risks are anticipated in the one black soldier fly sample that exceeds the SDL (Table 2). Metoprolol tartrate is one of the most frequently detected pharmaceuticals in drinking water and is used as a treatment for cardiovascular diseases as a selective beta-adrenergic receptor blocker (van den Brandhof & Montforts, 2010). Finally, nicarbazin is a commonly used antibiotic and is frequently detected in manure, where it remains

485 stable during storage and will end up in agricultural fields when the manure is eventually used as fertilizer. As such, it
486 will accumulate in the environment upon repeated use (Badea, Diacu, & Radu, 2013). For both metoprolol tartrate as well
487 as nicarbazin, no MRLs have been determined so far.

488 Even though excellent validation results were obtained for the class of the pesticides, only one pesticide was identified
489 after screening of insect samples. Therefore, the chemical safety of insects in relation to pesticide contamination can be
490 considered high. These observations are in line with Charlton, et al. (2015), who demonstrated limited chemical hazards
491 such as pesticides, veterinary drugs and mycotoxins.

492 Additionally, the potential of our developed methodology for future untargeted and suspect analysis of insect tissue
493 was evaluated. For the latter, the metabolomic fingerprint of the house cricket, grass hopper, yellow mealworm and black
494 soldier fly comprised respectively 8 773, 10 255, 7 418 and 7 959 (Table S12). Suspect analysis was performed by
495 screening the full scan HRMS spectra of the insect samples against our in-house database of hormones (n = 74) (Table
496 S4) and additional pesticides and (veterinary) drugs (n = 101) (Table S5). Using this approach, respectively 8 and 28
497 components were suggested, as visualized in Figures S27 – S28.

498 4. CONCLUSIONS

499 This study presents the development and validation of a novel semi-quantitative analytical UHPLC-Q-OrbitrapTM-HRMS
500 multi-residue method, for the simultaneous detection of 77 (veterinary) drugs, pesticides and mycotoxins in insect
501 samples. This is the first method to analyze a broad-range of physico-chemical diverging residues in insect tissue, with a
502 single screening methodology. To prove the method 'fit-for-purpose', a successful validation was performed, both
503 qualitatively, by determining the screening detection limit (SDL), selectivity and specificity as well as semi-
504 quantitatively, by assessing the precision (relative standard deviation (RSD)) and recovery. For the latter, acceptable
505 repeatabilities (RSD-values below 30 %) and good recoveries (ranging between 70 % and 120 %) were obtained,
506 determined by European regulation CD 2002/657/EC. The empirical SDL in insect matrices ranged from 1 to 100 µg kg⁻¹
507 with the majority of these in line with the MRL in edible matrices.

508 The potential of the newly developed method was demonstrated by screening several insect samples, including different
509 developmental stages and rearing conditions, for the contaminants of interest. The analyses revealed the presence of only
510 a limited number of contaminants above the SDL, but never exceeding current MRLs, established for edible matrices.
511 Not more than four contaminants, including paracetamol, salicylic acid, metoprolol tartrate and zearalenone were
512 regularly detected in the insect samples. This preliminary screening indicates limited chemical risks related to the
513 consumption of insect tissues. Gaining more insights in the chemical food safety of insects will contribute to the general
514 acceptance of entomophagy as alternative to the increasing food demand.

515

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521

522 6. CONFLICT OF INTEREST

523 The authors declare that they have no conflict of interest.

524

525 7. APPENDIX

526 **Table S1.** Additional information on the (veterinary) drugs included in this methodology. **Table S2.** Additional
527 information on the pesticides included in this methodology. **Table S3.** Additional information on the mycotoxins included
528 in this methodology. **Table S4.** In-house hormone database (n = 74) used for suspect screening. **Table S5.** In-house
529 database (n = 101- for additional pesticides and (veterinary) drugs, used for suspect screening. **Table S6.** Information on

530 the real insect samples (Figure S1). **Table S7.** Tested parameters for the preliminary screening. **Table S8.** Detailed results
531 of the method optimization by applying a 2-level fractional factorial resolution IV experimental design (n = 20). **Table**
532 **S9.** Physico-chemical properties of the target analytes. **Table S10.** Results of the qualitative validation: screening
533 detection limits in spiked insect matrices and their detection. The compounds are classified in increasing SDL order,
534 together with their respective internal standards (ISTD) and MRL-values. **Table S11.** Validation results; relative standard
535 deviation (RSD) on precision and the average recovery after fortification with 1 x SDL and 2 x SDL. **Table S12.** Results
536 of the untargeted metabolomics. **Figure S1.** Industrial rearing process of insects. **Figure S2.** Frozen yellow mealworm
537 prior to lyophilization (4x). **Figure S3.** Frozen yellow mealworm prior to lyophilization (40x). **Figure S4.** Frozen yellow
538 mealworm prior to lyophilization (100x). **Figure S5.** Yellow mealworm following lyophilization (4x). **Figure S6.** Yellow
539 mealworm following lyophilization (40x). **Figure S7.** Yellow mealworm following lyophilization (100x). **Figure S8.**
540 Yellow mealworm following homogenization by pestle and mortar (4x). **Figure S9.** Yellow mealworm following
541 homogenization by pestle and mortar (40x). **Figure S10.** Yellow mealworm following homogenization by pestle and
542 mortar (100x). **Figure S11.** Yellow mealworm following the first SLE with ACN (4x). **Figure S12.** Yellow mealworm
543 following the first SLE with ACN (40x). **Figure S13.** Yellow mealworm following the first SLE with ACN (100x).
544 **Figure S14.** Yellow mealworm following the second SLE with ethyl acetate (4x). **Figure S15.** Yellow mealworm
545 following the second SLE with ethyl acetate (40x). **Figure S16.** Yellow mealworm following the second SLE with ethyl
546 acetate (100x). **Figure S17.** Finalized extraction protocol. **Figure S18.** Chromatographic separation of (veterinary)
547 drugs. **Figure S19.** Chromatographic separation of pesticides. **Figure S20.** Chromatographic separation of
548 mycotoxins. **Figure S21.** Optimization of MS-parameters, with the evaluation of the optimum resolution and AGC-
549 target, based on the total number of recovered compounds and the summarized normalized area. **Figure S22.**
550 Fractional factorial resolution IV experimental design (FFR)-design for the optimization of the MS parameters.
551 **Figure S23.** LC-MS overview of the screening results in the lesser mealworm. **Figure S24.** LC-MS overview of the
552 screening results in the black soldier fly. **Figure S25.** LC-MS overview of the screening results in the house cricket.
553 **Figure S26.** LC-MS overview of the screening results in the grass hopper. **Figure S27.** Heat map of the suspect screening
554 using our in-house hormone database. **Figure S28.** Heat map of the suspect screening using our in-house database on
555 additional pesticides and (veterinary) drugs.

556

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658

659 9. FIGURE CAPTIONS

660 **Figure 1.** Response surface model (RSM), using a central composited faced-centered design of the mass spectrometric
 661 parameters sweep gas and S-lens (red = most optimal combination of parameters; blue = less optimal combination).

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 664 **Figure 2.** Detailed results of the method optimization by applying a 2-level fractional factorial resolution IV
 665 experimental design (n = 20).

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 667

668 **Figure 3.** Semi-quantitative validation results for pesticides, (veterinary) drugs and mycotoxins over the four matrices:
 669 average ($\pm$ SD) within-day precision determined at 1 x SDL and 2 x SDL (n = 6), expressed as relative standard
 670 deviation (RSD, %)).

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 672
 673 **Figure 4.** Semi-quantitative validation results for pesticides, (veterinary) drugs and mycotoxins over the four matrices:
 674 average ($\pm$ SD) within-day recovery after fortification with 1 x SDL and 2 x SDL (n = 6) (in %).

676 **Table 1.** Summary of the semi-quantitative validation results; relative standard deviation (RSD) on precision and the
677 average recovery after fortification with 1 x SDL and 2 x SDL (SD = Standard Deviation).

Compounds (%) with RSD below 30 % *Compounds (%) with recovery 70 - 120 %* *Mean RSD (%) ± SD* *Mean recovery (%) ± SD*

<i>Pesticides</i>	91.5 %	59.7 %	21.3 ± 8.5	104.0 ± 25.2
<i>(Veterinary) Drugs</i>	51.2 %	43.0 %	32.1 ± 15.4	116.6 ± 50.0
<i>Mycotoxins</i>	75.4 %	44.6 %	26.0 ± 12.0	125.9 ± 71.0

678

679 **Table 2.** Estimated concentrations of residues (semi-quantitatively determined), detected in the real samples, per matrix,
680 the number of samples the residue was detected in, between brackets (NF = Not found).

Lesser mealworm *Black soldier fly* *House cricket* *Grasshopper*
µg kg⁻¹ (n = 6) *µg kg⁻¹ (n = 3)* *µg kg⁻¹ (n = 6)* *µg kg⁻¹ (n = 1)*

<i>Salicylic acid</i>	> 1 (6/6)	3 (3/3)	1 (6/6)	1 (1/1)
<i>Alternariol</i>	< 15 (5/6)	NF	NF	NF
<i>Alternariol methyl ether</i>	NF	NF	< 100 (3/6)	NF
<i>HT2-Toxin</i>	> 15 (3/6)	NF	NF	NF
<i>Isoproturon</i>	NF	NF	NF	< 1 (1/1)
<i>Metoprolol</i>	> 1 (6/6)	< 3 (3/3)	1 (1/6)	NF
<i>Nicarbazin</i>	> 100 (6/6)	NF	NF	> 100 (1/1)
<i>Nivalenol</i>	NF	NF	NF	3 - 6 (1/1)
<i>Paracetamol</i>	NF	NF	< 60 (6/6)	NF
<i>Roquefortine C</i>	< 30 (1/6)	NF	NF	NF
<i>Zearalenone</i>	NF	60 (3/3)	NF	NF

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