Pesticide and veterinary drug residues in honey - validation of methods and a survey of organic and conventional honeys from Slovenia

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Pesticide and veterinary drug residues in honey - validation of methods and a survey of organic and conventional honeys from Slovenia

Four analytical methods for the determination of veterinary drug residues, as well as environmental pesticide residues in honey, were introduced and validated: a) the GC/MS method for the analysis of amitraz and all metabolites containing the 2,4-dimethylaniline moiety, b) the GC/MS method for the analysis of thymol, chlorfenvinphos and coumaphos, c) the GC/MS method for the analysis of 75 active substances and d) the LC-MS/MS method for the analysis of 60 active substances. Between GC/MS (point c) and LC-MS/MS method (point d) there was no overlap among active substances, meaning that with both methods 135 active substances originating from the environment in total were introduced and validated. The first method included hydrolysis of amitraz and its metabolites containing the 2,4-dimethylaniline moiety to 2,4-dimethylanilin and extraction of 2,4-dimethylanilin to n-hexane. The other three methods had the same extraction procedure with a mixture of solvents: acetone, dichloromethane and petroleum ether. All 4 methods were tested in practice. 60 samples were analysed: 22 from organic and 38 from conventional production. Overall, residues are mainly higher than reported in literature but do not exceed MRLs. Calculation of the risk assessment confirmed that the analysed samples are of no cause for concern for consumers.

Keywords: honey; organic honey; conventional honey; acaricides; pesticide residues; GC/MS; LC-MS/MS

Introduction

Nowadays, dietitians recommend honey not only because of its nutritional properties, but also as a natural sweetener, which can be used by diabetics as well. Consumers demand safe and high quality products, therefore analysis of honey on pesticide residues is required.

Calatayud-Vernich et al. (2016) found in honeybees, pesticide residues from two sources: the ones originating from the environment and the ones originating from veterinary drug use. This means that both types of pesticides can be found in honey as well.

Veterinary practices in conventional production expose honeybees to acaricides such as amitraz and coumaphos. These substances control *Varroa destructor* and *Varroa jacobsoni*, mites that attack honeybees, described by Anderson and Trueman (2000). In the case of organic honey production, only thymol, menthol, eucalyptol, camphor, formic acid, lactic acid, acetic acid and oxalic acid are used to supress varroa as laid down in Regulation (EC) 889/2008.

In both types of production (conventional and ecological) beekeepers cannot avoid possible contamination from the environment. Honeybees fly 4.8 km from their apiary (Eckert, 1933) during which they can pick contaminants from plants, soil and air (Zhou, 2018). Plant protection products (PPPs) do not only reach treated plants, but also drift to soil and air. In the air, aerosols are formed, containing PPP residues which can travel and deposit in surroundings where they can come into contact with bees.

In the European Union, maximum residue limits (MRLs) are set for both types of contaminants. In Regulation (EC) 396/2005, MRLs are set for pesticides from the environment. In Regulation (EC) 37/2010, MRLs are set for pharmacologically active substances.

Chiesa et al. (2016) already measured the active substances: aldrin, boscalid, captan, chlorpyrifos, coumaphos, diazinon, dieldrin, endrin, heptachlor, iprodione, methoxychlor, mevinphos, p,p'-DDD, p,p'-DDE, p,p'-DDT, quinoxyfen, and trifloxystrobin in organic honey samples from Italy. On the contrary, Panseri et al. (2014) found no pesticide residues in Italian honey from organic production. The

exceptions were Italian honey samples in the vicinity of apple orchards or industrialised areas, and from the market, where boscalid, captan, chlorpyrifos, dieldrin, heptachlor, iprodione, methoxychlor, p,p'-DDE, p,p'-DDD, p,p'-DDT, quinoxyfen and trifloxystrobin were found. In Polish honey, Bargańska et al. (2013) found the active substances azinphos-ethyl, azinphos-methyl, bifenthrin, carfentrazone-ethyl, chloridazon, coumaphos, diazinon, dimethoate, dimoxystrobin, fenpyroximate, haloxyfop-R-methyl, heptenophos, imidacloprid, indoxacarb, methidathion, methiocarb, methomyl, omethoate, oxamyl, oxydemeton-methyl, pirimicarb, profenofos, pyrazophos, qualiafos, spinosad, temephos, thiamethoxam and triazophos. Furthermore, Kujawski and Namieśnik (2011) found clothianidin, carfentrazone ethyl, fluroxypyr-meptyl, methidathion, imazalil and chlorpyrifos in Polish honey samples. Numerous active substances found in honey from Italy and Poland were included in our analytical methods.

The determination of environmental pesticide residues and veterinary drug residues in honey is nowadays performed with very sensitive equipment, like gas chromatography coupled with tandem mass spectrometry (GC/MS/MS) (Shendy et al. 2016), or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Jin et al. 2017; Juan-Borrás et al. 2016; Tette et al. 2016). Extraction for GC/MS/MS and LC-MS/MS determination is usually performed with acetonitrile, with the modified QuEChERs method (Jin et al. 2017; Juan-Borrás et al. 2016; Shendy et al. 2016; Tette et al. 2016). On the contrary, some laboratories still use extraction with large volumes of organic solvents like ethyl acetate and/or methanol (Panseri et al. 2014; Rodríguez López et al. 2014), dispersive liquid-liquid microextraction (DLLME) (Farajzadeh et al. 2014; Zacharis et al. 2012) or solid phase extraction (SPE) combined with DLLME (Shamsipur et al. 2016,) followed by determination with a gas chromatograph coupled

with a mass spectrometer (GC/MS) (Shamsipur et al. 2016; Wang et al. 2010; Zacharis et al. 2012), electron capture detector (ECD) (Malhat et al. 2015; Zacharis et al. 2012) or nitrogen phosphorus detector (NPD) (Farajzadeh et al. 2014; Rodríguez López et al. 2014).

Each solvent used for liquid-liquid extraction has some advanatages and some disadvantages. The weakness of solvent ethyl acetate are high co-extraction of interferences from matrix (Andersson and Pålsheden1991) and its non miscibility with water. The strength of ethyl acetate is that it gives good recoveries for numerous active substances with different properties (from polar like methamidophos to non-polar like chlorpyriphos) as observed by Vidal et al. (2006). To even improve its extraction abilities to polar active compounds, methanol can be added (Rodríguez López et al. 2014). The weakness of solvent acetonitrile is that in comparison with ethyl acetate and acetone it is less volatile, meaning that it takes longer to evaporate in injector of GC. Its strength is that in comparison to acetone and ethyl acetate it does not co-extract lipophilic materials such as wax, fat and lipophilic pigments (Anastassiades et al. 2003).

The drawback of liquid-liquid extraction is that large volumes of organic solvents are used. On the contrary in DLLME active substances are extracted with low volumes of an extraction solvent and a disperser solvent, meaning that the methods are environment friendly and low cost (Rezaee et al. 2006). The main weakness of DLLME method is, that it is suitable for trace analyses in range up to 0.05 mg kg^{-1} (Zacharis et al. 2012). DLLME in combination with SPE provides high preconcentration factors and is suitable for ultra-trace analyses up to 0.01 $mgkg^{-1}$ (Shamsipur et al. 2016). Since numerous MRLs for active substances in honey are 0.05 mg/kg, DLLME method is not suitable for measurement of possible MRL exceedances for numerous active substances.

In our laboratory, we introduced the single residue method for amitraz and all its metabolites containing the 2,4-dimethylaniline moiety, with GC/MS determination. For environmental pesticide residues, one extraction method was introduced for multiresidual GC/MS and LC-MS/MS determination. We have chosen liquid-liquid extraction with acetone, with purpose to introduce analytical method for measuring conformity of pesticide residues with valid MRLs. The strength of acetone is that it is more volatile than acetonitrile and it is therefore easier to concentrate it and remove it than acetonitrile. Besides, at extraction of materials containing high amount of sugar with acetone, no double layered extract is obtained like with acetonitrile (Luke et al. 1975). To acetone, dichloromethane and petroleum ether were added as extraction solvents, to achieve the extraction of very polar (for instance, thiamethoxam and trichlorfon) to non-polar (for instance, chlorpyrifos, cyhalothrin-lambda) pesticides at the same time. The same extraction procedure was tested and found to be suitable for the extraction of veterinary drug residues: thymol and coumaphos, and residues of acaricide chlorfenvinphos where determination was performed with GC/MS.

Once the methods were introduced, 60 samples of honey originating from organic and conventional production in Slovenia were analysed with all the methods presented in this paper and compared with data from literature. A risk assessment was performed for active substances found in honey samples.

Materials and methods

Sample collection

30 honey samples were collected in June and July 2017 and 30 in June and July 2018,

from Slovenian beekeepers from all 12 statistical regions in Slovenia. Sampling distribution is presented in Table 1. 38 samples originated from conventional production, meaning that beekeepers had the opportunity to use all registered veterinary products to supress varroa and 22 samples from organic production, meaning that beekeepers had the opportunity to use only thymol, formic acid and oxalic acid to supress varroa. Slovenia is one of the smallest producers of honey in the European Union, therefore in our survey we had a limited number of samples. Especially limited is the set of organic samples, since beekeepers in Slovenia rarely use only products approved in this type of production.

Chemicals and reagents

The certified standards were either from Sigma-Aldrich (Steinheim, Germany), or from Dr. Ehrenstorfer (Augsburg, Germany). Acetone p.a., acetone HPLC-grade, dichloromethane p.a., petroleum ether p.a., ethyl acetate p.a., ethyl acetate HPLC-grade, cyclohexane p.a., cyclohexane HPLC-grade, methanol p.a., methanol HPLC-MS-grade, n-hexane HPLC-grade were from J.T.Baker (Deventer, Netherlands). All other chemicals used were from Sigma-Aldrich (Steinheim, Germany). The water used was MilliQ deionised water.

For the preparation of the standard solutions for GC/MS, solvents of HPLC-grade were used. For the preparation of the standard solutions for LC-MS/MS, solvents of HPLC-MS grade were used.

Determination of amitraz and all metabolites containing the 2,4-dimethylaniline moiety

With this method, the samples were analysed immediately after arrival at the laboratory.

Preparation of solutions

Stock solutions of amitraz and 2,4-dimethylaniline (2,4 DMA) in acetone HPLC-grade were prepared with a concentration of 1000 μg mL^{-1}. From stock solutions, working solutions of amitraz and 2,4 DMA were prepared at the concentrations 100 μg mL^{-1} and 10 μg mL^{-1} respectively. From the working solution of 2,4 DMA (10 μg mL^{-1}), matrix match standards were prepared for calibration when samples were analysed, for linearity and LOQ determination. The working solution of amitraz (10 μg mL^{-1}) was used for determining other validation parameters.

Hydrolysis and extraction procedure

To 10g of honey, 40 mL of 2N HCl was added. The contents of the beaker were stirred for 1 hour. Afterwards, 2M NaOH was added until the solution reached pH 11. Then, 20 mL of n-hexane was added and the mixture was transferred into the separatory funnel. The water phase was re-extracted once again. Organic phases were collected and n-hexane was added until the final volume was 40 mL. 1.5 mL of extract was transferred into a tube. Afterwards, heptafluorobutyric anhydride (HFBA) was added. Derivatisation took place at room temperature for 5 minutes. Excess of HFBA was removed with 4 mL of 1M Na_2CO_3 water solution. The organic phase was transferred to a vial for GC/MS determination.

Determination with GC/MS

The samples were analysed using a gas chromatograph (Hewlett Packard 6890, Böblingen, Germany) and column, an DB-2255 MS (Agilent Technologies, 30 m, 0.25 mm i.d., 0.25 μm film thickness) with a constant flow of helium at 1.2 mL min^{-1}. The GC oven was programmed as follows: 50°C for 1 min, from 50 to 220°C at 4°C min^{-1} and held at 220°C for 5 min. For the determination of analytes, a mass spectrometer (Hewlett Packard 5973, Palo Alto, CA, USA) was used. The temperature of the ion source was 230°C, the auxiliary temperature was 240°C, and the quadrupole temperature was 150°C. For qualitative determination, retention time and mass spectrum in a selective ion monitoring mode (SIM) were used. One target and 2 qualifier ions, presented in Table 2, were used for the active substance. Calibration was performed to matrix match standards.

Determination of chlorfenvinphos, coumaphos and thymol

With this method, the samples were analysed immediately after arrival at the laboratory.

Preparation of solutions

Stock solutions in a mixture of ethyl acetate and cyclohexane at a ratio of 1 to 1 (v/v) of the individual active substances were prepared with the concentrations 100 μg pesticide mL^{-1}. From 3 stock solutions, three mixed solutions, each containing all 3 active substances, were prepared: one with a concentration of 5 μg mL^{-1} for all 3 active substances, the second wth a concentration of 5 μg mL^{-1} for chlorfenvinphos, 4.5 μg mL^{-1} for coumaphos and 35 μg mL^{-1} for thymol, and the third one at the limit of quantification (LOQ) of the active substances. From the first solution, diluted solutions

were prepared for calibration when samples were analysed for LOQ determination. The second solution was used for the preparation of diluted solutions for linearity determination. The third solution was used for determining other validation parameters.

Extraction procedure

20 g of honey and 15 ml of MilliQ water was added to the beaker. The honey was dissolved in the water, then 40 ml of acetone p.a. was added. The mixture was homogenised for 2 minutes with a mixer. Then, an 80 ml mixture of petroleum ether p.a. and dichloromethane p.a. at a ratio 1:1 (v/v) was added and mixed for another 2 minutes with a mixer. This mixture was transferred into the separatory funnel, containing 3g of NaCl. The vessel was rinsed with an 80 ml mixture of petroleum ether p.a. and dichloromethane p.a. at a ratio 1:1 (v/v). The solvent was added to the separatory funnel, which was shaken for 1 minute. The upper organic phase was filtered through 15g anhydrous Na_2SO_4 in a 500 ml Soxhlet flask. The lower water phase was re-extracted twice using the same procedure. Solvents in collected organic phases were evaporated to approximately 2 ml on rotavapor and dried with nitrogen flow. To dry the eluate, 2 ml of the mixture of ethyl acetate p.a. and cyclohexane p.a. at a ratio of 1:1 (v/v) was added in the case of a sample preparation. In the case of matrix match standards, 2ml of working solutions with proper concentrations were added.

Determination with GC/MS

The samples were analysed using a gas chromatograph (Agilent Technologies 7890A, Shanghai, China) equipped with a Gerstel MPS2 multipurpose sampler (Gerstel, Mülheim an der Ruhr, Germany) and column, an HP-5 MS (Agilent Technologies, 30 m, 0.25 mm i.d., 0.25 μm film thickness) with a constant flow of helium at 1.2 mL min^{-}

[1]. The GC oven was programmed as follows: 40°C for 1 min, from 40 to 150°C at 20°C min^{-1}, from 150 to 200°C at 5°C min^{-1}, from 200 to 280°C at 10°C min^{-1} then held at 280°C for 20 min. For the determination of analytes, a mass spectrometer (Agilent Technologies 5975C, upgraded with a triple-axis detector, Palo Alto, CA, USA) was used. The temperature of the ion source was 230°C, the auxiliary temperature was 280°C and the quadrupole temperature was 150°C. For qualitative determination, retention time and mass spectrum in SIM were used. One target and 2-3 qualifier ions, presented in Table 2, were used for each active substance. The calibration was performed to matrix match standards.

Multiresidual GC/MS method

With this method, the samples were analysed within a maximum period of six months after arrival at the laboratory. During that time, they were stored at -20°C.

Preparation of solutions

Stock solutions in a mixture of ethyl acetate and cyclohexane at a ratio of 1 to 1 (v/v) of individual active substances were prepared with the concentrations 625 µg pesticide mL^{-1}. From 75 stock solutions, two mix solutions of all 75 active substances were prepared: one with a concentration of 5 µg mL^{-1} and the second one at LOQ of active substances. All solutions used to determine linearity, LOQs and perform calibration when samples were analysed were prepared from a mix solution of 5 µg mL^{-1} with proper dilutions. For other validation parameters, a mixed solution with a concentration at LOQ was used.

Extraction procedure

The extraction procedure is the same as for the determination of chlorfenvinphos, coumaphos and thymol.

Determination with GC/MS

The samples were analysed using a gas chromatograph (Agilent Technologies 7890A, Shanghai, China) equipped with a Gerstel MPS2 multipurpose sampler (Gerstel, Mülheim an der Ruhr, Germany) and column, an HP-5 MS UI (Agilent Technologies, 30 m, 0.25 mm i.d., 0.25 µm film thickness) with a constant flow of helium at 1.2 mL min^{-1}. The GC oven was programmed as follows: 55°C for 2 min, from 55 to 130°C at 25°C min^{-1}, held at 130°C for 1 min, from 130 to 180°C at 5°C min^{-1}, held at 180°C for 30 min, from 180 to 230°C at 20°C min^{-1}, held at 230°C for 16 min, from 230 to 250°C at 20°C min^{-1}, held at 250°C for 13 min, from 250 to 280°C at 20°C min^{-1}, held at 280°C for 20 min. For the determination of analytes, a mass spectrometer (Agilent Technologies 5975C, upgraded with a triple-axis detector, Palo Alto, CA, USA) was used. The temperature of the ion source was 230°C, the auxiliary temperature was 280°C and the quadrupole temperature was 150°C. For qualitative determination, retention time and mass spectrum in SIM were used. One target and 2-3 qualifier ions, presented in Table 2, were used for each active substance. The calibration was performed to matrix match standards.

Multiresidual LC-MS/MS method

With this method, the samples were analysed within in a maximum period of six months after arrival at the laboratory. During this time, they were stored at -20°C.

Preparation of solutions

Stock solutions of individual active substances in methanol were prepared with the concentrations 625 µg pesticide mL^{-1}. From 60 stock solutions, two mix solutions of all 60 active substances were prepared: one with a concentration of 3.125 µg mL^{-1} and the second one at LOQ of active substances. All solutions used to determine linearity, LOQs and perform calibration when samples were analysed, were prepared from a mix solution of 3.125 µg mL^{-1} with proper dilutions. For other validation parameters, a mixed solution with a concentration at LOQ was used.

Extraction procedure

The extraction procedure is the same as for the determination of chlorfenvinphos, coumaphos and thymol, except that to dry eluate, 2 ml of methanol p.a. was added in the case of a sample preparation. In the case of matrix match standards, 2 ml of working solutions with proper concentrations were added.

Determination with LC-MS/MS

The samples were analysed using a liquid chromatograph (Agilent Infinity 1290, Palo Ato, USA) on a Titan™ C18 80A column (10 cm x 2.1 mm, 1.9 µm), Supelco with the gradient of 0.1% formic acid (A) and 0.1% formic methanol (B). Each sample was injected twice, once in ESI+ and one in ESI- mode. For ESI+ mode, the flow was 0.4 mL min^{-1} and the gradient was as follows: start at 3% B and hold for 3 min, increase to 100% B in 17 min, hold 100% B for 5 minutes, decrease to 3% B in 3 minutes, post run 2 minutes at 3% B. For ESI- mode, the flow was 0.5 mL min^{-1} and the gradient was as follows: start at 15% B, increase to 70% B in 4 min, increase to 100 B in 1 minute, hold 100% B for 1 minutes, decrease to 15% B in 1 minute, post run 3 minutes at 3% B. For

the determination of analytes, a triple quadrupole mass spectrometer Agilent 6460 (Agilent Technologies Palo Alto, CA, USA) was used. The source temperature was 250°C, gas flow 6 L min^{-1}, sheath gas flow 10 L min^{-1}, sheath gas temperature 375°C and nebuliser pressure 35 psi. Quadropole temperatures were 100°C. For each compound, two transitions were monitored, and therefore fragmentor and collision cell parameters were optimised. The data on possible MRM transitions were found in EURL Pesticides Data pool accessible on the internet at https://www.eurl-pesticides-datapool.eu/. Optimisation of fragmentor and collision cell voltages was performed using Agilent Optimizer software and standard solutions of active substances in methanol (1 mg L^{-1}). For quantitative determination, retention time and Multi Reaction Monitoring (MRM), peak area ratios were used. MRM transitions, fragmentor and collision energy are presented in Table 3. The calibration was performed to matrix match standards.

Validation of methods

LOQ and linearity

The linearity was verified by using the matrix match standards (five repetitions for one concentration level, three to eight concentration levels for the calibration curve). The linearity and range were determined by linear regression, using the F test.

LOQs were estimated from chromatograms of matrix match standards. LOQs were chosen at S/N = 10 at least.

MRLs for environmental pesticide residues are set in Regulation (EC) 396/2005. When MRLs are set at LOQ of the analytical method (this LOQ was gathered by different laboratories), in Regulation, * is added to mark this fact. Therefore, in cases where MRLs were marked with *, some of our LOQs were set at those MRLs.

Accuracy

Checking the recoveries was used to verify accuracy. Blank honey was bought in store and analysed to prove that it contains no pesticide residues. Ten extracts of spiked blank honey were prepared for each spiking level, in the shortest period possible. Each extract was injected twice. The average of recoveries was calculated. According to requirements for method validation procedures (SANTE/11813/2017), acceptable mean recoveries are those within the range 70-120%, with an associated repeatability of RSDr ≤ 20%.

According to the guidelines for single-laboratory validation (Alder et al. 2000), acceptable mean recoveries are:

- at level >0.01 mg kg^{-1} ≤ 0.1 mg kg^{-1} are those within the range 70-120%, with an associated repeatability RSDr ≤ 20% and
- at level >0.001 mg kg^{-1} ≤ 0.01 mg kg^{-1} are those within the range 60-120%, with an associated repeatability RSDr ≤ 30%.

The accuracy was also checked with participation in a proficiency testing scheme organised by BIPEA (Bureau interprofessionnel d´études analytiques).

Precision

For the determination of precision (ISO 5725), i.e. repeatability and reproducibility, the extracts of spiked blank honey were analysed at LOQ. Within a period of 10 days, two parallel extracts were prepared each day for each concentration level. Each one was injected once. Then, the standard deviation of the repeatability of the level and the standard deviation of reproducibility of the level were both calculated.

Uncertainty of repeatability and uncertainty of reproducibility

The uncertainty of repeatability and the uncertainty of reproducibility were calculated by multiplying the standard deviation of repeatability and standard deviation of reproducibility by the Student's t factor, for 9 degrees of freedom and a 95% confidence level ($t_{95;9} = 2.262$).

$U_r = t_{95;9} \times s_r$; $U_R = t_{95;9} \times s_R$

The measurement uncertainty for PPP residues should be 50%, as proposed in SANTE/11813/2017. With validation, analysts must prove that their measurement uncertainty is below or equal to the proposed measurement uncertainty.

Consumer risk assessment

Chronic exposure

The calculation of long-term exposure was performed with the EFSA PRIMo model revision 3, accessible on the internet at https://www.efsa.europa.eu/en/applications/pesticides/tools. The Supervised Trial Median Residue (STMR) was calculated from all samples analysed. It was compared to the Acceptable Daily Intake (ADI) of a single active substance. Chronic consumer exposure was expressed in % of the ADI. The acceptable limit for long-term exposure is 100% of the ADI.

Acute exposure

The calculation of short-term exposure was performed with the EFSA PRIMo model revision 3, accessible on the internet at https://www.efsa.europa.eu/en/applications/pesticides/tools.

The Highest Residue (HR) was compared to the Acute Reference Dose (ARfD) of a single active substance. Acute consumer exposure was expressed in % of ARfD. The acceptable limit for short-term exposure is 100% of the ARfD.

Results and discussion

Comparison of method for determination of amitraz and all metabolites containing the 2,4-dimethylaniline moiety from literature to our procedure

Our hydrolysis and extraction procedure is a modified procedure, as described by Jiménez et al. (2002). Our laboratory sample was bigger (10 g instead of 1 g), our pH adjustment was performed firstly with HCl and then with NaOH, instead of a one step adjustment with aqueous solution with pH 11. Our extraction with n-hexane took place in a separatory funnel instead by sonication. Afterwards in our procedure, hydrolysis with NaOH at 90°C was not performed. Hydrolysis in our procedure took place during pH adjustment at room temperature. Finally in both procedures, HFBA was used for derivatisation.

Validation of methods

LOQ and linearity

The linear model fits for all active substances presented in Tables 2 and 3. In Table 4 is presented how many substances have linear response in certain range for GC/MS and LC-MS/MS determination. In general linearity was checked in the range 0.01 – 0.2 mg kg^{-1} for GC/MS and in range 0.003-0.4 mg/kg for LC-MS/MS. R^2 ranged from 0.960 to 0.988 for GC/MS determination and from 0.991 to 0.999 for LC-MS/MS determination.

As expected, linearity ranges for active substances determined with GC/MS are mainly in a higher concentration range than the ones for active substances determined with LC-MS/MS.

LOQs are presented in Tables 2 and 3. For GC/MS determination 16 active substances have LOQ 0.01mg kg^{-1}, 6 of them 0.02mg kg^{-1}, 3 of them 0.03 mg kg^{-1} and 50 of them 0.05 mg kg^{-1}. For LC-MS/MS determination 1 active substance has LOQ 0.003 mg kg^{-1}, 46 of them 0.005 mg kg^{-1} and 13 of them 0.01 mg kg^{-1}. LOQs are lower or equal to MRLs set in Regulation (EC) 396/2005 and Regulation (EC) 37/2010.

As expected, LOQs for active substances determined with GC/MS are mainly substantially higher than the ones for active substances determined with LC-MS/MS.

Accuracy

Results for recoveries are given in Tables 2 and 3. Recoveries at LOQs for active substances scanned with GC/MS are in the range of 77.4 to 99.2%, with RSDs 5.4 to 16.7%. More precisely, recoveries at LOQs 0.01 mg kg^{-1} are within the range of 77.5 to 94.2% with RSDs 5.7 to 11.7% and recoveries at LOQs > 0.01 to ≤ 0.05 mg kg^{-1} are within the range of 77.4 to 99.2% with RSDs 5.4 to 16.7%.

Recoveries at LOQs, which are in the range of 0.003 to 0.01 mg kg^{-1}, for active substances scanned with LC-MS/MS are in the range of 83.1 to 103.7%, with RSDs 5.8 to 13.1%.

All recoveries and RSDs are within the required ranges from literature (Alder et al. 2000; SANTE/11813/2017).

Recoveries for active substances determined with GC/MS are slightly lower than the ones determined with LC-MS/MS.

Accuracy was also checked by collaboration in the inter-laboratory proficiency test BIPEA. All our results are in the required range (-2≥ z ≤2). 15 of 16 active

substances (93.8% of analysed active substances) have z value in the range -1 to 1, which is satisfactory. Only 1 active substance (acrinathrin) has z value lower than -1, but it is still in the required range for correct result. Results are presented in Figure 1.

Uncertainty of repeatability and uncertainty of reproducibility

Uncertainty of repeatability and uncertainty of reproducibility were determined at contents equal to LOQs. Results are presented in Tables 2 and 3. For GC/MS determination of active substances, uncertainty of repeatability ranging from 0.0007 to 0.01 mg kg^{-1}, which is 7.0 to 25.0% of LOQ and uncertainty of reproducibility ranging from 0.0007 to 0.02 mg kg^{-1}, which is 7.0 to 40.0% of LOQ.

For LC-MS/MS determination of active substances, uncertainty of repeatability ranging from 0.0003 to 0.0015 mg kg^{-1}, which is 6.2 to 18.0% of LOQ and uncertainty of reproducibility ranging from 0.0001 to 0.009 mg kg^{-1}, which is 13.4 to 29.8% of LOQ.

The highest percentages of uncertainties of repeatability and of reproducibility are higher for active substances determined with GC/MS than for the ones determined with LC-MS/MS.

Choosing of methods

According to Regulation (EC) 37/2010, amitraz has to be analysed as amitraz and all metabolites containing the 2,4-dimethylaniline moiety. This cannot be achieved with multiresidual method. Amitraz and metabolites in sample must be exposed to hydrolisys and derivatised to 2,4-dimethylaniline to enable measurement of valid residue definition. This is why the single residue method was introduced for amitraz.

Beside method for amitraz, our laboratory firstly introduced GC/MS and LC-MS/MS multiresidual methods for determination of environmental pesticide residues. GC/MS method was tested for 92 active compounds in one chromatographic run. When validation took place 75 active substances passed all validation criteria. Since this method is used in laboratory for analyses of fruit and vegetables as well, active substances with unsatisfactory results were not removed from chromatographic run. Analyses of these active substances was found to be suitable for anylses of fruit and vegetables. 92 compounds in one chromatographic run resulted in crowd, which did not allow to insert 3 more substances. This is why separate chromatographic run was set for thymol, chlorfenvinphos and coumaphos.

Applicability of methods

The methods described above can be used in every laboratory dealing with pesticide residues and acaricide residues in honey. All methods are simple to perform. The main advantage is that the three methods have practically the same extraction procedure, meaning that the time needed for the preparation of samples can be substantially reduced. The second advantage is that a laboratory performing these methods does not need to be equipped with the GC/MS/MS or GC/MS with large volume injection, as in the case of the QuEChERS method. During the extraction procedure, analytes are concentrated to such a level, that the determination with the GC/MS is sensitive enough to find out conformity with the valid MRLs. The third advantage is that the method can be extended to a larger scope of the active substances than presented in this paper. The

only disadvantage of the methods is that they require larger volumes of organic solvents, in comparison to the QuEChERS method.

Survey of pesticide residues in honey samples

The Slovenian Beekeepers' Association announced that in Slovenia, 11 veterinary drug residues, containing 6 active substances, are authorised to supress varroa. In our survey, we were checking for the presence of residues of most broadly used active substances: amitraz, coumaphos and thymol. Chlorfenvinphos was included, after ministry suspicion of its unauthorised use in the year 2016.

The Ministry of Agriculture, Forestry and Food reported that in Slovenia, 568 PPPs, containing 208 active substances, are authorised for use on different agricultural products. The Statistical office announced that in the year 2017, 1,087 tons of active substances were sold in Slovenia, where we have 476,000 hectares of cultivated agricultural area. This suggests broad use of PPPs among farmers. Since bees collect pollen, not only on flowers, acacia, spruce, sage, lime and chestnut, but also on agricultural products treated with PPPs, like oilseed rape, fruits,…, we wanted to research if that kind of pesticide residues are found in honey as well. We were searching for authorised (50% of active substances sought) and non-authorised active substances in Slovenia, to cover the possible misuse of PPPs.

The survey results are presented in Table 5 and Figure 2. In samples from organic production, the active substance amitraz was found in 2 samples at a content of 0.01 $mgkg^{-1}$, representing 9.1% of the analysed samples from organic production. The active substance thymol was found in only 1 sample from organic production at a content of 0.43 $mgkg^{-1}$, representing 4.5% of the analysed samples from organic

production. It was expected that thymol would be present in larger amount of samples in organic production, but beekeepers obviously prefer the use of formic acid and oxalic acid to supress varroa. The active substance thiacloprid was found in only 1 sample from organic production at a content of 0.018 $mgkg^{-1}$, representing 4.5% of the analysed samples from organic production. In Slovenia, thiacloprid is authorised (among others) for use on oilseed rape, apples, pears and ornamentals. These are the plants on which bees collect pollen. In spite of the organic production of honey, in which thiacloprid was found, bees obviously collected thiacloprid residues in their environment and brought it to their hive. Other measured active substances originating from veterinary drugs and other ones originating from the environment were <LOQ.

In samples from conventional production, only the active substances amitraz, coumaphos and thymol were found ≥LOQ. Other measured active substances originating from veterinary drugs and the ones originating from the environment were <LOQ. In 9 samples from conventional production, residues were <LOQ. Amitraz was measured in 14 samples (36.8% of the analysed samples from conventional production). Coumaphos was measured in 13 samples (34.2% of the analysed samples from conventional production). Thymol was measured in only 3 samples (7.9% of the analysed samples from conventional production). The reason why thymol was found not only in organic, but also in conventional production is, that beekeepers use more than one veterinary drug to supress varroa on many occasions. Beekeepers from conventional production are not restricted only to veterinary drugs containing amitraz and coumaphos. Since thymol is allowed in organic production and it is considered of no cause for concern for consumers, it is videly used also among beekeepers from conventional production. Multiple residues, more precisely residues of amitraz and

coumaphos, were found only in 1 sample (2.6% of the analysed samples from conventional production).

No MRL exceedances were observed in organic or in conventional production. The highest residue determined for amitraz represented 60% of the valid MRL. The highest residue determined for coumaphos represented 55% of the valid MRL. The residue determined for thiacloprid represented 9% of the valid MRL.

A consumer risk assessment was performed with EFSA PRIMo model rev. 3.0, where 36 national diets from EU countries are included. This model was used, since Slovenia has not created its own food basket yet. In the process of registration of PPPs in Slovenia, the same model is used. Calculations were conducted for amitraz, thiacloprid and thymol. For coumaphos, no ADI and ARfD were set. In the case of amitraz in honey, the highest chronic exposure was observed in the German diet for children. It represented 0.1% of ADI. Acute exposure for amitraz in honey represented 4% of ARfD. In the case of thymol in honey, the highest chronic exposure was observed in the German diet for children. It represented 0.1% of ADI. Acute exposure for thymol in honey represented 2% of ARfD. In the case of thiacloprid in honey, the chronic exposure was 0% of ADI. Acute exposure for thiacloprid in honey represented 0.2% of ARfD. Based on these calculations, the conclusion was that the analysed honey samples are of no cause for concern for consumers.

Our results were compared with results from other scientific papers. Juan-Borrás et al. (2016) reported that amitraz was found in 100% of honey samples in Spain, with a content range of 0.002 – 0.050 mg kg^{-1}, and coumaphos was found in 63.6% of samples measured in a range of 0.001 - 0.013 mg kg^{-1}. Amitraz was present in a higher ratio of analysed samples than in Slovenia, but at a lower content range. Coumaphos was present in a slightly lower ratio of analysed samples than in Slovenia, but at a lower

content range. On the other hand, in the same paper it was reported that chlorfenvinphos was present in 36.4% of samples in the range of 0.001 – 0.008 mg kg^{-1}. In Slovenia, no chlorfenvinphos was found in this study. The reason for a high ratio of positive samples (mainly for amitraz and chlorfenvinphos) could be reporting results between LD and LOQ in a survey from Spain. Chiesa et al. (2016) observed coumaphos maximum content in honey samples in Italy of 0.00206 mg kg^{-1}. Coumaphos was found in approximately 42% of samples. Ratio of positive samples is lower than in Slovenia but maximum content is not. Wiest et al. (2011) measured a maximum amitraz content of 0.026 mg kg^{-1}. Amitraz was found in 4% of the French honey samples analysed. The maximum coumaphos content was 0.029 mg kg^{-1}. Coumaphos was found in 77% of French honey samples analysed. Amitraz maximum content, as well as the ratio of positive samples, is lower than in Slovenia. The reason for a lower ratio of positive samples could be the higher LOQ for amitraz in France than in Slovenia. On the other hand, the maximum coumaphos content is lower than in Slovenia, but the ratio of positive samples is higher, in spite of a higher LOQ for the active substance in France in comparison to Slovenia. Gbylik-Sikorska et al. (2015) found neonicotionoide clothianidin in honey from Poland at a maximum content of 0.1928 mg kg^{-1}, but no neonicotinoids imidacloprid and thiacloprid. On the contrary, in Slovenia we found neonicotinoide thiacloprid. Neoniciotinoide imidacloprid was not found in Slovenia as well. Valverde et al. (2018) found neonicotinoide thiamethoxam at a maximum content of 0.144 mg kg^{-1} in 21.4% of the analysed honey samples from Spain and neonicotinoide clothianidin at an approximate content of 0.045 mg kg^{-1} in 3.6% of the analysed honey samples from Spain, but no neonicotinoide thiacloprid. In Slovenia, neonicotinoide thiacloprid was found in 1.7% of all analysed samples in this survey and neonicotinoide thiamethoxam was found in none of them.

Conclusions

In our research, 2 methods for the determination of veterinary drugs were introduced and validated: a) for the determination of amitraz, b) for the determination of coumaphos and thymol. Second method included determination of chlorfenvinphos, acaricide, which can originate from environment. Additionally 2 methods for the determination of pesticide residues originating from the environment were introduced and validated: a) multiresidual GC/MS method for the determination of 75 active substances, b) multriresidual LC-MS/MS method for the determination of 60 active substances. These methods were found to be fit for purpose.

All the methods were used in the analysis of 60 honey samples from Slovenian beekeepers: 22 from organic production and 38 from conventional production. One pesticide residue originating from the environment was found in one of these samples (insecticide thiacloprid), , while about half of the samples contained residues of veterinary drugs: amitraz, coumaphos and/or thymol. Although the contents of amitraz and coumpahos were mainly higher than observed in literature, a risk assessment revealed that Slovenian honey samples are of no cause for concern for consumers.

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Table 1: Number of samples collected from different statistical regions in Slovenia In 2017 and 2018.

	Conventional production		Organic production		Sum
Statistical region	2017	2018	2017	2018	
Goriška	1	1	1	1	4
Notranje kraška	1	0	1	2	4
Zasavska	0	0	0	1	1
Obalno kraška	1	1	0	4	6
Gorenjska	1	0	0	0	1
Koroška	4	3	1	0	8
Jugovzhodna Slovenija	0	4	0	0	4
Podravska	4	4	3	0	11
Spodnje posavska	1	0	0	1	2
Pomurska	2	1	1	1	5
Osrednja Slovenija	1	1	2	1	5
Savinjska	4	3	1	1	9
Sum	20	18	10	12	60

Table 2. Validation parameters for GC/MS determination, ions scanned and MRLs

Active substance	MRL [a,b] (mg kg^{-1})	Ions scanned (m/z) T, Q_1, Q_2, Q_3	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)	U_r (refer to legend) (mg kg^{-1})	U_r (refer to legend) (%)	U_R (refer to legend) (mg kg^{-1})	U_R (refer to legend) (%)
acrinathrin	0.05*[a]	181, 208, 289	0.02-0.15	0.978	0.02	94.7	6.6	0.003	15.0	0.003	15.0
aldrin	0.01[a]	263, 265, 261	0.01-0.15	0.984	0.01	88.4	6.1	0.0008	8.0	0.0007	7.0
amitraz	0.2[b]	148, 317, 120	0.01-0.3	0.994	0.01	102.9	4.6	0.001	10.0	0.002	20.0
azinphos-methyl	/	160, 132, 105	0.01-0.15	0.984	0.01	81.2	7.2	0.001	10.0	0.001	10.0
azoxystrobin	0.05*[a]	344, 388, 345	0.05-0.15	0.976	0.05	83.5	13.9	0.010	20.0	0.010	20.0
bifenthrin	0.05*[a]	181, 165, 166	0.01-0.15	0.984	0.01	88.8	6.9	0.001	10.0	0.001	10.0
boscalid	0.05*[a]	140, 342, 142	0.05-0.15	0.963	0.05	83.7	8.8	0.008	16.0	0.009	18.0
bromopropylate	0.01*[a]	183, 341, 185	0.01-0.15	0.982	0.01	92.4	7.5	0.001	10.0	0.001	10.0
bupirimate	0.05*[a]	273, 316, 208	0.05-0.15	0.972	0.05	77.6	11.3	0.008	16.0	0.009	18.0
captan	0.05*[a]	79, 107, 119, 149	0.05-0.15	0.972	0.05	88.9	5.9	0.009	18.0	0.015	30.0
carbaryl	0.05*[a]	144, 115, 116	0.05-0.15	0.973	0.05	85.8	8.2	0.006	12.0	0.007	14.0
carbofuran	0.05*[a]	164, 149, 131	0.03-0.15	0.987	0.03	82.1	7.2	0.004	13.3	0.004	13.3
chlorfenvinphos	0.01*[a]	269, 323, 325	0.01 - 0.5	0.989	0.01	84.9	7.5	0.001	10.0	0.001	10.0
chlorothalonil	0.05*[a]	266, 264, 268	0.05-0.15	0.974	0.05	84.7	6.2	0.005	10.0	0.005	10.0
chlorpropham	0.05*[a]	213, 127, 154	0.05-0.15	0.978	0.05	86.2	6.4	0.004	8.0	0.004	8.0
chlorpyriphos	0.05*[a]	314, 316, 197	0.05-0.15	0.976	0.05	89.3	5.8	0.004	8.0	0.004	8.0
chlorpyriphos-methyl	0.05*[a]	286, 288, 125	0.01-0.15	0.983	0.01	88.2	5.7	0.0008	8.0	0.0008	8.0
coumaphos	0.1[b]	362, 364, 226, 210	0.009 - 0.45	0.985	0.009	89.1	8.6	0.0013	14.4	0.0013	14.4
cyhalotrin-lambda	0.05*[a]	181, 197, 208	0.05-0.15	0.977	0.05	89.9	6.7	0.006	12.0	0.006	12.0
cypermethrin	0.05*[a]	181, 163, 165	0.05-0.15	0.967	0.05	93.5	7.1	0.007	14.0	0.008	16.0

Active substance	MRL [a,b] (mg kg^{-1})	Ions scanned (m/z) T, Q_1, Q_2, Q_3	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)	U_r (refer to legend) (mg kg^{-1})	U_r (refer to legend) (%)	U_R (refer to legend) (mg kg^{-1})	U_R (refer to legend) (%)
cyproconazole	0.05*[a]	222, 139, 224	0.05-0.15	0.973	0.05	82.7	9.0	0.008	16.0	0.008	16.0
cyprodinil	0.05*[a]	224, 225, 210	0.05-0.15	0.974	0.05	84.2	7.2	0.006	12.0	0.006	12.0
deltamethrin	0.05*[a]	181, 251, 255	0.03-0.15	0.983	0.03	92.9	7.8	0.004	13.3	0.004	13.3
diazinon	0.01*[a]	179, 304, 199	0.01-0.15	0.985	0.01	84.7	8.5	0.001	10.0	0.001	10.0
dichlofluanid	/	226, 123, 167	0.05-0.15	0.975	0.05	87.3	6.0	0.004	8.0	0.004	8.0
dichlorvos	/	109, 185, 145	0.02-0.1	0.982	0.02	83.0	16.7	0.005	25.0	0.006	30.0
dimethachlor	0.05*[a]	134, 197, 210	0.01-0.15	0.983	0.01	94.2	9.7	0.001	10.0	0.002	20.0
dimethoate	/	87, 229, 143	0.03-0.15	0.980	0.03	80.7	7.8	0.005	16.7	0.007	23.3
diniconazole	0.05*[a]	268, 270, 70	0.05-0.15	0.972	0.05	84.1	7.5	0.007	14.0	0.007	14.0
endrin	0.01[a]	263, 261, 265	0.01-0.15	0.984	0.01	89.3	6.2	0.001	10.0	0.001	10.0
esfenvalerate + fenvalerate	0.05*[a]	125, 167, 225	0.05-0.15	0.971	0.05	99.2	7.5	0.007	14.0	0.007	14.0
fenamidone	0.05*[a]	238, 268, 237	0.05-0.15	0.974	0.05	84.1	7.8	0.007	14.0	0.007	14.0
fenbuconazole	0.05*[a]	198, 129, 125	0.05-0.15	0.974	0.05	83.0	13.0	0.010	20.0	0.010	20.0
fenthion	0.01*[a]	278, 279, 280	0.01-0.15	0.985	0.01	89.6	6.0	0.001	10.0	0.001	10.0
flonicamid	0.05*[a]	174, 146, 229	0.05-0.2	0.982	0.05	78.2	11.4	0.009	18.0	0.010	20.0
fludioxonil	0.05*[a]	248, 154, 127	0.05-0.15	0.969	0.05	83.6	7.3	0.008	16.0	0.008	16.0
fluquinconazole	0.02*[a]	340, 342, 108	0.02-0.15	0.982	0.02	85.0	9.9	0.003	15.0	0.003	15.0
folpet	0.05*[a]	260, 262, 130	0.05-0.15	0.971	0.05	88.5	6.0	0.010	20.0	0.020	40.0
heptachlor	0.01[a]	272, 274, 270	0.01-0.15	0.985	0.01	89.0	6.0	0.0007	7.0	0.0008	8.0
hexachlorobenzene	0.01*[a]	284, 286, 282	0.01-0.15	0.985	0.01	84.7	7.2	0.0008	8.0	0.0009	9.0
indoxacarb	0.05*[a]	218, 264, 527	0.05-0.15	0.976	0.05	89.9	8.9	0.008	16.0	0.008	16.0
iprodione	0.05*[a]	314, 316, 187	0.05-0.15	0.974	0.05	86.4	7.2	0.006	12.0	0.007	14.0
kresoxim-methyl	0.05*[a]	116, 206, 131	0.05-0.15	0.974	0.05	87.3	5.7	0.005	10.0	0.005	10.0
malathion	0.05*[a]	173, 174, 211	0.05-0.15	0.974	0.05	86.6	5.6	0.005	10.0	0.005	10.0

Active substance	MRL [a,b] (mg kg^{-1})	Ions scanned (m/z) T, Q_1, Q_2, Q_3	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)	U_r (refer to legend) (mg kg^{-1})	U_r (refer to legend) (%)	U_R (refer to legend) (mg kg^{-1})	U_R (refer to legend) (%)
mecarbam	0.05*[a]	131, 159, 329	0.05-0.15	0.974	0.05	87.1	5.9	0.005	10.0	0.005	10.0
metalaxyl+metalaxyl-M	0.05*[a]	249, 206, 234	0.05-0.15	0.974	0.05	83.5	8.7	0.007	14.0	0.008	16.0
methacrifos	0.05*[a]	208, 180, 240	0.05-0.15	0.977	0.05	97.0	14.5	0.006	12.0	0.008	16.0
methidathion	0.02*[a]	145, 85, 125	0.02-0.15	0.977	0.02	84.0	10.5	0.002	10.0	0.002	10.0
metrafenone	0.05*[a]	393, 408, 379	0.05-0.15	0.977	0.05	88.3	6.7	0.006	12.0	0.006	12.0
myclobutanil	0.05*[a]	179, 288, 150	0.05-0.15	0.970	0.05	81.1	9.6	0.008	16.0	0.009	18.0
oxadixyl	0.01*[a]	163, 105, 132	0.01-0.15	0.982	0.01	77.5	11.7	0.002	20.0	0.002	20.0
parathion	/	291, 292, 235	0.01-0.15	0.984	0.01	87.6	8.1	0.001	10.0	0.001	10.0
penconazole	0.05*[a]	248, 159, 161	0.05-0.15	0.972	0.05	84.7	7.1	0.006	12.0	0.007	14.0
permethrin	/	183, 163, 165	0.02-0.15	0.987	0.02	89.8	5.9	0.002	10.0	0.002	10.0
phosalone	0.01*[a]	182, 367, 121	0.01-0.15	0.982	0.01	90.6	10.1	0.001	10.0	0.001	10.0
pirimicarb	0.05*[a]	166, 238, 167	0.05-0.15	0.979	0.05	79.5	11.6	0.009	18.0	0.010	20.0
pirimiphos-methyl	0.05*[a]	290, 305, 276	0.05-0.15	0.977	0.05	87.5	6.0	0.004	8.0	0.004	8.0
procymidone	0.05*[a]	283, 285, 96	0.05-0.15	0.973	0.05	86.7	5.5	0.005	10.0	0.005	10.0
profenofos	0.05*[a]	208, 139, 339	0.05-0.15	0.973	0.05	86.9	5.4	0.005	10.0	0.006	12.0
propargite	0.05*[a]	135, 173, 350, 201	0.05-0.2	0.971	0.05	86.6	5.9	0.006	12.0	0.006	12.0
propyzamide	0.05*[a]	173, 175, 145	0.05-0.15	0.976	0.05	84.0	6.4	0.005	10.0	0.005	10.0
pyridaphenthion	/	199, 340, 188	0.01-0.15	0.986	0.01	84.4	8.2	0.001	10.0	0.001	10.0
pyrimethanil	0.05*[a]	198, 199, 200	0.05-0.1	0.960	0.05	84.9	6.3	0.008	16.0	0.008	16.0
quinalphos	0.05*[a]	146, 298, 157	0.05-0.15	0.974	0.05	86.5	5.6	0.005	10.0	0.005	10.0
quinoclamine	0.05*[a]	207, 172, 209	0.05-0.15	0.964	0.05	77.4	5.9	0.005	10.0	0.008	16.0
quinoxyfen	0.05*[a]	237, 272, 307	0.05-0.15	0.977	0.05	85.1	6.1	0.006	12.0	0.006	12.0
tebuconazole	0.05*[a]	125, 250, 127	0.05-0.15	0.973	0.05	83.7	8.3	0.009	18.0	0.009	18.0
tetraconazole	0.02*[a]	336, 338, 337	0.02-0.15	0.979	0.02	83.8	9.1	0.003	15.0	0.003	15.0

Active substance	MRL [a,b] (mg kg^{-1})	Ions scanned (m/z) T, Q_1, Q_2, Q_3	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)	U_r (refer to legend) (mg kg^{-1})	U_r (refer to legend) (%)	U_R (refer to legend) (mg kg^{-1})	U_R (refer to legend) (%)
tetradifon	0.05*[a]	159, 229, 356	0.05-0.15	0.977	0.05	88.1	5.9	0.005	10.0	0.006	12.0
thymol	/	135, 150, 91	0.07 - 3.5	0.996	0.07	74.1	5.8	0.012	17.1	0.013	18.6
tolclofos-methyl	0.05*[a]	265, 267, 250	0.05-0.15	0.976	0.05	87.5	5.9	0.004	8.0	0.004	8.0
tolylfluanid	0.05*[a]	238, 137, 240	0.05-0.15	0.975	0.05	87.8	5.5	0.005	10.0	0.005	10.0
triadimefon	0.05*[a]	208, 210, 181	0.05-0.15	0.973	0.05	85.1	6.6	0.006	12.0	0.006	12.0
triadimenol	0.05*[a]	112, 168, 128	0.05-0.15	0.962	0.05	83.7	9.1	0.007	14.0	0.009	18.0
triazophos	0.05*[a]	161, 162, 285	0.01-0.15	0.988	0.01	86.5	6.0	0.002	20.0	0.002	20.0
trifloxystrobin	0.05*[a]	116, 222, 186	0.05-0.15	0.978	0.05	87.8	6.3	0.005	10.0	0.005	10.0
vinclozolin	0.05*[a]	285, 124, 187	0.05-0.15	0.976	0.05	87.9	5.5	0.004	8.0	0.004	8.0

[a] Regulation (EC) 396/2005

[b] Regulation (EC) 37/2010

* means that MRL is set at LOQ of analytical method

T is target ion

Q is qualifier ion

RSD was obtained during recovery analyses

U_r is uncertainty of repeatability

U_R is uncertainty of reproducibility

Table 3: Validation parameters for LC-MS/MS determination, fragmentor (F), collision energy (CE), MRM transitions and MRLs

Active substance	MRL [a,b] (mg kg^{-1})	F (V)	CE (V)	MRM transitions [c] (m/z)	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)	U_r (refer to legend) (mg kg^{-1})	U_r (refer to legend) (%)	U_R (refer to legend) (mg kg^{-1})	U_R (refer to legend) (%)
3-hydroxy carbofuran	0.05*[a]	100	5 10	238─►181 238─►163	0.005-0.16	0.997	0.005	91.4	5.8	0.0005	10.0	0.0007	13.4
acetamiprid	0.05*[a]	126	15 15	223─►126 223─►56	0.005-0.08	0.998	0.005	92.9	7.8	0.0004	7.8	0.0007	17.9
beflubutamid	0.05*[a]	115	20 40	356─►162 356─►91	0.005-0.08	0.996	0.005	94.9	8.9	0.0006	12.0	0.0011	21.5
benalaxyl	/	120	5 10	326─►294 326─►148	0.005-0.08	0.997	0.005	94.1	7.9	0.0006	10.2	0.0010	18.2
bitertanol	0.05*[a]	60	1 5	338─►269 338─►70	0.005-0.2	0.996	0.005	92.8	9.5	0.0007	13.9	0.0012	22.0
carbendazim	1.0[a]	120	15 35	192─►160 192─►132	0.005-0.08	0.991	0.005	83.1	8.5	0.0004	8.6	0.0008	18.0
chlorotoluron	0.05*[a]	100	15 15	213─►140 213─►72	0.01-0.08	0.991	0.01	103.7	8.4	0.0009	9.5	0.0020	21.0
demeton-S-methyl sulphone	0.01*[a]	120	15 25	263─►169 263─►125	0.005-0.20	0.995	0.005	92.9	8.6	0.0005	8.4	0.0011	19.9
desmedipham	0.05*[a]	130	5 25	301─►182 301─►136	0.005-0.08	0.993	0.005	94.1	9.0	0.0005	10.1	0.0011	20.8
difenoconazole	0.05*[a]	140	15 25	406─►337 406─►251	0.005-0.20	0.996	0.005	92.7	7.9	0.0008	15.1	0.0010	18.1
diflufenican	0.05*[a]	110	20 40	395─►266 395─►238	0.005-0.08	0.994	0.005	94.0	9.5	0.0008	15.6	0.0012	21.8
dimethenamid-P	0.05*[a]	120	10 15	276─►244 276─►168	0.005-.08	0.993	0.005	92.9	7.6	0.0006	10.7	0.0009	17.4
epoxyconazole	0.05[a]	130	25 50	330─►121 330─►101	0.01-0.16	0.992	0.01	95.2	7.8	0.0011	10.6	0.0019	18.0

Active substance	MRL [a,b] (mg kg^{-1})	F (V)	CE (V)	MRM transitions [c] (m/z)	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)	U_r (refer to legend) (mg kg^{-1})	U_r (refer to legend) (%)	U_R (refer to legend) (mg kg^{-1})	U_R (refer to legend) (%)
ethofumesate	0.05*[a]	130	5 15	287─►259 287─►121	0.005-0.08	0.998	0.005	94.7	8.4	0.0006	10.7	0.0010	19.5
ethoprophos	/	80	15 20	243─►131 243─►97	0.005-0.08	0.996	0.005	93.8	9.1	0.0007	13.4	0.0011	20.9
famoxadone	0.05*[a]	80	10 10	392─►331 392─►238	0.01-0.40	0.997	0.01	95.1	9.5	0.0015	14.3	0.0092	21.8
fenazaquin	0.01*[a]	120	15 20	307─►161 307─►57	0.003-0.08	0.997	0.003	92.5	12.7	0.0004	13.3	0.0010	29.4
fenhexamid	0.05*[a]	110	25 30	302─►97 302─►55	0.005-0.20	0.995	0.005	93.4	9.5	0.0009	17.0	0.0012	21.7
fenoxycarb	0.05*[a]	110	5 20	302─►116 302─►88	0.005-0.08	0.992	0.005	93.0	8.2	0.0005	8.7	0.0010	19.0
fenpropidin	0.05*[a]	155	30 60	274─►147 274─►117	0.005-0.08	0.995	0.005	92.9	6.7	0.0005	8.4	0.0008	15.4
fenpyroximate	0.05*[a]	130	15 40	422─►366 422─►165	0.005-0.08	0.993	0.005	92.3	9.0	0.0006	11.4	0.0011	20.8
fipronil	0.005*[a]	110	10 25	435─►330[c] 435─►278[c]	0.005-0.20	0.997	0.005	93.0	7.8	0.0005	9.3	0.0010	18.0
fluazifop butyl	0.05*[a]	120	15 20	384─►328 384─►282	0.005 - 0.08	0.991	0.005	95.3	8.3	0.0005	9.8	0.0009	19.3
fluazinam	0.05*[a]	135	15 10	463─►416[c] 463─►398[c]	0.005-0.08	0.996	0.005	89.6	8.7	0.0006	11.2	0.0011	20.2
flufenoxuron	/	80	10 15	489─►158 489─►141	0.005-0.20	0.998	0.005	93.8	11.9	0.0004	9.1	0.0013	27.4
flusilazole	0.05*[a]	120	15 30	316─►247 316─►165	0.005-0.08	0.994	0.005	94.1	10.8	0.0007	15.7	0.0012	24.9
flutriafol	0.05*[a]	100	15 15	302─►123 302─►109	0.01-0.40	0.997	0.01	93.2	10.8	0.0012	13.1	0.0023	24.6

Active substance	MRL [a,b] (mg kg^{-1})	F (V)	CE (V)	MRM transitions [c] (m/z)	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)	U_r (refer to legend) (mg kg^{-1})	U_r (refer to legend) (%)	U_R (refer to legend) (mg kg^{-1})	U_R (refer to legend) (%)
hexaconazole	/	80	10 10	314─►159 314─►70	0.01-0.40	0.998	0.01	93.1	10.6	0.0010	11.0	0.0023	24.4
hexythiazox	0.02*[a]	120	10 20	353─►228 353─►168	0.005-0.20	0.997	0.005	92.1	9.9	0.0006	12.4	0.0010	22.6
iprovalicarb	0.05*[a]	95	5 20	321─►203 321─►119	0.005-0.08	0.998	0.005	94.8	9.2	0.0003	12.0	0.0006	21.0
isoproturon	0.05*[a]	100	10 25	207─►165 207─►72	0.01-0.16	0.993	0.01	92.8	7.4	0.0007	7.8	0.0016	16.7
lufenuron	0.05*[a]	120	20 50	511─►158 511─►141	0.005-0.20	0.996	0.005	93.5	10.6	0.0006	11.9	0.0011	24.1
metazachlor	0.05*[a]	100	15 15	278─►210 278─►134	0.01-0.16	0.994	0.01	92.8	7.6	0.0009	10.1	0.0016	17.3
methoxyfenozide	0.05*[a]	90	0 15	369─►313 369─►149	0.005-0.08	0.991	0.005	93.8	7.9	0.0004	8.6	0.0009	18.5
metosulam	0.05*[a]	160	20 40	418─►175 418─►140	0.005-0.20	0.996	0.005	91.8	11.0	0.0005	10.8	0.0012	25.2
monocrotophos	/	100	0 10	224─►193 224─►127	0.005-0.08	0.994	0.005	93.3	7.9	0.0004	8.8	0.0009	18.7
napropamide	/	120	15 15	272─►171 272─►129	0.005-0.08	0.994	0.005	93.4	9.0	0.0006	12.5	0.0010	20.4
pendimethalin	0.05*[a]	80	5 10	282─►212 282─►194	0.010-0.40	0.996	0.01	93.3	9.0	0.0011	11.6	0.0019	20.8
phenmedipham	0.05*[a]	80	15 20	301─►168 301─►138	0.01-0.08	0.996	0.01	93.5	7.5	0.0009	8.6	0.0019	17.3
phorate sulfon	0.01*[a]	70	0 20	293─►171 293─►115	0.005-0.04	0.993	0.005	94.6	11.0	0.0006	11.8	0.0012	25.2
phoxim	0.02*[a]	80	15 15	299─►129 299─►77	0.005-0.08	0.995	0.005	91.6	9.8	0.0005	11.5	0.0010	22.5

Active substance	MRL [a,b] (mg kg^{-1})	F (V)	CE (V)	MRM transitions [c] (m/z)	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)	U_r (refer to legend) (mg kg^{-1})	U_r (refer to legend) (%)	U_R (refer to legend) (mg kg^{-1})	U_R (refer to legend) (%)
pyraflufen-ethyl	0.05*[a]	120	20 30	413─►339 413─►289	0.01-0.40	0.999	0.01	93.6	9.4	0.0012	12.5	0.0020	21.7
prochloraz	/	90	90 15	376─►308 376─►266	0.005-0.04	0.991	0.005	93.5	9.8	0.0003	7.2	0.0011	22.8
propaquizafop	0.05*[a]	60	20 15	444─►299 444─►100	0.005-0.20	0.997	0.005	92.5	10.3	0.0004	8.1	0.0011	23.4
propiconazole	0.05*[a]	140	25 25	342─►159 342─►69	0.005-0.20	0.996	0.005	91.2	9.5	0.0007	15.3	0.0010	21.8
pyraclostrobin	0.05*[a]	110	5 25	388─►194 388─►163	0.005-0.08	0.995	0.005	92.7	8.7	0.0005	10.1	0.0009	20.0
pyrazophos	0.05*[a]	125	15 50	374─►222 374─►70	0.01-0.40	0.997	0.01	93.2	10.0	0.0013	13.7	0.0022	23.4
pyridaben	0.02*[a]	100	10 30	365─►309 365─►147	0.005-0.08	0.994	0.005	94.7	8.9	0.0004	7.8	0.0010	20.6
pyriproxyfen	0.05*[a]	100	30 20	322─►185 322─►96	0.005-0.04	0.992	0.005	93.9	9.0	0.0004	9.3	0.0010	20.7
spinosyn A	0.05*[a]	140	35 35	732,5─►142 732,5─►98	0.005-0.16	0.996	0.005	93.3	7.8	0.0004	9.2	0.0008	18.1
spinosyn D	0.05*[a]	100	15 15	746,5─►142 746,5─►98	0.005-0.40	0.999	0.005	93.0	13.1	0.0008	18.0	0.0014	29.8
spirodiclofen	0.05*[a]	110	5 15	411─►313 411─►71	0.005-0.08	0.993	0.005	92.2	7.5	0.0005	10.0	0.0008	17.3
tebufenozide	0.05*[a]	90	0 15	353─►297 353─►133	0.005-0.08	0.992	0.005	92.8	7.6	0.0004	8.5	0.0008	17.7
terbuthylazine	/	120	15 40	230─►174 230─►68	0.005-0.08	0.998	0.005	93.1	6.4	0.0004	9.1	0.0007	14.7
thiacloprid	0.2[a]	90	10 20	253─►186 253─►126	0.005-0.08	0.995	0.005	92.1	7.5	0.0003	6.2	0.0008	17.1

Active substance	MRL [a,b] (mg kg^{-1})	F (V)	CE (V)	MRM transitions [c] (m/z)	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)	U_r (refer to legend) (mg kg^{-1})	U_r (refer to legend) (%)	U_R (refer to legend) (mg kg^{-1})	U_R (refer to legend) (%)
thiamethoxam	0.05*[a]	90	10 20	292─►211 292─►181	0.005-0.08	0.992	0.005	91.0	7.7	0.0004	9.7	0.0008	17.8
thiodicarb	0.05*[a]	90	10 15	355─►108 355─►88	0.005-0.08	0.994	0.005	92.0	8.3	0.0004	8.9	0.0009	19.3
triasulfuron	0.05*[a]	140	10 15	402─►167 402─►141	0.005-0.40	0.997	0.005	91.7	9.1	0.0005	11.8	0.0010	20.7
trichlorfon	0.01*[a]	105	10 10	402─►141 402─►141	0.01-0.16	0.993	0.01	92.1	6.5	0.0012	13.5	0.0014	15.1
trinexapac-ethyl	0.05*[a]	95	0 20	257─►221 257─►127	0.01-0.16	0.994	0.01	93.9	8.3	0.0010	10.3	0.0018	19.3

[a] Regulation (EC) 396/2005

[b] Regulation (EC) 37/2010

*means that MRL is set in Regulation (EC) 396/2005 at LOQ of analytical method of different laboratories

[c] means in negative Electro Spray Ionisation mode

RSD was obtained during recovery analyses

U_r is uncertainty of repeatability

U_R is uncertainty of reproducibility

Table 4: Linear response for GC/MS and LC-MS/MS determination

Determination with	Linear response in range (mg kg^{-1})	No. of active substances giving linear response
GC/MS	0.01-0.15	16
GC/MS	0.02-0.1	1
GC/MS	0.02-0.15	5
GC/MS	0.03-0.15	3
GC/MS	0.05-0.1	1
GC/MS	0.05-0.15	47
GC/MS	0.05-0.2	2
LC-MS/MS	0.003-0.008	1
LC-MS/MS	0.005-0.04	3
LC-MS/MS	0.005-0.08	28
LC-MS/MS	0.005-0.16	2
LC-MS/MS	0.005-0.2	11
LC-MS/MS	0.005-0.4	2
LC-MS/MS	0.01-0.08	2
LC-MS/MS	0.01-0.16	5
LC-MS/MS	0.01-0.4	6

Table 5: Pesticide residues in honey samples in 2017 and 2018

	amitraz	coumaphos	thiacloprid	thymol
MRL (mg kg^{-1})	0.2 (a)	0.1 (a)	0.2 (b)	/
conventional production				
Min content (mg kg^{-1})	0.01	0.009	<0.005	0.08
Max content (mg kg^{-1})	0.12	0.055	<0.005	0.22
Average (mg kg^{-1})	0.04	0.023	n.a.	0.13
SD (mg kg^{-1})	0.03	0.015	n.a.	0.07
No. of samples where residues were found	14	13	0	3
organic production				
Min content (mg kg^{-1})	0.01	< 0.009	0.018	0.43
Max content (mg kg^{-1})	0.01	< 0.009	0.018	0.43
Average (mg kg^{-1})	0.01	n.a.	n.a.	n.a.
SD (mg kg^{-1})	0.00	n.a.	n.a.	n.a.
No. of samples where residues were found	2	0	1	1

n.a. means not applicable

(a) Regulation (EC) 37/2010

(b) Regulation (EC) 396/2005

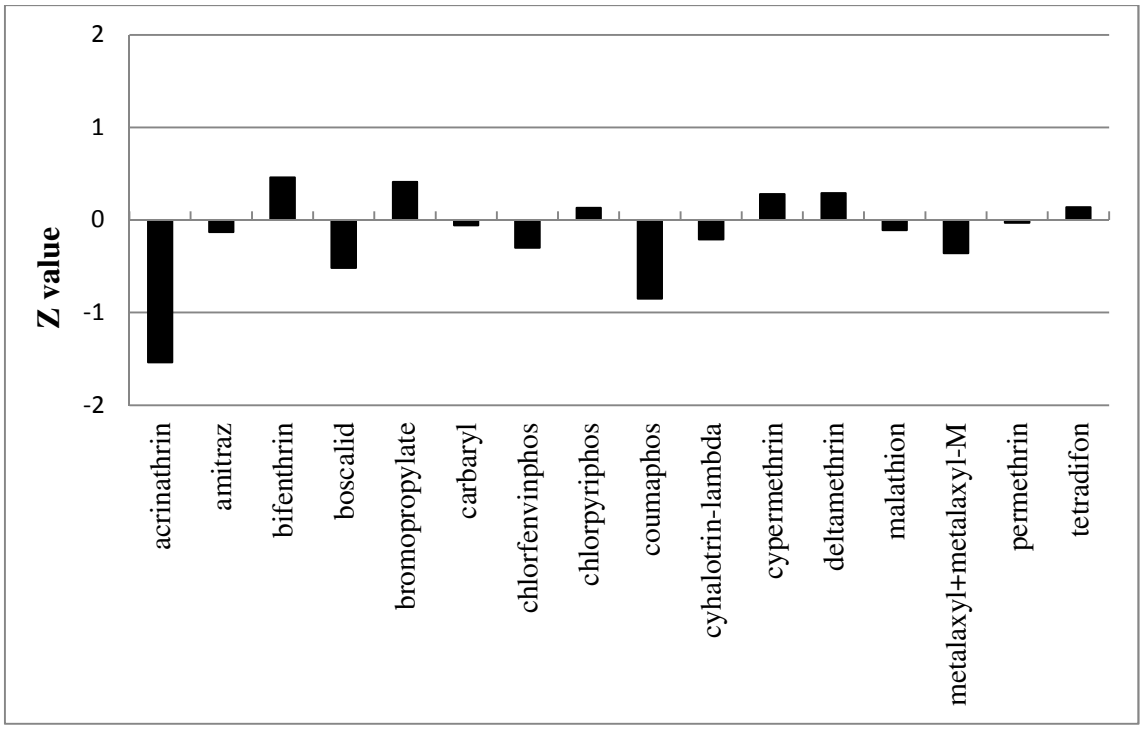

Figure 1: BIPEA interlaboratory comparisons (BIPEA 2017a and 2017b)

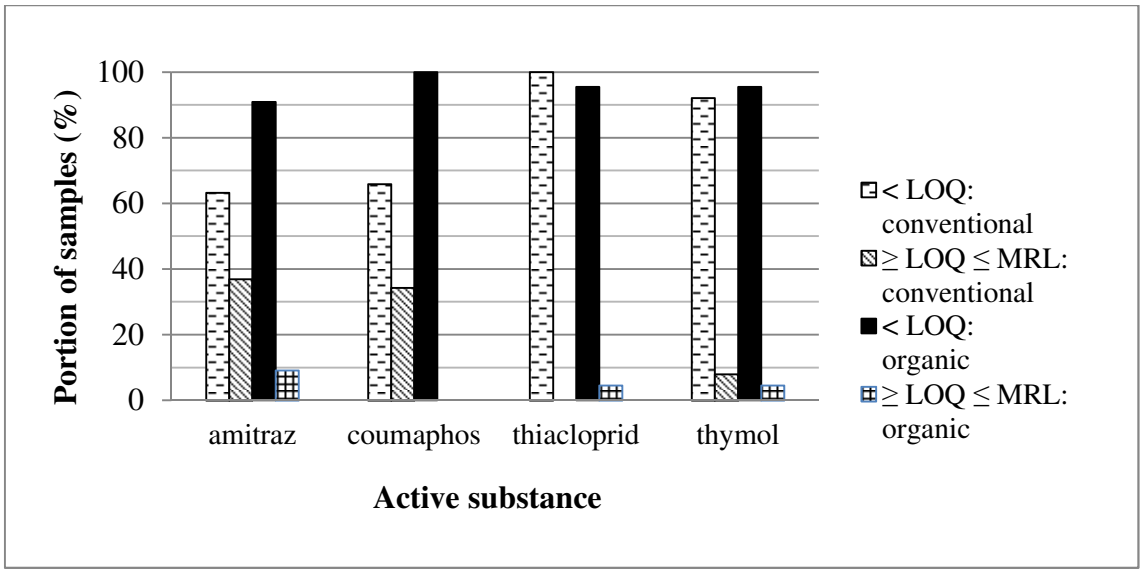

Figure 2: Portion of samples for conventional and organic production in 2017 and 2018