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Pesticide residues in bee pollen - validation of the gas chromatography-mass spectrometry multiresidual method and a survey of bee pollens from Slovenia

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Pesticide residues in bee pollen - validation of the gas chromatography-mass spectrometry multiresidual method and a survey of bee pollens from Slovenia

Abstract: A new analytical method for determining environmental pesticide residues in pollen was introduced and validated. The extraction was conducted using acetonitrile, the clean-up using Supelclean Ultra 2400 solid phase extraction cartridges, which contain Grapsphere, anion exchanger, C18 and zirconia-based sorbent, and the determination was conducted using gas chromatography coupled with mass spectrometry. The method was applied in practice. A total of 49 active substances (pesticides) were sought in 30 bee pollen samples gathered from Slovenian beekeepers from all 12 statistical regions of Slovenia. The fungicide azoxystrobin was the only active substance found and was found only in one sample with a concentration of < 0.05 mg kg^{-1}. The active substances sought were not detected in 96.7 % of the samples analysed. The risk assessment revealed that the analysed pollen samples do not represent an unacceptable risk for consumers. The results were compared with those from the literature and the outcome was that bee pollen from Slovenia contained a lower number of active substances at mainly lower contents as compared pollen from some other European countries.

Key words*:* bee pollen; GC-MS; pesticide residues; multiresidual method

Ostanki fitofarmacevtskih sredstev v cvetnem prahu - validacija multirezidualne metode s plinsko kromatografijo sklopljeno z masno spektrometrijo in preiskava cvetnega prahu iz Slovenije

Izvleček: Uvedli in validirali smo novo analizno metodo za določanje ostankov fitofarmacevtskih sredstev iz okolja. Ekstrakcijo smo izvedli z acetonitrilom, čiščenje z Supelclean Ultra 2400 koloncami za ekstrakcijo na trdni fazi, ki vsebujejo Grapsphere, anionski izmenjevalnik, C18 in sorbent na osnovi cirkonija, in določitev s plinsko kromatografijo sklopljeno z masno spektrometrijo. Metodo smo uporabili v praksi. V 30 vzorcih cvetnega prahu slovenskih čebelarjev iz vseh 12 statističnih regij Slovenije smo določali skupno 49 aktivnih spojin (pesticidov). Edina najdena aktivna snov je bil fungicid azoksistrobin in sicer le v enem vzorcu, pri koncentraciji < 0,05 mg kg^{-1} Iskanih aktivnih snovi nismo detektirali v 96,7 % analiziranih vzorcev. Z oceno tveganja smo ugotovili, da analizirani vzorci cvetnega prahu ne predstavljajo tveganja za potrošnika. Rezultate smo primerjali z literaturnimi podatki in ugotovili, da je cvetni prah v Sloveniji vseboval manjše število aktivnih spojin pri v glavnem nižjih vsebnostih fitofarmacevtskih ostankov kot cvetni prah iz nekaterih Evropskih dtžav.

Ključne besede: cvetni prah; GC-MS; ostanki fitofarmacevtskih sredstev; multirezidualna metoda

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1 INTRODUCTION

Bee pollen is a dietary supplement. It contains carbohydrates (mainly fructose, glucose and sucrose (13-55 %), proteins (10-40 %), lipids (1-13 %), and crude fibre (0.3-20 %)), minerals (mainly potassium, phosphorus, calcium, magnesium, zinc, manganese, iron and copper (2-6 %)), vitamins (0.005-5.6 mg kg^{-1}), and polyphenols (0.69-213.2 mg GAE g^{-1}) (Thakur and Nanda, 2020). Bee pollen has antioxidant activity, antimicrobial activity, anti-inflammatory activity, anticarcinogenic activity, cardioprotective effects, hepatoprotective effects, antiallergic activity and it boosts the immune system (Li et al., 2018). A diet supplemented with bee pollen strengthens muscles and improves the physical health of humans (Salles et al., 2014). Bee pollen also benefits those undertaking strenuous mental/physical work (Nakajima et al., 2009).

Honeybees fly up to 4.8 km from their apiary (Eckert, 1933) to collect pollen. When hives are located near agricultural fields, plants treated with plant protection products (PPP) are a possible source of contamination for bee pollen (Tosi et al., 2018). Honeybees may come into contact with PPP residues through the nectar, pollen or plant leaves of treated plants, or through air, soil and water where PPPs have drifted (Crenna et al., 2020).

Bee pollen is usually harvested by means of a trap fixed at the entrance of beehives (Thakur and Nanda, 2020). This type of pollen is called corbicular pollen. Some beekeepers also collect pollen from hives deposited in combs by bees. This type of pollen is called beebread.

Numerous analytical methods have been developed to analyse PPP residues in pollen. The more recent ones are based on the QuEChERS method, which has been introduced to analyse a wide range of PPP residues in fruit and vegetables (Anastassiades et al., 2003, Lehotay, 2007). In this method, acetonitrile is used as an organic solvent for the extraction. The advantage of acetonitrile is that it minimizes the co-extraction of lipids and proteins by precipitating the proteins (Wang et al., 2012) and limiting the lipid solubility (Lozano et al., 2014). This makes acetonitrile a suitable solvent for extracting PPP residues from pollen. In some cases (Tosi et al., 2018; Wiest et al., 2011), n-hexane was added to remove fatty acids and fatty acid esters.

The clean-up in the original QuEChERS method was conducted using primary secondary amine (PSA) sorbent and C18 (Anastassiades et al., 2003; Lehotay, 2007). In the case of pollen some authors used either PSA (Cabrera de Oliveria, 2016; Kasiotis et al., 2014), PSA and C18 sorbent (Mullin et al., 2010), PSA, C18 and graphitized carbon black (GCB) sorbent (David et al., 2016), or PSA, C18 and zirconia-based sorbents such as Z-Sep, which consists of a mixture of C18 and silica coated with zirconium dioxide sorbents (Hakme et al., 2017, Vázquez et al., 2015). In our laboratory we used Supelclean Ultra 2400 solid phase extraction (SPE) cartridges, which contain Grapsphere (graphitized spherical carbon), PSA, C18 and Z-Sep. PSA retains acidic interferences such as fatty acids. Grapsphere removes planar molecules such as pigments and at the same time enables better recovery of planar pesticides than GCB. The bottom layer of the cartridge contains Z-Sep, which removes oily residues and provides additional retention of some pigments (Stenerson, 2018). C18 retains lipids (Lehotay,2007). Thus, these SPE cartridges combine all the common clean-up procedures from the literature.

Determination of PPP residues can be performed using gas chromatography coupled with mass spectrometry (GC-MS) (Li et al.; 2015; Mullin et al.,2010; Raimets et al., 2020), GC coupled with tandem mass spectrometry (GC-MS/MS) (Cabrera de Oliveria, 2016), GC coupled with time-of flight mass spectrometry (GC-TOF) (Hakme et al., 2017), and/or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (David et al., 2016; Kasiotis et al., 2014; Raimets et al., 2020). Multiresidual methods are fit-for-purpose when their limits of quantification are lower or equal to the Maximum Residue Limits (MRLs) established for pollen in Regulation (EC) 396/2005. When the MRLs in this Regulation are set at the LOQ determined by the analytical method (this LOQ was gathered by different laboratories), an * is added to mark this fact. Many pesticides have an MRL and LOQ of 0.05 mg kg^{-1}, meaning that GC-MS is still suitable despite its smaller sensitivity than tandem mass detectors.

Numerous authors have analysed pesticide residues in pollen. García-Valcárcel et al. (2019) analysed 10 active substances in pollen samples in Spain. Vázquez et al. (2015) analysed 253 active substances in pollen samples in Spain. Hakme et al. (2017) tested pollen samples from Spain for 100 active substances. Wiest et al. (2011) introduced a method for determining 80 active substances in French pollen. Tosi et al. (2018) analysed 66 active substances in Italian pollen. Kasiotis et al. (2014) analysed 115 active substances in Greek pollen. Raimets et al. (2020) analysed 47 active substances in Estonian pollen. David et al. (2016) analysed 20 active substances in pollen from the United Kingdom. Many of active substances sought in these studies were introduced in our study as well. Our selection of active substances was based on both those authorised for use in Slovenia and those not authorised for use in Slovenia, the latter to cover misuse of PPP. Of those selected, 59 % were acaricides and/or insecticides, which may be the main reason for the death of bees.

The purpose of this paper is to present the multire-

sidual GC-MS method introduced for identifying 49 active substances in pollen using acetonitrile as the extraction solvent and Supelclean Ultra 2400 SPE cartridges for the clean-up. The validation parameters are summarised, as well as the practical use of the method on 30 samples of bee pollen gathered from Slovenian beekeepers. The contents of pesticide residues were compared with those from the literature. Finally, a risk assessment for consumers was conducted.

2 MATERIAL AND METHODS

2.1. MATERIALS

2.1.1 Chemicals

The certified standards were supplied by Dr. Ehrenstorfer (Augsburg, Germany). The acetonitrile HPLC-grade (used for the extraction procedure) and acetone HPLC-grade (used for preparation of standards) were supplied by J.T.Baker (Deventer, Netherlands). All other chemicals used were supplied by Sigma-Aldrich (Steinheim, Germany). The water used was MilliQ deionised water. The Ultra 2400 3 ml SPE columns were supplied by Supelco (Bellefonte, USA).

2.1.2. Preparation of the solutions

Stock solutions in acetone of individual active substances were prepared with the concentrations of 625 µg pesticide ml^{-1}. From 49 stock solutions, two mixed solutions of all 49 active substances were prepared: one with a concentration of 5 µg ml^{-1} and the second at the LOQ of active substances. All solutions used to determine the linearity and the LOQs and to perform calibration during sample analysis were prepared from a mixed solution of 5 µg ml^{-1} with proper dilutions. For other validation parameters, a mixed solution with a concentration at the LOQ was used.

2.2. EXTRACTION PROCEDURE

The samples were analysed within a maximum period of 27 days after arrival at the laboratory. During that time, they were stored at -20 °C.

To 10 g of pollen in the beaker, 50 ml of acetonitrile was added. The mixture was homogenised for 2 minutes with a mixer. The mixture was left for 30 minutes so that the sediment settled on the bottom of the beaker. The liquid part was transferred to a 50 ml centrifuge tube and centrifuged for 10 minutes at 7000 rpm. The supernatant was filtered through 15 g anhydrous Na_2SO_4 and black strip filter paper into a 100 ml Soxhlet flask. Then 30 ml of acetonitrile was added to the sediment in the beaker. The mixture was homogenised for 2 minutes with a mixer and transferred to a 50 ml centrifuge tube. Centrifugation followed for 10 minutes at 7000 rpm. This supernatant was combined with the first one after it was filtered through 15 g of anhydrous Na_2SO_4 and black strip filter paper into a 100 ml Soxhlet flask. The Na_2SO_4 was rinsed with 15 ml of acetonitrile. Then acetonitrile in Soxhlet flask was evaporated to approximately 2 ml on a rotavapor and dried with nitrogen flow. The dry eluate was dissolved in 1 ml of acetonitrile using ultrasound. The extract was transferred onto a column of Ultra 2400 3 ml, preconditioned with 3 ml of acetonitrile. The SPE column was rinsed with 16 ml of acetonitrile. The flow rate was 3-4 ml min^{-1} under vacuum. The whole eluate (partly taken from the SPE column after the sample was applied to the SPE column and partly eluate created during rinsing of the SPE column) was combined in a beaker. The content of the beaker was transferred to a 100 ml Soxhlet flask. The beaker was rinsed twice with 5 mL of acetonitrile and the content was transferred to the Soxhlet flask. The acetonitrile was then evaporated to approximately 2 ml on a rotavapor and dried with nitrogen flow. The dry eluate was dissolved in 1 ml of acetone using ultrasound in order to prepare a sample. For matrix match standards, 1 ml of the working solutions with proper concentrations was added and dissolved using ultrasound.

2. 3. DETERMINATION

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 a HP-5 MS UI column (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 °C to 130 °C at 25 °C min^{-1}, held at 130 °C for 1 min, from 130 °C to 180 °C at 5 °C min^{-1}, held at 180 °C for 30 min, from 180 °C to 230 °C at 20 °C min^{-1}, held at 230 °C for 16 min, from 230 °C to 250 °C at 20 °C min^{-1}, held at 250 °C for 13 min, from 250 °C to 280 °C at 20 °C min^{-1}, held at 280 °C for 20 min. In order to determine the 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, the retention time and

mass spectrum in the SIM were used. For each active substance, one target and two qualifier ions, presented in Table 2, were used. The calibration was performed to matrix match standards.

2. 4. VALIDATION OF METHODS

LOQ and linearity

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

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

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

Precision

Blank pollen was bought in store and analysed to prove that it contains no pesticide residues. For the determination of precision (ISO 5725), i.e. repeatability and reproducibility, the extracts of spiked blank pollen 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 the standard deviation of reproducibility by the Student's t factor, for nine 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. When validating, analysts must prove that their measurement uncertainty is below or equal to the proposed measurement uncertainty.

Accuracy

The accuracy was verified by checking the recoveries. The average of the recoveries from the tests for precision (10 days, 2 parallel samples each day) was calculated. According to the requirements for method validation procedures (SANTE/11813/2017), acceptable mean recoveries are those within the range of 70 % to 120 %, with an associated repeatability of RSDr ≤ 20 %.

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

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

2. 5. CONSUMER RISK ASSESSMENT

Long-term exposure was calculated using the EFSA PRIMo model revision 3.1, accessible online at https://www.efsa.europa.eu/en/applications/pesticides/tools. Chronic consumer exposure was expressed in % of the ADI. The acceptable limit for long-term exposure is 100 % of the ADI.

2. 6. SAMPLING

A total of 30 bee pollen samples (none of them beebread) were collected in May and June 2020 from Slovenian beekeepers that produce apiculture products sold on the market. Samples were gathered from all 12 statistical regions in Slovenia. The sampling distribution is presented in Table 1. All samples originated from conventional production.

3 RESULTS AND DISCUSSION

3. 1. COMPARISON OF QUECHERS METHOD WITH OUR METHOD

In the original QuEChERS method, 10 ml of acetonitrile was added to 10 g of the sample (Anastassiades et al.; 2003). In our method this ratio was different: 80 ml of acetonitrile was added to 10 g of the sample. The reason for increasing the solvent volume was that when we tested the addition of 10 ml of acetonitrile to 10 g of pollen, the recoveries were 20-30 % lower.

Table 1: Number of pollen samples collected from different statistical regions in Slovenia in 2020

Statistical region	Number of samples
Gorenjska	2
Goriška	2
Jugovzhodna Slovenija	1
Koroška	2
Notranje kraška	3
Obalno kraška	2
Osrednja Slovenija	7
Podravska	3
Pomurska	2
Savinjska	3
Spodnje posavska	1
Zasavska	2
Sum	30

The clean-up in our method was not conducted with dispersive SPE as in the original QuEChERS method (Anastassiades et al.; 2003, Lehotay; 2007), but with Ultra 2400 3 ml SPE columns.

In the QuEChERS method, aliquots of extracts were cleaned-up, while in our method, the transference of the extracts was quantitative.

3. 2. VALIDATION OF METHOD

LOQ and linearity

The linear model is valid for all active substances presented in Table 2. Linearity was proven in the range of 0.01 mg kg^{-1} to 0.15 mg kg^{-1} for six active substances, in the range of 0.05 mg kg^{-1} to 0.12 mg kg^{-1} for one active substance and in the range of 0.05 mg kg^{-1} to 0.15 mg kg^{-1} for 42 active substances. R^2 ranged from 0.974 to 0.996.

The LOQs are presented in Table 2. Six active substances have an LOQ of 0.01 mg kg^{-1} and 43 of them 0.05 mg kg^{-1}. The LOQs are equal to MRLs set in Regulation (EC) 396/2005.

Accuracy

The results for the recoveries are given in Table 2. The recoveries at LOQs for the active substances scanned with GC-MS are in the range of 73.0 % to 93.4 %, with RSDs of 5.6 % to 17.7 %. More precisely, the recoveries at LOQs of 0.01 mg kg^{-1} are within the range of 75.4 % to 93.4 % with RSDs of 9.3 % to 17.7 % and the recoveries at LOQs of 0.05 mg kg^{-1} are within the range of 73.0 % to 88.2 % with RSDs of 5.6 % to 15.9 %.

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

Uncertainty of repeatability and uncertainty of reproducibility

The uncertainty of repeatability and uncertainty of reproducibility were determined at contents equal to the LOQs. The results are presented in Table 2. Uncertainty of repeatability ranged from 0.001 mg kg^{-1} to 0.013 mg kg^{-1}, which is 10.0 % to 30.0 % of LOQ and uncertainty of reproducibility ranged from 0.002 mg kg^{-1} to 0.015 mg kg^{-1}, which is 12.0 % to 30.0 % of LOQ.

Table 2: Validation parameters, ions scanned, MRLs and activity type of active substances

Active substance	Activity type [a]	MRL[b] (mg kg^{-1})	Ions scanned[c] (m/z) T, Q_1, Q_2	Linearity range (mg kg^{-1})	R^2	LD (mg kg^{-1})	LOQ (mg kg^{-1})	Recovery (%)	RSD[d] (%)	U_r[e] (mg kg^{-1})	U_r[f] (%)	U_R[g] (mg kg^{-1})	U_R[h] (%)
acrinathrin	A, I	0.05*	181, 208, 289	0.05-0.15	0.991	0.015	0.05	87.8	13.8	0.010	20	0.014	28
azinphos-methyl	A, I	/	160, 132, 105	0.05-0.15	0.987	0.015	0.05	84.2	9.9	0.009	18	0.010	20
azoxystrobin	F	0.05*	344, 388, 345	0.05-0.15	0.987	0.015	0.05	85.3	11.8	0.011	22	0.011	22
bifenthrin	A, I	0.05*	181, 165, 166	0.05-0.15	0.992	0.015	0.05	76.6	8.0	0.010	20	0.010	20
boscalid	F	0.05*	140, 342, 142	0.05-0.15	0.984	0.015	0.05	85.0	11.9	0.009	18	0.012	24
carbaryl	A, I	0.05*	144, 115, 116	0.05-0.15	0.988	0.015	0.05	81.9	7.0	0.010	20	0.010	20
carbofuran	A, I	0.05*	164, 149, 131	0.05-0.15	0.992	0.015	0.05	80.6	8.2	0.007	14	0.008	16
chlorpropham	H	0.05*	213, 127, 154	0.05-0.15	0.983	0.015	0.05	74.9	8.0	0.007	14	0.007	14
chlorpyriphos	A, I	0.05*	314, 316, 197	0.05-0.15	0.982	0.015	0.05	76.0	8.5	0.010	20	0.010	20
chlorpyriphos-methyl	I	0.05*	286, 288, 125	0.05-0.15	0.996	0.015	0.05	77.6	7.0	0.010	20	0.010	20
clomazone	H	0.05*	125, 204, 127	0.05-0.15	0.988	0.015	0.05	75.2	10.9	0.006	12	0.009	18
cyhalotrin-lambda	I	0.05*	181, 197, 208	0.05-0.15	0.987	0.015	0.05	88.2	12.3	0.011	22	0.012	24
deltamethrin	I	0.05*	181, 251, 255	0.05-0.15	0.990	0.015	0.05	80.1	14.9	0.010	20	0.014	28
diazinon	A, I	0.01*	179, 304, 199	0.01-0.15	0.994	0.003	0.01	81.3	12.9	0.002	20	0.002	20
dichlofluanid	A, F	/	226, 123, 167	0.05-0.15	0.990	0.015	0.05	79.1	7.9	0.010	20	0.010	20
dimethachlor	H	0.05*	134, 197, 210	0.05-0.15	0.995	0.015	0.05	81.2	6.5	0.006	12	0.006	12
dimethoate	A, I	/	87, 229, 143	0.05-0.15	0.994	0.015	0.05	81.7	5.6	0.010	20	0.010	20
diphenylamine	F	0.05*	169, 167, 168	0.05-0.12	0.982	0.015	0.05	73.2	9.1	0.008	16	0.008	16
endosulfan-sulphate	A, I	0.01*	272, 274, 387	0.01-0.15	0.995	0.003	0.01	82.4	9.3	0.001	10	0.002	20
fenbuconazole	F	0.05*	198, 129, 125	0.05-0.15	0.989	0.015	0.05	78.9	15.9	0.013	26	0.014	28
fenitrothion	I	0.01*	277, 260, 109	0.01-0.15	0.981	0.003	0.01	84.7	14.7	0.002	20	0.003	30
flonicamid	I	0.05*	174, 146, 229	0.05-0.15	0.992	0.015	0.05	79.8	7.7	0.007	14	0.007	14
fludioxonil	F	0.05*	248, 154, 127	0.05-0.15	0.990	0.015	0.05	85.5	9.8	0.009	18	0.010	20
fluquinconazole	F	0.05*	340, 342, 108	0.05-0.15	0.989	0.015	0.05	82.6	10.7	0.010	20	0.010	20
HCH-alpha	I	0.01*	219, 181, 183	0.01-0.15	0.996	0.003	0.01	75.4	12.3	0.002	20	0.002	20

Active substance	Activity type [a]	MRL[b] (mg kg^{-1})	Ions scanned[c] (m/z) T, Q_1, Q_2	Linearity range (mg kg^{-1})	R^2	LD (mg kg^{-1})	LOQ (mg kg^{-1})	Recovery (%)	RSD[d] (%)	U_r[e] (mg kg^{-1})	U_r[f] (%)	U_R[g] (mg kg^{-1})	U_R[h] (%)
HCH-deltha	I	/	219, 181, 183	0.05-0.15	0.979	0.015	0.05	82.8	12.7	0.007	14	0.012	24
iprodione	F	0.05*	314, 316, 187	0.05-0.15	0.986	0.015	0.05	81.6	9.8	0.010	20	0.010	20
kresoxim-methyl	F	0.05*	116, 206, 131	0.05-0.15	0.992	0.015	0.05	84.5	9.3	0.008	16	0.009	18
mecarbam	A, I	0.05*	131, 159, 329	0.05-0.15	0.974	0.015	0.05	79.7	10.8	0.010	20	0.010	20
methacrifos	A, I	0.05*	208, 180, 240	0.05-0.15	0.993	0.015	0.05	73.0	8.4	0.006	12	0.007	14
metrafenone	F	0.05*	393, 408, 379	0.05-0.15	0.992	0.015	0.05	80.5	9.2	0.010	20	0.010	20
parathion	A, I	/	291, 292, 235	0.05-0.15	0.993	0.015	0.05	82.3	15.7	0.009	18	0.015	30
permethrin	A, I	/	183, 163, 165	0.05-0.15	0.989	0.015	0.05	78.3	10.2	0.008	16	0.008	16
phorate	A, I	0.01*	231, 260, 97	0.01-0.15	0.996	0.003	0.01	82.4	17.7	0.002	20	0.003	30
phosalone	A, I	0.01*	182, 367, 121	0.01-0.15	0.995	0.003	0.01	93.4	13.9	0.003	30	0.003	30
pirimicarb	I	0.05*	166, 238, 167	0.05-0.15	0.983	0.015	0.05	79.5	7.2	0.006	12	0.007	14
pirimiphos-methyl	A, I	0.05*	290, 305, 276	0.05-0.15	0.992	0.015	0.05	78.4	7.0	0.010	20	0.010	20
procymidone	F	0.05*	283, 285, 96	0.05-0.15	0.981	0.015	0.05	80.1	9.6	0.010	20	0.010	20
propyzamide	H	0.05*	173, 175, 145	0.05-0.15	0.993	0.015	0.05	78.3	9.8	0.007	14	0.009	18
pyridaphenthion	I	/	199, 340, 188	0.05-0.15	0.992	0.015	0.05	83.9	9.2	0.008	16	0.009	18
quinalphos	A, I	0.05*	146, 298, 157	0.05-0.15	0.992	0.015	0.05	81.2	7.8	0.010	20	0.010	20
quinoclamine	H	0.05*	207, 172, 209	0.05-0.15	0.990	0.015	0.05	76.9	12.8	0.010	20	0.010	20
tetradifon	A	0.05*	159, 229, 356	0.05-0.15	0.990	0.015	0.05	82.1	10.7	0.009	18	0.010	20
tolclofos-methyl	F	0.05*	265, 267, 250	0.05-0.15	0.994	0.015	0.05	78.4	7.9	0.010	20	0.010	20
tolylfluanid	F	0.05*	238, 137, 240	0.05-0.15	0.985	0.015	0.05	80.8	7.7	0.007	14	0.007	14
triadimefon	F	0.05*	208, 210, 181	0.05-0.15	0.993	0.015	0.05	80.4	9.0	0.007	14	0.008	16
triazophos	A, I	0.05*	161, 162, 285	0.05-0.15	0.982	0.015	0.05	84.1	10.4	0.010	20	0.010	20
trifloxystrobin	F	0.05*	116, 222, 186	0.05-0.15	0.992	0.015	0.05	83.7	8.7	0.008	16	0.008	16
vinclozolin	F	0.05*	285, 124, 187	0.05-0.15	0.995	0.015	0.05	83.9	8.1	0.008	16	0.008	16

[a] A = acaricide, I = insecticide, F = fungicide, H = herbicide
[b] Regulation (EC) 396/2005, * means that MRL is set at LOQ of analytical method
[c] T = target ion, Q = qualifier ion
[d] RSD was obtained during recovery analyses
[e,f] U_r = uncertainty of repeatability
[g,h] U_R = uncertainty of reproducibility

3. 3. SURVEY OF PESTICIDE RESIDUES IN BEE POLLEN SAMPLES

The Ministry of Agriculture, Forestry and Food reported that in Slovenia in 2020, 582 PPPs, containing 239 active substances, are authorised for use on different agricultural products. The Statistical Office announced that in 2018, 1,172 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, such as oilseed rape, fruits, etc., we wanted to research if these kinds of pesticide residues are found in bee pollen. We were searching for authorised (33 % of active substances sought) and non-authorised active substances in Slovenia, to cover the possible misuse of PPPs.

Of the 30 bee pollen samples analysed, only one contained one active substance: azoxystrobin, with a concentration of < 0.05 mg kg^{-1}. This means that in 96.7 % of all samples analysed, no pesticide residues were detected. The MRL for azoxystrobin in pollen is 0.05 mg kg^{-1} and it was not exceeded. In Slovenia, azoxystrobin is authorised as a fungicide for use on oilseed rape, vine and ornamentals (among others) in 14 different PPPs. These are the plants on which bees collect pollen.

A consumer risk assessment was performed using the EFSA PRIMo model rev. 3.1, in which 36 national diets from EU countries are included. This model was used since Slovenia has not created a model of its own. The same model is used in the process of registration of PPPs in Slovenia. Since azoxystrobin was the only substance found and it was only found in one sample at a concentration of < LOQ, the LOQ for this substance was used as the input value in PRIMo model. It was compared to the Acceptable Daily Intake (ADI) of azoxystrobin (0.2 mg $(kg\ bw)^{-1}\ d^{-1}$). The calculations of chronic exposure for azoxystrobin showed that the highest was observed in the German diet for children. It represented 0.003 % of ADI. Since no Acute Reference Dose was set for azoxystrobin, no acute exposure was calculated. Based on these calculations, the conclusion was that the analysed bee pollen samples are of no cause for concern for consumers.

Our results were compared with the results from other scientific papers. Azoxystrobin was found in the Estonian pollen by Raimets et al. (2020) in 3.4 % of all samples analysed up to a concentration of 0.04 mg kg^{-1}. Tosi et al. (2018) wrote that azoxystrobin was found in 2.9 % of the Italian pollen samples analysed, with a maximum concentration of 0.054 mg kg^{-1}. Vázquez et al. (2015) reported that azoxystrobin was found in a concentration of up to 0.235 mg kg^{-1} in the Spanish pollen. Azoxystrobin was found in 3.3 % of the Slovenian pollen samples analysed, which is comparable to Estonia and Italy. The concentration of azoxystrobin found in Slovenia is comparable to that found in Estonia and Italy, but much lower than in Spain.

Other active substances analysed in our laboratory, namely acrinathrin, bifenthrin, boscalid, carbaryl, carbofuran, chlorpyrifos, clomazone, dimethoate, fenitrothion, fludioxonil, iprodione, lambda-cyhalothrin, permethrin, trifloxystrobin and vinclozoline, were not detected in Slovenian pollen, but were found in samples analysed in Estonia, France, Greece, Italy, Spain and the United Kingdom.

All active substances sought by our laboratory and positively identified in Europe were measured up to concentrations higher than our LDs. The exception is carbofuran, which was found at a concentration 10-times lower than our LD. Literature results for these active substances are presented in Table 3.

4 CONCLUSIONS

In our research, a method for determining pesticide residues originating from the environment in pollen was introduced and validated. The limit of detection was 0.003 mg kg^{-1} for 6 active substances and 0.01 mg kg^{-1} for 43 active substances. The limit of quantification was 0.01 mg kg^{-1} for 6 active substances and 0.05 mg kg^{-1} for 43 active substances. The calibration curves gave a linear response with R^2 0.974 to 0.996. The recoveries ranged from 73.0 % to 93.4 % with RSDs from 5.6 % to 17.7 %. The measurement uncertainty of repeatability ranged from 10 to 30 % and the measurement uncertainty of reproducibility from 12 to 30 %. The method was found to be fit for purpose of measuring possible breaches of MRL for 49 active substances.

The method was used to analyse 30 bee pollen samples gathered from Slovenian beekeepers, all from conventional production. A total of 49 active substances were sought, but only the fungicide azoxystrobin was found in only one of these samples. In 96.7 % of the samples analysed, the active substances sought were not detected. A risk assessment revealed that the Slovenian bee pollen samples are no cause for concern for consumers.

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Table 3: Literature results for active substances sought, but not found in our laboratory

Active substance	Limit of detection (mg kg $^{-1}$)	Max content (mg kg $^{-1}$)	Ratio of positive samples (%)	Country of origin	Reference
acrinathrin	not reported	0.458	20.0	Spain	Calatayud-Vernich et al., 2018
acrinathrin	0.015	0.055	not reported	Spain	Vázquez et al., 2015
bifenthrin	0.015	0.015	not reported	Spain	Vázquez et al., 2015
boscalid	0.0025	0.058	0.7	Italy	Tosi et al., 2018
boscalid	0.00012	0.021	52.0	United Kingdom	David et al., 2016
boscalid	0.0015	0.03	not reported	Spain	Vázquez et al., 2015
carbaryl	0.0007	0.015	8.0	France	Wiest et al., 2011
carbaryl	0.00025	0.001	0.2	Italy	Tosi et al., 2018
carbofuran	0.0004	0.002	2.0	France	Wiest et al., 2011
chlorpyrifos	not reported	0.05	14.0	Spain	Hakme et al., 2017
chlorpyrifos	0.001	0.3982	not reported	Spain	García-Valcárcel et al., 2019
chlorpyrifos	not reported	0.1	31.1	Spain	Calatayud-Vernich et al., 2018
chlorpyrifos	0.0015	0.07	not reported	Spain	Vázquez et al., 2015
chlorpyrifos	0.008	0.14	4.0	France	Wiest et al., 2011
chlorpyrifos	0.0032	0.046	not reported	Greece	Kasiotis et al., 2014
chlorpyrifos	0.001	0.179	30.3	Italy	Tosi et al., 2018
clomazone	not reported	0.02	5.0	Spain	Hakme et al., 2017
dimethoate	not reported	0.042	20.7	Estonia	Raimets et al., 2020
dimethoate	0.0015	0.015	not reported	Spain	Vázquez et al., 2015
dimethoate	not reported	0.022	8.9	Spain	Calatayud-Vernich et al., 2018
dimethoate	0.00025	0.163	7.9	Italy	Tosi et al., 2018
dimethoate	0.0028	0.1445	not reported	Greece	Kasiotis et al., 2014
dimethoate	0.0091	0.0182	1.0	France	Wiest et al., 2011
fenithrothion	not reported	0.014	2.2	Spain	Calatayud-Vernich et al., 2018
fludioxonil	0.0015	0.033	not reported	Spain	Vázquez et al., 2015
iprodione	0.0156	0.0195	1.0	France	Wiest et al., 2011
lambda-cyhalothrin	not reported	0.077	17.2	Estonia	Raimets et al., 2020
permethrin	not reported	0.034	5.0	Spain	Hakme et al., 2017
permethrin	0.0015	0.0035	not reported	Spain	Vázquez et al., 2015
trifloxystrobin	0.0086	0.058	not reported	Greece	Kasiotis et al., 2014
trifloxystrobin	0.00024	0.01	40.0	United Kingdom	David et al., 2016
trifloxystrobin	0.00025	0.046	5.6	Italy	Tosi et al., 2018
trifloxystrobin	0.0015	0.0154	not reported	Spain	Vázquez et al., 2015
vinclozoline	0.0015	0.07	2.0	France	Wiest et al., 2011

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