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Risk assessment of antibiotic and pesticide residues associated with the consumption of honey bees (*Apis mellifera lamarckii*)

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ABSTRACT

In this study, a detection of antibiotic and pesticide residues in honey bees *(Apis mellifera lamarckii)* samples collected from the Egyptian local market followed by evaluation of human health risk assessment for both adults and children associated with honey consumption as a result of contamination with pesticide and antibiotic residues by calculation of human health risk assessment parameters such as HQ and HI. Modified method was used for antibiotics analysis shows acceptable recovery for 97% of the tested antibiotics between 70 and 115% while only 3% recovered between 60-69% with RSD of 21% in accordance with the EU guidelines 2002/657/EC. The limit of quantification for most of the target antibiotics was 10µg/kg except for Flumequine, trimethoprim (5µg /kg), Erythromycin (4µg/kg), and chloramphenicol (0.075µg/kg), LOD ranging from 0.002-0.004 µg/kg with method expanded uncertainty (Uexp) = 38 %. One-hundred-sixteen honey samples collected from (Giza, Cairo) Egyptian local markets were used to evaluate the human health risk assessment of both adults and children associated with honey consumption as a result of contamination with pesticide and antibiotic residues by calculation of human health risk assessment parameters such as HQ and HI. The results show that honey was contaminated with antibiotic residues belonging to five different chemical groups. The cumulative risk assessment parameters (cHI) for adults according to antibiotics chemical groups were calculated. The order of (cHI) was tetracyclines, quinolone, macrolide, and sulfonamides with values 1.559, 1.372, 0.001, 0.100 for children and 1.109, 0.976, 0.001, and 0.071 for adults respectively. For pesticide residues, 5% of the samples (n=6) out of 116 (mainly nigella sativa honey product containing wax) were contaminated with DMF, three less than the method limit of quantification, and the other contain residues of DMF less than EU-MRL. Hence, there is a need for continuous surveys and monitoring to protect adult and children from exposure to antibiotics, as well as beekeepers' education programs to control antibiotics uses during to treatment of honeybee colonies, in order to meet food safety standards and protect human health.

Keywords: Antibiotics, Pesticides, Hazard Quotient, Hazard Index, Risk assessment

1. Introduction

Honey is a naturally sweet and tasty food produced by honeybees after secretion from different flower nectars, due to its high contents of proline level, phenolic content, antioxidant activity and mineral profile, it can be used as an effective therapeutic medicinal agent in the treatment of numerous diseases also as vital source of healthy ingredients (Codex Alimentarius Commission, 2019). The presence of numerous environmental contaminants in honey such as heavy metals, pesticides, organic pollutants, antibiotics and genetically modified organisms (GMO) is considered to cause posing a risk to human health which might in most cases change disease resistance (Baša Česnik *et al*., 2019;

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Bedendo *et al*., 2010; Chiesa *et al*., 2018; Reybroeck, 2018). The monitoring methods playing an important role in improvement of food safety issue. The contamination of honey by antibiotic residues such as sulfonamides and tetracyclines has been reported, where the sulfonamides were dominant in the Egyptian and Saudi Arabian honey samples, while tetracyclines were the most predominant in Libya (Ahmed *et al*., 2022). Another study showed that the results from monitoring data of antibiotic residues, 2% of the analyzed samples contains oxytetracycline, 6% tetracycline and its 4% metabolite 4-tetracycline residues were detected in the honey samples (Yang *et al*., 2022). In 2020, more than 50 pesticide and veterinary drug residues were found in the four types of beeswax from 182 samples were collected from different Belgian local market (el Agrebi *et al*., 2020). The Egyptian honey samples analyzed in 2011 were contaminated by antibiotics residue of 89% tylosin, 47% chloramphenicol and 31% tetracycline(Asmaa E. Abd Alla, 2020a). Of total 43collceted honey samples, 44 % was contaminated with antibiotics and/or pesticide residues of one or more analytes with concentration ranged from 0.12 to 10 μg kg−1 in each sample (Orso *et al*., 2016a). Different analytical technique used for quantitative determination of antibiotics and pesticides residue in honey using LC-MS/MS and HPLC-DAD were used but the most competitive part is the presence of naturally occurring interferences also different physicochemical properties of target contaminants. One of the main problems was extraction step since different type of antibiotics belongs to different chemical groups. A development of liquid-liquid microextraction (DLLME) method and analysis by ultra-fast liquid chromatography coupled to diode array detector (UFLC-DAD) for determination of Antibiotic residues was performed by (Santana *et al*., 2018) and the results shows that method limits of detection (LODs) in the range of 3.1-6.8 μg kg and recoveries between 82.9 and 105.7%. Another method for the determination of 41 different types of antibiotics from 7 different groups and their different components in the honey using LC-MS/MS showed that 35 antibiotics acceptably recovered due to the difficulty of the stages, solvent strength and hyperbaric column problems(Namık BİLİCİ *et al*., 2019) The limits of detection and quantitation for six sulfonamide antibiotics ranged from 0.004–1.050 μg kg−1 and 0.014–3.499 μg kg−1, respectively as results of on-line solid phase extraction using molecularly imprinted polymers coupled and LC-MS/MS in honey samples with recovery ranged from 84.3 and 104.7% (RSD < 11.6%) (Baeza Fonte *et al*., 2018) Using of sample preparation that includes homogenization with McIlvaine buffer 0.1 mol L−1 (pH 4), followed by extraction with acetonitrile and cleanup using dispersive solid phase extraction (d-SPE) was used for determination of pesticides and antibiotics residue in honey using UPLC/MS-MS (Orso *et al*., 2016b). Maximum residue limits (MRLs) for antibiotic, pesticide, and environmental contaminants in honey established by food regulatory agencies. The European Union and codex Alimentarius set MRLs 10 μg kg−1 for some pesticides, and prohibited the use of antibiotics (Codex Alimentarius Commission, 2017, 2019; EU Pesticides Database (v.2.2), 2021). Therefore, the aim of study is to develop and validate a rapid and effective modified extraction QuEChERS method for extraction followed by detection using LC-MS/MS for antibiotic residues based on quality criteria of 2002/657/EC measuring linearity, accuracy, repeatability, within-laboratory reproducibility, decision limit (CCα) and detection capability (CCβ)(European Commission, 2002b) , and evaluating human health risk assessment of both antibiotics and pesticide residues considering the status of honey contamination with pesticide and antibiotic residues which is important issue related to human health risk.

2. Experimental

2.1. Certified reference materials

For pesticide residue analysis standard stock, working and calibration mixture solutions was prepared as prementioned in (Issa *et al*., 2020). Thirty target antibiotics certified reference materials of chloramphenicol, ciprofloxacin, enrofloxacin, erythromycin, sulfamerazine, sulfapyridine, trimethoprim, flumequine, sulfacetamide, sulfadiazine, sulfamethoxazole, sulfathiazole, sulfamethazine, doxycycline, tetracycline, oxytetracycline, chlortetracyclin, tylosin, oxolinicacid, sarafloxacin, sulfadoxine, sulfisoxazole, sulfanilamide, sulfamethoxypyridazine, sulfadimethoxine, sulfachlorpyridazine, sulfaguanidine, sulfamethizole, sulfamoxole, sulfamonomethoxine were purchased as active ingredient from (Dr. Ehrenstorfer-LGC GmbH, Augsburg, Germany) with a high purity ≥95%. Stock solutions for all antibiotics were prepared by compensating its salt (if found) to target analyte with concentration of 1000 μg/ml. Calibration mixtures of series 0.1, 0.25, 0.5,

1.00,2.00,5.00 MRL depending on LOQ of each target antibiotic were prepared in methanol for LC-MS/MS all stored at -18ºC.

2.2. Chemicals and reagents

Acetonitrile HPLC and LC-MS grade ≥ 99% was purchased from (Fisher, Loughborough, UK), acetone-HPLC grade ≥ 99% and methanol LC-MS grade ≥ 99% were from (Merck, Darmstadt, Germany). Citric acid monohydrate, Ammonium hydroxide and formic acid ≥ 99% (Sigma-Aldrich, Darmstadt, Germany). Ethylenediaminetetraacetic acid di-sodium salt Na2-EDTA (Merck, Darmstadt, Germany). De-ionized Water, generated by Millipore water purification system.

2.3. Apparatus

Geno/Grinder 2010- SPEX Sample Prep (UK) shaker, centrifuge up to 4500 rpm (Sigma, Germany 3-16KL). Hiedolph rotary evaporator (Heidolph Instruments GmbH & CO. KG). Calibrated micropipettes (Hirschman Laborgerate- Germany) for preparations of calibration in ranges (10-100, 100-100µl). Solvent dispenser with a 10mL capacity (Hirschman Laborgerate- Germany).

2.4. LC–MS/MS conditions

Separation was performed on a chromatographic column Zorbax-C18 (2.1 mm x 50 mm, 1.8 µm) (Merck, Darmstadt, Germany) using Agilent HPLC model 1200 system coupled to API 4000 Q-TRAP (Agilent, Santa Clara, USA). The injection volume was 25 µl. The elution flow rate was 0.8 ml/min. The API 4000 Q-TRAP System (Applied Biosystems, Foster City, CA, USA) with electrospray ionization (ESI) interface in both positive and negative electrospray ionization mode (ESI+) & (ESI-) was used and N2 nebulizer, curtain, and other gas settings were optimized according to recommendations made by the manufacturer. Source temperature was 300°C, ion spray potential 5500 V where multiple reaction monitoring (MRM) was applied, and two product ions was selected (for quantification and confirmation transition). Mobile phase solution consisting of: (A) 5mM ammonium format in methanol buffer (1:9) was prepared from 50mM ammonium hydroxide solution that was previously prepared and formic acid in water adjusted to pH= 2.8±0.1 and (B) methanol.

2.5. Sample handling

A total 116 Honeybee samples representing three varieties (40 sample from nigella sativa ten of them contain honey wax, 38 sample from each citrus and clover) were collected randomly from Egyptian local markets “Giza and Cairo” governorate during the period “June 2021 to July 2022” including season of the year used for random surveillance of Antibiotics and pesticide residues in honey samples. This aim particularly controlling the compliance with MRLs for residues of veterinary drugs fixed in Annexes I and III to Regulation (EEC) No 2377/90(European Commission, 2013), and the maximum levels of pesticides fixed in Annex III to Directive 86/363/EEC(European Commission, 2002a), and monitoring the concentration of environmental contaminants. The representative pre-packed in glass jar samples were collected randomly with minimum weight one-kilogram then labeled, maintained at 4°C using ice-box and sent to the laboratory for proceeding analysis. Disposable consumables and polypropylene tubes were used to prevent cross-contamination during analysis of samples.

2.6. Sample extraction

For pesticide residues analysis, the samples were subjected laboratory pre-validated acetonitrile-ethyl acetate extraction-based method for the residue analysis of 373 pesticides in beeswax using LC-MS/MS and GC–MS/MS applying method (Issa *et al*., 2020). For antibiotics residues analysis, two grams of well homogenized blank honey sample was weight in 50 ml polypropylene tube, then 1ml sodium citrate buffer and 0.5 ml Na2EDTA solution (0.5M) were added to the sample followed by ultrasonication for 30min to release the sulfonamide bonds from the honey matrix. The extraction was performed using 10ml of acetonitrile, then shake the sample for one-minutes using Geno-grinder and finally centrifugation was applied on 4500rpm for10minutes. The upper layer of aliquot completely transferred to 50ml flask. The acetonitrile extraction and centrifugation steps were repeated, and collect the other portion of aliquot to the same flask. Finally, evaporation using rotary evaporator at 40^0C till reach dryness and reconstitution with two-ml of dilution solvent, filter with syringe-filter into 2ml vial

and directly inject on LC-MS/MS. Complete validation for multiresidue antibiotics method of analysis in honey was performed to confirm method performance following EU guidelines 2002/657/EC(European Commission, 2002a) in performing the different validation parameters and uncertainty estimation.

3. Quality control and quality assurance

An intensive quality control program was applied on all target analytes where, LC-MS/MS instrument were conditioned with mobile phase followed by injection of blank sample, calibration standards series and laboratory fortified blank (LFB) with known concentration for quality control (QC). As a confirmation of target analyte retention times, linear curve accuracy for calibration series and recovery of (LFB) for each analyte was checked prior to evaluate the results of each injected batch. The linear regression coefficient (r) of each analyte calibration curve shall be more than 0.995 also, reproducibility confirmation via relative standard deviation (RSD) being less than 20%. All used glassware during samples preparation were cleaned with acetone then dried with oven prior use.

4. Human health risk assessment

A dietary portion size of honey recommended by Joint FAO/WHO Expert Committee on Food Additives (JECFA) the Twenty-first Session of CCRVDF during the seventieth meeting of JECFA that 55g/person per day for adults (95th percentile) and 22.1g/person per day for children (2-5 years).(Joint FAO/WHO Expert Committee on Food Additives (JECFA)on Food Additives & Organization, 2009). The risk was calculated through the assessment of detected residues recognized through the calculation of estimated daily intake (EDI) and use of acceptable daily intake (ADI) to calculate hazard quotient (HQ), where the estimated daily intake (EDI) of antibiotic residue was calculated as follows:

$$\text{EDI} = \sum \frac{Fi\ x\ Mi}{mean\ body\ wegiht} \qquad \text{Equation 1}$$

where: F_i - food consumption data consumption, M_{i-} mean concentration of detected antibiotics (Darko & Akoto, 2008)

For long-term risk assessment evaluation was performed via calculation of hazard quotient (HQ) associated with non-carcinogenic compared to the toxicological data (van der Velde-Koerts *et al*., 2021)of the intakes by dividing the estimated with the acceptable daily intake (ADI). (WHO, 1997)

$$HQ = \frac{EDI}{ADI} \qquad \text{Equation 2}$$

If the HQ is >1 then the food involved should be considered as violated with antibiotic residue, while if the index is <1, this would indicate that the food involved is considered acceptable. (U.S. EPA, 2005)

The chronic hazard index (cHI) was obtained by summation of HQs of antibiotics belonging to the same chemical group to assessing risk

$$cHI = \sum HQ \qquad \text{Equation 3}$$

i.e: Sulfonamide (Sulfamethoxazole, Sulfadiazine), Tetracycline (Tetracycline, Oxytetracycline, Doxycycline)

3. Results and Discussion

3.1. Validation of multiresidues method for antibiotics in honey

3.1.1. Recovery tests

Repeated spike honey samples at concentrations of 0.1 MRL, 0.25 MRL, 0.5 MRL, and 1 MRL were used to test the recovery for the chosen antibiotic. Chloramphenicol's MRPL is 0.3 g/kg, Trimethoprim and Flumequine's MRL is 50 g/kg, and the MRL for the remaining compounds is 100 g/kg. On each level, the average recoveries and the relative standard deviation were computed. The recognized recovery and precision are in compliance with the standards of the Codex guidelines and

the EU Commission Directive No. (2002/657/EC). Figure 1 shows real injection of recovery test at concentration level 0.1 MRL for all target analytes.

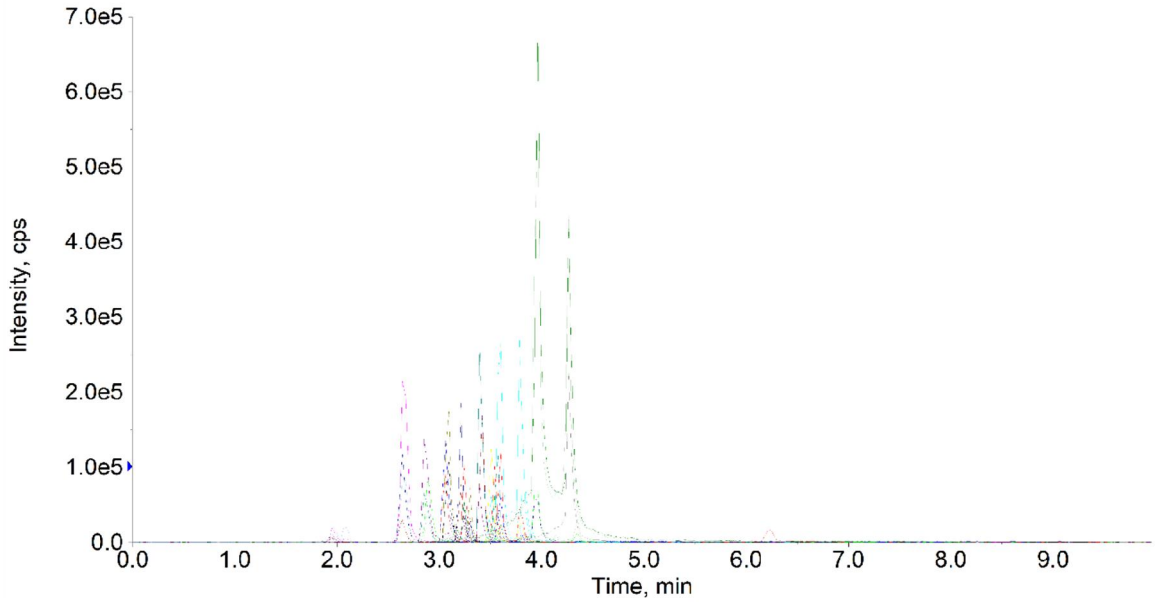


Fig. 1: Real injection MRM spectrum of recovery test at concentration level 0.1 MRL for all target analytes.

Multiple-reactions monitoring (MRM) conditions including target analyte retention time, de-clustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) are provided in supplementary data.

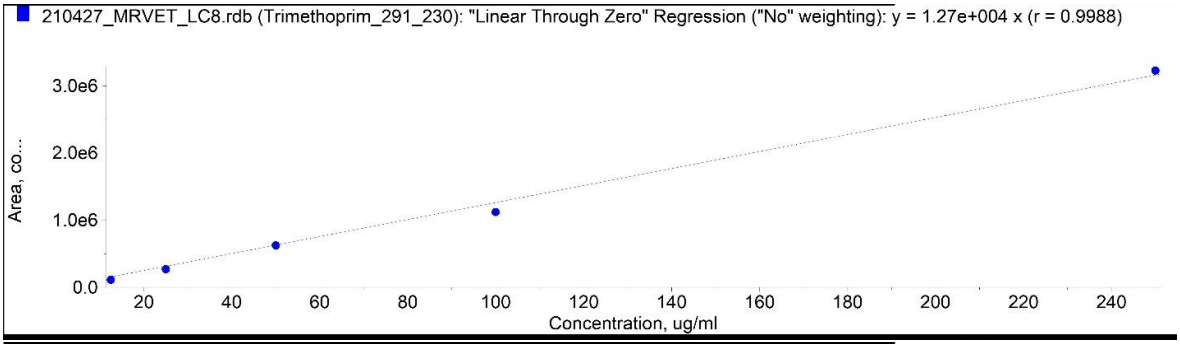
Using repeated spiked samples at the expected lowest quantitation level on honey samples, the limit of quantitation was estimated. All drugs had a limit of quantitation (LOQ) of 10 µg /kg with the exception of Flumequine and trimethoprim (5 µg /kg), Erythromycin (4µg/kg), and chloramphenicol (0.075µg/kg). According to the results in Table 1, 97% of the tested antibiotics recovered between 70 and 115%, while only 3% recovered between 60-69% at LOQ level of 0.1 MRL with an RSD of 21% in accordance with the EU guidelines 2002/657/EC.

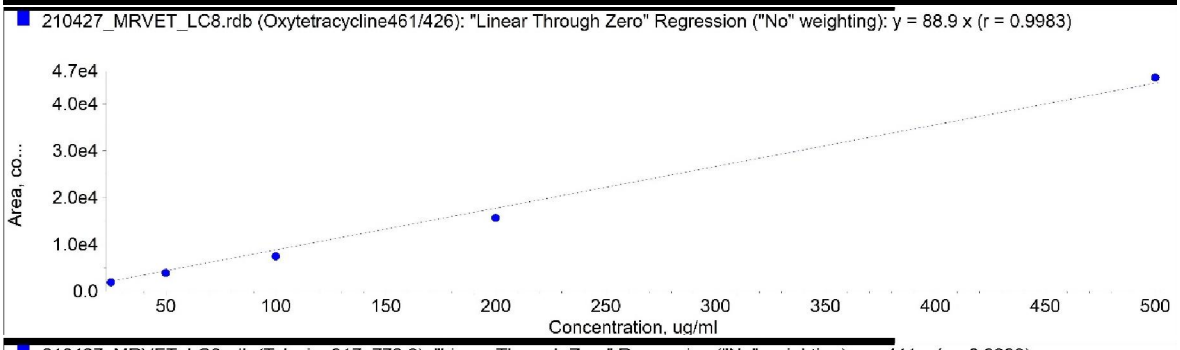
3.1.2. Linearity

Linearity was examined using a multi-point matrix matched calibration starting from 0.1, 0.25, 0.5, 1, 2, and 5 MRL mixture solutions containing the chosen antibiotics. The method's linearity was examined by running recovery tests on samples of honey at various concentration levels. From the LOQ up to 5 MRL, the method was found to be linear with linearity coefficient (r2) more than 0.999. Figure-2 shows calibration curve established using five-point calibration standards for different antibiotics. Linearity coefficient (r2) for all target antibiotics is provided in *supplementary data.*

Table 1: Performance characteristics: average recovery (Rec± RSD %), relative standard deviation, relative standard deviation pooled (RSDpooled %) and the average recovery (Q. Type%) for four fortification levels of honey samples (n=6).

	0.1 MRL	0.25 MRL	0.5 MRL	1 MRL		
Antibiotic Name	**Recovery ± RSD%**				**$Q._{Type}$ %**	**RSD pooled %**
Chloramphenicol	99±17	101±21	94±15	96±12	98	16
Ciprofloxacin	88±14	95±11	84±10	91±13	90	12
Enrofloxacin	71±12	100±13	95±9	95±9	90	11
Erythromycin	83±13	105±14	83±15	97±11	92	13
Sulfamerazine	90±13	80±13	90±13	89±12	87	13
Sulfapyridine	98±11	90±14	88±14	87±15	91	14
Trimethoprim	86±15	83±9	80±13	78±11	82	12
Flumequine	71±15	106±13	96±9	97±9	93	12
Sulfacetamide	89±13	94±13	95±14	98±14	94	14
Sulfadiazine	88±15	84±15	96±15	99±13	92	15
Sulfamethoxazole	95±9	96±13	99±11	102±13	98	12
Sulfathiazole	85±15	84±14	83±12	88±13	85	14
Sulfamethazine	86±10	96±14	96±11	86±12	91	12
Doxycycline	100±15	77±15	80±14	77±13	84	14
Tetracycline	113±15	64±16	67±14	85±11	82	14
Oxytetracycline	61±8	90±10	61±8	83±12	74	10
Chlortetracyclin	97±15	69±16	72±15	87±12	81	15
Tylosin	95±11	100±13	97±14	106±12	100	13
Oxolinic acid	97±15	98±13	99±11	99±15	98	14
Sarafloxacin	86±15	96±12	91±13	99±14	93	14
Sulfadoxine	87±13	75±14	99±14	91±14	88	14
Sulfisoxazole	89±15	78±16	98±15	86±14	88	15
Sulfanilamide	110±15	82±14	76±15	96±14	91	15
Sulfamethoxypyridazine	108±15	96±12	95±14	89±12	97	14
Sulfadimethoxine	93±13	102±12	99±11	100±10	99	12
Sulfachlorpyridazine	98±14	93±15	107±15	100±16	100	15
Sulfaguanidine	98±13	91±12	99±15	99±14	97	14
Sulfamethizole	86±14	92±15	96±13	91±15	91	14
Sulfamoxole	93±13	89±15	105±14	92±12	95	14
Sulfamonomethoxine_	84±15	83±14	101±13	93±16	90	15


210427_MRVET_LC8.rdb (Trimethoprim_291_230): "Linear Through Zero" Regression ("No" weighting): y = 1.27e+004 x (r = 0.9988)
Area, co...
3.0e6
2.0e6
1.0e6
0.0
20 40 60 80 100 120 140 160 180 200 220 240
Concentration, ug/ml


210427_MRVET_LC8.rdb (Oxytetracycline461/426): "Linear Through Zero" Regression ("No" weighting): y = 88.9 x (r = 0.9983)
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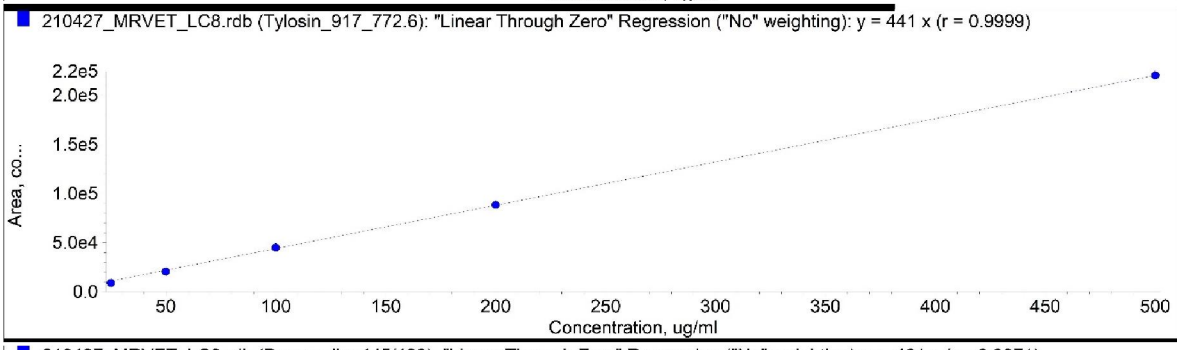
4.0e4
3.0e4
2.0e4
1.0e4
0.0
50 100 150 200 250 300 350 400 450 500
Concentration, ug/ml


210427_MRVET_LC8.rdb (Tylosin_917_772.6): "Linear Through Zero" Regression ("No" weighting): y = 441 x (r = 0.9999)
Area, co...
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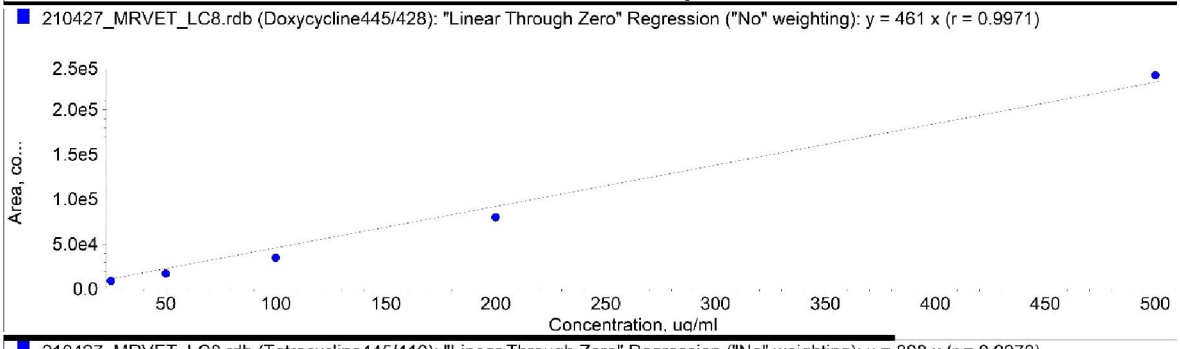
1.5e5
1.0e5
5.0e4
0.0
50 100 150 200 250 300 350 400 450 500
Concentration, ug/ml


210427_MRVET_LC8.rdb (Doxycycline445/428): "Linear Through Zero" Regression ("No" weighting): y = 461 x (r = 0.9971)
Area, co...
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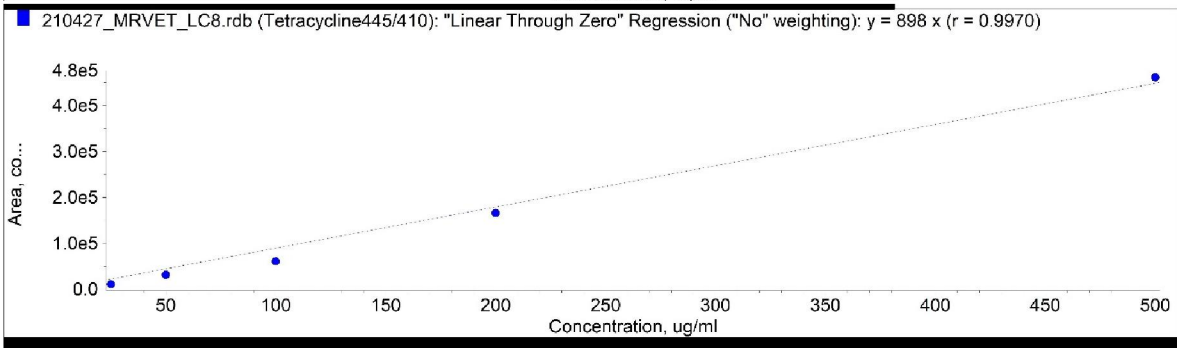
2.0e5
1.5e5
1.0e5
5.0e4
0.0
50 100 150 200 250 300 350 400 450 500
Concentration, ug/ml


210427_MRVET_LC8.rdb (Tetracycline445/410): "Linear Through Zero" Regression ("No" weighting): y = 898 x (r = 0.9970)
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4.0e5
3.0e5
2.0e5
1.0e5
0.0
50 100 150 200 250 300 350 400 450 500
Concentration, ug/ml

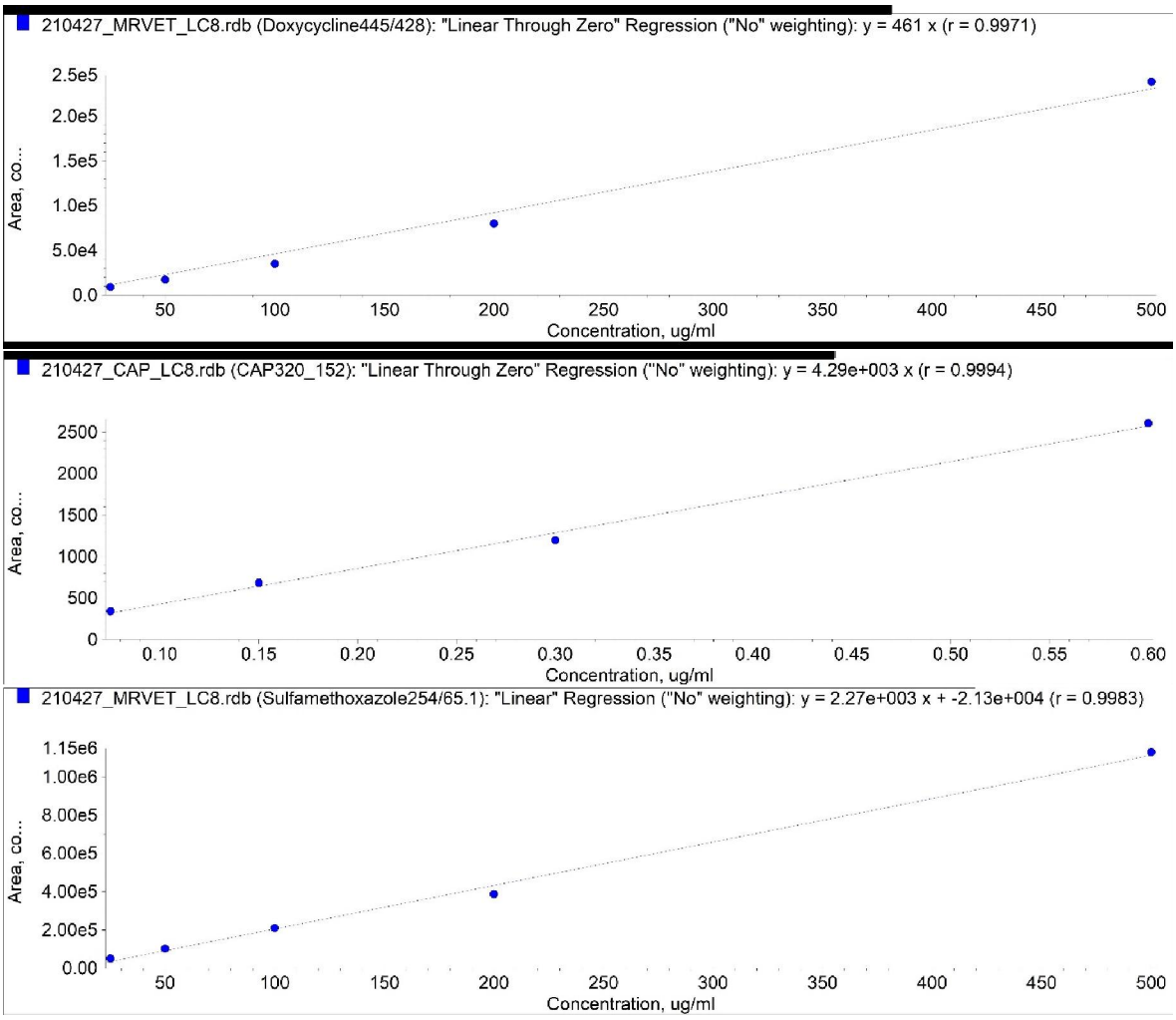

Fig. 2: Shows calibration curves for different antibiotics

3.1.3 Method Accuracy

Method accuracy was expressed in terms of both trueness (spiked samples used at different levels on honey samples or via the proficiency testing sample) and Precision which consists of two measures: repeatability (fortification of blank honey samples at different levels under the same conditions) and reproducibility (intra-laboratory reproducibility by spiking blank honey samples and application of extraction detection steps by different analysts on various days). The tested antibiotics' LOD and LOQ were also examined. LOD was found to be between 0.002-0.004 and LOQ's of all target antibiotics were 10 µg/kg, with the exception of chloramphenicol, whose LOQ was equal to 0.075 µg /kg. For all of the analytes calibration curves, the linear regression coefficients (R2) were more than 0.998, indicating good linearity. If the recovery is greater than or equal to 60%, the results will be corrected. Depending on the class of antibiotic, the limit of quantification (LOQ) began at 0.075 and 10 µg /kg. The validation results found that both recovery percent and RSD% in accordance with SANTE/11312/2021 (Pihlström *et al*., 2022).

The validated method was subjected to FAPAS proficiency test round and all results found satisfactory with acceptable z-score according to FAPAS reports summarized in tabl-2.

Table 2: Summarized Results of FAPAS Proficiency test round applying the validated method honey

FAPAS round	Compound	Assigned value (µg/kg)	Found (µg/kg)	Z-score	Status
02316	Tetracycline	130	132	0.1	Satisfactory
02321	Chloramphenicol	0.886	0.61	-1.4	Satisfactory
02328	Oxolinic acid	114.6	70.54	-1.7	Satisfactory
	Enrofloxacin	136.9	120.9	-0.5	Satisfactory

3.1.4. Reporting limit, decision limit (CCα) and the detection capability (CCβ)

For each examined antibiotic, the limit of reporting level (LRL) for the method—defined as the lowest fortified level for which recovery and precision were acceptable—was set at 0.5 MRL. The method decision limit (CCα) and the detection capability (CCβ) were evaluated in accordance with the EU guidelines 2002/657/EC.

3.1.5. Decision limit (CCα): is the limit above which it can be decided with a statistical certainty of 95% that the identified analyte concentration is truly above the MRL. (α error = 5% in the case of MRL compounds)

$$CC\alpha = MRL + 1.64(S.D.std^2 + S.D.R^2)\text{^}½ \quad equation\ 4$$

$$CC_\alpha = \mathrm{MRL} + 1.64\sqrt{S.D._{std}^2 + S.D._R^2}$$

MRL: is the maximum residue value (ug/kg);

S_{std}: the allowed variation in concentration of the standard solution, is typically set to 5%

S_{DR}: is the within-house reproducibility for the analysis of different samples.

3.1.6. The Detection Capability (CCβ): is the concentration of analyte, at which the method is able to detect MRL concentrations with a statistical certainty of 95%. (ß error = 5% in the case of MRL compounds), CC_β was calculated from CCα according to Commission Decision 2002/657/EC.
CC_β = CCα + 1.64 SD (within-laboratory reproducibility) equation 5

Table 3: The values of CCα and CCβ obtained from within-laboratory standard deviation

Antibiotic Name	Conc. (ug/kg)	SDR	SD_{std}	CCα (ug/kg)	CCβ (ug/kg)
Chloramphenicol	0.15	0.03	5	0.17	0.21
Chlortetracyclin	50	5.3	5	54	63
Ciprofloxacin	50	3.2	5	54	59
Doxycycline	50	5.4	5	55	64
Enrofloxacin	50	5.3	5	55	64
Erythromycin	20	6.7	5	24	35
Flumequine	25	1.8	5	27	30
Oxolinic acid	50	4.5	5	56	63
Oxytetracycline	50	3.6	5	57	63
Sarafloxacin	50	4.8	5	54	62
Sulfacetamide	50	6.5	5	60	71
Sulfachlorpyridazine	50	6.2	5	60	70
Sulfadiazine	50	3.1	5	60	65
Sulfadimethoxine	50	7.4	5	60	72
Sulfadoxine	50	7.4	5	60	72
Sulfaguanidine	50	7.9	5	60	73
Sulfamerazine	50	5.3	5	60	68

Sulfamethazine	50	7	5	60	72
Sulfamethizole	50	8	5	60	74
Sulfamethoxazole	50	7.4	5	61	73
Sulfamethoxypyridazine	50	8	5	61	74
Sulfamonomethoxine	50	7.5	5	59	71
Sulfamoxole	50	7	5	61	72
Sulfanilamide	50	6.7	5	59	70
Sulfapyridine	50	6.3	5	61	71
Sulfathiazole	50	7.6	5	59	72
Sulfisoxazole	50	8	5	61	74
Tetracycline	50	4.7	5	55	63
Trimethoprim	25	2.2	5	27	30
Tylosin	50	5.7	5	58	68

3.1.7. Measurement Uncertainty

From the data obtained for Qtype % (average recovery of the four levels of concentration) and pooled RSD % in table 1. Reproducibility was estimated by pooling the variances of the three different levels of concentration (Prudnikov, 1981)

RSD = Relative standard deviation

$$RSD_{pooled} = \sqrt{\frac{(RSD_1)^2(n_1-1)+(RSD_2)^2(n2-1)+.....}{(n_1-1)+(n_2-1)+....}} \quad \text{equation 6}$$

n = Number of samples.

The estimation of measurement uncertainty was carried out by applying the approaches that major part of uncertainty could be estimated form recovery and precision calculated form validation data using the following equations(Koesukwiwat *et al*., 2011; Walorczyk & Drożdżyński, 2012).

Relative standard uncertainty U_{Rec}

$$U_{(Rec)} = \frac{s}{\sqrt{n}}$$

Combined uncertainty U_c

$$U_C = \sqrt{(U_p)^2 + (U_{\text{Re}\,c})^2 + U_{\text{Re}\,f}} \quad \text{equation 8}$$

For 5 degrees of freedom for honey, U_{Rec} uncertainty due to recovery, $U_{processing}$ uncertainty due to sample processing, U_{Ref} uncertainty due to reference standard preparation and U_{pre} the uncertainty due to precision experiments were calculated and found equal to 2.2%, 10%, 0.7% and 16%, respectively. In this case (since t_{calc} = 15.92 is greater than t_{tab}= 2.07), the recovery is statistically significantly different from 100, but in the normal application of the method no correction is applied. The combined uncertainty estimation was 19%. Therefore, the expanded uncertainty was calculated at a confidence level of 95% and k=2 and found that Expanded Uncertainty (U_{exp}) = 38 %. The validated analytical method as part of a laboratory accreditation scope according to ISO/IEC17025:2017 by the Centre for Metrology and Accreditation, Finnish Accreditation Service (FINAS), Helsinki, Finland that used in routine work. Quality control sample at 40 MRL was subjected to method accompanied with each batch to monitor method performance. A daily routine recovery percentage (Rec.%) for each target analyte was tested through analysis of laboratory fortified blank (LFB), examined prior releasing results and evaluated using x-chart according to laboratory QC policy. It was found that daily routine recovery

for tetracyclines in range of (62-85%), quinolone (78-84%), macrolide (88-105%), sulfonamides (80-118%) and diaminopyrimidine (85-101%) with relative standard deviation (RSD%) less than 21%.

Table 4: Summarized the frequency of antibiotics & pesticides occurrence in honey samples, Mean, Median, range of detected concentration (µg/Kg), chemical group, and quality control recovery range (QC Rec%)

	Freq.	Mean (µg/Kg)	Median (µg/Kg)	Range (µg/Kg)		QC Rec%	Chemical group	Freq.
Sulfamethoxazole	47%	113.26	27.55	3.88	1043.64	80-118%	Sulfonamide	46%
Sulfadiazine	3%	158.89	7.30	1.74	1293.18			
Tylosin	16%	39.65	28.28	6.23	122.58	88-105%	Macrolide	16%
Ciprofloxacin	5%	2484.01	1367.50	286.90	7500.00	78-84%	Quinolone	5%
Trimethoprim	50%	103.54	18.49	4.58	902.99	85-101%	Diaminopyrimidine	50%
Oxytetracycline	2%	6851.05	29.20	13.45	68750.00	62-85%	Tetracycline	12%
Tetracycline	17%	177.04	177.04	80.46	273.62			
Doxycycline	3%	26.71	24.13	15.66	42.94			
Pesticides								
DMF	5%	12.02	10.00	<LOQ	16.00	94-110%	Acaricides	5%

For pesticide residues, 5% of the samples (n=6) out of 116 (mainly nigella sativa honey product that contain wax) contaminated with DMF which is the transformation products of amitraz, three of them less than method limit of quantification, while the other contain residues of DMF less than MRL=20µg/kg(European Commission, 2017). The results in table -4 showed that, fifty-eight samples (50%) of the total analyzed samples (n=116) were free from any antibiotics residue and (100%) free from any pesticides residues. The most frequently detected antibiotic group was diaminopyrimidine followed by sulfonamide, macrolide, tetracycline and quinolone. Fifty-eight samples of the total analyzed samples were contaminated with diaminopyrimidine group (50%), Fifty-three honeybee samples with sulfonamide group (46%), eighteen samples with macrolide group (16%), fourteen samples with tetracycline group (12%), and six samples with quinolone group (5%). Which was almost similar in sequence of occurrence of sulfonamides and tetracyclines group during the short brief about the Egyptian honeys in 2020study (Ahmed *et al*., 2022). Among the sulfonamide group, 100% of the sample were contaminated with sulfamethoxazole (n=53), 26% with sulfadiazine residue (n=14) and 25% of the samples contains both antibiotic residues. For tetracycline group, 86% of the sample were contaminated with oxytetracycline (n=12), 14% with tetracycline (n=2), 29% with doxycycline (n=4) and 36% of samples contain more than one antibiotic of tetracycline group. For quinolone group, 100% of the samples were contaminated with ciprofloxacin (n=6). For diaminopyrimidine group, 100% were contaminated with trimethoprim (n=58). For macrolide group 100% of the samples were contaminated with tylosin (n=18). The most detected antibiotic was trimethoprim (50%) form total analyzed samples with mean concentration of 103.539±2.20µg/kg followed by sulfamethoxazole (46%) at mean concentration of 113.256±7.40µg/kg, tylosin (16%) at 39.650±5.70µg/kg, sulfadiazine (12%) at 158.894±3.10µg/kg, oxytetracycline (10%) at 6851.05±3.60µg/kg, ciprofloxacin (5%) at 2484.007±3.20µg/kg, doxycycline (3%) at 26.713±5.40µg/kg, tetracycline (2%) at 177.040±4.70µg/kg lower than residues found for tylosin (89%) and tetracycline (31%).(Asmaa E. Abd Alla, 2020b)

Despite that, out the detected samples tetracycline and ciprofloxacin shows (100%) were violated exceeding the maximum residue limit, trimethoprim 97%, followed by tylosin and oxytetracycline both (83%), doxycycline (75%), sulfamethoxazole (38%) and sulfadiazine (21%). According to European Rapid Alert System for Food and Feed (RASSF) notifications of imported honeybee including royal jelly covering period 2016 till now, only twelve notifications concerning exceeding EU-MRLs of some antibiotics were elaborated with four notification of border rejection notification with serious risk related to the presence of residues of veterinary drugs. (RASSF Window

2022). As a result of these findings, the MRL's based on data of the detected antibiotic and pesticide residues combined with the estimated ADI exhibit great importance in evaluating the real risk of violation by comparing the results with ADI to find HQ for non-carcinogenic risk assessment using equations from (1,2 and 3) in order to investigate human health risk for violated antibiotics.

Table 5: summarized antibiotic individual & group acceptable daily intake (ADI), Estimated daily intake, (EDI) The hazard quotient (HQ) and the chronic hazard indexes (cHI)

		Individual	**Group**	***Estimated daily intake (EDI)* (µg/kg. bw day)**		***The hazard quotient (HQ)***		***The chronic hazard indexes (cHI)***	
	Antibiotic group	**ADI (µg/kg. bw day)**	**ADI (µg/kg. bw day)**	**Children**	**Adult**	**Children**	**Adult**	**Children**	**Adult**
Sulfamethoxazole	Sulfonamide	3	50	0.125	0.089	0.042	0.030	0.100	0.071
Sulfadiazine		3		0.176	0.125	0.059	0.042		
Tylosin	Macrolide	30	30	0.044	0.031	0.001	0.001	0.001	0.001
Trimethoprim	Diaminopyrimidine	*No ADI*	*No ADI*	0.114	0.081	----	-----	----	-----
Ciprofloxacin	Quinolone	2	2	2.745	1.952	**1.372**	0.976	**1.372**	0.976
Tetracycline	Tetracycline	5	30	0.196	0.139	0.039	0.028	**1.559**	**1.109**
Doxycycline		5		0.030	0.021	0.006	0.004		
Oxytetracycline		5		7.570	5.383	**1.514**	**1.077**		
DMF	Acaricide	3	3	0.018	0.013	0.006	0.004	-----	------

For children, the results in table 5 showed that HQs for individual antibiotic was 0.001 for tylosin, 0.006 for doxycycline, 0.039 for tetracycline, 0.042 for sulfamethoxazole, 0.059 for sulfadiazine, 1.372 for ciprofloxacin, 1.514 for oxytetracycline, while for pesticide residues of DMF was 0.006. For adults, the results showed that HQs for individual antibiotic was 0.001 for tylosin, 0.004 for doxycycline, 0.028 for tetracycline, 0.030 for sulfamethoxazole, 0.042 for sulfadiazine, 0.076 for ciprofloxacin, 1.077 for oxytetracycline, while for pesticide residues of DMF was 0.004. Trimethoprim HQ could not be calculated due to lack of information regarding ADI. This means that, long-term exposure assessment based on antibiotic detected levels in the honeybee analyzed in this study, confirms that the intake of the violated antibiotics by consumption in Egypt represent a health risk to children for ciprofloxacin and oxytetracycline while, oxytetracycline for adult. Trimethoprim HQ could not be calculated due to lack of information regarding ADI. This means that, long-term exposure assessment based on antibiotic detected levels in the honeybee analyzed in this study, confirms that the intake of the violated antibiotics by consumption in Egypt represent a health risk to children for ciprofloxacin and oxytetracycline. Cumulative risk assessment one of important approach reflects the potential effects from combined exposure to antibiotics that share similar chemical properties via calculation of the chronic hazard index (cHI) using equation (3). The chronic hazard index (cHI) for eight detected antibiotics were calculated by summation of his of antibiotics residues groups with similar chemical classes as well as mode of action such as (sulfonamide, tetracycline, quinolone, diaminopyrimidine, macrolide) to acquire the cumulative risk of such groups, the details of these values found in table-5. The results showed that there was a risk associated with the exposure via the consumption of honeybee for tetracycline and quinolone. Also, according to antibiotics even with lower individual HQ < 1 but when cumulate the exposure of different individual's antibiotics of the same group. The order of cHIs reflecting long term exposure of Tetracyclines, Quinolone, Macrolide and Sulfonamides according to antibiotic chemical groups was 1.559, 1.372, 0.001 and 0.100 for children and 1.109, 0.976, 0.001 and 0.071 for adults respectively. Due to lack of information for ADI regarding Trimethoprim belongs to Diaminopyrimidine chemical group only quantitation of the detected results was reported in spite of being detected in 100% of positive samples.

4. Conclusion

In this study, one-hundred sixteen honey samples were collected from Egyptian local market were analyzed and found contaminated with eight antibiotics residues belonging to five different chemical groups. The results illustrate potentially significant risks exposure for both non-carcinogenic risks on both adults and children after consumption of the contaminated honeybee from the selected locations in Egyptian local markets. Calculation of human health risk assessment parameters based on of the detected violated antibiotic residues in the honeybee in Egypt such as HQ and HI were performed and the results represent some health risk to adults and children. Additionally, cumulative risk assessment parameters (cHI) for adults according to antibiotics chemical groups were calculated and the order of (cHI) was tetracyclines, quinolone, macrolide and sulfonamides with value 1.559, 1.372, 0.001, 0.100 for children and 1.109, 0.976, 0.001, 0.071 for adults respectively. For pesticide residues, 5% of the samples (n=6) out of 116 (mainly nigella sativa honey product that contain wax) contaminated with DMF, three of them less than method limit of quantification, and the other contain residues of DMF less than EU-MRL. Hence, there is a need for continuous survey and monitoring to protect adult and children from exposure to antibiotic that could cause development of human resistance to antibiotics that increases the risk of death of the patient with strains of bacteria that are completely immune to antibiotics. As well as beekeepers' education programs to control antibiotics uses during to treatment of honeybee colonies, in order to meet food safety standards and protect human health.

Abbreviations

ADI, acceptable daily intake; ARfD, acute reference dose; HI, hazard index; HQ, hazard quotient; LC-MS/MS, liquid chromatography–tandem mass spectrometry; GC-MS/MS, gas chromatography–tandem mass spectrometry; EDI, estimated daily intake; cHI, chronic hazard index; LOQ, limit of quantitation; LOD, limit of determination; ESTI, Estimated Short-Term Intake; aHI, Acute Hazard Index; EFSA, European Food Safety Authority; RASSF, Rapid Alert System for Food and Feed; CAS-NO , Chemical Abstract Service Number; MOA, Mode of action; MRPL, Maximum residues permissible limit; MRL, Maximum Residue Level; QuEChERS, quick, easy, cheap, effective, rugged, and safe.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Ahmed, M. B. M., A. A. Taha, & F. M. S. Mehaya, 2022. Method validation and risk assessment for sulfonamides and tetracyclines in bees' honey from Egypt, Libya and Saudi Arabia. Environmental Geochemistry and Health. https://doi.org/10.1007/s10653-022-01258-0

Asmaa E. Abd Alla, 2020a) Residues of Tetracycline, Chloramphenicol and Tylosin Antibiotics in the Egyptian Bee Honeys Collected from Different Governorates. Pakistan Journal of Biological Sciences, 23(3), 385–390. https://doi.org/10.3923/pjbs.2020.385.390

Asmaa E. Abd Alla, 2020b. Residues of Tetracycline, Chloramphenicol and Tylosin Antibiotics in the Egyptian Bee Honeys Collected from Different Governorates. Pakistan Journal of Biological Sciences, 23(3), 385–390. https://doi.org/10.3923/pjbs.2020.385.390

Baeza Fonte, A.-N., Rodríguez Castro, G., & Liva-Garrido, M. (2018). Multi-residue analysis of sulfonamide antibiotics in honey samples by on-line solid phase extraction using molecularly imprinted polymers coupled to liquid chromatography-tandem mass spectrometry. Journal of Liquid Chromatography & Related Technologies, 41(15–16), 881–891. https://doi.org/10.1080/10826076.2018.1533477

Baša Česnik, H., V. Kmecl & Š. Velikonja Bolta, 2019. Pesticide and veterinary drug residues in honey - validation of methods and a survey of organic and conventional honeys from Slovenia. Food Additives & Contaminants: Part A, 36(9), 1358–1375. https://doi.org/10.1080/19440049.2019.1631492

Bedendo, G. C., I. C. S. F. Jardim & E. Carasek, 2010. A simple hollow fiber renewal liquid membrane extraction method for analysis of sulfonamides in honey samples with determination by liquid chromatography–tandem mass spectrometry. Journal of Chromatography A, 1217(42), 6449–6454. https://doi.org/10.1016/j.chroma.2010.08.030

Chiesa, L. M., S. Panseri, M. Nobile, F. Ceriani & F. Arioli, 2018. Distribution of POPs, pesticides and antibiotic residues in organic honeys from different production areas. Food Additives & Contaminants: Part A, 35(7), 1340–1355. https://doi.org/10.1080/19440049.2018.1451660

Codex Alimentarius Commission, 2017. Pesticide residues in food and feed. Plant Production and Protection Division. http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/pestres/commodities-detail/en/?c_id=512

Codex Alimentarius Commission, 2019. Standard for Honey CXS 12-19811 Adopted in 1981. Revised in 1987, 2001. 12, 1–7.

Darko, G., & O. Akoto, 2008. Dietary intake of organophosphorus pesticide residues through vegetables from Kumasi, Ghana. Food and Chemical Toxicology, 46(12). https://doi.org/10.1016/j.fct.2008.09.049

el Agrebi, N., K. Traynor, O. Wilmart, S. Tosi, L. Leinartz, E. Danneels, D. C. de Graaf, & C. Saegerman, 2020. Pesticide and veterinary drug residues in Belgian beeswax: Occurrence, toxicity, and risk to honey bees. Science of The Total Environment, 745, 141036. https://doi.org/10.1016/j.scitotenv.2020.141036

EU Pesticides Database (v.2.2)., 2021. EU Pesticides MRLs Database (v.2.2). https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/mrls/?event=search.pr

European Commission, 2002a. Commission Decision (EC) No 1481/2002. Official Journal of the European Communities. http://data.europa.eu/eli/dec/2002/657/oj

European Commission, 2002b. The performance of analytical methods and the interpretation of results 2002/657/EC. Official Journal of the European Union, 221(8). https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF

European Commission, 2013. Council Directive 96/23/EC. Official Journal of the European Communities. http://data.europa.eu/eli/dir/1996/23/2013-07-01

European Commission, 2017. Commission Regulations (EU) 2017/623. Official Journal of the European Union.

Issa, M. M., M. S. Taha, A. M. El- Marsafy, M. M. H. Khalil, , & E. H. Ismail, (2020). Acetonitrile-Ethyl acetate based method for the residue analysis of 373 pesticides in beeswax using LC-MS/MS and GC–MS/MS. Journal of Chromatography B, 1145, 122106. https://doi.org/10.1016/j.jchromb.2020.122106

Joint FAO/WHO Expert Committee on Food Additives (JECFA)on Food Additives, J. F. E. C., & Organization, (70th : 2008 : Geneve, Switzerland)., 2009. Evaluation of certain veterinary drug residues in food : seventieth report of the Joint FAO/WHO Expert Committee on Food Additives (p. 134 p.). World Health Organization. https://apps.who.int/iris/handle/10665/44085

Koesukwiwat, U., Lehotay, S. J., & Leepipatpiboon, N. (2011). Fast, low-pressure gas chromatography triple quadrupole tandem mass spectrometry for analysis of 150 pesticide residues in fruits and vegetables. Journal of Chromatography A, 1218(39), 7039–7050. https://doi.org/10.1016/j.chroma.2011.07.094

Namik Bilici, E. Kabil, Y. Altuner, K. Koc Topcuoglu, & Ü.Topcuoglu, 2019. simultaneous search of multiple groups of antibiotics and their different components in the honey. Sabuncuoglu Serefeddin Health Science, 1(1), 15–38.

Orso, D., L. Floriano, L.C. Ribeiro, N.M.G. Bandeira, O.D. Prestes, & R. Zanella, 2016a. Simultaneous Determination of Multiclass Pesticides and Antibiotics in Honey Samples Based on Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry. Food Analytical Methods, 9(6), 1638–1653. https://doi.org/10.1007/s12161-015-0339-8

Orso, D., L. Floriano, L. C. Ribeiro, N. M. G. Bandeira, O. D. Prestes & R. Zanella, 2016b. Simultaneous Determination of Multiclass Pesticides and Antibiotics in Honey Samples Based on Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry. Food Analytical Methods, 9(6), 1638–1653. https://doi.org/10.1007/s12161-015-0339-8

Pihlström, T., A. R. Fernández-Alba, C. Ferrer Amate, M. Erecius Poulsen, R. Lippold, L. Carrasco Cabrera, P. Pelosi, A. Valverde, H. Mol, M. Jezussek, O. Malato & R. Štěpán, 2022. SANTE 11312/2021. https://food.ec.europa.eu/system/files/2022-02/pesticides_mrl_guidelines_wrkdoc_2021-11312.pdf

Prudnikov, E. D., 1981. Theoretical calculation of the standard deviation in atomic emission spectroscopy. Spectrochimica Acta Part B: Atomic Spectroscopy, 36(4), 385–392. https://doi.org/10.1016/0584-8547(81)80039-0

RASSF Window, 2022. EU Rapid Alert System for Food and Feed (RASFF) portal. https://food.ec.europa.eu/safety/rasff-food-and-feed-safety-alerts_en

Reybroeck, W., 2018. Residues of antibiotics and chemotherapeutics in honey. Journal of Apicultural Research, 57(1), 97–112.
https://doi.org/10.1080/00218839.2017.1338129

Santana, A., Santana, M., & Pereira, P. (2018). Development of a Method Based on DLLME and UFLC-DAD for the Determination of Antibiotics in Honey Samples and the Study of Their Degradation Kinetics. Journal of the Brazilian Chemical Society. https://doi.org/10.21577/0103-5053.20180028

U.S. EPA. (2005). Guidelines for Carcinogen Risk Assessment, EPA/630/P-03/001F.

van der Velde-Koerts, T., Rietveld, A., & Boon, P. E. (2021). Use of food consumption data of food balance sheets and national food consumption surveys in deterministic long-term dietary exposure assessments of pesticides. Food and Chemical Toxicology, 151. https://doi.org/10.1016/j.fct.2021.112104

Walorczyk, S., & D. Drożdżyński, 2012. Improvement and extension to new analytes of a multi-residue method for the determination of pesticides in cereals and dry animal feed using gas chromatography–tandem quadrupole mass spectrometry revisited. Journal of Chromatography A, 1251.

https://doi.org/10.1016/j.chroma.2012.06.055
WHO, 1997. Guidelines for predicting dietary intake of pesticide residues. 2nd revised edition, GEMS/Food Document WHO/FSF/FOS/97.7, Geneva.
Yang, Y., G. Lin, L. Liu, , & T. Lin, 2022. Rapid determination of multi-antibiotic residues in honey based on modified QuEChERS method coupled with UPLC–MS/MS. Food Chemistry, 374, 131733.
https://doi.org/10.1016/j.foodchem.2021.131733.