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Residues of pesticides and veterinary drugs in diets of dairy cattle from conventional and organic farms in Austria

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ABSTRACT

Modern agriculture depends highly on pesticides and pharmaceutical preparations, so controlling exposure to these substances in the feed and food chain is essential. This article presents the first study on residues of a broad spectrum of pesticides and veterinary drugs in the diets of dairy cattle. One hundred and two representative samples of the complete diets, including basal feed rations and additional fed concentrate, were collected in three Austrian provinces (Styria, Lower and Upper Austria) in 2019 and 2020. The samples were tested for >700 pesticides, veterinary drugs and related metabolites using a validated method based on liquid chromatography/ electrospray ionization-tandem mass spectrometry (LC/ESI–MS/MS). In total, 16 residues (13 pesticides and three veterinary drug residues) were detected. > 90% of the diets contained pesticide residues and <10% veterinary drug residues, whereas banned pesticides were not found. The most frequent pesticide residues were fluopyram (62%), piperonyl butoxide (39%) and diethyltoluamide (35%). The following pesticides exceed the default EU maximum residue level (MRL) (10 μg kg^{-1}) for products exclusively used for animal feed production: Benzovindiflupyr (proportion of samples > MRLs: 1%), bixafen (2%), fluopyram (6%), ipconazole (1%) and tebuconazole (3%). Three residues (dinitrocarbanilide, monensin and nicarbazin) of veterinary drugs were identified, all below the MRLs. Over 60% of the evaluated samples contained mixtures of two to six residues/ sample. Only one pesticide (diethyltoluamide) presented a significant difference among regions, with higher concentrations in Upper Austria. Brewery's spent grains were the dietary ingredient that showed the strongest correlation to pesticide residues. These findings evidence the realistic scenario of highly occurrent low doses of pesticides cocktails in the feed/food chain, which may affect the animal, human and environmental health. Since the risk assessments are based on single pesticides, the potential synergistic effect of co-occurring chemicals ("cocktail effect") requires further investigations.

1. Introduction

Milk and dairy products represent one of the most important food commodities for all the age groups of the human population in several countries around the globe (Kubicova et al., 2019). The dairy industry is the second-largest agricultural sector in the European Union, corresponding to more than 12% of its total agricultural output (Augère-Granier, 2018). Specifically in Austria, the dairy industry is the most relevant agricultural sector, representing 18% of the national agricultural production (BMLRT, 2021). In modern agriculture (including dairy farming), the production of crops and animal-derived foods is highly dependent on pesticides and veterinary pharmaceutical preparations, which are the foundation of the called conventional agriculture systems. These substances have been essential for protecting

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crops and livestock from pest infestation and diseases (Beyene, 2016; Özkara et al., 2016).

Pre-and post-harvest use of pesticides safeguards crops and controls pests (like insects, weeds and plant pathogens), improving production quantity (Özkara et al., 2016). However, residues of pesticides can be accumulated in crops and the environment, affecting human, animal and environmental health (Igbedioh, 1991; Damalas and Eleftherohorinos, 2011; Cozma et al., 2017; Silva et al., 2019; Jepson et al., 2020; Kruse-Plaß et al., 2021; Zaller et al., 2022). For example, it is known that pesticides are stress factors affecting health and raising the mortality of bees and other insects worldwide (Hallmann et al., 2017; El Agrebi et al., 2020; Barmentlo et al., 2021; Bruinenberg et al., 2022). The global decline of insect populations is a big concern affecting complete ecosystems because of their critical role in several ecological functions like pollination, nutrient cycling, pest control and food sources for multiple species (Wilson et al., 1999; Yang and Gratton, 2014). Pesticides have also been related to the decline of bird populations (Goulson, 2014). Regarding the impacts on human health, chronic pesticide exposure has been linked to carcinogenicity, neurodegenerative diseases, infertility, malformation, hormonal disruption and alteration in the immune system (Parrón et al., 2011; Mai et al., 2014; Karalexi et al., 2021; de Barros Rodrigues et al., 2022; Palaniyappan et al., 2022; Singh et al., 2022).

The extensive use of veterinary drugs, which are added to the feed of food-delivering animals for prophylaxis and metaphylaxis purposes, and growth promoters is also a big concern (Anadón and Martınez-Larranaga, 1999; Beata, 2016; Anadón et al., 2018). Antibiotics, anti-parasitic drugs and non-steroidal anti-inflammatory drugs have been broadly utilized in livestock feeds, associated with the appearance of residues in animal products such as milk, meat and eggs (Beyene, 2016; Rana et al., 2019). Incorporating pharmaceutical preparations can affect feed/food safety, contributing mainly to public health problems like multidrug resistance, carcinogenicity, teratogenicity and disruption of normal gut microbiota (Ortelli et al., 2018; Rana et al., 2019). In particular, antimicrobial resistance represents an increasing threat to global public health that requires appropriate action across governments and society (Hao et al., 2014; Baynes et al., 2016; Lekshmi et al., 2017; Ortelli et al., 2018).

Organic agriculture has been developed to respond to problems generated by conventional industrial agriculture on the environment, animal and human health (Röös et al., 2018). In 2019, 8.5% of total EU agricultural land (approx. 13.8 million hectares) was under organic farming, which represented an increment of 66% compared with 2009 (8.3 million hectares). Austria presented the highest proportion of organic agriculture at the EU level, with 25.3% of the agricultural land under this productive system (Commission, 2022). The "organic" label guarantees a production that avoids synthetic fertilizers, hormones and pesticides as well as minimizing the use of veterinary drugs (Prache et al., 2022); however, pesticide and veterinary drugs residues have been detected in milk (Ghidini et al., 2005; Gutiérrez et al., 2012; Wanniatie et al., 2019), other commodities (Bursić et al., 2021; Schusterova et al., 2021) and soils of organic farming systems (Geissen et al., 2021). Monitoring the exposure to pesticides and veterinary drug residues in the feed and food chain is essential and required to enforce legislation and guarantee food safety (Masiá et al., 2016; Kumar et al., 2019). The European Union has one of the strictest legislation concerning pesticides and veterinary drug residues in the feed and food chain (EC, 2004; Anastassiadou et al., 2019; Kuchheuser and Birringer, 2022). The European Commission (EC) has been promoting low pesticide-input farming in the Member States and individual governments, and it has been expected to create the necessary conditions for farmers to implement Integrated Pest Management (IPM) (Hillocks, 2012).

More recently, the European Green deal, lined with the Farm to Fork and the Zero Pollution strategies, aims to reduce pesticide utilization by 50%, eliminate soil pollution and establish at least 25% organic farmland in Europe by 2030 (EC, 2020a, 2020b; Silva et al., 2022). To achieve the goals of these strategies, a diagnosis of the current situation and regular monitoring of the use of pesticides and veterinary drugs in different segments of the feed and food chain is crucial. Thus, this study aimed to characterize a broad spectrum (>700) of pesticide and veterinary drug residues in the complete dietary rations of lactating cows in Austrian organic and conventional dairy farms. It was achieved by employing a validated multi-metabolite liquid chromatography/electrospray ionization-tandem mass spectrometric (LC/ESI–MS/MS) method. Additionally, correlation analysis was performed between the most occurrent analytes and the main dietary ingredients. Moreover, the geographical distribution patterns of the residues were explored.

2. Material and methods

2.1. Sampling and data collection

This research was performed in the framework of a project that aimed to survey feed safety aspects in the Austrian dairy sector, which also included investigations on natural contaminants and metabolites (such as mycotoxins, phytoestrogens, plant toxins and other secondary metabolites) recently published (Penagos-Tabares et al., 2021, 2022a; 2022b, 2022c). After signing a confidentiality and data protection agreement with the involved Austrian dairy farmers, one representative sample of lactating cows' diet per farm was collected (n = 102, 93 rations of conventional and nine organic farms). The included organic farms followed the BIO AUSTRIA regulations for organic farming in Austria (available at. https://www.bio-austria.at/app/uploads/RiLiEnglish20121.pdf). The relation of organic/conventional farms was not balanced due to the low availability and acceptance of organic farms to participate in this study during the recruiting. Moreover, because it was not included in the project's overall goal. The sampling was performed between May 2019 and September 2020 in the three provinces with the country's major dairy production: Upper Austria (n = 53), Lower Austria (n = 32) and Styria (n = 17) (Fig. 1). On average, the herd sizes of the visited farms were 59 ± 15 standard deviation (SD) lactating cows per farm, fluctuating from 32 to 140. Each representative sample of the complete diet involved the separate collection of fresh mixed rations from the feeding table and concentrate feed from the automatic feeders. A minimum of 30 sub-samples of the above mentioned feeds were manually collected using nitrile gloves to avoid cross-contamination. The final sample of each kind of feed was at least 1 kg, which was vacuum-packed and stored at −20 °C until sample preparation. Additionally, information concerning the farming system (organic or conventional), basal feed composition (main components and their respective proportions), estimated total intakes (of mixed rations and concentrate feed), use of pesticides (in the feed crops) and veterinary drugs (in the rations) were obtained via a questionnaire-guided interview.

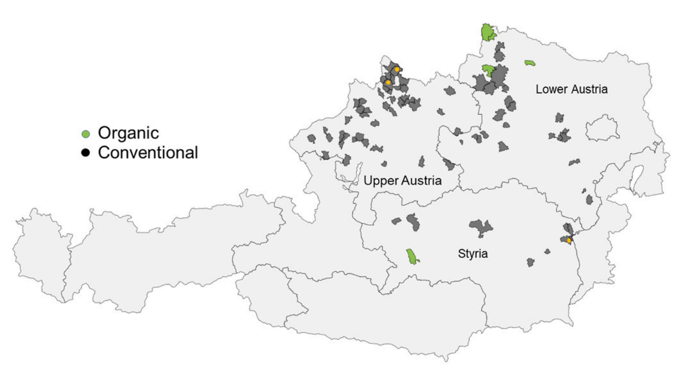

Fig. 1. Map showing the locations of the dairy farms of complete dietary rations (n = 102) of Austrian dairy cattle.

2.2. Sample preparation

Once the sampling period finished, the frozen mixed ration samples were dried at 65 °C in an electric fan oven for 48 h. Once dried, the mixed rations and concentrate feeds were milled to a final particle size of ≤0.5 mm. They were firstly milled using the cutting mill (SM 300, Retsch GmbH, Haan, Germany) at 1,500 rpm for approximately 1 min. Subsequently, using an ultra-centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) at 10,000 rpm for about 30 s, the remnants (non-milled residues, mainly corresponding to hard fragments of seeds) were milled. Both milled fractions were combined, mixed and packed in plastic bags. The processed, mixed rations and concentrated feeds were composited according to the average intake proportions (data provided by the farmers) to obtain 20 g (±0.01 g) of the whole diet representative sample. Finally, 5 g (±0.01 g) of the homogenized complete diet samples were stored in 50-mL polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) and kept at −20 °C until analysis.

2.3. Analysis of multiple pesticides and veterinary drug residues

Following the protocol described by Steiner et al. (2020), the previously prepared sample (5 ± 0.01 g) was put into a 250 ml Erlenmeyer flask with 20 ml of extraction solvent. Next, homogenization was performed using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) for 90 min. Quantification was established on external calibration utilizing a serial dilution of a multi-analyte stock solution. The solvent solution-sample mixture was centrifuged for 2 min at 2,012 × g on a GS-6 centrifuge (Beckman Coulter Inc., Brea, CA, USA). The extract, along with dilution solvent, was diluted at one to one proportion. The injection volume of both diluted sections of the samples and the standard analyte solutions was 5 μl. Identification and quantification of each analyte were performed in two separate chromatographic runs using a QTrap 5500 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a TurboV electrospray ionization (ESI) source coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Quantitative analysis of all the analytes was performed using a validated method based on LC-ESI-MS/MS described by Steiner et al. (2020). Results were corrected for apparent recoveries determined during method validation, according to Steiner et al. (2020). Values related to the method performance (apparent recoveries, the limit of detection (LOD) and the limit of quantification (LOQ) of each analyte as well as the specific chemical class are described in Table S1. The targeted pesticides (660), veterinary drugs (129) and their respective related metabolites along with the compound identification numbers (PubChem CID) are enlisted in Table S2 and Table S3. Analyses, analytical quality control and method validation were performed in accordance with DG SANTE guidelines for pesticide and veterinary drug residues analysis in food and feed. (EC, 2019).

2.4. Data analysis

Concentrations of all detected residues and related metabolites (i.e., markers such as dinitrocabanilide) were presented on a dry matter (DM) basis in μg kg^{-1}. Descriptive statistics, i.e., frequencies, mean, median and ranges of the concentration of analytes, were calculated considering only the positive results (x ≥ LOD). Results below the LOQ were computed as LOQ/2. Normatility test of the data was performed via D'Agostino & Pearson test, Anderson-Darling test, Shapiro-Wilk test and Kolmogorov-Smirnov test. All the tests indicated the non-normal distribution of the handled data. The Kruskal-Wallis test (the non-parametric alternative of the ANOVA) was runned to analyse significant differences in the concentration and number of compound residues among the three Austrian provinces. In case of significant differences among the three provinces, these differences were re-evaluated via non-parametric Mann-Whitney *U* test between pairs of provinces (Upper Austria Vs Lower Austria, Upper Austria Vs. Styria, and Lower Austria Vs. Styria, respectively). This applied only for diethyltoluamide. Additionally, to confirm our findings, was performed a two-stage step-up method of Benjamini, Krieger and Yekutieli test as multiple comparisons test for controlling the False Discovery Rate (FDR). Subsequently, Spearman correlation analysis between the compound residues as well as between compound residues and the proportions of the dietary ingredients were performed. The correlation analysis was interpreted considering only substantial correlations with coefficients (rho[ρ]) ≥ 0.3, based on Hinkle et al. (2003). The tables were made using Microsoft Excel®. The mentioned statistical analyses and figures were performed and elaborated using GraphPad Prism® version 9.1 (GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1. Diet composition (main ingredients)

The dairy farms in this investigation fed mixed rations (consisting mostly of forages but also mineral supplementation and concentrate feed) with an additional amount of concentrate feed (given to the animals via automatic feeders). The most common dietary components incorporated in the diets were: concentrate feed (with a frequency of inclusion of 100%), grass silage (97%), maize silage (84%), straw (58%), brewery's spent grains (26%), hay (19%) and other silages (including wheat, oats, barley, sunflower and beep pulp) (12%) (Fig. 2). Regarding the proportions in the diet, the most relevant dietary ingredient incorporated in the analysed dietary rations was grass silage, which represented on average 40.6% (SD ± 15%) of the complete ration, fluctuating from 10.4% to 86.8%. The inclusion rate of concentrate feeds was, on average, 35.3% (SD: ± 9.6), varying from 11% to 67.6%. On average, maize silage accounted for 26.7% (SD ± 10.6%) of the total diet, ranging from 1.7% to 59%. On average, the other mixed rations' ingredients corresponded to ≤5% of the diet. Such as other silages (average: 5%; SD: ± 4.8%; range: 0.5%–15.8%), hay (4.3%; ±5.9%; 0.6%–28.5%), brewery's spent grain (3.6%; ±1.8%; 0.3%–8.1%) and straw (2.7%; ±1.9%; 0.2%–10.1%). The mean proportion of forage in the ration (understood as the sum of silages, straw and hay) was 64.7% (SD: ± 9.6; range: 32.4%–89%) (Fig. 2). The respective rates and proportions of the conventional and organic farms are in Table 1. Since the unbalanced sample size of conventional and organic farms (due to the complexity and difficulty of recruiting organic farms for this study), no statistical comparison was performed. However, as a general trend, it can be observed that the diets of organic farms did not include brewery's spent grain and other silages in their formulations. Additionally, the diets of organic farms presented a higher inclusion rate of hay (33%) compared to conventional farms (17%). Regarding silage inclusion, the organic farms

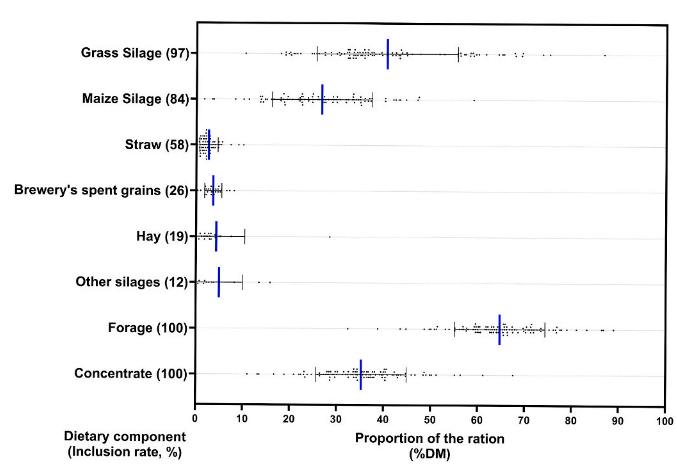

Fig. 2. Frequency and proportion of inclusion the main components of dietary rations of Austrian dairy cattle.

Table 1
Frequencies and proportion of inclusion of the main components incorporated in complete dietary rations of Austrian dairy cattle under conventional and organic farming systems.

Dietary ingredient	Conventional farms (n = 93)			Organic farms (n = 9)		
	Inclusion	Proportion in the diet (% DM)		Inclusion	Proportion in the diet (% DM)	
	(%)	Average ± SD	Range	(%)	Average ± SD	Range
Maize Silage	91	26.8 ± 10.6	1.7 – 59.0	11.1	18.5 ± 0.0	18.5 – 18.5
Grass Silage	97	38.4 ± 13.4	10.4 – 73.7	100	62.3 ± 12.4	43.7 – 86.8
Straw	58	2.6 ± 2.0	0.2 – 10.1	56	3.5 ± 1.4	1.6 – 5.0
Hay	17	4.5 ± 6.3	0.9 – 28.5	33	3.3 ± 3.0	0.6 – 7.5
Brewery's grains silage	29	3.6 ± 1.8	0.3 – 8.1	0	0	0
Other silages	13	5.0 ± 4.8	0.5 – 15.8	0	0	0
Forage	100	64.5 ± 9.3	32.4 – 89.0	100	67.4 ± 11.5	51.3 – 86.8
Concentrate	100	35.5 ± 9.3	11.0 – 67.6	100	32.6 ± 11.5	13.2 – 48.7

incorporated, on average, a higher proportion of grass silage (62.3%) and lower maize silage (18.5%) compared with the conventional diets (38.4% and 26.8%, respectively). As a general trend, the forage-to-concentrate ratio in both groups' was very similar; however, the organic farm contained slightly more forage (Table 1). For future studies, it would be required to have a balanced (higher sample size) of organic farms to get a more representative and accurate view of this farming system, which, as mentioned previously, should be promoted and established in at least 25% of the agricultural land of the European Union by 2030 (EC, 2020a, 2020b.; Silva et al., 2022). In 2020, 25.5% (6,631 of 25,872) of the Austrian dairy farms corresponded to organic farms, and 19.2% (649,368 of 3,384,412 t) of the milk produced was organic (BMLRT, 2021).

Based on our results and according to the FAO's report "world mapping of animal feeding systems in the dairy sector" (2014), the kind of diets analysed during this study are classified in the feeding system of "year-round silage", which is the most relevant in the country. This feeding system has been implemented in around 40% of the Austrian dairy farms, the equivalent to 50% of the national milk production, at the time of the report (FAO, IDF, IFCN, 2014). The other 50% of the production for 2014 was "green fodder + silage" (35%) and "haymilk" (15%)(FAO, IDF, IFCN, 2014). The forage proportion of this kind of feeding system (year-round silage) was in 2014 of 78%, and 22% of concentrate feeds (specifically, cereal grains (15%), by-products (6%) and compound feed (1%)). To the best of our knowledge, no current or more recent data on the proportion of the feeding system of the Austrian dairy sector are available. According to the cited report, grass silage (average: 53%), concentrated feeds (22%) (cereal grains (15%), by-products (6%) and compound feeds (1%)), maize silage (19%) and hay (6%) were the main dietary components (FAO, IDF, IFCN, 2014). Although the proportion of the main ingredients differs from the mentioned report, the order and relevance of the main dietary components are similar. The FAO's report also evidenced that the feeding system of the here targeted farms was (during the last decade) and surely is the most relevant in Austria in terms of the amount of produced milk and quantity of producing units (farms) (FAO, IDF, IFCN, 2014).

3.2. Information regarding the use of pesticides and veterinary drugs in Austrian dairy farms

Among the conventional farms, 62% of the interviewed farmers reported the application of pesticides. Around 33% of the farmers that confirmed the use of pesticides (equivalent to 19% of all the conventional farms) did not provide additional specific information (such as applied products or active substances). In total, 32 commercial pesticide products were indicated across the farms, consisting of 16 fungicides, 15 herbicides and one insecticide. According to the provided data, on average, two commercial pesticide products for feed crops per farm were applied, varying from one to ten (specific data not shown. As expected, the organic farmers stated that no pesticides were used in their crops. None of the farmers reported the incorporation of veterinary drugs in the rations.

Regarding the reported applied active substances, 12 were non-persistent, six were persistent, four were persistent and three were very persistent (PPDB, 2022). Of the reported active substances, 11 were fungicides, 13 were herbicides and one was an insecticide (Table S4). According to the interviews, the pesticides were applied on cereals (maize for silage, wheat, rye and triticale for concentrate feed). Three of the compounds described as applied (specifically, chlortoluron, esfenvalerate and S-metolachlor) were not targeted by implemented multi-pesticide analytic method (Table S2 and Table S4).

3.3. Occurrence and concentration of pesticides and veterinary drug residues in diets of Austrian dairy cattle

In total, residues of 15 active substances (13 pesticides and two veterinary drugs) were detected. Most of the samples (90%) presented some kind of residue. 89% of dietary rations contained pesticide residues and 8% of veterinary drugs. Among the pesticide residues were identified nine fungicides (benzovindiflupyr, bixafen, fluopyram, fluxapyroxad, ipconazole, metrafenone, pyraclostrobin, tebuconazole and trifloxystrobin), three insecticides (piperonyl-butoxide, pirimiphos-methyl, diethyltoluamide) and one herbicide (metolachlor). Two veterinary drug residues were detected: monensin and nicarbazin. The marker of nicarbazin, dinitrocarbanilide, was also detected. Dinitrocarbanilide [N,N′-bis(4-nitrophenyl)urea)] and 4,6-dimethyl-2(1H)-pyrimidinone in a ratio 1:1 conform to the molecular complex nicarbazin (an antiprotozoal compound used as a feed additive) (Tarbin et al., 2005). The pesticide residues detected in the highest occurrences were fungicides fluopyram (62%), the insecticide synergist piperonyl-butoxide (39%) and the repellent diethyltoluamide (35%). Residues of the other detected pesticides showed occurrences below 20%. Residues of veterinary substances (monensin, nicarbazin and dinitrocarbanilide) showed occurrences lower than 5%. The directive 2009/8/EC states that monensin and nicarbazin are authorised for use as feed additives by the regulation (EC) No 1831/2003 (EC, 2003, 2009; Anadón et al., 2018).

The pesticide residues with the highest average concentration were piperonyl-butoxide (27.1 μg kg^{-1}), diethyltoluamide (24.2 μg kg^{-1}) and fluopyram (7.07 μg kg^{-1}). Diethyltoluamide showed the maximum concentration detected among pesticides (1475 μg kg^{-1}). The average concentrations of the veterinary drug residues were less than 2.5 μg kg^{-1}. The highest concentration of the veterinary drug residues was 142 μg kg^{-1} of monensin. Concerning the maximum residue levels (MRLs), no veterinary drugs but five pesticides exceeded the EU-MRLs (EC, 2009; EU Pesticide Database, 2022). Specifically, the pesticides that exceeded the EU-MRLs definitely (taking into consideration the expanded measurement uncertainty of 50%) were: benzovindiflupyr (1% of the investigated samples), bixafen (2%), fluopyram (6%), ipconazole (1%) and tebuconazole (8%)(Table 2). In the European Union, pesticide

Table 2
Occurrences and concentrations of the pesticides and veterinary drug residues detected in complete dietary rations of lactating dairy cattle in Austria.

Analyte		Occurrence[a] (%)	> MRL[b] (%)	Concentrations (μg kg^{-1} DM)					Type[c]	Persistence[c,d]	WHO classification by hazard[e]/Enlisted as highly hazardous pesticides by PAN[f]
				Average ± SD		Range					
Pesticides	Benzovindiflupyr	10	1	1.05	±4.28	1.40	–	32.4	Fungicide	VP	II
	Bixafen	10	2	0.99	±4.21	4.65	–	29.3	Fungicide	VP	N/A
	Diethyltoluamide	35	N/A	24.2	±151	2.57	–	1475	Insecticide (repellent)	No data*	N/A
	Fluopyram	62	6	7.07	±11.2	2.30	–	78.3	Fungicide, nematicide	P	III
	Fluxapyroxad	10	0	0.46	±1.63	2.65	–	8.66	Fungicide	P	III
	Ipconazole	10	1	1.29	±4.43	2.40	–	25.5	Fungicide	MP	N/A
	Metolachlor	2	0	0.05	±0.37	2.65	–	2.65	Herbicide	MP	III
	Metrafenone	10	0	0.33	±1.43	0.90	–	12.8	Fungicide	P	U
	Piperonyl butoxide	39	N/A	27.1	±72.0	7.50	–	572	Insecticide (synergist)	NP	U
	Pirimiphos-methyl	13	0	0.73	±2.13	1.65	–	11.5	Insecticide	MP	II/+
	Pyraclostrobin	1	0	0.04	±0.38	3.85	–	3.85	Fungicide	MP	II
	Tebuconazole	12	3	3.87	±17.2	4.68	–	118	Fungicide, plant growth regulator	MP	U/+
	Trifloxystrobin	1	0	0.05	±0.46	4.62	–	4.62	Fungicide	NP	U
Veterinary drugs	Dinitrocarbanilide	4	N/A	2.3	±12.3	23.0	–	89	Marker of nicarbazin	N/A	N/A
	Monensin	4	0	1.75	±14.1	4.70	–	142	Antibiotic/ Anticoccidial	NP	N/A
	Nicarbazin	3	0	1.32	±8.35	19.8	–	69.4	Anticoccidial	P	N/A
Total pesticides		91	N/A	67.2	±164	2.30	–	1482	N/A	N/A	N/A
Total drug residues		8	N/A	5.36	±24.1	4.70	–	158	N/A	N/A	N/A
Total residues		90	N/A	72.6	±165	2.30	–	1482	N/A	N/A	N/A

[a] n = 102 representative samples of complete diets of lactating dairy cows from Austria, values considered as positive were > limit of detection (LOD); In case values > LOD and < limit of quantification (LOQ), LOQ/2 was used for the calculation.
[b] Maximal residue level of pesticides (MRL) for products or part of products exclusively used for animal feed production according to the European Union guidelines is 10 μg kg^{-1} expressed at 88% DM (11.36 μg kg^{-1} DM basis)(EU Pesticide Database, 2022). In Europe, MRL of the detected veterinary drugs are dictated by the Commission Directive 2009/8/EC of February 10, 2009 (EC, 2009). For instance the MRL of monensin and nicarbazin for compound feed for dairy are 1250 μg kg^{-1}, and 1500 μg kg^{-1}, expressed at 88% DM basis (and 1420 μg kg^{-1}, and 1705 μg kg^{-1} at DM basis).
[c] Data retrieved from Pesticide Properties DataBase (PPDB, 2022) and Veterinary Substance DataBase (VSDB, 2022) of the University of Hertfordshire.
[d] Based on the typical disappearance time 50 (DT50); VP = very persistent, P = persistent, MP = moderately persistent, NP = non-persistent; * No data found in the PPDB. According to an assessment report of the EU: "Diethyltoluamide does not meet any of the criteria for Persistent, Bioaccumulative and Toxic (PBT)" (Kem, 2010).
[e] WHO classification of pesticides by hazard. Ia (Extremely hazardous, Ib (highly hazardous), II (moderately hazardous), III (slightly hazardous) and U (unlikely to present an acute hazard) (WHO, 2019) (Organization, 2020).
[f] + = highly hazardous, according to pesticide action network international (PAN, 2021) (PAN, 2021), N/A: Not available/not apply.

residue levels, particularly in plant and animal-derived foods and feeds, have been set by Commission (EC) No 396/2005 (EC, 2022). Information concerning MRLs and toxicity is available in the EU Pesticide database (EU Pesticide Database, 2022). However, feedstuffs, compound feeds and dietary rations exclusively used for animal feed purposes have not yet established harmonized EU MRLs for pesticides. For that reason, the general default MRL value of 0.01 mg kg^{-1} (10 μg kg^{-1}) expressed at 88% DM applies (EU Pesticide Database, 2022). The distribution of the residue levels is illustrated in Fig. S1a.

Concerning the samples collected from conventional farms, 97% (90/93) contained pesticide residues and 8% (7/93) contained veterinary drug residues. On the other hand, only one of the nine dietary samples derived from organic farms (corresponding to 11%) was positive for pesticide residue, particularly for benzovindiflupyr (13.8 μg kg^{-1} DM). Likewise, other sample from an organic farm (also 11%) presented residues of dinitrocarbanilide (64.6 μg kg^{-1}) (Fig. S1a).

Regarding the environmental persistence, among the detected compound residues (15, not including the nicarbazin marker dinitrocarbanilide), two were classified as very persistent, three as persistent, four as moderately persistent and three as non-persistent (Table 2). Two of the detected pesticides are enlisted as highly hazardous by pesticide action network international, for instance pirimiphos-methyl (added since January 2009) and tebuconazole (added since March 2019) (PAN, 2021). According to the WHO classification by hazard, none of the detected compound residues were cataloged as extremely or highly hazardous. However, three of the detected pesticides (benzovindiflupyr, pyraclostrobin and pirimiphos-methyl) were considered moderately hazardous, also three (fluopyram, fluxapyroxad and metolachlor) as slightly hazardous and four as unlikely to present an acute hazard (WHO, 2019).

Four of the detected compound residues (metolachlor, piperonyl butoxide, pirimiphos-methyl and diethyltoluamide) are not approved as plant protection products on the European Union's market by the Regulation (EC) 1107/2009 (PPDB, 2022). The herbicide metolachlor is widely used in the USA and is linked to human carcinogenicity (EPA, 1995; Rusiecki et al., 2006). Metolachlor was also related to poor semen quality in men (Swan et al., 2003). Piperonyl butoxide, as an insecticide synergist, increases the potency of certain insecticides such as carbamates and pyrethrins (Basak et al., 2021). Piperonyl butoxide is not a cholinesterase inhibitor and has low toxicity; consequently, it is not only used for crop protection. Piperonyl butoxide-containing products are applied to crops both pre- and post-harvest. Facilities and storage areas where produce and livestock are processed may also be treated and can be a source of contamination (Daiss and Edwards, 2006; Keane, 1999). Its broader purpose of use may explain its higher detection frequency compared to the majority of detected residues. Pirimiphos-methyl is an organophosphate fumigant insecticide that controls many insects and mites (PPDB, 2022). This moderately persistent insecticide is considered highly toxic for bees (Berjawi et al., 2020) and was added to the HHP list in 2009 (PAN, 2021).

Diethyltoluamide is an insect repellent applied to human and animal skin to protect from insects. It is moderately toxic to aquatic life (PPDB, 2022). Not enough data is available regarding its environmental fate. According to an assessment report of the EU, this compound “does not meet any of the criteria for Persistent, Bioaccumulative and Toxic (PBT)” (Kem, 2010). The other detected pesticides approved as plant protection products are fungicides (see Table 2) and are usually used for crop protection against foliar diseases of cereals, legumes and other crops (PPDB, 2022). Residues of diethyltoluamide have been reported in several food commodities (such as chanterelle, blueberry and raspberry) in several countries like Germany, the Russian Federation, Poland, Belarus, Bulgaria and the Czech Republic (Scherbaum and Mraks, 2019). It was concluded that the residual contamination with diethyltoluamide was usually the result of contact with the hands of the picker who had sprayed himself with the repellent (Scherbaum and Marks, 2019). Diethyltoluamide has also been found in Avena in Poland (Malinowska et al., 2015) and was the most abundant “pharmaceutical and personal care product (PPCP)” in leachates of the USA and Poland, showing a high risk for the environment (Yu et al., 2020). In the case of our study, we speculate that spraying this substance on the stable and the animal is probably the source of the residues in the dietary rations.

3.4. Comparison of concentrations of residues by the geographical localization (province)

Table S5 shows the occurrences and concentrations of pesticide and veterinary drugs residues in Lower Austria, Styria and Upper Austria. The major occurrence of residues was in Upper Austria (96%), followed by Styria (82%). Lower Austria (78%). Relating to the average concentration of total residues, Upper Austria presented the highest concentration (89.7 μg kg^{-1}), subsequently Lower Austria (59.8 μg kg^{-1}) and finally Styria (43.3 μg kg^{-1}); however, no significant differences were evidenced (Tables S5 and S6, Fig. S1b). At the concentration of the individual analytes, only the diethyltoluamide levels presented substantial differences between provinces (Kruskal-Wallis test, p-value = 0.017). Upper Austria showed significantly higher levels compared with the respective levels of Lower Austria and Styria (Mann-Whitney Test, p-values = 0.016 and 0.046) (Fig. S1c, Table S5). The multiple comparisons test (two-stage step-up method of Benjamini, Krieger and Yekutieli) confirmed the findings suggesting that only diethyltoluamide levels presented significant differences among provinces (Table S6).

3.5. Cocktails of residues in diets of Austrian dairy cattle

This study shows that Austria dairy cattle’s complete diets usually contain mixtures of pesticides. For instance, 62% of the complete diets of lactating dairy cattle evaluated contained combinations (of two to six) of different residues. The diets contained, on average, two compounds, and no significant differences among Austrian provinces were detected (p-value = 0.4013) (Fig. 3a). Specifically, 23% of the samples contained

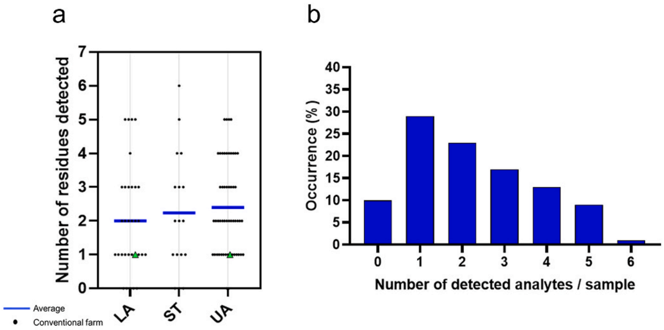

Fig. 3. Number of residues/sample of dairy cow’s diet. (a) In the provinces (LA: Lower Austria; ST: Styria; UA: Upper Austria). (b) Occurrence by number of residues/sample of dairy cow’s diet.

two residues, 17% three residues, 13% four residues, 9% five residues and 1% six different residues (Fig. 3b). These findings confirm once again the idea that multiple biocides are being incorporated at low levels in the feed/food chain and subsequently in the environment, implicating negative toxicological and ecological consequences (Márquez et al., 2005; Relyea, 2009; Mishra et al., 2014; Panico et al., 2022). Interactions of pesticide mixtures lead mainly to synergic effects, which differ depending on the dose and physiological target (Rizzati et al., 2016). Thus, although the detected levels of individual residues do not seem to be a risk, the effects of the detected biocide mixtures are unpredictable because such may imply multiple potential interactions amongst different pesticides. More research and data available in this exciting field are still highly required (Rizzati et al., 2016; Hernández et al., 2017). This kind of exposure to multiple pesticides could implicate adverse effects on health. It might contribute to an increased risk of long-term diseases, including cancer and neurodegenerative diseases, reproductive and developmental disturbances, and emerging threats such as developmental neurotoxicity and immunotoxic effects (Parrón et al., 2011, 2014; González-Alzaga et al., 2014; Mokarizadeh et al., 2015; Hernández et al., 2017).

3.6. Relationships between the detected residues and main dietary ingredients

Significant Spearman’s correlation coefficients (ρ) between residues as well as among the levels and the number of residues with the main

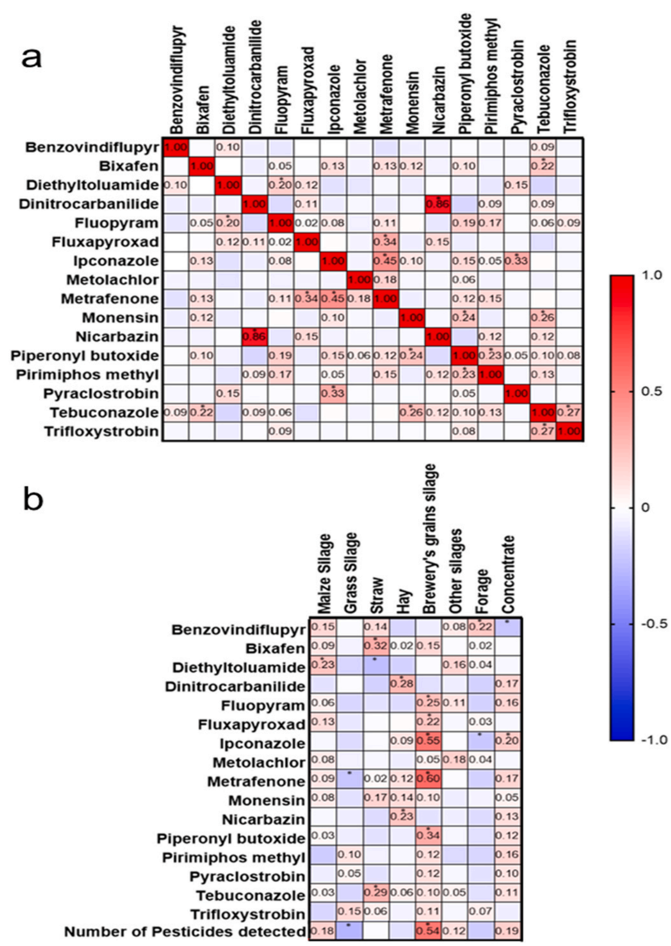

Fig. 4. Spearman’s correlation coefficients (ρ) (a) among residues as well as (b) among residues with the main dietary components. The asterisk (*) indicates a significant coefficient (p-value < 0.05). All Spearman’s correlation coefficients (ρ) and the exact p-values are available in Tables S7 and S8, respectively.

dietary components are shown in Fig. 4a and b, respectively. All Spearman's correlation coefficients (ρ) and all the exact p-values are available in Table S7 and Table S8, respectively. Firstly, the correlation analysis among the different compound residues showed a highly significant correlation ($\rho = 0.86$; p-value < 0.001) between nicarbazin and its marker residue dinitrocarbanilide (Danaher et al., 2008). Low positive correlations were detected between the fungicides metrafenone with ipconazole ($\rho = 0.45$; p-value <0.001), metrafenone and fluxapyroxad ($\rho = 0.34$; p-value = 0.001) as well as pyraclostrobin and ipconazole ($\rho = 0.33$; p-value = 0.001) (Fig. 4a). The correlation between the other compound residues were negligible ($\rho < 0.3$). Regarding the relationship between diet composition and residue levels, moderate positive correlations were found between brewery's spent grains with the fungicides metrafenone ($\rho = 0.60$; p-value < 0.001) and ipconazole ($\rho = 0.55$; p-value < 0.001). Brewery's spent grains also showed a moderate positive correlation with the number of detected pesticide residues ($\rho = 0.55$; p-value < 0.001). The other dietary components presented negligible correlations with the residues and the number of residues detected (Fig. 4b).

The most relevant dietary component related to pesticide residue levels was the brewery's spent grains, which was previously reported by its capacity of absorption (after mashing) of pesticides, which reduced the concentration of these substances in beer production (Inoue et al., 2011; Xi et al., 2014; Wei et al., 2020). Brewery's spent grains were not included in the rations of organic farms visited. The farms (n = 27) that incorporated this by-product presented average levels of ipconazole (4.87 $\mu g\ kg^{-1}$) and metranone (1.25 $\mu g\ kg^{-1}$), which are 3 times higher compared with general average (1.29 $\mu g\ kg^{-1}$ and 0.33 $\mu g\ kg^{-1}$). The number of detected residues per sample was also higher in farms with brewery's spent grains inclusion (four residues/sample) than the overall of the farms (two residues/sample). Several of the pesticides detected in this study such as benzovindiflupyr, bixafen, fluopyram, fluxapyroxad, metrafenone, piperonyl butoxide, pirimiphos-methyl, pyraclostrobin, tebuconazole and trifloxystrobin were also detected recently in samples of brewery's spent grains intended for feeding of dairy cows in Austria (Penagos-Tabares et al., 2022c). It is known that barley (cereal mostly used for beer production) is a crop frequently contaminated with traces of fungicides (Palladino et al., 2021). Given the incorporation of commercial concentrate feeds and other feedstuffs non-produced at the farm, the pesticides detected in this study can also be different from the ones reported by the farmers on the crop feeds (cereals, like maize, wheat, rye and others).

4. Conclusions

- To the best of our knowledge, this work is the first quantitative LC/ESI–MS/MS-based method covering a vast amount of pesticides (660) and veterinary drug residues (129) in complete dairy cattle diets. Consequently, it enabled data on the occurrences and levels of multiple residues in the diets of food-delivering animals.
- Mixtures of pesticides presented high occurrences (>60%) in the complete diets of Austrian dairy cows.
- Organic dairy farms presented lower occurrences (22%) and fewer residues (up to one per sample) than conventional dairy farms (97%, up to six per sample).
- In some cases, the complete diets of Austrian dairy cows exceed the default EU MRL (10 $\mu g\ kg^{-1}$) for pesticides in products or part of products exclusively used for animal feed production.
- Four detected compound residues (metolachlor, piperonyl butoxide, pirimiphos-methyl and diethyltoluamide) are not approved as plant protection products on the European Union's market.
- Veterinary drug residues in the diets of Austrian dairy cows were detected in very low frequencies (<10%) and were not detected above the EU MRLs.
- Brewery's spent grains were the most correlated ingredient to pesticide residues.
- Similar studies are required to estimate the current situation regarding pesticides and veterinary drug residues in animal feed and animal-derived products.
- Cocktails of pesticides are a realistic scenario in the diets of Austrian dairy cattle. Their potential long-term synergistic effects on animal, human and environmental health should be subject to further investigations.

Credit author statement

Felipe Penagos-Tabares: Conceptualization, sampling, sample preparation, data collection, data analysis, data interpretation, elaboration of tables and figures, writing original and final draft, **Michael Sulyok**: Pesticide and veterinary drug analysis, revising and editing the original draft. **Johannes Faas**: Conceptualization, funding acquisition, revision and editing of the original draft. **Rudolf Krska**: Pesticide and veterinary drug analysis, revising and editing the original draft. **Ratchaneewan Khiaosa-ard**: Revising and editing the original draft. **Qendrim Zebeli**: Conceptualization, resources, funding acquisition, review & editing of the original draft.

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Declaration of competing 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.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

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