UNIVERSITÀ DEGLI STUDI DI MILANO

PhD Course in Veterinary and Animal Science

Class XXXII

Study of environmental contaminants, veterinary drugs and residues throughout the food chain related to swine and poultry, and eventually other species of food-producing animals

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Abstract

Meat and meat products are the principal sources of human diets. The health of food-producing animals should under proper monitoring and control in order to reduce risks to the food supply chain. The studies presented in this thesis included the strategies of (i) Evaluate the outcome of feeding back the pathological report to the origin pig farms, and (ii) Build up the detection method and investigate the prevalence for environmental contaminants and veterinary drugs in pork, veal, chicken eggs and baby foods.

In Chapter 3, we collected meat inspection records at a national level. The number of large farms account for 9% of the total but produced 48.5% slaughtering pigs. About the percentage of pathological lesions in the carcass, its coefficients of variation (CVs) is of 42% in the class of large farms. It suggests that the health level in large farms were more homogenous than in small and medium ones. At the final of the study, we analysed the influences of pathological lesions after having sent the post-mortem result to pig producers. The results highlight that the percentages of liver and lung had gradually reduced by 0.02% per month. The feedback of post-mortem result improves the transparency of government information, the close collaboration between producers and official veterinarian, and the herd health, for safer food of animal origin.

In Chapter 4, 5 and 6, we developed highly sensitive detection methods on perfluoroalkyl substances (PFASs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs), pesticides, and antibiotics. The limit of quantification (LOQ) is 0.015-0.15 ng g^{-1} in PFASs, 0.5 ng g^{-1} in PBDE, which complied with Commission Decision 2002/657/EC. We applied our methods for the investigation of several animal matrices: pork, veal, and baby food.

The results suggest that the prevalence of environmental contaminants in pork, veal, and baby food are low and do not post risks to human health.

In Chapter 7, we developed a quick and easy-to-apply method to detect Fipronil and its metabolite and Amitraz from chicken eggs. The LOQ is 0.89 ng g^{-1} in Fipronil, and 2.4 ng g^{-1} in Amitraz.

The outputs described in this thesis consists of clear understanding of detection methods for environmental contaminants and veterinary drugs in swine, calves and poultry. Besides, via the feedback mail, the pig producers received continuous observations from the slaughterhouse. Thus they took actions to reduce pathological lesions. The results introduced in this thesis can be the future way to keep food safety throughout the food chain.

Riassunto

La carne e i prodotti a base di carne sono le principali fonti di diete umane. La salute degli animali da produzione alimentare dovrebbe essere adeguatamente monitorata e controllata al fine di ridurre i rischi per la catena alimentare. Gli studi presentati in questa tesi includevano le strategie di (i) valutare l'esito della spedizione del rapporto patologico agli allevamenti di suini di provenienza e (ii) sviluppare il metodo di rilevazione e studiare la prevalenza di contaminanti ambientali e farmaci veterinari nel maiale, vitello, uova di gallina e alimenti per bambini.

Nel capitolo 3, abbiamo raccolto i record delle ispezioni delle carni a livello nazionale. Il numero di grandi allevamenti rappresenta il 9% del totale ma hanno prodotto il 48,5% di suini da macello. Per la percentuale di lesioni patologiche nelle carcasse, i suoi coefficienti di variazione (CV) sono del 42% nella classe dei grandi allevamenti. Ciò suggerisce che il livello di salute nei grandi allevamenti era più omogeneo rispetto agli quelli piccoli e medi. Al termine dello studio, abbiamo analizzato le influenze delle lesioni patologiche dopo aver inviato il risultato post mortem agli allevamenti di provenienza. I risultati evidenziano che le percentuali di fegato e polmone sono gradualmente ridotte dello 0,02% al mese. Il feedback sui risultati post mortem migliora la trasparenza delle informazioni governative, la collaborazione tra produttori e veterinario ufficiale e la salute della mandria per alimenti più sicuri di origine animale.

Nei capitoli 4, 5 e 6, abbiamo sviluppato I metodi di rilevazione altamente sensibili su sostanze perfluoroalchiliche (PFAS), bifenili policlorurati (PCB), idrocarburi policiclici aromatici (IPA), eteri difenilici polibromurati (PBDE), pesticidi e antibiotici. Il limite di quantificazione (LOQ) è 0,015-0,15 ng g^{-1} in PFAS, 0,5 ng g^{-1} in PBDE, il che è conforme alla decisione 2002/657/CE della Commissione. Abbiamo applicato i nostri metodi per lo studio di diverse matrici animali: maiale, vitello e alimenti per bambini. I risultati

suggeriscono che la prevalenza di contaminanti ambientali in carne di maiale, vitello e alimenti per bambini sono bassi e non comportano rischi per la salute umana.

Nel capitolo 7, abbiamo sviluppato un metodo rapido e facile da applicare per rilevare il fipronil e il suo metabolita e l'amitraz dalle uova di gallina. Il LOQ è 0,89 ng g^{-1} in fipronil e 2,4 ng g^{-1} in amitraz.

I risultati descritti in questa tesi consistono nella chiara comprensione dei metodi di rilevazione di contaminanti ambientali e farmaci veterinari nei suini, vitelli e pollami. Inoltre, tramite la mail di feedback, i produttori di suini hanno ricevuto le osservazioni continue dal macello, quindi hanno intrapreso azioni per ridurre le lesioni patologiche. I risultati introdotti in questa tesi possono essere il modo futuro di mantenere la sicurezza lungo tutta la catena alimentare.

Chapter 1

General introduction

1.1 Food safety control in the EU

1.1.1 The treaties

In 1952, the 6 European countries, Belgium, France, Italy, Luxembourg, the Netherlands, and West Germany, had signed the Treaty establishing the European Coal and Steel Community (ECSC). The ECSC treaty invented a supranational authority to supervise the common market for coal and steel among the member countries. In 1958, the Treaties of Rome had become active, which created the European Economic Community (EEC) and the European Atomic Energy Community (EURATOM), had deepened the economic cooperation among the Member States. In 1987, the Single European Act (SEA) came into force, which enhanced European integration and established the internal market (where there is free movement of persons, goods, services, and capitals throughout member states). In 1993, the Treaty of Maastricht had legalised the established European Union and Euro. In 1999, the European Parliament endorsed the Treaty of Amsterdam, which re-formed the internal infrastructure of the European Union and improved its democratic principle. In 2003, the Treaty of Nice came into effect, which mainly focused on the composition and legislative procedures and methods of EU institutions. In 2009, the Treaty of Lisbon came into force, amending for more democratic consultations within the EU, streamlining the internal structure of the organisation, and strengthening and improving the decision-making power of the organisation itself.

Currently, food safety control in the EU is the remit of Directorates-General Health and Food Safety (DG SANTE). The Directorate F “Health and Food Audits and Analysis (the

former “Food and Veterinary Office” (FVO)) performs the audits, inspections and related non-audit activities for the DG SANTE. DG SANTE, the executive agency, works closely with the following agencies: European Food Safety Authority (EFSA), European Medicines Agency (EMA), European Centre for Disease Prevention and Control (ECDC)), the Community Plant Variety Office (CPVO), and the Consumers, Health and Food Executive Agency (CHAFEA). These agencies plan up with safety-related regulations, implementation, evaluation, animal disease prevention, education and training, in order to safeguard the meat safety and EU consumers’ health. Besides, national food safety authorities of Member States also work closely with the agencies mentioned above to ensure adequate and continuous protection of food safety and consumers' health.

1.1.2 Legislations for EU meat safety management

One of the essential management priorities regarding food safety is the meat hygiene management in slaughtering procedures. After World War II, the EU meat safety focused on stopping the porcine trichinosis and bovine tuberculosis. Followed with improved animal disease prevention and control technology, the diseases as mentioned above have reduced. In current meat inspection management, the most important control is to reduce the contamination of foodborne pathogens such as *Escherichia coli*, *Salmonella Spp.*, and *Campylobacter spp*. (Borch et al., 1996, and Edwards et al., 1997)

There are plenty of food-related regulations regarding food safety. The stringency of control on food regulations was in the third position, just behind the regulation of automobiles (Directorate-General for Enterprise and Industry, EC, 2006) and chemical products (Directorate-General for Internal Market, Industry, Entrepreneurship and

SMEs, EC, 2017).

Table 1. The number of regulations for Automobiles, chemical products and food and feed in Europe.

References	Category of industry	Number of regulations
Directorate-General for Enterprise and Industry, EC, 2006	Automobiles	Close to 100 regulations
Directorate-General for Internal Market, Industry, Entrepreneurship and SMEs, EC, 2016	Chemical products	More than 70 regulations
Directorate-General Health And Food Safety, EU, 2013	Food and feed	Almost 70 regulations

In the legislative contexts, EU regulations protect consumers on food safety very much. For instance, consumers can choose different TV companies, telecom service, mobile phone manufacturing companies, or choose nothing; however, consumers must eat food to survive. Once the food contains safety risks, it will pass to the consumer. Therefore, in the scene of food safety, the bargaining power of consumers is weaker than the Food business operator (FBO). Therefore, the food law shall not only be able to ensure food safety, but also protect consumer rights.

Before 2002, no legislation in the European Union could regulate the safety for all kinds of food, but individual regulations managed by a separate section by food name. However, since the FBO developed more and more innovative foods, the novel names of the food are different from those listed in the regulations so that the operators can escape from the control of the regulations. As a result, food safety problems are gradually spreading; eventually, consumers lost confidence in European food. Besides, since 1996, the incident of bovine spongiform encephalopathy in the United Kingdom has caused significant panic among consumers. Therefore, the establishment of the new management regulations has become an urgent task. Since 1997, the structure of food safety control in the EU has begun to undergo significant changes. After the EU's

efforts, the White Paper on Food Safety was published in 2000, emphasising the necessity for the EU to rebuild public confidence in the food supply, food science, food law, and food safety control. The basic principles of food safety policy supported by the consequences of prevention, precaution, traceability, and transparency. In 2002, promulgated the Regulations 178/2002, which defined the general principles and requirements of the Food Law, the FBO should trace food and procedures for food safety issues. That regulation also built up the European Food Safety Authority (EFSA) to strengthen food safety sciences and increase consumer confidence.

Regulation 178/2002 clearly defined the traceability of food. The regulations require all food and feed operators to be obligated to establish a traceability system; that is, the operator must keep records in all stage of producing. The food business operator must identify their own products, the supplier of ingredients, and the buyer of products. When there is a necessity, the FBO must quickly send the required record to the government authorities. Furthermore, the regulation also defined that the FBO has to record the name and address of the supplier and also the buyer, the contains and dates of the product. The FBO is also encouraged to preserve the volume or quantity of the product, the batch number on the package, and more detailed records, whether the product is fresh or processed. The FBO must get registered by the competent authority with approval, then obtain the registration number before it can operate. Through this step, the competent authority has noted what companies are registered, which numbers used in which addresses for producing what products. The FBO has to copy the registration number of supplier or buyer to keep any relevant records.

In 2004, the EU announced the legislative package on meat safety management, the Regulations 852, 853, and 854/2004, which set out matters to be implemented by the

FBO and the competent authority of the Member States. In the manufacture of food of animal origin, the FBO must record the details of the source of the animal. After the animals legally killed, the slaughterhouse number must label on the product. Among the Member States, the methods used for labelling (ear tags, passports, barcodes) may vary, but these methods must carry the same message. The official control on meat inspection is regulated in Regulation 852/2004, which the official veterinarian performed the post-mortem inspection, the inspection methods mainly based on visual inspection. When necessary, sensory examinations such as palpation can apply. Overall, the EU has put the FBO with the most fundamental obligations on food safety. The legal obligations are briefly listed as follows:

1. Safety: Unsafe food or feed shall not enter into the market.
2. Safety responsibility: FBO has the responsibility to keep food and feed safely in the manufacture, transportation, storage or sale.
3. Traceability: FBO should quickly identify the product of any supplier or buyer.
4. Transparency: Once there is a reason to believe that the product has a safety issue, the FBO should immediately inform the competent authority.
5. Contingency plan: Once there is a reason to believe that the product has a safety problem, the FBO shall immediately withdraw the food or feed from the market.
6. Preventive: FBO shall identify and regularly review important control points during manufacture, and confirm that control measures applied at critical control points.
7. Cooperative: The FBO shall work with the competent authorities to reduce food risks.

In order to deliver the traceability information to the consumer (Regulation

1760/2000), taking beef as an example, the product must be labelled the following information when it goes into the market:

1. Place of birth: The country where the animal was born.
2. Place of feeding: The country where the animal is raised or fattened.
3. Place and code of Slaughterhouse: Refer to the country and the code of slaughterhouse.
4. Place of cutting plant: Refers to the country and the code of cutting plant.

If any item is miss marked on the product, it will be considered illegal and can report to the competent authority.

In order to give consumers more detailed information, the FBO can print on the product with batch number, traceback number, farm of origin, and breed of cattle. All this information is intended to deliver the information on the food chain and encourage consumers to understand the food before choosing it.

Looking backwards to 1996 and the vital food crisis in the EU (which had been reported in major News media of the world), there are temporal changes in the new regulations, as follows:

1. 1996: Bovine Spongiform Encephalopathy (BSE) in the UK;
2. 1999: Dioxin contamination of chicken, pork and beef in Belgium;
3. 2000: The EU published the White Paper on Food Safety, emphasising the need for the EU to rebuild consumers' confidence in the food supply, the food science, the food law and food management. The basic principles of food safety policy need to support the precautionary measure, traceability and transparency.
4. 2002: Announcement of the General Food Law (GFL), Regulation (EC) No 178/2002, the general principles and requirements of the Food Law, the

establishment of the European Food Safety Agency (EFSA) and the relevant measures for food safety issues;

5. 2004: Announced Hygienic Package Regulations, whereas four bills: Regulation (EC) No 852/2004 Hygiene of food and foodstuffs, Regulations (EC) No 853/2004 specific hygiene rules, Regulations (EC) No 854/2004 official control on feed and food, and Regulation (EC) No 882/2004 official control on products of animal origin. The official control regulations ensure FBO compliant with feed and food law, animal health and animal welfare regulations;
6. 2005: To promote the protection of public health from microbial hazards, published Regulation (EC) No 2073/2005 the microbiological criteria for foodstuffs and No 2075/2005 official controls for *Trichinella* in meat;
7. 2006: H5N1 Avian influenza outbreak in India;
8. 2008: A case of Pork Dioxin contamination in Ireland;
9. 2008: Melamine incident in dairy products in China;
10. 2009: New influenza (Swine flu A, H1N1) infection occurred in Mexico and the United States;
11. 2011: *E. coli* O104 infection in Germany;
12. 2013: Horsemeat adulteration scandal in Europe;
13. 2017: Fipronil in chicken and egg in more than 45 countries (including the European countries, United States, Russia, Israel and Canada).

What will be the next one?

Although the prevailing food law has had performed from 2002, in the following 15 years, there are still many challenges on food safety control.

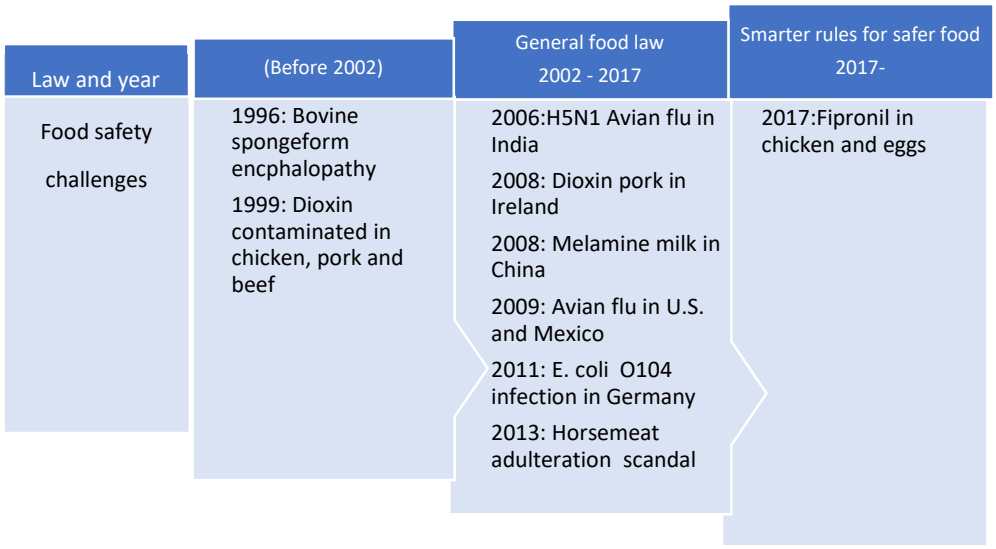

Figure 1. The comparative chronologic graph followed by current law and food safety events.

1.1.3 Smarter rules for safer food

Even though the EU's food safety regulations have performed from 2002, there still happened the horsemeat adulteration scandal in 2013. Each meat safety crisis or fraud issue always involved multiple levels of complicated works in one single incident. In order to put appropriate regulations in place to face increasingly complex meat safety incidents, the EU is about to improve its food safety regulatory framework, consolidate and strengthen food safety management provisions, and assist the Member States with more streamlined and easy-to-comply provisions. In the future, nearly 70 existing regulations will be re-constructed into eight parts to build the "Smarter rules for safer food", which has been announced as Regulation (EU) 2017/625.

Table 2. Summary of Regulation (EU) 2017/625. (Source: studies from the author)

Part	Article No.	Summary
1	1-3	Scopes and definitions
2	4-91	Official controls, border controls, financing and tariff
3	92-101	Qualifications of scientific laboratories
4	102-108	Collaboration among the Member States and EU while there must go the "cross-country" way.
5	109-115	Annual planning and reporting work for keeping continuously and regularly official control
6	116-136	Guideline for third countries. Official training. To set up the Information Management System for Official Controls (IMSOC)
7	137-140	Treatment for non-compliant cases. Temporary measure while any Member state was in "disruption" on official control.
8	142-145	Supplement about the relationship with other relevant laws

The Regulation (EU) 2017/625 has several characteristics:

1. Better self-finance: Since the Regulation (EC) No 882/2004 empowered competent authority to set up adequate financial resources for food safety official controls, Member States are free to set the tariff list. However, the member States met stresses on the financing of official controls, suggested to review the current fee system for inspections. (Christodoulou et al., 2009; Commission of the European Communities, 2009). The Regulation (EU) 2017/625 defined "Mandatory fees or charges", "The competent authorities ***shall*** collect fees or charges for the official controls…", which should be better financed than in the old regulation 852/2004 "Member States ***may*** collect fees or charges to cover the costs occasioned by official controls."
2. IMSOC is supporting to official controls: Through IMSOC, all documentary checks will be done electronically. The data exchange between the Member States and EU will be paperless as well. The digital file will become the original document, while the printed paper is a working copy. The official control

information of plant protection products, genetically modified organisms are also included. By IMSOC, Member States can streamline the administrative control process. Additionally, all collected data will be massive then can be analysed for predicting, preventing, or targeting potential/emerging problems.

3. Continuity and regularity: Member States are compulsory to design and implement the multi-annual national control plans to ensure the effective operation of regulation.
4. Intervention on “disruption” of official control: In order to ensure uniform food safety condition, while a Member State failed to maintain specific control then led to the emergence of risks, the EU shall be able to react by adopting measures to eliminate those risks from the food supply chain. Looking back at the Dioxin egg crisis, and Fipronil egg incident, the EU paid intense attention and supervised the Member States. Despite that, lots of unsafe products had entered into the market for quite an extended period. This intervention clause will authorise the EU to organise disposal resources and break the territory restriction in order to eliminate safety risks from the EU market.

1.2 Meat inspection information modernisation

The meat inspection is the most strategic position of food safety. The EU has had unified the organisation of meat inspection by Regulation 178/2002, followed with the hygiene package regulation 854/2004 and 882/2004. The Italian central advisory body is the Food Safety National Committee (“Il Comitato Nazionale per la Sicurezza Alimentare, CNSA”), which provide scientific and technical materials for risk assessment. The CNSA followed EU regulations, had proposed a Risk-based meat inspection system. The traditional meat inspection based on visual and sensory

(palpation or smell) inspection. However, while the inspector performed the palpation or incision on suspect organs, it facilitated the cross-contamination to the carcass. On the other hand, the traditional inspection practices were not able to discover meat-borne hazards, for instance, the *E.coli O157*, or organic pollutant substances.

In 2013, the EFSA published reviews and recommendations for meat inspection practices in order to modernise quickly. The EFSA recommended to use available options for the biological hazards at both farm and slaughterhouse level; to omit routine palpation or incision techniques in post-mortem inspection. For the chemical contaminants, it should be more integrated sampling, testing and intervention protocols for monitoring chemicals along the food chain. The EFSA also recommended to extended the use of other information collected throughout the food chain to compensate for lost information due to omitted palpation or incision practices. In the literature of known studies, in the Lithuania slaughterhouse, the ratio of pigs was found pathological lesion about 14.82%, which increased 1.42% annually, and all inspected animals that had no clinical signs of the disease (Januškevičiene et al., 2010). Another study suggested that in the chicken slaughterhouse, the detection of colisepticaemia and Infectious bursal disease (IBD) were minimal because the farmers were able to be aware of the disease thereof. Instead, the detection of ascites in the slaughterhouse is higher than in farms because the farmers were awkward to detect (Huneau-Salaün et al., 2014). It indicated that the inspection information of ante-mortem and post-mortem should be re-organised to satisfy the need for official control and the feedback of information also make it beneficial to farm managers.

1.3 Contaminants in the food chain

In January 1999, the Dioxin pollution incident occurred in Belgium. An animal feed producer used contaminated animal fat as ordinary ingredients to make chicken, pig and cattle feed. After the layer chicken having ingested contaminated feed, the laying performance declined. The abnormal scene caught the attention of the farm manager. After intensified investigation, the Belgium authority reported the alarm to the EU on 27 May 1999 (Haron, 1999). Unlike microbial risks, the dioxin cannot be eliminated through subsequent heating/frozen processing. The best choice is the use of uncontaminated materials. Besides, continuous monitoring of food contaminants is fundamental to eliminate pollution substance from the environment. On the other hand, in 1995, the United Nations Environmental Programme defined the persistent organic pollutants (POPs) as “chemical substances that persist in the environment, bio-accumulate through the food web, and pose a risk of causing adverse effects to human health and the environment”. According to its persistence characteristics in the environment, the water-soluble POPs gradually contaminated the drinking water. Animals drank polluted water and accumulate POPs in body tissues, eventually brought POPs through the food supply chain, from farm to the table, impacted human health. In 2004, the international initiative, “Stockholm Convention on Persistent Organic Pollutants” entered into force. Countries of co-signatories agreed to prohibit or limit the use of POPs. In the same year, the EU also promulgated Regulation (EC) 850/2004 on controlling on persistent organic pollutants. Notably, perfluoroalkyl substances (PFASs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs), and pesticides, are water-soluble and travelling into the animal or human drinking water supply, exposed risks to human health.

1.3.1 PFASs

PFASs is a collective name for vast organofluorine polymer compounds. The name of each compound was defined by the number of carbon atoms and functional group (acetate acid or sulfate acid). In 1947, the Minnesota Mining and Manufacturing Company (3M) synthesised perfluorooctanoic acid (PFOA), followed by the perfluorooctane sulfonic acid (PFOS) in 1949. The PFASs widely applied in waterproof textile, stickless fry pan, container, and various packaging materials. While manufacturing such products, the PFASs may spread into the air then contaminant peripheral soil and water. Gebbink et al. detected PFOS in human serum in 1997. Adinehzadeh et al. reported PFOA may be involved hepatotoxicity on the rat in 1998. The PFOA and PFOS had recognised as endocrine disruptors, with adverse effects on the reproduction system, performance suppression of the immune system on vulnerable groups of people, such as children, pregnant women or elders. While animals drank polluted water, the PFASs will eventually aggregate into the food supply chain. In 2014, the United States Environmental Protection Agency (USEPA) included the PFOA and PFOS into Emerging Risk substance list. Besides, the EFSA proposed tolerable daily intakes for PFOA (1,500 ng/kg b.w. per day) and PFOS (150 ng/kg b.w. per day). In the EFSA's scientific output, there are 25 different PFASs compounds in various foods. The EFSA has also published that perfluorinated compounds are the most frequently reported in fish, drinking water and meat products. Another European-wide survey evaluates that 11 rivers are continuously emitting PFOA and PFOS into rivers and eventually make estuarine exports to European oceans (Lindim et al., 2016). In 2002, the U.S. voluntary stopped producing PFOA and PFOS. In 2006, the EU banned the use of PFOA and PFOS. In 2019, the EU published Regulation (EU) 2019/1021, which repealed Regulation EC 850/2004, for more restriction on persistent

organic pollutants. In the regulation, the EU encourages the Member States to monitor environmental PFASs, collect results and exchange information, in order to evaluate the outcome after ratification for “Stockholm Convention on Persistent Organic Pollutants” on 17 May 2004.

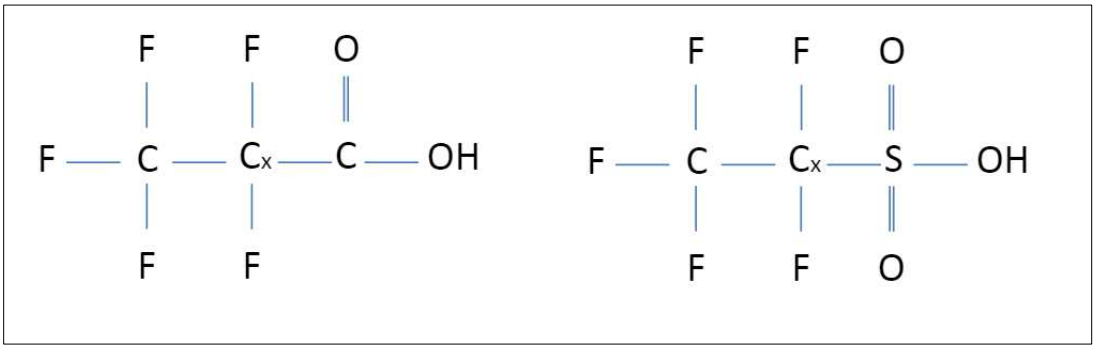

Figure 2. The molecular of PFCA(left) and PFCS(right) (Source :EFSA, 2012)

1.3.2 Polychlorinated Biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), Polybrominated Diphenyl Ethers (PBDEs), Pesticides

Besides the PFASs, the PCBs, PBDEs, and PAHs are contaminants commonly found in sediments, waters and wildlife (Erickson 1997; Safe 2003). These three classes of compounds have similar physicochemical characteristics of lipophilicity and resistance to degradation (Xua et al., 2013). Their high bioaccumulation potential added to a variety of toxic effects on humans and animals assesses their occurrence a crucial task (Van den Berg et al., 2006; Robertson and Hansen, 2001). The PCBs are synthesised organic biphenyl compound. To identify each PCB congeners, a “BZ number” which correlates the ascending order and number of chlorine within each sequential homologue is appended (Ballschmiter & Zell, 1980). It defines the PCBs being numbered from PCB 1 to PCB 209 (Ballschmiter & Zell, 1980). No natural PCBs were found. PCBs have no smell or taste. In the room temperature PCBs consists form light-colour liquid to black waxy solid. Because of its non-inflammable and excellent

electrical insulating characteristics, the PCBs were used as coolants, lubricants, electrical equipment, voltage regulators. The PCBs released into the environment from accidental leakage of manufacturing or handling, the broken container of equipment, or illegal landfill of PCB-contain products. The PCBs can stay in air, water and soil for quite a long time and do not naturally degrade in the environment (Haddaoui et al., 2016). PCBs can distribute in food crops (Liu et al., 2019). Therefore, human or animal ingested contaminated food or feed will accumulate in the tissue. From 1977 the U.S. halted the production of PCBs. The PCBs have been confirmed to make toxic hepatopathy to the rat. Workers have exposed to PCB contaminated plants were found a relation to hepatic cancer (Bosetti et al., 2003; Mallin et al., 2004; Ruder et al., 2014). In the animal model, PCBs can interfere with thyroid hormone levels (Gaum et al., 2016).

The PBDEs are made with a diphenyl ether structure which derived from combinations of bromine atoms on both rings. The identification of each PBDEs applies the BZ number rules as well (from PBDE 1 to PBDE 209). The primary use of PBDE is flame retardants which were added into textiles, electronic devices, and computer components. The enormous discarded computers and electronic products were dumped in the environment; therefore, the groundwater flowed through the waste, resolved PBDEs, went into the underground layer. The PBDEs caused endocrine disruption and thyroid hormone interference (Linares et al., 2015).

The PAHs are a group of polycyclic aromatic hydrocarbons, and generally not water-soluble (Choi et al., 2010). However, it favoured remaining in organic soil matter. The microorganism thus degraded the PAHs to soluble metabolites (Johnsen et al. 2005). The PAHs mainly cause the risks of human lung and bladder cancers (Mastrangelo et al., 1996).

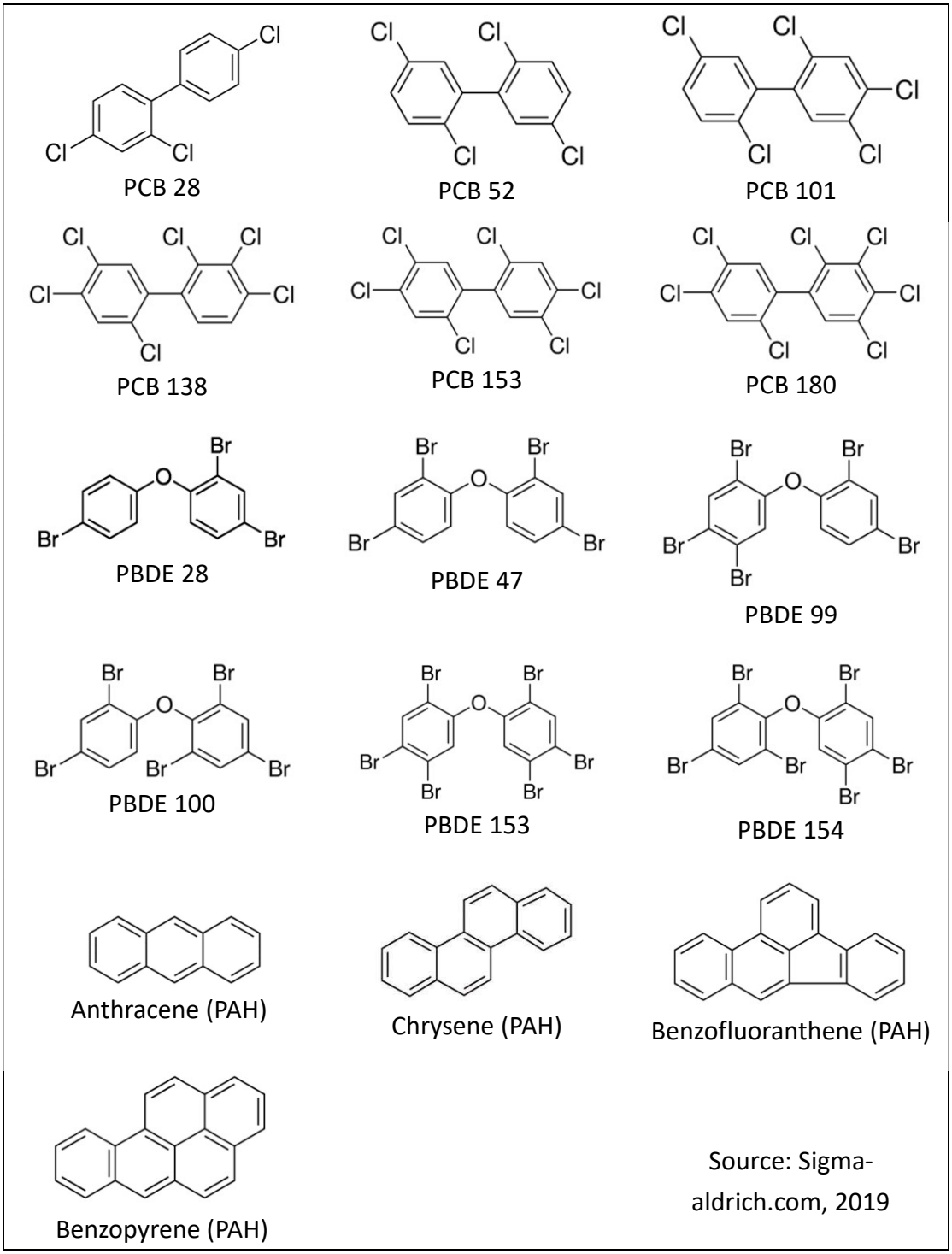

Figure 3: Molecular names and structures of PCBs, PAHs, and PBDEs.

1.3.3 Pesticides:

Pesticides are chemicals to kill pests or fungal microorganisms. They are sprayed on the entire growth field of crops, vegetables or fruits. The water and wind can bring the

pesticides to spread along with the farm field or the draining creek, thus extended the range of contamination. After the farm animals having ingested the contaminated water and crops, the pesticides distributed in the tissue. (Vijay & Vikas, 2011). The dichlorodiphenyltrichloroethane (DDT) is the significant pesticide which accumulated in the tissue of slaughtered goats and sheep (Nath et al., 1998). Past studies revealed the DDT and dichlorodiphenyldichloroethylene (DDE) might affect the children with neurodevelopmental risks (Eskenazi et al., 2006).

1.4 Veterinary drug residues in food

For promoting animals' growth, preventing disease, treatment for sickness and improving feed efficiency, farm managers might mix veterinary drugs into the feed. After the animals have ingested the feed, the original form of the drug or its metabolites may distribute and accumulate in the tissues, organs or edible products of the animal. The farm managers must respect the "withdrawal period", in order to give time for animals to full metabolite drugs intrinsically. Failure to control the withdrawal period, or illicit use of veterinary drugs will lead to the drug residue presented in food. For instance, the fipronil and amitraz are antiparasitic drugs and often prescribed for the fleas, lice and ticks infection on dog and cat. However, when it is illicitly administered to layer chicken, the fipronil will enter into the egg (Maclachlan, 2008), then the egg will be subsequently put into the food market. In the food supply chain, the drug residue is not visible and cannot be removed by the fabrication procedure. Eventually, it goes into the human's food, which also poses risks to human health.

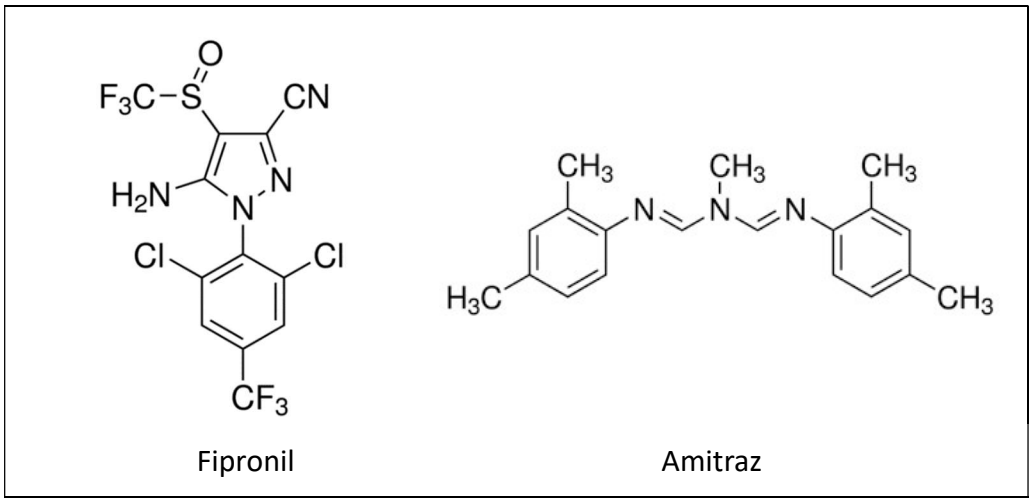

Figure 4. The molecular structures of antiparasitic drugs Fipronil and Amitraz. Source: Sigma-aldrich.com, 2019

Food with drug residues is harmful to human health. People who long-term ingested food of the same residue will gradually accumulate potential risks. Vulnerable groups, such as children, patient in grave condition, allergy for specific drug molecular, may occur diseases and even death. The veterinary drug residues with the principal risks of human health are as follows:

1. Drug hypersensitivity: β-Lactams (Penicillin family) drugs are responsible for most of the allergic syndrome after having ingested antibiotics.
2. Carcinogenic effect: Sulphamethazine, Oxytetracycline, Furazolidone
3. Teratogenic effect: Aminoglycosides, Polymyxins, Tetracycline, Vancomycin, Fipronil
4. Nephropathy: Aminoglycosides, Methicillin, Cephalosporins, Polymyxins or cyclins
5. Myelotoxicity: Vancomycin, Chloramphenicol. Due to the suppression of hematopoietic ability.
6. Poisoning (Clenbuterol, Amitraz): The clenbuterol acted as β- agonist and used for the treatment of respiratory diseases. However, it was misused as

growth promoters to obtain lean in meat. Patients appeared superventricular extrasystoles and atrial fibrillation (Sporano et al., 1998). The overdose of Amitraz leads to the depression of the central nervous system and respiratory system.

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Chapter 2

Aim and Outline of the thesis

2.1 Study for the feedback of inspection information

As mentioned in the introduction, the results of official control only kept in the competent authority. The results of official control contain the information of the origin of animals, defects found in post-mortem inspection. The records of defects are directly relative to the quality of animal health. In the north of Italy, the competent authorities collected results and sent toward superior authorities. In this stream-up flow, the farmer did not receive a copy of the results. The new food law has set the IMSOC but focused on official control works. We know the pigs need to stay on the farm around six months before slaughter, and the farmer is the practical person to keep pigs healthy. We hypothesised the ratio of lesions in pigs would not increase while the farmer received the results of post-mortem inspection. We collected data from Taiwan, which performed the feedback of post-mortem information to the farmer from 2004 to 2009, then retrieving what the pathological condition changes found in the slaughterhouse.

2.2 Develop methods to detect contaminant residues in European pork, veal, and baby food

The EU has banned the production and use of PFASs, PAHs, PBDEs, and PCBs, but in the literature, there are still traces in the fish samples. In our project, we develop detection and identification methods, then applied on pork, veal, and meat-containing baby food samples, in order to explore our hypothesis on the meat of livestock.

2.3 Develop methods to detect recent fipronil and amitraz in the chicken egg.

In the second year of research, an urgent food event, the illicit use of fipronil in layer chicken, happened in Belgium. Where possible, we developed a highly sensitive, easy-to-apply method to detect and identify the fipronil and metabolites, and the often prescripted insecticide amitraz as well.

Chapter 3

The feedback of meat inspection information from the slaughterhouse to pig producers may affect the health level of market pigs in the food supply chain. A nation-wide study.

(manuscript in preparation)

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In this study, I contributed to the experimental work planning, the execution of data collection and analyses, data processing and writing of the article.

Abstract

According to European Regulation 853/2004/EC, the message flow of food chain information is down-streamed. A veterinary inspector should inspect each animal carcass. After cutting off a lesion, the veterinarian registered a record of pathological result; thus, the health level of each herd can be monitored over time. This study retrieves data from the Taiwan national meat inspection system from the year 2003 to 2017. From 2005 to 2009, each farmer received the post-mortem inspection report monthly. Firstly, 54% of small farms that only account for 9.7% of the national production of pigs in 2011. Large farms are only in 9% of total and account for 48.5% of domestic production. Secondly, we collect the pathological counts/slaughtered ratio of each farm, then calculate the coefficient of variation (CV) value by farm-size. The CV indicates a high level of health: small farms are in high variation (79% to 60%), while medium and large farms are in lower variation (50% and 42%, respectively). By plotting with the monthly average, the pathological ratio of lung shows a declining trend from 2005; the liver trend reverses down from 2007 to 2011. This preliminary result suggests that the feedback of pathological result to animal producer reduces lung and liver lesions to market pigs.

Keyword: meat inspection, feedback, food chain, public health

3.1 Introduction

Meat inspection in the slaughterhouse is the modern official control for food safety and public health. The official veterinarian inspector performs the ante-mortem, post-mortem and offline inspection activities, which covers animal diseases, pathological findings, animal welfare, and public health (Regulation (EC) 852/2004; EFSA, 2011).

Besides public health, pathological records of meat inspection usually reflect the potential problem of animal herds. The Denmark authority made an agreement with associations of the slaughterhouse and the pig producers to construct a computer-based data system (Willeberg et cl., 1984). This system facilitates to identify the high prevalence of pathological lesion of the carcass and provide to veterinary experts. Another potential extension use of meat information is to evaluate animal welfare (Ellerbroek et al., 2011). According to welfare-related lesions in Danish sows, the highest prevalences are the abscesses and tail bites (Cleveland-Nielsen et al., 2004). Nevertheless, the slaughter pigs do not account for the national population (inclusive of weaning pigs, and sows), it can be estimated as constant and not affect the prevalence over time(EFSA, 2011; Harley et al., 2012). Despite there were benefits behind the meat inspection records, the Regulation (EC) 854/2004 does not set tasks to deliver the pathological report to pig producers and veterinarians (Harley et al., 2012). In Taiwan, the central authority built a voluntary feedback system to collect meat inspection records and then sent the post-mortem report to the farmer. Our objectives were to explore what the feedback affects the prevalence of pathological lesion, highlight the changes over time, and improve the use of meat inspection information.

3.2 Material and methods

3.2.1 Data source

The data of this study were retrieved from the Taiwan national meat inspection system from the year 2003 to 2017 in 69 pig slaughterhouse. A veterinary inspector inspected each pig carcass under national legislation. Once a pathological lesion present in carcass or organ, the veterinarian cut the lesion off and registered as a record of the

pathological result. From 2004 to 2005, the central authority performed a serial of on-job training measures to unify the judgement for each veterinarian. From 2003 to 2012, the farmer's address is traceable. Under the support of the budget from 2003 to 2009, each farmer had received the results of post-mortem inspection every month. This information is useful for farmers to review their policies of disease control and management of growth of pigs.

3.2.2 Analysis of data

The analyses were subdivided into the three following tasks:

Task 1: The levels of the dimension of farms

The dimensions of farms are heterogeneous. The quantity of pigs for slaughter ranges from 1 to 98,094 in 2005. Family members usually operated small farms. Large farms need more staff to manage the farm for more efficiency. As the market calls for 50 pigs at each batch of transportation, and consider possible lost, the class of the farm has set as 1-94, 95-599, 600-2,599, and larger than 2,600 pigs in the year 2011.

Task 2: The geographical distribution of farms, production, and slaughtering activity.

There are 6,129 traceable farms, 706,2407 pigs slaughtered in 19 counties in 2011. The dataset is collected and separated into each county.

Task 3: The percentage of the pathological lesion by organ

By checking post-mortem data, the most condemned organs are liver, kidney, heart and lung. Dataset has collected every month. We assumed that the percentage of the pathological lesion $P_{organ,month}$ is the number of pathological lesions of an organ (liver, kidney, heart, or lung) was condemned in one pig carcass over the numbers of pig slaughtered in one calendar month such that

$$P_{organ,month} = \frac{Number\ of\ pathologica\ \ records\ of\ the\ organ}{Numbers\ of\ pigs\ slaughtered} \times 100\%$$

The percentage of the pathological lesion by each organ was then plotted in Figure 3, 4 and 5.

3.3. Results

<u>Task 1</u>: The levels of the dimension of farms

By 2005, 56% of farms are small farms (which produce 1-94 and 95-599 pigs per year) and share 11% of the population. 37% are medium farms and produced 45% of the population. Large farms are only in 7% of the total, but share 45.4% of the population. The CV value suggests the level of health. Small farms are in high variation (75.7% to 60%), while medium and large farms are in lower variation (54% and 45%, respectively) (Figure 1 B). In 2011, the small farms shared less percentage (54%) and produced fewer pigs (9.7%) than in 2005. 37% are medium farms and produce fewer pigs (41.7%). The large farms increase by 2% and produce more pigs (48.5%). The CV of small farms is still high (79% to 60%), but both reduced in medium and large farms (50% to 42%) (Figure 1B).

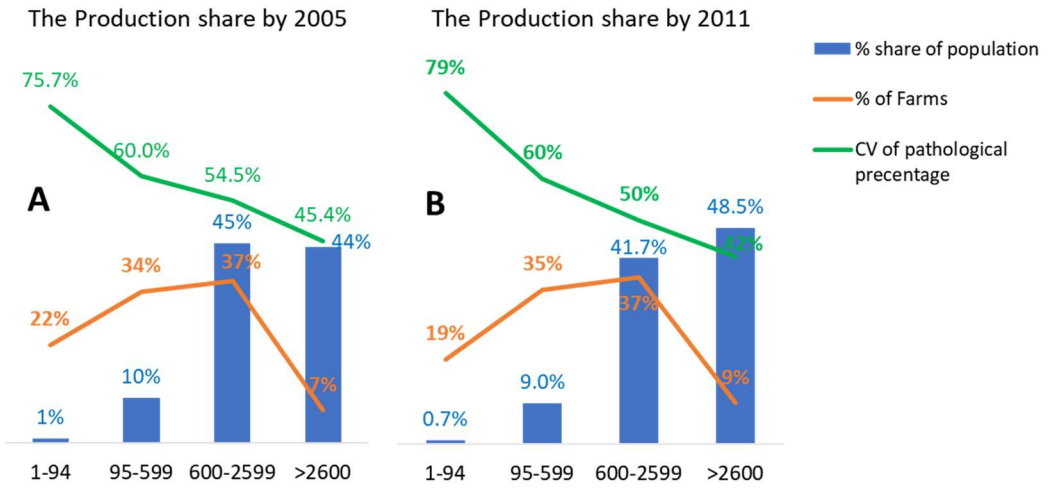

Figure 1. The proportion of the population and CV value of pathological counts by each class of farms in 2005 (A) and 2011(B), respectively.

<u>Task 2</u>: The geographical distribution of farms, production, and slaughtering activity.

Most of the farms located in the central and the south of Taiwan (Figure 2, A and B). Due to the needs of local consumption, pigs were transported to the north of Taiwan and then slaughtered (Figure 2, C).

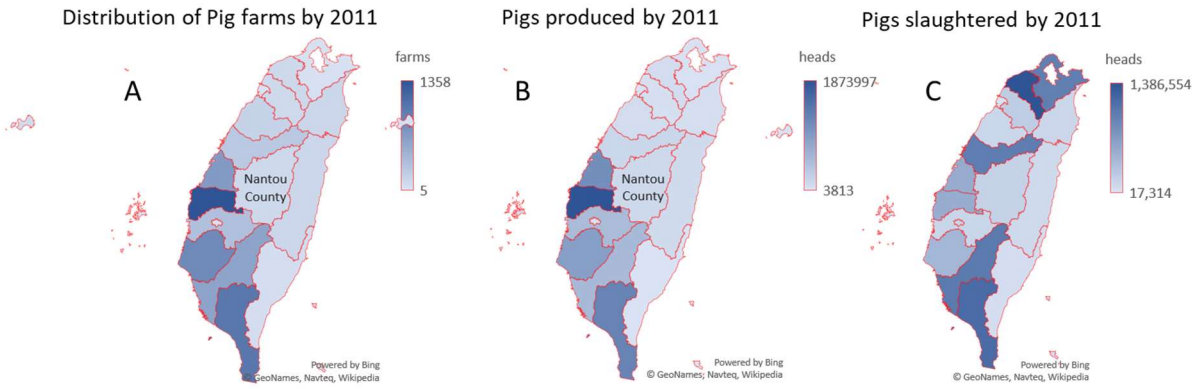

Figure 2. The geographical density of farms, pigs produced and slaughtered.

<u>Task 3</u>: The percentage of the pathological lesion by organ

By a yearly average, the lung lesion seems to decrease, while the condemnation ratio of kidney and heart are gradually increasing. The condemnation ratio of the liver is ranging from 5.38% to 12%. (Figure 3). The most common pathological findings are milk spot in the liver, cystic kidney/ hydronephrosis in the kidney, pericarditis in heart, pneumonia/pleuropneumonia in the lung.

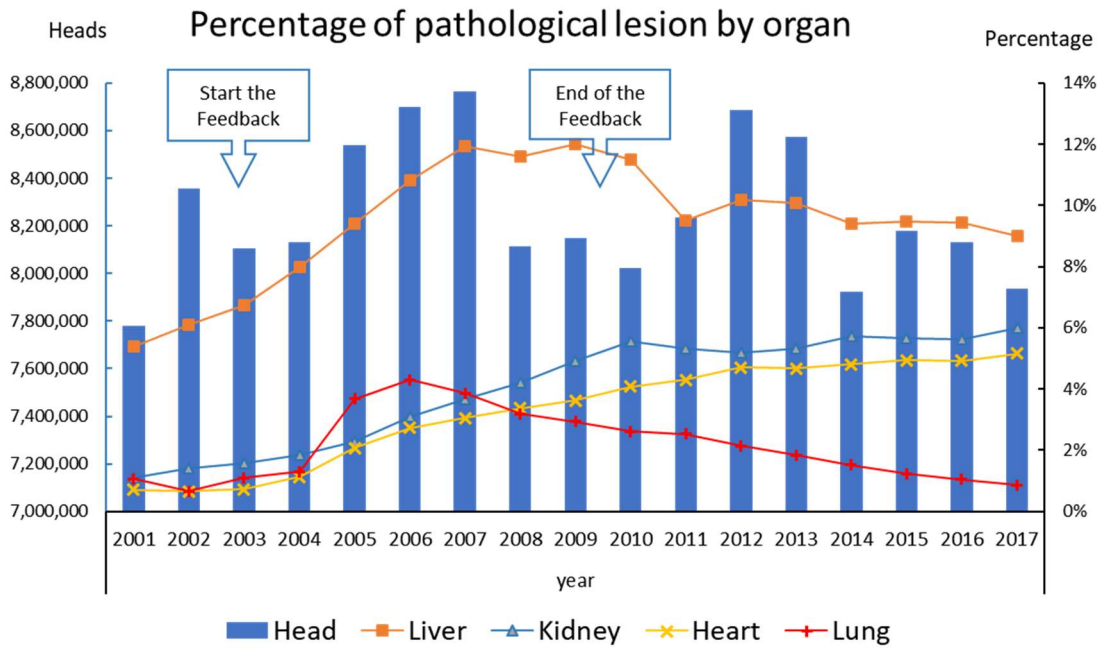

Figure 3. The percentage of the pathological lesions by organ.

By monthly plotting, the liver lesion was suggested to be seasonal circulation. Furthermore, the relative high season is in May or June, while the low season is in January or February. (Figure 4)

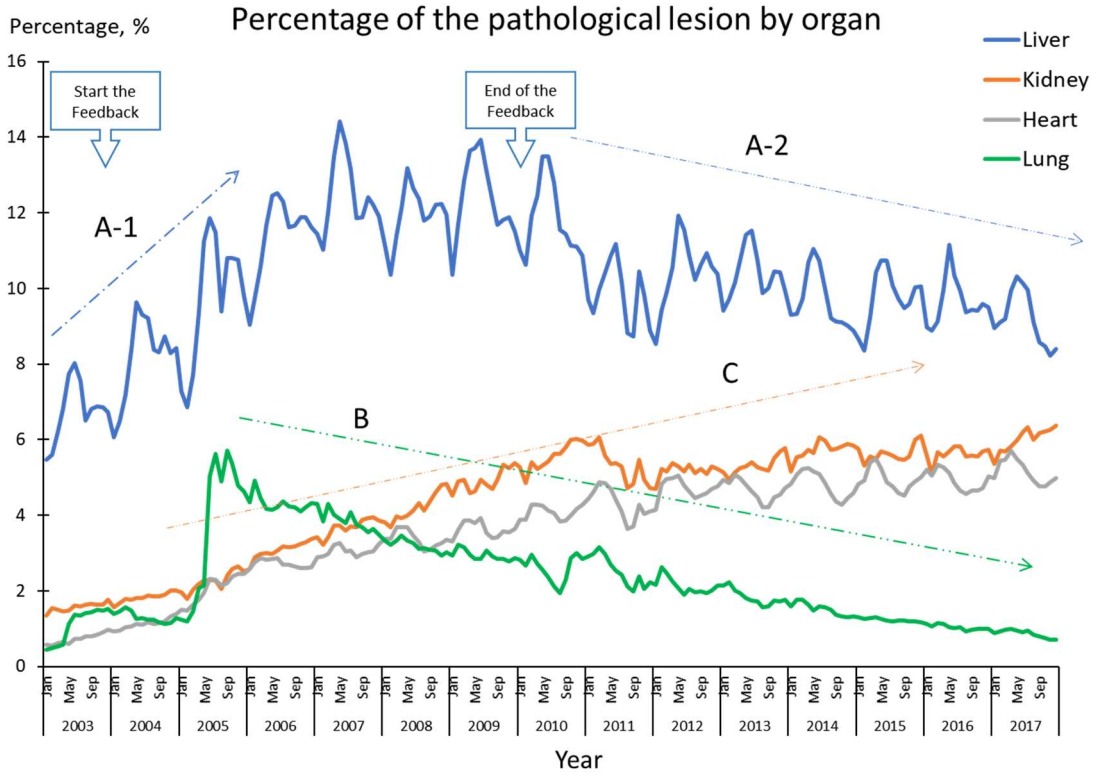

Figure 4. The condemnation ratios by organ.

In Figure 4, the liver lesion ratio was ascending from 2003 (line A-1). The ratio was high in May-June and is low in January-February. The liver lesion suggested a seasonal circulation, other than the kidney, heart, and lung records. After having stopped the feedback of information from 2009, the liver lesion ratio declined (line A-2). From 2009, after five years of information feedback, the circulation of liver and lung lesions steadily declined.

In 2005, the competent authority unified the practical request. The lung with lesion must cut off, and regardless the FBO will remove it autonomous or not. From this unified control, the lung lesion ratio was high in 2005 and thus informed the herd owner via the letter of the pathological report. Six months later, the lung lesion ratio was steadily declined (line B). Instead, the kidney and heart were still ascending after the feedback of pathological information.

Lesions assumed seasonal circulations. Liver lesions are low in January and February, high in May and June. Kidney lesions are low in January and February, high in November and December (Table 1).

Table 1. The seasonal appearance of lesions from 2003 to 2017.

Lesion	Month	
	Minimum	Maximum
Liver	January/February	May/June
Kidney	January/February	November/December
Heart	January/February/August	March/May/June/December
Lung	August/September/October/ November/December	February/March/April

From 2003 to 2009, the central authority sent pathological reports to pig producers by post mail. Depended on the trade activities, the number of mails is floating from 3,000 to 3,900 but had an average quantity of around 3,500 mail per month (Table 2).

Table 2. The number of mail sent to pig producers in each month of 2013.

2013											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
3,799	3,073	3,691	3,579	3,842	3,413	3,222	3,698	3,707	3,704	3,512	3,563

Average: 3,567

In Figure 5, despite the pathological letter stopped in December 2009, the farmers kept the liver and lung pathological ratio in a trend of decrease by 0.02% monthly. The pathological result of kidney and heart did not show a decline but present a seasonal circulation.

The preliminary result reflects that the descriptive output of the pathological result can be the principal parameter for the pig health level of the farm.

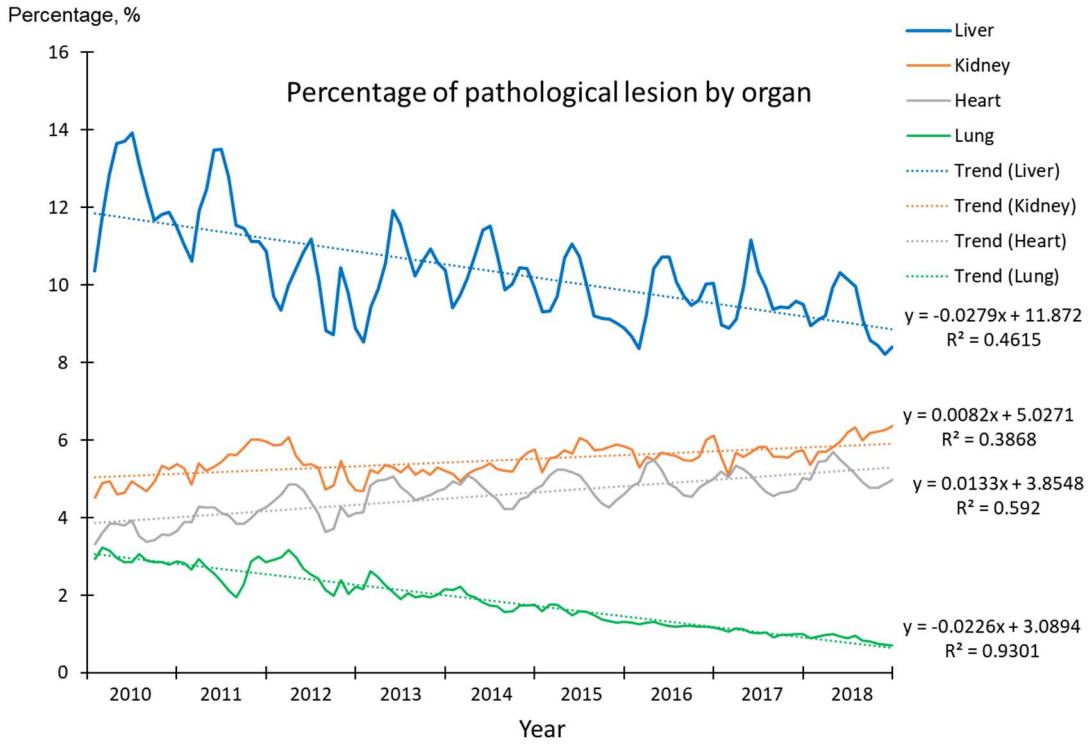

Figure 5. The trend line of the percentage of pathological lesions after having stopped the feedback of inspection results.

3.4 Discussion

<u>Task 1</u>: The levels of the dimension of farms

In Taiwan, the number of medium and large farms shared 46% of pig farms and produced 90.2% of pigs in 2011. According to the statistical data in 2014 from the Eurostat, in EU-28 (28 Member States), 1.7% were large farms and produced 77.9% of pigs in 2010. However, among the Member States exists considerable variations. In 12 Member States, large farms account 90% of pig farms, but in Romania, Croatia, Slovenia, Lithuania and Bulgaria, small units with less than ten pigs account for 62.8%,

45.3%, 31.4%, 28.8% and 25.8%, respectively. The CV values for EU small or large farms were absent. Even though, a Serbian study in slaughter pigs concluded that there exists a high prevalence in pathological lesions in smallholder farms (Čobanović et al., 2019). Regulation (EC) 854/2004 does not set tasks to reference the pathological record to the pig producer, perhaps Regulation (EU) 2017/625, which defined the IMSOC, is the legal basis for collecting both data of the pathological record and pig farms.

Task 2: The geographical distribution of farms, production, and slaughtering activity.

The land area of Taiwan is 36,197, km^2. Compare with EU countries, the size is similar to the Netherlands, 37,824 km^2. Taiwan is an island, and all slaughtering pigs are domestically produced. The pig farms highly clustered in central and south counties, in which the main agricultural producing areas. The pigs slaughtering activities focused on the north, central and south counties, in which the high population of habitats. Most consumers preferred acquiring pork, which just slaughtered within 24 hours. Compared with the EU countries, instead, the pig production activities are even cross borders. The highest pig production countries are Denmark, Germany, Spain, France, the Netherlands and Poland, which shared two-thirds of breeding pigs in 2013. For slaughtering, Germany and Poland are the highest pig importer countries (Eurostat, 2014).

Task 3: The percentage of the pathological lesion by organ

The percentage of pathological lesions is a useful parameter to evaluate the health level of the pig herds. The Danish health scheme was the preliminary practice to collect inspection data and use for improving the health of origin herds (Willeberg et al., 1984). The trend and the monthly scheme of the prevalence reflect epidemiology, risk factors and management strategies for disease control (Sanchez-Vazquez et al., 2011, and Correia-Gomes et al., 2017). In the UK, liver milk spot lesions are low in March and

April, high in September and October. Pericarditis lesions are low in December/January, high in May/June/July. The *Ascaris suis* infection leads to the milk spot in the liver and eventually being cut off in post-mortem inspection. Despite the pig producers having received the pathological report from 2003, the prevalence was ascending until 2006. It then subsequently reversed and gradually descending. The seasonal circulation is still present, but the trend of prevalence decreased by 0.02% monthly. This declining reflects the pig producers took the correct response to reduce Ascariasis. The lung lesion led to economic loss to pig producers. A study focused on pig pathological lesion and carcass weight highlighted that the average daily weight gain is 441.1 g, but the pigs suffered in lung disease were significantly lower, such as severe pneumonia (-39.4 g) and adhesive pleuropneumonia (-32.8 g) (Schuh et al., 2000).

Besides, the trend line of the lung also reflects the Taiwan pig producers in 2005 received high prevalence information (5.7%) from the pathological report, then took the correct action to reduce lung diseases. In 2017, the prevalence of pneumonia was 0.87%. For contrast, with the monitoring data on the Lithuania slaughterhouse, which works without information feedback to pig producers, pathology lesions increased by 1.42% annually (Januškevičienė et al., 2010). The feedback of post-mortem information to pig producers is undoubtedly the future trend to catalyse the reduction of pathological lesions right at the farm.

The pathological finding on the kidney is usually cystic or polycystic kidney. The lesion is congenital (Wells et al., 1980 and Wijeratne et al., 1980) and did not post a food safety issue.

The gross lesion of the heart is mostly the fibrinous pericarditis. The pathological changes are due to the infection of *Pasteurella multocida* and Mycoplasmas (*Mycoplasma hyopneumoniae, M. hyosynoviae* and *M. hyorhinis*) (Pors et al., 2011 and

Buttenschøn et al., 1997). *P. multocida* and Mycoplasmas are also the pathogens of porcine pneumonia. In contrast with the ascending trend of heart lesion, the lung lesion was declining. This comparison suggests that the commercial vaccine and antimicrobial treatment restricted the prevalence of lung lesion, but increase the potential infection to heart. Besides the visual inspection, an advanced monitoring method on the presence of veterinary drugs is necessary.

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Chapter 4

Levels and distribution of PBDEs and PFASs in pork from different European countries

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In this study, I contributed to the experimental work planning, the execution of preliminary trials and analyses, data processing and writing of the article concerning the LC-HRMS part.

The paper is reported by keeping the reference style indicated by the journal guidelines

Abstract

Meat and meat products are included in a great number of human diets. However, the great consumption of meat needs to be controlled for the presence of traces of contaminants. The European Commission has not stated maximum limits for some environmental pollutants such as the perfluoroalkyl substances (PFASs) and polybrominated diphenyl ether (PBDE); the European Food Safety Authority (EFSA) Scientific Panel has recommended that more occurrence data for PFASs in food should be collected to improve the accuracy of future exposure calculations. Therefore, the distribution of PFASs and PBDEs trace contaminants from eight EU Member States were investigated through liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) and Gas Chromatography-Mass Spectrometry (GC-MS/MS). No PFASs were detected, except perfluorooctanoic acid, in only one Austrian sample at the concentration of 0.531 ng g^{-1}. PBDEs were detected in 3 out of 77 samples: one from Germany showed the presence of all congeners analysed in the concentration range 0.53–0.77 ng g^{-1}, the others, from Netherland and Italy, respectively contained PBDE 153 (0.53 ng g^{-1}) and PBDE 100 (0.62 ng g^{-1}). The results show that the analysed samples do not pose a risk for human beings regarding PFASs and PBDEs. Further studies are needed to keep monitoring their presence in foodstuff, as it has been suggested by the European Commission.

Keywords: GC-MS/MS, LC-HRMS, PBDEs, PFASs, Pork, food safety

4.1 Introduction

Generally, food of animal origin plays an important role in determining the exposure of human beings to contaminants of chemical origin (Liem et al., 2000; Pastorelli et al. 2005; Törnkvist et al. 2011; Vogt et al. 2012). Perfluoroalkyl substances (PFASs) and polybrominated diphenyl ether (PBDE) contamination of food is a global issue of environmental pollution. PBDEs are one class of brominated flame retardants (BFRs) that can be released from manufacturing commercial products (e.g. acrylonitrile–

butadiene–styrene and polystyrene plastics, polyurethane foams), packaging materials, electronic devices, as computers or televisions. PBDEs can be released into the air, water, and soil at places where they are produced or used, but they have very low water solubility, and when these substances are released to water, they typically bind to sediment (ATSDR, 2011). These substances generally bind strongly to soil particles, and therefore, do not move easily through soil layers (Routti et al. 2015).

PFASs, such as perfluorooctane sulfonate (PFOS), represent a class of compounds showing high thermal, chemical, and biological inertness. Their application began in the early 1950s and, due to their widespread use, they are globally found in the environment, both in animals and in humans (Routti et al. 2015). Many countries, e.g. Germany, French, Denmark and Spain, reported the results of PFASs analysis from human serum samples (Ingelido et al. 2010) and other animals (Chiesa et al. 2018), where they found very low concertation with the average about 15 pg g^{-1} in pork and higher in fish where they reach 45 ng g^{-1} (Table 4). The highest concentrations are found near densely inhabited areas due to the discharge of industrial and municipal wastewater and fire-fighting operations (Lindstrom et al. 2011; Zacs and Bartkevics 2016).

Perfluorooctanoic acid (PFOA) and PFOS are recognised as endocrine disruptors with reproductive toxicity, and immunosuppression activity (Pèrez et al. 2014); several studies have shown that in experimental animals they have adverse effects including developmental toxicity, neurobehavioral toxicity and lung toxicity, as well as carcinogenic genotoxic potential (EFSA, 2012). On the basis of their properties, the EFSA proposed tolerable daily intake (TDI) levels for PFOA (1500 ng kg^{-1} body weight per day) and PFOS (150 ng kg^{-1} body weight per day) (EFSA 2012) due to their adverse effects in experimental animals and due to dietary exposure has been suggested as the

primary exposure route to PFASs.

Most information regarding the toxicity of PBDEs and their metabolites is from animal studies that show developmental neurotoxicity and endocrine disruption (Costa and Giordano 2007; Darnerud 2008). One study examined the effects of PBDEs in humans. The authors detected four congeners (PBDEs 47, 99,100, 153) in greater than 97% of women's serum samples analysed and found significant decreases infertility associated with PBDE exposure in women (Harley et al. 2010). The EFSA Panel on Contaminants in the Food Chain (EFSA 2011) considers eight PBDE congeners to be of primary interest: PBDE-28, −47, −99, −100, −153, −154, −183 and −209. In 2008, the United States Environmental Protection Agency (EPA, 2009) issued health assessments of four individual PBDE congeners, PBDE-47, −153, −99 and −209, within its Integrated Risk Information System (IRIS) programme. The dominant food category that is exposed to PBDE is food with high fat content because there is a relationship between the PBDEs levels and the fat content (EFSA 2011). In 2012– 2013, a U.S. meat and poultry (beef, pork, chicken, turkey) study reported that the mean summed concentrations of seven PBDE congeners from beef, pork, chicken and turkey were 0.40, 0.36, 0.19 and 0.76 ng g^{-1} lipid weight (lw), suggested that the U.S. consumer daily intake of PBDEs from meat and poultry was 6.42 ng day^{-1} (Lupton and Hakk 2017). Meat and meat products are included in a significant number of human diets. Their regular consumption means a significant intake of proteins and essential micronutrients. In addition, pork meat is used in many countries to produce derivative products (hams and cured meats) with high-qualitative value and relative recognition as Protected Designation of Origin (PDO) and the Protected Geographical Indication (PGI) products.

However, the enormous consumption of pork meat (Table 5) needs to be controlled

for the presence of chemical compounds. EU has not stated Maximum Levels (MLs) for PBDEs and PFASs; the EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) recommended that more occurrence data for PFASs in food should be collected to improve the accuracy of future exposure calculations (EFSA 2008). Subsequently, the European Commission issued the Commission Recommendation 2010/161/EU on the monitoring of PFASs in food in the Member States (EFSA 2012). It is therefore essential, to obtain information on the presence of these pollutants in food, mainly in those products whose consumption is highest (Table 5). EFSA's CONTAM Panel acknowledged that there were significant data gaps on issues such as the contribution of different foodstuffs, among which pork, to human exposure and that further research and data collection would be necessary (EFSA 2008).

Toxicological studies show that PFOS and PFOA are adsorbed after oral exposure and primarily accumulate in the serum, kidney and liver (EFSA 2008). Perfluoroalkyls tend to remain in the body unchanged for long periods. It takes approximately 4 years for the level to halve, so constant exposure could increase the levels in the organism resulting in adverse overcome (ATSDR 2009). People could be exposed to PBDEs in a wide variety of ways, including foods or dust/soils, air or through skin contact. The toxicokinetic of PBDEs depends on the number and position of the bromine atoms: the more toxic congeners are the lower brominated PBDEs, due to their ability to bioaccumulate, mainly in body fat. Consequently, decabromodiphenyl ether is expected to be less toxic than lower brominated PBDEs. Nowadays, the effects of PBDEs are not all well established and it is not known if PBDEs are carcinogens to human. However, the International Agency for Research on Cancer (IARC) has classified PBDE as a Group 3 carcinogen based on inadequate evidence of carcinogenicity in humans and inadequate or limited evidence in experimental animals

(Agency for Toxic Substances and Disease Registry 2011). So, based on the EFSA recommendation, in this paper we investigated the presence of PFASs through LC-HRMS and PBDE through GC-MS/MS in pork samples from eight EU Member States, to improve the knowledge on data gap of these compounds in literature.

4.2 Materials and Methods

4.2.1 Chemicals and reagents

The ^{13}C-labeled PFOS (MPFOS) and ^{13}C-labeled Perfluorononanoic acid (MPFNA), which were used as the internal standard (IS) in this study, and the 17 PFASs derivatives the Perfluorobutyric acid (PFBA), Perfluoropentanoic acid (PFPeA), (Perfluorobutane sulfonate acid), Perfluorohexanoic acid (PFHxA), Perfluoroheptanoic acid (PFHpA), Perfluorooctanoic acid (PFOA), Perfluorononanoic acid (PFNA), Perfluorodecanoic acid (PFDA), Perfluorooctane sulfonic acid (PFOS), Perfluoroundecanoic acid (PFUdA), Perfluorododecanoic acid (PFDoA), Perfluorodecane sulfonic acid (PFDS), Perfluorotridecanoic acid (PFTrDA), Perfluorotetradecanoic acid (PFTeDA), Perflugrohexadecanoic acid (PFHxDA), Perfluorooctadecanoic acid (PFODA), Perfluorobutane sulfonate acid (PFBS) and Perfluorohexane sulfonic acid (PFHxS), which were used for standard curve constructions, were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Mixtures of polybrominated diphenyl ether (PBDE) congeners (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 154) and 3-fluoro-2,2',4,4',6-pentabromodiphenyl ether (FBDE) as IS for PBDEs were purchased from AccuStandard (New Haven, USA). All standard purity was greater than 98%. Hexane and acetone (special grade for pesticide residue analysis (Pestanal) were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Each solvent is in HPLC or analytical grade. Purified water was supplied from the Milli-Q system (Millipore, Merck

KGaA, Darmstadt, Germany). The solid-phase extraction cartridges (Oasis WAX 3 mL, 60 mg) were bought from Waters™ (Milford, MA, USA). The ammonium formate, sodium acetate, acetic acid (99.9%) and 25% ammonia solution were purchased from Fluka. QuEChERS materials for the extraction were obtained from Supelco (Sigma-Aldrich, St. Louis, MO, USA); Supel™ QuE Citrate (EN) tubes, containing Sodium Citrate tribasic dihydrate and Sodium Citrate dibasic sesquihydrate. Magnesium Sulphate and Sodium Chloride were used for the extraction. Supel™ QuE-Z SEP (EN) tubes were used for the clean-up step.

4.2.2 Standard solutions

To make the stock solution, each of 17 standard PFASs compounds were prepared for 1 mg mL^{-1} concentration in methanol and store at -20 °C. The working solutions which were diluted from the stock solution at concentrations of 10 ng mL^{-1} and 100 ng mL^{-1} in methanol were freshly prepared before use and store at 4 °C.

Working solutions of PBDEs were prepared by diluting the stock solution in hexane for pesticides and then stored at -20 °C. An uncontaminated meat sample (previously checked for the presence of PBDEs and considered blank with a concentration of compounds less than limit of detection (LOD) used as control was selected for all procedure's optimization steps. For meat fortification, 1.0 g of the control sample was spiked by adding an appropriate volume of the standard working solution to cover the concentration range from 0.5 to 10 ng g^{-1} (five calibration points: 0.5, 1, 2, 5, 10 ng g^{-1}) for PBDEs in relation to literature to realise the matrix-matched calibration curves.

4.2.3 Sample collection

The muscle samples belonged to pigs from the food chain weighing 130 to 160 kg, and,

to minimize the damage to the carcass, the used muscles were obliquus internus abdominis and obliquus externus abdominis. Seventy-seven frozen samples from eight different European countries (Austria, Denmark, French, Germany, Holland, Italy, Poland and Spain) were collected. The samples were homogenized and then stored in -20 °C refrigerator and they were defrosted before being analyzed. The date of the sample collection was from 5 December 2016 to 5 May 2017.

4.2.4 Sample extraction of PFASs

Weight 1.0 g of homogenized sample into a 15-mL polypropylene screw-cap centrifuge tube. Add 50 µL of internal standard solution (which contains 100 ng mL^{-1} MPFNA and 100 ng mL^{-1} MPFOS in methanol) into the tube, to proceed a final concentration of 5 ng mL^{-1} over the matrix. Shake the tube by hand to mix it with the sample matrix. Add 10 mL of acetonitrile, vortex for 1 minute, then put the tube into the water tank with ultrasonication for 30 minutes in room temperature. Ultrasonicated samples were centrifuged at 4,612×g, 4 °C, for 10 minutes. Transfer all supernatant liquid into the evaporation flask and dried it with rotary vacuum evaporator at 35 °C. Add 10 mL of Milli-Q water into the flask and resuspend the analyte by vortex for 10 seconds. Load the resuspended liquid into Waters™ WAX SPE cartridge, which was previously conditioned with 3 mL of 0.05 mL mL^{-1} NH_4OH in methanol, followed with 3 mL of methanol, and 3 mL of Milli-Q water. After sample liquid running out through the cartridge, flush the cartridge through 3 mL of 25mM acetate buffer pH 4.5 to release proteins and lipids from the cartridge, followed with 2 mL of methanol. Elute the cartridge with 3 mL of 0.05 mL mL^{-1} NH_4OH in methanol and transfer the eluted liquid into evaporation flask then dried it with rotary vacuum evaporator at 35 °C. The dried analyte was solved with 100 µL of methanol:ammonium formate 20 mM (10:90 v/v)

to reconstruct the final volume. Transfer liquids into a screw vial and perform for the analysis with LC-HRMS.

For the estimation of recovery ratio, use blank pork samples of 1.0 g, divided into group A and B. In Group A, spike into matrix with 50 µL of internal standard solution (which contains 100 ng mL^{-1} MPFNA and 100 ng mL^{-1} MPFOS in methanol) into the tube, to proceed a final concentration of 5 ng mL^{-1}, and with 10 µL of 17 PFASs mixture (each single compound contains 100 ng mL^{-1}) to proceed a final concentration of 1 ng mL^{-1}, then run the extraction procedure. In Group B, spike the internal standard solution of 50 µL into the matrix, then run the extraction procedure. While solid phase extraction finished, spike the 10 µL of 17 PFASs mixture into the eluted liquid. Use LC-HRMS to determine the concentration of each PFASs then calculate the ratio of same PFASs between Group A and B.

For coefficient of variation of intra-day (repeatability), and inter-day (reproducibility) evaluation, use blank pork samples of 1.0 g, spike into matrix with 50 µL of same internal standard solution into the tube, to proceed a final concentration of 5 ng mL^{-1}, and 10 µL of 17 PFASs mixture (each single compound contains 100 ng mL^{-1}) to proceed a final concentration of 1 ng mL^{-1}, then run the extraction procedure. Use LC-HRMS to determine the concentration of each PFASs from each tube and calculate the value of each PFASs for the coefficient of variation of intra-day (repeatability) and inter-day (reproducibility).

4.2.5 Sample extraction of PBDEs

The extraction of PBDEs was performed using the QuEChERS (quick, easy, cheap, effective, rugged and safe) method. Briefly, 1.0 g of sample was homogenized and transferred to a QuEChERS extraction tube, then a solution containing the ISs (FBDE)

was added to the sample to a final concentration of 100 ng g^{-1}. Ten mL of acetonitrile was added as the extraction solvent; the tube was shaken for 1 min using a vortex and centrifuged for 10 min at 4,612×g at 4 °C. Later, the supernatant was transferred to a QuEChERS clean-up tube, shaken and centrifuged at the same conditions described above. The extract was collected, divided into two aliquots and dried under vacuum in a centrifugal evaporator at a temperature of 35 °C. The residue was dissolved in 200 µL hexane for the analysis by GC-MS/MS.

4.2.6 LC-HRMS Orbitrap analyses

The LC-HRMS analysis was performed by an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), composed with a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with a 20-µL loop. Chromatographic separation was carried out using a Synergi Hydro RP reverse-phase HPLC column (150 x 2.0 mm, internal diameter 4 µm), with a C18 guard column (4 x 3.0 mm; Phenomenex, Torrance, CA, USA). To minimize PFASs background contamination in the system, use stainless steel column tubes and peeks. Moreover, since PFOA and PFOS were always present in the blank of the chromatographic system, we mounted a small Megabond WR C18 column (5 cm x 4.6 mm, i.d. 10 µm) between pump and injector to delay our analytes of two minutes than those already present in the system.

The mobile phase used for the gradient consisted of a programmed mixture of solvents A (aqueous ammonium formate 20 mM), and B (Methanol). The elution started at 10% B, which increased to 40% in 4 min. Subsequently, the mobile phase B was gradually increased to 95% at the 12^{th} minute, which remained constant up to the 18^{th} minute. The initial conditions were reached at the 20^{th} minute, with an equilibration time of 7

min. The run was performed at a flowrate of 0.3 mL min^{-1}.

The detector was a Thermo Q-Exactive Plus (Thermo Scientific, San Jose, CA, USA), equipped with heated electrospray ionization (HESI) source. Capillary temperature and vaporizer temperature were set at 330 °C and 280 °C, while the electrospray voltage was set at 3.50 kV operating in negative mode. Sheath and auxiliary gas (nitrogen) were set at 35 and 15 arbitrary units, with S lens RF level of 60. Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the LC-HRMS system. The exact mass of the compounds was calculated using Qualbrowser program in Xcalibur 3.0 software. Instrument calibration was done every analytical session with a direct infusion of an LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA).

The Full scan (FS) acquisition was combined with an Independent Data Acquisition (DIA) mode, providing the MS2 spectra for a confirmatory response, based on an inclusion list.

The resolving power of FS was set at 70,000 FWHM. In consideration of molecular weight to our compound list, a scan range of m/z 200–950 was chosen; the automatic gain control (AGC) was set at 1 x 10^6 and the maximum injection time was 200 ms. The DIA segment operated in negative mode at 35,000 FWHM.

Detection of analytes was based on the retention time of target compounds, on the calculated exact mass of the deprotonated molecular ions, and at least one specific and typical fragment (Table 1). The formula of the compounds, with the exact theoretical mass of the parents and the diagnostic transition used to confirm the different PFASs are reported in Table 1. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

Table 1. The formula, exact theoretical mass of the parents, diagnostic transitions and validation parameters of the selected PFASs. The electrospray ionization (ESI) is set as negative.

Compound*	Name	Formula	Exact mass [m/z]	Transition [m/z]	LOD (pg g^{-1})	LOQ (pg g^{-1})	Recovery (%)	intra-day CV (%) (n=5)	inter-day CV (%) (n=7)
PFBA	Perfluorobutyric acid	$C_4HF_7O_2$	212.9792	168.98836	10	30	99	6	20
PFPeA	Perfluoropentanoic acid	$C_5HF_9O_2$	262.97601	218.98560	10	30	104	15	14
PFBS	Perfluorobutane sulfonate acid	$C_4F_9HO_3S$	298.94299	98.95434	5	15	119	19	20
PFHxA	Perfluorohexanoic acid	$C_6HF_{11}O_2$	312.97281	268.98288	10	30	112	11	15
PFHpA	Perfluoroheptanoic acid	$C_7HF_{13}O_2$	362.96962	318.97949	5	15	109	7	10
PFHxS	Perfluorohexane sulfonic acid	$C_6F_{13}HO_3S$	398.9366	98.95437	5	15	101	19	20
PFOA	Perfluorooctanoic acid	$C_8HF_{15}O_2$	412.96643	368.97681	8	24	114	8	11
PFNA	Perfluorononanoic acid	$C_9HF_{17}O_2$	462.96323	418.97385	20	60	110	8	11
PFOS	Perfluorooctane sulfonic acid	$C_8F_{17}HO_3S$	498.93022	79.95598	10	30	84	13	17
PFDA	Perfluorodecanoic acid	$C_{10}HF_{19}O_2$	512.96004	468.97064	28	84	87	5	9
PFUdA	Perfluoroundecanoic acid	$C_{11}HF_{21}O_2$	562.95684	518.96729	30	90	87	13	20
PFDS	Perfluorodecane sulfonic acid	$C_{10}F_{21}HO_3S$	598.92383	79.95593	50	150	81	10	15
PFDoA	Perfluorododecanoic acid	$C_{12}HF_{23}O_2$	612.95365	568.96436	5	15	80	12	20
PFTrDA	Perfluorotridecanoic acid	$C_{13}HF_{25}O_2$	662.95046	618.96094	30	90	80	8	16
PFTeDA	Perfluorotetradecanoic acid	$C_{14}HF_{27}O_2$	712.94726	668.95795	50	150	83	10	15
PFHxDA	Perfluorohexadecanoic acid	$C_{16}HF_{31}O_2$	812.94088	768.95093	50	150	80	9	13
PFODA	Perfluorooctadecanoic acid	$C_{18}HF_{35}O_2$	912.93449	868.94507	50	150	80	16	20

*= reported in alphabetic order

4.2.7 GC-MS/MS analyses

Triple quadrupole mass spectrometry (QqQ) in electronic impact (EI) mode was used for the simultaneous detection and quantification of PBDE in meat samples.

A GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) was used to confirm and quantify residues in meat samples by using a fused-silica capillary column Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane (35 m x 0.25 mm i.d., 0.25 µm film thickness, Restek, Bellefonte, PA, USA). The oven temperature program was as follows: initial temperature of 80 °C, held for 3 min, and increased to 170 °C at 10 °C min^{-1}; then, increased from 170 °C to 190 °C at 3°C min^{-1}, and raised to 240 °C at 2 °C min^{-1}, before

being ramped to 280 °C at 3 °C min^{-1} and finally from 280 °C to 310 °C at 10°C min^{-1} and held at this temperature for 5 min. The carrier gas (Helium, purity higher than 99.999%) was in constant flow mode at 1.0 mL min^{-1}. A volume of 1 µL was injected using a programmed temperature vaporiser injector (PTV) in splitless mode with a 1-min splitless period and the following inlet temperature programme: 80 °C (0.05 min), 14.5 °C s^{-1} to 200 °C (1 min) and 4.5 °C s^{-1} to 320 °C (12 min – cleaning phase). A baffle liner (2 mm × 2.75 mm × 120 mm, Siltek-deactivated; Thermo Fisher Scientific) was used. The transfer line was maintained at 270 °C and the ion source at 250 °C. The electron energy and emission current were set to 70 eV and 50 µA, respectively. The scan time was 0.3 s and the peak width of both quadrupoles was 0.7 Da full widths at half maximum. Argon was used as a collision cell gas at a pressure of 1.5 mTorr. The QqQ mass spectrometer was operated in selected reaction monitoring mode (SRM) detecting two-three transitions per analyte. Identification of PBDEs was carried out by comparing sample peak relative retention times with those obtained for standards under the same conditions and the MS/MS fragmentation spectra obtained for each compound.

The Xcalibur™ processing and instrument control software program and Trace Finder 3.0 for data analysis and reporting (Thermo Fisher Scientific) were used.

Table 2. The retention times (Tr), precursor ions (m/z), product ions (m/z), Collision Energy (V), Recovery (%), LOQ (ng g^{-1}) of investigated polybrominated diphenyl ethers (PBDE).

Compound	Name	Formula	Mass [m/z]	Tr (minute)	Precursor ion [m/z]	Product ion [m/z]	Collision energy (V)	LOQ (ng g^{-1})	Recovery (%)	Intra-day CV (%) (n=6)	inter-day CV (%) (n=6)
PBDE 28	2,4,4'-Tribromodiphenyl ether	$C_{12}H_7Br_3O$	406.9	32.35	248 **246** 408	139 **139** 246	30 30 10	0.5	88	4	8
PBDE 33	2,3',4'-Tribromodiphenyl ether	$C_{12}H_7Br_3O$	406.9	31.95	246 **248** 406	139 **139** 246	30 30 10	0.5	89	4	10
PBDE 47	2,2',4,4'-Tetrabromodiphenyl ether	$C_{12}H_6Br_4O$	485.8	38.52	326 328 **482**	217 219 **326**	30 30 20	0.5	91	3	7
PBDE 99	2,2',4,4',5-Pentabromodiphenyl ether	$C_{12}H_5Br_5O$	564.7	41.27	404 406 **563**	297 297 **404**	30 30 20	0.5	89	1	5
PBDE 100	2,2',4,4',6-Pentabromodiphenyl ether	$C_{12}H_5Br_5O$	564.7	42.01	404 406 **564**	297 297 **404**	30 30 10	0.5	90	7	10
PBDE 153	2,2',4,4',5,5'-Hexabromodiphenyl ether	$C_{12}H_4Br_6O$	643.6	43.70	482 **484** 642	324 **377** 482	30 30 20	0.5	93	3	6
PBDE 154	2,2',4,4',5,6'-Hexabromodiphenyl ether	$C_{12}H_4Br_6O$	643.6	44.91	**484** 486 644	**324** 326 484	30 30 20	0.5	92	3	2

*=The precursor ion and product ion value reported in **bold** indicates the diagnostic transition.

4.2.8 Analytical performances and method validation

The validation was carried out following the European Commission SANTE/2015 guideline (SANTE, 2015). SANTE/2015 has been superseded by SANTE/2017 (SANTE,2017). For the PFASs, the method showed high specificity, without interference signals close to the retention time of the analytes, and consequently showed a high signal-to-noise (S/N) ratio in the presence of analytes even at concentrations in the order of pg g^{-1}. Selectivity demonstrated good compliance with the relative retention times for each analyte, which in our case were within 2.5% tolerance, with an S/N ratio greater than 3 when compared with the standard solution mix, both in FS and MS2 chromatograms. Moreover, diagnostic fragments showed an ion ratio within the recommended tolerances (European Commission, 2002).

The mean recoveries for all analytes ranged between 80 and 117%, indicating the efficiency of the extraction protocol.

By searching the S/N ratio of each sample, the LOD values of 17 PFASs were from 5 pg g^{-1} to 50 pg g^{-1}, the limit of quantification (LOQ) values were from 15 pg g^{-1} to 150 pg g^{-1}.

Matrix validation curves were linear over the working range demonstrating a good fit for all analytes with an R^2 value greater than 0.99. Precision in terms of intra- and inter-day repeatability (Thompson et al., 2010) was calculated using one-way analysis of variance ANOVA, expressed as coefficients of variation (CVs), and was below 19 and 21%, respectively.

About PBDE, the selectivity of the method was evaluated by injecting extracted blank meat samples. The absence of interferences was proved by the lack of peaks with an S/N ratio higher than 3 at the retention times of the target compounds. Pork samples, previously analysed and checked for the absence of all PBDEs, were used as control samples during the optimisation and validation procedure. For the LOQ of the methods, we used the lowest validated spiked level meeting the requirements of recovery within the range of 70–120% and an RSD less than or equal to 20%, as defined by the European Commission (EC, 2002). Finally, the extraction methods were also evaluated for their repeatability, linearity and recovery. Recoveries were calculated at LOQ for all compounds. (Table 1). The repeatability as CV% was calculated by analysing six replicates at the same fortification level.

4.3 Results and discussion

4.3.1 Method validation parameters

The methods showed high specificity, without any interference close to the retention

time of each compound, and consequently a S/N ratio great than or equal to 3 in the presence of analytes was confirmed, even at the lowest detectable concentration demonstrating good selectivity. Matrix validation curves show good linearity over the working range with a good fit (R^2 greater than 0.99) for all compounds. The mean recoveries (from 80 to 119%), with the other validation parameters, are reported in Tables 1 and Table 2. The CVs % are below 19% and 21%, satisfying the criteria required by the European Commission (EC, 2002) and specified by Thompson et al., (2010). Regarding the LOD and LOQ for PFASs and for PBDEs, our satisfactory results show high method sensitivity for the selected compounds both for LC-HRMS and GC-MS/MS analyses.

4.3.2 Application to pork samples

Overall results in terms of number detected, concentration levels and distribution of contaminant residues in the pork samples investigated are summarised in Table 3.

Based on the results of 77 samples, only PFOA was detected in an Austrian sample with a concentration of 0.531 ng g^{-1}. PBDEs were detected in three out of 77 samples; only one, coming from Germany, showed the presence of all congeners analysed with the range concentration from 0.53 to 0.77 ng g^{-1}. In the other two samples, coming from Netherland and Italy, only one congener was detected, respectively PBDE 153 (0.53 ng g^{-1}) and PBDE 100 (0.62 ng g^{-1}).

Table 3. Quantification results of chemical residues from different production areas.

Production area	N° of Sample	Analyte detected		Concentration (ng g^{-1} fresh weight)
Austria	7	PFOA	(n=1)	0.53
Denmark	8	n.d.		-
France	8	n.d.		-
Germany	10	PBDE 28		0.57
		PBDE 33		0.73
		PBDE 47		0.60
		PBDE 99	(n=1)	0.74
		PBDE 100		0.77
		PBDE 153		0.70
		PBDE 154		0.53
Netherland	8	PBDE 153	(n=1)	0.53
Italy	20	PBDE 100	(n=1)	0.62
Poland	8	n.d.		-
Spain	8	n.d.		-

n.d.=Not detected

Based on our results, some consideration could be made. EU has not stated MLs for PBDEs in food, due to the risk characterization has not been defined about PBDEs in people, though recent studies have evaluated associations between PBDE concentrations in human tissues (e.g., blood, human milk) and health effects (immunological, reproductive, developmental, genotoxic and carcinogenic effects) (ATSDR, 2011). Comparing our results on literature, in this work has been found very low concentration than other study about the presence of PBDEs in pork meat coming from Spain (109 ng g^{-1}) (Bocio et al., 2003), Catalonia (32.3 ng g^{-1}) (Perellò et al., 2009); Sweden (63.6 ng kg^{-1}) (Domingo et al., 2004) and (8.074 ng g^{-1}) China (Gong et al., 2014).

Due to their lipophilicity, Törnkvist et al., 2011 have shown that the highest contributors to the total of PBDEs intake were fish (39%) and dairy products (31%),

followed by meat (17%). Vouriner et al. 2012, studied the biomagnification of PBDEs in Atlantic salmon from three areas of the Baltic Sea and they demonstrated that PBDE accumulation is dependent on both age and fat content.

Humans can be exposed to PBDEs in a wide variety of ways. The main routes of exposure are from contaminated foods, environment (air, soils) and skin contact with contaminated products. Several studies indicate that infants and toddlers have higher exposures to PBDEs compared to children or adults, due to their smaller weight and their frequent skin contact with the floor dust (ATSDR, 2011). Information on PBDE dietary intake is very scarce in the literature. It is also important to note that we analysed fresh meat, whereas preparation and different cooking methods can influence the levels of contaminants and so also consumers exposure. It has been observed that during the cooking process, PBDE losses were higher than other POPs probably to lipid remove during the process. (Perello et al.,2009). Pork is widely used in the market, mostly due to its products. In almost all the places that we have included in our paper we have distinctive derived products in which the amount of PBDEs could be increased/reduced to the industrial processing method (ATSDR, 2011). On the base on this consideration we could suppose that the human intake, on the base on our results, don't pose a risk for human beings. It reasonable defined that a risk could be present due to the long exposure to this compound.

About PFOA we could do a similar consideration. The PFOA is a class of chemical compounds that due to their chemical structure, are very stable in the environment and resistant to biodegradation and hydrolysis (ATSDR, 2009). In living organisms, perfluoroalkyls, unlike PBDEs, bind to protein albumin in blood, liver, and eggs, but do not accumulate in fat tissue. Due to their hydrolytic properties are more present in the

water environment and tend to be much present in fish than other products.

PFOS in our samples did not appear concerning, in fact, it was found to be predominant compound in fish samples (Chiesa et al., 2018; Squadrone et al., 2015; Guerranti et al., 2013), although other studies have found low concentrations in pork (15 pg g^{-1}; 0.74 ng g^{-1}) (Guerranti et al., 2013; Noorlander et al., 2011).

Table 4. The literature data on PFOA, PFOS and PBDE.

Investigated Compounds	Author	Analytical technique	Sample matrix	Producing area	Concentration or range
PFOA	Ingelido et al. 2010	HPLC-MS	Human serum	Italy	5.77 ng g^{-1} h.w.
	Noorlander et al. 2011	LC-MS/MS (ESI)	Pork	Netherland	Average 15 pg g^{-1} w.w.
	Domingo et al. 2012	UPLC-MS/MS	Meat and meat products	Spain	<300 pg g^{-1} f.w.
.	Guerranti et al. 2013	HPLC-MS/MS	Pork	Italy	n.d. <LOD w.w.
PFOS	Noorlander et al. 2011	LC-MS/MS (ESI)	Pork	Netherland	14 pg g^{-1} w.w.
	Domingo et al. 2012	UPLC-MS/MS	Meat and meat products	Spain	34 pg g^{-1} f.w.
	Guerranti et al. 2013	HPLC-MS/MS	Pork	Italy	0.74 ng g^{-1} w.w.
PBDE	Bocio et al. 2003	GC/MS	Pork and pork products	Spain	172 ng g^{-1} w.w.
	Perelló et al. 2009	HRGC/HRMS	Loin of pork	Spain	7.05 ng kg^{-1} f.w.
	Törnkvist et al. 2011	GC-MS/MS	Meat	Sweden	0.023 ng g^{-1} f.w.
	Gong et al. 2014	GC/MS	Pork	China	0.32173±0.75326 ng g^{-1} w.w.

Analytical technique:
f.w.: fresh weight
h.w.: whole weight
w.w.: wet weight
n.d.: not detected

PFOA was found at low concentration in only one sample coming from Austria (0.531 ng g^{-1}). Our results provide reasons for low concern. Based on what has been reported

in literature, our concentration looks under than other study made in Italy (less than 500 pg g^{-1}) (Guerranti et al., 2013); Belgium and Spain (55 pg g^{-1}) (Corneli et al., 2012; Ericson et al., 2008); and Norway (15 pg g^{-1}) (Haug., 2010). The Highest concentration has been found in Fat fish (1.678 pg g^{-1}) (Berger et al., 2009). EFSA Scientific Panel on Contaminants in the Food Chain recommended that more occurrence data for PFASs in food should be collected to improve the accuracy of future exposure calculations (EFSA, 2008). Subsequently, the European Commission issued the Commission Recommendation 2010/161/EU on the monitoring of PFASs in food in the Member States (EFSA, 2012). This paper gives a contribute to the knowledge of their presence in foodstuff. On the bases of these results, there is no risk for human beings, but further studies are needed to keep monitoring their presence in foodstuff, as it has been suggested by the EU.

Table 5. Intake of pork meat of European countries (g/capita/day) and fresh water fish.

Region	Pork intake: year 2000	Pork intake: year 2013	Freshwater Fish intake 2000	Freshwater Fish intake 2013
European Union	113	107	6	10
Austria	165	144	5	11
Denmark	74	68	8	2
France	103	91	9	12
Germany	145	142	6	12
Netherland	149	100	5	8
Italy	103	110	5	8
Poland	131	127	4	5
Spain	175	134	5	11

Data sources: Food Supply - Livestock and Fish Primary Equivalent, provided by Food and Agriculture Organization of the United Nations (FAO).

4.4 Conflicts of Interest Statement

No potential conflict of interest was reported by the authors.

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Chapter 5

Presence of emerging contaminants in baby food

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In this study, I contributed to the experimental work planning, the execution of practical work and analyses, and data report concerning the antibiotics and PFASs on LC-HRMS part.

The paper is reported by keeping the reference style indicated by the journal guidelines

Abstract

Food safety becomes imperative when it aims to protect infants. The objective of this study was to investigate the presence of emerging contaminants of which some act as endocrine-disruptors in baby food. Persistent organic pollutants (POPs), perfluoroalkyl substances (PFASs), parabens and antibiotics were analysed in 112 baby food of different categories (meat, fish, vegetables, fruit, cheese). As regard POPs, PFASs and antibiotics, no residues were detected, while one sample showed methyl-paraben (4.14 ng g^{-1}), whereas another three contained propylparaben (median 1.70 ng g^{-1}). Special attention must be paid on parabens metabolites, as 4-hydroxybenzoic acid, the principal parabens metabolite, was detected in all samples (median 176.7 ng g^{-1}). It may be present as a degradation product, but also, it can be released from vegetables and fruits during food processing. It is recommended to collect more data on natural vs non-natural occurrence of parabens and metabolites to evaluate the exposure of sensitive population vs ADI published by the European Food Safety Authority and European Medicines Agency.

Keywords: baby food, POPs, PFASs, antibiotics, parabens, food safety.

5.1 Introduction

Baby food is homogenised food, packaged in sterile conditions, made from fruit, vegetables, fish, meat, or combining different of these matrices, directly ready for use. An alternative to traditional baby food is organic baby food, even if it is more expensive. In general, baby foods are produced by subjecting the selected substances to a sophisticated procedure of homogenisation that makes them digestible for infants between 4–6 months and 2 years old. Infant formulas are very useful in the first

months of life, in the so-called weaning phase, when milk is gradually replaced with a practical and functional solution to ensure a complete supply of nutrients. Food safety checks are very important and challenging when the aim is to detect simultaneous residue analysis of different compounds belonging to a wide variety of different classes, in selective foodstuffs both of vegetable and animal origins (Pérez-Ortega et al. 2012), especially to protect a vulnerable and most at-risk population group, such as infants.

On the other hand, the presence of emerging contaminants and/or endocrine disruptors such as persistent organic pollutants (POPs), perfluoroalkyl substances (PFASs), parabens, human and veterinary drugs (e.g. antibiotics) has been recently reported in processed food deriving from environmental contamination and/or farming/crop practices (Chiesa et al. 2018a, 2019).

As the European Food Safety Authority (EFSA) states in its guidance on risk assessment for substances in baby food (EFSA 2017), the immune system in immediate post-natal life is particularly sensitive and exposure to immunotoxicants may result in persistent effects on the immune system that last or appear only long after exposure, but may also occur at lower doses than adult exposure. Different compounds or types of exposure may produce different severities and unpredictable alterations depending on the time of exposure during the immune system development. They may be associated with chronic immunological conditions such as immune deficiency, autoimmunity, inflammation and allergic reactions.

Although the guidance addresses specifically the risk assessment of infants less than 16 weeks of age, the matters affects infants and young children above 16 weeks of age. To ensure appropriate nutritional composition and safety of foods for infants and young children, the European Commission has defined specific rules for such foodstuffs.

The Directive 99/39/EC encompasses the specific rules on the presence of pesticide residues in processed cereal-based baby foods and baby foods and requires that this type of food contains no detectable levels of pesticide residues, meaning not more than 0.01 mg kg^{-1}, as consumed. In addition, the Directive prohibits the use of certain very toxic pesticides in the production of processed cereal-based baby foods and baby foods and establishes levels lower than the general maximum level of 0.01 mg kg^{-1} for a few other very toxic pesticides.

In addition, the Directive 2006/125/EC, indicates that cereal-based foods and baby foods must also comply with other specific provisions laid down in the relevant measures of EU law on hygiene, on the use of food additives, on the presence of contaminants and on the use of materials intended to come into contact with the products.

As well known, food is considered as a cumulative daily source of parabens and other legislation was established to ensure consumers' safety. A risk assessment of parabens was recommended by the EFSA (2004) and was set an acceptable daily intake (ADI) of 10 mg kg^{-1} body weight (bw)/day for methyl paraben (MeP) and ethyl paraben (EtP), but for a long time safety evaluations have not been defined for other parabens. In recent years, special attention has been paid to propyl-paraben (PrP) and ADI of 1.25 mg kg^{-1} bw was established just a few years ago (European Medicines Agency 2015). The levels of residues that might occur following its utilisation in veterinary products are expected to be too low to impact on industrial food processing and therefore maximum residual limits (MRL) were not setup, as was declared in EU regulations (European Commission 2015).

The question about paraben presence in processed foods is even more complicated when the parabens transformation products, namely 4-hydroxybenzoic acid (*p*-

hydroxybenzoic acid, *p*-HBA), 3,4-dihydroxybenzoic acid (protocatechuic acid, 3,4-DHB), methyl-protocatechuate (OH-MeP) and ethyl-protocatechuate (OH-EtP), are taken into consideration (Xue et al. 2015, 2017). Those (di) hydroxybenzoic acids have been recognised as metabolites of parabens and thus might serve as potential markers of parabens incidence (Wang et al. 2018; Chiesa et al. 2018e). Nevertheless, the parabens are not their unique, exclusive source: *p*-HBA and 3,4-DHB are also naturally present in many plants and vegetables (Tomás-Barberán and Clifford 2000; Kakkar and Bais 2014). Also, both *p*-HBA and 3,4-DHB appear as intermediates in several industrial processes with potential biotechnological applications in food production (Wang et al. 2018), and if not managed properly they could represent a risk for baby food, as well. Additionally, OH-MeP and OHEtP are recognised as hydroxylation products of MeP and EtP, respectively, and generally, they are produced by biotic and abiotic transformation of many xenobiotics (Xue et al. 2017). There is no available literature data about their origin, level and risk assessment in the baby food.

Salicylic acid, a structural isomer of *p*-HBA, is a compound that is naturally present in foods can cause adverse reactions to persons who are intolerant. Salicylate sensitivity is not as common as other type of food intolerance, but it should be taken into consideration especially when its quantity in baby food is concerned. Studies on the salicylic content of foods are sparse and have produced distinctly different results, giving rise to controversy (Malakar et al. 2017).

As regards veterinary drugs or other class of substances, there is not any current legislation for MRL in baby food, so a zero-tolerance policy is applied establishing that the presence of these compounds is illegal at any level (Aguilera-Luiz et al. 2012).

As regards PFASs, EFSA recommended the analysis of this class of compounds in different food items to assess a reliable risk evaluation, and this appears essential

when the highest chronic dietary exposure to perfluorooctanesulfonic acid (PFOS) was estimated for the youngest population groups (EFSA 2018b).

Therefore, in the light of these considerations, the application of these preventive policies require the development of sensitive analytical methods to determine the presence of these compounds and of their metabolites, useful as markers, at very low concentrations to protect infant health.

There are few works in literature on the multiresidue analysis of emerging contaminants and endocrine-disrupting chemicals in baby food, and those deal with single or only a few classes of compounds, as reported in Table 1, a summary table on the state of art on this topic.

In this regard, our aim was to analyse different baby food on the basis of the matrix type (meat, fish, cheese, vegetables and fruit) for the detection of POPs, PFASs, antibiotics and parabens evaluating the possible direct or indirect contamination of residues, relative to the different breeding/crop practices or environmental contamination, to evaluate infant health risk.

Table 1. Literature summary on emerging contaminants and endocrine-disrupting chemicals detected in baby food.

Antibiotics						
Reference	**Compounds**	**Baby food typologies**	**Extraction Technique**	**Detection techniques**	**LOD/LOQ CCα/CCβ (ng g^{-1})**	**Min and Max Conc. detected (Application) (ng g^{-1})**
Gentili et al. (2004)	Sulfonamides	Bovine (veal and beef), Porcine (pig and ham), Poultry meat (chicken	ASE	LC-ESI-MS/MS	LOD: 0.4 – 1.7 LOQ: 1.2 – 5.1	<LOQ- 3.5

		and turkey),				
Díaz-Alvarez (2009)	Quinolones Fluoroquinolones	Chicken meat and vegetables	ultrasound-assisted extraction + solid-phase extraction	HPLC-UV	LOD: 30-110 LOQ: 100-350	No application
Rodriguez et al. (2011)	Fluoroquinolones	Baby food purées ham, chicken, turkey, lamb, beef, sole, hake	MISPE (molecularly imprinted solid phase extraction)	LC-FLD (liquid chromatography with fluorescence detection)	CC_{α}:11-19 CC_{β}:18-32	n.d.-3
Aguilera-Luiz et al. (2012)	multiresidue veterinary drugs	meat-based baby food and powdered milk-based infant formulae	QuEChERS	UHPLC-MS/MS	CCα:0.5-16.2 CCβ:1.4-22.4	<5-25.2
Jia et al. (2014)	MULTI-RESIDUES (333 veterinary drugs and pesticides included antibiotics, OCs and OPs)	Baby food (93 including VBF, MBF, CBF, FBF and PMBIF)	QuEChERS	UHPLC-Q-Orbitrap	CCα:0.01-5.35 CCβ:0.01-9.27	1.45-22.34
Nasr et al. (2014)	Macrolides (Tylosin and josamycin)	(chicken muscles, chicken liver, bovine muscles, liver, milk and eggs) Chicken-based baby food and baby formulae	liquid–liquid extraction	MLC–monolithic method with UV	LOD: 1100 - 3000 LOQ:3600-9900	No application
Nebot et al. (2014)	Tetracyclines	Meat /vegetables	liquid–liquid extraction	HPLC-MS/MS	LOQ: 5.0	5.0-9.0
Vakh et al. (2018)	Fluoroquinolones	chicken, beef or turkey	automated magnetic dispersive micro-solid phase extraction	HPLC-FLD	LOD: 1.5- 3.0 LOQ: 5.0-10.0	No application
Persistent Organic Pollutants - POPs (PBDEs, PCBs, OCs, IPA, OPs)						
Pandelova et al. (2011)	PCBs OCs	fruits, vegetables, meat, fish,	ASE for PCBs Soxhlet extraction for OCs	HRGC/HRMS	LOD: 0.0005 LOQ: 0.0035	0.001-0.04

Jeong et al. (2014)	PBDEs	homemade baby food	Soxhlet extraction	HRGC/HRMS	LOQ: 0.0001-0.01	0.245-6.00
Jeong et al. (2014)	OCs PCBs	homemade baby food	Soxhlet extraction	HRGC/HRMS	LOD: 0.00012 - 0.0015 LOQ: 0.0004 – 0.005	0.00028-3.338
Liu et al. (2014)	PBDEs	baby food (formula, cereal, and puree)	ASE	GC/MS	-	n.d.- 0.94
Schecter et al. (2010)	PBDEs	Meat based baby food (Ham/veal, beef)	Soxhlet extraction	HRGC-HRMS GC–ECD	LOD: 0.0002-0.1	0.012-0.62
Notardonato et al. (2018)	OPs	freeze-dried products (chicken, rabbit, turkey) and soft baby foods (chicken, rabbit, sea bream, plaice)	Ultrasound–vortex-assisted DLLME (liquid–liquid microextraction)	GC–IT/MS	LOD: 0.2 - 4.7 LOQ: 2.3 – 8.5	<LOQ
Toms et al. (2016)	PBDEs OCPs PCBs	fruit-, vegetable-, meat-, fish- and dairy-based baby foods	ASE	GC/MS	LOD: 0.0001-0.0005	<LOD – 0.095
Lorán et al. (2010)	PCBs	processed cereal baby food, meat (chicken, beef and lamb), fish (sole and hake)	Soxhlet extraction	HRGC coupled to Ion Trap MS/MS	0.1- 0.5	0.03-0.29
Leandro et al. (2005)	OCs and OPs	Fruit and rice, fish and pasta, potato and pork	QuEChERS	Large volume injections LVI–GC–MS/MS LC-MS GC-MS	0.5-10	No application
Fontcuberta et al. (2008)	OCs	not specified	Liquid extraction	GC/MS	LOQ: 5 - 10	n.d
Dobrinas et al. (2011)	OCs	fruit, vegetable, meat–vegetable + fish–vegetable based purée	Soxhlet extraction	GC-ECD	-	<LOD – 304

Radford et al. (2014)	OPs	Vegetable and fruit	Solid phase extraction	HPLC-MS/MS	LOD: 0.18 – 2.7	0.08 –1.43
Al-Zahraa et al. (2016)	OCPs, OPPs	fruit-vegetables and rice cereal-based baby foods	QuEChERS	GC/MS	LOD:0.0001-0.0191	n.d.- 13.97
Santonicola et al. (2017)	PAH	Meat (chicken, turkey, calf, pig, lamb, horse) and fish (trout, flounder, salmon, hake, sea bass, gilthead bream)	Liquid extraction	HPLC-FD	LOD: 0.005 - 0.11	n.d -72.88
Perfluoroalkyl Substances - PFASs						
Ullah et al. (2012)	PFASs	Vegetables, meat, and fish	Liquid extraction +SPE C18+ SPEC8	HPLC/HRMS (qTOF)	LOD 0.0018-0.2 LOQ 0.006-0.066	n.d.-1.84
Lorenzo et al. (2016)	PFASs	meat, poultry, fish, offal, vegetables and fruit	Liquid extraction +SPE Strata X	UHPLC-MS/MS.	LOD 0.75-4.5 LOQ 3.75-15.00	0.017-5.013
Parabens						
Chiesa et al. (2018e)	methyl- (MeP), ethyl-, propyl-, butyl-, benzylparaben, 4-hydroxybenzoic acid (*p*HBA)	fish and fish products (including baby food)	simple liquid-liquid extraction	LC-HRMS	LOD 0.65-3.50 LOQ 2.15-11.70	*p*HBA 27.40-94.00

5.2 Material and methods

5.2.1 Chemicals and reagents

All solvents were purchased from Merck and water was purified by a Milli-Q system (Merck KGaA, Darmstadt, Germany). SupelTM QuE Citrate (EN) tubes and SupelTM QuE-ZSEP (EN) tubes were from Supelco (Sigma Aldrich, St. Louis, MO, USA). The Oasis HLB 3 mL, 60 mg and Oasis WAX 3 mL, 60 mg cartridges were from Waters (Milford, MA, USA). Non-dioxin like-polychlorinated biphenyls (NDLPCB) (PCB 28; −52; −101; −138; −153 and −180) [congener 209 as internal standard (IS)] and PBDEs (PBDE 28; −33; −47; −99; −100; −153 and −154) [3-fluoro-2,2,4,4,6- pentabromodiphenyl ether (FBDE) as IS] were from AccuStandard (New Haven, USA). Organochlorine pesticides (OCs) (aldrin; α-hexachlorocyclohexane (α-HCH); β-hexachlorocyclohexane (β-BHC); hexachlorobenzene (HCB); dichlorodiphenyldichloroethylene (DDE); dichlorodiphenyltrichloroethane (DDT); dichlorodiphenyldichloroethane (DDD); endosulphan I; endosulphan II; endosulphan sulphate; endrin; heptachlor; heptachlor epoxide; lindane and trans chlordane) were from Restek (Bellefonte, PA, USA). Organophosphorus pesticides (OP): chlorpyriphos diazinon, disulphoton, ethoprophos, mevinphos and phorate, and 4-nonylphenol (IS for OCs and OPs) were from Sigma-Aldrich. The four PAHs: chrysene, benz(a)anthracene, benzo(b)fluoranthene and benzo-(a)pyrene were from Restek (Bellefonte, PA, USA).

PFASs: perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorobutane sulphonic acid (PFBS), perfluoroheptanoic acid (PFHpA), PFOA, perfluorohexane sulphonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), PFOS, perfluorododecanoic acid (PFDoA), perfluoroundecanoic acid (PFUnDA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA),

perfluorohexadecanoic acid (PFHxDA), and perfluorooctadecanoic acid (PFODA) were from Chemical Research 2000 Srl (Rome, Italy) and ISs perfluoro-[1,2,3,4,5-^{13}C5]nonanoic acid (MPFNA) and perfluoro-[1,2,3,4-^{13}C4]octanesulfonic acid (MPFOS). Antimicrobial agents: amoxycillin, ampicillin, benzylpenicillin, cefquinome, ceftiofur, cefalexin, ciprofloxacin, chloramphenicol, chlortetracycline, cloxacillin, danofloxacin, dicloxacillin, dimetridazole, doxycycline, enrofloxacin, florfenicol, florfenicol amine, flumequine, furaltadone, furazolidone, lincomycin, lomefloxacin, marbofloxacin, nalidixic acid, nitrofurazone, oxolinic acid, oxytetracycline, ronidazole, spiramycin, sulphadiazine, sulphathiazole, sulfadimethoxine, sulphadimidine, sulfamerazine, tetracycline, thiamphenicol, tiamulin, tilmicosine, tinidazole, trimethoprim, tylosin and enrofloxacin-d5 (IS) were from Merck.

Parabens: MeP, EtP, propyl-(PrP), butyl-(BuP) and benzylparaben (BzP), 4-hydroxybenzoic acid (*p*HBA), 3,4-DHB, OH-MeP and OH-EtP including 4-fluorobenzoic acid (4-FB) used as IS, were from Merck (KGaA, Darmstadt, Germany).

5.2.2 Standard solutions

For stock and working solutions, kept at -20 °C, hexane was used as the solvent for GC-MS/MS and methanol for HPLC-HRMS analyses.

5.2.3 Sample collection

The total number of collected samples was 112. In detail: 45 meat (veal, swine, horse, lamb, rabbit, chicken, turkey), 13 fish (plaice, salmon, sea bream, hake, trout, bass, cod), 47 fruit (apple, pear, plum, blueberry, apricot, peach, mixed fruit) and vegetable (legumes, zucchini, carrots, potatoes, sweet potato, tomato, broccoli, peas, spinach, mixed vegetables) and 7 cheese baby food. They were from different commercial

Italian brands, present in the international market, and bought in different Italian supermarkets. Moreover, 11 samples of different matrices were bought in some supermarkets of Serbia, to extend the international scope. The sample details are specified in Table 2.

Table 2. Sample collection details according to food categories

Meat	**Fish**	**Fruit/vegetables**	**Cheese**
veal	plait	apple	cheese (bovine milk)
swine	hake	plum	
horse	plait and potatoes*	pear	
lamb	trout and vegetables*	pear and blueberry	
rabbit	bream and vegetables*	apple and blueberry	
chicken	bream and potatoes*	apple and banana	
turkey	bass and vegetables*	apple and peach	
veal and ham	cod and potatoes*	apple and apricot	
chicken and carrots*	cod and vegetables*	banana and kiwi	
chicken with green beans and zucchini*	salmon and vegetables*	mixed fruit	
veal and vegetables*		carrot and apple	
veal and carrots*		legumes	
veal and potatoes*		zucchini	
veal, broccoli and carrots*		broccoli	
veal, potatoes and mushrooms*		carrots, potatoes and zucchini	
turkey, corn and potatoes*		sweet potato and carrots	
		tomato and vegetables	
		peas and spinach	
		mixed vegetables	
Total **n=45**	**n=13**	**n=47**	**n=7**

*for mixed categories, meat and fish represented the major component as declared in the label

5.2.4 Sample treatment protocol for POPs

Two g samples were extracted by the QuEChERS protocol described in Chiesa et al. (2018a).

5.2.5 Sample treatment protocol for PFASs

Two g samples were extracted as in our previous works (Chiesa et al. 2018b).

5.2.6 Sample treatment protocol for antibiotics

One g samples were extracted as described by Chiesa et al. (2017), (2018c) and (2018d).

5.2.7 Sample treatment protocol for parabens and metabolites

The sampling procedure performed for parabens is reported by Chiesa et al. (2018e).

5.2.8 GC-MS/MS analyses for POPs and pesticides

The instrument was a GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) with electronic impact (EI) mode set in selected reaction monitoring mode (SRM). The column was a fused-silica capillary Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane (35 m x 0.25 mm i.d., 0.25 µm film thickness, Restek, Bellefonte, PA, USA). The oven temperature program and all operation parameters were the same as for our previous work (Chiesa et al. 2018a). Xcalibur software was used to control instrument and Trace Finder 3.0 for Table 1. data processing (Thermo Fisher Scientific).

5.2.9 LC-HRMS Orbitrap analyses for PFASs, antibiotics, and parabens

A Q-Exactive Orbitrap equipped with a heated electrospray ionisation source (HESI) was used. The HPLC system was a Surveyor MS quaternary pump (Thermo Fisher Scientific, San Jose, CA, USA) with a Synergi Hydro-RP reverse-phase HPLC column (150 × 2.0 mm, i.d. 4 μm) and a C18 guard column (4 × 3.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase used for PFASs was a gradient of aqueous NH_4COOH (20 mM) and MeOH; for antibiotics and parabens separation a binary mixture of aqueous HCOOH (0.1%) and MeOH was used. All the parameters are described in our previous works (Chiesa et al. 2018a, 2018d, 2018e).

For each analytical method, we combined a full scan (FS) with a data-independent acquisition (DIA), providing the MS2 spectra for confirmatory analysis.

Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA) acquired and elaborated data.

5.2.10 Validation parameters

Antibiotic validation was assessed following the Commission Decision guidelines 657/2002/CE, while for the other analytes SANTE/11813/2017 guidelines were followed. All the validation parameters are described in our previous works (Chiesa et al. 2018a, 2018d). Regarding parabens, our analytical procedure published earlier (Chiesa et al. 2018e) was followed strictly, including also the determination of validation parameters for 3,4-DHB, OH-MeP and OH-EtP that were not previously elaborated.

5.2.11 Statistical evaluation

Preliminary statistical evaluation (Shapiro-Wilk Test) revealed that data were not normally distributed. Therefore, non-parametric Kruskal-Wallis One Way analysis followed by all pairwise multiple comparison processes (Dunn's method) were used to check the differences between the medians of the datasets. Statistical analyses were performed using Sigma Stat (Statistical Analysis System, version 12.5) software (Jandel Scientific GmbH, Erkrath, Germany). A *P*-value of 0.05 was set as statistically significant.

5.3 Results and discussion

No POPs were found in samples analysed. In literature, one of the compounds detected with highest frequency were PCBs, where concentrations ranged up to 95 pg g^{-1} (Toms et al. 2016), 0.03 ng g^{-1} and 0.29 ng g^{-1} for fish and gluten-free cereals products (Lorán et al. 2010), 7.78–270 pg g^{-1} (Jeong et al. 2014) while negligible PCB levels were detected in another study, in line with our results (Table 1). Literature results showed PBDEs were found with. median concentrations at 21 pg $^{g-1}$ in United States samples and 36 pg g^{-1} in Chinese samples (Liu et al. 2014). In one study, conducted on homemade Korea samples, PBDEs were found with highest frequency in 90% of samples at concentrations from 24.5 to 6000 pg g^{-1} (Jeong et al. 2014), higher than those found in commercial formulae from the United States where median concentration were 1725 pg g^{-1} for meat samples, 283 pg g^{-1} fish, 31.5 pg g^{-1} in dairy products (Schecter et al. 2004). The lower levels were found in European products, with whom our results are in line suggesting a safety of the products. Moreover, according to European Community in 2006 (European Commission 2006), baby food should be free of pesticides residues and EFSA panel set a Maximum Residue Level of 0.01 mg kg^{-1} in food for infant, as consumed (EFSA 2018a). In one study conducted in

Spain (Fontcuberta et al. 2008), the authors observed a gradual disappearance of regulated chlorinated organic pesticides from 1989–2000 period and 2001–2006 period, suggesting that this could reflect an improvement of worldwide regulation (Fontcuberta et al. 2008). In our study, no pesticides residues were found and this reflects what has been reported in other studies (Fontcuberta et al. 2008) on the progressive lower detection of pesticides as a consequence of the improvement of industrial processes and regulation. So, on the base of our results, a growing enhancement of regulation could be linked to an improvement of product safety and therefore an absence of contaminants (EFSA 2018a).

As regard PFASs, none were detected, demonstrating that this kind of contamination in the different baby food analysed may currently not be of concern. In particular if we compare our results to the few studies present in literature, in that of Ullah et al. (2012) the detection frequency (percentage detects) for the 13 investigated PFASs was 77% in fish, 64% in meat, 49% in vegetables at concentrations below the respective minimum detectable level of 7 to 20 pg g^{-1}, and could thus only be estimated semi-quantitatively. Quantifiable concentrations of several PFASs were found in pig liver and fish and the highest level of PFOS (1.8 ng g^{-1}) was quantified in fish from The Netherlands, if compared to 13 pg g^{-1} found in those from Bangladesh. In the study of Lorenzo et al. (2016), PFBA and PFOA were detected in 100% of analysed samples with concentrations up to 5013 ng g^{-1}, followed by PFDA (83%) up to 387 ng g^{-1} and PFOS detected only in 17% of the samples and they stated they can derive from the production chain since many parts of the equipment were made of perfluoroalkylated materials.

As regard antibiotics, also in this case we found no residues in any analysed baby food. In the study of Gentili et al. (2004), among 30 analysed infant foods for sulphonamides,

one, whose formulation was based on veal meat, was positive to sulfamethizole (1.4 ng g^{-1}) and other two samples were <LOQ. In the work of Aguilera-Luiz et al. (2012) only one meat baby food sample out of 21 showed the presence of levamisole at 9.5 ng g^{-1}. In the work of Nebot et al. (2014) 31 baby food samples containing between 15% and 20% beef analysed for tetracyclines, only 3 samples showed doxycycline with concentrations between 5 and 9 ng g^{-1}, one tetracycline (5.4 ng g^{-1}) and another chlortetracycline (7.2 ng g^{-1}). In the other few works reported in Table 1, no compounds were present.

Parabens affect reproductive or endocrine endpoints at high concentrations in both male and female immature experimental animals, and with exposure, both boys and girls may be at risk of endocrine disruption. Oestrogenic effects in boys may increase the risk for incomplete masculinisation resulting in decreased sperm quality. In girls, an increased oestrogenic load may increase the risk of early puberty, and premature mammary development (Boberg et al. 2010). The great majority of samples enrolled in this study did not reveal measurable levels of parabens (Table 3), except one plum preparation that contained 4.14 ng g^{-1} of MeP and one apple, one pear and one turkey sample that contained PrP at the concentrations of 1.2, 3.4 and 1.33 ng g^{-1}, respectively. Although having such low incidence, this kind of contamination should not be underestimated as it is not clear what might be the origin of those two parabens discovered randomly in 4 out of 112 samples (<3.5%). The range of concentration detected herein corresponds to the daily intake which is 2–3 orders of magnitude (about 1000 times) below ADI recommended by European Medicines Agency (2015) which was set at 1.25 mg kg^{-1}.

Table 3. Concentration levels (ng g^{-1}) of parabens and their analogues/possible metabolites in all baby food sample analysed.

	MeP[a]	EtP	PrP	BtP	BzP	*p*-HBA	3,4-DHB	OH-MeP	OH-EtP
Positive (%)	1 (0.9%)	n.d.	3 (2.7%)	n.d.	n.d.	112 (100%)	86 (76%)	10 (8,9%)	3 (2.7%)
Mean	4.14	n.d.	1.70	n.d.	n.d.	321.7	162.2	3.7	7.5
Median	/	n.d.	1.33	n.d.	n.d.	176.6	10.1	2.1	7.3
Min	/	n.d.	1.20	n.d.	n.d.	14.4	2.1	0.8	7.2
Max	/	n.d.	3.24	n.d.	n.d.	2149	3638	14.4	8.2
Percentile 25% (Q1)	/	n.d.	1.33	n.d.	n.d.	93.9	3.3	1.1	7.2
Percentile 75% (Q3)	/	n.d.	3.24	n.d.	n.d.	455.9	52.6	4.6	8.2

[a] Refer to text (materials and methods section) for full names of the abbreviated compounds.

*n.d.= not detected

Special attention needs to be directed towards PrP because legislation concerning this compound has been rather confusing in the past and an ADI has been recommended recently (European Medicines Agency 2015). PrP is an antimicrobial preservative used in veterinary medicinal products, and it was previously classified as additive E216. As a result of EFSA's re-evaluation (2004) of parabens with E numbers E214-E219, the E classification of PrP (and its sodium salt) were successively suspended. This decision was based on the scientific data indicating that administration of PrP to male rats resulted in adverse effects on the hormonal system and male reproductive functions. It is therefore recommended to collect more occurrence data for parabens and transformation products to conduct a thorough exposure and safety assessment.

Unfortunately, the literature data regarding parabens' occurrence in processed food intended for infants' diet is rather limited, apart from the preliminary results reported by our group that concerns exclusively baby food containing fish (Chiesa et al. 2018e) where no parabens were detected.

p-HBA was found in all samples which is why results obtained here in regard to different type of infant food preparation were obtained. It is wellestablished that *p*-HBA does not exclusively derive as degradation product and potential indicator of parabens treatment, but it is also naturally present in many vegetables (Tomás-Barberan and Clifford, 2000). Indeed, when samples from four food groups were taken into consideration there were evident differences in the *p*-HBA level (Figure 1). The vegetable samples possessed an extremely high amount of *p*-HBA most probably due to the endogenous origin of *p*-HBA, with preparations based on carrot and plum showing the highest levels. However, the reason why samples that consisted of meat only, contained a substantial amount of this metabolite is uncertain (n = 36, median = 89,3, 25–75 ng g^{-1} percentile = 50.9–99.1 ng g^{-1}). One possible explanation might lay down in the fact that those samples were subjected to more elaborate technological processes (such as cooking) including the addition of water treatment that might be source of parabens, as well. Also, it remains to be defined what would be the safe levels of *p*-HBA because regardless of its origin it has been reported (independently from other parabens) to exhibit oestrogenic activity (Boberg et al. 2010). Actually, *p*-HBA is used as a flavouring additive, with no safety concern declared at current levels of intake.

Regarding 3,4-DHB, recent studies indicate its potential to act as a protective antioxidant polyphenolic compound against various diseases including neoplasms (Xie et al. 2018) while the findings about the positive correlation between its urinary concentration and childhood obesity call for caution (Xue et al. 2015). The differences between infant food based on meat or vegetables/fruit is also apparent when the amount and distribution of 3,4-DHB is concerned (Table 3, Figure 2): the median level (with 25^{th}-27^{th} percentile) in 22 meat/meat+veg samples was 3.4 ng g^{-1} (1.8–4.6 ng g^{-1})

vs 38 ng g^{-1} (5.2–177.8 ng g^{-1}) for all 46 fruit and vegetables preparations. Considerable variability within each class and limited number of fish/fish+veg samples disabled any statistical confirmation regarding the fact that fish/fish+veg samples contained notably lower levels when compared with veg/fruit samples. Cheese samples did not reveal any measurable amount of 3,4-DHB. Extremely high contents of 3,4-DHB were found in all three pure plum specimens (2148, 2471 and 3638 ng g^{-1}). A high concentration of 3,4-DHB was found in one sample that was plum-apple homogenate (943.2 ng g^{-1}). The endogenous origin of 3,4-DHB in those samples is apparent, as plum has been shown to contains a substantial amount of polyphenolic compounds, 3,4-DHB included (Kakkar and Bais 2014). The same samples contained OH-EtP and also here their natural origin as part of polyphenolic pertinence is more plausible. Random occurrence of OH-MeP in meat and vegetable also points towards its endogenous origin. On the other hand, a very important finding concerning OH-MeP is highlighted by its frequent appearance in preparations that contained fish as a main constituent: 7 of 13 fish samples showed OH-MeP presence. Considering that OH-MeP is the main hydroxylated MeP derivate in aquatic biota (Xue et al. 2017) the content of OH-MeP especially in infant food preparation based on fish (without any other ingredient) might be a reliable indicator of parabens contamination.

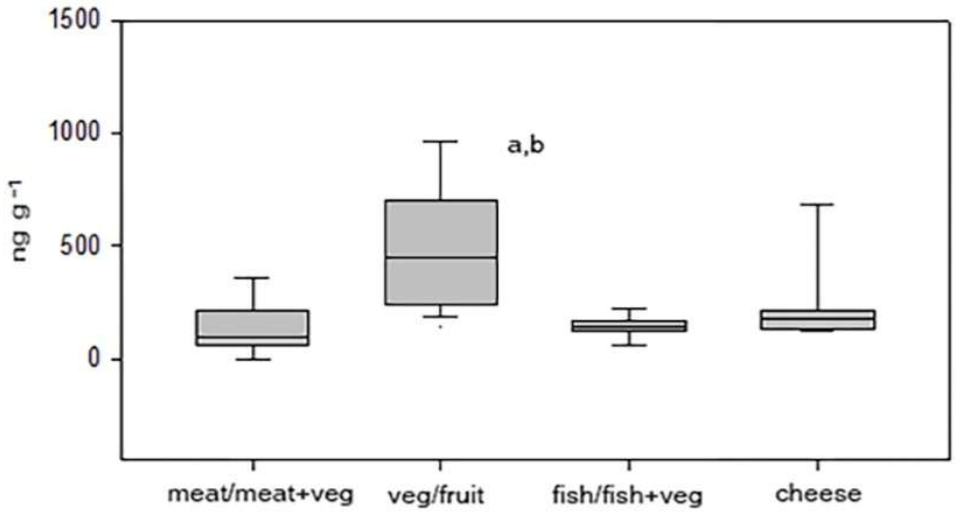

Figure 1. Distribution of *p*-HBA according to baby food category: animal, vegetable/fruit, cheese and mixed matrix for which meat or fish represented the major component as declared on the label. Data are reported as median with 25^{th}–75^{th} percentile range. Comparison was done using Kruskal-Wallis One Way Analysis of Variance on Ranks, followed by Dunn's test for pairwise multiple comparison procedures: a - stands for $p<0.001$ when meat/meat +vegetables samples were compared with vegetables/fruit preparation; b - stands for $p<0.001$ when fish/fish +vegetables samples were compared with vegetables/fruit preparation.

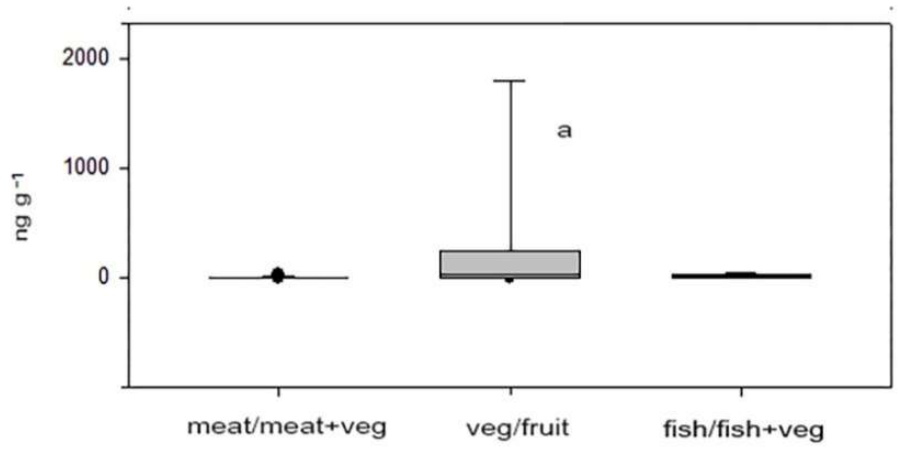

Figure 2. Distribution of 3,4-DHB of the samples where it was detected. N (meat/meat+veg) = 22; N (veg/fruit) = 46; N (fish/fish+veg) =9. Data are reported as median with 25^{th}–75^{th} percentile range. The comparison was made using Kruskal-Wallis One Way Analysis of Variance on Ranks that revealed statistical significance (a stands for $p<0.001$ vs meat/meat+veg group).

The analysis conducted for parabens confirmed the presence of salicylic acid in all infant food samples and its distribution is presented in Figure 3. This is due to the addition of ingredients rich in salicylates, such as vegetables where salicylates are naturally present in high quantities (Malakar et al. 2017). Plant salicylates have an important role against pathogens, herbivores, and abiotic stresses, mediating physiological and biochemical processes. Several studies reported the beneficial action of the salicylates on the human health, due to the antiinflammatory and antioxidative activities (Malakar et al. 2017). However, the concentration of salicylic acid is species-dependent and different plants could produce high amounts of these substances that could be a potential health risk (Cunningham 2010), especially for infants as particularly vulnerable category. In this regard, infants are a matter of concern because

some of them may have adverse reactions to even a small quantity of salicylates. Salicylates are well-known food additives and therefore an analytical strategy that would distinguish between naturally occurring and industrially introduced salicylic acid is needed for further investigation. This is especially because of increased incidence of allergic reaction to salicylate. Our data regarding the salicylic acid concentration in food items for infant diet are the first of this kind; therefore it was not possible to make a comparison with similar studies. Our results indicate the much lower content compared to fresh food items as recently was reported by Kęszycka et al. (2017). Therefore, it remains to be elucidated whether the concentration found in the samples enrolled in this study represents a safety risk for some paediatric categories and in which extend food processing influences its final quantity.

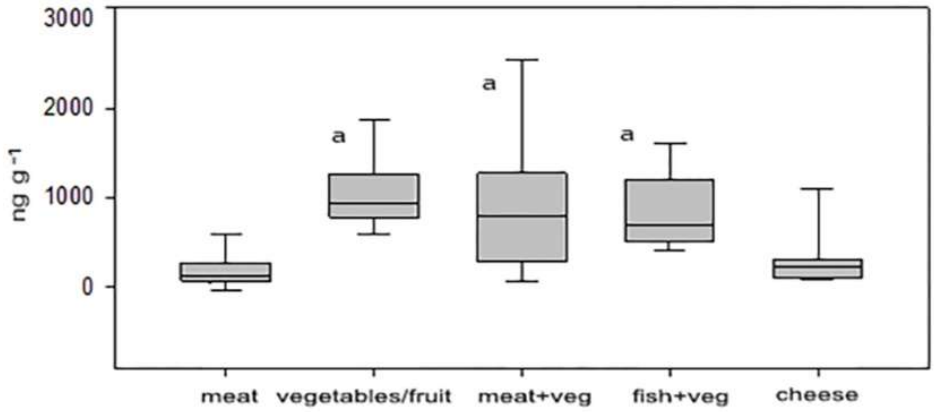

Figure 3. Distribution of salicylic acid according to baby food category: meat, vegetable/fruit, mixed meat + vegetables, mixed fish + vegetable and cheese. Data are reported as median with 25^{th}–75^{th} percentile range. The comparison was done using Kruskal-Wallis One Way Analysis of Variance on Ranks that revealed statistical significance (a stands for $p<0.001$ vs meat group)

5.4 Conclusions

POPs, PFASs or antibiotics were not detected and all samples were compliant with European legislation. Confirmation of negative data is also important, particularly for the indications and needs dictated by EFSA and other competent authorities in expanding a database on residual analyses of emerging contaminants in different types of food for a reliable risk assessment. On the other hand, some parabens and their metabolites, which are classified as endocrine disruptors, were detected at trace levels and significantly below the ADI recommended by EFSA and the European Medicines Agency. This study shows the importance of collecting more data on the occurrence of parabens and transformation products to assess exposure and possible health impact for sensitive populations such as infants and young children.

5.5 Disclosure statement

No potential conflict of interest was reported by the authors.

5.6 Funding

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Chapter 6

Levels of antibiotics and environmental contaminants in veal from Belgium, Italy, and the Netherlands in 2018

(manuscript in preparation)

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In this study, I contributed to the experimental work planning, the execution of practical work and analysis of samples, data processing and writing of the article.

Abstract

In the dairy cow industry, many calves are arranged to produce veal meats. In the case of the calves are not fully mature; they are weak for infectious diseases. On the other hand, the diet of calves is usually milk, fibrous feed, or grass. Calves can ingest environmental pollutants from the diet, then eventually distribute into the human food supply chain. This study invested 108 veal samples acquired from the local supermarket in Milan, of which three from Belgium, 53 from Italy, and 52 from the Netherlands. We performed the analysis for antibiotics, perfluoroalkyl substances (PFASs), and polar pesticides through the liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) and the analysis for persistent organic pollutants (POPs) through gas chromatography-mass spectrometry (GC-MS/MS). No traces of antibiotics, PFASs, POPs, and polar pesticides were detected in our sample. The results suggest the veal samples do not contain the residues of antibiotics and environmental pollutants.

Keyword: GC-MS/MS, LC-HRMS, antibiotics, POPs, veal

6.1 Introduction

Veal is the meat from calves which the age is usually less than one year. In 2017, the veal shared 13.1% of bovine meat production in the EU. The most productive countries are Spain (25.1%), the Netherlands (23%), France (19.3%) and Italy (10.2%) (EUSTAT, 2017). Due to the calves are still young and required transportations on long trips, the antimicrobial drugs are often used for prevention or control for the disease (Pardon et al., 2012; Lava et al., 2016). In a Belgium study, the season of the year and the veal

company are the significant factors which affect the use of antimicrobial drugs (Bokma et al., 2019).

About the origin of environmental contaminants in veal or beef, it mainly comes from industrially produced animal feed. In 1999, it happened the dioxin-contaminated feed in Belgium, then in 2008, dioxin-contaminated pork happened in Irland. A study in the literature discovered that in the PCB contaminated area of Brescia, Italy, food consumers and plant workers had higher levels in serum than all other groups (Turrio-Baldassarri et al., 2008). Another study invested polled cattle samples from the city Brescia had contamination levels 75-103 pg WHO-TE/g fat (La Rocca et al., 2004). The competent authority and the animal feed industry, therefore, applied stringent control to prevent risks of contamination in feed. However, in recent decades, more and more dioxin or dioxin-like PCB concentrations were found from free ranched chicken, sheep, and beef, which the source of contamination is unknown. The calves may intake more pollutant substances than adult cattle. In this chapter, we performed the investigation on the presence of antibiotics, environment pollutants (PFASs, POPs, and polar pesticides) through LC-HRMS and GC-MS/MS in veal samples from Belgium, Italy, and the Netherlands to give the monitoring result for the data gap of these compounds in literature.

6.2 Materials and methods

6.2.1 Materials

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid (98–100%) was obtained from Riedel-de Haën (Sigma-Aldrich, St. Louis, MO, USA). Trichloroacetic acid (TCA) powder and the ingredients for the EDTA-McIlvaine extraction buffer solution, pH 4 (disodium

hydrogen phosphate dihydrate, citric acid monohydrate and EDTA) purchased from Fluka. Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The Solid-phase extraction cartridges (Oasis HLB 3 ml, 60 mg) purchased from Waters (Milford, MA, USA). The standard reference Amoxicillin, Ampicillin, Benzylpenicillin, Cefalexin, Cefquinome, Ceftiofur, Chloramphenicol, Chlortetracycline, Ciprofloxacin, Danofloxacin, Dimetridazole, Doxycycline, Enrofloxacin, Florfenicol, Florfenicol amine, Flumequine, Furaltadone, Furazolidone, Lincomycin, Lomefloxacin, Marbofloxacin, Nalidixic acid, Nitrofurazone, Oxolinic acid, Oxytetracycline, Ronidazole, Spyramicin, Sulfadiazine, Sulfadimethoxine, Sulfadimidine, Sulfamerazine, Sulfathiazole, Tetracycline, Thiamphenicol, Tiamulin, Tilmicosine, Tinidazole, Trimethoprim, Tylosin, and Enrofloxacin D5 as the internal standards (IS) were purchased from Merck. Supel^{TM} QuE Citrate (EN) tubes and Supel^{TM} QuE-ZSEP (EN) tubes were from Supelco (Sigma Aldrich, St.Louis, MO, USA). The Oasis HLB 3 mL, 60 mg and Oasis WAX 3 mL, 60 mg cartridges were from Waters (Milford, MA, USA). A mixture of PCB congeners (PCB 28; -52; -101; -138; -153 and -180), PCB 209 (internal standard [IS] for PCBs), a mixed solution of PBDEs (PBDE 28; -33; -47; -99; -100; -153 and -154) (numbered according to IUPAC) , and fluoro-bromodiphenyl ether (FBDE), IS for flame retardants, were purchased from AccuStandard (New Haven, USA). A standard solution of 15 organochlorine pesticides (OCs) and their metabolites (α- HCH, Aldrin, β – BHC, Dichlorodiphenyldichloroethane (DDD), Dichlorodiphenyldichloroethylene (DDE), Dichlorodiphenyltrichloroethane (DDT), Endrin, Endosulfan I, Endosulfan II, Endosulfan sulfate, Heptachlor, Hexachlorobenzene, Heptachlor epoxide, Lindane, Trans Chlordane), and a standard solution of four PAH congeners (Benz(a)anthracene, Benzo(b)fluoranthene, Benzo(a)pyrene, and Chrysene) were purchased from Restek (Bellefonte, PA, USA).

Organophosphorous pesticides (OP): chlorpyriphos diazinon, disulfoton, ethoprophos, mevinphos and phorate, and 4-nonylphenol (IS for PAHs, OCs and OPs), seventeen acid and sulfonate perfluorinated compounds and ISs as we previously described in Chapter 4 (Chiesa et al., 2018c), were purchased from Sigma-Aldrich. Triphenylphosphate (TPP, as the internal standard) were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). The 40 polar pesticides listed in Table 2 were purchased from Sigma-Aldrich.

6.2.2 Sample collection

The veal samples were collected from local supermarkets of Milan. The date of slaughtering is from May 2018 to May 2019. The calves were up to 8 months old. One hundred eight veal samples were collected, of which three from Belgium, 53 from Italy, and 52 from the Netherlands. The veal samples were homogenised and stored by -20 °C refrigerator. The homogenised samples were defrosted before analysis.

6.2.3 Sample extraction for antibiotics

Put 1 g of homogenised veal muscle into a 15 mL PP centrifuge tube. Add with IS at a final 5 ng g^{-1}. Mix with 5 mL of McIlvaine buffer, 100 µL of 20%TCA. The samples were vortexed and then put into the ultrasonication water bath for 10 minutes. After having centrifugated (at 4,612×g, 4 °C, for 10 minutes), the supernatant was transferred to a new centrifuge tube and added with 3 mL of hexane. Vortex and centrifugate the sample under the same condition above. Discard the upper layer and repeated with 3 mL of hexane once again. Discard the hexane liquid after having vortexed and centrifugated under the same condition. The obtained liquid was then purified by SPE Oasis HLB cartridges, which were preconditioned the cartridge with 3 mL methanol

and 3 mL Milli-Q water. Load the sample into the cartridge and then control the flow under vacuum. Wash the cartridge two times with 3 mL methanol:water (5:95 v/v). Elute the cartridge with 5 mL methanol. Use the rotary vacuum evaporator to evaporate the eluent. Resuspend the dried extracts by 200 µL methanol:water (10:90 v/v) in 0.1% formic acid. Transfer to an autosampler vial, then analyse with LC-HRMS. Set 10 µL as the injection volume.

6.2.4 Sample extraction for PFASs

One g of samples were extracted as in our previous built method in Chapter 4 (in the session **4.2.4 Sample extraction of PFASs**, Chiesa et al., 2018c).

6.2.5 Sample extraction for POPs and polar pesticides

Two g samples were extracted based on the QuEChERS protocol described in previous works (Chiesa et al., 2018a).

The extraction of PCBs, PBDEs, OCPs, PAHs, and polar pesticides was performed using the QuEChERS approach. Two g of homogenised sample was transferred to a QuEChERS extraction tube, then the four ISs (TPP, PCB 209, FBDE, and 4-nonylphenol) were added. 20 mL acetonitrile was added as extraction solvent; the tube was shaken vigorously then followed with a vortex for 1 min and centrifuged for 10 min at 4,612×g at 4°C. The supernatant was transferred to a Z-sep cleanup tube, shaken and centrifuged for 10 min at 4,612×g at 4°C. The extract was divided into two aliquots, and each aliquot was transferred in a flask and evaporated under vacuum in a centrifugal evaporator at 35°C. The residue of one of the aliquot was dissolved in 1 mL of hexane and analysed by GC/MS-MS; another was resuspended with 200 µL of methanol:ammonium formate 20mM (10:90 v/v) and analysed with LC-HRMS.

6.2.6 LC-HRMS Orbitrap analyses for antibiotics, polar pesticides, and PFASs

All the parameters are described in our previous works (Chiesa et al., 2018b and 2018c). Detection of analytes was based on the retention time of target compounds, on the exact calculated mass of the deprotonated molecular ions, and at least one specific and typical fragment (Table 1). The formula of compounds, with the exact theoretical mass of the parents and the diagnostic transition used to confirm the different antibiotics (reported in Table 1), polar pesticide (Table 2) and PFASs. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

Table 1. The formula, exact theoretical mass of the parents, and diagnostic transitions of selected antibiotics.

Compound name (39 compounds)	Formula	Exact mass [m/z]	Transition [m/z]	ESI mode +/-
Amoxicillin	$C_{16}H_{19}N_3O_5S$	366.11182	114.00109	+
Ampicillin	$C_{16}H_{19}N_3O_4S$	350.11690	106.06545	+
Benzylpenicillin	$C_{16}H_{18}N_2O_4S$	335.10600	176.06030	+
Cefalexin	$C_{16}H_{17}N_3O_4S$	348.10125	158.02704	+
Cefquinome	$C_{23}H_{24}N_6O_5S_2$	529.13224	134.09634	+
Ceftiofur	$C_{19}H_{17}N_5O_7S_3$	524.03629	126.01212	+
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	321.00505	257.03409	+
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	479.12157	444.08377	-
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	332.14050	288.15005	+
Danofloxacin	$C_{19}H_{20}FN_3O_3$	358.15615	314.16579	+
Dimetridazole	$C_5H_7N_3O_2$	142.06110	112.06335	+
Doxycycline	$C_{22}H_{24}N_2O_8$	445.16054	410.12305	+
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	360.17180	316.18188	+
Florfenicol	$C_{12}H_{14}Cl_2FNO_4S$	355.99319	185.02769	+
Florfenicol amine	$C_{10}H_{14}FNO_3S$	248.07512	130.06515	-
Flumequine	$C_{14}H_{12}FNO_3$	262.0874	244.07686	+
Furaltadone	$C_{13}H_{16}N_4O_6$	325.11426	100.07608	+
Furazolidone	$C_8H_7N_3O_5$	226.04585	95.03703	+
Lincomycin	$C_{18}H_{34}N_2O_6S$	407.22103	126.12775	+
Lomefloxacin	$C_{17}H_{19}F_2N_3O_3$	352.14672	265.11438	+
Marbofloxacin	$C_{17}H_{19}FN_4O_4$	363.14631	320.10410	+
Nalidixic acid	$C_{12}H_{12}N_2O_3$	233.09207	205.06041	+

Nitrofurazone	$C_6H_6N_4O_4$	199.04618	152.96921	+
Oxolinic acid	$C_{13}H_{11}NO_5$	262.07100	244.06044	+
Oxytetracycline	$C_{22}H_{24}N_2O_9$	461.15546	426.11816	+
Ronidazole	$C_6H_8N_4O_4$	201.06183	140.04529	+
Spyramicin	$C_{43}H_{74}N_2O_{14}$	422.26428	174.11231	+
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	251.05972	156.01120	+
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	311.08085	156.07666	+
Sulfadimidine	$C_{12}H_{14}N_4O_2S$	279.09102	149.02325	+
Sulfamerazine	$C_{11}H_{12}N_4O_2S$	265.07537	156.01135	+
Sulfathiazole	$C_9H_9N_3O_2S_2$	256.02089	156.01120	+
Tetracycline	$C_{22}H_{24}N_2O_8$	445.16054	410.12305	+
Thiamphenicol	$C_{12}H_{15}Cl_2NO_5S$	353.99752	185.02805	+
Tiamulin	$C_{28}H_{47}NO_4S$	494.32986	192.10501	-
Tilmicosine	$C_{46}H_{80}N_2O_{13}$	435.2903	174.11232	+
Tinidazole	$C_8H_{13}N_3O_4S$	248.06995	121.03193	+
Trimethoprim	$C_{14}H_{18}N_4O_3$	291.14517	245.10294	+
Tylosin	$C_{46}H_{77}NO_{17}$	916.52643	174.11229	+

Table 2. The formula, exact theoretical mass of the parents, and diagnostic transitions of the selected polar pesticides.

Compound name (40 compounds)	Formula	Exact mass [m/z]	Transition [m/z]	ESI mode +/-
Atrazin	$C_8H_{14}ClN_5$	216.10105	174.05385	+
Azinphos-ethyl	$C_{12}H_{16}N_3O_3PS_2$	346.04435	114.96143	+
Azinphos-methyl	$C_{10}H_{12}N_3O_3PS_2$	318.01305	142.99245	+
Azoxystrobin	$C_{22}H_{17}N_3O_5$	404.1241	372.09729	+
Benalaxyl	$C_{20}H_{23}NO_3$	326.17507	148.11185	+
Bitertanol	$C_{20}H_{23}N_3O_2$	338.1863	70.04069	+
bupirimate	$C_{13}H_{24}N_4O_3S$	317.16419	108.01172	+
Buprofezin	$C_{16}H_{23}N_3OS$	306.16346	201.10551	+
Cadusafos	$C_{10}H_{23}O_2PS_2$	271.09498	158.96980	+
Chlorfenvinphos	$C_{12}H_{14}Cl_3O_4P$	358.97681	155.04663	+
Cyproconazol	$C_{15}H_{18}ClN_3O$	292.12112	70.04073	+
Cyprodinil	$C_{14}H_{15}N_3$	226.13387	108.08103	+
Diazinon	$C_{12}H_{21}N_2O_3PS$	305.10833	169.07928	+
Ethoprophos	$C_8H_{19}O_2PS_2$	243.06368	130.93852	+
Ethoxyquin	$C_{14}H_{19}NO$	218.15394	190.12244	+
Fenamiphos	$C_{13}H_{22}NO_3PS$	304.11308	217.00816	+
Fenarimol	$C_{17}H_{12}Cl_2N_2O$	331.03994	81.04534	+
Fludioxonil	$C_{12}H_6F_2N_2O_2$	266.07356	227.04482	+
Flusilazole	$C_{16}H_{15}F_2N_3Si$	316.10761	165.06987	+
Furalaxyl	$C_{17}H_{19}NO_4$	302.13868	95.01640	+
Kresoxim-methyl	$C_{18}H_{19}NO_4$	314.13868	222.09219	+

Malathion	$C_{10}H_{19}O_6PS_2$	331.04334	99.00809	+
Metalaxyl	$C_{15}H_{21}NO_4$	280.15433	220.13306	+
Methidathion	$C_6H_{11}N_2O_4PS_3$	302.96913	145.00656	+
Oxadixyl	$C_{14}H_{18}N_2O_4$	279.13393	219.11262	+
Paraoxon-methyl	$C_8H_{10}NO_6P$	248.03185	234.02864	+
Phosalone	$C_{12}H_{15}ClNO_4PS_2$	367.99414	182.00029	+
Piperonyl butoxide	$C_{19}H_30O_5$	356.24315	177.09122	+
Pirimicarb	$C_{11}H_{18}N_4O_2$	239.15025	72.04513	+
Pirimiphos-ethyl	$C_{13}H_{24}N_3O_3PS$	334.13488	198.1058	+
Pirimiphos-methyl	$C_{11}H_{20}N_3O_3PS$	306.10358	108.05595	+
Profenophos	$C_{11}H_{15}BrClO_3PS$	372.94242	344.91083	+
Propachlor	$C_{11}H_{14}ClNO$	212.08367	170.03662	+
Propargite	$C_{19}H_{26}O_4S$	368.18901	231.17419	+
Pyrazophos	$C_{14}H_{20}N_3O_5PS$	374.0934	194.55950	+
Quinalphos	$C_{12}H_{15}N_2O_3PS$	299.06138	147.05527	+
Simazine	$C_7H_{12}ClN_5$	202.0854	132.03226	+
Tetrachlorvinphos	$C_{10}H_9Cl_4O_4P$	364.90653	127.01553	+
Tetraconazole	$C_{13}H_{11}Cl_2F_4N_3O$	372.02881	91.05791	+
Triazophos	$C_{12}H_{16}N_3O_3PS$	314.07228	162.06616	+

6.2.7 GC-MS/MS analyses for POPs and pesticides

The oven temperature program and all operation parameters were the same as our previous work (Chiesa et al., 2018a). All the parameters are described in our previous works (Chiesa et al., 2018a, 2018d and 2018e).

Table 3. The formula, exact theoretical mass of the parents, and diagnostic transitions of the selected POPs and pesticides.

Compound name (38 compounds)	Formula	Retention time (min)	Precursor ion [m/z]	Transition ion [m/z]	Collision energy (V)
α HCH	$C_6H_6Cl_6$	17.83	180.9	145	10
β BHC	$C_6H_6Cl_6$	19.35	180.9	145	10
Aldrin	$C_{12}H_8Cl_6$	23.83	260.9	191	30
Anthracene	$C_{14}H_{10}$	37.77	226.1	224.1	10
Benzofluoranthene	$C_{18}H_{10}$	42.02	252.1	250.1	30
Benzopyrene	$C_{20}H_{12}$	42.02	252.1	250.16	30
Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	24.33	278	109.1	20
Chrysene	$C_{18}H_{12}$	37.76	228.1	226.2	30
o,p'-DDT	$C_{14}H_9Cl_5$	33.06	235	165.1	20
p,p'-DDD	$C_{14}H_{10}Cl_4$	32.53	235	165.1	20

p,p'-DDE	$C_{14}H_8Cl_4$	33.06	246	176.1	30
p,p'-DDT	$C_{14}H_9Cl_5$	34.22	235	165.1	20
Diazinon	$C_{12}H_{21}N_2O_3PS$	19.00	304.1	179.2	10
Disulfoton	$C_8H_{19}O_2PS_3$	19.64	142	81	10
Endosulfan I	$C_9H_6Cl_6O_3S$	28.53	372.8	265.9	20
Endosulfan II	$C_9H_6Cl_6O_3S$	28.54	240.9	205.9	10
Endrin	$C_{12}H_8Cl_6O$	31.31	262.9	193	30
Ethoprophos	$C_8H_{19}O_2PS_2$	15.83	158	97	20
Heptachlor	$C_{10}H_5Cl_7$	22.21	271.8	236.9	10
Heptachlor epoxide	$C_{10}H_5Cl_7O$	26.39	352.9	262.9	10
Hexachlorobenzene	C_6Cl_6	18.13	283.8	248.9	20
Lindane	$C_6H_6Cl_6$	21.03	219	183	10
Mevinphos	$C_7H_{13}O_6P$	12.33	127	109	10
PBDE 28	$C_{12}H_7Br_3O$	32.39	246	139	30
PBDE 33	$C_{12}H_7Br_3O$	31.98	247.9	139	30
PBDE 47	$C_{12}H_6Br_4O$	38.33	483.7	325.9	20
PBDE 99	$C_{12}H_5Br_5O$	40.90	563.6	403.8	20
PBDE 100	$C_{12}H_5Br_5O$	41.60	563.6	403.8	10
PBDE 153	$C_{12}H_4Br_6O$	43.13	483.7	376.8	30
PBDE 154	$C_{12}H_4Br_6O$	44.20	483.7	323.8	30
PCB 28	$C_{12}H_7Cl_3$	22.09	256	186	20
PCB 52	$C_{12}H_6Cl_4$	23.54	291.8	222	25
PCB 101	$C_{12}H_5Cl_5$	28.32	325.8	255.9	25
PCB 138	$C_{12}H_4Cl_6$	33.23	359.8	289.9	25
PCB 153	$C_{12}H_4Cl_6$	34.82	359.8	289.9	25
PCB 180	$C_{12}H_3Cl_7$	38.01	393.8	323.8	25
Phorate	$C_7H_{17}O_2PS_3$	17.07	121.1	65	10
Trans chlordane	$C_{10}H_6Cl_8$	28.29	372.8	265.9	20

Figure 1 demonstrated the chromatograph in GC-MS/MS of Chrysene, PBDE 28, and PCB 28.

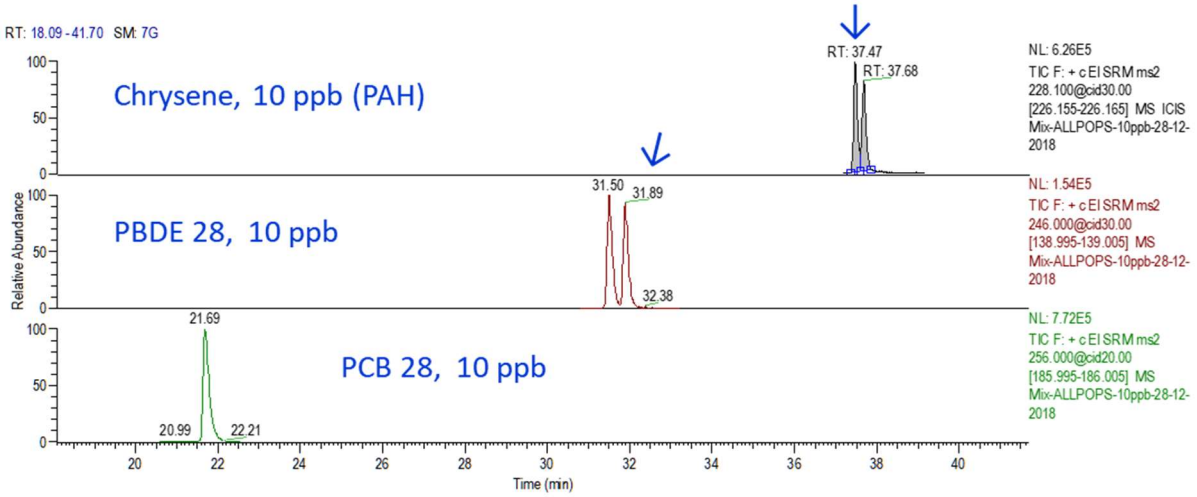

Figure 1. Chromatograph of standard reference of Chrysene, PBDE 28, and PCB 28.

6.3 Results and discussion

No antibiotics, PFASs, POPs, and polar pesticides were found in the veal samples we have analysed (Table 3).

Table 3. The results of veal samples

Country of origin	N° of Sample	Analysis			
		Antibiotics (39 compounds)	PFASs (17 compounds)	POPs (38 compounds)	Polar pesticide (40 compounds)
Belgium	3	n.d.	n.d.	n.d.	n.d.
Italy	53	n.d.	n.d.	n.d.	n.d.
Netherlands	52	n.d.	n.d.	n.d.	n.d.
Total samples	108				

n.d.=Not detected

The collection of veal was correlated with the distribution network of local supermarkets. Ten veal samples from Brescia and Italy are also analysed. No traces of PCB were detected from the Brescia veal.

In the RASFF system, there have been seven notifications on antibiotic residues. The chlortetracycline residue (358 ng g^{-1}) was reported in Italy veal, where the MRL is

100ng g^{-1} (RASFF portal, 2012). The sulfadimethoxine residue (318 ng g^{-1}) was reported in veal of Italy, where the MRL is 100 ng g^{-1} (RASFF portal, 2011). The sulfadimidine residue (greater than 200 ng g^{-1}) was reported in veal of Belgium, where the MRL is 100ng g^{-1} (RASFF portal, 2013). The doxycycline residue was reported in 120 ng g^{-1} in Belgium veal, where the MRL was 100 ng g^{-1} (RASFF portal, 2017). The tilmicosin and lincomycin-spectinomycin residue were reported in Italy, but the concentrations were not disclosed, where the MRLs are 50 ng g^{-1}, 100 ng g^{-1}, and 300 ng g^{-1}, respectively (RASFF portal, 2017). The prohibited furazolidone residue was reported in 41 ng g^{-1} in Netherlands veal (RASFF portal, 2014). Another prohibited furaltadone residue was reported in 4.92 ng g^{-1} in Italy veal (RASFF portal, 2015). Besides, no PFASs, POPs and pesticides notifications in the RASFF portal system. The RASFF data showed the farm managers failed to control the use and the withdrawal period of veterinary drugs resulted in the residue in the veal meat. As a result, the veal meat is free of environmental contaminants, but future monitoring studies should be kept on in order to reflect background levels of contaminations. Furthermore, under the Regulation (EU) 2019/1021 each Member State shall continuously report EU the presence of POPs in the environment.

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Chapter 7

Preliminary evaluation results of the extraction methods for Fipronil and its metabolites and Amitraz in chicken eggs.

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In this study, I contributed to the experimental work planning, the execution of preliminary trials and analyses, data processing and writing of the article.

Abstract

Fipronil and Amitraz are broadly used insecticides for the treatment or prevention for animal health, indoor pest control, and commercial crop protection. As the use of Fipronil or Amitraz on food-producing animals was not allowed by the EU legislation, the Maximum Residue Limit (MRL) values of Fipronil and Amitraz were set at the detection limit of 5 ng mL^{-1} and 10 ng mL^{-1}, respectively. According to the database of Rapid alert system for food and feed (RASFF), after the Belgian authority reported Fipronil residues in chicken eggs in 2017, there were 719 follow-up reports from 34 countries. Fipronil and Amitraz are included in the Italian National Residue Program, so it is necessary to develop a selective, sensitive, specific and rapid method. Three extraction methods were evaluated on fresh egg blank samples to determine the presence of Fipronil, as well as its metabolites and Amitraz. In the solvent-salt method the sample was added by water, NaCl and formic acetonitrile, followed by hexane to remove potential fat. In the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method, the sample was extracted by Superl® Que Citrate powder and acetonitrile, followed by Superl® PSA powder. In the water-associated QuEChERS method, the sample was mixed with water and acetonitrile, followed by Superl® Que Citrate powder; then the supernatant was collected and mixed with CaCl2. The analyses of the extracts were performed with high-performance liquid chromatography coupled to Q-Exactive Orbitrap high-resolution mass spectrometer (LC-HRMS). Furthermore, Thompson (2000) mentioned that the Coefficient of variation (CV) is acceptable if it is lower than 22%. Based on the obtained recovery values (72 to 113%) and CV (1.67 to 14.69%), the water-associated QuEChERS method was selected because the recoveries rates obtained with the other methods were lower than 70%.

Calibration curves exhibited correlation values ranging from 0.9653 to 0.9999(Figure

1); the limits of detection ranged from 0.08 to 1.21 ng mL^{-1}, and the limits of quantification were from 0.28 to 4.04 ng mL^{-1}. The preliminary results fulfilled the European criteria for the validation of the analytical methods.

Keywords: Fipronil, Amitraz, QuEChERS, LC-HRMS

7.1 Introduction

The fipronil can block the Gamma-aminobutyrate (GABA) receptor of the central nervous system of insects (Poppenga et al., 2010). The fipronil was firstly registered for commercial use by the United States Environmental Protection Agency (US EPA) in May 1996. However, several studies suggested fipronil has potential to reproductive efficiency and thyroid gland tumour in the rat (Dalsenter et al., 1997, US EPA, 1997, and Hurley et al., 1998). About the amitraz, it kills insects by blocking monoamine oxidase of CNS (Gupta, 2007). In 1986, the US EPA first registered amitraz as commercial use for controlling ticks on cattle and lice on hogs. In another study, the amitraz involved gross dysmorphology during the pregnancy in rats (Lazarini et al., 2001). A combination of fipronil and amitraz can significantly remove ticks from dogs (Prullage et al., 2011).

In recent years, some works of literature reported the detection of fipronil and its metabolites and amitraz through liquid chromatography or gas chromatography coupled with mass spectrometry. The extraction methods and limits of detection had listed in Table 1. In this study, we compare and evaluate from three extraction methods and use the best one to develop multiple detection methods for identifying and quantify fipronil, and its metabolites, and amitraz in chicken eggs.

Table 1. The literature data on Fipronil and its metabolites and Amitraz

Reference	Compounds	Sample matrix	Extraction Technique	Detection techniques	LOD/LOQ CCα/CCβ (ng g^{-1})	Min and Max Conc. detected (Application) (ng g^{-1})
Duhan et al., 2015	Fipronil, fipronil desulfinyl, fipronil sulfide, fipronil sulfone	Cauliflower crop	QuEChERS	GC-MS/MS	LOD: 1 LOQ: 3	No application
Kaur et al., 2015	Fipronil, fipronil desulfinyl, fipronil sulfide, fipronil sulfone	brinjal, cabbage, capsicum, cauliflower, okra, tomato	QuEChERS	GC-MS	LOD: 0.01 LOQ: 0.003	No application
Shen et al., 2017	Fipronil, fipronil desulfinyl, fipronil sulfide, fipronil sulfone	Eggs and egg products	QuEChERS	GC-NCI-MS	LOQ: 0.1	No application
Biswas et al., 2019	Fipronil, fipronil desulfinyl, fipronil sulfone	sugarcane	QuEChERS	GC-MS/MS	LOD: 1.5-2 LOQ: 5	No application
Zhang et al., 2016	Fipronil	Chicken egg, muscle	$MgSO_4$+NaCl	LC-MS/MS	CCα:0.002 CCβ:0.01	0.24
Kiljanek et al., 2016	Fipronil, Fipronil carboxamide, Fipronil-desulfinyl, Fipronil-sulfide, Fipronil-sulfone	honeybees	QuEChERS	LC-MS/MS	LOQ: 1-5	1.8-433
Wu et al., 2017	Fipronil, fipronil desulfinyl, fipronil sulfide, fipronil sulfone	Cotton	QuEChERS	UPLC-MS/MS	LOQ: 0.005-0.01	No application
Zheng et al., 2018	Fipronil, amitraz	honey	QuEChERS	LC–MS/MS	LOD: 0.0004 LOQ: 0.001	No application
Chen et al., 2018	Fipronil, fipronil desulfinyl, fipronil sulfide, fipronil sulfone	tea , chrysanthemum	QuEChERS	UPLC Q-Exactive Orbitrap	LOQ: 2	6.6-600
Chou et al., 2018	Fipronil, fipronil desulfinyl, fipronil sulfide, fipronil sulfone	feeds	QuEChERS	UHPLC-MS/MS	LOD: 0.05 LOQ: 0.2	No application
Zhang et al., 2018	Fipronil, fipronil desulfinyl, fipronil sulfide, fipronil sulfone	Eggs	Solvent and salt	UPLC-MS/MS	LOD: 0.01-0.43	No application
Guo et al., 2018	Fipronil, fipronil desulfinyl, fipronil sulfide, fipronil sulfone	Chicken egg, muscle, cake	QuEChERS	LC-MS/MS	LOD: 0.1 LOQ: 0.2	0.005-4.1
Song et al., 2019	Fipronil, fipronil sulfone	eggs	QuEChERS	LC-MS/MS GC-MS/MS	LOD: 1 LOQ: 4	No application

7.2 Material and Methods

7.2.1 Chemical and reagents

Fipronil, fipronil-sulfone, fipronil-sulfide, fipronil-desulfinyl, Amitraz, Triphenylphosphate (TPP, as the internal standard) were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). The hydroxyl fipronil kindly provided from the University of California Davis, Davis, United States. QuEChERS materials for the extraction were obtained from Supelco (Sigma Aldrich, St. Louis, MO, USA); Supel^{TM} QuE Citrate (EN) tubes, containing Sodium Citrate tribasic dihydrate and Sodium Citrate dibasic sesquihydrate.

7.2.2 Standard solutions

Each standard, the stock solution was prepared (1 mg g^{-1}) in methanol and kept at -20 °C. Working solutions at 10 and 100 ng g^{-1}, were prepared daily. Each working solution was maintained at 4 °C during the method validation procedure. In order to make the stock solution, each of 6 standard compounds was prepared for 1 mg g^{-1} concentration in methanol and store at -20 °C. The working solutions which were diluted from the stock solution at concentrations of 10 ng g^{-1} and 100 ng g^{-1} in methanol freshly prepared before use and store at 4 °C.

7.2.3 The hydrolysis effect of Amitraz

During the preparation work of materials, the amitraz has observed that there is an auto-degrade effect while amitraz was in acidic solutions. In Figure 3, the Amitraz was stable in methanol (A, B), but not stable in 1% formic acid (C, D). Furthermore, the molecular for the peak in D was the metabolite form of amitraz. We discard the use of formic acid for transferring the extracts of amitraz, the abundance of the signal is still

high, without interference, and identifiable. We use methanol and water as the resuspension solution to reconstruct the final volume of extraction.

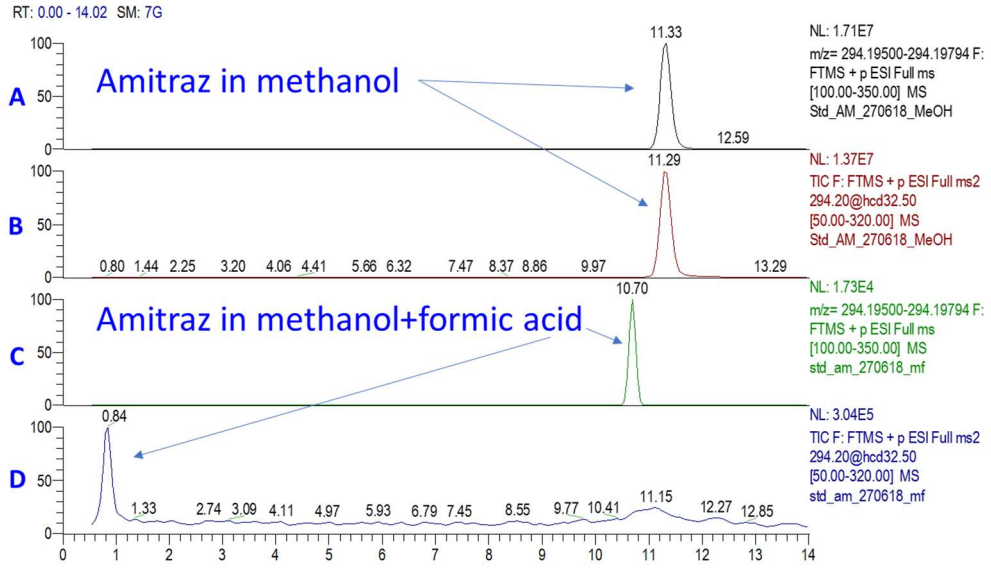

Figure 3. Signal abundances of amitraz in different solvents. A and B, the amitraz resolved in methanol. C and D, the amitraz resolved in acidic methanol.

7.2.4 Sample extraction

Solvent and salt method: One g of homogenised chicken egg sample was weighed and put into a 15-mL centrifugation tube. Milli-Q water 2.5 mL, 1 g of NaCl and 7.5 mL 1% formic Acetonitrile were added. Vortexed the tube for 1 minute then ultrasonicated for 15 mins. The ultrasonicated tube was centrifugated at 4,612x g 4°C for 5 minutes. The supernatant liquid was collected and added with 5 mL hexane. The tube was vortexed for 1 minute then centrifugated at 4,612x g 4°C for 5 minutes. Discard all hexane liquid, then transferred the lower layer liquid into the evaporation flask and dried with rotary vacuum evaporator at 35 °C. 500 µL of mobile phase (Methanol: H_2O=50:50) was added to reconstruct the final volume. All liquids were transferred into a screw vial and perform the analysis with LC-HRMS.

QuEChERS method: One g of homogenised chicken egg sample was weighed and put into a 15-mL centrifugation tube. Pour the Supel Que Citrate extraction powder into the tube, then 10 mL Acetonitrile was added. Shake strongly. Vortex for 1 minute, and then centrifugated at 4,612x g 4°C for 10 minutes. The supernatant liquid was collected and add with Supel PSA powder. The tube was shaken vigorously, vortexed for 1 minute, then centrifugated at 4,612x g 4°C for 10 minutes. All liquid was transferred into the evaporation flask and dried with rotary vacuum evaporator at 35 °C. 200 µL of mobile phase (Methanol: H_2O=90:10) was added to reconstruct the final volume. All liquids were transferred into a screw vial, and the analysis with LC-HRMS was performed.

Water-associated QuEChERS method: Five g of homogenised chicken egg sample was weighed into a 15-mL centrifugation tube. Five mL Milli-Q water and 10 mL acetonitrile was added then the tube was vortexed for 1 minute. Supel Que Citrate extraction powder was added. The tube was shaken vigorously and vortexed for 3 minutes, then centrifugated at 4,612x g 4°C for 10 minutes. The supernatant was transferred and 1.0 g $CaCl_2$ was added. The tube was shaken vigorously and vortexed for 3 minutes, then centrifugated at 4,612x g 4°C for 10 minutes. All liquid was transferred into the evaporation flask and dried with rotary vacuum evaporator at 35 °C. Two hundred µL of mobile phase (Methanol: H_2O=90:10) was added to reconstruct the final volume. All liquids were transferred into a screw vial, and the analysis with LC-HRMS was performed.

7.2.5 LC-HRMS Orbitrap analyses

The LC-HRMS analysis was performed by an HPLC system (Thermo Fisher Scientific, San jose, CA, USA), coupled with a Q-Exactive Orbitrap mass spectrometry. The mobile phase was a gradient of aqueous NH_4COOH (20 mM) and MeOH. All the parameters

are described in our previous works in Chapter 4 (in the session **4.2.6 LC-HRMS Orbitrap analyses**, Chiesa et al., 2018). Detection of analytes was based on the retention time of target compounds, on an exact calculated mass of the deprotonated molecular ions, and at least one specific and typical fragment (Table 1). The formula of the compounds, with the exact theoretical mass of the parents and the diagnostic transition used to confirm the different analytes, are reported in Table 1. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

Table 1. The formula, exact theoretical mass of the parents, diagnostic transitions, ESI mode for Fipronil its metabolites and amitraz.

	Analyte	Formula	Retention time (min)	Exact mass [m/z]	Transition [m/z]	ESI mode +/-
1	Fipronil	$C_{12}H_4Cl_2F_6N_4OS$	8.79	434.93143	329.95961	-
2	Fipronil sulfone	$C_{12}H_4Cl_2F_6N_4O_2S$	9.56	450.92634	414.94959	-
3	Fipronil sulfide	$C_{12}H_4Cl_2F_6N_4S$	9.10	418.93651	170.00953	-
4	Fipronil desulfinyl	$C_{12}H_4Cl_2F_6N_4$	8.43	386.96444	350.98726	-
5	Hydroxyl-Fipronil	$C_{11}H_5Cl_2F_3N_4O$	2.95	334.97197	298.99516	-
6	Amitraz	$C_{19}H_{23}N_3$	11.31	294.19647	163.12296	+
7	Triphenylphosphate	$C_{18}H_{15}O_4P$	9.99	327.07807	233.03615	+

7.3 Results

7.3.1 Analytical performances and method validation

The LC-HRMS showed high specificity, without any interference close to the retention time of each compound as shown in Figure 1, and consequently an S/N ratio greater than or equal to 3 in the presence of analytes was confirmed, even at the lowest detectable concentration demonstrating good selectivity.

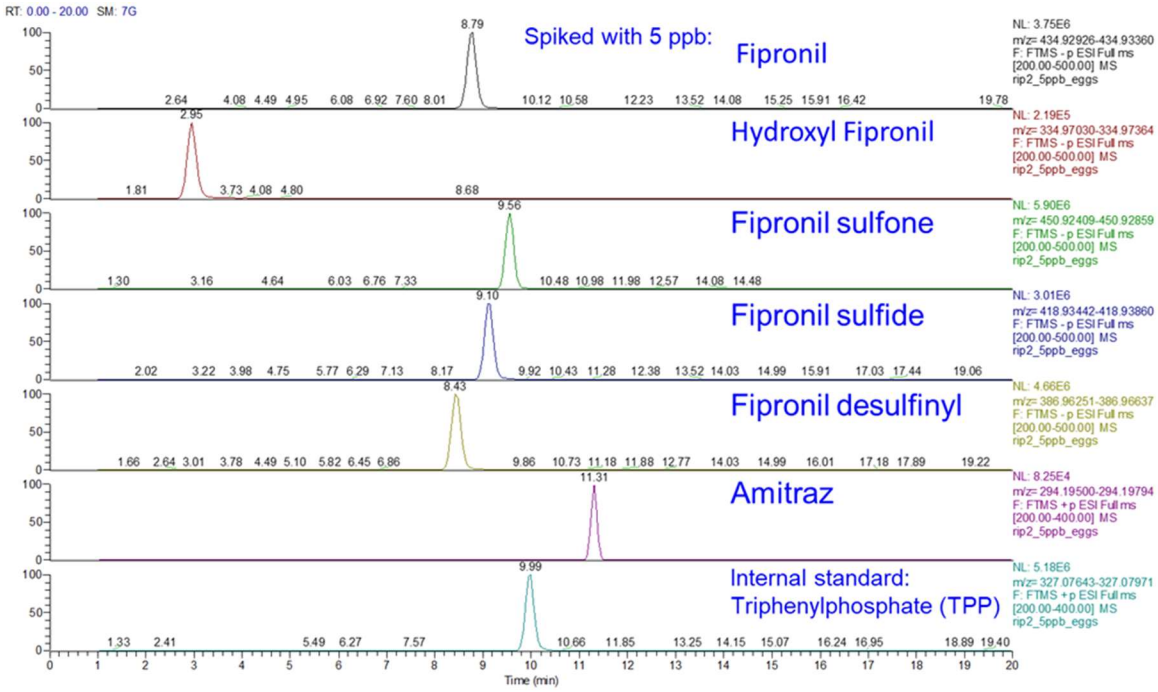

Figure 1. The chromatography of standard reference of fipronil, its metabolite and amitraz in concentration 5 ng g^{-1}.

In the Solvent-salt method, only a shallow signal of fipronil and fipronil sulfone was detected after extraction. The recovery ratio is 0.26% and 0.001%, respectively. In the QuEChERS method, the recovery ratio is around 45% to 52%, except amitraz (7%) and hydroxyl-fipronil (not detected). In the water-associated QuEChERS method, the recovery ratio was in a range of 72%to 113%, which were higher than the previous two methods. (Table 2).

Based on the recovery values are between 72% and 113% were obtained, the water-associated QuEChERS method was selected to validate, whereas recoveries values obtained with the other two methods were lower than 70%.

Table 2. The recovery ratio of different extraction method.

	Analyte	Recovery ratio		
		Solvent-salt	QuEChERS	Water-associated QuEChERS
1	Fipronil	0.26 %	45 %	99 %
2	Fipronil sulfone	0.001 %	47 %	106 %
3	Fipronil sulfide	n.d.	52 %	103 %
4	Fipronil desulfinyl	n.d.	49 %	101 %
5	Hydroxyl-Fipronil	n.d.	n.d.	72 %
6	Amitraz	n.d.	7 %	113 %

n.d.: Not detected

The water-associated QuEChERS extraction is an exothermic reaction. The QuEChERS powder contains citric acid. Although the amitraz is an acid-sensitive pesticide, the influences of heat should not be the problem of method. The recovery ratio (72-113%) estimates that the exothermic heat does not affect the quality of extraction.

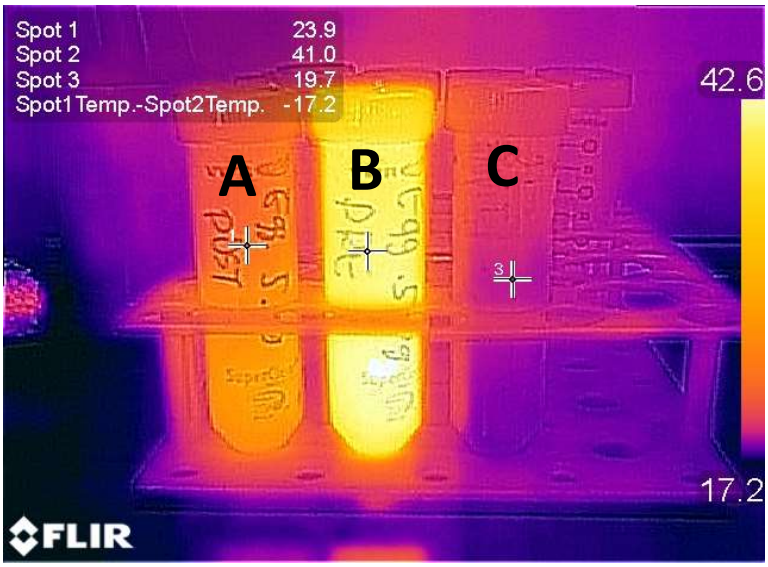

Figure 4. Infrared thermography during the extraction procedures. A. Samples mixed with water and ACN in room temperature (23˚C). B. Sample solution mixed with QuE Citrate (41˚C). C. Fresh homogenised egg. Compare with the temperature of tube A and B; the difference is 17.2˚C.

Calibration curves demonstrated correlation values ranging from 0.9653 to 0.9999 (Figure 2), the limits of detection were from 0.08 ng g^{-1} to 1.21 ng g^{-1}, and the limits of quantification were from 0.28 ng g^{-1} to 4.04 ng g^{-1} (Table 3). The preliminary results satisfied the European criteria for the validation of the analytical methods. Further analyses have performed to evaluate the repeatability and reproducibility.

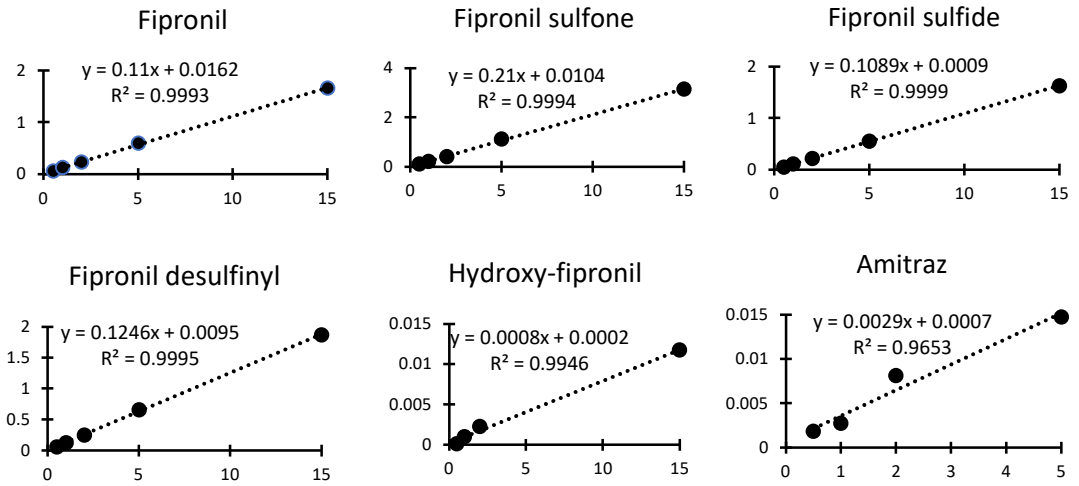

Figure 2. The calibration curves of fipronil, its metabolites and amitraz, respectively.

Table 3. The repeatability. Limit od detection and limit of quantification for water-associated QuEChERS extraction method.

	Analyte	Limit of detection (LOD) (ng g^{-1})	Limit of quantification (LOQ) (ng g^{-1})	Recovery	Repeatability (CV) (n=5)	Reproductivity (CV) (n=9)
1	Fipronil	0.27	0.89	99%	1.67 %	3.65%
2	Fipronil sulfone	0.24	0.78	106%	4.78 %	8.99%
3	Fipronil sulfide	0.08	0.28	103%	4.96 %	5.41%
4	Fipronil desulfinyl	0.21	0.72	101%	4.62 %	6.08%
5	Hydroxyl-Fipronil	1.21	4.04	72%	14.45 %	18.93%
6	Amitraz	0.72	2.4	113%	14.69 %	20.8%

7.4 Discussion

Fipronil is highly selective and effective against wide-ranged agricultural pests (US EPA, 1996). It also recognised as the suspect in mass mortalities of honey bees (Holder et al., 2018). In the veterinary practice, the Fipronil is only prescribed on non-food producing animals, because the fipronil is lipophilic and mainly distributed in fat and egg. However, illicit use of fipronil on layer chicken resulted in the fipronil scandal in 2017. Besides European countries, in the Asian continent, the Taiwan, Hongkong and South Korea also discovered contaminated eggs in the market. An investigation in China reported the residues existed in market samples, of which 4.94 ng g^{-1} in chicken eggs, 3.34 ng g^{-1} in muscle and 8.99 ng g^{-1} in fat. The contamination in the chicken egg is not a public health issue but had impacted consumer confidence. After the Fipronil scandal in 2017, the Italian Ministry of Health included the fipronil detection into the National Residue Program. In this study, we build up a quick and easy-to-apply method consist of the water-associated QuEChERS extraction and LC-HRMS analysis. The sensitivity is high, and the limit of detection complied with the European regulations. This method is valid for the test of fipronil and its metabolite and amitraz in the egg.

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Chapter 8

General conclusion

From the study of the inspection records, we found that the percentage of pathological lesion of the liver is in ascending with seasonal circulation. This finding is as the same as the study in Lithuania. After having fed back the post-mortem result to the farmers for five years, the percentage of liver and lung are both declined. The result is useful for the future development of EU official control. The IMSOC is the unified database for the registering, transmitting, and exchanging of ante-mortem and post-mortem inspection results since the farmer should have registered under the legal requirement. In European, the use of PFOA and PFOS had banned from 2006. We have performed high sensitivity detection method to analyse environmental pollutants and veterinary drugs. In our study above, we found there were traces of PFOA and PBDEs found in pork. The traces were low and did not pose risks to human health. EU pork is quite safe. The EU also encourages the Member States for monitoring food samples continuously in order to review the risk status of PFASs.

Under the limitation of resources, we have performed the two extraction protocol (QuEChERS and solid-phase extraction) in two multi-residue analysis (by LC-HRMS and GC-MS/MS). We have performed 134 analytes (of which 39 antibiotics, 17 PFASs, 38 POPs, and 40 polar pesticides) from 108 veal samples. In the veal samples, we did not find antibiotics and pollutants. It reflected that the control activities for veterinary drugs and environmental pollutants on calves are sufficient.

In the study of fipronil and amitraz in chicken eggs, we have successfully achieved the best extraction method. Water-associated extraction has the best recovery ratio and best sensitivity than the solvent-solvent method or the conventional QuEChERS

method. The LOD is 0.27 ng g^{-1}, which complied the Commission Decision 2002/657/EC, and lower than the listed MRL value (5 ng g^{-1} for Fipronil).

The human's health principally relies on excellent food safety, and excellent food safety relies on excellent animal health. Thus, protecting animal health is protecting human health. In conclusion, we have proved that the feedback of meat inspection could reduce the percentage of pathological lesions in market pigs. The feedback is a way to improve the transparency of animal public health, that filled up the knowledge gap of animal producers, eventually keep animal health under low prevalence. Secondly, we also have explored the current food contaminant issues and have developed analytical methods to identify and quantify known molecules in the food of animal origin. Definitely, the EU food safety is still under challenging from variant industrial innovations, environmental problems and climate changes. The associated scientific research in this thesis is undoubtedly vital for discovering potential chemical residues in the food matrix, safeguard the safety of food of animal origin.

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Cheers !

Lin, Shih-Kuo

Milan, Italy