UNIVERSITÀ DEGLI STUDI DI MILANO

School of Veterinary Medicine

PhD course in Veterinary and Animal Sciences

Class XXX

A spread study on the presence, in different animal matrices, of residues of different origin: pseudo-endogenous substances and environmental contaminants.

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Preface

The presence of xenobiotic residues in food of animal origin represents an issue for both producers and consumers. Many are the classes of substances, which could be present as residues; the most important are veterinary drugs, substances having anabolic effects, or those not authorized, and environmental contaminants. Several European and National legislations are available with the aim of proposing monitoring plans and maximum residue levels.

Over the years, new substances become the subject of Control Authorities, which require the development of state-of-the-art methods for the detection of these compounds and, where necessary, the evaluations of their occurrence and the related risk for the consumer's health. Based on these considerations, this PhD thesis is focused on the development and validation of new analytical methods for the analyses of these compounds in different matrices of animal origin, considering that innovative and sophisticated techniques are always required in order to investigate their presence. The first part of the project is focused on two "pseudo-endogenous": prednisolone investigated in urine and adrenal gland of pigs and thiouracil in urine and thyroid gland of cows. Concerning prednisolone, it was detected in urine both at the farm and at the slaughterhouse, with a concentration and frequency higher at slaughter, while in the adrenal glands it was detected in 89% of the samples. Regarding thiouracil, and for other thyreostatic drugs, two simple methods without the derivatisation step were developed for their analyses in both cow urine and in thyroid glands.

The validated methods showed satisfactory results for the recovery (96–104 % for both the matrices), precision (coefficients of variation were less than 20 % for urine and 21 % for thyroid glands). The decision limit and detection capability for all the compounds were lower than the recommended values. In urine, the decision limit ranged from 6.9 to 7.3 μg L^{-1}, and the detection capability from 8.5 to 9.7 μg L^{-1}, while in thyroid glands these values varied from 6.6 μg kg^{-1} to 7.4 μg kg^{-1} and from 8.0 μg kg^{-1} to 9.7 μg kg^{-1}, respectively.

The second part of the project takes in consideration the presence of environmental contaminants in food of animal origin (in particular fish and honey).

The first study was focused on the evaluation of the distribution of persistent organic pollutants (POPs) in tuna samples from different FAO areas. The results obtained showed that POPs contamination of tuna reflects FAO area contamination, in particular for FAO area 37, Mediterranean and Black seas, which is an enclosed bacin, with heavily populated shores.

The second study assessed the occurrence of different classes of contaminants in 59 organic honeys. Residues of many contaminants were found in most of the samples investigated. The majority of honey samples contained at least one of the contaminants, even if their concentrations were found to be lower

than its maximum residue level (MRL). Diazinon, Mevinphos, Coumaphos, Chlorpyrifos and Quinoxyfen were the pesticide residues frequently detected in samples.

The third study evaluated the effectiveness of accelerated solvent extraction (ASE) compared to QuEChERS methods for the analysis of pesticides in organic honey by gas chromatography-triple quadrupole mass spectrometry. Two simple and rapid ASE methods with "in-line" clean-up, with two different extraction solvents and fat retainers, were optimized and then compared to QuEChERS. The three methods were validated and showed that QuEChERS and ASE with PSA as retainer had better repeatability than ASE with Hexane:EtylAcetate and Florisil. In particular, QuEChERS and ASE (ACN and PSA) showed good recovery, according to the SANTE criteria, for the majority of investigated pesticides. Conversely, when ASE with Hexane:EtylAcetate and Florisil was used as the retainer, several compounds showed recoveries lower than the acceptable value of 70%

The last study considered the presence of environmental contaminants in mussels and clams. As done in the previous studies, the analytical methods were validated, showing recovery in the range 70-100 %, coefficients of variation between 2-20 %, and good linearity. The contaminants were detected in most of the samples with the highest prevalence (58 %) in mussels for polychlorinated biphenyls (PCBs), which were also the contaminants with the highest concentration (Σ PCBs = 49.02 ng g^{-1}).

A part of the honey project was carried out at the Special Solution Center Europe of Thermo Fisher Scientific (Dreieich, Germany).

Chapter I

FOREWORD

1.1 Prednisolone

1.1.1 Introduction

Prednisolone is a corticosteroid drug with predominant glucocorticoid and low mineralocorticoid activity, making it useful for the treatment of a wide range of inflammatory and autoimmune conditions (Czock et al., 2005). It has a structure similar to cortisol, which differs from prednisolone by the absence of a double bond in position 1 of ring A of the steroidal nucleus of the molecule, as shown in figure 1.

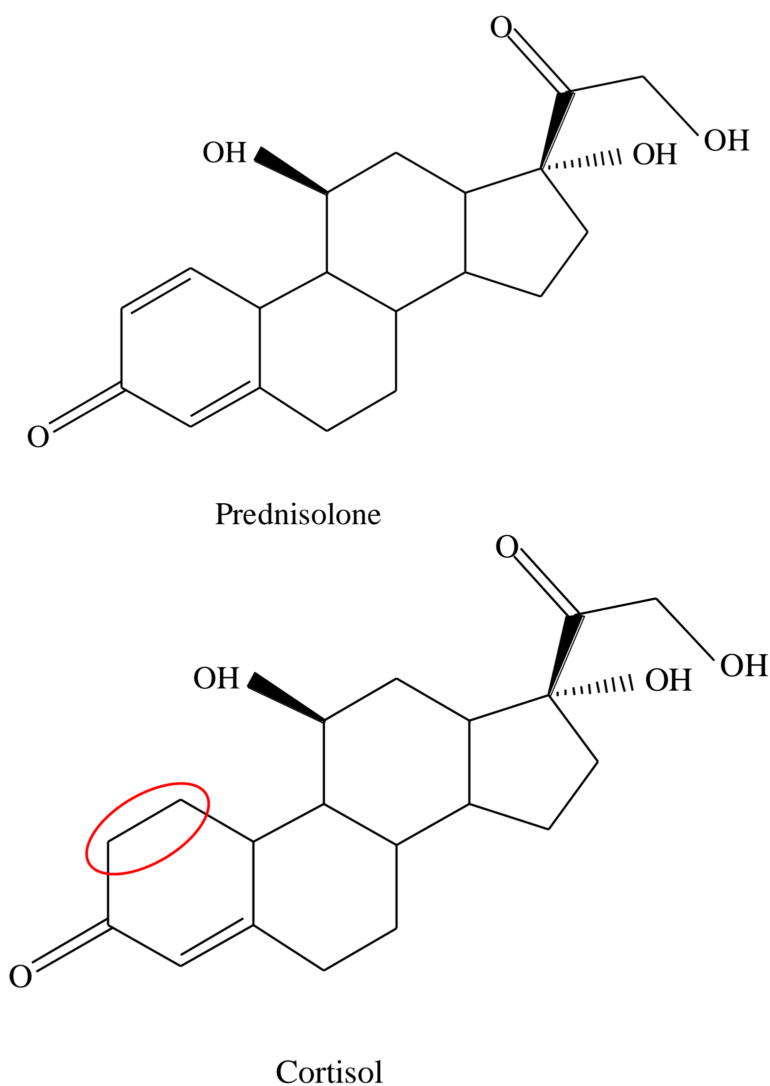

Figure 1. Structure of prednisolone and cortisol.

Prednisolone was discovered in 1955 (Kim et al, 2016) and, in the same year, Bunim et al. (1955) demonstrated that its anti-inflammatory activity is four to five times higher than that of cortisol and cortisone.

1.1.2 Clinical uses in veterinary medicine

Prednisolone, as well as other corticosteroids, is mainly used for anti-inflammatory therapy, with particular attention to locomotor and cutaneous apparatuses. It is also involved in pregnancy-related pathologies (gravidic toxicosis, ketosis), in case of metabolic disorders, shock and intoxications. Typically, the IM or IV viae are the ones preferred for its administration. Topically, prednisolone is used for conjunctivitis, blepharoconjunctivitis, keratoconjunctivitis and inflammations at the back of the eye and, by intramammary infusion, for the treatment of bovine mastitis.

1.1.3 Prednisolone regulations and its "pseudo-endogeneous" nature

The presence and metabolism of synthetically produced substances with hormonal activity in live animals and animal products has been a matter of discussion for many researchers over the years. Only four corticosteroids, prednisolone, methylprednisolone, betamethasone and dexamethasone are allowed for therapeutic or prophylaxis use in food producing animals, but an illicit use, as growth promoters cannot be excluded. The EU Council Directive 96/23/EC (European Commission 1996) takes in consideration the monitoring of certain substances and their residues, separating the substances in two main categories and allocating corticosteroids in the group B2f (defined "*other pharmacologically active substances*"). Focusing on prednisolone, the therapeutic use is regulated by Commission Regulation (EU) No. 37/2010 (European Community 2010) which sets maximum residue limits (MRLs) in, muscle, fat, liver, kidney and milk but only in bovine. No MRLs have been set for urine but, following the indications of the EU Reference Laboratory (RIKILT) of Wageningen and the National Institute for Public Health and the Environment (RIVM) of Bilthoven (de Rijke et al. 2014), a cut-off level of 5 ng ml^{-1} has been recommended by the Italian Ministry of Health (2012). This value was suggested after several studies in which prednisolone was detected also without any treatment; Arioli et al. (2010) showed the formation of prednisolone by cortisol after a 24 hours' fecal contamination while Pompa et al (2011) demonstrated that stress could induce the presence of prednisolone in untreated bovine urine.

Also Delahaut et al. (2014) detected prednisolone in not-treated pig urine, after a somministration of tetracosactide hexaacetate (synthetic analogue of ACTH) while Ferranti et al. (2011) detected a corticosteroid residue in untreated bovine urine samples collected from control bovines especially at the slaughterhouse, but did not conclude it was prednisolone, even if the Rt and the parent and product ion were the ones of prednisolone.

For these reasons, prednisolone could be defined a 'pseudo-endogenous' or 'grey zone substance' due to its dual synthetic/endogenous nature (Van Thuyne, 2006).

1.2 Thyreostats

1.2.1 Introduction

Thyreostats (TS) are a various group of substances that inhibit the thyroid function, resulting in a decreased production of thyroid hormones triiodothyronine (T3) and thyroxine (T4) (Courtheyn et al. 2002; De Brabander et al. 1984). They are also called thyreostatic drugs or antithyroid agents.

Thyreostats can be divided into two main groups, respectively the xenobiotic and the naturally occurring sulfur compounds (Courtheyn et al. 2002). Chemically, they are polar amphoteric compounds with a heterocyclic tautomeric structure, consisting of nitrogen–carbon–sulphur, known as thioamide, which is also considered responsible for the thyroid-inhibiting activity (Vanden Bussche et al. 2009). Thyreostatic drugs are characterized with a low molecular weight and the best known are: 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU) and 1-methyl-2-mercaptoimidazole, also called tapazole, (TAP), reported in figure 2. (De Wasch et al. 2001; Vanden Bussche et al. 2009).

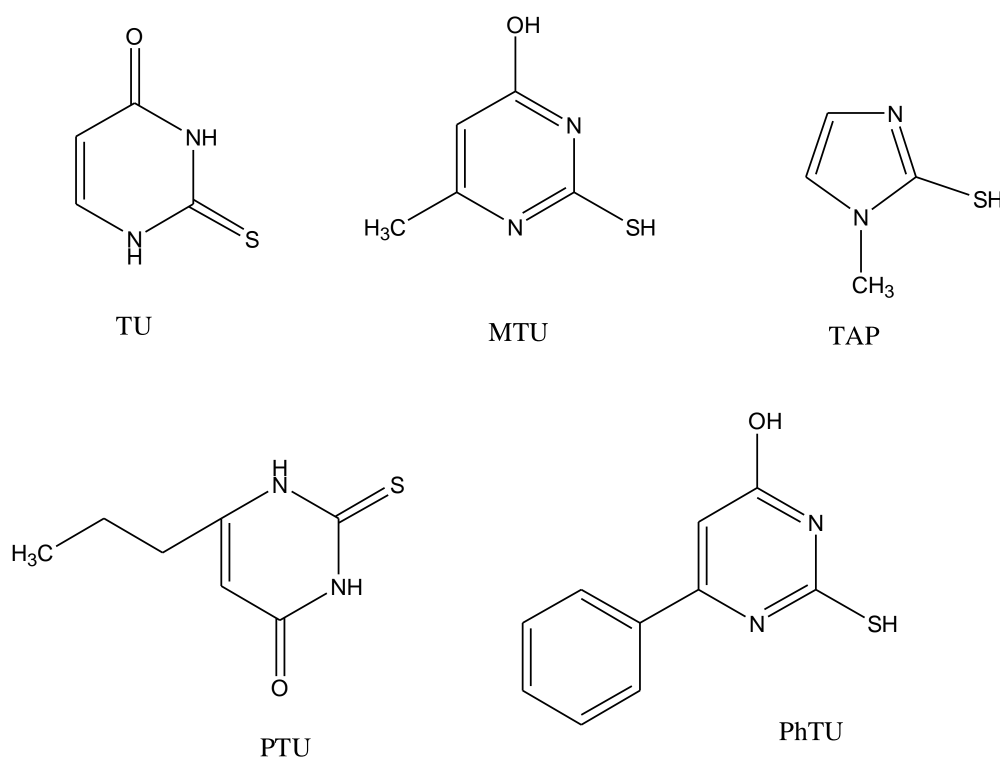

Figure 2. Structures of the most common thyreostatic drugs.

1.2.2 Thyreostats regulations and "pseudo-endogeneous" nature of thiouracil

During the years, thyreostats were used in animal production not only as therapeutic agents but, due to the capability to increase absorption and extracellular retention of water in the edible tissues and in the gastrointestinal tract (Hall et al. 2010), so causing an improvement in bodyweight gain, they were used also as growth promoters. This fraudulent use produces low-quality meat, could represent a risk to the consumer's health due to the presence of residues and their teratogenic and carcinogenic effects (Martinez-Frias et al. 1992; Vanden Bussche et al. 2010).

The European Union, in 1981, banned their use and in the Council Directive 96/23/ CE they were classified into the group A2 defined "*substances having anabolic effects and unauthorized substances*" (European Commission 1996). Among thyreostas, thiouracil can be considered a 'pseudo-endogenous' or 'grey zone substance', in fact, as reported by Pinel et al. (2006), Vanden Bussche et al. (2011) and Kiebooms et al. (2014) thiouracil was detected in urine of not-treated animals; In particular, its detection is strongly related to the presence (in feed) of Brassicaceae (syn. Cruciferae), which contains possible precursors of thiouracil and other natural thyreostats (as thiocyanates and oxazolidine-2-thiones) synthesis. As decribed by Vanden Bussche et al. (2009), glucosinolates which are present Brassicaceae, have different metabolic pathways which could lead to the formation of natural tyreostats, due to the hydrolysis of the glucosinolates catalyzed by myrosinase (produced by the bacterial microflora of the gastro-intestinal tract). The metabolic pathways are described in Figure 3.

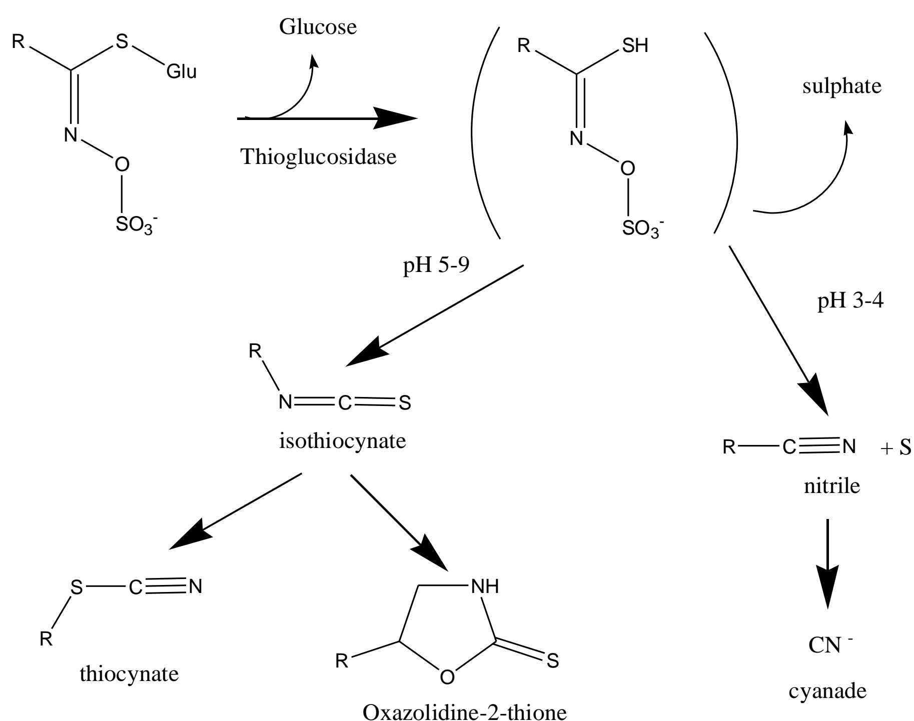

Figure 3. Possible metabolic pathways of glucosinolates. Figure from Vanden Bussche et al. (2009).

The different reactions strongly depend on the pH. With an acidic pH (3-4), due to the loss of sulfur, the nitrile formation could be observed, then cyanide (CN-) could be generate. At pH between 5 and 9, isothiocyanates, which later can generate, could be produced. The natural TSs formation depends exclusively on the lateral chain structure (R).

On the basis of these considerations, the Community Reference Laboratories (CRLs) proposed, in 2007, a suggested concentration of 10 μg L^{-1} in urine and 10 μg kg^{-1} in thyroid tissue for the purpose of control. However, recent studies demonstrated in untreated animals the presence of thiouracil at values higher than those raccomandated by CRLs. In particular, Le Bizec et al. (2011) evaluated the presence of thiouracil in more than 1300 urine samples from different animal species and suggested new threshold values to differentiate compliant from suspect urine samples. Moreover, a large-scale retrospective epidemiologic study involved six European member states (France, Poland, The Netherlands, United Kingdom, Norway, and Belgium), which have shared their official data regarding the concentration of thiouracil in urine samples, collected from bovines, porcines, and small livestock in the two-year period 2010‒2012. As results, Wauters et al. (2015) suggested a new recommended concentration of 30 μg L^{-1}. In fact, the 2015 Italian National Residue Plan already provides this concentration as the limit of detection for thyreostats in urine (Ministry of Health, 2015).

1.3 Environmental contaminants

1.3.1 Introduction

Environmental contaminants are chemical substances (synthetically or naturally produced) which are present in the environment both naturally and due to the anthropogenic activities. The massive industrial development, occurred in the nineteenth century Industrial Revolution, had a profound impact on the amounts and types of compounds released into the environment. During this period, many thousands of novel materials were produced, used, stored, and transported, increasing exponentially the number of discharge products which led to the release of massive amounts of contaminants into the environment (Berkowitz et al. 2014)

Several are the pollution sources that can introduce contaminants in the environment; for instance, combustions are a primary source as well as the oil spills caused by maritime transport of petroleum products, which interest marine (particularly coastal) pollution (Gonzalez-Doncel et al. 2008). Moreover, the development, the production and the use of new compounds (mainly pesticides), that reformed agriculture and industry, (Blais et al. 2015) played an important role in the contamination of the environment. In particular, they have a strong influence on the food and feed contamination, posing

a potential risk to animal and human health, as reported by the European Food Safety Agency (EFSA), who defines contaminants as: *"chemical substances that have not been intentionally added to food or feed"* (EFSA).

Focusing the attention on food, many are the classes of compounds involved in contamination processes (e.g. metals, organochlorine pesticides, polycyclic aromatic hydrocarbons, brominated compounds) and many of them can be included in the category of Persistent Organic Pollutants (POPs).

POPs represent the best-known contaminants; they are mostly man-made chemicals that are resistant to environmental degradation through chemical, biological, and photolytic processes (Ritter et al. 2007). Due to their highly stability, low volatility and lipophilic nature, they are able to accumulate in the environment for a significant time and bioaccumulate. (Gui et al. 2014). The effects of POPs on human and environmental health are discussed, aiming to eliminate or restrict their production, by the international community at the Stockholm Convention on Persistent Organic Pollutants beginning from 2001.

The most studied POPs are: polychlorinated biphenyls (PCBs), organochlorine and organophosphate pesticides (OCPs and OPPs), polybrominated diphenyl ethers (PBDEs), polycyclic aromatic hydrocarbons (PAHs). All these classes were described and examined in the following paragraphs.

1.3.2 Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are organic chlorine compounds with formula $C_{12}H_{10-x}Cl_x$ structure as reported in figure 4:

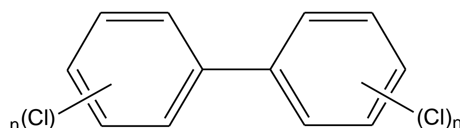

Figure 4. PCBs general structure.

They are man-made chemicals with a wide range of physicochemical properties, as chemical inertness, low electrical conductivity, heat-resistance and low vapor pressure. They are very lipophilic, with log K_{ow} (octanol-water partitioning coefficients) ranging from 4.3 to 8.3 (Dobson and van Esch, 1993).

Because of this broad range, PCBs have been used in a variety of applications, e.g. as organic diluents, plasticizers, adhesives, heat transfer and dielectric fluids in transformers, dielectric fluids in capacitors, hydraulic lubricants and in carbonless copy paper (Safe, 1984).

Considering their structure, PCBs can be divided in two main groups: coplanar (or non-ortho and mono-ortho) and non-coplanar (or di-ortho) PCBs, resulting in 209 different congeners. These difference in structure is possible because the benzene rings can rotate around the bond connecting them, depending on the chlorine atom positions on the rings.

Non-ortho substituted PCBs, as well as mono-ortho substituted PCBs, may assume a planar conformation while, when hydrogen atoms are substituted by large chlorine atoms in ortho position of both rings, the two benzene can not assume a coplanar conformation, resulting in non-planar congeners.

These two different types of structure determine the possible PCB toxicity. Coplanar PCBs are similar to polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans, in fact they are also called PCB dioxin like (PCBs-DL), so they have agonist properties on the aryl hydrocarbon receptor (AhR) in organisms. Therefore, PCBs-DL could produce toxic effects as immunotoxicity, endocrine disrupting effects, reproductive and developmental toxicity, or carcinogenic responses (McFarland and Clarke, 1989; Safe, 1993). Twelve PCB congeners are PCBs-DL, four non-ortho PCBs and eight mono-ortho PCBs.

All other congeners are non-planar congeners (also called PCBs-NDL) and they do not activate the AhR. The effects not correlated to AhR activation are mainly neurotoxic and immunotoxic effects; in particular, PCBs interfere with the thyroid signalling pathway by reducing levels of thyroid hormones 3,3',5-triiodothyronine (T3) and 3,3',5,5'-tetraiodothyronine (T4) and increase thyroid stimulating hormone (TSH) levels in blood from wildlife and humans (Debier et al. 2005; Sormo et al. 2005). Several studies also shown the alteration of neurotransmitter (dopaminergic and cholinergic) processes, Ca^{2+} homeostasis, signal transduction and cell death of neuronal cells (Mariussen and Fonnum, 2006).

Due to these possible toxic effects, PCBs have been classified by the International Agency for Research on Cancer (IARC) as probably carcinogenic to humans (group 2A) (IARC, 1987). Moreover, the European Community in 1976 by Council Directive 76/403/EEC banned the use of PCBs in open applications and in 1985 by Council Directive 85/467/EEC (6th amendment of Directive 76/769/EEC) the use as a raw material or chemical intermediate. As described previously, PCBs are listed by the Stockholm Convention in the persistent organic pollutants. Being considered POPs, several legislations were provided in order to reduce their presence in the environment. In particular, focusing the attention on foodstuff, the most recent is the Commission Regulation (EC) No 1259/2011 (amendment of Commission Regulation (EC) N° 466/2001) which set maximum levels for dioxins,

dioxin-like PCBs and non dioxin-like PCBs in foodstuffs. Regarding PCBs-NDL, only six PCBs-NDL (CB 28, 52, 101, 153, 138, and 180) were selected as indicators by the European Union Community Bureau of Reference, due to their relatively high concentrations in technical mixtures and their wide chlorination range (3–7 chlorine atoms per molecule); these PCBs-NDL are also called PCBs-ICES (Webster et al. 2013).

1.3.3 Polybrominated diphenyl ethers

Polybrominated diphenyl ethers (PBDEs) are organobromine compounds with formula $C_{12}H_{(10-x)}Br_xO$ and structure as shown in figure 5:

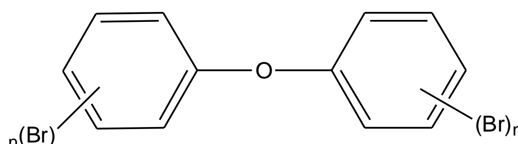

Figure 5. PBDEs structure. The values of m and n range between 1 to 10**.**

PBDE structure is similar to PCBs, apart for an oxygen atom between the aromatic rings, therefore 209 congeners are possible.

PBDEs are lipophilic compounds, with logarithm of n-octanol/water partition coefficient (log $K_{ow)}$ ranging from 3.7 to 11 (Palm et al. 2002) and low vapor pressure. The larger the number of bromine atoms, the heavier the molecule and consequently the less volatile the molecule is. Considering the number of bromine atoms, PBDEs can be divided in lower brominated PBDEs and higher brominated PBDEs. Lower brominated PBDEs have 1 to 5 bromine atoms per molecule and are regarded as more dangerous because they more efficiently bioaccumulate, while higher brominated PBDEs have more than 5 bromine atoms per molecule.

PBDEs represent a large group of brominated flame retardants (BFRs) used in a wide array of products as building materials, electronics, furnishings, motor vehicles, airplanes, plastics, polyurethane foams and textiles (Stapleton et al. 2011).

Although PBDEs are similar to PCBs, they could enter the environment in different ways. They can leach in the environment during natural operating life of television sets, computers and also during processing, recycling or combustion process (D'Silva et al. 2004).

Concerning the toxicity of PBDEs, few data are present in literature and, as for their toxicokinetic, is strictly correlated to congener structure. Many studies were carried out using commercial PBDE mixtures and no information were available on the possible presence of dioxin-like impurities. The

acute toxicity of commercial PBDEs (administered orally, dermally or by inhalation) for laboratory animals is low (LD50 > 0.5 – 28 g kg^{-1} body weight).

PBDEs (in particular lower-brominated PBDEs) have been known to affect hormone levels in the thyroid gland. Zhou et al. (2001) reported the alterations in thyroid hormone homeostasis. The mechanism of thyroid hormone disruption by PBDEs has not been fully characterized, but different mechanisms have been suggested by Hallgren and Darnerud (2002), Zhou et al. (2001) and Meerts et al. (2000). Also neurobehavioural and receptor mediated effects were observed (Eriksson et al. 2006; Chen and Bunce, 2003).

Following the few data on PBDE toxicity and distribution in the environment, no many legislations are available. The European Union (EU) in 1976 by the Directive 76/769/EEC established the limitations on the marketing and use of dangerous substances. This directive has been amended several times in order to update its scope of application to other dangerous substances until obtaining Directive 2002/95/EC (also called "the Restriction of certain Hazardous Substances in electrical and electronic equipment (RoHS)"). In 2001, the EU identified a priority list of 33 substances in the field of water policy (Decision 2455/2001) while, concerning foodstuff, only Commission Recommendation 2014/118/UE on the monitoring of traces of brominated flame retardants in food were provided to suggest a monitoring program on the presence of brominated flame retardants in food, during the years 2014 and 2015. As cited in the legislation "*The monitoring should include a wide variety of individual foodstuffs reflecting consumption habits in order to give an accurate estimation of exposure and different food commodities should be included for the different classes of brominated flame retardants*".

1.3.4 Pesticides

A pesticide, as defined by the Food and Agriculture Organization (FAO), is: "*any substance or mixture of substances intended for preventing, destroying, or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals, causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances that may be administered to animals for the control of insects, arachnids, or other pests in or on their bodies. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant, or agent for thinning fruit or preventing the premature fall of fruit. Also used as substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport*" (FAO, 2002).

Among the years, pesticides were largely used to kill mosquitoes (Chadwick, 1975) that can transmit potentially deadly diseases (e.g. yellow fever, transmitted by *Aedes aegypti(L.)*), to kill parasites (e.g in the control of Varroa disease in bee hives) (Panseri et al. 2014), and nowdays also to the pest management in urban environment (Masciocchi et al. 2017). Despite the benefits listed above, some pesticides have potential toxicity to humans and the environment. Concerning health effects, pesticides can cause a variety of adverse health effects, ranging from simple irritation of the skin and eyes to more severe effects such as affecting the nervous system, endocrine disruption (e.g. mimicking hormones causing reproductive problems), and also causing cancer (EPA, 2006). Bassil et al. (2007) reported most studies on non-Hodgkin lymphoma and leukemia that showed positive associations with pesticide exposure, in particular with the cosmetic use of pesticides (Bassil et al. 2007). Several epidemiological studies also demonstrated the association between organophosphate insecticide exposure and neurobehavioral alterations (Jurewicz and Hanke, 2008; Weselak et al. 2007; Wigle et al. 2008; Mink et al. 2011).

Regarding environmental effects, the main one is the potential contamination of air, water and soil. Over 98% of sprayed insecticides and 95% of herbicides are able to reach a destination other than their target species (Miller, 2004) and, considering that some of pesticides are POPs, the process of accumulation in the environment and the bioaccumulation (mainly in marine organisms) is favorited, posing a risk for the environment and also, as described before, for the human health.

Pesticides can be classified according to chemical structure (e.g., organic, inorganic, synthetic, or biological (biopesticide) or according to the target organism. Following this second classification, the main classes are herbicides, insecticides, in which organochlorine and organophosphate compounds belong to, fungicides, rodenticides. In the next paragraphs the attention will be focused on organochlorine and organophosphate pesticides.

1.3.4.1 Organochlorine pesticides

Organochlorine pesticides (OCPs) are organic compounds containing at least one covalently bound atom of chlorine. Many are the compounds belonging to this class, but the most representatives are listed in table 1:

Compound
α-hexachlorocyclohexane (α-HCH)
β- hexachlorocyclohexane (β-BHC)
γ- hexachlorocyclohexane (γ-HCH)
Hexachlorbenzene

Trans-chlordane
α-endosulfan
β- endosulfan
Endosulfan sulfate
Heptachlor
Heptachlor epoxide
Aldrin
Endrin
Dieldrin
Dichlorodiphenyltrichloroethane (DDT)
Dichlorodiphenyldichloroethylene (DDE)
Dichlorodiphenyldichloroethane (DDD)

They were commonly used in the past in many countries because of their low cost and versatility against pests, but many have been removed from the market due to their health and environmental effects. In fact, they are POPs able to accumulate in the environment for a significant time and bioaccumulate. (Gui et al. 2014). These pesticides are still present in the natural ecosystem (as residues), although they have been already banned in different countries (Kannan et al. 1997) because considered endocrine disruptors, which interfere with the body's endocrine system producing adverse effects in humans (e.g. developmental, reproductive, neurological, cardiovascular, metabolic and immune effects.) (Schug et al. 2011).

Pesticide residue presence still represents an issue for human health and environment, therefore many are the regulations aiming to monitor their presence. Focusing on foodstuff, several studies on pesticide exposition, distribution and toxicity were carried out, in order to establish the Maximum Residue Level (MRL) for each compound in each matrix. As European Commission defines, a maximum residue level (MRL) is "*the highest level of a pesticide residue that is legally tolerated in or on food or feed when pesticides are applied correctly (Good Agricultural Practice)"* (European commission); all MRLs are available on-line on the European Pesticides Database.

1.3.4.2 Organophosphate pesticides

Organophosphate pesticides (OPPs) are organic compounds having a phosphate, as shown in figure 6:

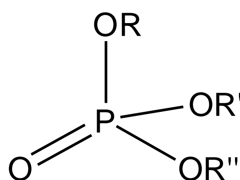

Figure 6. General structure of an organophospate.

Most organophosphates are insecticides. They were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932.

OPPs largely replaced OCPs because they degrade rapidly by hydrolysis on exposure to sunlight, air, and soil. Although organophosphates degrade faster than the organochlorides, some of them, being lipophilic compounds, such as chloropyrifos, diazinon, parathion, and coumaphos, can accumulate in body fat, and remain in the body for many days (Abend et al. 1994).

OPPs inhibit the enzyme acetylcholinesterase, allowing acetylcholine to transfer nerve impulses indefinitely and causing a variety of symptoms from weakness to paralysis and death (Colovic et al. 2013). Their inhibitory effects on the acetylcholinesterase enzyme could lead to a pathological excess of acetylcholine in the body causing neurotoxic effects, especially on developing organisms as fetuses and young children, where brain development depends on a strict sequence of biological events (Jurewicz et al. 2008).

The IARC, found that some organophosphates may increase cancer risk, and classified tetrachlorvinphos and parathion as "possibly carcinogenic", and malathion and diazinon as “probably carcinogenic to humans” (IARC, 2015).

As for OCPs, many legislations are present and, focusing on foodstuff, all MRLs are available on-line on the European Pesticides Database.

1.3.5 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds containing only carbon and hydrogen, which are organized in multiple aromatic rings. The simplest PAHs are naphthalene (two aromatic rings), anthracene and phenanthrene (three aromatic rings) and their structures are reported in figure 7:

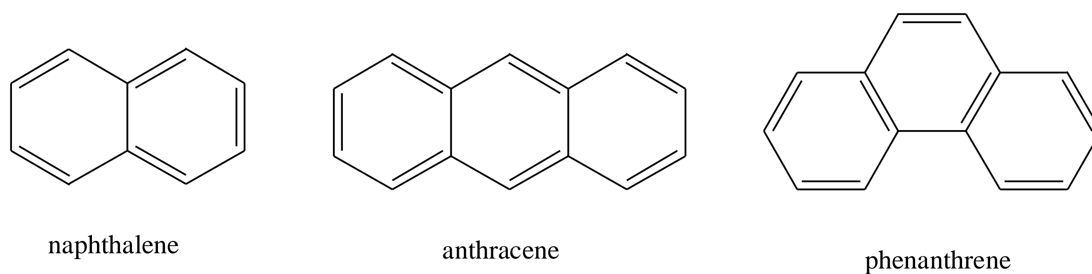

Figure 7. Structures of the simplest PAHs

PAHs are nonpolar and lipophilic compounds, generally insoluble in water, which limits their mobility in the environment (Choi et al. 2010), but some of them are soluble and could contaminate drinking water. (Xinliang et al. 2009; WHO, 1998). Aqueous solubility of PAHs decreases approximately logarithmically with the increasing of molecular mass. (Johnsen et al. 2005), in fact two-ring PAHs, and to a lesser extent three-ring PAHs, dissolve in water, making them more available for biological uptake and degradation (Choi et al. 2010; Johnsen et al. 2005).

Unlike PCBs and PBDEs, PAHs are mainly found in natural sources such as creosote and coal (Sörensen and Wichert, 2009), or can be produced by the incomplete combustion of organic matter in engines and incinerators, when biomass burns in forest fires, etc., in particular wood-burning and combustion contribute more than half of annual global PAH emissions, principally due to biofuel use in India and China. (Ramesh et al. 2011). It is interesting to note that lower-temperature combustion, such as tobacco smoking or wood-burning, tends to generate low molecular weight PAHs, while high-temperature industrial processes typically generate PAHs with higher molecular weights. (Tobiszewski and Namieśnik, 2012).

Concerning toxicity, the main effect associated to PAHs exposition is carcinogenesis. Some carcinogenic PAHs are genotoxic and induce mutations that initiate cancer; others are non-genotoxic affecting cancer promotion or progression (Baird et at. 2005; Slaga, 1984). Nebert et al. (2004) showed that PAHs that affect cancer initiation are modified by enzymes (usually in the cytochrome family) into metabolites (diol epoxides) that react with DNA, leading to mutations; Ramesh et al. (2004), instead, described the co-carcinogenic activity during the promotional stage of cancer, most manifested by low molecular weight PAHs. Being these PAHs prevalent in the environment, significant risk to human health is related to them.

Also cardiovascular diseases are associated to PAHs exposition (Korashy and El-Kadi, 2006), mainly through cigarette smoke and particulate air pollution (Lewtas, 2007). Ramos et al. (2005) explained the

increasing of plaques (atherogenesis) development within arteries in animals exposed to several PAHs with the same mechanisms involved in the carcinogenic and mutagenic properties of PAHs.

Due to their carcinogenicity, some of PAHs are classified as probable or possible human carcinogens by IARC, while EFSA, EPA and other governmental bodies investigates their toxicity in order to enact legislations to reduce risk for human health.

Focusing the attention on foodstuff, the Commission Regulation (EC) No 1881/2006 set maximum levels for certain contaminants, including PAHs in foodstuffs. In particular, only for benzo(a)pyrene maximum levels are listed, because *"benzo(a)pyrene is used as a marker for the occurrence and effect of carcinogenic polycyclic aromatic hydrocarbons. These measures therefore provide full harmonisation on polycyclic aromatic hydrocarbons in the listed foods across the Member States."* (European Commission, 2006).

In 2008, a Scientific Opinion on PAHs in food was presented by EFSA. The exposition, and the related possible toxicity, through food were evaluated for the 16 PAHs reported in table 2.

Table 2. List of PAHs considered in EFSA opinion.

Compound	Abbreviation
Benz[a]anthracene	*BaA*
Benzo[b]fluoranthene	*BbFA*
Benzo[j]fluoranthene	*BjFA*
Benzo[k]fluoranthene	*BkFA*
Benzo[ghi]perylene	*BghiP*
Benzo[a]pyrene	*BaP*
Chrysene	*CHR*
Cyclopenta[cd]pyrene	*CPP*
Dibenz[a,h]anthracene	*DBahA*
Dibenzo[a,e]pyrene	*DBaeP*
Dibenzo[a,h]pyrene	*DBahP*
Dibenzo[a,i]pyrene	*DBaiP*
Dibenzo[a,l]pyrene	*DBalP*
Indeno[1,2,3-cd]pyrene	*IP*
5-methylchrysene	*MCH*
Benzo[c]fluorene	*BcFL*

In particular, concerning carcinogenesis, in addition to using only benzo[a]pyrene as a marker for the carcinogenic PAHs in food, the CONTAM Panel explored additionally the use of:

- benzo[a]pyrene and chrysene (PAH2),
- benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene and chrysene (PAH4),

- the sum of the eight carcinogenic PAHs (benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-cd]pyrene) (PAH8)

At the end of all evaluations, The CONTAM Panel concluded that: "*benzo[a]pyrene is not a suitable indicator for the occurrence of PAHs in food. Based on the currently available data relating to occurrence and toxicity, the CONTAM Panel concluded that PAH4 and PAH8 are the most suitable indicators of PAHs in food, with PAH8 not providing much added value compared to PAH4.*" (EFSA, 2008)

1.4 General discussion

Based on these considerations, it is clear that the presence of residues of veterinary drugs, pesticides and environmental contaminants, is a global problem, involving all the ecosystem. Many are the possible contamination pathways, depending mainly on the physico-chemical properties of the contaminants.

In this global contest, my attention has been focused on the presence of residues in animal matrices, with a particular interest regarding the contamination of food of animal origin, which may represent an issue for producers and a risk for consumer's health.

In detail, this PhD thesis is focused on the development and validation of innovative and sophisticated analytical methods to investigate and evaluate the presence of contaminants residues in different matrices of animal origin. The three-years PhD project was divided in two main parts, the first one focused on two "pseudo-endogenous" substances: prednisolone and thiouracil, investigated in urine and adrenal gland of pigs, and in urine and thyroid gland of cows, repectively, and the second one on the evaluation of the presence of environmental contaminants in tuna collected from different FAO areas, in organic and industrial honey, in mussels and clams.

During the PhD period, I actively contributed in planning works, developing novel analytical methods and applying them to samples from different animal matrices, in the evaluation of data and in writing the papers.

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Chapter II

Pseudoendogenous origin of prednisolone in pigs from the food chain

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2. Pseudoendogenous origin of prednisolone in pigs from the food chain

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2.1 Abstract

The debate about the origin of prednisolone in animal organisms has lasted for 5 years. Bovine species have been the most studied, but studies on humans and horses are also present in the literature. Even if prednisolone in pigs does not yet represent a problem for control agencies, interest has recently increased with regard to this species. To date, there has been just a single study in the literature about this topic, performed on 10 sows treated with prednisolone or a synthetic analogue of adrenocorticotropic hormone. We therefore initiated a study on 80 pigs, a number considered representative in relation to the expected frequency (prevalence) of prednisolone detection in urine collected at slaughter. Prednisolone was detected in urine both at the farm and at the slaughterhouse, with a concentration and frequency higher at slaughter. The presence of prednisolone was also studied in the adrenal glands, where the corticosteroids are produced in response to stress, and it was detected in 89% of the samples. These results, together with the similar behaviors of prednisolone and cortisol, i.e. a mutual rise in the two corticosteroids in urine collected at the slaughterhouse and the correlation between the concentrations of the two corticosteroids in the adrenal glands, seem to indicate an endogenous origin of prednisolone in pigs.

2.2 Introduction

Cortisol is a steroid hormone produced and released by the adrenal cortex. Cortisol is involved in physiological processes such as immune reactions, the regulation of inflammatory states and carbohydrate metabolism (Osamu 2001; Shimada et al. 2001). Prednisolone is a glucocorticosteroid whose anti-inflammatory activity is 3–4 times higher than cortisol. The therapeutic use of prednisolone in bovine is regulated by Commission Regulation (EU) No. 37/2010 (European Community 2010) which sets maximum residue limits (MRLs), even if its illicit use as a growth promoter agent cannot be discarded (Pavlovic et al. 2013). No MRLs have been set for urine, but a 5 ng ml^{-1} cut-off level has

been recommended, e.g. by the Italian Ministry of Health (2012), following the indications of the EU Reference Laboratory (RIKILT) of Wageningen and the National Institute for Public Health and the Environment (RIVM) of Bilthoven (de Rijke et al. 2014). Corticosteroids are allocated to group B2f (other pharmacologically active substances) by EU Council Directive 96/23/EC (European Commission 1996) and monitoring of their administration to livestock is carried out both on urine collected at the farm and urine or liver at the slaughterhouse (European Community 2010; Italian Ministry of Health 2014). In recent years, an increase in cases positive for prednisolone, reported by some EU Member States, has been observed in bovine urine, particularly when sampled at the slaughterhouse (European Commission Staff Working Document 2010). The possibility of in vitro formation of prednisolone from cortisol has been reported, possibly due to poor collection and storage conditions of the urine samples (Arioli et al. 2010; Ferranti et al. 2011; Bredehöft et al. 2012). Pompa et al. (2011) investigated the relation of stress to the formation of prednisolone from cortisol in dairy cows. The role of stress in cortisol production is well-known. The cortex of the adrenal glands is stimulated by the adrenocorticotropic hormone (ACTH) secreted from the anterior pituitary in response to corticotropinreleasing hormone (CRH) from the hypothalamus. Under unstressed conditions, prednisolone was found sporadically in urine. When the cows were stressed with intramuscularly (i.m.) administered tetracosactide hexaacetate, a synthetic analogue of ACTH, or physically by transport and slaughter, cortisol concentration increased and prednisolone was consistently found in urine, demonstrating the possibility of its endogenous formation. The possibility of the endogenous origin of prednisolone has also been described for equine and human urine (Fidani et al. 2012, 2013). Finally, Delahaut et al. (2014) reported that the Belgian Federal Agency for the Safety on the Food Chain (FASFC) found prednisolone at a mean concentration of 0.96 ng ml^{-1} in 73% of 393 samples of porcine urine collected at the slaughterhouse. The same authors described the results of a preliminary study concerning the presence of prednisolone in sows before and after i.m. administration of prednisolone or tetracosactide hexaacetate. The urine collection was performed at the farm before and after the treatment and at the slaughterhouse, where the liver was collected as well. The presence of prednisolone in porcine urine was confirmed in all samples prior to the treatment and in most of them after the treatment, but, in liver, prednisolone was only found after administration of prednisolone or tetracosactide hexaacetate. The authors proposed the prednisolone/cortisol ratio in liver samples as an indicator for detecting illicit prednisolone administration to pigs and suggested confirming these observations in a study on a larger number of animals (Delahaut et al. 2014). In order to clarify the possible endogenous origin of prednisolone and the influence of stress on the production of this corticosteroid in pigs, the present study investigated the presence of prednisolone in urine samples collected from the same 80 pigs at the farm and at the slaughterhouse; we also analysed the

adrenergic glands of the same animals, supposing an endogenous production of prednisolone in this organ.

2.3 Materials and methods

2.3.1 Chemicals and reagents

Cortisol and prednisolone were purchased from Sigma– Aldrich (St. Louis, MO, USA). The internal standard prednisolone- d6 was from CDN Isotopes (Pointe-Claire, Quebec, Canada). All other chemicals were from Fluka Chemie GmbH (Buchs, Switzerland). Ultrapure water was obtained through a Milli-Q system (Millipore, Molsheim, France). Standard stock solutions were prepared in methanol (1 mg ml^{-1}) and stored at −40°C. Working solutions were prepared daily by diluting the stock solutions with methanol:water (50:50, v/v).

2.3.2 Animals and sampling procedure

The study was carried out on 80 pigs of both genders weighing 100–150 kg, coming from farms in northern Italy and slaughtered in different abattoirs of Lombardy. Urine and adrenal gland samples, not used for routine analyses, were collected by Official Veterinarians of Lombard Veterinary Services. They also verified the lack of treatments in the 90 days before slaughter, by checking the records of purchase, possession and administration of veterinary medicinal products of the animal treatments, maintained by the owners of food-producing animals as required by Directive 2001/82/EC (European Community 2001). Urine samples were collected for the first time at the farm into long-handled sterile containers, approximately 1 week before transport of the animals to the slaughterhouse. Only clean urine, i.e. clear and without raw materials, was sampled, frozen and taken to the laboratory for storage at −40°C until extraction and analysis. A second collection was made at the slaughterhouse: urine samples were collected directly from the urinary bladder immediately after slaughter, as well as the adrenal glands. All samples were immediately frozen and taken to the laboratory for storage at −40°C. Each pig, randomly selected and followed from farm to slaughterhouse, provided three different samples (urine at the farm, urine at the slaughterhouse and adrenal glands) in order to have matched data.

2.3.3 Sample size

The urine and adrenal glands investigated in this work came from pigs that were under veterinarian control for 90 days before slaughter. The sampling therefore had to be made on an appropriate number of animals that would assure detection of the predicted prevalence, i.e. the expected frequency of endogenous prednisolone detection in urine and adrenal glands. The sample size calculation was made

according to Bottarelli and Ostianello (2011) using the following equation: $n = Z^2 \times [P (1 - P)]/D^2$, where n is the sample size, Z is the Student's t value (1.96, when the level of significance is 5%), P the expected prevalence and D the required precision. At the time the experimental protocol was designed, we had no data on the frequency of prednisolone detection in pig urine, but only from bovines (cows at slaughter = 71% positive; Bertocchi et al. 2013) and race horses (78.5%; Fidani et al. 2012). Based on these frequencies, on their difference between cows and horses, and because the pigs, i.e. a different species, are studied in this work, we supposed a prevalence for prednisolone detection in pig urine collected at the slaughterhouse of 70% (P = 0.7); and a precision of ±10% (D = 0.1). The necessary sample size predicted was 80 animals.

2.3.4 Pig urine sample extraction

Sample preparation was conducted as previously reported in Arioli et al. (2010) with slight modifications. An aliquot of 2 ml of each urine sample was spiked with prednisolone- d6 as internal standard to a concentration of 2 ng ml^{-1}. A 4 ml mixture of tert-butyl methylether:ethyl acetate (4:1, v/v) was then added. After shaking in a vertical rotary shaker for 20 min, the sample was centrifuged at 1300 g for 15 min. The upper organic layer was collected and dried under vacuum in a centrifugal evaporator at a temperature of 30°C. The residue was dissolved in 200 µl of the mixture of methanol/aqueous formic acid 0.1%, 50:50 v/v and transferred to an autosampler vial for the LC–MS^2 analysis. The injection volume was 10 µl.

2.3.5 Pig adrenal gland sample extraction

Sample preparation was conducted as previously reported in Bertocchi et al. (2013). A 5 g portion of the adrenal gland was transferred to a 50 ml tube and spiked to a concentration of 10 ng ml^{-1} with the internal standard prednisolone-d6. After the addition of 10 ml water, the sample was homogenised in a dispersing machine operating at a speed of 13,500 rpm for 1 min. A 4 ml mixture of tert-butyl methyl ether:ethyl acetate (4:1, v/v) was then added. After shaking in a vertical rotary shaker for 20 min, the sample was centrifuged at 3000 g for 15 min. The tube was then put in a freezer for about 1 h until lipid solidification. The organic liquid supernatant was transferred to a glass 10-ml tube; the solid lipid layer was placed in a polypropylene 15-ml tube and centrifuged again to recover residual liquid, which was transferred to the glass tube. The aqueous phase was then re-extracted (as described above) and the supernatant liquid was added to the two portions already placed in the glass tube. The sample was then dried under vacuum in a centrifugal evaporator. The residue was dissolved in 250 µl of methanol:

aqueous formic acid 0.1%, 50:50 v/v, 1.5 ml of petroleum ether was added, and then the sample was vortexed for 30 s and centrifuged for 5 min at 3000 g. The lower aqueous phase was then quantitatively (200 µl) transferred to an autosampler vial. The injection volume was 20 µl.

2.3.6 LC-MS² analysis

Chromatographic separation was performed with a Thermo Finnigan LC system consisting of a Surveyor MS quaternary pump (Thermo Fisher Scientific, San Jose, CA, USA) operating at a flow rate of 250 µl min^{-1} and a Synergi Hydro RP column 150 × 2.0 mm, internal diameter 4 µm (Phenomenex, Torrance, CA, USA), kept at 30°C. The mobile phase was aqueous formic acid 0.1% (eluent A) and methanol (eluent B). The gradient programme, lasting 31 min, was as follows: A was at 75% at minute 0, decreased to 20% over 20 min, then to 5% for 1 min, maintained for 3 min, and increased again to 75% from the 24th to the 26th minutes; the last 5 min were in an isocratic elution (A = 75%). The mass spectrometer was a TSQ Quantum (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray interface (ESI) set in the negative ionisation mode. The capillary voltage was 3.2 kV, ion transfer capillary temperature was 340°C, while the sheath and auxiliary gas (nitrogen) had arbitrary units of 30 and 10, respectively. The collision gas was argon at 1.5 mTorr. Three diagnostic transitions were

Table 1. MS² conditions for the MRM acquisitions of analytes and the internal standard. Ions for quantification are in bold. CE, collision energy expressed in electron volts (eV).

Analyte	Precursor ion $[M+HCOO]^-$ (m/z)	Product ions$_{CE}$ (m/z)	ESI
Cortisol	407	282_{37}, **297_{33}**, 331_{30}	(-)
Prednisolone	405	187_{30}, **280_{35}**, 329_{19}	(-)
Prednisolone-d6 (IS)	411	284_{37}, 299_{32}, **333_{19}**	(-)

monitored, in multiple reaction monitoring (MRM), for the analytes and internal standard. The quantification was performed on transition with the higher signal-to-noise ratio. Table 1 shows the precursor ions, i.e. the formiate adducts ($[M+HCOO]^-$), the product ions and the collision energies. Data were acquired using Xcalibur™ software from Thermo.

2.3.7 Method validation

The method was validated for prednisolone and cortisol, according to Commission Decision 2002/657/EC requirements (European Community 2002). The instrumental linearity was evaluated by preparing eight point calibration curves in the mobile phase containing a fixed amount of internal standard prednisolone-d6 (2 ng ml^{-1}) and analytes at concentrations corresponding to 0.01–0.05–0.1–0.2–0.5–1–2–5 ng ml^{-1}. Matrix calibration curves were obtained by spiking urine samples and adrenal glands with the analytes, resulting in three analytical series, each with three concentration levels (0.05–0.1–0.2 ng ml^{-1} for urine and 0.1–0.2– 0.3 ng g^{-1} for adrenal glands) and six samples per concentration level (6 samples × 3 concentration levels × 3 series = 54 analyses for each matrix). Method recovery and precision were evaluated using these matrix curve results; recovery was expressed in terms of percentage of measured concentration to fortified concentration and precision as the coefficient of variation (CV) calculated by applying one-way analysis of variance (ANOVA) for the intra-day and inter-day repeatability. The decision limit (CCα) and detection capability (CCβ) were calculated according to the procedure described in the Commission Decision 2002/657/EC as clarified in the document SANCO/2004/2726-revision 4 (European Union 2008). Specificity identification was achieved by detecting the peaks in the blank matrix chromatograms matching the relative retention time observed for the spiked analytes, compared to standard analytes in methanol, with a tolerance of ± 2.5%.

2.3.8 Statistical analysis

The Kolmogorov–Smirnov test was performed to check the normality of data sets: depending on whether this test was positive or negative, the correlation of the data sets was verified through the Pearson or the non-parametric Spearman test. The results obtained from farm and slaughterhouse urine were compared using one of the following tests depending on correlation, standard deviation (equal or different), and normality of the data sets: the unpaired t-test; the Wilcoxon matched-pairs signed-ranks test; and the Mann–Whitney test. When three data sets were compared, the non-parametric analysis of variance (Kruskal– Wallis test for unpaired data and non-normal distributions) with Dunn's Multiple Comparisons post-test was used. The null hypothesis was set at $P > 0.05$. GraphPad InStat version 3.10 for Windows (GraphPad Software, San Diego, CA, USA) was used.

2.4 Results and discussion

2.4.1 Method validation

The instrumental linearity for prednisolone ($r^2 = 0.991$) and cortisol ($r^2 = 0.994$) were both satisfactory. The validation parameters, shown in Table 2, demonstrated the good performance of the analytical methods in urine and adrenal glands. As regards specificity, blank and spiked samples did not show any interference (signals, peaks, ion traces) in the region of interest where peaks for cortisol and prednisolone were expected.

2.4.2 Sample analysis

The hypothesis made by Delahaut et al. (2014) that prednisolone can be endogenously produced was checked on 80 pigs, a number calculated as already described in the "Sample size" section. In Figure 1, a representative chromatogram and the relative ion spectra of cortisol and prednisolone in a urine sample are shown. The overall results obtained in this study are reported in Tables 3 and 4. Cortisol was always detected in urine and its concentration was significantly different (higher) when the sample was collected at the slaughterhouse ($P < 0.0001$). Despite the wide variability (relative standard deviations greater than

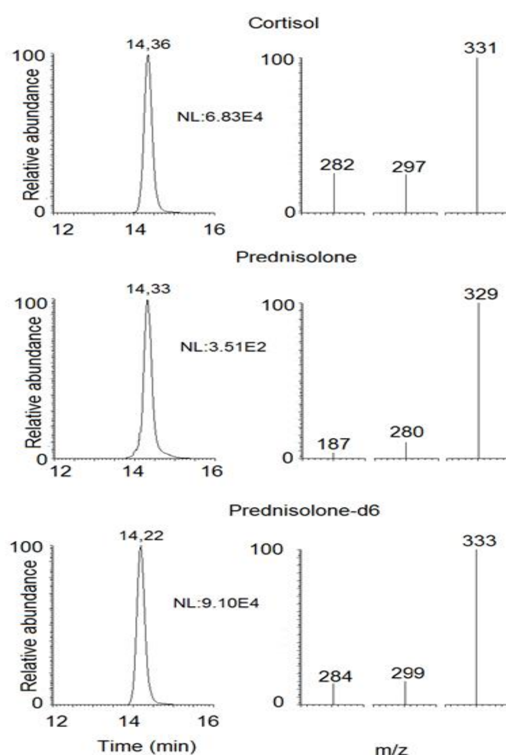

Figure 1. Reconstructed LC-MS2 chromatograms and respective ion spectra of the analytes detected in a urine sample. The calculated concentration of cortisol and prednisolone are 9.2 and 0.11 ng ml^{-1}, respectively

Table 2. Validation parameters of the analytical method. CCα and CCβ are expressed in ng ml−1 for urine and ng g−1 for adrenal glands

Matrix	Analyte	Recovery (%)	CV%		CCα	CCβ
			Intra-day	Inter-day		
Urine	Cortisol	98	7.3	9.6	0.06	0.07
	Prednisolone	100	9.3	13.3	0.07	0.09
Adrenal glands	Cortisol	96	13.5	15.3	0.20	0.25
	Prednisolone	93	7.8	12.3	0.12	0.20

Table 3. Overall analytical results

	Prednisolone			Cortisol		
	Farm urine	Slaughter urine	Adrenals	Farm urine	Slaughter urine	Adrenals
Samples	80	80	80	80	80	80
Positive (%)	44 (56%)	68 (85%)	71 (89%)	80 (100%)	80 (100%)	80 (100%)
Mean ± SD (ng ml^{-1})	0.23 ± 0.48[a,b]	0.42 ± 0.29[c]	1.4 ± 1.6	8.1 ± 6.4[a]	71.4 ± 64.9	2001 ± 1405

[a]Different from the corresponding corticosteroid in urine at the slaughterhouse (Mann–Whitney Test, P < 0.0001); [b] no correlation with cortisol in farm urine (Spearman r = −0.02, P > 0.05); [c] correlation with cortisol in slaughter urine (Spearman r = −0.38, P > 0.01).

80%), the difference was extremely significant, making the data even more meaningful for the influence of stress due to transport and slaughter on cortisol release. The observed variability could be explained by the circadian rhythm of cortisol secretion in pigs (Ruis et al. 1997) and by inter-individual variability in its urinary excretion. The selection of pigs and the time at which urine samples were collected at the farm and slaughterhouse followed the Official Collection Schedule, so we could not control these factors. As regards prednisolone, the concentration of this corticosteroid in urine from the farm was different with respect to urine from the slaughterhouse (P < 0.0001), as well as the frequency of its detection. For both parameters, the value at the farm was lower than that at the slaughterhouse, so demonstrating the influence of stress as for cortisol. Based on the positivity of urine for prednisolone at the farm or slaughterhouse, the data were divided into four groups as shown in Table 4. Group 1 consisted of 10 animals negative both at the farm and at the slaughterhouse; Group 2 consisted of 26 pigs negative at the farm but positive at the slaughterhouse; Group 3 consisted of 42 pigs positive in both cases; Group 4 consisted of just 2 animals positive at the farm and negative at the slaughterhouse. Table 5 shows the mean ± SD of urine and adrenal concentrations of cortisol and prednisolone in these groups.

Table 4. Relationship between prednisolone detection and the place of urine collection for the same pig.

Place of urine collection and detection of prednisolone			
	Farm	Slaughterhouse	No. of pigs
Group 1	Not detected	Not detected	10
Group 2	Not detected	Detected	26
Group 3	Detected	Detected	42
Group 4	Detected	Not detected	2

Group 4 was not considered due to the very small number of values. In Table 6 the normality, correlation and comparison tests are reported. Either the Pearson or Spearman test was performed to check the correlation between different data sets, in the first case between data sets that included the results from the same urinary corticosteroid collected at the farm or at the slaughterhouse, respectively (Table 6A); in the second case, between data sets that included the results from the two different urinary corticosteroids collected at the same place (Table 6B); in the third case, between data sets that included the results from the two different corticosteroids

Table 5. Cortisol and prednisolone urinary (ng ml−1) and adrenal (ng g−1) levels expressed as mean ± SD values considering three groups, partitioned accounting for prednisolone detection in urine and place of urine collection for the same animal

	Group 1: always negative to prednisolone	Group 2: negative to prednisolone at the farm and positive at the slaughterhouse	Group 3: always positive to prednisolone
Urine samples	10	26	42
Cortisol farm	7.7 ± 4.9	5.7 ± 3.7	9.4 ± 7.9
Prednisolone farm	nd	nd	0.24 ± 0.49
Cortisol slaughterhouse	24.6 ± 9.0[a,b]	73.4 ± 51.8[c]	83.1 ± 75.5[d]
Prednisolone slaughterhouse	nd	0.37 ± 0.24	0.46 ± 0.31[e]
Adrenal samples positive to prednisolone	10	23	36
Adrenal cortisol	2180 ± 1084	2184 ± 1022	2175 ± 1546
Adrenal prednisolone	1.3 ± 0.6	1.3 ± 1.1	1.6 ± 1.3

nd, not detected. [a] Different from Group 2 and Group 3 (Kruskal–Wallis test and Dunn's Multiple Comparisons post-test, $P < 0.05$); [b] different from Group 1 urinary cortisol at the farm (unpaired t-test, $P < 0.0001$); [c] different from Group 2 urinary cortisol at the farm (Mann–Whitney test, $P < 0.0001$); [d] different from Group 3 urinary cortisol at the farm (Wicoxon test, $P < 0.0001$); [e] different from Group 3 urinary prednisolone at the farm (Mann–Whitney test, $P < 0.0001$).

Table 6. Statistical analyses, performed on Groups. (A) Normality, correlation and comparison of the urinary concentration of the same corticosteroid at the farm and at the slaughterhouse; (B) normality and correlation of the urinary concentrations of the two distinct corticosteroids at the farm and or the slaughterhouse; (C) normality and correlation of the concentration of the same corticosteroid in adrenal glands. The Groups considered are related to prednisolone detection in urine collected at the farm and at the slaughterhouse as shown in Table 4

		Matrix	Analyte	Normality	Correlation	Mean or median comparison
A	**Group 1**	Farm urine	Cortisol	yes	Pearson r=-0.43 P>0.05, NS	Unpaired t-test P<0.0001, S
		Slaughter urine	Cortisol	yes		
	Group 2	Farm urine	Cortisol	no	Spearman r=0.04 P>0.05, NS	Mann-Whitney test P<0.0001, S
		Slaughter urine	Cortisol	yes		
	Group 3	Farm urine	Cortisol	no	Spearman r= 0.30 P>0.05, NS	Wilcoxon test P<0.0001, S
		Slaughter urine	Cortisol	no		
		Farm urine	Prednisolone	no	Spearman r=0.11 P>0.05, NS	Mann-Whitney test P<0.0001, S
		Slaughter urine	Prednisolone	no		
B	**Group 2**	Slaughter urine	Cortisol	yes	Spearman r=0.47 P<0.05, S	Distinct corticosteroids
		Slaughter urine	Prednisolone	no		
	Group 3	Farm urine	Cortisol	no	Spearman r=0.04 P>0.05, NS	Distinct corticosteroids
		Farm urine	Prednisolone	no		
		Slaughter urine	Cortisol	no	Spearman r=0.33 P<0.05, S	Distinct corticosteroids
		Slaughter urine	Prednisolone	no		
C	**Group 1**	Adrenal glands	Cortisol	yes	Pearson r= 0.81 P<0.01, S	Distinct corticosteroids
		Adrenal glands	Prednisolone	yes		
	Group 2	Adrenal glands	Cortisol	yes	Spearman r= 0.67 P<0.001, S	Distinct corticosteroids
		Adrenal glands	Prednisolone	no		
	Group 3	Adrenal glands	Cortisol	no	Spearman r=0.73 P<0.0001, S	Distinct corticosteroids
		Adrenal glands	Prednisolone	no		

S: significant
NS: non significant

in the adrenal glands (Table 6C). A comparison of means or medians (depending on the result of the Kolmogorov– Smirnov normality test) was made only between data sets for the same urinary corticosteroid collected at the farm and at the slaughterhouse (Table 6A). A difference between urinary cortisol collected at the farm or at the slaughterhouse was always found, independent of the group. Urinary cortisol concentrations in the three groups at the farm ranged between 7.7 ± 4.9 and 9.4 ± 7.9 ng ml^{-1} and were not significantly different by the Kruskal–Wallis test. However, when the same test was made for urinary cortisol at the slaughterhouse, a difference was found ($P < 0.05$) between groups: Group 1 differed from Groups 2 and 3 ($P < 0.05$) (Table 5). It must be noted that in Group 1 urine, prednisolone was never detected, while in Group 2 it was found at the slaughterhouse (0.37 ± 0.24 ng ml^{-1}) and in Group 3 a significant rise ($P < 0.0001$) in prednisolone concentration was observed

between farm (0.24 ± 0.49 ng ml^{-1}) and slaughterhouse (0.46 ± 0.31 ng ml^{-1}) urine. Also in Group 2, a rise in prednisolone concentration was actually observed, from "not detected" to 0.37 ± 0.24 ng ml^{-1}. The similar behavior of prednisolone in Groups 2 and 3 could be related to their similar urinary cortisol concentrations at the slaughterhouse: this could be interpreted as further evidence for the relationship between prednisolone and cortisol. A difference between the urinary levels of each corticosteroid collected at the different places is also observable within the same group (Table 6A). As regards the adrenal glands, no difference was observed between Groups 1, 2 and 3 for both cortisol and prednisolone levels, as the P value of the Kruskal–Wallis test was higher than 0.05. It is worthy of note that in the three groups the Spearman and Pearson tests evidenced significant correlations between cortisol and prednisolone levels in the adrenal glands, thus demonstrating an endogenous origin of prednisolone. (Table 6C). Our data on urine collected at the farm do not completely agree with Delahaut et al. (2014), who found a very good correlation coefficient value of 0.81 between prednisolone and cortisol levels in untreated pigs. We could not find this correlation at the farm, but only at the slaughterhouse (Tables 3 and 6B). The positive correlation between prednisolone and cortisol at the slaughterhouse seems to demonstrate a mutual rise in their concentrations, a condition that should exclude treatment with prednisolone, as checked by Official Veterinarians. A doubt about one sample out of 80 could arise: the concentration of prednisolone in urine collected at the farm was 3.3 ng ml^{-1} and that of cortisol at the slaughter was 0.69 ng ml^{-1}. However, the levels of cortisol and prednisolone in the adrenal glands were 3691 and 2.6 ng g^{-1}, respectively, quite a bit higher than the mean values found in this study, showing no inhibition due to treatment with corticosteroids. Confirmation of the presence of prednisolone in the adrenal glands was made by LC-MS^3 on 8 samples already analysed by LC-MS2. The

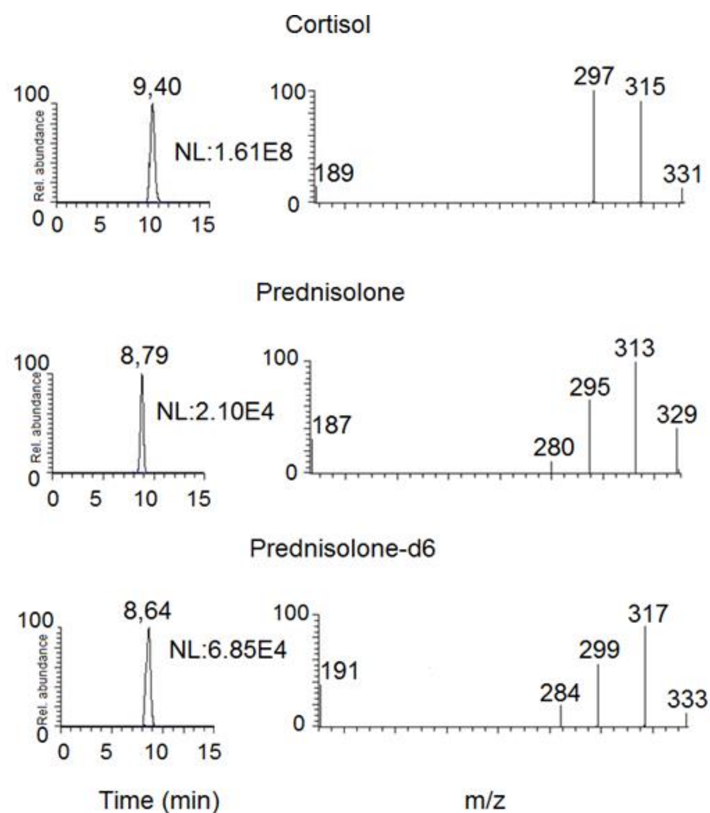

Figure 2. Reconstructed LC-MS3 chromatograms and respective ion spectra of the analytes detected in an adrenal gland sample. The calculated concentration of cortisol and prednisolone are 4.4 µg $g^{-1}$and 1.4 ng g^{-1}, respectively

analysis, already used for bovine adrenal glands by Bertocchi et al. (2013) was performed with an ion trap in the negative ESI mode. The results, performed only through qualitative determination, fully confirmed those reported in this work. A reconstructed chromatogram with the relative ion spectra is shown in Figure 2. Finally, the possibility of setting a cut-off level, calculated in an analogous way to the one proposed by de Rijke et al. for cattle (2014) should not be discarded. The threshold level for a finding of prednisolone in pig urine would be equal to the mean value of 80 urine samples + 3 × SD. Accounting for both urine at the farm and at the slaughterhouse, the average concentration is 0.35 ng ml^{-1} and the standard deviation is 0.38 ng ml^{-1}. The cut-off value would be 1.51 ng ml^{-1} (0.35 + 3 × 0.38).

2.5 Conclusions

The possibility that prednisolone is endogenously produced in pigs was directly demonstrated by its presence in the adrenal glands, the organ in which cortisol is produced. Indirect evidence was also provided about the origin of prednisolone that considered its relationship to cortisol levels under different conditions. First, both prednisolone and cortisol urinary concentrations were higher at the slaughterhouse than at the farm because of the stress the animals underwent. Second, Groups 2 and 3, in which a rise in prednisolone urinary concentration was observed at the slaughterhouse, showed a higher concentration of cortisol with respect to Group 1, in which prednisolone was never found. Third, in the adrenal glands, cortisol and prednisolone levels were positively correlated in the three groups. The similar trends in their concentrations and the positive correlation demonstrate the endogenous nature of prednisolone. Due to these considerations, a cut-off level was calculated as a starting point for regulatory control purposes. Moreover, in order to understand the mechanism leading to the formation of prednisolone in pigs, further studies on its metabolites, like 6β-hydroxyprednisolone, 20α-hydroxyprednisolone, and 20β-hydroxyprednisolone, must be carried out.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Chapter III

Determination of Thyreostats in Bovine Urine and Thyroid Glands by HPLC–MS/MS

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3. Determination of Thyreostats in Bovine Urine and Thyroid Glands by HPLC–MS/MS

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3.1 Abstract

The use of thyreostats in livestock is strictly forbidden by European legislation since 1981. The investigation of thyreostats is commonly performed by their detection as derivatives with 3-iodobenzylbromide. Although it has advantages, the derivatisation procedure can generally cause a decrease in analyte concentrations. With the aim of simplifying the analysis of five thyreostats in both bovine urine and in thyroid glands, two methods were developed without the derivatisation step. Salting-out assisted liquid– liquid extraction was carried out for both matrices, followed by high-performance liquid chromatography coupled with triple-quadrupole mass spectrometry analysis. The methods were validated in agreement with the guidelines of Commission Decision 2002/657/EC. For all the thyreostats evaluated, satisfactory results were achieved; the recovery was within 96–104 % for both the matrices, while precision (coefficient of variation) was less than 20 % for urine and 21 % for thyroid glands. The limits of decision and capacities of detection for all the compounds were lower than the recommended values of 10 μg L^{-1} and 10 μg kg^{-1}, respectively. In urine, the limits of decision ranged from 6.9 to 7.3 μg L^{-1}, and the capacities of detection ranged from 8.5 to 9.7 μg L^{-1}, while in thyroid glands these values varied from 6.6 μg kg^{-1} to 7.4 μg kg^{-1} and from 8.0 μg kg^{-1} to 9.7 μg kg^{-1}, respectively. The results obtained show that the methods described are suitable for the direct detection of thyreostats in bovine urine and thyroid glands.

3.2 Introduction

Thyreostats are drugs that interfere with the mechanism involved in the synthesis of thyroid hormones and cause a condition of deficiency of circulating thyroxine (T4) and triiodothyronine (T3) [1, 2], whose production and release are controlled by the hypothalamus–anterior pituitary axis. The hypothalamus secretes thyrotropin-releasing hormone (TRH), which in turn stimulates the anterior pituitary gland to release thyroid-stimulating hormone (TSH) that induces the production of T3 and T4 by the thyroid, which releases them into the bloodstream. These hormones activate the nuclear

transcription of a large number of genes, thus causing the synthesis of enzymes, as well as structural and transport proteins. This leads to an increase in metabolism and maintains the physical and psychological development of the organism. The administration of thyreostats causes an improvement in bodyweight gain mainly due to increased absorption and extracellular retention of water in the edible tissues and in the gastrointestinal tract [3]. Thyreostats are polar amphoteric thionamides with a heterocyclic tautomeric structure, and are mostly derived from thiouracil and mercapto-imidazole. The sequence consisting of nitrogen–carbon–sulphur, known as thioamide, is considered responsible for the thyroid-inhibiting activity (Fig. 1). The best known thyreostatic drugs include the very potent thyroid-inhibiting compounds 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU) and 1-methyl-2-mercaptoimidazole (tapazole, TAP) [4–6]. The chemical structures of these substances are shown in Fig. 1.

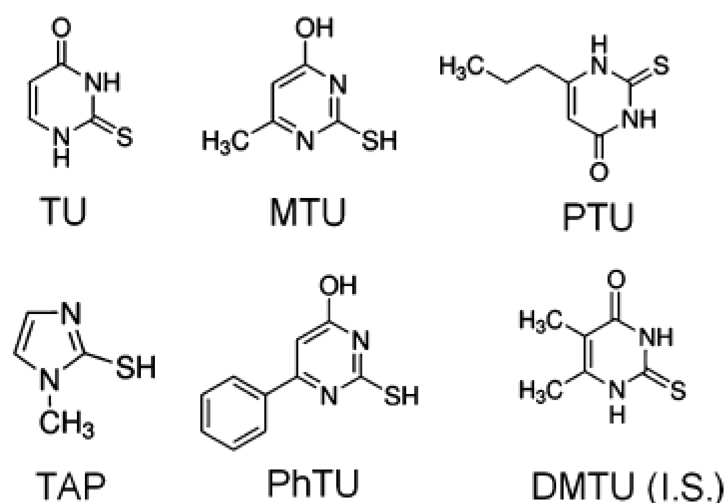

Fig. 1 Chemical structure of thyreostats. TU (2-thiouracil), MTU (6-methyl-2-thiouracil), PTU (6-propyl-2-thiouracil), PhTU (6-phenyl- 2-thiouracil), TAP (1-methyl-2-mercapto-imidazole; tapazole), DMTU (5,6-dimethyl-2-thiouracil; internal standard)

The fraudulent use of thyreostats produces low-quality meat. Moreover, the edible tissues derived from treated animals might represent a potential risk to the consumer's health due to the presence of residues and their teratogenic and carcinogenic effects [7–11]. In 1981, the European Union banned their use in animal production both as growth promoters and therapeutic agents [12] and classified them as "substances having anabolic effects and unauthorized substances" belonging to the group A2 as described by the Council Directive 96/23/ CE [13]. However, a relationship between the presence of Brassicaceae in feed and thiouracil in urine has been demonstrated by Pinel et al. [9], Vanden Bussche

et al. [14] and Kiebooms et al. [15, 16]. The Community Reference Laboratories (CRLs) in 2007 proposed a recommended concentration of 10 μg L^{-1} in urine and 10 μg kg^{-1} in thyroid tissue for the purpose of control, as "low concentrations of thiouracil have been detected in bovine animals fed with cruciferous plants, however, there is scientific evidence showing that levels above 10 ppb in urine cannot be linked to natural origin due to this contamination" [17]. Recently, Wauters et al. reported concentrations of up to 18.2 μg L^{-1} in the 99 % percentile from 3894 bovines and they suggested that the recommended concentration should be increased to 30 μg L^{-1} [18]. In fact, the 2015 Italian National Residue Plan already provides this concentration as the limit of detection for thyreostats in urine [19]. Thyreostats analyses typically consist of separation methods based on gas or liquid chromatography associated with a mass spectrometry system of detection. Normally, the extraction of the substances is carried out using polar solvents more suitable to the chemical characteristics of the thyreostats, such as methanol, acetonitrile or ethyl acetate. Further steps of purification or clean-up with different kinds of solid-phase extraction (SPE) have been reported. Due to the low molecular mass and high polarity of the thyreostats, several authors have proposed a derivatisation step before or after the clean-up, mainly using 3-iodobenzylbromide (3-IBBr) in the case of HPLC–MS/MS analysis [6]. In the case of GC methods, derivatisation is an unavoidable step to convert the analytes into volatile compounds. When HPLC is applied as the separation technique, analytes may be derivatised and, in the analysis of thyreostats, this procedure induces the stabilisation of the chemical structure of the molecule in a specific and single tautomeric form, the reduction of the molecular polarity to increase the separation characteristics on the reversed-phase column in the case of HPLC–MS detection, and an increase in the molecular mass [20]. The low molecular mass, particularly, could be disturbed by the chemical noise. In term of sensitivity, the derivatisation leads to an improvement of the signal to noise ratio, and subsequently of the detection capabilities [21]. Despite these advantages, the derivatisation procedure can generally cause a loss in analyte concentrations. Furthermore, removing derivatisation step simplifies, shortens and makes cheaper the whole analysis procedure [22, 23]. Based on these observations, we developed the extraction without derivatisation of the five above-mentioned thyreostats in bovine urine and thyroid glands followed by a sensitive, specific and reproducible HPLC–MS/MS analysis. For the full identification and quantification of the analytes, the criteria established in the 2002/657/EC Commission Decision were followed and the decision limit (CCα) and the detection capability (CCβ) were calculated according to the matrix calibration curve procedure as clarified in the document SANCO/2004/2726 rev. 4 [24, 25].

3.3 Materials and Methods

3.3.1 Reagents and Chemicals

All solvents were of HPLC–MS grade quality and purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid (98–100 %) was from Riedel-de Haën (Sigma- Aldrich). Ultrapure water was obtained through a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). KH_2PO_4 and NaCl were from Sigma-Aldrich. The analytes 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), 2-mercaptobenzimidazole or tapazole (TAP) were acquired from Sigma-Aldrich, as well as 5,6-dimethyl-2-thiouracil (DMTU), used as internal standard (IS). A stock solution of 1 mg mL^{-1} was prepared by dissolving the compounds in methanol. Serial dilutions were prepared by diluting the stock solution in the mobile phase, which were then stored at −40 °C.

Table 1 Gradient table for HPLC method

Time (min)	Eluent A (%)	Eluent B (%)	Flow rate (µl min^{-1})
0	90	10	200
2	90	10	200
20	30	70	200
24	10	90	200
27	90	10	200
30	90	10	200

A 0.1 % aqueous formic acid, B methanol

Phosphate buffer, prepared by dissolving 0.25 M KH_2PO_4 in ultrapure water, was adjusted to pH 7 and then saturated with 0.1 % DL-dithiothreitol (DTT; Sigma- Aldrich) as in Vanden Bussche et al. [11].

3.3.2 Sample Collection

Urine and thyroid gland samples from Friesian Cows aged 32–63 months were collected in a Lombard abattoir after slaughtering, immediately frozen and taken to the laboratory for storage at −40 °C until analysis.

3.3.3 Sample Extraction

Urine

One millilitre of bovine urine was transferred to a 15-mL glass tube and spiked with 10 ng of internal standard (DMTU) to give a final concentration of 10 µg L^{-1}, then vortexed and left for 5 min to equilibrate. The samples then underwent denaturation conditions at 65 °C for 30 min, after the addition

of 1 mL of PBS buffer with 0.1 % DTT at pH 7. NaCl (2 g) was added to the solution to mixture as a salting-out reagent. The extraction was performed by twice repeating these steps: addition of 5 mL tert-butyl methyl ether, centrifugation at 2000×g for 5 min at 4 °C, and collection and transfer of the upper organic layer to a 10-mL polypropylene tube. The extract was dried under vacuum in a rotary evaporator apparatus (Heidolph Instruments GmbH & Co., Schwabach, Germany) at a temperature of 40 °C. The residue was dissolved in 200 µL of the mobile phase (methanol: 0.1 % aqueous formic acid, v/v 50:50) and transferred to vials for HPLC. The injection volume was 10 µL.

Thyroid Gland

The thyroid gland samples were minced with surgical scissors and homogenised. The sample (1 g) was weighed in a polypropylene tube and 10 ng of internal standard (DMTU) were added, and then the sample was vortexed and left for 5 min to equilibrate, then 5 mL of methanol was added. The samples were vortexed, placed in an ultrasonic bath for 10 min and then centrifuged at 2000×g at 4 °C for 10 min. The organic liquid supernatant was then filtrated and transferred to a 15-mL glass tube and 5 mL of PBS buffer with 0.1 % DTT at pH 7 were added. The samples underwent denaturation conditions at 65 °C for 30 min. To carry out the extraction of the analytes, 2 × 10 mL of Tert-butyl methyl ether and 4 g of NaCl (used as a salting-out reagent) were added to the solution. The sample was centrifuged at 2000×g for 5 min at 4 °C. The upper organic layer was collected and transferred to a 50-mL glass evaporating flask. Lastly, the extracts were combined and dried under vacuum in a rotary evaporator apparatus at 40 °C. The residue was dissolved in 200 µL of the mobile phase and transferred to vials for the autosampler. The injection volume was 10 µL.

3.3.4 HPLC–MS/MS analysis

A Synergi Hydro RP reverse-phase HPLC column C18 (150 × 2.0 mm, i.d. 4 µm) with a C18 4 × 3.0 mm guard column (Phenomenex, Torrance, CA, USA) at a column oven temperature of 30 °C was used for the separation, which was performed by an HPLC system that included a Surveyor MS quaternary pump with a degasser, a Surveyor AS autosampler with a column oven, and a Rheodyne valve with a 20-µL sample loop (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase consisted of 0.1 % aqueous formic acid (solvent A) and methanol (solvent B), and the flow rate was set at 200 µL/min. The gradient program is shown in Table 1. The overall run time was 30 min. The HPLC system was connected to a TSQ Quantum (Thermo Fisher Scientific, San Jose, CA, USA) triple-quadrupole mass spectrometer with an electrospray interface (ESI) set in the positive (ESI+) ionization mode. The acquisition was made in the multiple reaction-monitoring (MRM) mode. The

specific acquisition parameters of all the analytes were optimised by means of direct infusion of standard solutions of the analytes at a concentration of 1 µg mL^{-1}, a flow rate of 50 µL min^{-1} and a flow rate of the MS pump of 100 µL min^{-1}. The capillary voltage was 3.2 kV; the capillary temperature was 340 °C; nitrogen was used as the sheath and auxiliary gas at 30 and 10 arbitrary units, respectively, and argon as the collision gas at 1.5 mTorr; peak resolution was 0.70 Da FWHM. The parent ions, product ions, and collision energy values for each analyte are shown in Table 2. The scan time for each monitored transition was 0.1 s and the scan width was 0.5 amu. The mass spectrometer data acquisition and processing were carried out using Xcalibur™ 2.0.7 SP1 software from Thermo Fisher Scientific Inc.

Table 2. MS/MS conditions for the MRM acquisitions of analytes and the internal standard.

Analyte	**Precursor ion [M-H]$^+$ (m/z)**	**Product ions$_{CE}$ (m/z)**	**ESI**
TAP	115	56_{22}, 57_{20}, $\mathbf{74_{17}}$, 83_{17}, 88_{16}	(+)
TU	128	57_{35}, 60_{34}, $\mathbf{70_{17}}$, 83_{27}, 111_{16}	(+)
MTU	143	60_{32}, 72_{34}, $\mathbf{84_{17}}$, 86_{23}, 126_{16}	(+)
PTU	171	60_{35}, 67_{26}, 86_{27}, $\mathbf{112_{19}}$, 154_{17}	(+)
PhTU	205	77_{41}, 86_{27}, $\mathbf{103_{26}}$, 105_{25}, 146_{19}	(+)
DMTU (IS)	157	60_{35}, 72_{29}, 86_{22}, $\mathbf{98_{18}}$, 140_{16}	(+)

Ions for quantification are in bold

IS internal standard, CE (eV) collision energy

3.3.5 Method Validation

The HPLC–MS/MS method was validated according to the guidelines of Commission Decision 2002/657/EC [24]. MS identification criteria were verified throughout the validation study by monitoring relative retention times, signal-to noise ratios (S/N) and ion ratios. The instrumental linearity was evaluated through calibration curves in solvent at six levels (1.0, 5.0, 10, 20, 50, 80, 100 µg L^{-1}) and 10 µg L^{-1} of DMTU as IS The method validation parameters were determined with fortified blank urine and thyroid gland samples at three concentration levels (5.0, 10, 15 µg L^{-1} and µg kg^{-1}) in six replicates on three different days (6 samples × 3 concentration levels × 3 series = 54 analyses). Method recovery and precision were evaluated using the matrix curves; recovery is calculated as ratio between the measured concentration to fortified concentration, corrected by internal standard and expressed in percentage; precision is calculated in terms of intra- and inter-day repeatability expressed as the coefficient of variability (CV). The same data from the matrix calibration

curves were used to calculate the decision limit (CCα) and the detection capability (CCβ) according to the matrix validation curve procedure described in the Commission Decision 2002/657/EC and clarified in the document SANCO/2004/2726-rev. 4 [24, 25].

3.4 Results and Discussion

3.4.1 Sample Preparation

Despite the diversity of the matrices analysed, we carried out two similar methods to prepare urine and thyroid glands to have the same steps for each matrix. A preliminary denaturation step of matrix proteins was carried out to disrupt the protein–thyreostat interaction, as reported by Vanden Bussche et al. [11], through the cleavage of the disulfide bonds of the proteins by the addition of a reducing agent, such as DTT. Differently from the above mentioned study, which considered only urine, we adopted this step for both urine and thyroid glands, with a ten-time lower concentration of DTT. The polarity of the thyreostats requires the use of an organic polar solvent to extract them from the matrices: we evaluated the applicability of different solvents by several tests using ethyl acetate, chloroform and tert-butyl methyl ether. Comparing the signal intensity of the analytes extracted with the three different solvents, tert-butyl methyl ether was chosen as the best solvent for the extraction. The poorest results were obtained by the extraction performed with ethyl acetate by which we could not extract most of the thyreostats. To facilitate the phase separation and to reduce the miscibility of the analytes in the aqueous phase, this protocol adopted the approach of salting-out-assisted liquid–liquid extraction (SALLE), adding salt (NaCl) prior to the liquid– liquid extraction to favour the transfer of the analytes into the organic solvent [26–28].

3.4.2 Method Validation

The analytical procedures developed were subjected to the validation process according to the Commission Decision 2002/657/EC and clarified in the document SANCO/2004/2726-rev. 4 [24, 25]. The HPLC–MS/MS-reconstructed chromatograms of the thyreostats in urine and thyroid glands are shown in Fig. 2. DMTU as the internal standard (10 μg L^{-1}) is also reported. The analytes were detected and confirmed based on their proper relative retention times and their ion ratios. The relative retention times were within a tolerance limit of 2.5 % and the relative ion intensities were within the maximum permitted tolerances [24]. The chromatograms in Fig. 3 show the absence of interference peaks at the expected retention times of the thyreostats, hence illustrating a good specificity and

selectivity of the method. For the HPLC–MS/MS confirmation of substances listed in Group A of Annex I of Directive 96/23/EC [13], a minimum of four identification points (IPs) is required [24]. In the present work, we monitored five products ions with the highest intensity. Each one of the five product ions is equal to 1.5 IPs, making a total of 7.5 IPs. The ion giving the highest signal-to-noise ratio was selected for the quantification. The MRM transition intensities were compliant with the maximum tolerances permitted. The parameters obtained for the method validations are given in Tables 3, 4, and 5. Linearity was verified using squared correlation coefficients (r^2): The regression coefficients of the curves that were built to check the instrumental linearity were

Fig. 2 HPLC-MS/MS chromatograms and ion spectra of a blank urine (a) and a thyroid gland (b) sample spiked with thyreostats at a final concentration of 5 μg L^{-1} or μg kg^{-1}, respectively. TU (2-thiouracil), MTU (6-methyl-2-thiouracil), PTU (6-propyl-2 thiouracil), PhTU (6-phenyl-2-thiouracil), TAP (1-methyl-2-mercaptoimidazole; tapazole). The concentration of DMTU (5,6-dimethyl-2-thiouracil; internal standard) is 10 μg L^{-1} or μg kg^{-1}, respectively

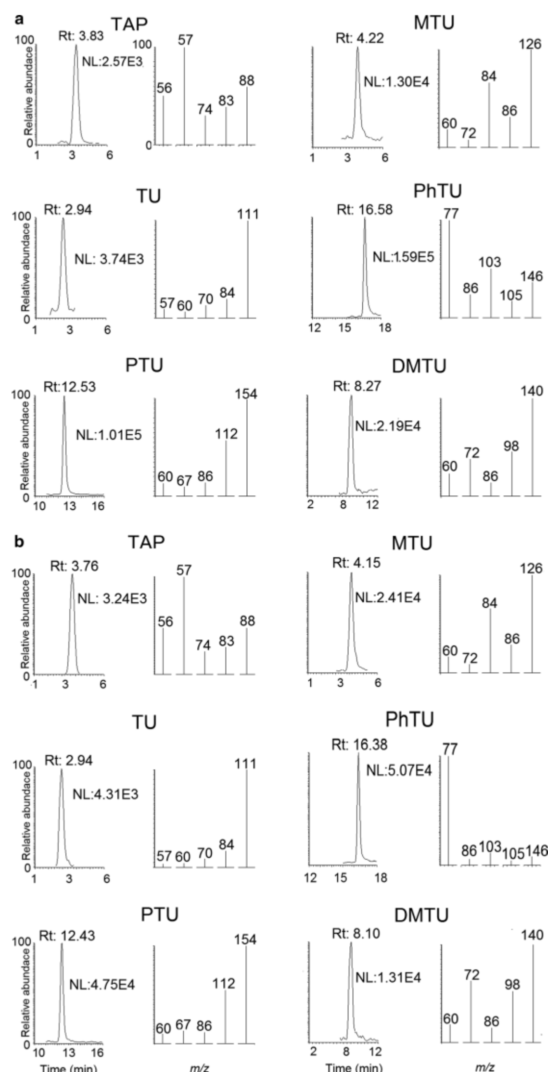

Fig. 3 HPLC-MS/MS chromatograms of a blank urine (a) and a thyroid gland (b) sample, showing the absence of interfering compounds. TU (2-thiouracil), MTU (6-methyl-2-thiouracil), PTU (6-propyl-2-thiouracil), PhTU (6-phenyl-2-thiouracil), TAP (1-methyl-2-mercapto-imidazole; tapazole)

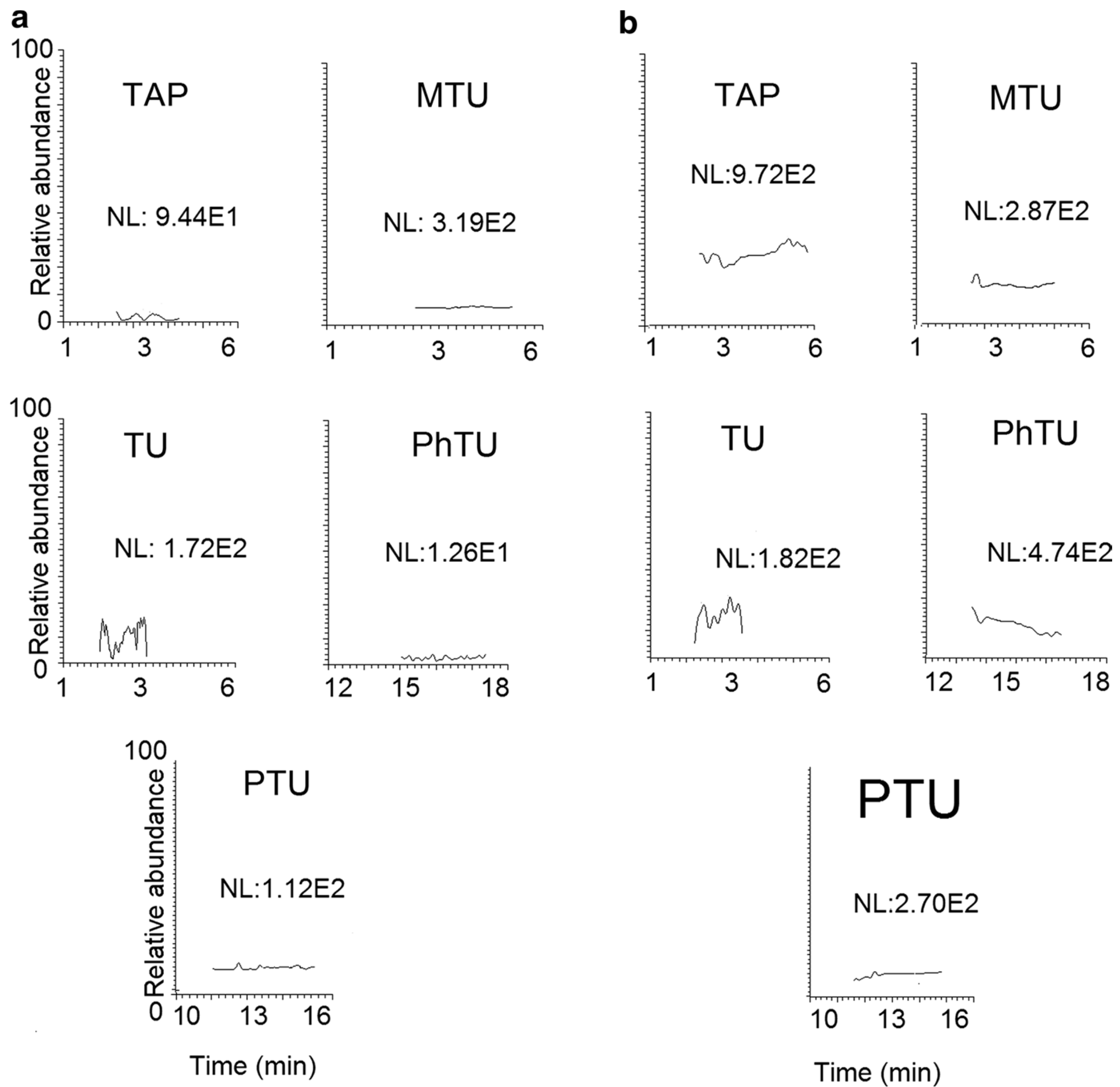

Table 3 Analytical performance (method trueness and precision) data for thyreostat determination in urine

Analyte	Concentration level (µg/L)	Recovery % (n= 18)	Repeatability	
			intra-day (CV; n=6)	inter-day (CV; n=18)
TAP	5	99	8	20
	10	101	5	19
	15	100	5	8
TU	5	104	15	20
	10	98	10	11
	15	101	5	5
MTU	5	104	6	19
	10	96	9	20
	15	101	7	9
PTU	5	104	12	19
	10	96	7	16
	15	101	5	7
PhTU	5	100	11	16
	10	100	5	13
	15	100	3	5

CV coefficient of variation

Table 4 Analytical performance (method trueness and precision) data for thyreostat determination in thyroid glands

Analyte	**Concentration level (µg/kg)**	**Recovery % (n= 18)**	**Repeatability**	
			intra-day (CV; n=6)	**inter-day (CV; n=18)**
TAP	5	104	7	19
	10	96	10	20
	15	101	8	10
TU	5	101	15	21
	10	99	9	17
	15	100	7	9
MTU	5	99	14	17
	10	103	9	10
	15	103	9	18
PTU	5	102	12	20
	10	98	6	17
	15	101	8	9
PhTU	5	100	12	14
	10	100	9	12
	15	100	9	9

CV coefficient of variation

Table 5 Decision limits (CCα) and detection capabilities (CCβ) calculated for thyreostats in urine and in thyroid glands

Analyte	CCα ($\mu g\ L^{-1}$ and $\mu g\ kg^{-1}$)		CCβ ($\mu g\ L^{-1}$ and $\mu g\ kg^{-1}$)	
	Urine	Thyroid gland	Urine	Thyroid gland
TAP	7.3	7.3	9.7	9.7
TU	7.3	7.4	9.2	9.7
MTU	7.2	7.0	9.5	8.7
PTU	7.2	7.4	9.2	9.6
PhTU	6.9	6.6	8.5	8.0

higher than 0.982, which indicates a satisfactory linearity for all the analytes. Good linearities were also achieved in urine and in thyroid glands and showed values higher than 0.978 and 0.973, respectively, thus demonstrating a suitable and adequate correlation between the concentration and the acquired response in the sample for both matrices. The precision of the method, which was calculated by applying one-way analysis of variance (ANOVA), was evaluated in terms of intra- and inter-day repeatability, and is expressed as the coefficients of variation (CV) from the replicate samples. Their values were lower than 23 %, as proposed by Thompson [29], demonstrating an acceptable precision for the method. The recoveries showed good values ranging from 96 to 104 % in urine and from 96 to 104 % in thyroid glands. The results regarding the precision, even if similar, are not comparable with the results obtained by Abuìn et al. [22, 30], who developed methods for the detection of underivatised thyreostats in thyroid, because of the lower concentrations used in this paper. The decision limit (CCα) and detection capability (CCβ) are very important, debated and decisive points to evaluate. For the estimation of these values, the document of the Commission Decision 2002/657/EC [24] explains both the definition and procedure. However, the approach proposed in the document to evaluate these limits—based on the extrapolation of the calibration curve procedure according to ISO 11843—may lead to an underestimation of the parameters, as already explained by Galarini et al. [31] and other authors [32, 33]. Therefore, CCα (and, consequently, CCβ) was determined using a parallel extrapolation to the x-axis at the lowest experimental concentration as clarified in the document SANCO/2004/2726-rev. 4 [25]. Decision limits achieved with this approach were thus experimentally determined, and therefore not underestimated. A comparison with previously published data concerning the detection of non-derivatised thyreostats should consider the differences in the method

of CCα determination. Table 5 shows the obtained CCα and CCβ values, which are lower than the minimum required performance limits (MRPLs) proposed in the CRL guidance document of 2007 in urine and in thyroid glands [17]. Moreover, the TAP analytical limits are lower than those reported in literature for the two matrices, such as MTU in the thyroid gland [11, 22, 30, 34]. Finally, it is worth noting that the validation parameters obtained with our method are comparable between the two different matrices.

3.5 Conclusion

The methods for the simultaneous direct identification and quantification of five thyreostats without derivatisation in both urine and thyroid gland samples were specific and sensitive. Moreover, the validated methods guarantee a better performance for TAP in both matrices than those reported in the literature. The choice to develop a method without derivatisation and clean-up steps was made due to the advantages in terms of costs and the time of analysis. The simultaneous determination of five thyreostats in two matrices using similar methods could be useful to make comparative analyses more reliable, because the process variables are the same for urine and thyroid glands. Furthermore, the measurement of the endogenous TU in urine and thyroid is possible as the analytical limits are all below 10 μg L^{-1} and 10 μg kg^{-1}, and particularly considering that the CCα which was determined as clarified by the document SANCO/2004/2726-rev. 4 [25] is not an estimate, but an experimentally verified concentration with all the characteristics required by the Commission Decision 2002/657/EC [24] for a substance to be quantified.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

Informed consent Not applicable.

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Chapter IV

Distribution of persistent organic pollutants (POPs) in wild Bluefin tuna (*Thunnus thynnus*) from different FAO capture zones

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4. Distribution of persistent organic pollutants (POPs) IN wild Bluefin tuna (*Thunnus thynnus*) from different FAO capture zones

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4.1 Abstract

Residues of environmental contaminants in food represent a concern in food safety programs. In this study, the distribution of persistent organic pollutants (POPs) were evaluated in 79 tuna samples from FAO areas 51 (Indian Ocean), 71 (Pacific Ocean), 34 (Atlantic Ocean), and 37 (Mediterranean Sea). 6 polychlorinated biphenyls (PCBs), 16 organochlorines (OCs) and 7 polybrominated biphenyl ethers (PBDEs) were selected as representative compounds according to EFSA POPs monitoring guidelines. An analytical method, based on Accelerated Solvent Extraction (ASE), with an "in-line" clean-up step and GC-MS/MS detection, was developed, validated and applied. PCBs were detected in all FAO areas, with a prevalence of 100% for most of them. In the FAO area 37, only, all PBDEs were detected. Only 5 OCs were detected. The results showed that POPs contamination of tuna reflects FAO area contamination; in particular FAO area 37 was the most polluted. Moreover, tuna muscle was an appropriate matrix for monitoring contamination and for obtaining information about food safety.

4.2 Introduction

Since the second half of the past century, a particular care has been devoted to the analysis of various essential elements and toxic contaminants in seafood in order to limit exposure of consumers to contaminants while maximizing the benefits of seafood consumption. (Herceg-Romanic et al., 2014). Fish possess clear nutritional benefits providing high quality protein, minerals, essential trace elements, fat-soluble vitamins (Vitamin D) and essential fatty acids (Da Cuna et al., 2011). However, fish is also known to bioaccumulate contaminants, such as toxic metals and Persistent Organic Pollutants (POPs), which can represent a risk for human health. Anthropogenic inputs of POPs into the marine environment have increased their levels to large extent within past a few decades.

The waters of estuaries, coastal areas and “enclosed” seas as the Mediterranean Sea are often characterized by high concentrations of variably toxic POPs among which are commonly found pesticides and heavy metals (Di Bella et al., 2006; Ansari et al., 2004). POPs represent the best-known contaminants; they are mostly man-made chemicals that might accumulate in the environment for a significant time (Gui et al., 2014) and bioaccumulate in organisms (due to their highly stability, low volatility and lipophilic nature), leading to the contamination of foodstuffs, even those not directly treated (Panseri et al., 2014). In fact, concerning seafood, once in the marine water, these compounds become distributed betweenwater phase and particulate matter, which acts as a sorbent and transports them into sediment, which serves both a sink and a source of contamination to the surrounding biota (Storelli and Perrone, 2010). So, marine organisms occupying a top trophic position in the marine ecosystem accumulate great concentrations of these lipophilic contaminants and can become more vulnerable to their toxic effects. Among POPs, polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCs), are two groups of the most studied contaminants. Although the production and usage of these compounds, in most industrialized countries, some of them, as DDT, were banned in the1970s, but they still persist in all parts of the environment because they are resistant to environmental degradation (Boethling et al., 2009). In effect, although PCBs and OCs levels in the environment are steadily declining (Albaiges et al., 2011), they continue to bioaccumulate in human and animal tissues and biomagnify in food chains, and may have potentially significant impacts on human health and the environment (Kljakovic-Gaspic et al., 2015). All these compounds are regulated by Stockholm Convention (2001), which aims to eliminate or restrict the production and use of POPs. In term of emerging classes of POPs, it is interesting to pose the attention to the presence of polybrominated diphenyl ethers (PBDEs), also known as brominated flame retardants (BFRs) that share a number of chemical features characteristics as well as bioaccumulation mechanisms similar to PCBs (De Boer et al., 2000). They are widely used industrial chemicals added to various materials important in manufacture of electronic equipment, upholstered furniture, construction materials, textiles to minimise or even suppress the combustion process. Thus given the ubiquity of plastics in the modern world, it is not surprising that PBDEs are being found in all environmental compartments, including aquatic ecosystem. Not only the capacity of PBDEs to bioaccumulate in biotic fatty tissues and biomagnify up the food chain (several studies demonstrated their occurrence in wildlife and human tissue) in combination with their resistance to degradation, but also their toxicity makes this class of chemicals of a high concern to the environment and human health. Furthermore, the European Commission has asked Member States to monitor the presence of BFRs in food over the next two years. The move is in response to EFSA's recommendation that more data on the levels of BFRs in food should be gathered. (Bragigand et al., 2006; McDonald, 2002; Commission Recommendation 3

March 2014). The Bluefin tuna, *Thunnus thynnus* (Linnaeus 1758), has a relevant importance for the sea ecosystems not only from an economic but from an ecological point of view as well. Bluefin tuna shows interesting and peculiar features that may affect their contaminant bioaccumulation. In fact, Bluefin tuna are the best example of a fast growing, long-lived, wide-ranging fish, capable of migrating from the Mediterranean Sea to the Atlantic Ocean and back. Then, they are top predators of the benthic-pelagic trophic web from the time they are yearlings, feeding on several species of small fish, crustaceans, and cephalopods; once adults, their diet becomes more specific, relying on large cephalopods and pelagic fish On the basis of above mentioned considerations the purpose of the present research was to evaluate the presence of different POPs (PCBs, OCs and PBDEs), in Bluefin tunas arising from four different FAO catch areas, in order to have an overview and mapping of their distributions. Tunawas chosen as fish species because is principally distributed from the offshore waters to the open seas in tropical and temperate regions almost all over the world, as in the Pacific, Atlantic, and Indian Oceans (Wilson et al., 2005) This species represent an important commercial fish product, and its ecology and biology has been well-studied (Fromentin and Powers, 2005). Then, the obtained values can be used to fill the database of levels of organic contaminants in seafood, in particular for flame retardants presence about which scarce literature exists and used for future risk assessment of the Italian population. Lastly the paper describes a rapid, accurate and sensitive method to determine multi-residues by GC-MS/MS (PCBs, organochlorines (OCs) and PBDEs) by using the Accelerated-Solvent-Extraction sample preparation method with "in-line" clean up purification approach. The attention regarding the sample preparation method should increase the overall sample laboratory throughput by decreasing time and cost requirements and at the same time be environmentally friendly.

4.3 Experimental procedure

4.3.1 Chemicals and reagents

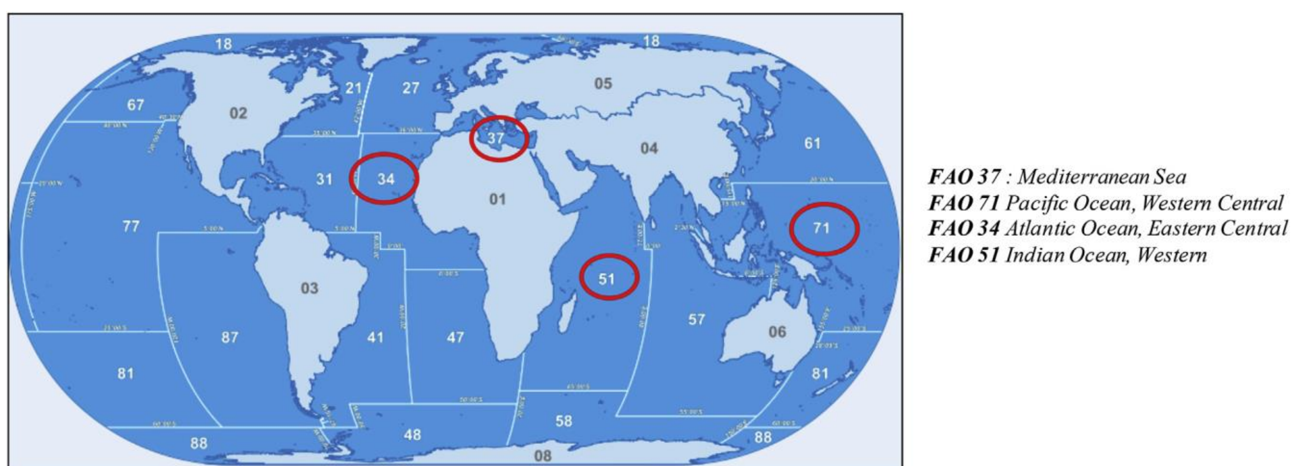
Mix solution of PCBs congeners (PCB 28; PCB 52; PCB 101; PCB 138; PCB 153 and PCB 180), PCB 209 (internal standard (IS) for PCBs), mix solution of PBDEs (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 154) and fluoro-bromodiphenyl ether (FBDE), IS for flame retardants, were purchased from AccuStandard (New Haven, USA). Standard solution of 16 OCs (α-HCH; Hexachlorobenzene; β-BHC; Lindane; Heptachlor; Aldrin; Heptachlor epoxide; Trans Chlordane; 4,4'-DDE; Endosulfan I; 2,4'- DDT; Endrin; 4,4'-DDD; Endosulfan II; 4,4'-DDT and Endosulfan sulfate) was purchased from Restek (Bellefonte, PA, USA). Silica gel 60 (0.063-0.200 mm) was purchased from Merck (Darmstadt, Germany). Hexane, isooctane, acetone (special grade for pesticide residue analysis (Pestanal)) and 4-nonylphenol (IS for OCs) were purchased from Fluka

(SigmaeAldrich, St.Louis, MO, USA). However, since a wide range of contaminants were included in the study, for some the Maximum Levels (MLs) were still below this concentration and for others they were well above this concentration.

4.3.2 Sample collection

A total of 79 Bluefin wild tunas (*Thunnus thynnus*) originating from different FAO catch areas were selected for this study. Details of sampling and biometric data are reported in Table S1. All tuna samples were provided by the most important tuna industry at the national level and by the Fish Market of Milan, from different FAO catch areas. All samples were captured and collected during April- May 2015. An overview of the sampling areas according to its FAO capture zone was shown in Fig. 1. Representative sample from each tuna was obtained by sampling fish tissue from 3 different anatomic zones (proximal, ventral and caudal); each sample was then stored at - 22 °C until the analyses.

Fig. 1. World FAO fishing zones and sampling sites (highlighted in red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



4.3.3 Accelerated solvent extraction (ASE) procedure with clean-up "in line"

In order to analyse a large number of pesticides from different classes, a simple extraction and clean up in single step ("in-line") method was optimised to expand range applicability. The extraction was performed using an ASE 350 (Thermo-Fisher Scientific, Waltham, MA, USA). A 33mL cells for accelerated solvent extraction (ASE) were used for the analysis. A representative portion (300 g) of tuna was obtained from each fish and minced, then 3 g were homogenized with an equal weight of Diatomaceous earths, sodium sulfate and transferred into the cell. 1 mL of isooctane solution

containing the three ISs was added (20 ng g^{-1} PCB 209; 2 ng g^{-1} FBDE and 50 ng g^{-1} 4-nonylphenol). To fill the remaining empty part of the cell diatomaceous earths were added. The cells were packed with one cellulose filter at the bottom followed by the fat retainer (10 g silica gel). The dried samples were transferred to the ASE cells. Temperature (80 °C), pressure (1500 psi), number of static cycles (3 min each), purging time (90 s with nitrogen) and rinse volume (90%) were fixed throughout the study. The extraction solvent was a mixture of hexane/acetone (4:1, v/v). Organic extracts were finally collected in 66 mL vials and treated with sodium sulfate to remove any possible humidity. Afterwards, the extract was collected and dried under vacuum in a centrifugal evaporator at a temperature of 30 °C. The residue was dissolved in 200 µL of isooctane and submitted to analysis by GC/MS-MS. An uncontaminated tuna sample (previously checked for the presence of POPs and considered blank with a concentration of compounds < LOD) used as control was selected for all procedure's optimization steps. For fish fortification, 3 g of the control sample was spiked by adding an appropriate volume of the standard working solution to cover the concentration range from 1 to 100 ng g^{-1} (six calibration points: 1, 10, 20, 40, 80, 100 ng g^{-1}) for PCBs; from 0.5 to 10 ng g^{-1} (five calibration points: 0.5, 1, 2, 5, 10 ng g^{-1}) for PBDEs and from 5 to 1000 ng g^{-1} for OCs (eight calibration points: 5, 10, 25, 50, 100, 200, 400, 1000 ng g^{-1}), in relation to pesticide maximum residue levels (MRLs) to realise the matrix-matched calibration curves.

4.3.4 GC-MS/MS analysis of POPs

Triple quadrupole mass spectrometry (QqQ) in electronic impact (EI) modewas employed for the simultaneous detection and quantification of POPs in tuna 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 fish 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 mm film thickness, Restek, Bellefonte, PA, USA). The oven temperature program was: initial temperature 80 °C, hold 3 min, increased to 170 °C at 10 °C min^{-1}, then from 170 °C to 190 °C at 3 °C min^{-1}, then raised to 240 °C at 2 °C min^{-1}, then 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 mL was injected using programmed temperature vaporizer injection (PTV) in splitless mode with a 1-min splitless period and the following inlet temperature programme: 80 °C (0.05 min), 14.5 °Cs^{-1}-200 °C (1 min) and 4.5 °C s^{-1}-320 °C (12 min-cleaning phase). A baffle liner (2 mm x 2.75 mm x 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 the emission current were set to 70 eV and 50 mA, respectively. The

scan time was 0.3 s and the peak width of both quadrupoles was 0.7 Da full width 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, which are listed together with the particular collision energies in Table S2. Identification of pesticides 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 XcaliburTM processing and instrument control software program and Trace Finder 3.0 for data analysis and reporting (Thermo Fisher Scientific) were used.

4.3.5 Validation parameters and quality control

The method was evaluated for its repeatability, linearity, recovery, limit of detection and quantification. The limits of detection (LOD) and quantification (LOQ) were calculated from the calibration curve in the concentration range corresponding to the lower concentration levels according to MRL for each pesticide. LOD was calculated using the equation LOD = 3.3 SD_0/slope, where SD_0 is the residual standard deviation. The limit of quantification was calculated as LOQ = 3 LOD. Working solution were prepared by diluting the stock solution in hexane for pesticides and then stored at -40 °C. Mixed compound calibration solution, in hexane, was prepared from the stock solutions (10m mL^{-1}) and used as spiking solutions as well. Recovery of the analytes studies were carried out at fortification level of 10 ng g^{-1}, while the method repeatability (expressed as coefficient of variation, CV, %) was evaluated analyzing six replicates each by adding known quantities of POPs standard solution (50 ng g^{-1}) to 3 g of homogenized fish (SANTE/ 11945/2015; Panseri et al., 2011).

4.3.6 Statistical analyses

All statistical analyses performed used SPSS 15.0 (SPSS Inc., Chicago, Illinois). Because of the skewed distribution of all measured parameters, the results are presented with range, the 25th, the 50th (median), and the 75th percentile values (Table 2). Based on the examination of normal scores plots of residuals, most of contaminant concentration data were transformed to achieve normality prior to statistical analysis. Natural log-transformations achieved best normal approximation for organic contaminants presented in Fig. S1. Wilcoxon matched pairs test was used to test for differences of POPs levels among FAO capture zone. Significance was accepted at probabilities of 0.05 or less. Also, Spearman correlation analyses were used to assess the relationship between ΣPCBs and ΣOCs and the lipid percentage of tuna form different zones. Results were considered significant at a 5% critical level ($p < 0.05$).

4.4 Results and discussion

4.4.1. Validation parameters

The proposed method has been optimised for the multi-residue analysis of 29 persistent organic pollutants. A GC-MS/MS chromatogram of tuna sample naturally contaminated was shown in Fig. S2. An overview of the quantitative and confirmation MS/MS transitions and the collision energies selected for each compound in EI mode is given in Table S2. Notwithstanding that a highly selective QqQ mass spectrometer is used, since GC-MS instruments are generally rather intolerant to non-volatile matrix impurities, the choice of an appropriate sample preparation strategy is also important to avoid poor ionization, background noise and contamination of the whole GC-MS system. All results obtained for all compounds confirm the efficacy of the present method for the determination of multi-residue pollutants in fish tissue. The method showed a good linearity with determination coefficients equal or higher than 0.99 for all the compounds investigated and good repeatability confirming the present method as useful to monitor compounds belonging to different chemical classes (Table S3). The recoveries ranged from 108 to 119% for PCBs; from 91 to 102% for PBDEs and from 75 to 96. % for OCs. The CVs were all in the range from 4 to 14%. The one-step ASE method using silica as fat retainer is both rapid and cost-effective and minimizes waste generation compared to the classic methods. The time required in the laboratory is reduced to half by combining the extraction and the two clean-up steps (i.e., GPC and SPE) in one single ASE step. Silica impregnated with sulphuric acid is the most frequently used fat retainer for integrated extractions of organic contaminants but florisil and neutral alumina have also been used (Muller et al., 2001). A recent study of the fat-retention capacity of sulphuric-acid- impregnated silica, florisil, and basic, neutral, and acidic alumina showed that all fat retainers, except basic alumina (1.4%), yielded fat-free or nearly fat-free extracts (<1%) (Sun et al., 2012; Ghosh et al., 2011). So the final selection of neutral silica was preferred in order to minimise the laboratory waste. Our results are then in accordance with Zhang et al. (2011) that used neutral silica as fat retainer to extract and clean-up polybrominated diphenyl ethers and polychlorinated biphenyls from sheep liver tissue obtaining good validation parameters in term of recovery and precision for all investigated compounds.

Table 1 Detection Frequency (number of POPs and percentage) of contaminants in Bluefin tuna samples from different FAO catch areas

POPs Contaminants	**FAO area 51**	**FAO area 71**	**FAO area 34**	**FAO area 37**
	Indian Ocean	***Pacific Ocean***	***Atlantic Ocean***	***Mediterranean Sea***
	(n=20)	(n=20)	(n=20)	(n=19)
Polychlorinated biphenyls (PCBs)				
PCB 28	20 (100%)	20 (100%)	20 (100%)	19 (100%)
PCB 52	20 (100%)	20 (100%)	20 (100%)	19 (100%)
PCB 101	20 (100%)	20 (100%)	20 (100%)	19 (100%)
PCB 138	20 (100%)	20 (100%)	20 (100%)	19 (100%)
PCB 153	3 (15%)	0	2 (10%)	19 (100%)
PCB 180	20 (100%)	20 (100%)	20 (100%)	19 (100%)
Polybrominated diphenyl ethers (PBDEs)				
PBDE 28	0	0	0	5 (26%)
PBDE 33	0	0	0	5 (26%)
PBDE 47	3 (15%)	1 (5%)	1 (5%)	19 (100%)
PBDE 99	1 (5%)	0	0	13 (68%)
PBDE 100	4 (20%)	6 (30%)	5 (25%)	18 (95%)
PBDE 153	1 (5%)	0	0	11 (55%)
PBDE 154	13 (65%)	12 (60%)	17 (85%)	17 (89%)
Organochlorines (OCs)				
α HCH	0	0	0	0
Hexachlorobenzene	0	0	0	0
β BHC	0	0	0	0
Lindane (γ HCH)	0	0	0	0
Heptachlor	0	0	0	0
Aldrin	0	0	0	0
Heptachlor epoxide	0	0	0	0
Trans chlordane	0	0	0	0
Endosulfan I	0	0	0	0
pp' DDE	0	0	0	9 (47%)
Endrin	5 (25%)	6 (30%)	1 (5%)	0
Endosulfan II	0	0	0	0
pp’ DDD	0	0	0	0
op’ DDT	0	0	0	1 (5%)
Endosulfan sulfate	17 (85%)	16 (80%)	13 (65%)	17 (89%)
pp' DDT	11 (55%)	0	3 (15%)	17 (89%)

Table 2. Concentration of POPs (ng g^{-1} lipid weight) in Bluefin tuna samples from different catch FAO areas.

POPs Contaminants	FAO area 51 (*Indian Ocean, Western*)					FAO area 71 (*Pacific Ocean, Western Central*)				
	Min	Percentile			Max	Min	Percentile			Max
		25th	50th	75th			25th	50th	75th	
Polychlorinated biphenyls (PCBs)										
PCB 28	24.50	25.24	25.62	26.39	36.12	23.31	23.96	24.44	25.33	28.31
PCB 52	8.90	9.21	9.55	10.10	17.11	8.48	8.76	9.07	9.57	12.21
PCB 101	5.62	6.14	6.39	7.45	13.25	5.22	5.50	5.90	6.13	9.07
PCB 138	5.27	5.81	6.26	7.65	12.72	5.03	5.28	5.61	5.84	9.13
PCB 153	0.00	1.23	0.00	1.66	1.94	n.d.	n.d.	n.d.	n.d.	n.d.
PCB 180	5.49	5.71	5.86	6.71	11.90	5.09	5.15	5.21	5.43	6.13
Polybrominated diphenyl ethers (PBDEs)										
PBDE 28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBDE 33	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBDE 47	n.d.	0.27	n.d.	0.81	1.22	n.d.	0.97	n.d.	0.97	0.97
PBDE 99	n.d.	1.20	n.d.	1.20	1.20	n.d.	n.d.	n.d.	n.d.	n.d.
PBDE 100	n.d.	0.65	n.d.	1.89	2.27	n.d.	n.d.	n.d.	0.62	0.95
PBDE 153	n.d.	3.76	n.d.	3.76	3.76	n.d.	n.d.	n.d.	n.d.	n.d.
PBDE 154	n.d.	1.93	1.93	1.95	4.66	n.d.	1.80	1.79	1.81	1.84
Organochloriens (OCs)										
α HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hexachlorobenzene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β BHC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lindane (γ HCH)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heptachlor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Aldrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heptachlor epoxide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trans chlordane	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Endosulfan I	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
pp' DDE	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Endrin	n.d	40.72	n.d.	425.13	928.81	n.d.	243.96	0.00	524.43	680.31
Endosulfan II	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
pp’ DDD	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
op’ DDT	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Endosulfan sulfate	n.d	163.12	163.24	164.26	166.26	n.d.	151.68	151.71	151.94	152.31
pp' DDT	n.d	159.85	158.00	163.60	170.52	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.= not detected (<LOD).

POPs Contaminants	FAO area 34 (Atlantic Ocean, Eastern Central)					FAO area 37 (Mediterranean Sea)				
	Min	Percentile			Max	Min	Percentile			Max
		25th	50th	75th			25th	50th	75th	
Polychlorinated biphenyls (PCBs)										
PCB 28	24.16	24.60	25.27	26.14	28.09	25.07	30.58	43.62	55.92	124.07
PCB 52	8.83	9.18	9.53	10.48	11.00	11.90	19.01	25.96	56.84	329.31
PCB 101	5.49	6.31	6.87	7.80	10.47	14.43	27.39	35.87	130.71	628.11
PCB 138	6.01	6.64	7.82	9.01	14.04	35.29	67.30	107.33	355.36	1549.64
PCB 153	n.d.	0.74	n.d.	1.02	1.16	16.94	32.72	56.22	198.90	789.53
PCB 180	5.39	5.65	6.13	6.64	8.45	14.05	22.45	32.53	90.23	292.67
Polybrominated diphenyl ethers (PBDEs)										
PBDE 28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.71	n.d.	36.19	98.27
PBDE 33	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.19	n.d.	15.66	38.24
PBDE 47	n.d.	0.06	n.d.	n.d.	0.06	0.95	3.67	10.02	40.72	139.76
PBDE 99	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.36	0.36	5.44	9.06
PBDE 100	n.d.	0.01	n.d.	n.d.	0.21	n.d.	0.10	0.33	0.62	2.23
PBDE 153	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	0.05	0.04	1.07	1.85
PBDE 154	n.d.	1.80	1.80	1.82	1.85	n.d	1.82	1.84	1.90	2.00
Organochlorines (OCs)										
α HCH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
Hexachlorobenzene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
β BHC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
Lindane (γ HCH)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
Heptachlor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
Aldrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
Heptachlor epoxide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
Trans chlordane	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
Endosulfan I	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
pp' DDE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	30.75	n.d.	395.18	785.13
Endrin	n.d.	180.07	n.d.	180.07	180.07	n.d	n.d.	n.d.	n.d.	n.d.
Endosulfan II	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
pp’ DDD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
op’ DDT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d	24.14	n.d.	24.14	24.13
Endosulfan sulfate	n.d.	151.50	151.51	151.64	152.04	n.d	151.57	151.64	151.78	151.96
pp' DDT	n.d.	145.94	n.d.	149.58	152.56	n.d	150.78	155.49	187.43	325.33

n.d.= not detected (<LOD).

4.4.2 Application to tuna sample from different FAO catch areas

The method developed was applied to the analysis of 79 tunas from different FAO areas, in order to evaluate the occurrence of persistent organic pollutants (POPs) to have an overview and mapping on their distribution. The results of detection frequency and concentration levels of POPs residues, found

in tuna samples, are presented in Tables 1 and 2. Because of the skewed distribution of all measured parameters, the results are presented with range, the 25th, the 50th (median), and the 75th percentile values. Spatial distribution of PBDEs and PCBs among FAO catch areas is shown in Fig. S1 and an overview of the profile of detected POPs in tuna samples are presented in Fig. 2. All the PCBs investigated were detected in all tuna samples, with the exception of the PCB 153, which tends to be always present in the FAO 37 area, while in the other three areas was only detected in five samples (two in FAO 34 area, three in FAO 51 area). In this study, we found a positive correlation between ΣPCBs and lipid percentage of tunas from all investigated FAO zones. Due to the lipophilic nature of PCBs, they are generally well correlated to the lipid content in biota samples (Xia et al., 2012). In particular, the correlation coefficients calculated were $R^2 = 0.71$ in FAO zone 51; $R^2 = 0.73$ in FAO zone 71; $R^2 = 0.79$ in FAO zone 34 and $R^2 = 0.83$ in FAO zone 37; P value was lower than 0.05 for all FAO areas. The relationship between ΣPCBs (ng g^{-1} wet weight) and lipid percentage among FAO investigated zones is showed in Fig. S3. The concentrations of PCBs in the samples from the FAO 37 area were much higher than those of the other three areas; in fact, they range from 25.07 to 1649.64 ng g^{-1} lipid weight, while in the other areas ranged from 5.09 to 36.12 ng g^{-1} lipid weight. Being a semiclosed basin, the Mediterranean Sea has limited exchange with the open ocean (Gimenez et al., 2013) and this facilitates the accumulation of these pollutants. The Mediterranean marine environment has been exposed to a handful of adverse events, which greatly threaten marine organisms. One of the most significant occurred in the 1990s, when tens of thousands of striped dolphins died in the Mediterranean. Analyses revealed high levels of polychlorinated biphenyls in the fish's tissue as well as in liver and other organs (Kannan et al., 1993; Borghesi et al., 2009). The POPs pollution of Mediterranean Sea ecosystem is attributable to the many sources of agricultural, municipal, and industrial contamination in the adjacent regions. In particular, these chemicals mainly arrive in the sea as a consequence of evaporation, atmospheric fallout, surface run-off, and wastewater discharges from the intensively cultivated areas, the densely populated urban centres, the large industrial complexes, and the many waste dumps clustered along the coasts. This hypothesis is confirmed by the presence of the highest concentrations of organochlorine and PCBs pollutants in the sea bass and the grey mullet, two strictly resident and benthic species, which inhabit nearshore marine areas (Bailey, 2001; Naso et al., 2005). Moreover, in FAO 37 area, PCBs 101, PCB 138, PCB 153 and PCB 180 are at higher concentrations compared to PCB 28 and PCB 52; the abundance of these congeners is consistent with their high prevalence in technical mixtures, high lipophilicity, stability and persistence, which facilitate adsorption to sediments and accumulation in the aquatic ecosystem, and to their molecular structure. PCBs 101, 138, 153 and 180, being refractory to metabolic attack by monooxygenases, tend to be more slowly eliminated because of their high degree of chlorination and the lack of adjacent

unsubstituted H-atoms in ortho-meta and/or meta-para position on the aromatic ring. (Storelli et al., 2009; Masci et al., 2014). In fish, PCBs decreased growth; caused ionic imbalance, hyperglycemia, anemia, toxicopathic lesions in tissues, such as gill, liver, and spleen; disrupted reproduction; and ultimately affected population levels (Khan, 2011; Miranda et al., 2008). The fate of individual PCB congeners is determined by both environmental processes and physical-chemical properties of individual congeners, and differential rates of uptake, metabolism and elimination will influence the congener profile to which target tissues are ultimately exposed.

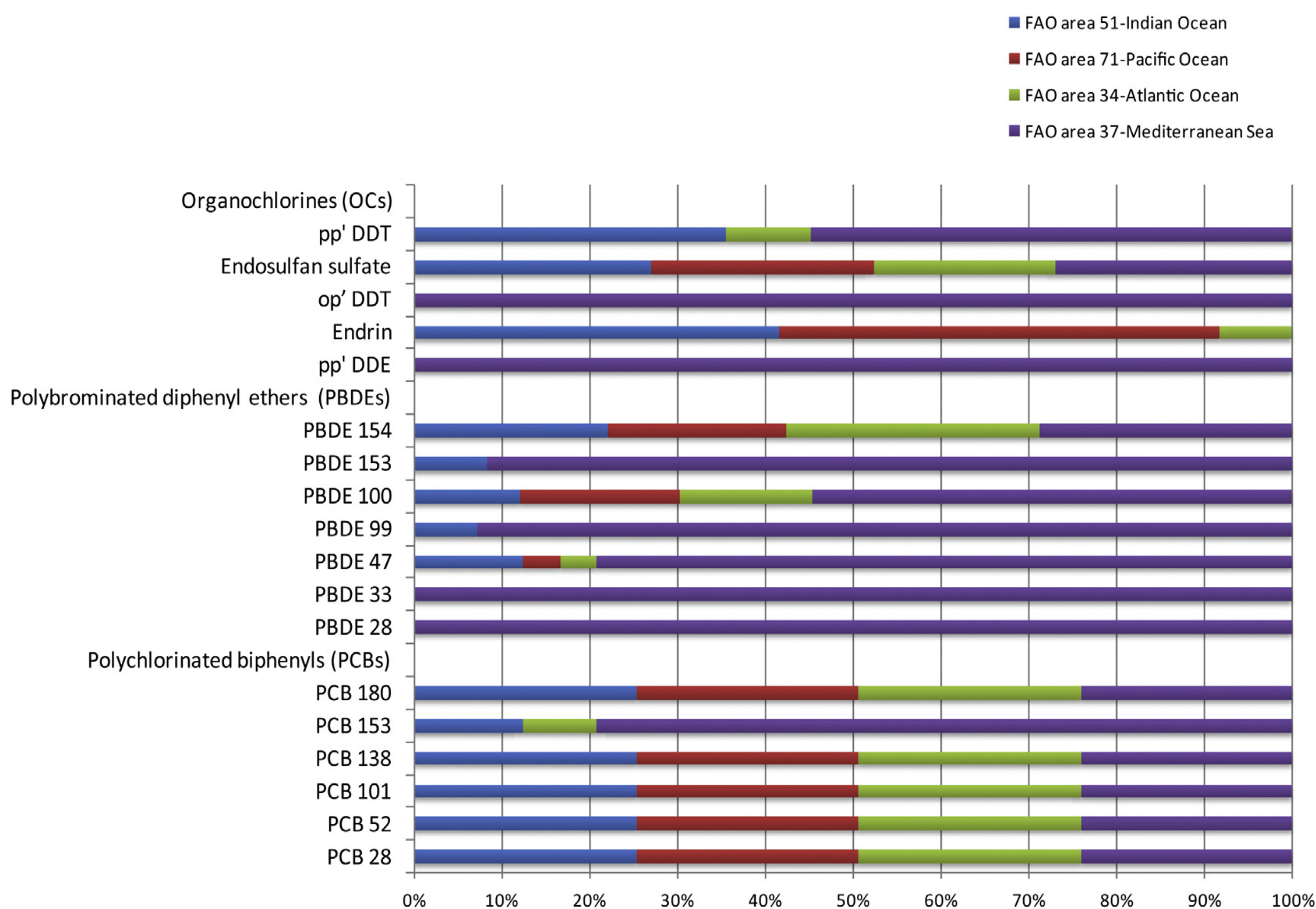


Fig. 2. Profiles of detected POPs in tuna samples from different capture areas.

Except for dioxins and dioxin like PCBs, EU regulation on maximum permissible levels (MPL) for organochlorine compounds in fish for human use (EFSA, 2010; Decision (EC) No 2455/2001) prescribes only the concentrations of six indicator PCBs in fish and mussels (<75 ng g^{-1} fresh tissue), while concentrations of OCs are not regulated by any law. The sum of the six indicator PCBs can be used as an appropriate marker for occurrence and human exposure to NDL-PCBs because this value

represents about 50% of the total NDLPCBs in food (EFSA, 2010). Since the sum of indicator PCBs in our study (2.49-38.25 ng g^{-1} wet weight; 55.33-910.71 lipid weight) was lower than proposed MPL, results of this research suggested that the consumption of analysed tunas does not pose a health risk when considering exposure to NDL-PCBs even if the concentration in tuna from FAO 37 was closer to MPL. Concerning PBDEs, the 47, 100, and 154 congeners were detected in all samples with concentrations between 0.06 ng g^{-1} and 139.76 ng g^{-1} lipid weight; PBDE 99 and PBDE 153 were found in the FAO 51 area and FAO 37 area, while the remaining congeners (28 and 33) were only detected in FAO 37 area. These data show that, as for PCBs, all the PBDEs investigated have been detected in the Mediterranean Sea, probably because of the reasons mentioned previously. Another interesting aspect is that the prevalence of PBDEs in the FAO 37 area is higher than the other areas, in fact it ranges from 25 to 100%, while in the other three ones the frequency is between 5 and 65% except for PBDE 154, which was detected with a prevalence of 85% in the FAO 37 area. Unfortunately, there are no many studies regarding the concentration of PBDEs in foodstuff, so few data are available. A study of Corsolini et al. (2008), focused on the presence of PBDEs in different swordfish tissues in the Mediterranean Sea, shows that PBDEs were detected in the swordfish muscles in a range from 4 pg g^{-1} to 1.91 ng g^{-1}, concentrations lower than tuna samples. These results are in according to ours because tuna has a fat content greater than the swordfish, and being their lipophilic character responsible for their bioaccumulation in fatty tissues, this involves in a higher concentration in tuna samples. Also for OCs a positive correlation between ΣOCs and lipid percentage of tunas from all investigated FAO zones was found. The correlation coefficients obtained were $R^2 = 0.73$ in FAO zone 51; $R^2 = 0.86$ in FAO zone 71; $R^2 = 0.87$ in FAO zone 34 and $R^2 = 0.92$ in FAO zone 37; P value was lower than 0.05 for all FAO areas. This result was in accordance with Erdogrul et al. (2005) that investigated the levels of organochlorines, polychlorinated biphenyls and polybrominated diphenyl ethers in fish species from Kahramanmaras, Turkey. The relationship between ΣOCs (ng g^{-1} wet weight) and lipid percentage among FAO investigated zones is showed in Fig. S4. Regarding OCs, only five compounds were detected in tuna samples. Endosulfan sulfate was detected in all FAO areas, with a mean concentration of about 156.67 ng g^{-1} lipid weight in each area; the prevalence for this OC was between 65 and 89%; $p < 0.05$. Endrin was present in FAO 51, 71 and 34 areas, with a concentration ranges from 40.72 to 928.81 ng g^{-1} lipid weight and with a frequency range from 5 to 30%. No studies showed the presence of Endolsulfan sulfate and Endrin in tuna samples, therefore this is the first study to indicate their possible presence. pp-DDT (one of the two congeners of DDT investigated) was found in all areas, except the 71; op-DDT (the second congener) was only detected in the Mediterranean Sea. The prevalence of pp-DDT was higher than that of op-DDT, in fact it ranged from 15 to 89%, while for op-DDT the frequency was 5% (it was found in just one sample of FAO 37

area). In addition to DDT, also its metabolite pp-DDE was detected, but only in the FAO 37 area, where its concentration ranged from 30.75 to 785.13 ng g^{-1} lipid weight and its prevalencewas 47%. These data are in according to many other studies, in which DDT and its metabolites were detected in different marine organisms. Storelli et al. (2009) studied the presence of OCs in deep-sea from the Mediterranean Sea, and they found both DDT (op0 and pp0) and DDE (pp0) in their samples. Also Ueno et al. (2003) demonstrated the presence of DDT in Skipjack tuna. All these data show that DDT and its metabolites, due to hydrophobic properties, are absorbed by aquatic organisms and bioaccumulate, leading to the final contamination of foodstuffs. The organochlorines pollution is attributable to many sources: atmospheric fallout, intensive agriculture, densely populated urban centres and large industrial complexes; these factors probably play a key role in pollution of FAO areas, especially for the Mediterranean Sea. This study shows that, investigating three different classes of POPs, is possible to have an overview and mapping on their presence in four FAO areas. Furthermore, much information was provided for further studies, especially for PBDEs, for which many data are not yet available in literature.

4.5 Conclusions

An analytical methodwas developed and applied to evaluate the POPs residues in tuna samples from different FAO areas. The method proved to be simple and rapid, requiring small sample sizes, minimizing solvent consumption, due to the ASE with an “in line” clean up step. MS/MS detection provides both quantitative information and confirmation of POPs residues in tuna confirming the one-step ASE method a valid alternative to classical extraction methods because the analytical quality is comparable. The determination of POPs in foods is necessary to ensure that human exposure to contaminants does not exceed tolerable levels for health. The results of this study show that POPs contamination of tuna is strictly related to the FAO area of origin, reflecting the specific pollution of a given environment, as most stressed for the Mediterranean Sea. Moreover, as expected, it was possible to obtain an accurate profile of persistent organic pollutants in order to have an overview and to map the distribution of POPs in fish for the consumer's food safety purpose. Indeed, further experimental plans will be designed extending the anayses to other compounds belonging to flame retardant chemical class to add new knowledge about contamination and presence of these emerging contaminants in fish.

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The Authors dedicate this work to their good friend and colleague Guglielmo Dusi, who recently passed away.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.03.010.

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Chapter V

The occurrence of pesticides and persistent organic pollutants in Italian organic honeys from different productive areas in relation to potential environmental pollution

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5. The occurrence of pesticides and persistent organic pollutants in Italian organic honeys from different productive areas in relation to potential environmental pollution.

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5.1 Abstract

Bee products, such as honey, are widely consumed as food and consumer interest is currently oriented towards organic foods. Regarding this, the European Commission establishes that the qualification of organic honey and other beekeeping products as being from organic production is closely bound with the characteristics of hive treatments as well as the quality of the environment. Agricultural contamination with pesticides is a challenging problem that needs to be fully addressed, in particular in the field of organic production systems. In this study, the occurrence of different classes of contaminants selected as representative of potential contamination sources were investigated in 59 organic honeys: organochlorines, OCs; organophosphates, OPs; polychlorobiphenyls, PCBs and polybromodiphenylethers, PBDEs. A method based on Accelerated Solvent Extraction with “in line” clean-up and GC-MS/MS detection was developed to detect contaminants. Residues of many pesticides were found in most of the samples investigated. The majority of honey samples contained at least one of the pesticides, even if their concentrations were found to be lower than its MRL. Diazinon, Mevinphos, Coumaphos, Chlorpyrifos and Quinoxyfen were the residues frequently detected in samples coming from the apple and citrus orchard areas. Furthermore, the results of the present study show that the presence of the residue in organic honey may also be affected by the geographical area (e.g. the presence of an agricultural system) confirming honey bee and beehive matrices as appropriate sentinels for monitoring contamination in the environment. The optimized method proved to be simple and rapid, requiring small sample sizes and minimising solvent consumption, due to the ASE having an “in line” clean-up step.

5.2 Introduction

Honey is a natural food product, made of nectar, secretions of livings parts of plants or excretions of insects sucking on the living parts of plants, which Apis millifera bees collect, transform by combining with specific substances and deposit in honeycombs (Giorgi et al., 2011; Wilczynska and Przybylowski, 2007; Panseri et al., 2014). Honeybees (Apis mellifera L.) perform the vital task of pollinating agricultural crops and native species and are important for the commercial products of honey and beeswax. Honey composition mainly depends on the floral origin of nectar, climate conditions, bee physiology, honey harvesting and post-collection processing (Panseri et al., 2013). Today, consumer interest regarding honey and its derived products is oriented towards organic foods. Regarding this, the European Commission establishes that the qualification of organic honey and other beekeeping products as being from organic production is closely bound to the characteristics of hive treatments as well as the quality of the environment. This qualification also depends on the conditions of extraction, processing and storage of beekeeping products. The Council Regulation 1804/1999 EC is very restrictive with regard to the production of organic honey in terms of the origin of bees, siting of the apiaries, feed, disease prevention and veterinary treatments. In particular, it establishes that plants that can be foraged by bees, either biological or spontaneous, must be at least 3 km from any source of pollution and from any non-agricultural production sources, possibly leading to contamination, such as industrial areas, urban centres or motorways. Also, the use of veterinary medicinal products in beekeeping is regulated by the European Council (EC, 1804/1999). Usually, beekeepers administered insecticides, fungicides, and acaricides to control some infestations such as Varroa destructor, Acarapis woodi and Paenibacillus larvae (Lopez et al., 2014; Fell and Cobb, 2009; Genersch et al., 2010). According to the Council Regulation 1804/1999, the use of allopathic chemicallysynthesised medicinal products for preventive treatments in organic beekeeping is prohibited, since these fat-soluble and nonvolatile compounds can accumulate in the stored honey, where they are able to migrate from the wax comb (Panseri et al., 2014). In the cases of Varroa infestation, formic acid, acetic acid and oxalic acid can be used, as well as menthol, thymol, eucalyptol or camphor (Council Regulation, 1804/1999 EC). Therefore, in organic honey production, direct pollution by beekeeping practices as well as indirect contamination from the environment must be prevented. Many pollutants in the environment may contaminate bee matrices, comprising bee, honey and pollen. Environmental pollutants include pesticides (Chauzat et al., 2011), heavy metals (Tuzen et al., 2007), bacteria and radioactive materials (Al-Waili et al., 2012). Honeybees are able to cover a wide area and come into contact with contaminated food sources, such as pollen, nectar and water during foraging. Therefore, honeybees and beehive products are considered potential indicators for

environmental biomonitoring (Malhat et al., 2015; Kasiotis et al., 2014). Lambert et al. described the use of bees, honey and pollen as sentinels for environmental chemical contaminants in France (Lambert et al., 2012). Porrini et al. described the use of honey bees and bee products as bioindicators of pesticide, heavy metal and radionucleotide pollution (Porrini et al., 2003); Panseri et al. (2014) demonstrated the high direct relation between the contaminant source and pesticide residues found in honey samples. Among the environmental contaminants, different studies have documented the occurrence of organochlorines (OCs), polychlorobiphenyls (PCBs), organophosphates (OPs) and polybromodiphenylethers (PBDEs) in honey. In particular organochlorine, and to a minor extent organophosphorous pesticides, are highly stable, minimally volatile, lipophilic and persistent organic pollutants. Due to these characteristics, the compounds tend to accumulate and bioaccumulate, representing important groups of dangerous organic contaminants, since they can contaminate foodstuffs if not directly treated (Panseri et al., 2014). Organophosphorus pesticides (OPs) represent important environmental and food contamination sources, as they are widely used in agriculture for the control and protection of crop-eating insects. In addition, OPs are acetylcholinesterase inhibitors leading to acute poisoning via food consumption (He et al., 2015). Recently EFSA (European Food Safety Authority) has realised scientific opinion on the risks to public health related to the presence of brominated flame retardants in food (EFSA, 2010). Thus, the Commission used the Recommendation of 3 March 2014 ask European countries to monitor traces of brominated flame retardants in food. Brominated flame retardants (BFRs), especially polybromodiphenyethers (PBDEs), are organobromine compounds applied to products in order to reduce their flammability (COMMISSION RECOMMENDATION, 2014). They contaminate the environment and food chain because of their persistent, lipophilic, bioaccumulative and toxic nature, and are suspected of causing neurobehavioral effects and endocrine disruption (Mohr et al., 2014). In general, the European Commission set the maximum residue levels values (MRLs) for feed as well as for food of animal origin (Commission Regulation 396/2005; Commission Regulation 839/2008). Critical steps in the determination of contaminants residues in food are the extraction from matrices and the following sample clean-up (Rissato et al., 2007; LeDoux, 2011). Among the many extraction techniques, accelerated solvent extraction (ASE) is characterised by shorter extraction times and reduced solvent consumption. The accelerated solvent extraction utilises high temperatures combined with high pressure. A high temperature allows a higher rate of extraction due to a reduction of the viscosity and surface tension, and increases the solubility and diffusion rate into the sample. At the same time, high pressure prevents the solvents from reaching their boiling point and promotes penetration into the sample (Beyer and Biziuk, 2008). Recently, the ASE technique has also been tentatively used combining the clean-up step during the extraction process, generating an “in line” extraction-clean-up method in which the sample

purification is directly performed in the ASE cell. Until now, only three studies reported the use of ASE for the extraction of pesticides from honey without “in line” clean-up (Kort et al., 2002; Wang et al., 2010; Lambert et al., 2012). Considering the lack of information in the literature about the presence of pesticides and other contaminants in organic bee products, the aim of the present study was to investigate the presence of POPs in organic honeys arising from different Italian regions. Our attention was focused on the residues of pesticides used in citrus and apple orchards for crop protection [organochlorines (OCs) and organophosphates (OPs)] as well as other POPs present in the environment as a possible consequence of antrophic activities [polychlorobiphenyls (PCBs) and polybromodiphenylethers (PBDEs)]. Lastly, this paper presents a rapid, accurate and sensitive method to evaluate multiple residues by using the accelerated solvent extraction (ASE) sample preparation method with “in line” clean-up purification followed by GC-MS/MS (triple quadrupole - QqQ) analysis.

5.3 Material and methods

5.3.1 Chemicals and reagents

Mixtures of PCB congeners (PCB 28; PCB 52; PCB 101; PCB 138; PCB 153 and PCB 180) and PBDE congeners (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 154), PCB 209, internal standard (IS) for PCBs, and 3-fluoro-2,2,4,4,6- pentabromodiphenyl ether (FBDE), and IS for flame retardants, were purchased from AccuStandard (New Haven, USA). A mixture of 19 standard OCs (α-HCH; Hexachlorobenzene; β-BHC; Lindane; Heptachlor; Aldrin; Heptachlor epoxide; Trans Chlordane; 4,4’-DDE; Endosulphan I; 2,4’-DDT; Endrin; 4,4’-DDD; Endosulphan II; 4,4’-DDT and Endosulphan sulphate, Dieldrin, Endrin Aldehyde and Methoxychlor) was purchased from Restek (Bellefonte, PA, USA). OP pesticide standards of Mevinphos, Ethoprophos, Phorate, Diazinon, Disulphoton, Methyl Paration, Fenchlorphos, Chlorpyriphos, Fenthion, Sulprofos, Coumaphos, Tetrachlorpirophos, Protiofos, Tribuphos, Anzifos metile, Chlorpyriphos, Penconazol, Captan, Bupiramate, Quinoxyfen, Fluazinam, Trifloxystrobin, Iprodion, Chlorantraniliprol, Spirodiclofen, Boscalid, and Pyraclostrobin were purchased from Sigma-Aldrich, St Louis, Mo, USA. Florisil (100-200 96 mesh) was provided by Promochem (Wesel, Germany). Hexane, isooctane, acetone, ethyl acetate (special grade for pesticide residue analysis (Pestanal)) and 4-nonylphenol (IS for OCs and OPs) were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA).Working solutions were prepared by diluting the stock solution in hexane for pesticides and then stored at -40

°C. Mixed compound calibration solution, in hexane, was prepared daily from the stock solutions (10 m mL^{-1}) and the proper volume was used as a spiking solution as well.

5.3.2 Sample collection

Fifty-nine organic honey samples were provided by the beekeepers from three different Italian regions: Calabria, South Italy (14 samples); Trentino Alto Adige, North Italy (18 samples) and Lombardia, North Italy (27 samples), as summarised in Table 1. All samples were stored at -20 °C until analysis to prevent any possible matrix alteration (fermentation phenomena).

5.3.3 Extraction and clean-up

The extractionwas performed using an ASE 350 (Thermo-Fisher Scientific,Waltham, MA, USA). The extraction conditions are shown in Table 2. Here, 33 mL cells for accelerated solvent extraction (ASE) were used for the analysis. A 2 g sample of honey was homogenized with an equal weight of Diatomaceous earths, sodium sulphate and transferred into the cell. Then, 1 mL of isooctane solution containing the three ISs was added. In order to fill the remaining empty part of the cell, Diatomaceous earths were added. The cells were finally packed with a cellulose filter at the bottom followed by Florisil (5 g). The dried samples were transferred to the ASE cells. Temperature (80 °C), pressure (1500 psi), number of static cycles (3 min each), and purging time (90 s with nitrogen) were fixed throughout the study. The extraction solvent was a mixture of hexane/ethyl acetate (4:1, v/v). Organic extracts were finally collected in 66 mL vials and treated with sodium sulphate to remove any possible humidity. Afterwards, the extract was collected and dried under vacuum in a centrifugal evaporator at 30 °C. The residue was dissolved in 200 mL of isooctane and submitted to analysis by GC/MS-MS. An uncontaminated honey sample used as control was selected for the optimisation of all procedures. For honey fortification, 2 g of the control sample was spiked by adding an appropriate volume of the standard working solution to cover the concentration range from 1 to 100 ng g^{-1} for PCBs, from 0.5 to 10 ng g^{-1} for PBDEs, and from 5 to 100 ng g^{-1} for OCs and OPs, and also in relation to pesticide MRLs when available in order to realise the matrix-matched calibration curves.

5.3.4 GC-MS/MS analysis of pesticides and POPs

Triple quadrupole mass spectrometry (QqQ) in electronic impact (EI) mode was used for the simultaneous detection and quantification of pesticides and POPs in honey 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 honey 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 mm 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 mL was injected using a programmed temperature vaporiser injector (PTV) in splitless mode with a 1-min splitless period and the following inlet temperature program: 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 e cleaning phase). A baffle liner (2 mm x 2.75 mm x 120 mm, Siltek-deactivated; Thermo Fisher Scientific) was used. The transfer linewas maintained at 270 °C and the ion source at 250 °C. The electron energy and emission current were set to 70 eV and 50 mA, 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, which are listed together with the particular collision energies in Table 3. Identification of POPs 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.

5.3.5 Validation parameters and quality control

The method was evaluated for its repeatability, linearity, recovery, limit of detection and quantification. The limits of detection (LOD) and quantification (LOQ) were calculated from the calibration curve in the concentration range corresponding to the lower concentration levels according to MRL for each pesticide when available. LOD was calculated using the equation LOD = 3.3 SD_0/slope, where SD_0 is the residual standard deviation. The limit of quantification was calculated as LOQ = 3 LOD. Recovery of the

Table 1 Origin of 59 organic honey samples from different Italian areas.

Sample no.	Origin information	Botanical source	Potential environment's contamination sources
27	North Italy[b] (Lombardia)	Multifloral	Industrialized area (OCPs, PCBs, PBDEs source)
14	South Italy[c] (Calabria)	Citrus - monofloral	Intensive citrus orchard (pesticides utilized in IPMa plan)
18	North Italy[d] (Trentino)	Multifloral	Intensive apple orchard (pesticides utilized in IPMa plan)

[a] IPM = integrated pest management.

[b] Lombardia (north).

[c] Calabria (south east)).

[d] Trentino (north west).

Table 2 POPs Recoveries (% RDS), LOD, LOQ, determination coefficients (r^2) of the proposed method and precursor ions, product ions and collision energy of investigated contaminants

Contaminants	Rt (min)	LOD (ng g^{-1})	LOQ (ng g^{-1})	Recovery % (RDS)	Determination coefficient (r^2)	Precursor Ions, product Ions[a] (m/z)	Collision Energy (eV)
Polychlorobiphenyls (PCBs)							
PCB 28	18.76	0.08	0.24	102 (7)	0.9994	256, 186	20
PCB 52	20.25	0.07	0.21	103 (7)	0.9999	292, 222	25
PCB 101	24.46	0.04	0.12	97 (4)	0.9999	326, 256	25
PCB 138	28.99	0.05	0.15	105 (4)	0.9999	360, 290	25
PCB 153	30.25	0.02	0.06	102 (4)	0.9999	360, 290	20
PCB 180	34.06	0.06	0.18	98 (9)	0.9999	394, 324	25
Polybrominated diphenyl ethers (PBDEs)							
PBDE 28	27.95	0.01	0.03	100 (9)	0.9991	248, 139	10
PBDE 33	28.05	0.02	0.06	98 (9)	0.9999	248, 139	30
PBDE 47	34.34	0.02	0.06	97 (8)	0.9996	484, 326	30
PBDE 99	38.17	0.03	0.09	102 (7)	0.9998	564, 404	20
PBDE 100	39.05	0.01	0.03	103 (7)	0.9998	564, 404	10
PBDE 153	40.88	0.03	0.09	97 (10)	0.9992	642, 482	10
PBDE 154	41.76	0.02	0.06	100 (12)	0.9999	644, 484	20
Organochlorines (OCs)							
α HCH	15.27	0.99	2.97	78 (10)	0.9959	181, 145	10
Hexachlorobenzene	15.45	1.26	3.78	80 (12)	0.9945	284, 249	20
β BHC	16.69	1.17	3.51	85 (12)	0.9995	181, 145	10
Lindane (γ HCH)	16.44	0.79	2.39	96 (10)	0.9985	181, 145	10
Heptachlor	19.27	0.95	2.84	93 (12)	0.9996	272, 237	10
Aldrin	20.84	0.85	2.55	75 (14)	0.9991	261, 191	30
Heptachlor epoxide	22.77	0.91	2.73	77 (14)	0.9994	353, 263	10
Trans chlordane	23.96	1.48	4.44	92 (10)	0.9993	373, 266	20
Endosulphan I	24.64	1.13	3.38	80 (13)	0.9992	373, 266	20
pp' DDE	25.96	0.85	2.55	97 (12)	0.9994	246, 176	30
Endrin	27.06	0.99	2.98	88 (11)	0.9998	263, 193	10
Endosulphan II	27.65	1.14	3.42	90 (10)	0.9993	295, 159	10

pp DDD	28.18	0.91	2.74	87 (14)	0.9986	235, 165	20
op DDT	28.27	0.94	2.83	82 (14)	0.9963	235, 165	20
Endosulphan sulphate	29.88	1.07	3.22	85 (12)	0.9921	272, 237	10
pp' DDT	30.36	0.91	2.74	95 (12)	0.9992	235, 165	20
Organophosphorus (OPs)							
Mevinphos	11.28	0.75	2.25	75 (12)	0.9996	192, 109	20
Ethopropos	14.22	0.44	1.32	86 (10)	0.9991	158, 114	10
Dichlorvos	14.53	0.33	0.99	93 (10)	0.9997	145, 113	20
Phorate	15.19	0.52	1.56	75 (13)	0.9993	213, 129	20
Demephron (-O and -S)	15.75	1.12	3.36	77 (14)	0.9992	231, 129	20
Diazinon	17.01	1.1	3.3	90 (10)	0.9994	304, 179	10
Disulphoton	17.36	0.9	2.7	80 (14)	0.9998	142, 109	10
Parathion-methyl	19.09	0.83	2.49	97 (8)	0.9993	263, 127	10
Fenchlorphos	19.58	1.12	3.36	88 (11)	0.9986	287, 242	20
Chlorpyrifos	20.99	0.95	2.85	90 (9)	0.9963	278, 125	20
Fenthion	21.17	0.78	2.34	87 (12)	0.9996	245, 213	10
Tricloronat	21.76	0.98	2.94	85 (14)	0.9998	297, 269	10
Tetrachlorpyrifos	24.43	1.12	3.36	85 (12)	0.9998	329, 109	20
Prothiofos	25.55	0.75	2.25	92 (12)	0.9992	309, 239	10
Terbufos	26.17	0.68	2.04	90 (12)	0.9963	258, 146	10
Fensulphotion	27.82	1.09	3.27	88 (10)	0.9921	292, 156	10
Sulprofos	29.13	0.98	2.94	87 (10)	0.9992	156, 141	10
Azinphos methyl	34.95	1.07	3.21	87 (12)	0.9978	160, 51	30
Coumaphos	38.08	0.78	2.34	84 (14)	0.9962	226, 198	10

[a] Precursor ions and product ions (quantifier ions).

analytes studied were carried out at a fortification level of 10 ng g^{-1}, while the method repeatability (expressed as coefficient of variation, CV, %) was evaluated analysing six replicates each by adding known quantities of POPs standard solution (10 ng g^{-1}) to 2 g of honey (SANTE/11945/2015; Panseri et al., 2011).

5.3.6 Statistical analysis

As residue concentrations in honey do not follow a normal distribution, the non-parametric Kruskal-Wallis ANOVA test was used to evaluate the differences of contaminants in samples among the investigated regions. The level of significance was set as $p \leq 0.05$ throughout this study. Data were analysed using SPSS 15.0 software (SPSS, Inc., Illinois, USA). In addition, it must be pointed out that, for the calculations, ½ LOD was used for those compounds whose concentration was below LOD.

5.4 Result and discussion

5.4.1 Method development and validation

A multi-residue method for the analysis of organic contaminants and pesticides was developed. The ASE procedure with cleanup in a single step with an "in line" was necessary for the removal of interfering substances from honey samples. For this purpose, Florisil was used since it proved to be very efficient for the clean-up of different foods (Sun et al., 2012) as well as for honey samples (Rissato et al., 2004; Amendola et al., 2010; Panseri et al., 2014).

Table 3 Concentration of pesticides, POPs residues (ng g^{-1}) and detection frequency in organic honeys from different geographic areas of Italy

Contaminants	**South Italy (Calabria)**					
	Min	**Percentile**			**Max**	**Detection frequency (n=14)**
		25th	50th	75th		
Polychlorobiphenyls (PCBs)						
PCB 28	0.27	0.28	0.29	0.30	0.34	100%
PCB 52	0.47	0.48	0.48	0.48	0.50	100%
PCB 101	0.75	0.75	0.76	0.76	0.78	100%
PCB 138	0.90	0.90	0.90	0.90	0.92	100%
PCB 153	0.54	0.54	0.54	0.54	0.55	100%
PCB 180	0.87	0.87	0.87	0.87	0.87	100%
Polybrominated diphenyl ethers (PBDEs)						
PBDE 28	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 33	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 47	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 99	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 100	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 153	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 154	n.d	n.d	n.d	n.d	n.d	n.d
Organochlorines (OCs)						
α HCH	n.d	n.d	n.d	n.d	n.d	n.d
Hexachlorobenzene	n.d	n.d	n.d	n.d	n.d	n.d
β BHC	n.d	n.d	n.d	n.d	n.d	n.d
Lindane (γ HCH)	n.d	n.d	n.d	n.d	n.d	n.d
Heptachlor	n.d	n.d	n.d	n.d	n.d	n.d
Aldrin	n.d	n.d	0.58	1.22	1.25	50%
Heptachlor epoxide	n.d	n.d	n.d	n.d	n.d	n.d
Trans chlordane	n.d	n.d	n.d	n.d	n.d	n.d
Endosulphan I	n.d	n.d	n.d	n.d	n.d	n.d
pp' DDE	n.d	n.d	n.d	n.d	n.d	n.d

Endrin	1.95	2.26	3.95	5.59	18.90	100%
Endosulphan II	n.d	n.d	n.d	n.d	n.d	n.d
pp DDD	n.d	n.d	n.d	n.d	n.d	n.d
op DDT	n.d	n.d	n.d	n.d	n.d	n.d
Endosulphan sulphate	n.d	n.d	n.d	n.d	n.d	n.d
pp' DDT	n.d	n.d	n.d	n.d	n.d	n.d
Dieldrin	n.d	n.d	n.d	n.d	n.d	n.d
Endrin Aldehyde	n.d	n.d	n.d	n.d	n.d	n.d
Methoxychlor	n.d	n.d	n.d	n.d	n.d	n.d
Organophosphorus (OPs)						
Mevinphos	n.d	10.04	10.15	10.42	10.67	86%
Ethopropos	n.d	n.d	n.d	n.d	n.d	n.d
Phorate	n.d	n.d	n.d	1.78	5.83	29%
Diazinon	n.d	n.d	1.13	1.13	1.14	64%
Disulphoton	n.d	n.d	n.d	n.d	n.d	n.d
Parathion-methyl	n.d	n.d	n.d	n.d	n.d	n.d
Fenchlorphos	n.d	n.d	n.d	n.d	n.d	n.d
Chlorpyrifos	n.d	n.d	n.d	8.67	389.50	29%
Fenthion	n.d	n.d	n.d	n.d	n.d	n.d
Sulprofos	n.d	n.d	1.13	2.26	2.27	50%
Coumaphos	n.d	1.25	1.42	1.51	1.76	79%
Tetrachlorpyrifos	n.d	n.d	n.d	n.d	1.878	21%
Prothiofos	n.d	n.d	n.d	n.d	n.d	n.d
Terbufos	n.d	n.d	n.d	n.d	n.d	n.d
Azinphos methyl	n.d	n.d	n.d	n.d	2.85	17%
Penconazol	n.d	n.d	n.d	n.d	n.d	n.d
Captan	n.d	n.d	n.d	n.d	n.d	n.d
Bupiramate	n.d	n.d	n.d	n.d	n.d	n.d
Quinoxyfen	n.d	n.d	n.d	n.d	n.d	n.d
Fluazinam	n.d	n.d	n.d	n.d	n.d	n.d
Trifloxystrobin	n.d	n.d	n.d	n.d	n.d	n.d
Iprodion	n.d	n.d	n.d	n.d	n.d	n.d
Chlorantraniliprol	n.d	n.d	n.d	n.d	n.d	n.d
Spirodiclofen	n.d	n.d	n.d	n.d	n.d	n.d
Boscalid	n.d	n.d	n.d	n.d	n.d	n.d
Pyraclostrobin	n.d	n.d	n.d	n.d	n.d	n.d

Contaminants	North Italy (Trentino)					
	Min	Percentile			Max	Detection frequency (n=18)
		25th	50th	75th		
Polychlorobiphenyls (PCBs)						
PCB 28	0.27	0.29	0.29	0.30	0.30	100%
PCB 52	0.48	0.48	0.48	0.48	0.49	100%
PCB 101	0.75	0.75	0.75	0.76	0.76	100%
PCB 138	0.90	0.90	0.90	0.90	0.91	100%
PCB 153	0.54	0.54	0.54	0.54	0.54	100%
PCB 180	0.87	0.87	0.87	0.87	0.88	100%
PBDE 28	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 33	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 47	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 99	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 100	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 153	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 154	n.d	n.d	n.d	n.d	n.d	n.d
Organochlorines (OCs)						
α HCH	n.d	n.d	n.d	n.d	n.d	n.d
Hexachlorobenzene	n.d	n.d	n.d	n.d	n.d	n.d
β BHC	n.d	n.d	n.d	n.d	n.d	n.d
Lindane (γ HCH)	n.d	n.d	n.d	n.d	n.d	n.d
Heptachlor	n.d	n.d	n.d	n.d	0.15	5%
Aldrin	n.d	n.d	n.d	n.d	1.17	5%
Heptachlor epoxide	n.d	n.d	n.d	n.d	n.d	n.d
Trans chlordane	n.d	n.d	n.d	n.d	n.d	n.d
Endosulphan I	n.d	n.d	n.d	n.d	n.d	n.d
pp' DDE	n.d	n.d	n.d	n.d	1.47	17%
Endrin	n.d	n.d	n.d	3.39	13.34	44%
Endosulphan II	n.d	n.d	n.d	n.d	n.d	n.d
pp DDD	n.d	n.d	n.d	n.d	n.d	n.d
op DDT	n.d	n.d	n.d	n.d	n.d	n.d
Endosulphan sulphate	n.d	n.d	n.d	n.d	5.43	22%
pp' DDT	n.d	n.d	n.d	n.d	0.09	17%
Dieldrin	n.d	n.d	n.d	n.d	0.94	5%
Endrin Aldehyde	n.d	n.d	n.d	n.d	n.d	n.d
Methoxychlor	n.d	n.d	n.d	n.d	0.07	22%
Organophosphorus (OPs)						
Mevinphos	n.d	n.d	10.02	10.27	12.14	67%
Ethopropos	n.d	n.d	n.d	n.d	n.d	n.d
Phorate	n.d	n.d	n.d	n.d	n.d	n.d
Diazinon	1.13	1.13	1.14	1.14	1.15	100%

Disulphoton	n.d	n.d	n.d	n.d	n.d	n.d
Parathion-methyl	n.d	n.d	n.d	n.d	2.939	11%
Fenchlorphos	n.d	n.d	n.d	n.d	n.d	n.d
Chlorpyrifos	n.d	n.d	n.d	n.d	1.99	17%
Fenthion	n.d	n.d	n.d	n.d	n.d	n.d
Sulprofos	n.d	n.d	n.d	n.d	2.26	22%
Coumaphos	n.d	1.28	1.38	1.73	2.06	78%
Tetrachlorpyrifos	0	0	0	0	1.88	11%
Prothiofos	n.d	n.d	n.d	n.d	n.d	n.d
Terbufos	n.d	n.d	n.d	n.d	n.d	n.d
Azinphos methyl	n.d	n.d	n.d	n.d	n.d	n.d
Penconazol	n.d	n.d	n.d	n.d	n.d	n.d
Captan	n.d	n.d	n.d	5.85	11.79	33%
Bupiramate	n.d	n.d	n.d	n.d	n.d	n.d
Quinoxyfen	3.09	3.36	3.43	3.77	4.23	100%
Fluazinam	n.d	n.d	n.d	n.d	n.d	n.d
Trifloxystrobin	n.d	n.d	n.d	n.d	n.d	n.d
Iprodion	n.d	n.d	n.d	n.d	6.28	11%
Chlorantraniliprol	n.d	n.d	n.d	n.d	n.d	n.d
Spirodiclofen	n.d	n.d	n.d	n.d	n.d	n.d
Boscalid	n.d	n.d	n.d	4.39	4.80	33%
Pyraclostrobin	n.d	n.d	n.d	n.d	n.d	n.d

Contaminants			**North Italy (Lombardia)**			
	Min		**Percentile**		**Max**	**Detection frequency (n=27)**
		25th	50th	75th		
Polychlorobiphenyls (PCBs)						
PCB 28	0.27	0.28	0.29	0.30	0.32	100%
PCB 52	0.47	0.48	0.48	0.48	0.49	100%
PCB 101	0.75	0.75	0.75	0.76	0.77	100%
PCB 138	0.90	0.90	0.90	0.90	0.91	100%
PCB 153	0.54	0.54	0.54	0.54	0.55	100%
PCB 180	0.87	0.87	0.87	0.87	0.87	100%
Polybrominated diphenyl ethers (PBDEs)						
PBDE 28	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 33	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 47	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 99	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 100	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 153	n.d	n.d	n.d	n.d	n.d	n.d
PBDE 154	n.d	n.d	n.d	n.d	n.d	n.d
Organochlorines (OCs)						
α HCH	n.d	n.d	n.d	n.d	n.d	n.d

Hexachlorobenzene	n.d	n.d	n.d	n.d	n.d	n.d
β BHC	n.d	n.d	n.d	n.d	n.d	n.d
Lindane (γ HCH)	n.d	n.d	n.d	n.d	n.d	n.d
Heptachlor	n.d	n.d	n.d	n.d	1.19	11%
Aldrin	n.d	n.d	n.d	n.d	n.d	n.d
Heptachlor epoxide	n.d	n.d	n.d	n.d	n.d	n.d
Trans chlordane	n.d	n.d	n.d	n.d	n.d	n.d
Endosulphan I	n.d	n.d	n.d	n.d	n.d	n.d
pp' DDE	n.d	n.d	n.d	1.45	1.62	33%
Endrin	n.d	n.d	n.d	n.d	n.d	n.d
Endosulphan II	n.d	n.d	n.d	n.d	n.d	n.d
pp DDD	n.d	n.d	n.d	n.d	1.99	22%
op DDT	n.d	n.d	n.d	n.d	n.d	n.d
Endosulphan sulphate	n.d	n.d	n.d	n.d	n.d	n.d
pp' DDT	n.d	n.d	n.d	0.05	1.9	41%
Dieldrin	n.d	n.d	n.d	0.93	2.93	41%
Endrin Aldehyde	n.d	n.d	n.d	n.d	n.d	n.d
Methoxychlor	n.d	n.d	n.d	n.d	0.08	22%
Organophosphorus (OPs)						
Mevinphos	n.d	n.d	n.d	n.d	n.d	n.d
Ethopropos	n.d	n.d	n.d	n.d	n.d	n.d
Phorate	n.d	n.d	n.d	n.d	n.d	n.d
Diazinon	n.d	n.d	n.d	n.d	n.d	n.d
Disulphoton	n.d	n.d	n.d	n.d	n.d	n.d
Parathion-methyl	n.d	n.d	n.d	n.d	n.d	n.d
Fenchlorphos	n.d	n.d	n.d	n.d	n.d	n.d
Chlorpyrifos	n.d	n.d	n.d	n.d	1.58	4%
Fenthion	n.d	n.d	n.d	n.d	n.d	n.d
Sulprofos	n.d	n.d	n.d	n.d	n.d	n.d
Coumaphos	n.d	n.d	n.d	n.d	n.d	n.d
Tetrachlorpyrifos	n.d	n.d	n.d	n.d	n.d	n.d
Prothiofos	n.d	n.d	n.d	n.d	n.d	n.d
Terbufos	n.d	n.d	n.d	n.d	n.d	n.d
Azinphos methyl	n.d	n.d	n.d	n.d	n.d	n.d
Penconazol	n.d	n.d	n.d	n.d	n.d	n.d
Captan	n.d	n.d	n.d	10.54	20.56	37%
Bupiramate	n.d	n.d	n.d	n.d	n.d	n.d
Quinoxyfen	n.d	n.d	n.d	n.d	n.d	n.d
Fluazinam	n.d	n.d	n.d	n.d	n.d	n.d
Trifloxystrobin	n.d	n.d	n.d	n.d	9.43	18%
Iprodion	n.d	n.d	n.d	n.d	n.d	n.d
Chlorantraniliprol	n.d	n.d	n.d	n.d	n.d	n.d
Spirodiclofen	n.d	n.d	n.d	n.d	n.d	n.d
Boscalid	n.d	n.d	n.d	n.d	5.23	4%
Pyraclostrobin	n.d	n.d	n.d	n.d	n.d	n.d

n.d.= not detected (<LOD)

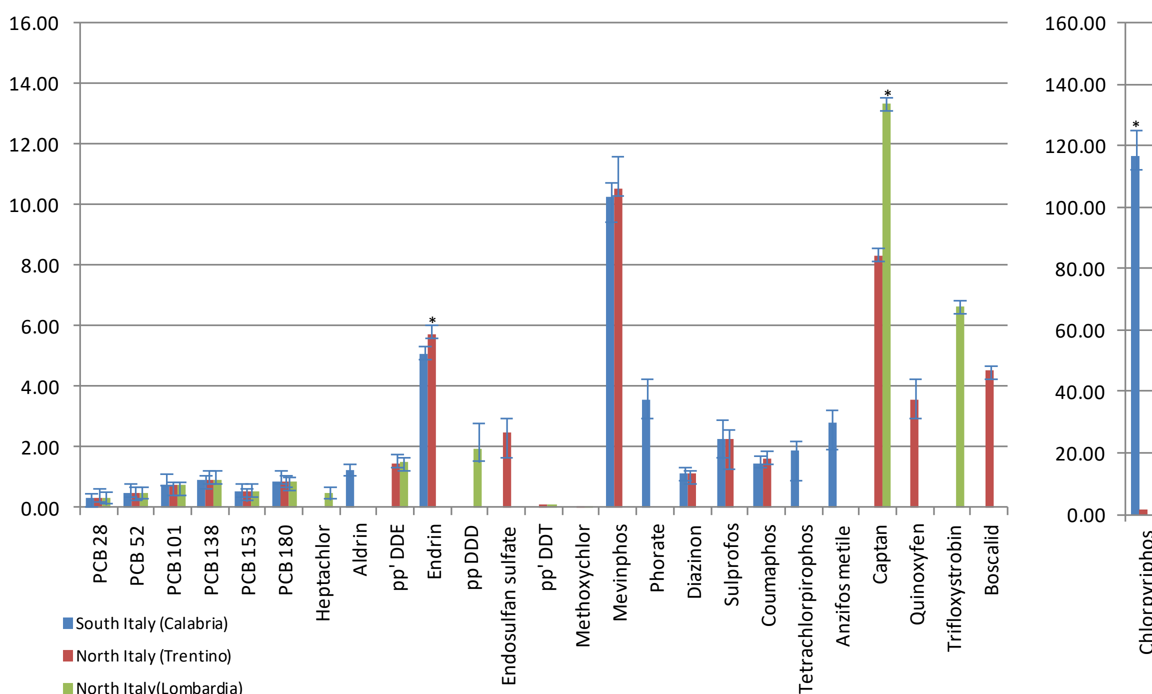
A total ion current (GC-MS/MS) chromatograms of blank honey samples spiked with investigated compounds and a naturally contaminated sample are shown in Figs. S1 and S2 (supplementary materials section). Optimisation of the MS/MS method consisted of (1) acquisition of respective MS spectra in full-scan mode (m/z 100-1000 mass range), (2) selection of precursor ions, (3) product ion scans at different collision energies (10, 20 and 30 eV) and (4) final tuning of the collision energy in selected reaction monitoring mode. For each compound, two MS/MS transitions were chosen to fulfill the generally applied identification criteria: according to the SANTE document, one precursor ion with two product ions or two precursor ions with one product ion should be available for the unbiased identification of the target analyte. An overview of the quantitative and confirmation MS/MS transitions and collision energies selected for each compound in EI mode are given in Table S1.

The method showed good linearity with determination coefficients equal to or higher than 0.99 for all of the compounds investigated; there was also good repeatability, demonstrating that it is useful for monitoring compounds belonging to different chemical classes (Table 2). The recoveries ranged from 97 to 102% for PCBs and PBDEs, from 75 to 95% for OCs and from 75 to 97% for OPs. The CVs were all in the range from 4 to 14%. The one-step ASE method using Florisil as an interference retainer is both rapid and cost-effective and minimises waste generation compared to the classic methods. The time required in the laboratory is reduced to half by combining the extraction and the two clean-up steps (i.e., GPC and SPE) in one single ASE step (Panseri et al., 2014). Our results are in accordance with Lambert et al. (2012), who used Florisil as an interference retainer to extract and clean-up polycyclic aromatic hydrocarbons (PAHs) from bees using ASE extraction techniques combined with in line clean-up obtaining good validation parameters in term of recovery and precision for all PAHs. At present this research represents the first ASE application using an in line clean-up step to screen the presence of different pesticides and organic contaminants from honey.

5.4.2 Application to honey samples

In the present study, the developed method was applied for the analysis of 59 honey samples produced in different Italian geographic areas in order to screen and tentatively relate the presence of pesticide residues to their potential contamination source, also confirming organic honey as a suitable indicator of environmental pollution as well as an indicator of the presence of pesticides utilized in crop protection management. This topic is crucial, especially for organic productions in which the use of allopathic chemically synthesised medicinal products for preventive bee treatments is prohibited and

specific guidelines are given in order to minimise the impact of environmental pollution on bee products like honey (e.g. siting of the apiaries). Overall, the results of detection frequency, concentration levels and distribution of pesticide residues found in organic honey samples according to their sampling area are presented in Table 3 and Fig.1. This research represents the first investigation on the presence of different classes of pesticides and POPs in organic honey. As a consequence, it is difficult to compare our results with those obtained from other monitoring programs, because only a few are published, and the range of pesticides considered is different. The six PCBs examined were detected in all samples, with similar concentrations for each molecule in the three different regions ranging from 0.27 to 0.92 ng g^{-1}. These data show that there are no significant differences in concentrations among the three areas; therefore, the PCB contamination of honey is not influenced by the sample origin. Our results reflect the fact that these regions were characterised by the presence of several harmful industries in the past. Moreover, our data, even at higher concentrations, agree with those of Erdogul (2006), who found PCBs in honey samples from Kahramanmarao, Turkey. Concerning flame retardants, no PBDEs were detected. Unfortunately, there are not many studies regarding the concentration of PBDEs in organic honey, so few data are available. The study byWang et al. (2010), which focused on the presence of PBDEs in developing and developed countries, detected all of the investigated PBDEs and showed that the average concentration of PBDEs in developed regions is always higher than the corresponding PBDE in developing countries, except for PBDE 209, which was not considered in our study. Mohr et al. (2014) also provided data on the presence of PBDEs; according to our data, PBDE 28 and 154 were not detected in their samples, while PBDE 33, 99, 100 and 153 were detected at concentrations in the order of pg g^{-1}. This incongruity is probably due to the fact that our samples are made of organic honey, so the environment and conditions of production have probably significantly reduced the presence of this class of pollutants. Several OC pesticides were present; all honey samples from Calabria showed the presence of Eldrin, with a concentration ranging from 1.95 to 18.9 ng g^{-1}. In one sample, the concentration value was higher than the MRL, while in the other two, the values were close to the MRL. Aldrin, whose prevalence was 50%, was also found at concentrations up to 1.24 ng g^{-1}. Honey is considered unfit for human consumption if residues surpass the maximum residue level (MRL) [Regulation (EC) No 396/2005]. Samples from Trentino Alto Adige are those in which therewas a greater number of OCs. This situation is probably related to the fact that Trentino Alto Adige, in particular Trento Province, is one of the major apple growing areas of Europe (Marini et al., 2012). Intensively cultivated apple plantations are subject to the extensive use of pesticides to control most agricultural pests, even if the integrated pest management system is applied during the growing season (Berrie and Cross, 2005; Tresnik and Parente, 2007). Aldrin



Vertical bar= mean standard deviation

Fig. 1. Distribution of detected contaminants (ng g^{-1}; expressed as mean concentration and standard deviation) in organic honey samples according to their sampling area

and Endrin were detected again, with a frequency of 5% and 44% and a maximum concentration of 1.174 ng g^{-1} and 13.343 ng g^{-1}, respectively. In addition, Dieldrin, an Aldrin metabolite produced by insects, was found. The prevalence of this compound was 5% and the maximum concentration was 0.94 ng g^{-1}; with the same frequency, Heptachlor was detected at a concentration levels up to 0.15 ng g^{-1} pp DDT and its metabolite pp DDE were also present, both with a prevalence of 17%, but with a maximum concentration of 0.09 ng g^{-1} for DDT and 1.47 ng g^{-1} for DDE. Endosulphan sulphate was found, with a frequency of 22% and a maximum concentration of 5.43 ng g^{-1}. Although many OC pesticides are prohibited, the presence of their residues further underlines the persistent nature of these compounds; it also shows that they can enter the food chain not only via fatty products, but also via nonfatty products such as honey. The concentrations of OC pesticides of all samples from Trentino Alto Adige are lower than the MRLs. The situation is analogous for the honey samples from Lombardia, in which all of the concentration values were lower than MRLs. Here, pp DDT and its metabolites pp DDD and pp DDE, were present at concentrations up to 1.99 ng g^{-1} and with prevalence of 41%, 22% and 33% respectively. These results are due to the metabolic degradation of DDT after microbial catabolism, even if the mechanisms have not yet been clarified (Panseri et al., 2014).

Heptachlor was detected with a frequency of 11% and a maximum concentration of 1.19 ng g^{-1}; Dieldrin was also found, with a prevalence of 41% and a maximum concentration of 2.93 ng g^{-1}. Some OP pesticides were also investigated. They are insecticides that are typically used for crop protection in the geographical area characterised by intensive apple orchards (Panseri et al., 2014). Many of them have been found in honey samples, especially those from Trentino Alto Adige, where 12 different pesticides were detected. In particular, Quinoxifen, usually employed in the control of oidium infections, was detected with a prevalence of 100% and a concentration ranging from 3.09 to 4.23 ng g^{-1}. Agricultural activities can be a source of contamination by a variety of pesticides. The pesticide pollution in intensively cultivated areas represents a matter of concern because these products accumulate in vegetation, water and soil and cause damage to beneficial organisms such as honey bees (Apis mellifera L.) (Wallner,1999; Codling et al., 2016). Diazinon was always found in samples from Trentino Alto Adige at concentrations ranging from 1.13 ng g^{-1} to 1.15 ng g^{-1}, while in samples from Calabria this was detected with a prevalence of 64% and a maximum concentration of 1.14 ng g^{-1}. Mevinphoswas found in honey from both Trentino Alto Adige and Calabria, with a prevalence of 67% and 86%, respectively. The samples from Lombardia showed the fewest number of OPs; the highest prevalence (37%) was for Captan, a fungicide that is mainly used for diseases of apples during the growing season (Berrie and Cross, 2005; Blasco et al., 2003, 2008), with a maximum concentration of 20.56 ng g^{-1}. All of the values of pesticides are lower than their MRLs. Only Chlorpyrifos has been detected in some samples of all three regions, showing the highest prevalence (29%) and the highest concentration (389.5 ng g^{-1}) in honey from Calabria: as it is one of the most commonly used insecticides worldwide (Environmental Protection Agency, 738-R-01-007, 2002), such high concentrations are justified. Furthermore, no MRLs are provided for this compound (Cutler et al., 2014). Intensively cultivated apple and citrus plantations are subject to an extensive use of pesticides to control most agricultural pests, even if the integrated pest management system is applied during the growing season, leading to the contamination of bee products (Carpentier and Faucon, 2011; Berrie and Cross, 2005; Ponikvar et al., 2005). Also, Coumafos was detected with high and similar frequencies in honey from Calabria and Trentino (78% and 79%, respectively). Coumafos followed by amitraz and carbendazim are the most commonly used fungicide and acaricide, used by beekeepers to control Varroa destructor. This result is surprising considering that the use of allopathic chemically synthesised medicinal products for preventive bee treatments is prohibited for organic system productions. Several other studies have previously demonstrated that the chemicals used by beekeepers inside the hives are frequently found in the apicultural matrices (Pedersen et al., 2006; Lambert et al., 2013). Coumaphos, another acaricide extensively used against Varroa in recent decades, was also frequently detected in apicultural matrices (Haarmann et al., 2002). In addition, many studies indicate

that coumaphoswas persistent inwax and diffused from wax to honey in high proportions (Haarmann et al., 2002; Blasco et al., 2011).

5.5 Conclusion

An analytical method was developed and successfully applied to evaluate pesticides and POP residues in organic honey samples produced in three different Italian regions characterised by different contamination sources. The method proved to be simple and rapid, requiring small sample sizes, minimising solvent consumption, due to the ASE with an "in line" clean-up step. MS/MS detection provides both quantitative information and the confirmation of POP residues in honey confirming the one-step ASE method as a valid alternative to classical extraction methods because the analytical quality is comparable. The determination of chemical residues in the environment and foods is necessary to ensure that human exposure to contaminants, especially by dietary intake, does not exceed tolerable levels for health. The presence of residues of a number of pesticides in the honey samples and organic contaminant residues indicate that bee colonies in the investigated regions are probably exposed to chronic impacts of pesticides. Furthermore, the results of the present study showed that the presence of the residue in organic honey may also be affected by the contaminant's geographical area (e.g. the presence of an agricultural system) confirming honey bee and beehive matrices as appropriate sentinels for monitoring contamination in the environment. In agricultural areas with developed apiculture, useful information about the occurrence and distribution of pesticide residues due to crop protection treatments can be obtained from the analysis of collected honey samples, which were used as bioindicators. This approach is pivotal and could help beekeepers to select production areas, in particular if dedicated for organic honey production.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.04.004.

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Chapter VI

Accelerated solvent extraction by using an ‘in line’ clean-up approach for multiresidue analysis of pesticides in organic honey

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6. Accelerated solvent extraction by using an 'in line' clean-up approach for multiresidue analysis of pesticides in organic honey

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6.1 Abstract

The worldwide loss of honeybee colonies may be due to their exposure to several contaminants (i.e., pesticides); such contamination may also have impacts on consumers' health. Therefore, is essential to develop quick and new methods to detect several pesticide residues in honey samples. In this study, the effectiveness of accelerated solvent extraction was compared to QuEChERS methods for the analysis of 53 pesticides in organic honey by gas chromatography-triple quadrupole mass spectrometry. Two simple and rapid ASE methods with "in-line" clean-up were optimized and then compared to QuEChERS. Hex:EtAc and Florisil were chosen as extraction solvent and retainer for the first ASE method; ACN and PSA were selected for the second ASE method. The methods were validated according to the European Union SANTE/11945/2015 guidelines. The validation parameters showed that QuEChERS and ASE with PSA as retainer had better repeatability than ASE with Hex:EtAc and Florisil. In particular, QuEChERS and ASE (ACN and PSA) showed good recovery, according to the SANTE criteria, for the majority of investigated pesticides. Conversely, when ASE with Hex:EtAc and Florisil was used as the retainer, several compounds showed recoveries lower than the acceptable value of 70%. The ASE "in-line" method was finally applied to evaluate pesticide concentration in organic honey samples.

6.2 Introduction

The Codex Alimentarius (2001) define honey as: the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts that the bees collect, then transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature" (Wilczynska & Przybylowski 2007; Panseri et al. 2013). Honeybee populations are in worldwide decline, which has been referred to as colony collapse disorder (CCD) and colony weakening (Porrini et al. 2003). Several reasons of colony losses were uphold (Panseri et al. 2014; Chiesa et al. 2016). For instance, pesticides are strongly presumed by the scientific and beekeeping communities to have a great impact on honeybee mortality and colony weakening. Bee matrices, such honey, could be contaminated by environment pollutants including pesticides (Chauzat et al. 2011), heavy metals (Tuzen et al. 2007), bacteria and radioactive materials (Al-Waili et al. 2012). Honeybees are supposed to be possible indicators for environmental biomonitoring (Kasiotis et al. 2014; Malhat et al. 2015) since they cover an extensive area and so are subjected to contaminated food sources, such as pollen and nectar, or water during foraging. Several authors described the use of bees, honey, and pollen as bioindicators of pesticide, heavy metal, and radio nucleotide pollution (Porrini et al. 2003). Moreover, Panseri et al. 2014 and Chiesa et al. 2016 demonstrated the relationship between the contaminant source and the pesticide residues conventional and organic honey samples. It is well known that pesticides (mainly acaricides) can be used in beehives for the control of *Varroa jacobsoni* and *Ascosphaera apis*, so attention is focused on the examination of these pesticides in honey; however, few studies have centred they attention on pesticides (as insecticides) used to treat plants and those introduced in the hive by bees; the relative contribution of pesticides to colony losses remains unknown (Lambert et al. 2013). In order to improve the knowledge of the role that pesticides could have in colony losses it is necessary to develop analytical methods for the detection of pesticides in bee products as honey, to guarantee the food safety (Tette et al. 2016). Moreover, the examination of pesticides in honey could provide information about the use of pesticides in crop fields and in neighbourhoods (Krupke et al. 2012). This is crucial for organic productions systems in which the treatment with synthetic medicinal products in organic beekeeping is not allowed, as these compounds are able to migrate from the wax comb to stored honey and to accumulate in it, being lipophilic and non-volatile substances (Council Regulation 1999; Panseri et al. 2014). Generally, the crucial steps for the identification of contaminant residues in foodstuff are strictly connected with extraction and clean up procedures. (Kujawski et al. 2014). Traditional techniques, including liquid extraction (LLE) and subsequent clean up by using SPE are often carried out, but they are expensive and require a large amounts of organic solvents, which may toxic and cause environmental contamination. (Kamle 2010).

In order to have an adequate program of monitoring, multiresidual methods are required for the detection and quantification of pesticides when attempting to reduce the duration of analyses, extraction and clean up steps (Lambert et al. 2012).

Several extraction and purification techniques, including QuEChERS method and accelerated solvent extraction (ASE), have become popular extraction and purification methods characterised by short extraction times and reduced solvent consumption (Lesueur et al. 2008).

At present, in non-fatty matrices such as honey, QuEChERS represents the most adopted extraction and clean up method for the analysis of pesticides (Wilkowska & Bizuk 2009). Different changes to the traditional QuEChERS have been made depending on the nature of pesticides analysed and the sample characteristics (Wiest et al. 2011). Nonetheless, the QuEChERS approach has disadvantages (i.e. the samples need to have more than 75% of water, otherwise an initial dissolution is required (Carneiro et al. 2013), which lowers the analyte concentration in the sample. ASE uses high temperature and high pressure to reach higher extraction rates due to reduced viscosity and surface tension, but this also increas the diffusion rate and solubility into the matrix. (Wiest et al. 2011). Recently, the extraction and the clean-up steps were combined, creating “in line” methods in which the purification is performed in the ASE cell simultaneously with the extraction. No many studied have examined the extraction of pesticides with an “in line” ASE method, (Kort et al. 2002; Wang et al. 2010; Lambert et al. 2012; Chiesa et al. 2016) therefore, the present study was undertaken to evaluate the effectiveness of ASE compared with QuEChERS for the detection of multiclass pesticides in organic honey samples. Fifty-three pesticides were evaluated including acaricides and insecticides, both chlorinated and non-chlorinated, as they are representative of dangerous chemicals due to their possible toxic effects to honey bees at low environmental concentrations (Lambert et al. 2013; Kasiotis et al. 2014; Malhat et al. 2015). In addition, these compounds have widespread use in plant protection or on the bee hive directly (Porrini et al. 2003; Chiesa et al. 2016). Finally, two ASE preparation methods, based on clean-up with different interferences sorbents were tested and the best one subsequently used for the detection of pesticides in organic honeys by GC–MS/MS

6.3 Materials and methods

6.3.1 Chemicals and reagents

Acrinathrin, bifenthrin, boscalid, bromopropylate, buprofezin, chlorfenvinphos, chlorpyrifos-methyl, chlorothalonil, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, diazinon dichlorvos, difenoconazole, dimethoate, endosulfan (α, β and sulphate), ethion, ethoprophos, fenamiphos, fenitrothion, fenpropathrin, lindane (γ HCH), iprodion, malathion, methamidophos, oxadixyl,

permethrin, phosalone, pirimiphos-methyl, procymidon, propargite, propiconazole, pyridaben, quinoxyfen, tebuconazole, tetradifon, triadimefon and vinclozolin were purchased from Sigma–Aldrich (St. Louis, MO, USA). 3-fluoro-2,2,4,4,6-pentabromodiphenyl ether (FBDE), was used as internal standard (IS) and purchased from AccuStandard (New Haven, USA). Fifty-mL QuEChERS extraction tubes (4 g MgSO4, 1 g NaCl, 1 g Na Cit Tri & 500 mg Na Cit Dibasic), 15-mL QuEChERS clean-up tubes (1200 mg MgSO4, 400 mg CUPSA / 400 mg CE C-18), Acetonitrile LC-MS Grade, hexane, distol-pesticide residue grade, Florisil® 60–100 mesh for column chromatography, 1-mL NORM-JECT® Tuberkulin syringes, and NYLON 0.2-µm filters were purchased from Fisher Scientific (Schwerte, Germany). Diatomaceous Earth, ASE extraction glass fibre filters were purchased from (Thermo-Fisher Scientific, Waltham, MA, USA), Ethylacetate, Lichrosolv for liquid chromatography was purchased from Merck (Darmstadt, Germany), Supelclean ™ PSA (Primary-Secondary Amine) was purchased from Supelco Analytical (Bellofonte, PA, USA). To prepare the stock solution, 10 µg mL-1 reference standard powders of all analysed pesticides were dissolved in hexane or acetonitrile for ASE extraction and QuEChERS, respectively. Stock solutions were stored at −40°C. Calibration solutions containing all substances investigated, in hexane or acetonitrile, were prepared daily from the stock solutions (10 µg mL^{-1}). The appropriate volume was used as a spiking solution.

6.3.2 Honey Samples

Overall, 45 organic honey samples were involved in the present study. In particular, 10 orange blossom honey samples from a German beekeeper were selected and 35 organic honey samples were also provided by Italian beekeepers from two different Italian regions: Calabria, South Italy (15 samples) where intensive citrus orchards were present and Trentino Alto Adige, North Italy (20 samples) where the geographical area is characterised by intensive apple orchards. All samples were stored at -20 °C until analysis to prevent any possible matrix alteration (fermentation phenomena).

6.3.3 Accelerated Solvent Extraction (ASE) procedure with "in-line" clean-up

All extractions were performed using an ASE 350 (Thermo-Fisher Scientific, Waltham, MA, USA). A 34-mL cell was used for ASE. A total of 5 g of honey was weighed and then homogenised with a same weight of diatomaceous earth and transferred into the cell. Then, 1 mL of IS solution was added. To fill the remaining empty part of the cell, diatomaceous earths were added. Two sorbents were tested to evaluate the effectiveness of clean-up: Florisil and PSA. The last one was selected in order to better compare ASE and QuEChERS by using the same extraction solvent and clean-up sorbent. Therefore, the extraction cells were packed with one glass filter at the bottom followed by Florisil® (for the first

test) and PSA (for the second test), which was used as an interference retainer. For the first test, the interferences retainer, solvents, solvent composition, and flush volume had already been optimised in a previous research of Chiesa et al. (2016). The overall extraction parameters are summarised in Table 1. All extracts were collected in 66 mL vials and dried under vacuum using a Rocket evaporator.

The residues obtained from the two investigated tests were evaporated to dryness and reconstituted in 1 mL of hexane or acetonitrile, then transferred to a GC vial for GC-MS/MS analysis;1 µL was then injected.

6.3.4 QuEChERS extraction

QuEChERS extraction tubes (50 ml) were used for extraction. Honey (5 g) was weighed and transferred into the extraction tube. Then, 1 mL of acetonitrile solution containing the IS was added. acetonitrile (10 mL) was added and the tube was shaken for 10 min and centrifuged for 5 min at 5000 rpm.

Table 1. Overview of accelerated solvent extraction (ASE) with '*in line*" clean-up parameters for the two tests adopted in the study.

ASE parameters	ASE (Hex:EtAc and Florisil) Test 1	ASE (ACN and PSA) Test 2
Temperature (°C)	100	Amb.
Heat (min)	5	0
Static Time (min)	5	5
Cycles (n°)	2	2
Rinse volume (%)	90	90
Purge (s)	100	100
Solvent	Hexane:Ethylacetate (4:1)	Acetonitrile
Interferences sorbent retainer (g)	Florisil (2 g)	PSA (2 g)

Supernatant (8 mL) was then transferred in a 15 mL QuEChERS clean-up tube and then centrifuged for 5 min at 5000 rpm. An aliquot of 1 mL was filtered using a nylon 0.2-µm filter directly in a GC vial; 1 µL was injected.

6.3.5 GC-MS/MS analysis of pesticides

Gas chromatography coupled to triple quadrupole mass spectrometry (QqQ) in electronic impact (EI) mode was employed for the simultaneous identification and quantification of pesticides in honey. A GC Trace 1310 coupled to a TSQ8000 triple quadrupole mass detector, (Thermo–Fisher Scientific,

Palo Alto, CA, USA) was used, and a fused-silica capillary column TG-5SilMS (30 m x 0.25 µm x 0.25 mm, Thermo-Fisher Scientific, Waltham, MA, USA) was chosen for the separation of analytes. All the instrumental parameters are reported in our previous work (Chiesa et al. 2016). The QqQ mass spectrometer was operated in SRM mode, detecting two or more transitions per analyte, which are listed together with the particular collision energies in Table S1 in the supplementary data on line. For data analysis The Xcalibur^{TM} processing and instrument control software program and Trace Finder 3.2 (Thermo–Fisher Scientific) were utilised.

6.3.6 Validation parameters

Validation was carried out following the European Union SANTE/2015 guideline (European Union, European Commission 2015; Malhat et al. 2015; Chiesa et al. 2016). The selectivity of the method was evaluated by injecting extracted blank honey samples. The absence of signal above a signal-to-noise ratio of 3 at the retention times of the target compounds was the parameter used to show that the method was free of interferences.

An uncontaminated honey sample was selected as control and then for the procedures' optimisation and validation (QuEChERS and ASE "in line"); 5 g of the control honey were spiked by adding different volumes of the standard working solution in order to have the following concentration :1, 5, 10, 25, 50, 100 ng g^{-1}, in relation to pesticide MRLs, when available, and to generate a matrix-matched calibration curve (MMC). As defined in SANTE guidelines, the LOQ of the methods was the lowest validated spiked level meeting the requirements of recovery within the range of 70–120% and an RSD ≤ 20%. Finally, the extraction methods were evaluated for their repeatability, linearity and recovery as well. Recoveries were calculated by comparing the concentrations of the extracted compounds with those from the MMC calibration curves at three different fortification levels (10, 50 and 100 ng g^{-1}). The repeatability (evaluated as coefficient of variation, CV %) was calculated by analysing six replicates at a concentration level of 50 ng g^{-1}.

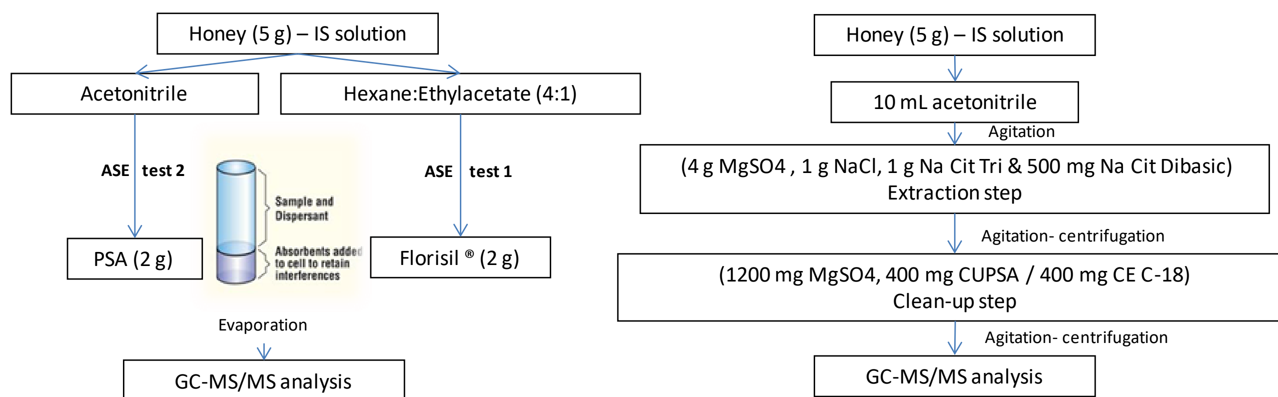
6.4 Result and discussion

6.4.1 Method development and validation

A multi-class method for the analysis of 53 pesticides using ASE with "in-line" clean-up was developed. The ASE with "in-line" clean-up and QuEChERS method's workflows applied for the analyses of organic honey are shown in Figure 1. An overview of investigated compounds concerning their chemical classes, practical use in agriculture and respective LMRs is presented in Table S2 in the

supplemental data online. Our interest was primarily focused on the detection of pesticides utilized in integrated pest

Figure 1. Accelerate dsolvent extraction (ASE) with "in line" clean-up and QuEChERS method's workflow applied for the determination of pesticides in organic honey.



management (IPM) programs adopted in intensive orchards because they may contaminate bee products, as demonstrated by several authors (Panseri et al. 2014). The detection of pesticides in foodstuff is strongly influenced by the presence of fats and requires clean-up steps before analysis (Chiesa et al. 2016). The ASE procedure by using a clean-up in-line approach has been revealed to be effective for removing interferences from honey samples (Chiesa et al. 2016), and in particular Florisil was used as a sorbent for this purpose. Then, the effectiveness of ASE in-line method was evaluated by using the QuEChERS conditions in term of extraction solvent (acetonitrile) and interference retainer sorbent (PSA) for clean-up. QuEChERS was selected for the comparison analysis of pesticides in honey as the efficiency of this approach, for the extraction of pesticides in honey, has been demonstrated by several studies (Kujawski & Namiesnik 2008; Tomasini et al. 2012; Barganka et al. 2013).

The GC-MS/MS chromatograms of naturally contaminated organic honey samples extracted by using ASE in line with PSA as interferences retainer are shown in Figures S1 in the supplemental data online. In general, the complexity of matrices like food and the physical and chemical characteristics of pesticides render the development of analytical methods adequate for the identification of a multiclass of contaminants challenging. Therefore, sample preparation represents a key role in food analysis, because it may cause inaccuracy, furthermore, the efficiency of the extraction is strictly linked with the nature of the matrix and with the characteristics of the analyte (Orso et al. 2016). The desorption of compounds from solid samples can be carried out via three steps during the extraction:

(1) desorption from a solid particle; (2) diffusion through the solvent located inside a particle pore and (3) transfer to the bulk of the flowing fluid (Orso et al. 2016). An ideal extraction method should be fast and easy to perform, yielding satisfying recoveries without loss or degradation of investigated analytes; moreover, fully automated extractions and little laboratory waste are also desiderble characteristics. Concerning ASE technology, many analytical methods to detect residues of many pesticides in food are present in the literature but few studies regarding ASE with in-line clean-up into the cell are reported (Sun et al. 2012).

The linearity of the three investigated methods was in general very good, with correlation coefficients (R^2) > 0.98 for most of the compounds detected both in solvent and in matrix (Table 2). In addition, evaluating the average correlation coefficients and LOQ values obtained in matrix, the in-line ASE method using PSA as interference retainer showed the best results compared with QuEChERS (0.9916, 7 ng g^{-1} for ASE and 0.9480, 22 ng g^{-1} for QuEChERS).

Another criterion used to choose a sample preparation technique was acceptable recovery for all analytes (Panseri et al. 2011). Recoveries (a measurement of accuracy) of the investigated compounds were measured at three different levels (10, 50 and 100 ng g^{-1}). Matrix-matched standards coupled with internal standard calibration were used to compensate potential matrix effects and to avoid any under/overestimation during quantification.

The recoveries calculated at three different concentration levels and clustered in three different groups (<70 %; 70–120 % and >120) are presented in Figure 2. The percent recoveries obtained for all analytes are reported in Table S3 in supplemental data online. According to SANTE, recovery is considered acceptable when the values are 70–120%. Extraction and clean-up using both QuEChERS and ASE (ACN and PSA) showed recovery of the majority of the compounds in this range. In contrast, when ASE was performed with Hex:EtAc and Florisil as retainer, several compounds showed recoveries lower than the acceptable range. It has to be highlighted that, considering the ASE with ACN and PSA in-line method, the recoveries did not depend on the concentrations of analytes. Conversely, when QuEChERS was used, the recoveries were strongly related to concentrations; in particular, we observed a substantial decrease in the number of compounds characterised by satisfactory recovery when the spiking concentration decreased.

Temperature and pressure in ASE represent the predominant parameters influencing the analytes' recoveries. High pressure is applied during the extraction in order to maintain the solvent in a liquid state at elevated temperatures (Sun et al. 2012), and also to enhance the extraction efficiency because solvent is forced into the pores, thus making the analytes available. High temperatures (commonly > 80 °C) during the extraction process has an impact

Table 2. LOQ and determination coefficients, r^2 (solvent and matrix) of QuEChERS and ASE "in line"methods used for pesticides analysis in organic honey.

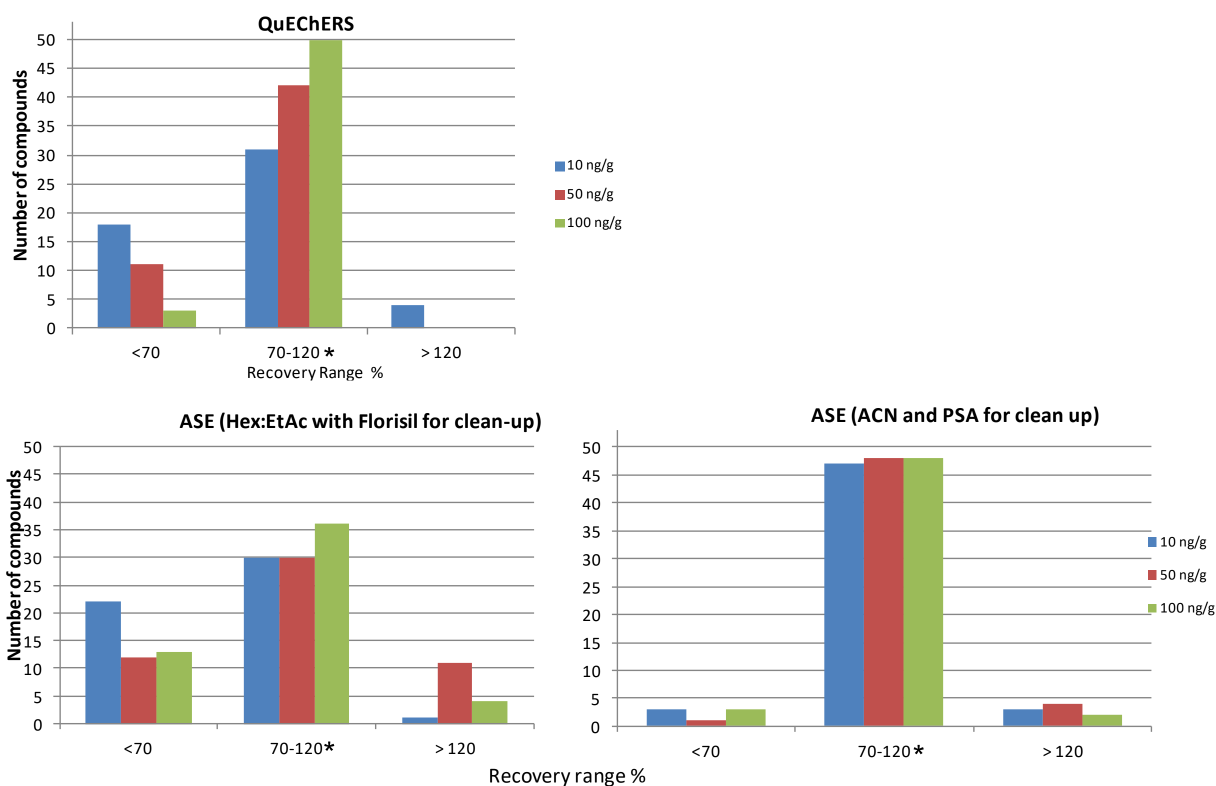
Compounds	QuEChERS R^2		QuEChERS LOQ	ASE (Hex:EtAc and Florisil) Test 1 R^2		ASE Test 1 LOQ	ASE (ACN and PSA) Test 2 R^2		ASE Test 2 LOQ
	Solvent	Matrix (MMC)	(ng g^{-1})	Solvent	Matrix (MMC)	(ng g^{-1})	Solvent	Matrix (MMC)	(ng g^{-1})
Acrinathrin	0.7699	0.9030	50	0.9879	0.9975	1	0.9807	0.9901	10
Bifenthrin	0.9912	0.9369	25	0.9981	0.9902	10	0.9687	0.9927	1
Boscalid	0.9945	0.9932	1	0.9935	0.9913	1	0.9838	0.9969	10
Bromopropylate	0.9566	0.9876	50	0.9969	0.9944	10	0.9694	0.9923	1
Buprofezin	0.9940	0.9286	50	0.9877	0.9932	10	0.9892	0.9915	1
Chlorfenvinphos	0.9973	0.9587	10	0.9904	0.9614	25	0.9799	0.9953	1
Chlorfenvinphos-isomer 1	0.9960	0.9860	10	0.9979	0.9886	25	0.9720	0.9924	1
Chlorfenvinphos-isomer 2	0.9960	0.9860	10	0.9979	0.9886	25	0.9799	0.9956	1
Chlorothalonil	0.4117	0.5698	25	0.9891	0.9796	25	0.9806	0.9780	10
Chlorpyrifos-methyl	0.9877	0.9796	10	0.9987	0.9918	10	0.9536	0.9951	1
Cyfluthrin isomer 1	0.9782	0.9372	25	0.9830	0.9958	10	0.9800	0.9900	10
Cyfluthrin isomer 2	0.9356	0.9605	10	0.9905	0.9958	10	0.9800	0.9800	10
Cyfluthrin isomer 3	0.9696	0.9531	10	0.9795	0.9879	10	0.9900	0.9900	10
Cyfluthrin isomer 4	0.9913	0.9900	10	0.9935	0.9984	10	0.9900	0.9800	10
Cyhalothrin-R	0.9391	0.9746	10	0.9929	0.9976	1	0.9700	0.9939	10
Cyhalothrin-S	0.6904	0.8765	25	0.9944	0.9974	10	0.9631	0.9955	10
Cypermethrin isomer 1	0.9608	0.9565	50	0.9906	0.9900	10	0.9900	0.9900	10
Cypermethrin isomer 2	0.9858	0.9651	25	0.9907	0.9924	10	0.9800	0.9800	10
Cypermethrin isomer 3	0.9729	0.9693	10	0.9929	0.9938	10	0.9900	1.0000	10
Cypermethrin isomer 4	0.9888	0.9674	25	0.98	0.9940	10	0.9900	0.9900	10
Deltamethrin	0.9227	0.9522	25	0.9964	0.9963	10	0.9800	0.9921	10
Diazinon	0.9912	0.9899	10	0.9933	0.9924	25	0.9938	0.9959	1
Dichlorvos	0.9845	0.9889	50	0.9962	0.9978	10	0.9891	0.9807	1
Difenoconazole isomer 1	0.9362	0.9481	50	0.9893	0.9753	25	0.9970	0.9992	10
Difenoconazole isomer 2	0.8928	0.9758	100	0.9929	0.9806	1	0.9860	0.9991	10
Dimethoate	0.9971	0.9827	1	0.9988	0.9977	10	0.9816	0.9775	10
Endosulfan I	0.9534	0.4055	50	0.9965	0.9906	25	0.9947	0.9910	10
Endosulfan II	0.9836	0.7619	25	0.9963	0.9860	25	0.9752	0.9909	10
Endosulfan sulfate	0.9841	0.9697	10	0.9800	0.9958	1	0.9299	0.9871	10
Ethion	0.9971	0.9895	10	0.9941	0.9969	10	0.9829	0.9931	1
Ethoprop (Ethoprophos)	0.9968	0.9917	10	0.9930	0.9939	10	0.9834	0.9935	1
Fenamiphos	0.9719	0.9675	25	0.9901	0.9310	10	0.9360	0.9955	10
Fenitrothion	0.9966	0.9896	1	0.9900	0.9901	10	0.9851	0.9950	10
Fenpropathrin	0.9935	0.9678	25	0.9946	0.9871	25	0.9800	0.9989	1
HCH gamma_Lindane	0.9864	0.9758	10	0.9954	0.9942	10	0.9835	0.9948	1
Iprodione	0.9776	0.9655	25	0.9924	0.9897	1	0.9953	0.9869	10
Malathion	0.9676	0.9719	50	0.9952	0.9892	10	0.9931	0.9950	10
Methamidophos	0.9261	0.8723	50	0.9977	0.9954	10	0.9945	0.9965	25
Oxadixyl	0.9809	0.9897	1	0.9978	0.9945	10	0.9414	0.9723	10

Permethrin isomer 1	0.9889	0.9834	1	0.9982	0.9918	10	0.9837	0.9945	10
Permethrin isomer 2	0.9939	0.9957	10	0.9981	0.9919	10	0.9766	0.9936	10
Phosalone	0.9907	0.9814	10	0.9932	0.9941	10	0.9806	0.9969	10
Pirimiphos methyl	0.9944	0.9973	10	0.9906	0.9934	10	0.9964	0.9942	1
Procymidone	0.9884	0.9671	25	0.9948	0.9960	25	0.7000	0.9931	10
Propargite	0.9765	0.9457	50	0.9961	0.9838	10	0.9959	0.9957	10
Propiconazole isomer 1	0.9877	0.9937	1	0.9937	0.9957	10	0.9821	0.9924	10
Propiconazole isomer 2	0.9877	0.9937	1	0.9937	0.9957	10	0.9785	0.9974	10
Pyridaben	0.9904	0.9915	1	0.9992	0.9964	10	0.9794	0.9909	1
Quinoxyfen	0.9980	0.9953	1	0.9973	0.9953	10	0.9514	0.9939	1
Tebuconazole	0.9867	0.9913	1	0.9885	0.9919	10	0.9693	0.9976	10
Tetradifon	0.9944	0.9920	10	0.9906	0.9946	10	0.9945	0.9907	10
Triadimefon	0.9972	0.9888	25	0.9899	0.9938	10	0.9590	0.9893	1
Vinclozolin	0.9902	0.9896	25	0.9898	0.9958	1	0.9900	0.9900	1
Average value	**0.9587**	**0.9480**	**22**	**0.9931**	**0.9906**	**12**	**0.9740**	**0.9916**	**7**

on the properties of the solvent, increasing, for instance, the diffusion rates and the capacity to solubilize analytes. The analytical procedures become more complex as the number of organic substances present in the matrix increases; when using polar solvents as acetonitrile, ethyl acetate, or a mixtures of polar and non-polar solvents (e.g. n-hexane-acetone, n-hexane- ethyl acetate) the extraction of wet samples could be facilitated (LeDoux 2011).

The precision (repeatability) for each compound, expressed as % CV and evaluated measuring six replicates, is reported in Figure 3. The % CV obtained were, for all three methods, lower than 20 % for most of the compounds, even if the results showed that the QuEChERS and ASE with ACN and PSA had repeatability superior to that of the ASE with Hex:EtAc and Florisil as retainer. Our results underline that for the determination of these

Figure 2. Recovery of the 53 pesticides in organic honey at three spiked concentration levels (10, 50, 100 ng g^{-1}) evaluated for ASE with “in line” clean-up and QuEChERS methods. * Recovery range criteria according to SANTE 2015.



compounds, ACN with PSA into the extraction cell can be considered suitable solvent/sorbent pair for the ASE extraction and clean-up, comparable with QuEChERS, demonstrating that it is useful for monitoring compounds belonging to different chemical classes.

6.4.2 Application to organic orange honey samples

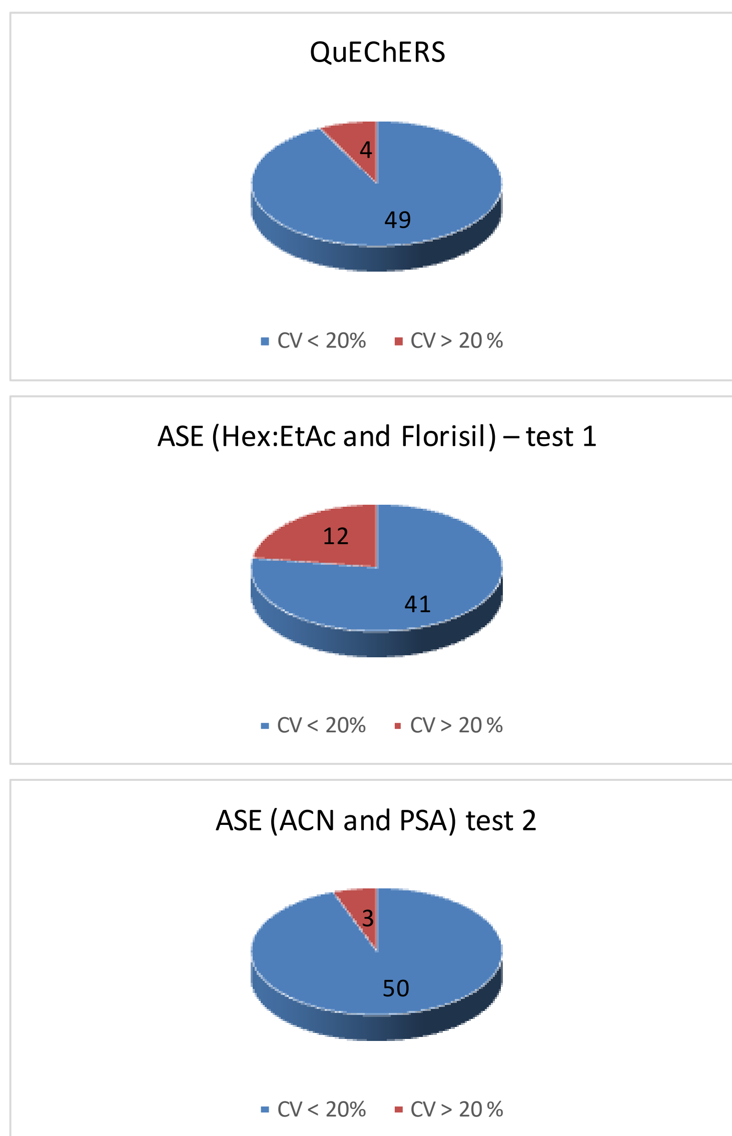
The developed ASE method with ACN and PSA as retainer was used to evaluate the presence of pesticides in organic orange honey samples. An overview of the most recent extraction-purification methods adopted to monitor contaminants in honey and bee products is shown in Table 3. The concentration levels and frequencies of detected pesticide residues in honey samples are presented in Table 4.

Among 53 investigated pesticides, boscalid, diazinon and chlorpyrifos-methyl were found in honey samples; these compounds are used in apple and citrus orchards (Krupke et al. 2012; Cutler et al. 2014; Panseri et al. 2014), but, in general, all pesticides used in cultivated area represent an issue as they are substances able to damage organisms like honey bees (*Apis mellifera L.*) (Porrini et al. 2003; Krupke et al. 2012; Wang et al. 2010). Organic productions are strongly concern about this topic because in this case the use of pharmaceutical drugs for preventive bee treatments is prohibited; specific guidelines are provided in order to reduce the impact of environmental pollution on bee products like honey

(Chiesa et al. 2016). Diazinon and boscalid were found in all samples from Trentino Alto Adige at concentration ranging from 1.13 to 1.15 ng g^{-1}, while in samples from Calabria they were detected with a prevalence of 64% and a maximum concentration of 1.14 ng g^{-1} (Porrini et al. 2003; Krupke et al. 2012; Cutler et al. 2014). Intensively cultivated apple and citrus plantations are subject to an extensive use of pesticides to control most agricultural pests even if the IPM system is applied during the growing season, leading to a contamination of bee products (Chiesa et al. 2016).

Chlorpyrifos-methyl has also been detected at high concentrations in honey samples from German (Table 3). This finding is explainable by

Figure 3. Repeatability expressed as coefficient of variation (CV%) obtained fore ASE with “in line” clean up and QuEChERS methods.



Reference	Number of pesticides analyzed	Separation/Detection	Extraction method	Clean-up	Recovery (%)	LOQ (µg/kg)
Malhat et al. (2015) [9]	18	GC-mECD	QuEChERS	dSPE	85–115	2-60
Eissa et al. (2014) [41]	46	GC-ECD GC/NPD	QuEChERS	dSPE	84–120	1-168[a]
Orso et al. (2014) [32]	24	GC-ECD	QuEChERS	dSPE	71–119	10-20
Wiest et al. (2011) [20]	80	GC-TOF	QuEChERS	dSPE	23-136	3-65.8
Wang et al. (2010) [25]	11	GC–IT/MS	ASE	SPE	52–95	0.001-0.01[a]
Chiesa et al. (2016) [23]	48	GC-MS/MS	ASE	“in line”/Florisil	75-103	0.03-3.36
Chiesa et al. (present study)	53	GC-MS/MS	ASE	“in line”Florisil-PSA	76-142	1-25

[a] when LOQ was non available, LOD is reported

the compound's large worldwide use (US Environmental Protection Agency (USEPA) 2006). Furthermore, no MRLs are provided for this compound (Cutler et al. 2014).

In general, to avoid the presence of agricultural pests, many pesticides are involved in the cultivation of citrus and apple orchards, resulting in a possible contamination of bee products (Wallner 1999; Haarmann et al. 2002; Berrie & Cross 2005; Rissato et al. 2007).

In agricultural areas with developed

Table 3. Overview of the most recent extraction-purification methods adopted to monitor contaminants in honey and bee products

Pesticides	Minimum	Percentile 25th	50th	75th	Maximum	Detection frequency (%)
Organic honey samples-Germany						(n=10)
Boscalid	n.d	10.04	10.15	10.42	10.67	86%
Diazinon	n.d	n.d	1.13	1.13	1.14	64%
Chlorpyrifos-methyl	n.d	n.d	n.d	8.67	389.50	29%
Organic honey samples – South Italy (Calabria)						(n=15)
Boscalid	n.d	n.d	3.12	3.18	6.68	74%
Diazinon	n.d	n.d	n.d	2.54	5.44	38%
Organic honey samples – North Italy (Trentino)						(n=20)
Boscalid	1.13	1.13	1.14	1.14	1.15	100%
Diazinon	n.d	n.d	n.d	n.d	n.d	17%

n.d.= not detected (<LOD)

Table 4. Pesticides concentration (ng g^{-1}) and detection frequency in organic honeys detected with ASE "in line" using ACN and PSA as interferences retainer

apiculture, information about the occurrence and distribution of pesticide residues due to crop protection treatments is essential to prevent and also to manage the production process, especially for organic production system.

6.5 Conclusion

Two in-line ASE extraction methods using Florisil and PSA as an interference retainer were developed and compared with QuEChERS to isolate pesticides residues from organic honey samples. The ASE with in line clean up is cost-effective and minimises waste generation compared with traditional methods, combining the extraction and the clean up in a single step, the time required for the analysis is halved. In particular, ACN with PSA as interferences retainer presented excellent performance in terms of recovery, linearity, and repeatability for all investigated pesticides according to the SANTE 2015, so it was suitable for multiresidue detection and the quantification of 53 pesticides in organic honey. This study is the first to use in-line ASE methods clean-up conditions in comparison with QuEChERS for the evaluation of the presence of different pesticides in honey.

The characterisation of pesticide residues in honey is essential to ensure that human exposure to pollutants, particularly by dietary intake, does not exceed tolerable levels. The presence of residues of a number of pesticides in the honey samples and organic contaminants residues indicate that bee colonies in the investigated regions are probably exposed to chronic impacts of pesticides. Furthermore, the results of the present study show that in organic honey the presence of residues may be affected by the prevalence of a pesticide in a geographical area (e.g. the agricultural system employed) confirming honey bee and beehive matrices as appropriate markers for monitoring contamination in the environment.

Additionally, it is potentially useful for the evaluation of a possible environmental contamination by pesticides, which represents a pivotal task for organic apiculture and other organic production systems.

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spectrometry. We also thank Michal Godula and Katerina Bousova, whose expertise was fundamental to the research.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Chapter VII

Evaluation of the distribution of PCBs, PBDEs, OCPs, PAHs and PFASs in mussels and clams using innovative approaches: HPLC-HRMS analysis and modified QuEChERS extraction followed by GC-MS/MS

Submitted to *Food Chemistry*

7. Evaluation of the distribution of PCBs, PBDEs, OCPs, PAHs and PFASs in mussels and clams using innovative approaches: HPLC-HRMS analysis and modified QuEChERS extraction followed by GC-MS/MS

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7.1 Abstract

Reviewing the presence of contaminant residues is important both for food safety and monitoring of environmental pollution. Here, the occurrence of 6 polychlorinated biphenyls (PCBs), 15 organochlorine pesticides (OCPs), 7 polybrominated diphenyl ethers (PBDEs), 4 polycyclic aromatic hydrocarbons (PAHs) and 17 perfluoroalkyl substances (PFASs) was evaluated in mussels and clams. A liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) and an innovative QuEChERS extraction followed by gas chromatography-tandem mass spectrometry (GC-MS/MS) methods were developed, validated and applied. We demonstrate good linearity, repeatability and accuracy of these methods, confirming that these methods are suitable for the analyses of mollusc samples. The prevalence of PCBs, OCPs and PAHs was higher in mussels than clams. For PFASs, the contamination was higher in clams than in mussels. The samples were all compliant with the regulations and, for the compounds without limit, a risk assessment confirmed that the values were lower than the tolerable intake suggested by EFSA.

7.2 Introduction

Marine ecosystems are subjected to continuous pollution events because of increasing anthropogenic activities and the releasing of various sources of contaminants (Van De Vijver et al., 2003). Bivalve molluscs are considered good environmental contamination indicators because their tissues accumulate contaminants with little metabolic transformations (Roesijadi, Young, Drum, & Gurtisen, 1984; Sericano, 1993). In fact, mussels and clams are filter-feeding organisms. Therefore, most of the contaminants are directly bioavailable and can accumulate across gills and by ingestion of particles (Kimbrough, Johnson, Lauenstein, Christensen, & Apeti, 2008). Mussels were often used as sentinel indicator species to monitor the environmental accumulation of various persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) (Webster et al., 2008) and perfluoroalkyl substances (PFASs). Concerning PFASs, research attention has rapidly increased because of their worldwide spread in multiple environmental areas (Kannan, 2011). Global monitoring of PFAS contamination has identified perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) as the predominant compounds, ubiquitously distributed in several animal tissues (Giesy & Kannan, 2001; Van de Vijver et al., 2003). The persistence in the environment of PFOS and PFOA was demonstrated, such as their capability to bioaccumulate in the trophic chain (Valsecchi, Rusconi, & Polesello, 2013) but, as emerging contaminants, no maximum residue levels (MRLs) have yet been set. Among other POPs, PCBs, PBDEs and PAHs are contaminants commonly found in sediments, waters and wildlife (Erickson, 1997; Safe, 2002). These three classes of compounds have similar physicochemical characteristics of lipophilicity and resistance to degradation (Xua, Wanga, & Caia, 2013). Their high bioaccumulation potential added to a variety of toxic effects on humans and animals makes the evaluation of their occurrence a pivotal task (Van den Berg et al., 2006; Robertson, & Hansen, 2001). PAHs, PCBs and PBDEs produced by anthropogenic activities can undergo long-range atmospheric transport and could be, therefore, found in the marine environments (Fernandez, & Grimalt, 2003; Teil, Blanchard, & Chevreuil, 2004; Chiesa, Labella, Panseri, Pavlovic, Bonacci, & Arioli, 2016 a). PFASs have no MRLs, whereas PCBs and PAHs have maximum limits that are recommended by Commission Regulation No 1259/2011 (European Union, 2011) and Commission Regulation No 1881/2006 (European Commission, 2006). Also, no MRLs have been established for PBDEs, but the European Commission recommended their monitoring in food, especially of animal origin (European Union, 2014). Organochlorine pesticides (OCPs) have a similar behavior to the other contaminants described. OCPs reach the marine environment from surface runoff and ground leachate but can also be found in stormwater and wastewater discharges (Clendening, Jury, & Ernst, 1990). Although

several pesticides (as DDT) are prohibited, they and their metabolites are still found in coastal waters, sediment and biota (Richardson, & Zheng, 1999). Monitoring guidelines for OCPs in fish are reported by the Food and Drug Administration (2011).

Because of the very low limits reported by legislations (in the order of ng g $^{-1}$) and considering the large number of compounds that have to be monitored, novel analytical protocols are necessary to allow the quantification of these compounds with high sensitivity, selectivity and specificity. Among the analytical techniques available, high-pressure liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) and gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) represent the best choice for the detection of ultra-trace levels of different compounds in heterogeneous matrices. In particular, the Orbitrap HRMS resolving power, combined with the fast scan speed, results in high accuracy (lower than 1 ppm), sensitivity and specificity, providing all the characteristics for confirmatory methods, while GC-MS/MS guarantees the high performances required for the analyses of lipophilic compound, such as PBDEs and PCBs, as reported by Chiesa et al. (2016 b).

Based on the considerations discussed above, the aim of this study was to develop and validate two analytical methods, a HPLC-HRMS method for the analysis of PFASs and a GC-MS/MS method with an innovative Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction, for the analysis of PCBs, PBDEs, PAHs and OCPs. Later, the occurrence of these five classes of POPs was evaluated in mussels and clams. The two mollusk species were selected because they are the most consumed in EU (European Commission, 2016) and live at different depths: the mussel habitat is epipelagic whereas the clam habitat is benthonic, therefore possibly representing different levels of contamination.

7.3 Material and methods

7.3.1 Sampling

Mussels and clams were collected at the wholesale fish market of Milan, the most important Italian fishery market. The sample collection, randomly made, was representative of the contamination levels of mollusks available to Italian consumers. Multiple species were selected: *Mytillus Galloprovincialis, Mytillus Edulis* and *Mytillus Chilensis* for mussels, and *Venerupis philippinarum*, *Perna Canaliculus, Tapes decussatus, Tapes Semidecussatus, Meretrix Meretrix* and *Meretrix Iyrata* for clams. All

molluscs were collected from June 2016 until February 2017, and the sampling areas are shown in Fig. 1.

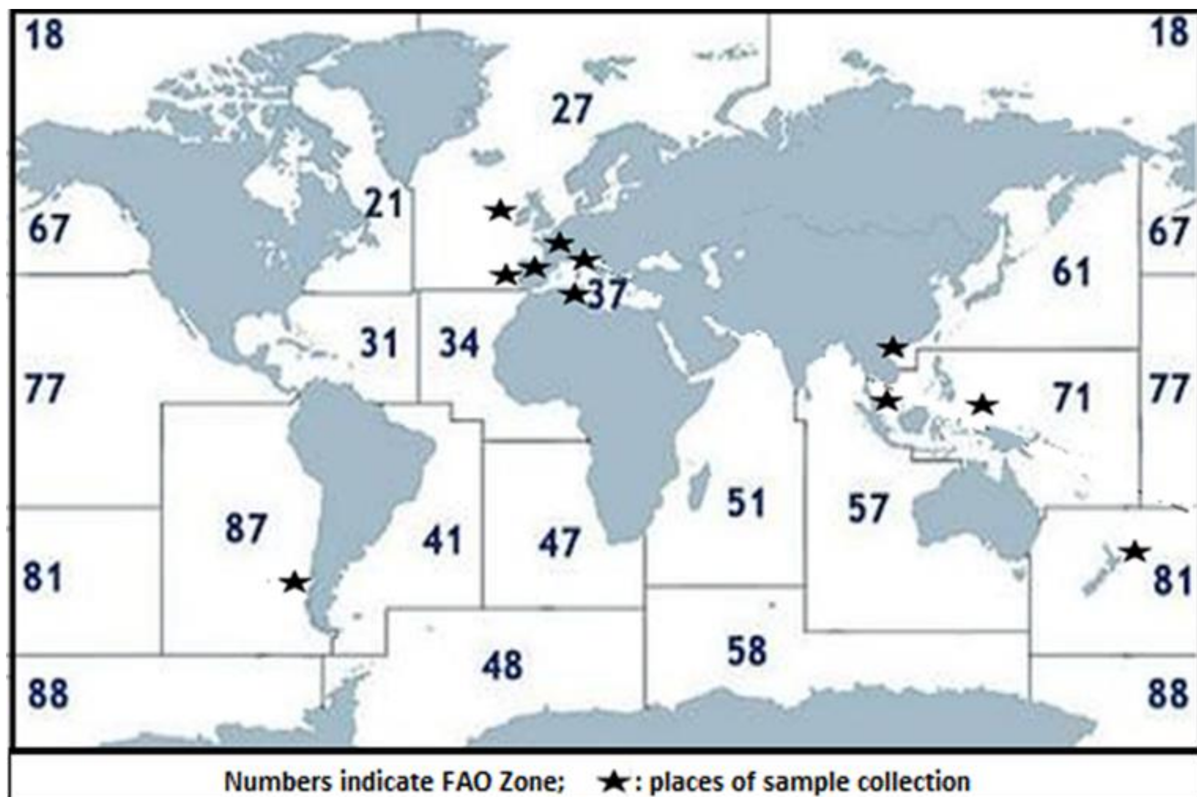

Fig. 1. Map of sample collection sites.

A total of 50 mussel and 39 clam samples were made: the soft tissue was separated from the shells and pools of about 50 individuals were prepared for each sample; after homogenization, the samples were stored at -20°C until analyses.

7.3.2 Chemicals and reagents

A mixed solution of PCB congeners (PCB 28; PCB 52; PCB 101; PCB 138; PCB 153 and PCB 180), PCB 209 (internal standard [IS] for PCBs and PAHs), a mixed solution of PBDEs (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 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 OCPs and their metabolites (α- HCH; Hexachlorobenzene; β-BHC; Lindane; Heptachlor; Aldrin; Heptachlor epoxide; Trans Chlordane; 4,4'-Dichlorodiphenyldichloroethylene [4,4'- DDE]; Endosulfan I; Endosulfan II, Endosulfan sulfate; Endrin, 4,4'Dichlorodiphenyldichloroethane [4,4'-DDD], 2,4'-Dichlorodiphenyltrichloroethane [2, 4'-DDT]) and a standard solution of four PAH congeners (Chrysene, Benz(a)anthracene,

Benzo(b)fluoranthe and Benzo(a)pyrene) were purchased from Restek (Bellefonte, PA, USA). Seventeen acid and sulfonate perfluorinated compounds were examined in this study: perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorobutane sulphonic acid (PFBS), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorohexane sulphonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluorooctane sulfonic acid (PFOS), perfluorododecanoic acid (PFDoA), perfluoroundecanoic acid (PFUnDA), Sodium perfluoro-1-decanesulfonate (PFDS), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA) and perfluorooctadecanoic acid (PFODA). All of these compounds and the two ^{13}C-labeled internal standards (ISs) MPFNA and MPFOS were purchased from Fluka (SigmaeAldrich, St. Louis, MO, USA), as well 4-nonylphenol (IS for OCs) and all GC and HPLC solvents. Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). For the extraction and clean-up of POPs, QuEChERS materials were obtained from Supelco (SigmaeAldrich, St. Louis, MO, USA); Supel^{TM} QuE Citrate (EN) tubes, containing Sodium Citrate tribasic dihydrate and Sodium Citrate dibasic sesquihydrate. Magnesium Sulfate and Sodium Chloride were used for the extraction. Supel^{TM} QuE-Sep tubes were used for the clean-up step. For the extraction of PFAs, the extraction cartridges (Oasis HLB WAX 3 mL, 60 mg) were provided by Waters (Milford, MA, USA). Ammonium formate, sodium acetate, acetic acid (99.9%) and 25% ammonia solution were purchased from Fluka.

7.3.4 Standard solutions

Stock solutions (1 mg mL^{-1}) of each standard used for HPLC-HRMS analyses, were prepared in methanol and stored at -20°C. Working solutions at the concentrations of 10 and 100 ng mL^{-1} were prepared during each analytical session and maintained at 4°C throughout the method validation. For GC-MS/MS analyses, working solutions were prepared daily in hexane from various stock solutions containing a mix of standards. The storage conditions of the solutions were the same as described for HPLC analyses.

7.3.5 Extraction procedure

For the extraction of PFASs, 2 g of sample was spiked with the two internal standards at the concentration of 5 ng mL^{-1}. After the addition of 10 mL of acetonitrile for the protein precipitation and analytes extraction, the sample was vortexed and sonicated for 15 min. After centrifugation (2500×g,

4°C for 10 min), the supernatant was collected into a glass flask and evaporated in a rotary vacuum evaporator at 35°C. The extract was suspended in 10 mL of water and underwent the SPE extraction using the Oasis WAX Cartridges under vacuum, for further purification and extraction. The SPE cartridges were preconditioned with 3 mL of 0.5% ammonium hydroxide in methanol, 3 mL of methanol, and 3 mL of Milli-Q water. The sample was loaded, and then the cartridges were washed with 3 mL of 25 mM acetate buffer pH 4.5 to remove interferences, as well as lipid or proteins, and to increase the adsorption of target anions to the cartridge, followed by 2 mL of methanol. Finally, the compounds were eluted using 3 mL of 0.5% ammonium hydroxide in methanol and were collected in a 15 mL polypropylene tube. The eluate was dried in a rotary vacuum evaporator at 35°C. The dried extract was suspended in 100 µL of methanol:ammonium formate 20 mM (10:90 v/v), and then transferred to an auto-sampler vial. The injection volume was 10 µL. The method was developed and optimized taking into consideration the work of Taniyasu et al. (2005), considering the different effect of pH of acetate buffer, the percentage of ammonium hydroxide in methanol and the influence of elution volume of ammonium hydroxide in methanol on recoveries of PFASs.

Moreover, taking into account the ubiquity of PFAS in the environment of analytical laboratories, several precautions were taken, such as washing glassware with methanol, the execution of at least 10 procedural blanks at days to subtract any background contamination.

The extraction of PCBs, PBDEs, OCPs and PAHs was performed using the QuEChERS approach. A 5 g of sample was homogenized and transferred to a QuEChERS extraction tube, then the three ISs were added. Ten milliliters of a mixture of hexane/acetone (4:1 v/v) was added as extraction solvent; the tube was shaken for 1 min using a vortex and centrifuged for 10 min at 2000×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 transferred in a flask and evaporated under vacuum in a centrifugal evaporator at 35°C. The residue was dissolved in 1 mL of hexane and analysed by GC/MS-MS.

7.3.6 HPLC-HRMS analyses

The HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), consisted of 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. A Synergi Hydro-RP reverse-phase HPLC column (150 × 2.0 mm, 4 µm particle size), with a C18 guard column (4 × 3.0 mm) (Phenomenex, Torrance, CA, USA) was used for

the chromatographic separation. Stainless steel capillary tubes were used for minimising PFAS background contamination in the system. Moreover, since PFOA and PFOS were always present in the chromatographic system, we introduced a small Megabond WR C18 column (5 cm × 4,6 mm, i.d. 10 μm) between pump and injector, allowing us to delay our analytes by 2 min relative to those already present in the system.

Solvents A (aqueous ammonium formate 20 mM) and B (MeOH) were the mobile phases used for the gradient. The elution started with 10% B, which increased to 40% at the 4th minute and more gradually to 95% at the 12th minute, then remaining constant up to the 18th minute. The initial conditions were reached at the 20th minute, with an equilibration time of 7 min. The flow was 0.3 mL min^{-1}. The detector was a Thermo Q-Exactive Plus (Thermo Scientific, San Jose,CA, USA), equipped with a 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. The sheath and auxiliary gas were set at 35 and 15 arbitrary units. S lens RF level of 60 instrument calibration was done for 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 acquisition was combined with a DIA Independent Data Acquisition mode, providing the MS2 spectra for the confirmatory response, based on an inclusion list. The resolving power of FS was set at 70,000 FWHM. On the basis of our compound list, a scan range of m/z 200–950 was chosen; the automatic gain control (AGC) was set at 1×10^{-6} and the maximum injection time was 200 ms. The DIA segment operated in negative mode at 35,000 FWHM. The AGC target was set to 5×10^{-4}, with the maximum injection time of 100 ms. the quadrupole filtered the precursor ions with an isolation window of 2 m/z. Fragmentation of precursors was optimised as two-stepped normalized collision energy (NCE) (10 and 70 eV). The mass tolerance window was set to 2 ppm. Detection of analytes was based on the retention time of target compounds, on calculated exact mass of the deprotonated molecular ions, and at least one specific and typical fragment. 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. Xcalibur™ 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HPLC-HRMS system, the exact mass of the compounds, record and elaborate data.

7.3.7 GC-MS/MS analysis of contaminants

The GC analysis was described in a previous study of ours (Chiesa, Labella, Panseri, Pavlovic, Bonacci, & Arioli, 2016 a). Briefly, GC-MS/MS in electronic impact (EI) mode was carried out by a

GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) using a fused-silica capillary column RXi-XLB (30 m, 0.25 mm i.d., 0.25 mm film thickness, Restek, Bellefonte, PA, USA).

Selected reaction monitoring mode (SRM) was used to detect two ot three transitions per analyte according to European Commission (2015). Compound identification was performed by comparing relative retention times of samples and standard solutions and mass fragmentations obtained for each compound. All fragments are reported in Table 1. Xcalibur™ and Trace Finder™ 3.0 (Thermo Fisher Scientific) were the software used as instrument control and data processing, respectively.

Table 1. Retention time (tr), precursors, main products, polarity and collision energies of the compounds analysed by LC-HRMS and GC-MS/MS.

Compound LC-HRMS	**Formula**	**t_r (min)**	**Precursor (m/z)**	**Main product (m/z)**	**Polarity**
PFBA	$C_4HF_7O_2$	9.07	212.97920	168.98836	(-)
PFPeA	$C_5HF_9O_2$	11.68	262.97601	218.98560	(-)
PFBS	$C_4F_9HO_3S$	12.02	298.94299	98.95434	(-)
PFHxA	$C_6HF_{11}O_2$	13.22	312.97281	268.98288	(-)
PFHpA	$C_7HF_{13}O_2$	14.36	362.96962	318.97949	(-)
PFHxS	$C_6F_{13}HO_3S$	14.39	398.93660	98.95437	(-)
PFOA	$C_8HF_{15}O_2$	15.27	412.96643	368.97681	(-)
PFNA	$C_9HF_{17}O_2$	16.03	462.96323	418.97385	(-)
PFOS	$C_8F_{17}HO_3S$	16.00	498.93022	79.95598	(-)
PFDA	$C_{10}HF_{19}O_2$	17.96	512.96004	468.97064	(-)
PFUdA	$C_{11}HF_{21}O_2$	18.48	562.95684	518.96729	(-)
PFDS	$C_{10}F_{21}HO_3S$	17.35	598.92383	79.55599	(-)
PFDoA	$C_{12}HF_{23}O_2$	18.98	612.95365	568.96387	(-)
PFTrDA	$C_{13}HF_{25}O_2$	19.50	662.95046	618.96057	(-)
PFTeDA	$C_{14}HF_{27}O_2$	20.06	712.94726	668.95823	(-)

PFHxDA	$C_{16}HF_{31}O_2$	20.80	812.94088	768.95184	(-)
PFODA	$C_{18}HF_{35}O_2$	21.81	912.93449	868.94513	(-)
MPFNA	$[13]C_5C_4HF_{17}O_2$	16.03	467.98001	422.98703	(-)
MPFOS	$[13]C_4C_4F_{17}HO_3S$	16.00	502.94364	79.95592	(-)

7.3.8 Validation parameters

Validation was carried out following the European Commission (2015) SANTE/2015 guideline. The selectivity of the method was evaluated by injecting extracted blank mollusc samples. The absence of interferences was proved by the lack of peaks with a signal-to-noise ratio higher than 3 at the retention times of the target compounds. Mollusk sample, previously analysed and checked for the absence of all POPs, were used as control samples during optimization and validation steps. For mollusc fortification, 5 g of the control sample was spiked in order to cover the concentration range from 0.5 to 100 ng g^{-1} (five calibration points: 0.5, 1, 10, 50 and 100 ng g^{-1}) for PCBs and PAHs; from 0.5 to 50 ng g^{-1} (five calibration points: 0.5, 1, 10, 25, 50 ng g^{-1}) for PBDEs and from 5 to 1000 ng g^{-1} for OCs (five calibration points: 5, 50, 100, 500 and 1000 ng g^{-1}). For PFASs, 2 g of control sample was spiked to cover the concentration range from LOQ to 10 ng g^{-1} (six calibration points LOQ, 0.05, 0.1, 3, 5, 10 ng g^{-1}), except for PFBA, PFOA and PFUdA (up to 50 ng g^{-1}, six calibration points: LOQ, 0.05, 0.1, 5, 10, 50 ng g^{-1}) in order to realize the matrix-matched calibration curves. For the limit of quantification (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 ≤ 20%, as defined by the European Commission (2015). Finally, the extraction methods were also evaluated for their repeatability, linearity and recovery. Recoveries were calculated by comparing the concentrations of the extracted compounds with those from the MMC calibration curves at LOQ for all compounds. The repeatability (evaluated as the coefficient of variation, CV%) was calculated by analysing six replicates at the same fortification level.

7.4 Results and discussion

7.4.1 Validation parameters

The methods showed high specificity, without any interferences close to the retention time where the investigated compounds were expected to elute, and consequently showed a high S/N ratio in the presence of analytes, even at the lowest detectable concentration. The mean recoveries ranged between

70 and 120%, indicating the efficiency of the extraction protocol. Matrix validation curves demonstrated a good linearity over the working range with a good fit (R^2 > 0.985) for all compounds. Repeatability was calculated using one-way analysis of variance (ANOVA), the CV was lower or equal to 20 % for all POPs, satisfying the criteria required by the European Commission (2015).

Regarding the LOQs, our satisfactory results showed high method sensitivity for the selected contaminants both for LC-HRMS and GC-MS/MS analyses. In particular, the analytes detected with GC-MS/MS showed LOQs equal or lower than those reported by Pizzini et al. (2016), for example, benzo(b)fluoranthe has an LOQ of 0.5 ng g^{-1}, which is lower than the 3.54 ng g^{-1} reported by Pizzini et al. (2016). For PFASs, the LOQs were much lower than those reported by Nania et al. (2009) and Wille et al. (2011), which have for PFOS an LOQ of 6 ng g^{-1} and 0.1 ng g^{-1} respectively, compared to our LOQ of 0.005 ng g^{-1}.

All of the validation parameters for GC-MS/MS and HPLC-HRMS are reported in Table 2.

Table 2. Validation parameters of the investigated POPs

Compounds by GC-MS/MS	**LOQ (ng g^{-1})**	**CV %**	**Recovery %**
PCB 28	0.5	11	85
PCB 52	0.5	9	87
PCB 101	0.5	9	83
PCB 138	0.5	12	97
PCB 153	0.5	12	85
PCB 180	0.5	10	88
PBDE 28	0.5	2	93
PBDE 33	0.5	3	79
PBDE 47	0.5	9	94
PBDE 99	0.5	7	81
PBDE 100	0.5	11	80
PBDE 153	0.5	7	70

PBDE 154	0.5	9	84
α HCH	5	18	119
β BHC	5	20	120
Hexachlorbenzene	5	16	100
Lindane	5	20	116
Heptachlor	5	20	120
Aldrin	5	12	89
Heptachlor epoxide	5	10	93
Trans chlordane	5	12	94
Endosulfan I	5	12	95
Endosulfan II	5	10	84
pp' DDE	5	16	90
Endosulfan Sulfate	5	14	75
Endrin	5	10	120
op DDT	5	20	120
pp DDD	5	3	102
Chrysene	0.5	3	82
Antracene	0.5	6	75
Benzofluoranthene	0.5	3	75
Benzopyrene	0.5	2	77

Compounds by HPLC-HRMS	**LOQ (pg g^{-1})**	**CV %**	**Recovery %**
PFBA	5	7	82
PFPeA	10	10	114
PFBS	10	11	102
PFHxA	20	6	110
PFHpA	5	5	112
PFHxS	15	9	103
PFOA	5	5	113
PFNA	5	10	95

PFOS	5	12	83
PFDA	20	13	84
PFUdA	20	6	85
PFDS	20	8	83
PFDoA	20	8	89
PFTrDA	15	8	87
PFTeDA	5	15	91
PFHxDA	5	18	85
PFODA	5	18	84

7.4.2 Mussel and clam sample POP distribution

Results on the prevalence and concentration of contaminants are reported in Table 3.

Table 3 Prevalence and concentration ranges of the selected contaminants.

Compounds	**Prevalence (%)**		**Concentration range (ng g^{-1})**	
	Mussels	**Clams**	**Mussels**	**Clams**
Σ PCBs	58	n.d.	n.d. - 49.02	n.d.
Σ PAHs	36	28	n.d. - 13.95	n.d. - 4.35
Σ DDTs	12	8	n.d. - 16.34	n.d. - 14.96
Σ PBDEs	8	2	n.d. - 0.5	n.d. - 0.5
Σ PFAs	70	100	n.d. - 91.80	n.d. - 120.75

n.d. = not detected

PCBs were found with the highest prevalence in mussels, while they were not found in clams, as showed in Fig. 2.

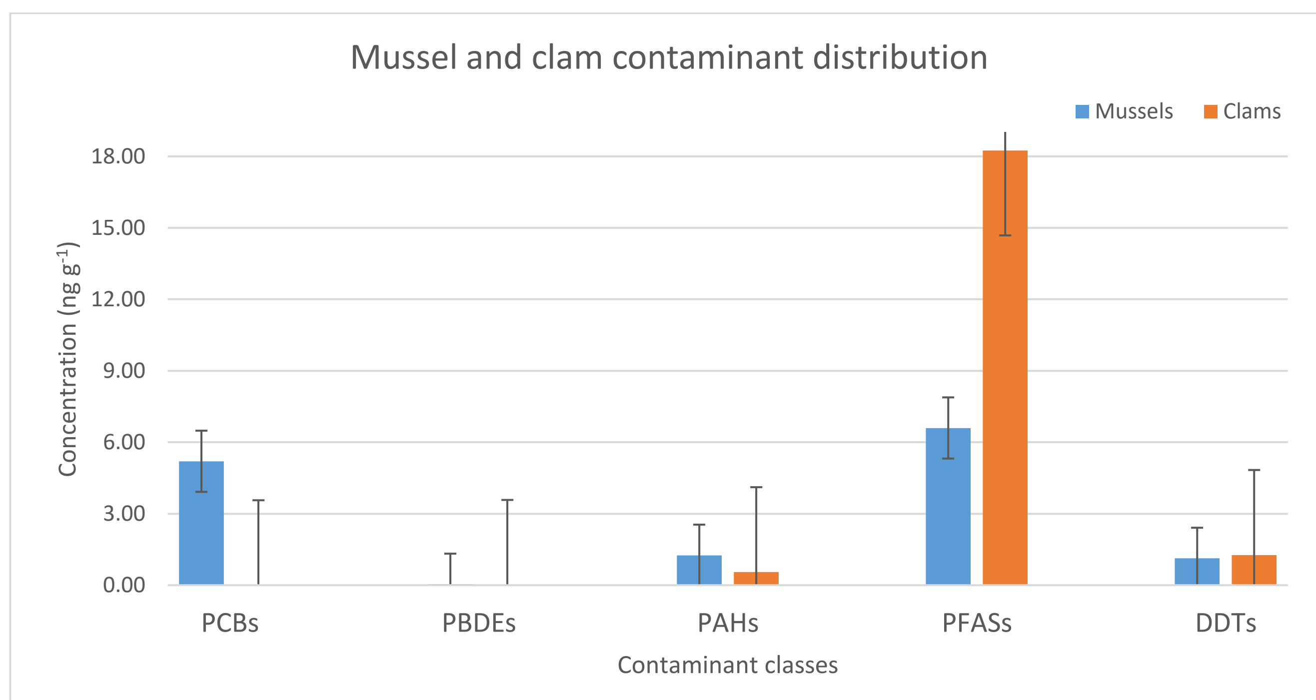

Fig. 2. Mean values of the Σ PCBs, Σ PBDEs, Σ PAHs, Σ PFASs and Σ DDTs in mussels and clams.

In particular, the most abundant congener was PCB 138, showing the highest concentration of 25.34 ng g^{-1}. The concentrations were all lower than the maximum levels of 75 ng g^{-1} required by the European Union (European Commission, 2011). Referring to the overview of the literature studies reported in Table 4, the concentration of PCBs in mussel samples were in according to those found by Herceg-Romanic´ et al. (2014), which found PCB 138 as one of the most abundant congeners, but with a lower maximum concentration compared to our results (6.34 ng g^{-1}).

Table 4. Literature data on POPs distribution in mollusks.

Reference	Compounds investigated	Analytical technique	Concentration range in ng g^{-1} (average values)	
			Mussels	**Clams**
Choi et al. (2016)	18 PCBs	GC-ECD	70.6-159 [a]	69.3-109 [a]
	DDTs		38.6-102 [a]	40.3-49.3 [a]
	α- , β- , γ- and δ-HCH		9.00-13.5 [a]	6.25-17.8 [a]
Pizzini et al. (2016)	127 PCBs	GC-MS	< LOD - 4.57 [b]	< LOD - 3.68 [b]
	16 PAHs		< LOD - 7.03 [b]	2.32 - 5.67 [b]

Dodder et al. (2014)	2 PFASs	LC-MS/MS	< LOD - 29 [c]	not investigated
	11 PBDEs	GC-MS/MS	< LOD - 68 [c]	not investigated
Herceg-Romanic´ et al. (2014)	17 PCBs	GC-ECD	1.12 - 23.86 [b]	not investigated
	α- , β- and γ-HCH		0.40 - 1.61 [b]	not investigated
	Hexachlorobenzene (HCB)		0.01 - 0.12 [b]	not investigated
	DDTs		0.15 - 2.61 [b]	not investigated
Wille et al. (2011)	14 OCPs	LC-MS/MS	< LOD - 28 [c]	not investigated
	10 PFASs	LC-ToF	< LOD - 4 [c]	not investigated
Nania et al. (2009)	2 PFASs	LC-MS/MS	< 1.5 – 3 [b]	< 2 – 16 [b]

a = expressed as lipid weight
b = expressed as wet weight
c = expressed as dry weight

PAHs were detected both in mussels and clams, with the highest prevalence in mussels. This could be because the discharges of maritime transport of petroleum products (oil spills) are mainly composed of PAHs and are viscous fluid mixtures having a density lower than water, so PAHs tend to remain on the water surface (Gonzalez-Doncel, Gonzalez, Fernandez-Torija, Navas, & Tarazona, 2008; Fingas, 2016). The most frequent compound detected was Benzo(a)pyrene, with a maximum concentration of 7.05 ng g^{-1}. Also for these contaminants, all of the samples were compliant to Regulation No 1259/2011 (European Commission, 2011). As also reported by Pizzini et al. (2016), the PAHs concentration is higher in mussels than in clams, but in our study, the difference was greater, maybe due to the reasons described above. In fact, the level of PAHs in mussels was 13.95 ng g^{-1}, while this value was 4.35 ng g^{-1} in clams (approximately three times lower than in mussels).

Concerning OCPs, only DDT metabolites were found. In particular, 4,4’-DDE was detected only once in mussels, and was never detected in clam samples; 4,4’-DDD was found both in mussels and clams with a low prevalence of 10 and 8 %, respectively and a highest concentration of 16.34 ng g^{-1}. The concentration of DDTs found in our mussel samples was higher compared to the results of Herceg-Romanic´ et al. (2014), who found a highest concentration of 2.61 ng g^{-1}.

PBDEs were found only in four mussel samples and one clam sample at the LOQ. Despite low prevalence, the concentrations found are higher than reported by Hu et al. (2010), which detected PBDEs at concentrations ranging from 25.4 to 58.9 pg g^{-1}.

Regarding PFASs, up to 11 compounds (both acid and sulfonate forms) were detected in almost all clam samples, showing an evident higher contamination in terms of frequency and concentration than in mussels. The most contaminated clam pool was fished in the FAO area 37.2, confirming the pollution of this area, as reported by Vianello et al. (2013). The most abundant compound in clams was PFOA, with 97% of positivity and the highest concentration of 31.03 ng g^{-1}. Of the tested compounds, PFBA was present at the highest concentration (both for mussels and clams). This is because, as discussed by Water Research Foundation project #4322 (Fulmer, 2016), conventional treatment at wastewater treatment plants and most drinking water treatment plants are ineffective at removing this shorter chain PFAS. It should be emphasized that this analyte was always present even in the background contamination of the extractive procedure at a maximum concentration of 4 ng g^{-1}, evaluated through the analysis of a batch of 10 procedural blanks during each analytical session. The evidence of a major contamination in clams (Fig. 2) is present also in the study of Nania et al. (2009). This could be explained by the fact that clams can absorb both from seawater and sediments, as reported by Berger et al. 2004 and Nakata et al., 2006. PFOA prevalence was found to be higher than PFOS, which is in line with the results of Nakata et al., 2006. In clams, the PFOA concentrations were also higher than those of PFOS, as reported in the last study about sea sediments. However, in mussels, this trend is reversed, even if the concentrations of PFOA and PFOS were quite similar.

7.4.3 Risk assessment

Considering the absence of maximum limits for PFASs, a risk assessment was carried out on the basis of our results referring to the established tolerable daily intake [TDI] for PFOA and PFOS (1.5 µg Kg^{-1} b.w. per day and 150 ng Kg^{-1} b.w. per day, respectively (EFSA, 2008)). Considering a person of 70 Kg, the threshold dose is 105 µg per day for PFOA and 10.5 µg per day for PFOS; on the basis of data reported by EUMOFA (European Commission, 2016), the annual per capita consumption is 1.27 Kg for mussels and 0.33 Kg for clams. Considering these tolerable intakes and, with a conservative approach, the highest concentration of PFOA and PFOS found in our samples were 0.55 and 3.64 ng g^{-1} (in mussel) and 31.03 and 7.20 ng g^{-1} (in calm) respectively. These concentrations could result in a daily intake of 1.91 ng of PFOA and 12.66 ng of PFOS in mussels and 27.93 ng of PFOA and 6.48 ng of PFOS in clams. These intake values are well below the suggested TDI. Thus, in this case, the consumption of mollusks does not represent a risk for consumers. This consideration could also be extent by taking into account the other contaminants, which have MRLs. In fact, all of the concentrations found were well below the limits provided by the legislations, confirming that all samples were compliant.

7.5 Conclusions

Due to anthropogenic activities, various contaminants could be present in the environment, increasing the pollution of marine ecosystems. Bivalve molluscs have been used as contamination indicators of the marine ecosystem. For this purpose, we used mussels and clams, belonging to diverse areas, to evaluate the occurrence of PCBs, PFASs, OCPs, PAHs and PBDEs, related to the different habitats of the two mollusc species. Mussel cultures are generally suspended to hard substrates placed at 2 to 5 m in the seawater, while clams usually live buried in the sand or the muddy seabed in brackish waters (Nania et al., 2009). Considering the different chemical-physical properties of the selected contaminants, two sensitive, specific and robust analytical methods, based on LC-HRMS and GC-MS/MS, were developed and validated for the analysis of mussel and clam samples. The results showed a greater contamination of PCBs, OCPs and PAHs in mussels than clams, whereas this trend was reversed for PFASs. These data could be accounted for by the different contamination sources, different chemical-physical properties of the selected classes, and different distribution in the marine layers.

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Conflict of Interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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Summary and conclusion

The presence of residues in the environment is a global issue. The massive industrial development has a deep impact on the amounts and types of compounds released into the environment; in fact, many are the classes of substances that could be present as residues: environmental contaminants, metals, pesticides, veterinary drugs, or more generally substances having pharmacological activities, personal care products and other compounds. Over the years, all these substances, and the new ones periodically discovered, have become the subject of Control Authorities, which require the development of state-of-the-art methods for their detection and the assessments of their occurrence in order to evaluate all the implications related to their presence. In this context, the presence of residues in animal matrices represents an important problem both for consumer's health, due to their possible toxic effects, and for producers, which could incur in legal and economic problems.

For all these reasons, this PhD thesis was focused on the development and validation of new analytical methods for the analyses of these compounds in different matrices of animal origin.

Chapter 1 is an overiew on the substances investigated in this thesis was reported, with the aim to present the specific issue. Two "pseudo-endogenous" (prednisolone and thiouracil) and five classes of contaminants were discussed. In Chapter 2 and 3 the "pseudo-endogenous" substances were considered in animal matrices. In Chapter 2, the pseudoendogenous origin of prednisolone in urine and adrenal gland of pigs was investigated. It was detected in urine both at the farm and at the slaughterhouse, with a concentration and frequency higher at slaughter, while in the adrenal glands it was detected in 89% of the samples. The presence in the adrenal glands, the organ in which cortisol is produced, demonstrated the possible endogenous nature of prednisolone. Moreover, indirect evidence was also provided about the origin of prednisolone that considered its relationship to cortisol levels under different conditions. In Chapter 3, two methods were developed for the analyses of thiouracil and other thyreostatic drugs without the derivatisation step in both cow urine and in thyroid glands. The validated methods showed satisfactory recovery (96–104 % for both the matrices) and precision (coefficients of variation were less than 20 % for urine and 21 % for thyroid glands) values. Furthermore, the decision limits and detection capabilities of all the compounds were lower than the recommended values. In the other four chapters, the presence of environmental contaminants and pesticides in food of animal origin was discussed.

In Chapter 4 the distribution of persistent organic pollutants (POPs) in tuna samples from different FAO areas was evaluated. An analytical method was developed and applied to evaluate the POPs

residues in tuna samples from different FAO areas. The method proved to be simple and rapid, based on ASE with an "in line" clean up step. The results of this study show that POPs contamination of tuna is related to the FAO area and also reflects the specific pollution of that area. Moreover, it was possible to have a profile of the POPs detected in order to have an overview and to map their distribution in tuna for the consumer's food safety purpose. In Chapter 5 was described the occurrence of different classes of contaminants in 59 organic honeys. An analytical method was developed and successfully applied, showing the presence of residues of many contaminants in most of the samples. Diazinon, Mevinphos, Coumaphos, Chlorpyrifos and Quinoxyfen were the pesticide residues frequently detected in samples, even if their concentrations were found to be lower than their MRL. In Chapter 6 two in-line ASE extraction methods using Florisil and PSA as an interference retainer were developed and compared with QuEChERS to isolate pesticides residues from organic honey samples. Two extraction solvents were used. The three methods were validated and showed that: QuEChERS and ASE with PSA as retainer had better repeatability than ASE with Hex:EtAc and Florisil; QuEChERS and ASE (ACN and PSA) had good recovery for the majority of investigated pesticides while ASE with Hex:EtAc and Florisil had recoveries lower than the acceptable value of 70% for several compounds. A part of this project was carried out at the Special Solution Center Europe of Thermo Fisher Scientific (Dreieich, Germany). The final chapter (Chapter 7) described the presence of environmental contaminants in mussels and clams. As done in the previous studies, analytical methods were validated, showing recoveries in the range of 70-100 %, coefficient of variations between 2-20 %, and good linearity, and applied to the samples. The results showed a greater contamination of PCBs, OCPs and PAHs in mussels than clams, whereas this trend was reversed for PFASs. These data could be accounted for by the different contamination sources, different chemical-physical properties of the selected classes, and different distribution in the marine layers.

In conclusion, we have developed, validated and applied to real samples new analytical methods for the analysis of residues in animal matrices. Sophisticated ad innovative metodics were provided to contribute to the state-of-the-art of methods for residue detection. We were also able to present data regarding the occurance and the distribution of most of the main persistent organic pollutants and other emerging contaminants, in relation to the different matrices analysed and to the different area in which they were collected, contributing to add new knowledge about contamination in animal matrices.