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# Study and evaluation of key factors in food and environment for human health concerns

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Alla mia famiglia e

al Dott. Alfredo Calabrese

*Il tempo*
*cambia molte cose nella vita*
*il senso, le amicizie, le opinioni*
*che voglia di cambiare che c'è in me…*
*F.B.*

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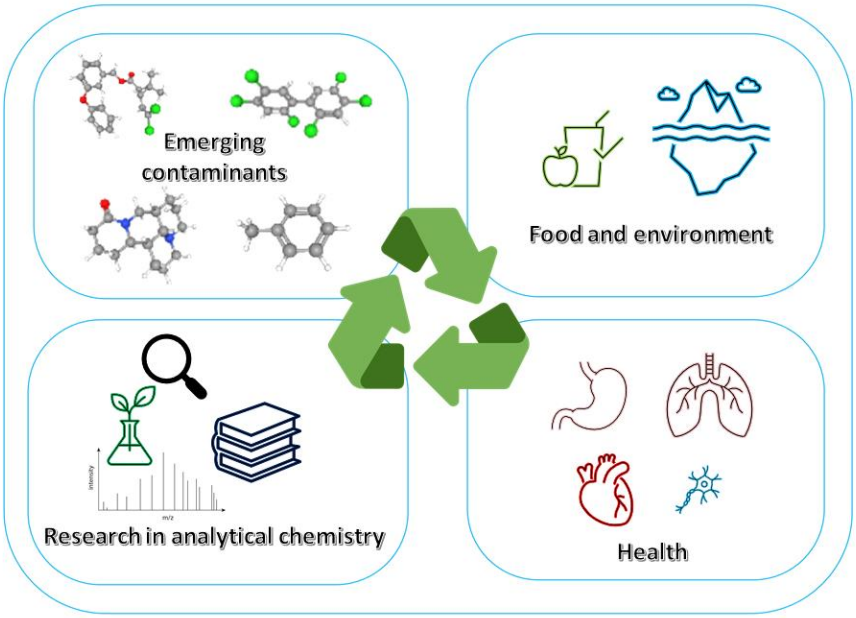
# Chapter 1 - Introduction



## 1.1 Emerging contaminants

Emerging contaminants are considered a fashionable research topic to the present day and most of them give a challenge for laboratories and regulatory agencies. The interest of the governments regarding this point is intensifying due to the high chance of public health impairment for the contaminants of air, lands, and seas. Furthermore, considering the elevated risk for human health in case of the assumption of these dangerous substances, the interest in food safety of consumer organizations and producers has increased and is leading to the approval of more restrictive laws about the determination and quantification of emerging contaminants. Once the topic is being targeted, we need to better define the term "emerging contaminants'': a large class of contaminants which have appeared only recently, and/or contaminants which have been in the environment for a while but for which concerns have been raised much more recently. The research in this field is then focused on: *a) "true or really new" emerging contaminants*, new compounds or molecules that were not previously known or that just recently appeared in the scientific literature; *b) well-known contaminants of emerging interest*, to whom the environmental contamination issues were not fully realized or apprehended; *c) emerging issues about well-known contaminants*, studies on new information that can improve the understanding of environmental and human health risks related to these contaminants [1].

The countless side effects for human health and the environment of emerging contaminants are still not clear so this has led an extension of their definition to include *A) "compounds that are known but whose toxicity mechanisms are still not fully understood*"; *B)* compounds that need legislation revisions to include new information not previously taken into account; *C)* compounds analyzed with new tested and improved methodologies [2].

More in-depth studies on this topic appear to be necessary to achieve the knowledge, as clear as possible, about the hazard profile of these compounds for public health. To give our contribution in this research field, the aim of this work is to show the results of five novel analytical methods for the determination and quantification of pyrethroids (PYRs), polychlorinated biphenyls (PCBs), matrine and oxymatrine (MT, OMT) and benzene derivatives (BDs) that can be considered widely disseminated emerging contaminants with high chance to contaminate food and the environment.

## 1.2 Pyrethroids (PYRs)

Synthetic pyrethroids (PYRs) are a class of insecticides that are one of the most used in the world (Fig. 1.1). They are used in agriculture to control pest infestations of row crops, forestry, horticulture, and livestock. PYRs are also employed in medicines to treat scabies, louse, and flea infestations. PYRs rank second only to organophosphates

in terms of total insecticide use in USA agricultural practices. Furthermore, PYRs are widely used indoors in residential and occupational settings [3]. They are very versatile compounds for their fundamental importance in the control of *Plasmodium* parasites and the Zika virus, which can be transmitted into a human host by insect vectors [4]. The widespread and improper use of PYR insecticides has resulted in the evolution of resistance in many mosquito and pest species [5]. Therefore, it is very important to watch over this kind of compound due to its considerable use, especially in producing food as a pesticide, which tends to influence public health and the economy. These compounds could contribute to developmental and reproductive toxic effects in vertebrates following chronic low-dose environmental exposures. For example, the high levels of urinary pyrethroid metabolites detected in children have been classified as an important risk factor for their exposure to PYRs based pesticides. Furthermore, the development of pest resistance to PYRs may not be effective in fighting malaria diffusion, particularly in sub-Saharan Africa [3].

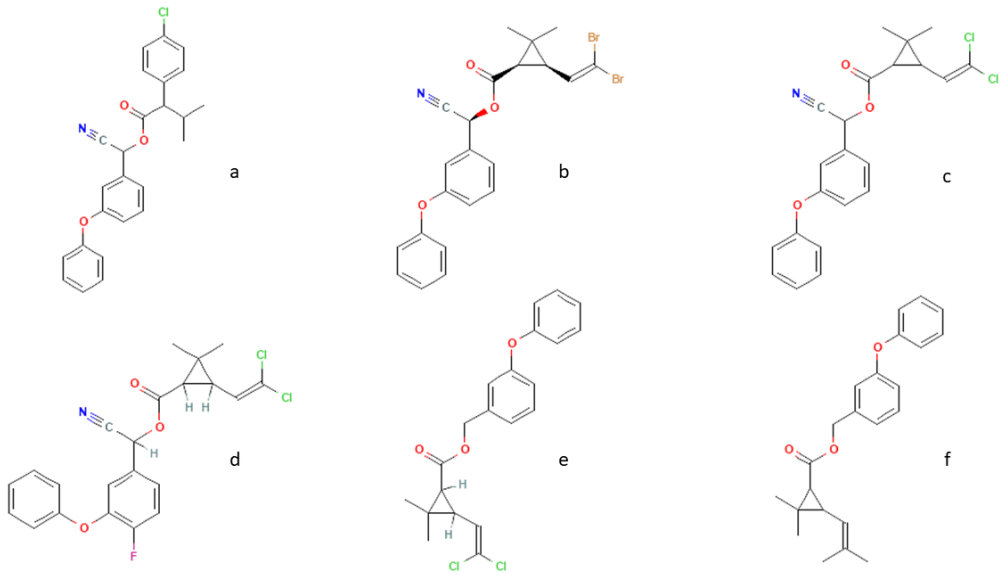



*Fig.1.1 Chemical structures of phenothrin (a), permethrin (b), cyfluthrin (c), cypermethrin (d), deltamethrin (e) and fenvalerate (f).*

## 1.2.1 Health Effects of PYRs

Multiple toxicological studies have been carried out about PYRs starting from the Encyclopedia of Environmental Health that describes in detail the chemical structure, metabolism, toxicity and describes data on human exposure [3]. Below, is a focus on *in vivo* studies using animal models and *in vitro* studies with human cells. Studies of effects on human health are still ongoing.

### *1.2.1.1 Neurodegenerative effects*

The type II compound deltamethrin is the most toxic compound among PYRs for the central nervous system. Indeed, endoplasmic reticulum (ER) stress could be induced by deltamethrin in neuronal cell lines and the rat hippocampus. Deltamethrin is associated with a molecular and cellular disruption in the brain. Indeed, the apoptotic cell death in the hippocampus with deficits in hippocampal precursor proliferation are both associated with learning deficits and more studies are still focusing on this aspect [6,7]. Furthermore, the treatment of murine microglial cells (active immune defense cells in the central nervous system) with deltamethrin caused an inflammatory state with the release of proinflammatory cytokine TNFα [8]. Some correlation studies between PYRs and hyperactivity and attention deficit syndrome (ADHD) have been treated but require further investigation due to its significant impact on public health [9,10]. An association between PYRs and neurodevelopmental disorders is supported by *in vivo* studies, for example, an interesting study using laboratory mice exposed to deltamethrin during development showed some reminiscent traits of ADHD including high levels of dopamine transporters (DAT), hyperactivity, working memory, attention deficit, and impulsive behaviour [9]. Some studies suggest that the dopaminergic system, glutamatergic and serotonergic systems (fundamentals for information processing in neuronal networks) are targets for PYR compounds like deltamethrin, λ-cyhalothrin, fenpropathrin, cypermethrin and bifenthrin [11–16], including [12,16,17]. Consequential neurotoxic effects cause an increase in inflammation and/or oxidative stress [18]. Furthermore, changes in the behaviour and variation of biomarker levels of oxidative stress and neuroinflammation of the hippocampus, frontal cortex and striatum in rats subchronically treated with bifenthrin were documented [19].

### *1.2.1.2 Endocrine and reproductive effects*

Some PYRs are responsible for destructive effects against the endocrine system that can lead to early alterations of sexual characteristics, for example, with the early onset of puberty in children, as demonstrated in the Chinese boys and girls population [20,21]. Cypermethrin accelerates puberty in male mice by destroying the hypothalamic-pituitary-gonadal axis and promoting gonadotropin biosynthesis in a murine LβT2 pituitary gonadotropic cell line [22,23]. PYRs were suspected of producing destructive endocrine compounds, as suggested by estrogenic effects in fish models treated with permethrin [24]. PYRs can act as endocrine disruptors, as agonists or antagonists of the estrogen receptors, androgen receptors or thyroid hormones and can influence the regulation of the cell cycle [25–27]. Additional evidence about the disruption of the endocrine function with DNA damage by chemicals with hormonal activity was described in a large cohort study known as the “NewGeneris Project” (Newborns and Genotoxic exposure risks). This study showed that exposure to this kind

of chemicals in utero is associated with micronuclei in the umbilical cord blood mononuclear lymphocytes of newborns. The detection of micronuclei in these cells gives an estimate of the genome damage accumulated in stem cells as well as in circulating lymphocytes [28].

### *1.2.1.3 Metabolic and cardiovascular effects*

Recent studies suggest that chronic and acute exposure to PYRs could contribute to the development of metabolic and heart diseases, after the investigation in "*in vivo"* systems with living animals. However, further confirmations are needed. For example, a study on 12 weeks of permethrin exposure have found a high-fat diet-induced weight gain and insulin resistance in male C57BL/6J mice when compared to pair-fed controls not exposed to permethrin and similar effects were found on the metabolic phenotype of female C57BL/6J mice [29,30]. Further tests in "*in vitro'' systems* suggest that PYRs might act as obesogens and could promote insulin resistance. For example, permethrin treatment of cultured murine 3T3-L1 preadipocytes promotes adipogenesis and increases intracellular calcium levels with concentration-dependent stress markers [31,32]. In addition, treatments with deltamethrin on *Caenorhabditis elegans* have shown increases in triglyceride accumulation levels [33]. Furthermore, permethrin can induce insulin resistance in murine C2C12 myotubes [32]. Studies about the early prenatal exposure of rats to permethrin have shown that it led to damage to the cardiac cell, DNA, reduction of membrane fluidity and increase of cholesterol content and lipid, and protein oxidation. Similar effects on membrane fluidity have been shown on isolated rat cardiac cells treated with permethrin metabolites [34,35]. Moreover, the early exposure to permethrin can lead to cardiac hypotrophy, an increase of calcium and increase the expression levels of the Nrf2 gene that regulate the physiological and pathophysiological outcomes of oxidant exposure inducing oxidative damage to purine bases in the cardiac cells of adults [36–38]. Based on a case report of a 28-year-old female after suicidal consumption of 20 ml of prallethrin, PYRs can modify neuronal channels as they induce persistent, steady-state sodium current within depolarized membranes leading to cardiac hypotrophy [39].

### *1.2.1.4 Cancer and Pyrethroids*

Different environmental factors, like tobacco, alcohol and ultraviolet irradiation, have been associated with cancer. Before the classification as a carcinogen, any kind of compounds need to be observed and systematically reviewed by the International Agency for Research on Cancer (IARC). However, there are certain groups of environmental factors, such as pesticides, whose carcinogenic effects are still controversial and considered not classifiable as carcinogens [40,41]. Already in 1991, the role of permethrin and deltamethrin in the evolution of cancer was discussed

thanks to IARC. At that time, both compounds were not classified as carcinogens and were applied in Group 3 [41]. More recent studies have shown relationships with cancer incidence, especially for permethrin [42,43]. Based on a classification carried out on 2 types of benign tumors (liver and lung) in mice, the US EPA classified permethrin as "likely to be carcinogenic to humans" when ingested orally [44]. In addition, the permethrin induced breaks and rearrangements in genes during the *in vitro* experiments suggest that it could be associated with childhood and adult leukaemia and lymphoma with the possibility to induce chromosomal abnormalities. like aneuploidy [45]. Finally, PYRs can increase the proliferation of the breast carcinoma cell line MCF-7 through the activation of the estrogen receptor [25].

## 1.3 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are synthetic organochlorine chemicals and anthropogenic compounds that were manufactured in the United States of America between 1930 and 1977 (Fig. 1.2) [46]. PCBs are mixtures of aromatic chemicals, synthesized by the chlorination of biphenyl in the presence of a suitable catalyst. The chemical formula can be represented as $C_{12}H_{10-n}Cl_n$, where $_n$ is the number of chlorine atoms between 1 and 10. PCBs are colorless to light yellow, have no smell or taste and can be either an oily liquid or a solid. They do not crystallize, even at low temperatures, but turn into solid resins. Some PCBs are volatile and may exist as vapor in the air. PCBs have very low electrical conductivity, rather high thermal conductivity, and extremely high resistance to thermal break-down. The physicochemical properties vary widely and depend on the number and positions of chlorine atoms in the biphenyl rings. PCBs resist both acids and alkalis and are thermally stable [47]. PCBs are members of the halogenated aromatic group of environmental pollutants that have been identified worldwide in diverse environmental matrices. Furthermore, they are well known environmental contaminants due to their persistence and bioaccumulation related to human health concerns [48]. They can affect the immune, reproductive, nervous, and endocrine systems and are carcinogens that were banned in the United States in 1977 [46].

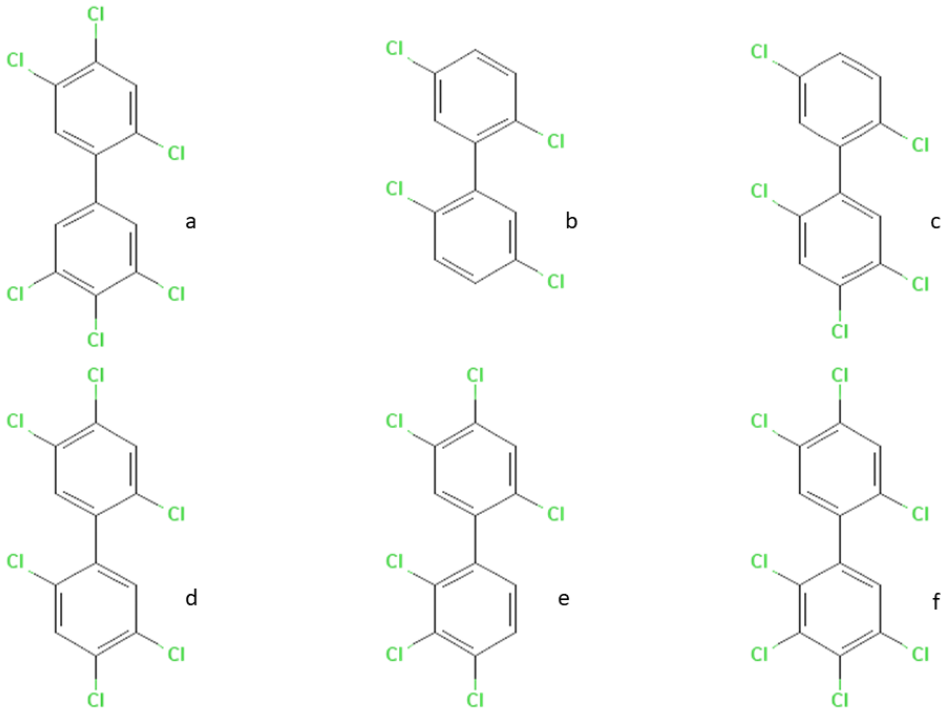



*Fig.1.2 Chemical structures of PCB 28 (a), PCB 52 (b), PCB 101 (c), PCB 138 (d), PCB 153 (e) and PCB 180 (f).*

### 1.3.1 Health effects of PCBs

There is a lot of evidence about PCBs and their correlation with health problems in humans thanks to many studies that have shown their high lipophilic tendency. This characteristic allows their accumulation in animals and human body fats including cellular membranes [49]. Moreover, PCBs are known as persistent organic pollutants (POPs), since they are not easily broken down and keep the general environment persistently contaminated [50,51]. Historically, PCB exposure in humans was either occupational or accidental, however, the most relevant source of PCB exposure today is through ingestion of PCBs contaminated food and water. In fact, studies have shown that PCBs are present in fish globally, which results in human exposure through food [52–55].

#### *1.3.1.1 Neurodegenerative effects*

Polychlorinated biphenyls are among the most well-studied endocrine-disrupting chemicals (EDCs) for their neurobehavioral effects, especially for neurodevelopment and cognitive performances. Exposure to PCBs has been associated with a wide range of neurological effects, including neurobehavioral abnormalities in newborns and young children and neurochemical alterations in laboratory animals [56–62]. Several

PCBs congeners are responsible to be cytotoxic on cerebellar granule cells. With the support of experiments on rats, a rapid induced loss of membrane integrity of cerebellar granule cell neurons related to some congeners was discovered. The relative toxicity of the *ortho-substituted* congeners varied with the number of *ortho* chlorines. Among this dangerous non-coplanar, ortho-substituted congeners, that are capable to kill neurons, there are: PCB 8 (2,4'-dichlorobiphenyl), PCB 28 (2,4,4' trichlorobiphenyl), PCB 47 (2,4,2',4'-tetrachlorobiphenyl), and PCB 52 (2,5,2',5'-tetrachlorobiphenyl) [63]. Moreover, the exposure to PCBs damages multiple membrane components, including the plasma membrane, mitochondria, and endoplasmic reticulum [49]. It has also been reported that PCBs are linked to dementia and cognitive decline [58,64]. Furthermore, the functioning of the thyroid hormone is reported to be adversely affected by the PCBs that may lead to cognitive impairment [51]. Epidemiological studies have shown an increased incidence of Parkinson's disease, especially in females who were occupationally exposed to PCBs [65].

### *1.3.1.2 Endocrine and reproductive effects*

Chemicals with hormonal activity can be divided into three main groups: (i) synthetic compounds used in industry and agriculture as well as in consumer products, (ii) synthetic compounds used in pharmaceutical drugs, and (iii) natural compounds present in the food chain. Within this class, compounds can be further subcategorized into those that are persistent in all elements of the environment, bio-accumulative, transportable over long distances, and capable of adversely affecting life forms that reside within short and long distances from the site of contamination. These compounds list include PCBs, which may disrupt follicular steroidogenesis either by mimicking natural hormones as agonists or antagonists, altering the pattern of hormone synthesis, modulating hormone receptor affinities or numbers or by altering enzymes involved in hormone secretion [66]. PCBs have also been reported to adversely affect puberty, development, and oocyte viability and some studies point to unpredictable changes of translational regulation within the oocyte under the influence of PCBs [67,68]. Hormone-mimicking compounds can bind to cell receptors, interfere with hormone action, and affect ovarian function that is controlled by the hypothalamus, pituitary, and autoparacrine factors. How endocrine disruptors affect ovarian function is not clear yet but a disruption in gonadotropin secretion and feedback mechanisms involving estrogens and progesterone may be involved [66]. Moreover, early puberty, earlier menarche and thelarche ages have been reported in girls after exposure to PCBs [69,70]. In addition, even the exposure to PCBs for male reproductive functions has been associated with reproductive dysfunction, including decreased sperm motility and a decrease in fecundity [71,72].

### *1.3.1.3 Metabolic and cardiovascular effects*

Cardiovascular diseases are multifaceted, with links to many modifiable and non-modifiable risk factors. Epidemiological evidence now implicates even the exposure to PCBs, with an increased risk of developing diabetes, hypertension, and obesity; all of which are clinically relevant to the onset and progression of cardiovascular disease. Recent studies showed that serum PCB levels were significantly associated with hypertension and were higher among individuals with hypertension [73,74]. PCBs and heart disease have a relationship found in correlation with high levels of serum lipids (cholesterol and triglycerides) considered as a major risk factor for cardiovascular disease due to possible endothelial cell damage resulting from oxidative stress [75]. In a population-based cohort study of 992 individuals aged 70 to 80 years, 18 persistent organic pollutants were measured in plasma. Elevated levels of highly chlorinated polychlorinated biphenyls (PCBs) were associated with increased mortality risk, mainly from cardiovascular diseases, during 10 years of follow-up [76]. Concerning the metabolic detrimental effects, the alteration of the leptin pathway after NDL (Non-dioxin-like)-PCBs exposure (PCB 101, PCB 153, and PCB 180) showed damages against adipocyte activity *in vitro*. NDL-PCBs exposure increased lipid content in mature adipocytes, disrupting leptin sensitivity through the downregulation of its functional receptor. Besides, signalling transduction, and leptin transcription was increased in 3T3-L1 cells after PCB exposure, further confirming their detrimental effects on hormone functions [77]. Moreover, increasingly data and an expanding body of literature are associating PCBs exposure with heightened risk and incidence of type 2 diabetes development. These studies involve a variety of cohorts and subject ages, suggesting that PCBs exposure increases the risk of type 2 diabetes development regardless of age or cohort [78–89]. In addition, PCBs also have been implicated in the development of gestational diabetes and can contribute to insulin resistance in expecting mothers [90].

### *1.3.1.4 Cancer and Polychlorinated biphenyls (PCBs)*

In 2016, the IARC upgraded the classification of the PCBs to Group 1 “Carcinogenic to humans” from the previous Group 2A classification “Probably carcinogenic to humans” [91]. Although the results of some studies are supportive of an association with melanoma and non-Hodgkin lymphoma, on the other hand, there are no studies that provide strong evidence that PCBs exposure can increase the risk of this kind of cancer in humans [92,93]. The role of PCBs in breast cancer has been investigated intensively and data suggests a correlation may exist between high levels of PCBs in mammary tissues or sera and breast cancer risk [94], even if there is critical evidence [95]. A study demonstrated that PCBs congeners like PCBs 101, 118, 138, 153, and 180increasedthe proliferation of MCF-7 cells (a breast cancer cell line isolated in 1970 from a 69-year-

old Caucasian woman) [96,97]. PCB 138 and 153 had the highest stimulatory effects on basal MCF-7 cell proliferation as well as the highest inhibitory actions on basal caspase-9 activity (an enzyme critical to the apoptotic pathway found in many tissues) [66,98]. Moreover, another study showed that PCBs 138 and 153 contribute to the action of endogenous 17$\beta$-estradiol on cell proliferation and apoptosis in the breast cancer cell line MCF-7 [99]. *In vivo* experiments have also shown that PCBs can increase metastasis by triggering the production of reactive oxygen species (ROS) [100,101]. There are currently controversial pieces of evidence about the correlation between PCBs and cancer, so it is necessary to wait for new scientific publications on this topic.

## 1.4 Matrine and Oxymatrine (MT and OMT)

Traditional Chinese Medicine is well known for its natural medical remedies that were widely utilized for centuries in clinical practice especially using herbs or roots like *Sophorae tonkinensis* or "*Dogel ebs*" also known as *Sophora flavscens Ait* belonging to the family of Fabaceae. This plant and its biological effects, induced by more than 70 matrine-type alkaloids known to date, were already used in ancient times by the medical sage Zhang Zhongjing to alleviate some disorders of the urinary tract. Currently, matrine-type alkaloids are the main topic of a considerable number of convincing and important studies that support many positive effects on human health and the treatment of many diseases [102]. In recent years, anticancer effects were well documented especially on some mechanisms like apoptosis autophagy and metastasis [103–105] beyond the treatment of some of the most diffused tumors like breast, colorectal or pancreatic cancer [106–108]. Even if the biological effect of matrine-type alkaloids remains to be further explored and explained, there is much scientific evidence in different clinical fields about the benefits of its use. It was found that these compounds have a significant role in the prevention and treatment of the fibrotic response of the human coronary smooth muscle cells induced by AGEs (advanced glycation end-products) [109]. Furthermore, a study has recently been published on oxidative stress induced by preclinical administration in a model of liver injury due to carbon tetrachloride ($CCl_4$) resulting in a reduction of cell apoptosis [110]. In addition, these compounds showed impressive antibacterial and antiviral properties [111,112]. Matrine and oxymatrine are the main constituents of extracts from *Sophora flavescens Ait.* Well known also as "botanical insecticides" used against various insect pests, pathogenic fungi, bacteria, and nematodes (Fig. 1.3). This kind of polar and more easily degradable insecticides are replacing more persistent and synthetic ones due to the high demand for alternatives as plant protection products (PPPs). Globally, health and safety concerns are not clearly defined by the law. Hence, to satisfy new market demand, the matrine based products are labelled as fertilizers or as corroborant plant extracts to be tradable in Europe as required by Regulation (EC) No. 2003/2003

avoiding the complex law pathway required by Regulation (EC) No. 1107/2009 for the PPPs [113–115].

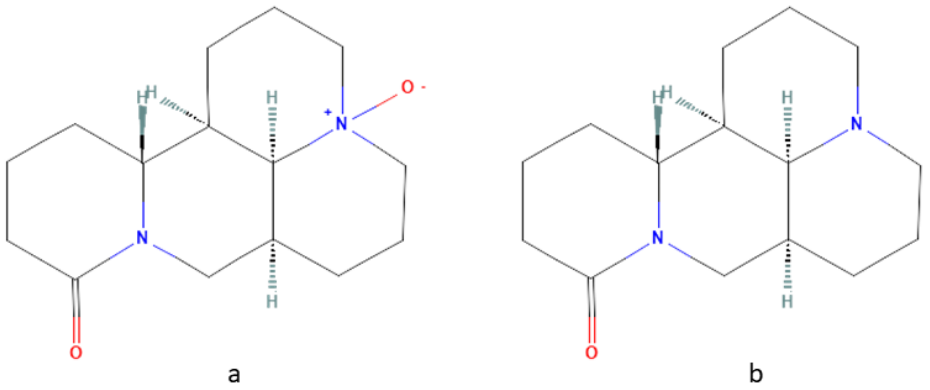



*Fig. 1.3 Chemical structures of matrine (a), and oxymatrine (b).*

### 1.4.1 Health effects of MT and OMT

The multiple utilization of matrine-type alkaloids in medicine and for clinical trials does not exclude the possibility of side effects, especially with their use as a pesticide whose different sources can reach the human body. Indeed, the intake of contaminated foods is the principal pathway. To date, the toxicity and safety of matrine and oxymatrine in overdose amounts have sparked intense debate and research for its characteristics as an emerging contaminant. While in the United States and China, the FDA and CFDA respectively authorized the use of matrine-type alkaloids in the dermatological sector as well as in the agri-food field and to treat various kinds of cancers with minimum toxicity, in Europe, due to the lack of scientific evidence about their safety, MT and OMT have been included in the list of non-approved substances for which the legal Maximum Residue Limit (MRL) is set by default at 0.01 mg $kg^{-1}$ according to Regulation (EU) No. 396/2005. However, the potential toxicity of MT and OMT and their possible toxic mechanisms are still unclear and there is not a consistent number of studies about the side effects for their utilization and about the public health-related consequences as a result of food or environmental contamination due to the use of these compounds as biopesticides.

#### *1.4.1.1 Neurotoxic effects*

Studies about Chinese medicinal herbs are largely confined to China and reports about neurotoxic effects are uncommon, especially in the English language. In an interesting

case study [116], MT and OMT in high dose injections were suspected to be responsible for patients' intoxications that showed primarily convulsions, mental changes, dystonia syndromes between complex neurological manifestations with lesions in the stratium and globus pallidus with the risk of damage of the basal ganglia. In a recent *in vivo* study [117], MT was investigated about its developmental toxicity and neurotoxicity in zebrafish (*Danio rerio*) displaying teratogenic and lethal effects, altering their spontaneous movement and inhibiting swimming performances and causing, in certain cases, death and malformations.

### *1.4.1.2 Endocrine effects*

Although MT is widely used in clinical treatments for liver diseases, including HBV infection, the mechanisms remain largely unknown, and hepatotoxicity is the most diffuse endocrine side effect in the *in vivo* experiments with treated mice. Previous studies *in vitro* showed a high concentration (>140 mg $L^{-1}$) of MT related to decreasing albumin secretion of hepatocytes. The dose-dependent enzymatic activity showed the toxic effect of MT on the liver with the damaging of the membrane system of hepatocytes, reduction of LDH (lactate dehydrogenase) and AST (aspartate aminotransferase) levels and the expression of cytochromes CYP2A6, CYP2B6 and CYP3A4 resulting in lessening cell viability [118]. A study about high dose MT-treated rats revealed dysfunctional levels of biomarkers in urine related to a disorder in the gut microbiota, liver, and renal damages with high levels of hippurate, taurine, and oxidation products of triethylamine (TMAO) due to MT induced toxicity [119]. Further results concerning hepatotoxicity through oral administration of MT and OMT extracted from radix of *S. tonkinensis* related to centrilobular hypertrophy in the liver recognized as an indicator of hepatocytes dysfunction in mice has been released [120]. A recent study investigated the possible dose-dependent toxicity of the same radix identifying serum cholinesterase (CHE) as a potential supplemental biomarker for liver injury [121]. In addition, the involvement of ROS-dependent mechanism of HL-0772 cells treated with MT showed its hepatotoxic effects exerted through the inhibition of the Nrf2 pathway, activation of ROS-mediated mitochondrial apoptosis pathway, and cycle arrest at S phase. In addition, the suppression of cell viability, the increase of cytotoxicity, the alteration in the expression of apoptotic-related proteins, and the activation of proteins that plays an essential role in the signalling cascade leading to apoptosis, such as caspase-3 and caspase-9, have been shown in MT-treated mouse liver NCTC cells [122,123].

## 1.5 Benzene derivatives (BDs)

Benzene derivatives (BDs) belong to the family of volatile organic compounds (VOCs), polluting materials mainly generated by anthropogenic activities like fossil fuel extraction, urban waste incineration and synthesis of organic compounds for many consumers (Fig. 1.4; Fig. 1.5; Fig. 1.6). The occurrence and physiochemical behaviour of these organic contaminants mostly depend on their original phase in the environment, which can persist for a long time due to their immiscibility in aqueous media. Similarly, other physicochemical properties make their degradation fast in the upper atmosphere and a relevant portion may be removed by rain to contaminate surface water and soil [124–126]. With the increase of scientific knowledge over the last century, BDs health-related problems, ecological apprehensions, and socio-economic awareness have been well-studied topics. The presence and persistence of BDs in water bodies, in the environment, and food are of continuing and burning interest worldwide especially for the acute and chronic exposures that are directly associated with observed hematologic effects in humans and animals [127–129]. Furthermore, high indoor concentrations levels of benzene have been detected in buildings due to persistence in the construction materials. Even the work tasks performed inside the companies for specific occupational purposes probably exposed many workers to BDs for a prolonged time contributing to the onset of chronic disease. In addition, BDs did not always receive the necessary legislative attention, despite their importance in environmental chemistry. Moreover, the use of benzene alternatives, such as toluene and xylene, may not be safe even at low levels [126,130–132]. Then, BDs monitoring plays an important role in the exposure assessment of the worldwide population potentially exposed to these chemicals.

### 1.5.1 Health effects of BDs

Benzene and its derivatives have been well-studied compounds due to their direct association with chronic and acute exposure to human and animal health whose metabolism is quite similar. Even if the first observations about the effects of benzene were very difficult for the lack of knowledge and adequate technology, many reports have been produced since the late 1800s starting from the acute human exposures to benzene in the workplace at concentrations relating to certain toxic endpoints [128]. The qualitative liver metabolism occurs through the cytochrome P 450 2E1 system and the main metabolites like phenols, catechols, and hydroquinones are also found in the bone marrow of laboratory animals [133]. Although environmental exposure through inhalation is more dangerous compared to oral exposure, benzene and its derivatives represent a chemical hazard even in food, whose regulation is not always complete and a global approach on indoor levels are still missing [127,134].

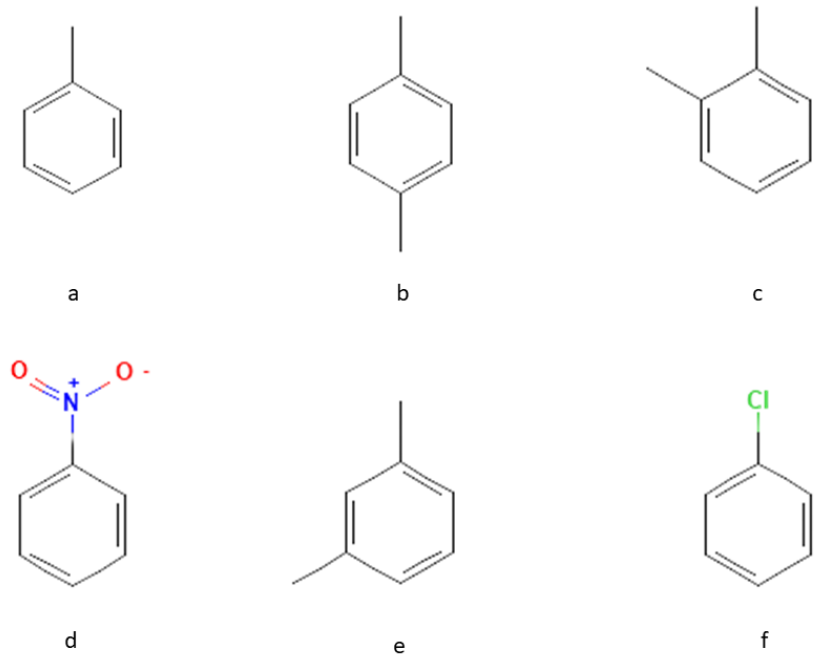



*Fig.1.4 Chemical structures of toluene (a), chlorobenzene (b), m-xylene (c), p- xylene (d), o-xylene (e) and p-dichlorobenzene (f).*

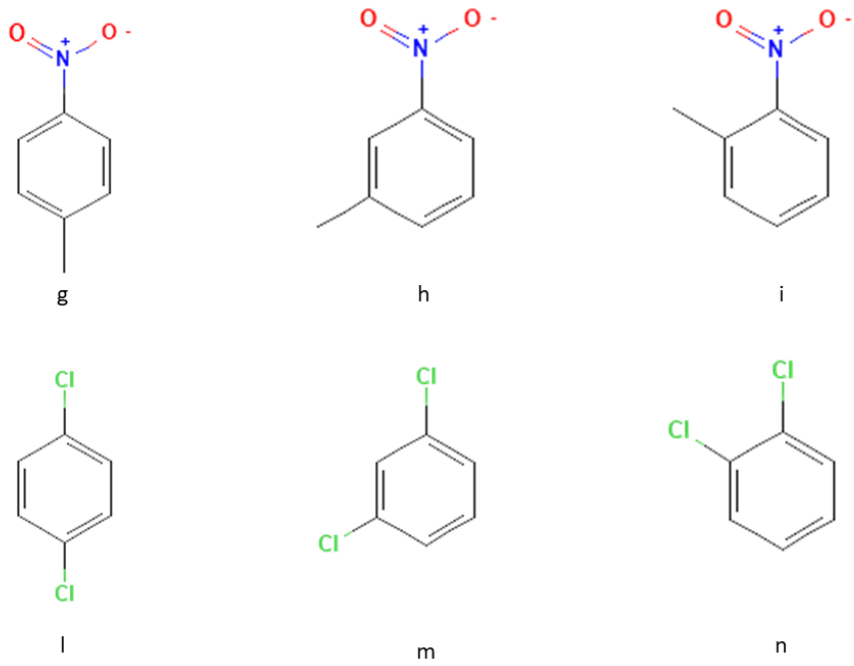



*Fig.1.5 Chemical structures of m-dichlorobenzene (g), o-dichlorobenzene (h), nitrobenzene (i), o-methylnitrobenzene, (l), m-methylnitrobenzene (m) and p-methylnitrobenzene (n).*

### *1.5.1.1 Toxic effects*

The toxicity of benzene is well known, especially the haematological effects as a leukemic agent via occupational and environmental exposure [135]. Other immunotoxic and hematotoxic effects related to the chlorobenzene were already studied after occupational exposures and showed aplastic anaemia, porphyrias, and decreasing neutrophil enzyme activity [136]. Further evidence of hematotoxicity and genotoxicity of toluene and xylene were carried out through biochemical assays showing that their induced oxidative stress and apoptosis had a major role in the DNA damage and haematological parameters [137]. Even low levels of chronic exposure to benzene can alter biomarkers that may represent early signals of DNA damage [138].

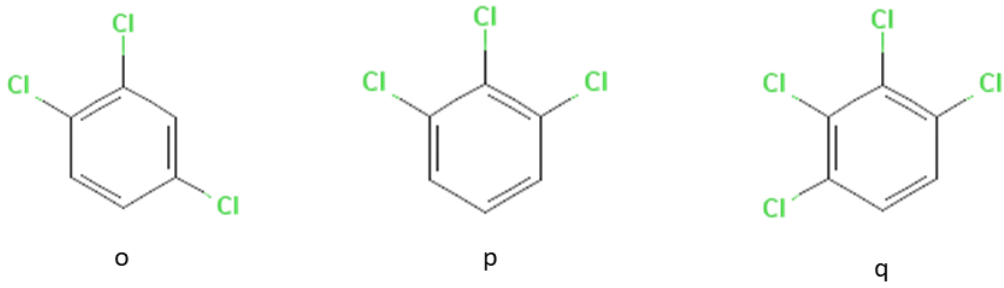



*Fig.1.6 Chemical structures of 1,2,4-trichlorobenzene (o), 1,2,3-trichlorobenzene (p) and 1,2,3,4-tetrachlorobenzene (q).*

### *1.5.1.2 Endocrine and reproductive effects*

The effects of benzene and its metabolites include inhibition in sperm mobility, viability, and sperm DNA damage that is an important step between spermatogenesis and malfunctions such as infertility [139,140]. Furthermore, effects on pregnancy through high exposure to toluene are well known and include intrauterine growth retardation, premature delivery, congenital malformations, and postnatal developmental retardation [141]. Other endocrine interfering properties of benzene and toluene are represented by altered menstrual cycles and altered concentrations of reproductive hormones like LH, FSH, and testosterone [142,143]. In addition, further evidence of infertility due to an impaired hormonal balance and alteration of the physiological functions of the reproductive organs was tested on rats through nitrobenzene administration as an induced hormonal disruptor [144]. Given the above, both estrogenic and androgenic interfering activity suggest benzene and its derivatives as EDCs (endocrine disruptive chemicals) [145].

### *1.5.1.3 Metabolic and cardiovascular effects*

Many epidemiological and experimental studies showed that BDs exposure can lead to numerous effects associated with malfunction and abnormalities of the vital systems in the body including the cardiovascular one. Indeed, cardiovascular tissues are highly sensitive to inhaled pollutants and workers occupationally exposed to high concentrations of benzene and xylene had an increased chance of onset hypertension and pathologic changes in ECG; as well as the exposition to benzene of smokers and non-smokers induced deficits in circulating angiogenic cells after measuring urinary levels of the benzene metabolite t,t-MA (trans,trans-muconic acid) [146–148]. Excessive benzene exposure in rats experimental design showed the alteration of gut microbiota and metabolic disorders due to the significant influence on the composition of cecal contents and faeces at both the phylum and genus levels and the exact mechanism remains unclear [149].

### *1.5.1.4 Cancer and Benzene derivatives (BDs)*

The hypothesis of benzene-induced leukaemia was debated over the years and many studies were published to confirm its role in the carcinogenic process. Starting by proposing benzene as a non-classic carcinogen due to the ability through its metabolite to induce DNA damages, many recent reports were issued about its primary role in the damage of bone marrow and circulating cells up to leukaemia via apoptosis [150–152]. Taking this into account, the risk of cancer could increase for occupationally exposed workers due to the specific work activity near benzene sources. [153,154]. The key characteristics of benzene carcinogenicity are the easy absorption, the extensive metabolism, and the wide distribution that involves multiple metabolic pathways in various tissues, like bone marrow, that triggers a large variety of cancers, for example, acute non-lymphocytic leukaemia, multiple myeloma, non-Hodgkin lymphoma, and chronic myeloid leukaemia. [155]. Exposure to other BDs, like toluene and xylene, is highly correlated with an increased risk of prostatic cancer in men subjected to work near BDs sources. High DNA adducts were found in the urine of men with prostatic cancer under the assumption that its initiation results from oxidation of the metabolites catechols of estrogens or benzene to quinones, which react with DNA to form depurination adducts allowing DNA mutations and initiating related tumors [156].

## 1.6 Chromatography and mass spectrometry methodologies and applications

The undergoing radical increase of human diseases related to pollution and the changes of world climate due to globalization are concerning the scientific community to develop increasingly innovative and performing analytical methods to update the

classification of emerging contaminants and to monitor them in as many matrices as possible. The analysis of emerging contaminants is a very broad and widespread field with a large variety of analytical approaches that start specifically based on the chemical characteristics of the studied molecules. A qualitative analysis must be characterized by repeatability and reliability while a quantitative determination is crucially defined by selectivity and sensitivity. Starting from the very first step of sample preparation and extraction of the studied compounds to the data obtained through the large variety of detectors, each analytical stage is crucial and must not be neglected.

### 1.6.1 Extraction trends

Due to their cruciality in any analytical application, the sample preparation and extraction are developing even more to minimize material and solvent use in the laboratory and to develop on-site capabilities to reach more efficiency. To ensure the unchanged composition of the sample, the analytical scientists have to constantly develop, apply and upgrade suitable samples preparation protocols. The mission of these starting steps is to reduce the sample size, simplify the analyte release from the matrix, clean up and remove interferences to make the sample compatible with the analytical protocol. Obviously, to achieve these steps is necessary to apply several sub-steps, such as dilution, filtration, centrifugation, purification, derivatization, evaporation and so on. To date, the past prevailing extraction techniques, like liquid-liquid extraction (LLE) and solid-liquid extraction (SLE) have been substituted by the newest extraction techniques improved in sensitivity and rapidity such as the solid-phase extraction (SPE), the solid-phase microextraction (SPME) or the microextraction by packed sorbent (MEPS) that allows working with lower solvent consumption procedures, less exposure to toxic agents and less sample handling that contribute to reducing cross-contamination, even during direct field sampling (Fig. 1.7). Some big disadvantages of these methods are small sample volumes that could easily clog the system and the possible lack of suitable adsorbent materials.

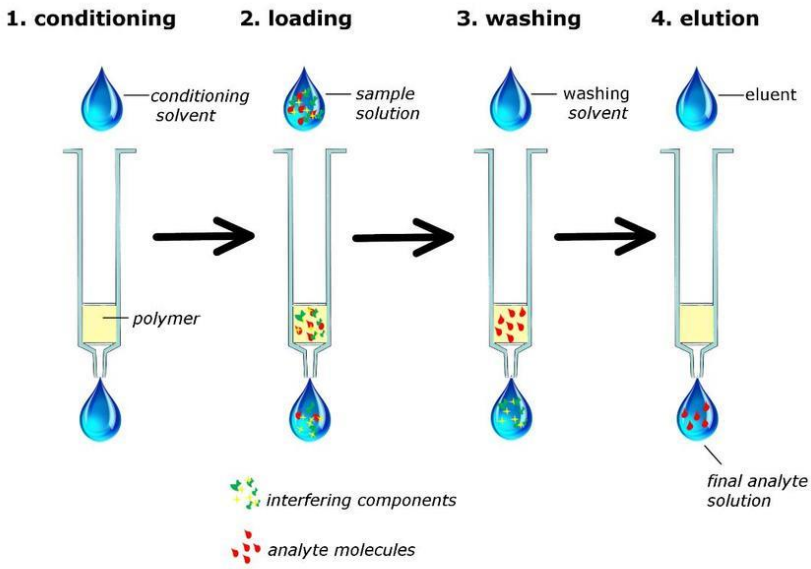



*Fig. 1.7 General steps for solid-phase extraction (SPE). Courtesy of https://www.researchgate.net/profile/Maria-Sandoval-R*

Since their low concentration in food samples, the preconcentration of PYRs is a necessary step often carried out with SPME, dispersive liquid−liquid microextraction (DLLME), magnetic solid-phase extraction (MSPE), QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) and matrix solid-phase dispersion (MSPD) [157–162]. As well for PCBs, QuEChERS was also applied including other methods like a pressurized solvent (PLE), microwave-assisted (MAE) and SLE techniques for trace analyses [163–167]. Due to their solubility in the most common organic solvents like methanol or acetonitrile, MT and OMT were treated with extraction solvents coupled with clean-up steps like PSA sorbents or SPE cartridges [113,115]. In addition, water, ultrasonic extraction (UE) with ethanol, LLE with chloroform and MAE were also investigated as extraction methods for the alkaloids in the study [168–171]. Extraction techniques associated with the wide range of BDs are quite different due to the variation of matrices and efficiency of techniques. SPME, due to its reduction of complications associated with matrix effect, small sample amount requirement and solvent use minimization, was the best choice for this step, especially for biological fluids [172]. To date, the recent trends of microextraction techniques, especially MEPS, are the methods of choice for benzene, toluene, ethylbenzene, xylene (BTEX) and their metabolites [173] (Table 1.1).

*Table 1.1 Extraction methods overview per analyte in the study.*

| Analyte | Extraction methods |
|---|---|
| PYRs | SPME; DLLME; MSPE; QuEChERS; MSPD |
| PCBs | QuEChERS; PLE; MAE; SLE |
| MT, OMT | PSA; SPE; UE; LLE; MAE |
| BDs | SPME; MEPS |

## 1.6.2 Chromatography trends

Chromatography is a biophysical process for separating, purifying and identifying components of a mixture for qualitative and quantitative analysis based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) are separating from each other while moving with the aid of a mobile phase. The effective separation between the molecules is carried out thanks to the adsorption (liquid-liquid), the partition (liquid-solid), and the affinity among their molecular weights. Due to these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into the mobile phase, and leave the system faster. The basics of the chromatography techniques are found in the stationary phase, which is the phase always composed of a solid phase or a layer of a liquid adsorbed on the surface solid support, or in the mobile phase, which is always composed of liquid or a gaseous component (Fig. 1.8). Hence, through the type of interaction between the stationary phase, mobile phase and the compounds of the mixture the separation finally occurs.

*Fig.1.8 Liquid chromatography (left) and gas chromatography systems (right). Courtesy of Thermo Fisher and Agilent.*

Liquid and gas chromatography coupled with mass spectrometry remains the most valuable tools for the determination of all analytes in the study. In many papers, liquid chromatography was proposed as a separation technique for PYRs even if gas chromatography is the best choice for the PYR analyses since these compounds have sufficient volatility that does not need derivatization; indeed, many reports were published about the use of GC coupled with electron capture detection (ECD) [160,174–180]. Furthermore, the gas chromatographic approach is the best process for the PCB analyses due to the volatility of the most common congeners [181,182]. Changing perspective, high-pressure liquid chromatography (HPLC) is the technique of choice due to its application simplicity for matrine-type alkaloids between other used techniques like high-performance capillary electrophoresis (HPCE), GC, and thin-layer chromatography (TLC) [109,114,170,183–186]. Depending on the matrix composition and the type of BDs, the chromatographic methods are alternatively chosen between GC and HPLC where the first is often the most used method [172,173] (Table 1.2).

*Table 1.2 Chromatography modes overview per analyte in the study.*

| Analyte | Chromatography modes |
|---|---|
| PYRs | GC; LC |
| PCBs | GC |
| MT, OMT | LC |
| BDs | GC; LC |

### 1.6.3 Mass spectrometry trends

Mass spectrometry is one of the most powerful tools in chemistry, biochemistry, pharmacy, and medicine. Thanks to the enormous quantity of data available in the online libraries, mass spectrometry can be employed in both elucidations of unknowns or quantitation of known compounds. Basically, a mass spectrometer generates, separates, and detect (quantitatively and qualitatively) the ions by their mass-to-charge ratio (m/z). The analytes, which can be single ionized atoms, molecules, or their fragments, needs to be ionized before their identification and quantitation and this step can be done thermally, by electric fields or by impacting energetic electrons, ions or protons. Finally, the separation is affected by magnetic fields. Between the various mass spectrometer types that are used in combination with gas or liquid chromatography the quadrupole type, probably, is the simplest one. These kinds of instruments are relatively cheap but excellent for quantitation. Even, as conceptually simple as the single-stage variants, the triple quadrupole instruments are more advanced than the first-mentioned type due to the advanced techniques available like tandem mass spectrometry (MS/MS) that provides structural information or multiple

reaction monitoring (MRM) that can be available coupled with multi-stage analysers allowing simultaneous monitoring of a large number of analytes in complex matrices with outstanding sensitivity. The most sensitive instruments among the previous mentioned remain the ion trap instruments (IT). Relatively inexpensive and performative, their cost could increase if in combination with orbitrap (Fig. 1.9) but with extremely high resolution as result. Therefore, high-resolution mass spectrometry (HRMS) using advanced spectrometric techniques continues to be the hottest trend for the unequivocal identification for most of the compounds classified as emerging contaminants.

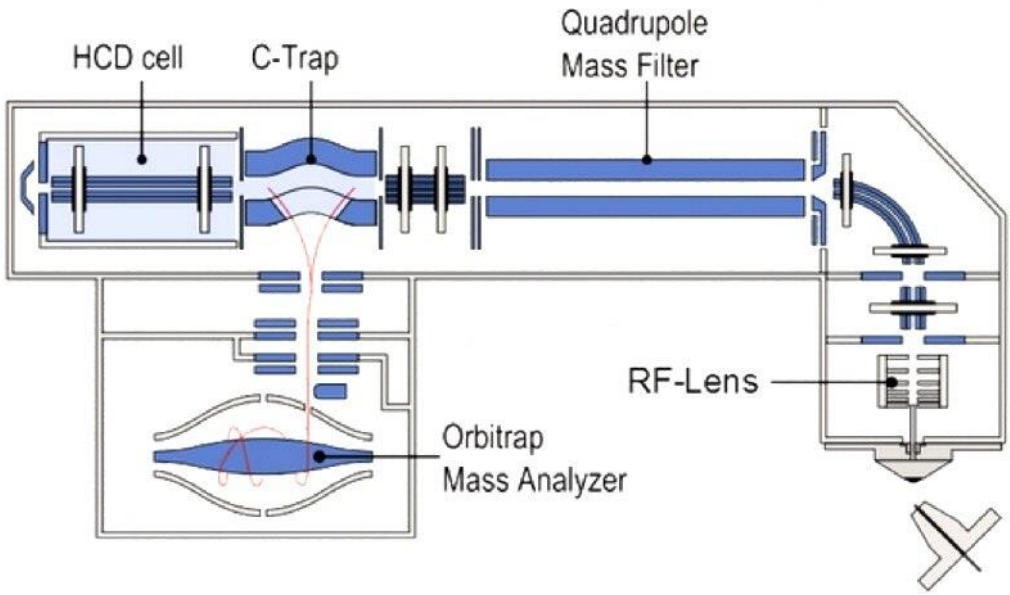



*Fig. 1.9 Orbitrap mass analyzer technology overview. Courtesy of Thermo Fisher.*

This choice is fundamental due to the lipophilicity of compounds like PYRs and PCBs in complex matrices thanks to the versatile scanning modes suitable for a wide range of methods that allow monitoring more than one reaction in a fast succession, especially in MS/MS mode [182,187,188]. The detection of the real compositions of Chinese medicines and their clear qualitative and quantitative assessment for food control assurance was accomplished with great results through the MS/MS experiments for matrine like alkaloids analyses [114,169,184,189–194]. Given the high sensitivity of MS/MS assays for quantitation of BDs in a wide range of matrices, this method was often used for routine practice in many supervision assays. [172,173] (Table 1.3).

*Table 1.3 Mass spectrometry modes overview per analyte in the study.*

| Analyte | Mass spectrometry modes |
|---|---|
| PYRs | MS/MS |
| PCBs | MS/MS |
| MT, OMT | MS/MS |
| BDs | MS/MS |

## 1.7 Aim and PhD thesis structure

Pollution, emerging contaminants, and their effects on human health remain one of the main topics of today's scientific research, due to the high demand for healthy food since diet is still the principal carrier for bioaccumulation in the human body. Considering the lack of regulation of compounds of emerging interest, their frequent undetectability with conventional or outdated analytical tests, and their persistence in the environment with potential effects on ecosystems and living organisms upon long-term exposition, constant studying and updating of analytical methods are required in order to develop new strategies to contain their spread through simple, fast and effective monitoring making the most of innovative analytical technologies. Given this, this PhD thesis aimed to develop and validate analytical methods for the determination of some classes of emerging contaminants in food and environmental matrices focusing on pesticides (pyrethroids, matrine and oxymatrine) and persistent environmental contaminants (PCBs and benzene derivatives).

This work is divided into seven chapters. Chapter 1 contains the introduction and a description of the most interesting health effects of the studied compounds. Chapters 2,3,4,5 and 6 focus on the experimental works carried out during the PhD research project. In particular, chapter 2 deals with the development of a screening method for the determination of PYRs in chicken eggs and meat; Chapters 3 and 4 deal with the development of analytical methods for the determination of PCBs in eggs and milk samples. Chapter 5 presents the experimental work undertaken at “BonassisaLab SRL” - s.s 16 Km. 684.300, Zona Industriale ASI, Incoronata – 71122 Foggia (Italy), during a nine-month research internship. In this chapter, a rapid, reliable and cheap analytical method for novel emerging contaminants like the alkaloids MT and OMT is assessed. Chapters 6 contains a study for the development of two innovative extraction methods for the determination of BDs in seawater samples from Changxing Island (Dalian, China) during six months of collaboration with “Key Laboratory of Natural Resources of the Changbai Mountain and Functional Molecules”, Yanbian University, Yanji, China. In the final Chapter, 7 conclusions of this PhD work are drawn.

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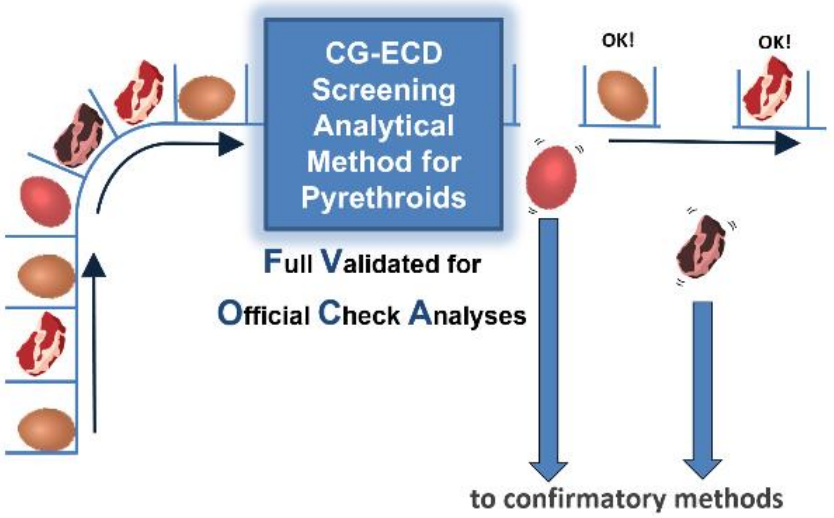
# Chapter 2 - A Sensitive Screening Method for the Determination of Pyrethroids in Chicken Eggs and Various Meat Samples by Gas Chromatography and Electron Capture Detection



## 2.1 Introduction

Pyrethroids (PYRs) are synthetic insecticides, frequently used in agriculture, in veterinary medicine and also for indoor/outdoor pest control because of their low cost, efficacy, low mammalian toxicity and environmental hazard.[1] Nevertheless, the wide use of PYRs has resulted in widespread distribution of residues in ground and surface water and then along the food chain.[2,3] Although PYRs are thought to be safe for humans, toxicological tests have shown that excessive exposure to PYRs can cause serious health effects, such as paraesthesia, headache, dizziness, nausea, and skin irritation.[4,5] For these reasons, pyrethroids have been included in the Group B substances (Veterinary drugs and contaminants), listed in Annex I of Directive No. 96/23 of the European Council. Maximum residue levels (MRLs) have been established for these compounds in food products of animal origin (European Commission Regulation 149/2008/EC; Regulation 839/2008/EC). Therefore, validated and robust analytical methods to measure concentrations of pyrethroids in a variety of food matrices are necessary for human health and research purposes. In chicken eggs, an MRL of 50 μg $kg^{-1}$ has been set for phenothrin, permethrin, cypermethrin, deltamethrin, and 20 μg $kg^{-1}$ for cyfluthrin and fenvalerate; in adipose tissue and meat of different animal origin, a maximum level of 50 μg $kg^{-1}$ has been established for phenothrin, permethrin, cyfluthrin, cypermethrin, fenvalerate, while a less restrictive limit of 500 μg $kg^{-1}$ has been fixed for deltamethrin.

In recent years, the problems and risks associated with PYRs contamination of animal feed and foods have promoted the development of a variety of analytical methods for their determination[6] including high-performance liquid chromatography[7–10] and gas chromatography (GC).[11–16] As recommended by the European Directives (European Commission Decision 657/2002/EC) for the official control programs of organic residues or contaminants, GC-MS is currently used for confirmatory PYR analyses.[13,17–19] On the other side, screening analytical methods for the determination of pyrethroids in food samples are required to provide reliable results and quick turnaround of data within- and inter-official control laboratories involved in monitoring and risk-assessment studies. Therefore, for high throughput applications, chromatographic methods based on GC-ECD could represent a valid solution for a screening evaluation of PYR contents, ensuring good results in terms of selectivity, instrumental costs and simplicity.[19–22]

Quite recently, in a previous paper published by our research group, the experimental conditions in GC coupled with mass spectrometry were optimized and the method was validated for its use in the confirmatory analyses of pyrethroids in chicken egg samples.[23] In this work, a reliable and sensitive screening method by GC-ECD is proposed for the multiresidual determination of six pyrethroids (phenothrin,

permethrin, cyfluthrin, cypermethrin, deltamethrin, fenvalerate), selected because of their relevance to agricultural and commercial uses and in disinfestations in the poultry houses. Compared with MS, electron capture detection could suffer from sample matrix interferences, due to the impossibility of monitoring potential co-eluted compounds. For this reason, in this work, more attention has been devoted to the optimization of the experimental conditions (both chromatographic and sample preparation). A good compromise between fast temperature gradients (to ensure a rapid and high-throughput analysis) and satisfactory peak resolutions has been also achieved. Through the validation procedure, linearity, selectivity, recovery, precision, detection and quantitation limits (LODs, LOQs), and measurement uncertainty were evaluated. Results demonstrated the conformity of this screening method with provisions of the European directives for PYRs analysis in monitoring programs along the food production chain.

# 2.2 Materials and Methods

## 2.2.1 Chemicals

Pyrethroids reference standards (Mix 118: phenothrin, permethrin, cyfluthrin, cypermethrin, deltamethrin, fenvalerate, purity from 94.0% to 99.0%) were purchased from Dr Ehrenstorfer, Reference Materials (Augsburg, Germany). For each pyrethroid, individual stock solutions at a concentration of 10 mg $L^{-1}$ were prepared in isooctane in Pyrex glass vials and stored at -20°C in the dark, where they resulted stable for 6 months, as confirmed by stability tests. Intermediate standard solutions were prepared just before injection by diluting stock solution in isooctane to obtain concentrations of 50, 100, 250 and 500 µg $L^{-1}$ for each pyrethroid. Standard working solutions were stored in a freezer at -20°C and used for not more than a week (as suggested by stability tests performed on the calibration standards in solution). Stock solutions of PCB 209 (Dr Ehrenstorfer, > 99.0%), used as internal standard, were prepared in isooctane at a concentration of 10 mg $L^{-1}$ and added to pyrethroid standard calibration solutions to a final concentration of 100 µg $L^{-1}$. Calibration curves were obtained by plotting the ratio between analyte peak area and IS peak area against the PYR concentration. Analogously, the identification of the target compounds was accomplished by calculating the relative retention time as the ratio between the analyte and IS retention times.

Glassware was treated with a sulphochromic mixture (Carlo Erba Reagenti, Milano, Italy) for organic and inorganic residues removal. Then, it was washed with different solvents (water, acetone, and n-hexane of HPLC grade) to eliminate cross-contamination.

Solid-phase extraction C18 (500 mg/6 mL) and Extrelut NT3 cartridges were supplied by Varian (Sunnyvale, CA, USA) and Merck (Darmstadt, Germany), respectively.

### 2.2.2 Samples and sample preparation

All the samples (chicken egg and bovine, pork, and equine meat) were collected from local farms regularly inspected by veterinary services. The Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extraction system (Thermo Fisher Scientific, Sunnyvale, CA, USA) with centrifugal evaporation was used to gravimetrically determine the fat content. A representative portion of each sample (about 30 g, with a fat percent of c.a. 30% in egg and ranging from 3 to 20% in meat) was thoroughly blended using a food processor. The homogenized samples were stored at -20°C. The samples were thawed at 4°C overnight before use. A 5 g portion of the sample (whole egg, albumen-free yolk and meat) was placed in a polypropylene flask, then 40 or 20 mL of extraction mixture (hexane/acetone, 95/5, v/v) were added for the extraction of pyrethroids from egg or meat, respectively, aided by a magnetic stirrer for 30 min. After centrifugation for 20 min at 1500 rpm, the clear supernatant was collected and evaporated under a stream of nitrogen at 45°C by a Turbovap system (Caliper Mod. LV, Hopkinton, MA, USA). The residue was then dissolved in 3 mL of hexane and transferred into an Extrelut NT3 cartridge. After about 10 min (*i.e.* the time necessary to obtain uniform adsorption of the solution on the cartridge-bed), the pyrethroids were eluted with 3 x 5 mL of acetonitrile saturated with *n*-hexane (80/20, v/v). Then, 15 mL of eluate were loaded on a C18 cartridge (500 mg/6 mL) previously conditioned with 10 mL of acetonitrile. The elution of pyrethroids was obtained using 10 mL or 15 mL of acetonitrile for the analysis of egg and meat, respectively. After evaporation under a stream of nitrogen at 45°C, the residue was dissolved in 1 mL of isooctane containing 100 µg $L^{-1}$ PCB 209 and then injected into the GC/ECD system. Compared to the original sample intake, the analytical procedure of sample extraction and clean up resulted in a concentration factor equal to 5 for each pyrethroid.

### 2.2.3 Gas chromatography - electron capture detection analysis

Chromatographic separations were performed by an AutoSystem XL GC (Perkin Elmer, Waltham, MA, USA) equipped with a programmable injector and coupled with an Electron Capture Detector. The glass liner siltek© deactivated (Perkin Elmer) was used in split/splitless mode and equipped with a Carbofrit plug (Restek, Bellefonte, PA, USA). The chromatographic separations were carried out using a FactorFour™ analytical column (VF-5 ms, 30 x 0.25 mm inner diameter, 5% diphenyl-95% dimethylsiloxane liquid phase, film thickness: 0.25 µm; Varian Instruments). An untreated, fused-silica capillary column (2 x 0.25 mm i.d.; Supelco, Oakville, Ontario, Canada) was used as a guard column. A volume of 2.5 µL of the final extract was injected into the

chromatographic system in splitless mode. The flow rate of the carrier gas (Helium, 99.999%, pressure-pulse mode: 30 psi for 1 min) was 1.0 mL $min^{-1}$. The temperature of the electron capture detector (ECD) was 375°C. The injector temperature started at 70°C and after 0.5 min ramped to 300°C at the maximum rate; after 5.0 min decreased to 200°C (hold for 10.0 min). The oven temperature was initially set at 70°C for 2.0 min, then increased to 160°C at a rate of 20°C $min^{-1}$, to 260°C at 4°C $min^{-1}$ and 300°C at 20°C $min^{-1}$. The final temperature of 300°C was kept for 5.5 min. The total run time was 39.0 min. Acquisition and data processing were performed by the TotalChrom workstation (Perkin Elmer).

### 2.2.4 Validation procedure

The linearity test in egg and meat was performed by three series of analyses on three different days, by injecting four mixed standard solutions of pyrethroids, each at concentrations of 50, 100, 250 and 500 µg $L^{-1}$. Method selectivity was tested by the analysis of 20 independent blank samples of egg and meat samples. The absence of interfering peaks in the retention time window of interest was checked for each analyte within the retention time tolerance of 0.2 min. As reported in Dec 657/2002/EC, the trueness of measurements was assessed through the recovery of additions of known amounts of the analytes to a blank matrix. Then, spiked samples with PYRs were prepared by adding proper amounts of PYRs standard solutions to the food sample before the extraction process. Precision and recovery were determined by performing tests on two sets of blank egg yolk (six replicates each), fortified with phenothrin, permethrin, deltamethrin, cypermethrin, cyfluthrin and fenvalerate, each at a concentration of 20 µg $kg^{-1}$ and 50 µg $kg^{-1}$, in order to include the maximum residue level of all PYRs in the explored range. For the method validation in meat samples, spiked samples (six replicates) with phenothrin, permethrin, cypermethrin, cyfluthrin, fenvalerate and deltamethrin, each at a concentration of 50 µg $kg^{-1}$ were analyzed. Since a different Maximum Residue Limit (MRL) has been established for deltamethrin in meat samples, additional analyses on a set of spiked samples (six replicates) with deltamethrin at a fortification level of 450 µg $kg^{-1}$ (slightly lower the legal limit of 500 µg $L^{-1}$, in order to increase the method reliability during screening analyses) were also performed. The experiments were carried out on different days with the same instruments but by different operators, to ensure the greater variability of results. Precision and recovery data have been previously processed by the Shapiro–Wilk test[24] to verify normal distribution. Afterwards, a one-way ANOVA test was performed to verify the homogeneity of the mean concentration values evaluated among the validation sessions at each fortification level. Results from ANOVA were used to calculate intra-laboratory repeatability relative standard deviations ($RSD_r$) in accordance with Decision 2002/657/EC.

## 2.3 Results and Discussion

### 2.3.1 Optimization of sample clean-up and chromatographic separation conditions Optimization of the chromatographic conditions

Sample pretreatment for trace PYRs analysis can be considered a key step in the whole analysis process since the concentrations of pyrethroids in food samples are quite low [25]. Different sample preconcentration methods have been proposed for the enrichment of the target analytes, such as solid-phase microextraction (SPME),[26–29] dispersive liquid-liquid microextraction (DLLME) [30], magnetic solid-phase extraction,[8] QuEChERS [25] and matrix solid-phase dispersion (MSPD).[31] In this study, the extraction of PYRs from egg and meat was easily performed by a quick clean-up procedure consisting of an extraction step of the sample chopped up into small pieces with an organic solvent, followed by two sequential SPE steps.

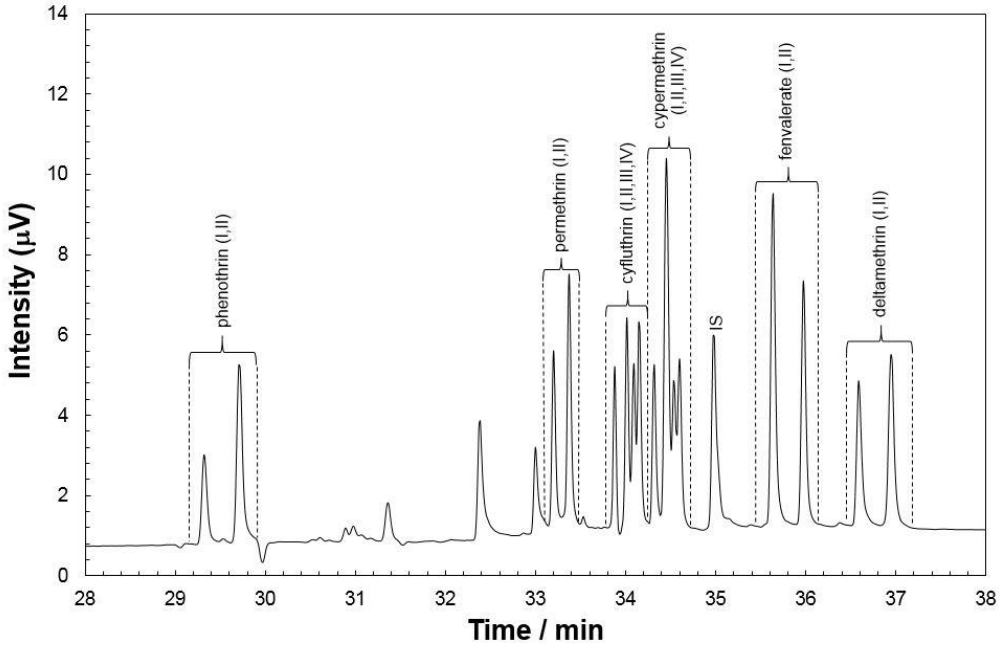



*Fig. 2.1 Chromatographic separation of a mixed standard solution of phenothrin, permethrin, cyfluthrin, cypermethrin, deltamethrin, and fenvalerate at a concentration of 250 µg $L^{-1}$ each. IS: internal standard (PCB 209)*

As previously reported [23], the ruggedness of the method for the extraction of PYRs from chicken eggs when minor and major changes are applied (extraction volume and time, centrifugation time, SPE cartridges characteristics, elution volume, and final

extract evaporation temperature) was confirmed by using the Youden experimental design, as described in Decision 2002/657/EC. A good selectivity was obtained for the proposed screening method, adopting the same sample clean-up procedure of the previous work, where MS is used instead of ECD. The optimized extraction-purification protocols are not time consuming nor labor-intensive and do not require the use of expensive equipment, with the possibility of multiple and parallel sample extractions. Moreover, the use of mixtures hexane/acetone during the extraction procedure has led to high recovery values of these pesticides. As far as chromatographic separation is concerned, most of the methods for the determination of pyrethroids make use of electron capture detection, which provides great sensitivity for halogenated compounds.[29,32–37] The optimization of the chromatographic conditions were performed in order to achieve a good compromise between low chromatographic times and adequate peak resolution. Details of the employed analytical method are summarized in the experimental section and a typical chromatogram of a standard mix of pyrethroids is shown in Fig. 2.1. The use of a Programmed Temperature Vaporising (PTV) inlet offered several benefits compared to the classical hot injection technique. Indeed, memory effects among repeated injections were minimized (due to the high temperatures reached at the end of the injection program) and, moreover, the different analytes boiling temperatures produced a shift in the injection time, improving the peak resolution. The PTV injection was combined with an oven temperature program optimized in terms of temperature values, ramp, and duration, in order to separate matrix components from the target analytes. Under the optimized chromatographic conditions, a satisfying separation was achieved with symmetrical and narrow peaks in the retention time window between 28 and 38 min.

Pyrethroid standards are available as mixtures of more than one isomer and, in addition, isomerization processes could occur during chromatographic separation and sample extraction. Therefore, due to the presence of two or three carbon chiral centres, multiple peaks associated with different diastereoisomers were observed (double peaks for phenothrin, permethrin, fenvalerate, and deltamethrin, and quadruple peaks for cyfluthrin and cypermethrin). For quantitative analyses, then, the sum of the area of the peaks of the different diastereoisomers has been considered.

### 2.3.2 Method validation

Validation of the analytical methods is essential to provide accurate results with a high within- and inter-laboratory reproducibility, which are very important parameters in monitoring and risk-assessment studies, as well as in official controls. In agreement with Decision 657/2002/EC and Regulation 882/2004/EC, which describe the analytical parameters to be tested to assure the method reliability, validation parameters such as

selectivity, linearity, detection, and quantitation limits, precision, recovery, and measurement uncertainty have been evaluated.

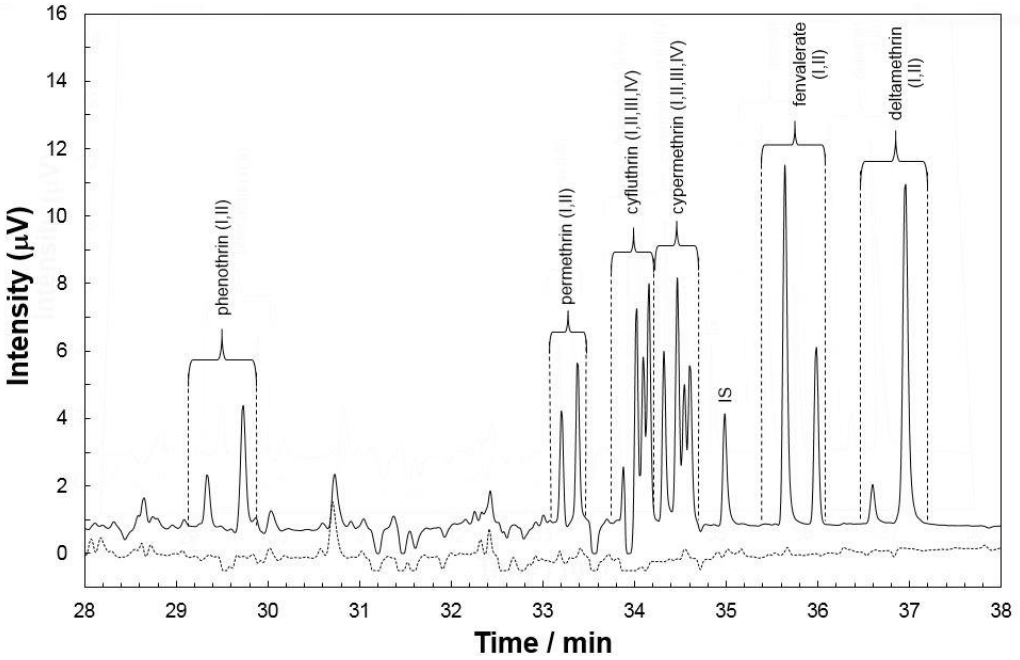



*Fig. 2.2 Chromatograms of a blank sample (dotted line) and a spiked egg yolk sample (solid line) with phenothrin, permethrin, cyfluthrin, cypermethrin, fenvalerate, and deltamethrin, each at a concentration of 50 µg kg$^{-1}$. IS: internal standard (PCB 209)*

### *2.3.2.1 Selectivity towards interferences*

For the assessment of the method selectivity, samples of egg and meat of different animal origins were processed by the proposed method. The comparison of typical chromatograms obtained for blank and spiked samples (see and 2.3) evidenced that the proposed method can distinguish the analytes from other matrix components since for each pyrethroid in the maximum tolerance range (±0.2 min) no interfering peaks were observed.

### *2.3.2.2 Calibration curves and limits of detection and quantitation*

Good linearity was found for all target analytes in the range 50-500 µg L$^{-1}$ (corresponding to 10-100 µg kg$^{-1}$ in the matrix) with correlation coefficients always higher than 0.9992. The goodness-of-fit of the data to the calibration curve was evaluated through the response factor distribution, by calculating the signal-to-concentration ratio (y/x) for each experimental point. Then the $x_i/y_i$ ratios were

checked to ensure that their deviation from the mean value of the signal-to-concentration ratio did not exceed ±10%.

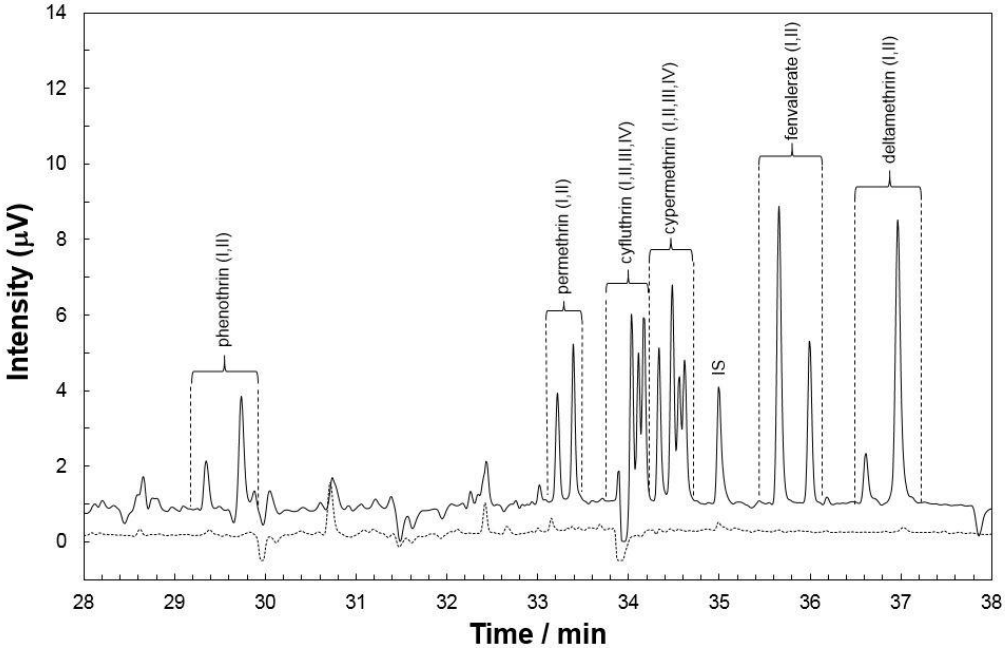



*Fig. 2.3 Chromatograms of a blank sample (dotted line) and spiked meat sample (solid line) with phenothrin, permethrin, cyfluthrin, cypermethrin, fenvalerate, and deltamethrin, each at a concentration of 50 µg kg$^{-1}$. IS: internal standard (PCB 209)*

Furthermore, any systematic instrumental bias can be ruled out since the confidence interval of the intercept includes the zero value at 95% confidence level (n=4). The suitability of the regression model was also proven by the statistical lack-of-fit-test, by using all the calibration datasets (12 data pairs, 4 calibration points, 3 replicates at each calibration point). Then, the residual sum of squares of regression is separated into two components: the sum of squares due to lack of fit and the pure "error" sum of squares.[38] The experimental F values for all PYRs (ranging from 1.011 to 3.630), calculated by the lack-of-fit and pure error sums of squares, divided by the corresponding degrees of freedom, respectively 2 and 8, was clearly lower than tabulated $F_{crit}(0.99; 2; 8)$ equal to 8.65, confirming that the hypothesis of non-linearity referring to the calibration model has to be rejected. Finally, by Mandel's fitting test,[39] the residual variances, resulting from the linear and the quadratic calibration function, were compared by an F-test. Considering the experimental F values, the hypothesis $H_0$ (no significant difference between the residual variances) was accepted for all the target analytes. Straight-line calibration curves should always be preferred over curvilinear or non-linear calibration models if equivalent results can be gained.[40] In our case, linear regression curves well fitted the experimental data whose calibration

parameters, evaluated for each pyrethroid, are reported in Table 2.1. By the chromatograms of the pyrethroid standard solutions obtained for the lowest calibration level (50 μg $L^{-1}$), the instrumental limits of detection (LOD) and quantitation (LOQ) were estimated at a signal-to-noise ratio of 3 and 10, respectively. LODs and LOQs in the range 0.22-0.63 μg $L^{-1}$ and 0.72-2.1 μg $L^{-1}$ were observed (corresponding to 0.04-0.13 μg $kg^{-1}$ and 0.14-0.42 μg $kg^{-1}$ in the matrix, respectively). These results suggested that the proposed method provides higher sensitivity than other methods reported in the literature for PYRs analysis, allowing their determination at trace levels. Indeed, our LOD values are better than those obtained by analytical methods designed for the analysis of PYRs by GC and MS[15–17,25] or ECD.[19,22,26,41] This aspect is a key factor for screening analytical methods, because the lower are LOD values, the lower is the probability of false negative results.

Predictable matrix effects in real samples, due to loss or enrichment of analyte during extraction and cleanup steps, have been evaluated, for each pyrethroid, by fitting the data obtained by analyses of spiked eggs and meat samples at five concentration values (from 0 to 300 μg $kg^{-1}$; six replicates at each validation level in the two different working sessions), as a function of the nominal fortification level. As expected, the slopes obtained by standard solutions (corrected by the concentration factor) and the ones obtained by spiked real samples were significantly different at 95% confidence level, confirming the presence of matrix effects in these analyses (data not shown). Matrix effects and changes in baseline noise can also have a strong impact on LOD and LOQ values: for this reason, their evaluation under these conditions is mandatory. LOD values ranging from 0.05 to 0.25 μg $kg^{-1}$ and from 0.07 to 0.23 μg $kg^{-1}$ were obtained from the chromatograms at the lowest fortification level in egg and meat samples, respectively. Analogously, LOQs ranged from 0.18 to 0.84 μg $kg^{-1}$ for eggs and from 0.22 to 0.79 μg $kg^{-1}$ in meat samples. These values demonstrated that, even in the case of real sample analyses, the proposed GC-ECD method returns LODs and LOQs considerably lower than legal limits.

### *2.3.2.3 Precision and recovery*

The method was tested for accuracy, intra-day assay within-laboratory repeatability, and the relevant data determined for each pyrethroid by spiked egg and meat samples are summarized in Table 2.2 and 2.3, respectively. As can be noted from the tables, the obtained intra-day $RSD_r$ values are well below the reference values of 15, derived by the Horwitz equation for a mass fraction $\leq$ 0.1 mg $kg^{-1}$, under repeatability conditions.[42]

*Tab. 2.1 Performance and chromatographic parameters of pyrethroids analyzed by the proposed GC-ECD method. [a]Retention time; tolerance range ±0.2 min. Instrumental LOD and LOQ values referred to standard solutions prepared in solvent[b] and their estimation in matrix[c].*

| Analyte | $t_R$[a] (min) | Linear Range (R) (µg $L^{-1}$) | Sensitivity (µV µg $^{-1}$ L) | Instrumental | | | | Method | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | LOD (µg $L^{-1}$) | LOQ (µg $L^{-1}$) | LOD (µg $kg^{1}$) | LOQ (µg $kg^{-1}$) | LOD (µg $kg^{-1}$) | LOQ (µg $kg^{-1}$) | LOD (µg $kg^{-1}$) | LOQ (µg $kg^{-1}$) |
| | | | | solvent[b] | | matrix[c] | | Chicken Eggs | | Meat | |
| Phenothrin I | 29.3 | 2.1-500 (0.9994) | 940±20 | 0.63 | 2.1 | 0.13 | 0.42 | 0.20 | 0.68 | 0.23 | 0.79 |
| Phenothrin II | 29.7 | | | | | | | | | | |
| Permethrin I | 33.2 | 0.89-500 (0.9992) | 931±30 | 0.27 | 0.89 | 0.05 | 0.18 | 0.08 | 0.27 | 0.10 | 0.35 |
| Permethrin II | 33.4 | | | | | | | | | | |
| Cyfluthrin I | 33.9 | 0.93-500 (0.9997) | 2380±40 | 0.28 | 0.93 | 0.06 | 0.19 | 0.07 | 0.22 | 0.08 | 0.25 |
| Cyfluthrin II | 34.0 | | | | | | | | | | |
| Cyfluthrin III | 34.1 | | | | | | | | | | |
| Cyfluthrin IV | 34.2 | | | | | | | | | | |
| Cypermethrin I | 34.3 | 1.3-500 (0.9996) | 2550±140 | 0.40 | 1.3 | 0.08 | 0.26 | 0.06 | 0.21 | 0.09 | 0.30 |
| Cypermethrin II | 34.4 | | | | | | | | | | |
| Cypermethrin III | 34.5 | | | | | | | | | | |
| Cypermethrin IV | 34.6 | | | | | | | | | | |
| Fenvalerate I | 35.6 | 0.72-500 (0.9995) | 1840±40 | 0.22 | 0.72 | 0.04 | 0.14 | 0.05 | 0.18 | 0.07 | 0.22 |
| Fenvalerate II | 36.0 | | | | | | | | | | |
| Deltamethrin I | 36.6 | 1.5-500 (0.9999) | 1370±50 | 0.46 | 1.5 | 0.09 | 0.30 | 0.25 | 0.84 | 0.18 | 0.61 |
| Deltamethrin II | 37.0 | | | | | | | | | | |

*Tab. 2.2 Recovery and repeatability data for the determination of pyrethroids in spiked egg samples. Mean values ± standard deviations (n=6). [b] $RSD_r$: Relative Standard Deviation under repeatability conditions*

| Analyte | Fortification Level | | | |
|---|---|---|---|---|
| | 20 μg $kg^{-1}$ | | 50 μg $kg^{-1}$ | |
| | Recovery (%)[a] | $RSD_r$ (%)[b] | Recovery (%)[a] | $RSD_r$ (%)[b] |
| **Phenothrin** | 85±11 | 13 | 74.4±0.8 | 1.1 |
| **Permethrin** | 106±5 | 5 | 93±2 | 2 |
| **Cyfluthrin** | 90±8 | 8 | 89±4 | 5 |
| **Cypermethrin** | 80±7 | 8 | 84±2 | 3 |
| **Fenvalerate** | 85±6 | 7 | 87.6±0.8 | 0.9 |
| **Deltamethrin** | 96±9 | 10 | 78.3±1.5 | 2 |

*Tab. 2.3 Recovery and repeatability data for the determination of pyrethroids in spiked meat samples.[a] Mean values ± standard deviations (n=6). [b] $RSD_r$: Relative Standard Deviation under repeatability conditions*

| Analyte | Fortification Level | | | |
|---|---|---|---|---|
| | 50 μg $kg^{-1}$ | | 450 μg $kg^{-1}$ | |
| | Recovery (%)[a] | $RSD_r$ (%)[b] | Recovery (%)[a] | $RSD_r$ (%)[b] |
| **Phenothrin** | 74±4 | 5 | - | - |
| **Permethrin** | 80±6 | 8 | - | - |
| **Cyfluthrin** | 88±8 | 9 | - | - |
| **Cypermethrin** | 88±4 | 5 | - | - |
| **Fenvalerate** | 86±6 | 7 | - | - |
| **Deltamethrin** | 83±8 | 10 | 79±6 | 8 |

Recovery percentages were evaluated by comparing the concentration of spiked samples, determined by the external calibration regression line, with the nominal fortification level. For each analyte, it was verified that the calculated mean recovery was in compliance with the recovery range of 70-120%, reported in the official

documents SANTE/11945/2015 and 2013/12571, dealing with the method validation and quality control procedures for Pesticide Residues Analysis in Food and Feed. Recoveries ranging from 74% to 106% were obtained, demonstrating a good accuracy of the method, which can be considered a useful tool for the screening evaluation of pyrethroids in egg and meat.

*Tab. 2.4 Measurement uncertainty values at MRLs in eggs[a] and meat[b]. [a]50 µg kg $^{-1}$ for phenothrin, permethrin, cypermethrin and deltamethrin and 20 µg kg $^{-1}$ for cyfluthrin and fenvalerate. [b] 50 µg kg $^{-1}$ for phenothrin, permethrin, cyfluthrin, cypermethrin, fenvalerate and 450 µg kg $^{-1}$ for deltamethrin.*

| Analyte | Chicken Eggs (%) | Meat Products (%) |
|---|---|---|
| **Phenothrin** | 2.7 | 8.1 |
| **Permethrin** | 2.2 | 10 |
| **Cyfluthrin** | 11 | 25 |
| **Cypermethrin** | 4.8 | 4.6 |
| **Fenvalerate** | 12 | 9.3 |
| **Deltamethrin** | 2.6 | 11 |

### *2.3.2.4 Measurement uncertainty*

For the evaluation of uncertainty of analytical results, the metrological approach was adopted, using the validation data obtained from each step of the total analytical procedure [43]. On the basis of uncertainties propagation law, for each PYR, the concentration relative uncertainty has been calculated taking into account the analyte concentration in the analyzed spiked samples, the volume of the final extract, and the sample weight before extraction and clean-up. Then, the determination of the measurement uncertainty was performed by considering four sources of uncertainty: (a) preparation of the standard; (b) method reproducibility; (c) method recovery; (d) instrumental calibration curve. A relative expanded measurement uncertainty was calculated using a coverage factor k of 2, corresponding approximately to a 95% confidence level. Values in the range 2.2-12% and 4.6-25% were determined in egg and meat, respectively, as reported in Tab. 2.4.

## 2.4 Analyses of food samples

Method feasibility has been demonstrated by the analyses of eggs and meat from cows, pigs, and horses, commercially available in local markets. Generally, the sample preparation procedure is not labor-intensive, confirming the potential of the proposed method in screening analysis. Then, the optimized experimental conditions allow to satisfy the demand of a sensitive and reliable determination of PYRs in complex food

matrices, in compliance with the European directives. Although for a single sample the time required for the total analysis is quite long (~90 min of sample preparation + 39 min of chromatographic run), a high sampling rate process was obtained taking advantage of the automated SPE system, due to the parallel preparation of multiple extracts. The sample preparation process can start during the first minutes of the previous chromatographic run, including the food extract samples progressively in the sample sequence of the autosampler system: in a working day, then, more than 10 samples can be processed and analyzed. Therefore, a high-throughput analysis is ensured, with a total analysis time of one hour, calculated by dividing the number of hours of a day working session by the number of the analyzed samples.

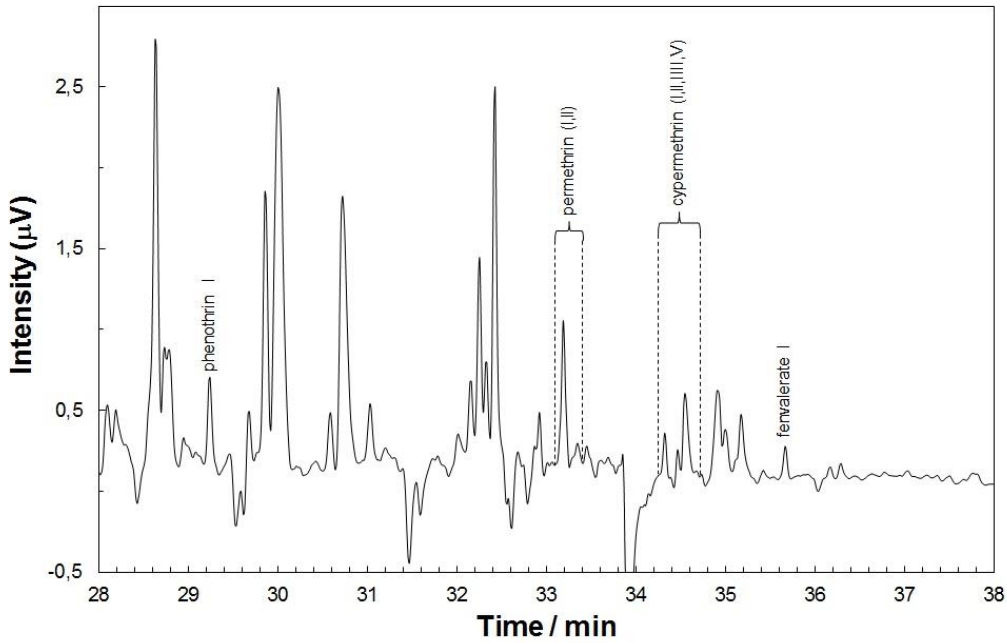



*Fig. 2.4 Chromatogram of a fatty tissue sample from bovine meat.*

Despite the relatively high complexity of the meat matrix, a good selectivity was obtained. No sample showed a value higher than the maximum level fixed by the European Community, indeed lower levels of PYRs than LOQs was observed in all the analyzed samples. As an example, in Fig. 2.4 the chromatographic separation of a fatty tissue sample from bovine meat (fat content 19%) is shown. No peaks were observed in the time-window where deltamethrin elutes. The peaks of phenothrin I and fenvalerate I are observed at 29,3 and 35.6 min, respectively. The two peaks at around 33 min correspond to permethrin, while the peaks between 34-35 min correspond to cypermethrin. Peak identity was confirmed by standard additions.

## 2.5 Conclusions

In conclusion, in this work, a sensitive and reliable screening analytical method for the determination of six pyrethroids by GC-ECD in egg and meat was validated through the evaluation of linearity, detection and quantitation limits, selectivity, recovery, precision, and measurement uncertainty. Optimized chromatographic conditions, combined with a minimal sample preparation clean-up, assure good results in terms of rapidity, response sensitivity, and separation efficiency. The results of the method validation, performed according to the European Commission directives, demonstrated that the proposed method is well suited to satisfy the demand for the accurate screening of pyrethroids in complex food matrices.

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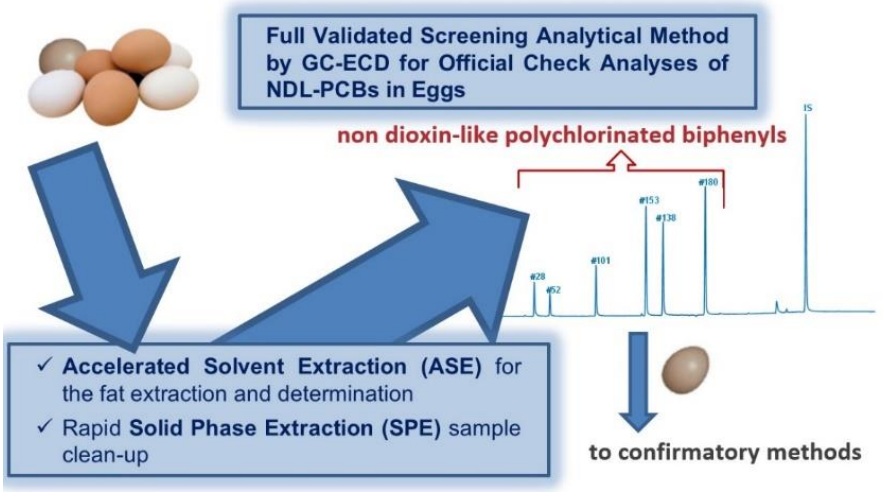
# Chapter 3 - Development of a screening analytical method for the determination of non dioxin-like polychlorinated biphenyls in chicken eggs by gas chromatography and electron capture detection



## 3.1 Introduction

Polychlorinated biphenyls (PCBs) are a group of anthropogenic environmental pollutants with serious implications for human health, due to their great toxicity, bioaccumulative character and resistance to metabolic degradation [1]. Due to their lipophilic nature, PCBs tend to accumulate in fatty tissues, resulting in a widespread distribution along the food chain. Even if the toxicity equivalency factor (TEF, based on the toxicity of the 2,3,7,8-tetraclorodibenzo-p-dioxin as a reference) has been determined only for dioxin-like compounds (DL-PCBs), six non dioxin-like polychlorinated biphenyls (NDL-PCBs, congener 28, 52, 101, 138, 153, and 180) are typically used as indicators to monitor the contamination levels in foodstuffs, due to their higher contents respect to other congeners [2]. Maximum Residue Levels (MRLs) have been established for both DL- and NDL-PCBs in food products of animal origin as well as in vegetable oils and fats (European Commission Regulation 1259/2011/EC), taking into account recent occurrence data compiled in the EFSA scientific report [3]. In hen eggs and egg products, an MRL of 40 ng $g^{-1}$ fat has been set for the sum of the six NDL-PCBs.

Considering the importance of NDL-PCBs determination in food samples, during the last decade efforts have been done to develop accurate methods for determining the levels of NDL-PCBs in food matrices [2,4–8]. In particular, for high throughput applications in monitoring and risk-assessment studies, screening analytical methods based on electron capture detection [9–12] have been developed to provide quick and reliable results, ensuring at the same time good results in terms of selectivity, instrumental costs and simplicity. Furthermore, due to the high cost and time-consuming sample preparation requested for NDL-PCBs official analyses, researchers are nowadays focused on the development of faster and more cost-effective alternatives. Among the others, accelerated solvent extraction (ASE) procedures have received the attention of researchers due to a series of advantages, including automation, reduced extraction time and simultaneous purification steps[13–18]. ASE procedures have been successfully employed for food analyses[13–18]. In this work, a reliable and sensitive screening method by GC-ECD for the determination of six NDL-PCBs (congener 28, 52, 101, 138, 153, 180) in chicken egg samples is proposed. An ASE procedure for the fat extraction and determination from eggs and a solid phase extraction process for an efficient and rapid sample clean-up has been also optimized, evaluating the validation procedure, linearity, selectivity, recovery, precision, detection and quantification limits (LODs, LOQs), ruggedness, and measurement uncertainty of the whole analysis procedure.

## 3.2 Materials and Methods

### 3.2.1 Chemicals

High purity (≥ 97%) NDL-PCB standards (IUPAC congeners 28, 52, 101, 153, 138, 180) were provided by Dr Ehrenstorfer (Augsburg, Germany). For each NDL-PCB, individual stock solutions at a concentration of 10 mg $L^{-1}$ were prepared in isooctane and stored at -20°C in the dark, where they resulted stable for 6 months, as confirmed by stability tests. Working standard solutions were prepared just before injection by dilution in isooctane. Stock solutions of NDL-PCB #209 (Dr Ehrenstorfer, > 99.0%), used as internal standard (IS), were prepared in isooctane at a concentration of 10 mg $L^{-1}$ and added to NDL-PCB standard calibration solutions to a final concentration of 100 µg $L^{-1}$. ENVI-carb (120-140 mesh, 100 $m^2$ $g^{-1}$) was purchased from Supelco Inc. (Bellefonte, PA, USA): impurities eventually present were removed washing the ENVI-carb plentifully with different solvents, in the following order: n-hexane, cyclohexane and toluene, and finally again n-hexane. Solid-phase extraction Bond Elut-PCB cartridges (1 mg, 3 mL) were supplied by Agilent Technologies (Inc. Folsom, CA, 95630). Glassware was treated with a sulphochromic mixture (Carlo Erba Reagenti, Milano, Italy) for organic and inorganic residues removal. Then, it was washed with different solvents (water, acetone, and n-hexane of HPLC grade) to eliminate cross-contamination.

### 3.2.2 Samples and sample preparation

Chicken eggs were collected from local farms regularly inspected by veterinary services. The extraction-cleanup procedure for the determination of NDL-PCB in fresh egg samples involves the treatment of 5 g of albumen-free yolk by 40 mL of n-hexane/acetone 85:15 (v:v). The extraction was carried out for three hours by a magnetic stirrer. After sonication for 30 min, the clear supernatant was evaporated to 4 mL under a nitrogen stream at 45ºC in a Turbovap system (Caliper Mod. LV, Hopkinton, MA, USA). Then, 4 ml of sulphuric acid (98%) and 0,1 g of ENVI-carb® were added and the mixture was kept overnight at room temperature. After centrifugation at 4ºC for 20 min at 3000 rpm, the upper clear phase was transferred into a tube and evaporated to dryness under a nitrogen stream at 45ºC. Then, the residue was dissolved in 2 mL of n-hexane and loaded into a Bond Elut-PCB cartridge (1 mg, 3 mL), previously conditioned with 3 mL of n-hexane. The elution was obtained using 10 mL of n-hexane. Finally, after evaporation to dryness under a stream of nitrogen at 45 °C, the residue was dissolved in 1 ml of isooctane containing 100 µg $L^{-1}$of NDL-PCB #209, used as an internal standard, before GC-ECD analysis.

The Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extraction system (Thermo Fisher Scientific, Sunnyvale, CA, USA) was used to gravimetrically determine and extract the fat content. The ASE conditions were as follows: oven temperature

100°C; pressure 1500 psi; three 15 min cycles with 5 min of heat and static time, each; flush volume 60%; 100 s purge time; carrier gas nitrogen; stainless steel extraction cells 10 mL; collection vials 60 mL. An amount of 1 g of the yolk was mixed with Extrelut® (Merck, Darmstadt, Germany) in a ratio of 1:4, and air-dried in the oven at 100°C for 15 min. A cellulose filter was placed at the bottom of a 10 mL extraction cell and the sample was transferred directly into the cell. The 60 mL collection vials were weighted and labelled before being placed into the ASE system. For the extraction, a mixture of chloroform/methanol/petroleum ether (2:1:1) was used (60 mL $g^{-1}$ of the sample). The resulting extracts were evaporated to dryness at 40 °C by a rotavapor system.

### 3.2.3 Gas chromatography - electron capture detection analysis

Chromatographic separations were performed by an AutoSystem XL GC (Perkin Elmer, Waltham, MA, USA) equipped with a split/splitless injector and coupled with an Electron Capture Detector. The glass liner Siltek deactivated (Perkin Elmer) was used in splitless mode at 250°C. The chromatographic separations were carried out using a FactorFour™ analytical column (VF-5 ms, 30 x 0.25 mm inner diameter, 5% diphenyl-95% dimethylsiloxane liquid phase, film thickness: 0.25 µm; Varian Instruments). A volume of 1.0 µL of the final extract was injected into the chromatographic system. The flow rate of the carrier gas (Helium, 99.999%, pressure-pulse mode: 30 psi for 1 min) was 1.0 mL $min^{-1}$. The temperature of the electron capture detector (ECD) was 375°C. The oven temperature was initially set at 130°C, then increased to 190°C at a rate of 20°C $min^{-1}$ and 280°C at 5°C $min^{-1}$. The final temperature of 280°C was kept for 14 min, with a total run time of 35.0 min. Acquisition and data processing were performed by the TotalChrom Workstation (Perkin Elmer).

### 3.2.4 Validation procedure

The linearity test was performed by three series of analyses on three different days, by injecting six mixed standard solutions of NDL-PCBs, at concentrations of 2.5, 5.0, 10, 20, 40, and 60 µg $L^{-1}$. Calibration curves were obtained by plotting the ratio between analyte peak area and IS peak area against the NDL-PCB concentration. Analogously, the identification of the target compounds was accomplished by calculating the relative retention time as the ratio between the analyte and IS retention times. Method selectivity was tested by the analysis of 20 independent blank samples of egg samples. The absence of interfering peaks in the retention time window of interest was checked for each analyte within the retention time tolerance of $\pm$ 0.5%. As reported in European Commission Decision 657/2002/EC and SANTE 2017/11813/EC, in absence of official and certified reference material (CRM), trueness of measurements was assessed through the recovery of additions of known amounts of the analytes to a blank matrix. Then, spiked samples with NDL-PCBs were prepared by adding proper amounts of NDL-

PCBs standard solutions to the food sample before the extraction process. Precision and recovery were determined by performing tests on three sets of blank egg (six replicates each), fortified with NDL-PCBs at a total concentration of 20, 40, and 80 ng $g^{-1}$ fat, in order to include in the explored range the maximum residue level set for the sum of NDL-PCBs. Experiments were carried out on different days with the same instruments but by different operators, to ensure the greater variability of results. Precision and recovery data have been previously processed by the Shapiro–Wilk test[19] to verify normal distribution. Afterwards, a one-way ANOVA test was performed to verify the homogeneity of the mean concentration values evaluated among the validation sessions at each fortification level. Results from ANOVA were used to calculate intra-laboratory repeatability relative standard deviations (RSDr) in accordance with Decision 2002/657/EC.

## 3.3 Results and Discussion

### 3.3.1 Sample clean-up and fat extraction optimization

In fatty and highly viscous food matrices such as eggs, the sample clean-up process is a challenging step, due to the high content in lipids and non-volatile compounds of the raw extracts. Nowadays, in the European Union, dedicated sample preparation methods are applied for pesticide residue analysis in food of animal origin (BS EN 1528-3:1997. Fatty food. Determination of pesticides and polychlorinated biphenyls (PCBs) Clean-up methods). These official sample processing methods are characterized by long time, delicate and labor-intense steps. As an alternative, the extraction-cleanup is typically performed by a multistep purification process based on the use of QuEChERS [20] or various adsorbents such as Florisil, C18, Alumina, and Silica gel [21–23]. To reduce the total sample preparation time and to simplify this process, which represents the bottleneck of the NDL-PCB analysis, a procedure that allows a higher sample throughput and lower solvent consumption is here proposed. The optimization of the extraction and purification process took into account the most influencing parameters, such as the extraction solvent composition (based on binary mixtures of hexane/acetone at different percentages), the extraction time (ranging from 150 min to overnight) and the nature of SPE cartridges used (SPE/PCB or conventional C18). Then, the optimization of each factor was carried out on blank egg samples spiked at 40 ng $g^{-1}$ fat. The recovery percentages (as the sum of the 6 NDL-PCBs) obtained from the extracts of the spiked samples under the different extraction conditions were plotted against all the parameters that need to be optimized. The first set of experiments were performed to select the extraction solvent composition, considering different hexane:acetone mixtures at different v:v ratio (100:0; 95:5; 90:10; 85:15; 80:20). A one-way ANOVA test was performed to compare the recovery data,

calculated using the different extraction solvent mixtures. Statistical differences were verified at a 95% confidence level, with the highest recovery value obtained by using the mixture hexane: acetone 85:15, as reported in Fig. 3.1.

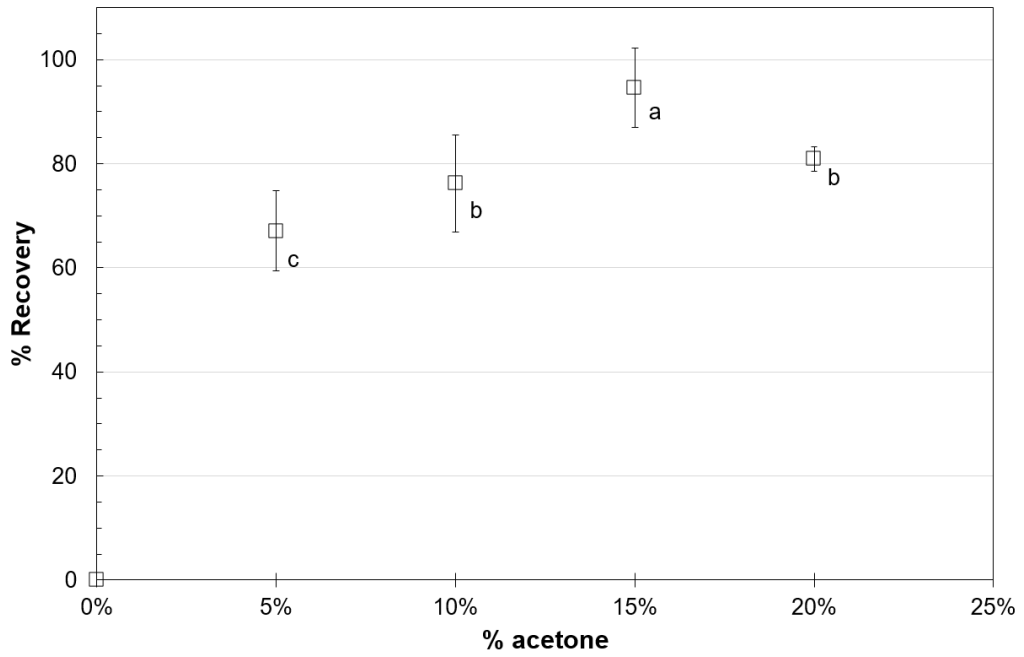



*Fig. 3.1 Recovery values calculated by using solvent extractions at different acetone percentages. Data with different letters (a, b, c) are significantly different (one-way ANOVA test, P < 0.05).*

The best compromise between recovery values and total sample preparation time was obtained reducing the extraction time from overnight to 180 min: with these experimental values, a decrease of the background noise was also observed. Another important factor was the choice of the SPE, which resulted fundamental to remove interfering peaks in the retention window close to the NDL-PCB#52 peak. When SPE/PCB was used instead of the conventional SPE/C18, the recoveries increased from 52.6% (± 0.1%, n=4) to values higher than 72%. Finally, the duration of the treatment by sulphuric acid (on the same working day or overnight) was tested. Significant differences in recovery were observed between the analytical results of these two sets of experiments (at 95% confidence level, data not shown), therefore, the overnight treatment, which additionally does not represent an analysis extra-time, was chosen.

In order to express the final measurement results of NDL-PCBs in the samples as ng $g^{-1}$ of fat, as specified by European Commission Regulation 1259/2011/EC, an ASE procedure was optimized to automate the fat extraction process from egg samples. Several factors affecting the efficiency of the ASE process (sample weight, packing

material, extraction solvent volume and composition, temperature and number of extraction cycles) were tested on market egg samples. The fat recovery was evaluated by comparison with the labelled values. The first experiments were performed to select the typology of diatomaceous earth to be used and to determine the most appropriate ratio of sample-to-bonded-phase solid support. Starting from a sample amount of 1g, the commercially available packing material Extrelut® was chosen as a drying and dispersive agent and added to the sample before homogenization (in a ratio 4:1), due to its capability of reducing the formation of emulsions, thus ensuring better extraction efficiencies. The oven temperature for the drying process was explored in the range 100-125°C (5°C steps) and the optimal value of 100°C was set for 15 minutes. Then, binary and ternary mixtures of apolar solvents, generally used for the fat extraction from food samples (hexane, petroleum ether, isopropanol, chloroform and methanol) at different combination percentages were compared for the liquid-liquid extraction. The best results in terms of recovery (92-115%) were obtained by using the ternary mixture chloroform/methanol/petroleum ether (2:1:1), associated with 3 extraction cycles of 5 min, each. Indeed, the number of cycles was varied from 3 to 8, but no statistical differences were observed ($\alpha$=95%) and a number of 3 cycles was sufficient to recover the fat amount reported in the label (RSD% lower than 9,4%, n = 8, under reproducibility conditions in different working days, with different operators and reagent lots). Finally, for the evaporation process, the Soxhlet and Turbovap systems were compared at a temperature of 60°C; complete evaporation by Soxhlet mode was obtained in 60 min, also allowing an efficient fat extraction and high recovery values. To summarize, the whole sample preparation procedure proposed for the sample clean-up and fat extraction from eggs offers several advantages, compared to the currently established official methods, in terms of ease to use and analysis time, allowing the simultaneous preparation of several samples. In addition, the proposed clean-up procedure requires small amounts of samples and solvents, providing rapid isolation of the target analytes with a high degree of selectivity. Therefore, the optimized extraction and purification steps have been proved as a suitable and convenient alternative to the traditionally accepted protocols for the routine analysis of NDL-PCBs in food samples.

### 3.3.2 Optimization of the chromatographic conditions

The optimization of the chromatographic conditions is a critical stage in the development of a multiresidue method for screening analysis, in particular when ECD is used instead of MS, because it could suffer from sample matrix effects, due to possible and unverifiable interferent coelutions. For this reason, in this work particular attention has been devoted to the optimization of the experimental chromatographic conditions.

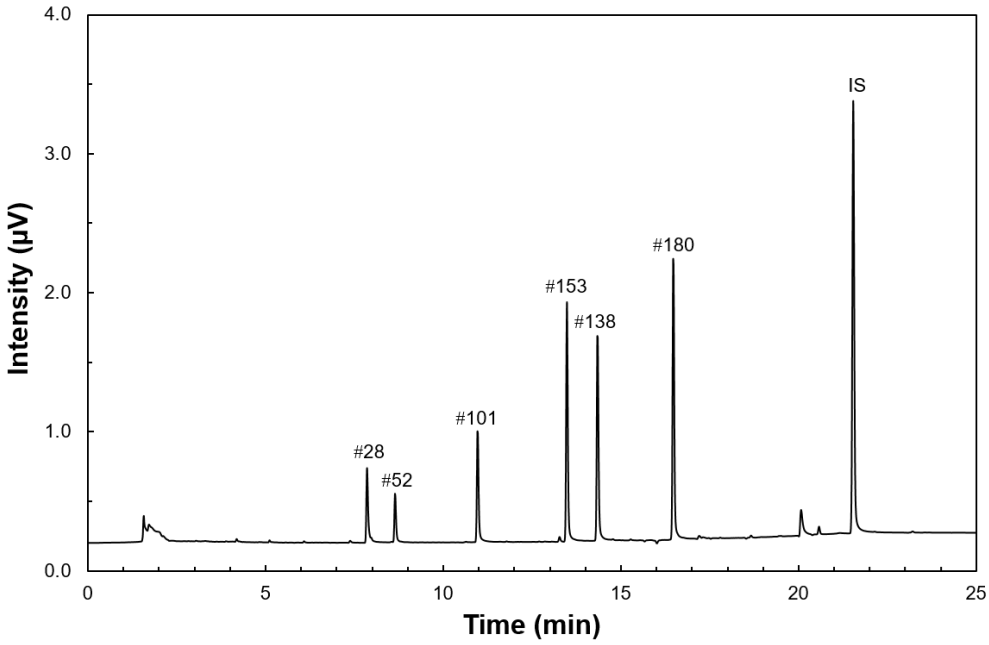



*Fig. 3.2 Chromatographic separation of a 40 µg $L^{-1}$ mixed standard solution of NDL-PCBs (congeners 28, 52, 101, 138, 153, 180). IS: internal standard (NDL-PCB #209).*

A fast temperature gradient was optimized to ensure a rapid and high-throughput chromatographic analysis and, at the same time, guaranteeing good peak resolutions. A typical chromatogram of a standard mix of NDL-PCBs is reported in Fig. 3.2. Under the optimized operational conditions, a satisfactory separation takes place in the time window of 7-22 min, with a total run time of 35 min. Excellent peak shapes with low peak tailing were observed. Overlapping with interfering peaks from the matrix component was avoided, as evident from Fig. 3.3, showing the chromatograms obtained for blank and spiked egg samples.

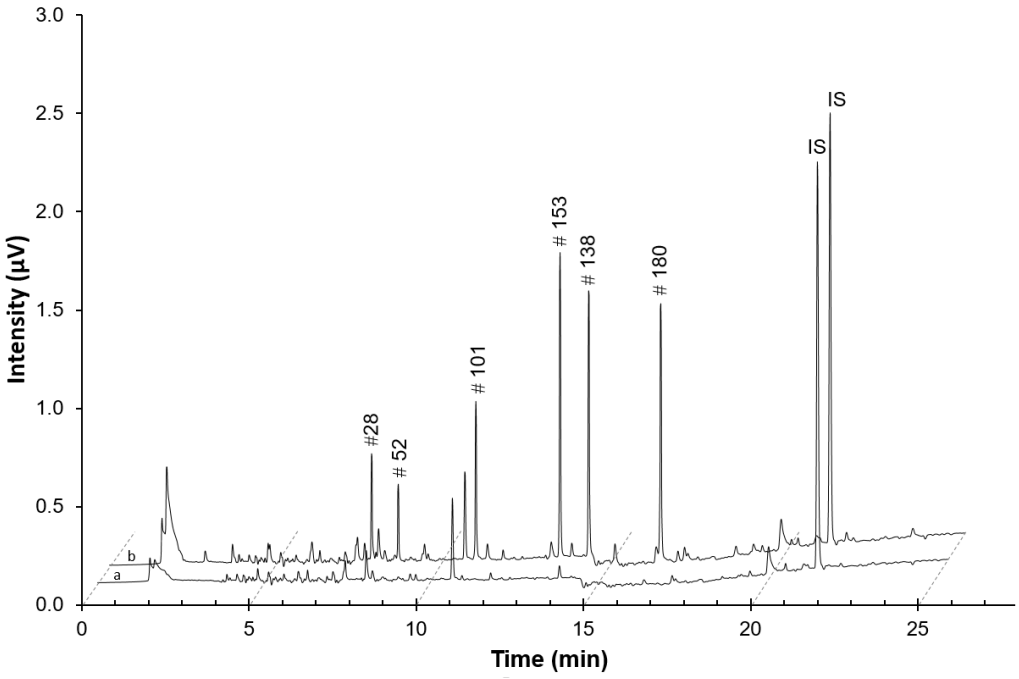



*Fig. 3.3 Chromatograms of blank samples (a) and spiked egg yolk sample (b) with NDL-PCBs at 40 ng $g^{-1}$ fat. IS: internal standard (NDL-PCB #209).*

## 3.3.3 Method validation

In monitoring and risk-assessment studies as well as in official controls, the validation of the analytical methods is essential to provide accurate and reliable results, as recommended by the European regulations (European Commission SANTE 2017/11813/EC, European Commission Regulation 2017/644/EC and European Commission Decision 657/2002/EC). Therefore, the analytical performances in terms of selectivity, linearity, detection and quantification limits, precision, recovery, ruggedness and measurement uncertainty have been evaluated.

### *3.3.3.1 Selectivity towards interferences*

For the assessment of the method selectivity, 20 independent blank samples of eggs, purchased in local markets were processed under the optimized experimental conditions. Comparison of blank and spiked samples chromatograms evidenced that the proposed method is able to distinguish the analytes from other matrix components and the optimized sample clean-up allowed an efficient elimination of possible endogenous matrix interfering substances. Then, the absence of interfering peaks in the retention time-window of each NDL-PCB, with a maximum tolerance range of ±0.5%, confirmed the selectivity of the method toward other naturally present compounds.

### *3.3.3.2 Calibration curves and limits of detection and quantification*

Good linearity was found for all NDL-PCBs in the range 2.5-60 µg $L^{-1}$ (corresponding to 1.6-39.6 ng $g^{-1}$ fat in the matrix) with correlation coefficients always higher than 0.9995. The goodness-of-fit of the data to the calibration curve was evaluated in terms of response factor distribution (signal-to-concentration ratio, y/x, calculated for each experimental point). Then the $x_i/y_i$ ratios were checked to ensure that their deviation from the mean value of the signal-to-concentration ratio did not exceed ±10%. Furthermore, the confidence interval for the intercept, including the zero value (α=95%, ν=5), indicates the absence of systematic instrumental bias. The fitness of the regression model was also confirmed by the statistical lack-of-fit-test, by using all the calibration datasets (18 data pairs, 6 calibration points, 3 replicates at each calibration point). The residual sum of squares of regression was calculated as the sum of squares due to lack of fit and the pure "error" sum of squares [24]. The hypothesis of non-linearity of the calibration model was rejected, since the experimental F values for all NDL-PCBs (ranging from 0.654 to 4.381), calculated by the lack-of-fit and pure error sums of squares, divided by the corresponding degrees of freedom, respectively 4 and 12, was clearly lower than tabulated $F_{crit}(0.99; 4; 12)$ equal to 5.412. Moreover, by Mandel's fitting test [25], the residual variances, resulting from the linear and the quadratic calibration function, were compared by an F-test and the hypothesis of no significant difference between the residual variances was accepted for all the target analytes. Therefore, straight-line calibration curves rather than over curvilinear or non-linear calibration models well fitted the experimental data, whose calibration parameters, evaluated for each NDL-PCB, are reported in Tab. 3.1.

*Tab. 3.1 Performance and chromatographic parameters of PCBs analyzed by the proposed GC-ECD method. [a]Retention time; tolerance range ± 0.5%. Instrumental LOD and LOQ values referred to standard solutions prepared in solventb and their estimation in matrixc. Method Detection and Quantification Limits calculated on spiked egg samples at 20 ng/g fat.*

| Analyte | $t_R$[a] (min) | Linear Range | | | Sensitivity ($\mu V\ \mu g^{-1}\ L$) | Instrumental | | | | Method | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ($\mu g\ L^{-1}$) | ($ng\ g^{-1}$ fat) | R | | LOD ($\mu g\ L^{-1}$) | LOQ ($\mu g\ L^{-1}$) | LOD ($ng\ g^{-1}$ fat) | LOQ ($ng\ g^{-1}$ fat) | LOD ($ng\ g^{-1}$ fat) | LOQ ($ng\ g^{-1}$ fat) |
| | | | | | | Solvent[b] | | Matrix[c] | | Chicken Eggs[d] | |
| PCB#28 | 7.9 | 0.75-60 | 0.49-40 | 0.9995 | $(325\pm5)10^{-5}$ | 0.22 | 0.75 | 0.15 | 0.49 | 2.7 | 11.6 |
| PCB#52 | 8.7 | 1.1-60 | 0.76-40 | 0.9995 | $(212\pm3)10^{-5}$ | 0.35 | 1.06 | 0.23 | 0.76 | 3.5 | 14.5 |
| PCB#101 | 11.0 | 0.50-60 | 0.33-40 | 0.9994 | $(489\pm9)10^{-5}$ | 0.15 | 0.50 | 0.10 | 0.33 | 2.2 | 4.6 |
| PCB#153 | 13.5 | 0.29-60 | 0.19-40 | 0.9999 | $(1043\pm9)10^{-5}$ | 0.09 | 0.29 | 0.06 | 0.19 | 0.8 | 1.7 |
| PCB#138 | 14.3 | 0.32-60 | 0.21-40 | 0.9999 | $(973\pm7)10^{-5}$ | 0.09 | 0.32 | 0.06 | 0.21 | 1.6 | 6.3 |
| PCB#180 | 16.5 | 0.27-60 | 0.18-40 | 0.9996 | $(1298\pm18)10^{-5}$ | 0.08 | 0.27 | 0.05 | 0.18 | 1.6 | 6.6 |

By the chromatograms of the NDL-PCB standard solutions obtained for the lowest calibration level (2.5 μg $L^{-1}$), the instrumental limits of detection (LOD) and quantification (LOQ) were estimated at a signal-to-noise ratio of 3 and 10, respectively. LODs and LOQs in the range 0.082-0.346 μg $L^{-1}$ and 0.27-1.1 μg $L^{-1}$ were observed (corresponding to 0.05-0.23 ng $g^{-1}$ fat and 0.18-0.76 ng $g^{-1}$ fat in the matrix, respectively), which are considerably lower than the legal limits of 40 ng $g^{-1}$ fat (as the sum of the six NDL-PCBs), allowing NDL-PCB determination at trace levels. These results suggested that the proposed method provides higher sensitivity than other methods reported in the literature for NDL-PCBs analysis. Indeed, our LOD values are better than those obtained by analytical methods designed for the analysis of NDL-PCBs by GC-ECD [26–29], reducing the risk of false negative results. Moreover, since matrix effects and changes in baseline noise can also have a strong impact on LOD and LOQ, their estimation in the matrix was also performed by the chromatograms of spiked egg samples at a concentration level of 20 ng $g^{-1}$ fat. LOD and LOQ values ranging from 1.6 to 3.5 ng $g^{-1}$ fat and 6.6 to 14.5 ng $g^{-1}$ fat were obtained, respectively. These values demonstrated that, even in the case of real sample analyses, the proposed GC-ECD method returns LODs and LOQs considerably lower than legal limits.

### *3.3.3.3 Precision and recovery*

Method precision and recovery were determined by performing a series of analysis of spiked egg samples. In Tab. 3.2 the results of repeatability and recovery values evaluated for each NDL-PCB are summarized. As can be noted, the obtained intra-day RSDr values are well below the reference values of 15%, derived by Horwitz equation [30] for a mass fraction $\leq$ 0.1 mg $kg^{-1}$, under repeatability conditions.

Recovery percentages were calculated by comparing the concentration of spiked samples, determined by the external calibration regression line, with the nominal fortification level. For the sum of NDL-PCBs, it was verified that the calculated mean recovery at each spiking level was in compliance with the recovery range of 60-140%, reported in the official documents [3] dealing with the method validation and quality control procedures for Pesticide Residues Analysis in Food and Feed. Recoveries ranging from 85% to 99% were obtained, demonstrating a good accuracy of the method.

*Tab. 3.2 Repeatability and recovery data for the determination of NDL-PCBs in spiked egg samples. [a] $RSD_r$: Relative Standard Deviation under repeatability conditions (n=6). [b] Evaluated for the sum of PCBs. Mean values ± standard deviations (n=6).*

| Fortification Level | $RSD_r$(%)[a] | | | | | | Total Recovery[b] |
|---|---|---|---|---|---|---|---|
| | PCB#28 | PCB#52 | PCB#101 | PCB#153 | PCB#138 | PCB#180 | |
| **20 ng $g^{-1}$ fat** | 6.3 | 4.4 | 9.0 | 6.9 | 8.6 | 7.0 | 85±6 |
| **40 ng $g^{-1}$ fat** | 8.5 | 8.9 | 14 | 9.9 | 11 | 3.7 | 98±9 |
| **80 ng $g^{-1}$ fat** | 5.2 | 9.8 | 7.8 | 5.3 | 4.5 | 4.8 | 99±4 |

### *3.3.3.4 Method Ruggedness*

The ruggedness of the method for the extraction of NDL-PCBs from chicken eggs was confirmed by using the Youden experimental design, as described in Decision 2002/657/EC. The seven factors chosen as variables for the Youden test (identified by preliminary experiments as factors that could influence the results if their nominal values slightly change) were: extraction volume, sonication time, centrifugation time, centrifugation speed, extraction solvent mixtures (hexane/acetone ratio), volume of sulphuric acid and final extract evaporation temperature. As shown in Tab. 3.3, for the selected seven factors, alternative upper and lower values than the nominal mean value are denoted with the upper case letters A, B, C, D, E, F, G, and the corresponding lower case letters a, b, c, d, e, f, and g, respectively. The Youden test results in 128 (i.e. 27) different combinations, but it is possible to choose a subset of eight of these combinations that have a balance between capital and small letters. Therefore, only eight determinations are sufficient to study the influence of the seven Youden factors. The experiments were performed on spiked eggs and the results were determined by the sum of the observed NDL-PCB amounts as ng $g^{-1}$ fat. Then, the standard deviation of the differences ($SD_i$) between the averages of the results associated with the capital letter experiments and the averages of their corresponding small letter experiments were calculated. An $SD_i$ value lower than the standard deviation of the method determined under within-laboratory reproducibility conditions ($SD_r$) at the same fortification level demonstrated that none of the factors has an effect on the result, therefore the method can be considered robust against the chosen modifications in the extraction procedure.

*Tab. 3.3 Youden experiment design for ruggedness studies (Dec 657/2002/EC)*

| FACTOR | NOMINAL VALUE | DESCRIPTION | | COMBINATION OF DETERMINATIONS NUMBER | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| **Extraction Volume** | 40 mL | A/a | 41/39 mL | A | A | A | A | a | a | a | a |
| **Sonication Time** | 30 min | B/b | 40/20 min | B | B | b | b | B | B | b | b |
| **Centrifugation Time** | 20 min | C/c | 25/15 min | C | c | C | c | C | c | C | c |
| **Centrifugation Speed** | 3000 rpm | D/d | 3500/2800 rpm | D | D | d | d | d | d | D | D |
| **Hexane/Acetone ratio** | 85:15 | E/e | 83:17/87:13 | E | e | E | e | e | E | e | E |
| **Volume of Sulphuric Acid** | 4 mL | F/f | 4.5/3.5 mL | F | f | f | F | F | f | f | F |
| **Evaporation Temperature** | 45°C | G/g | 50/40°C | G | g | g | G | g | G | G | g |
| **OBSERVED RESULTS: SUM of PCBs (ng $g^{-1}$ fat)** | | | | **32.5** | **33.5** | **30.8** | **33.6** | **36.8** | **31.8** | **34.7** | **31.2** |
| **Recovery %** | | | | **83.3** | **83.8** | **77.0** | **84.1** | **91.9** | **79.4** | **86.8** | **78.0** |

### *3.3.3.5 Measurement uncertainty*

The evaluation of uncertainty of analytical results was performed by the metrological approach, using the validation data obtained from each step of the total analytical procedure [31]. Four sources of uncertainty were considered: preparation of the standard, method reproducibility, method recovery, and instrumental calibration curve. The determination of the measurement uncertainty was performed by the uncertainties propagation law. For each NDL-PCB, the concentration relative uncertainty has been calculated taking into account the analyte concentration in the analyzed spiked samples, the volume of the final extract, and the sample weight before extraction and clean-up. A relative expanded measurement uncertainty was calculated using a coverage factor k of 2, corresponding approximately to a 95% confidence level. Values in the range 5.3-15.0 % were determined, as reported in Tab. 3.4.

*Tab. 3.4 Measurement uncertainty values at 40 ng $g^{-1}$ fat in chicken eggs.*

| Analyte | Measurement uncertainty (%) |
|---|---|
| PCB#28 | 14 |
| PCB#52 | 11 |
| PCB#101 | 15 |
| PCB#153 | 9.9 |
| PCB#138 | 8.0 |
| PCB#180 | 5.3 |

## 3.4 Analysis of food samples

The validation process demonstrated that the proposed analytical method could be considered a useful tool for the screening evaluation of NDL-PCBs in eggs; then, it was successfully applied to the analysis of 102 market samples. Each batch of egg samples was processed together with a matrix blank to reduce the risk of false positives as a result of contamination in the extraction process or chemicals used. Therefore, for every batch of 10 samples, a reagent blank (prepared according the co tomplete analytical procedure using an equivalent amount of suitable solvent in place of the test

portion) and a sample blank (i.e. a compliant control sample, prepared from a test portion taken from a sample from which the analyte is absent) were included in the sample list to be analysed. Although for a single sample the time required for the total analysis is quite long, a high sampling rate process was obtained considering that multiple extracts can be prepared in parallel and progressively inserted in the sample sequence of the autosampler system. Therefore, a high-throughput analysis is ensured, with a total analysis time of one hour, calculated by dividing the number of hours of a day working session by the number of the analysed samples. Peak identity was confirmed by standard additions. As reported in the EFSA report [3], the most predominant NDL-PCB congeners were #153, #138, and #180 whose concentration in the analysed eggs ranged from 1.7 to 11.4 ng $g^{-1}$ fat. Moreover, 2 samples contaminated by the presence of NDL-PCBs well above the MRL of 40 ng $g^{-1}$ fat were found: one sample with a level of PCB #153 and #138 of 26.8 ng $g^{-1}$ fat and 21.3 ng $g^{-1}$, and a second one contaminated by PCB#153 and #180 at 35.2 ng $g^{-1}$ fat and 32.0 ng $g^{-1}$, respectively. NDL-PCB residues (LOD < NDL-PCBs < LOQ) were detected in 43 samples, while 6 egg samples showed NDL-PCB amounts above the quantification limits.

## 3.5 Conclusions

A rapid, sensitive and reproducible screening method for the analysis of 6 NDL-PCBs (congener 28, 52, 101, 138, 153, 180) in egg samples by GC-ECD has been developed, using a clean-up procedure based on solid phase extraction that provides a simplified approach for the removal of interferences from raw samples. A fat extraction process by accelerated solvent extraction was also optimized for the determination of the fat content. The simple and fast sample preparation procedure allowed to perform the simultaneous extraction and clean up of more than 12 samples in a single day working session. The instrumental GC-ECD analysis of the target compounds was carried out in a chromatographic run of less than 25 min. Finally, the method was validated through the evaluation of linearity, detection and quantification limits, selectivity, recovery, precision, ruggedness and measurement uncertainty, demonstrating its conformity with provisions of the European directives for the accurate screening of NDL-PCBs in complex food matrices.

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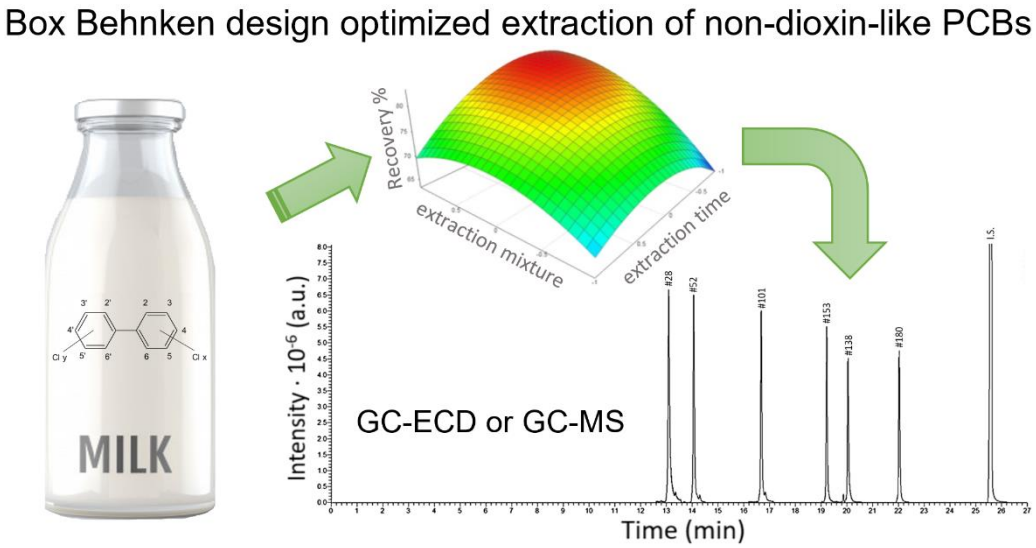
# Chapter 4 - Box Behnken design-based optimized extraction of non-dioxin-like PCBs for GC-ECD and GC-MS analyses in milk samples



## 4.1 Introduction

In recent years, multivariate optimization has been frequently applied to the development of analytical methods, considering its advantages in the reduction of the number of required experiments and consequently resulting in lower reagent consumption and laboratory work [1]. Furthermore, the multivariate approaches allow the assessment of the statistical significance of the factors under investigation as well as the evaluation of the interaction effects between the factors on the response. Then, a large amount of information can be obtained from a minimum number of experimental runs. On the contrary, in the univariate strategies, the effect of each variable is singularly studied, independently of the level of the other factors involved in the optimization process. Therefore, if there are significant interaction effects between factors, the optimal conditions determined by the univariate studies could be very different from the (correct) results found by the multivariate optimization, in which the levels of all the variables are changed simultaneously. Among the different chemometric tools currently applied to analytical chemistry, the Box Behnken design (BBD) is often used to optimize the most influential parameters involved in the method development. Several applications include the optimization of the extraction process in pesticide residue analysis from food samples [2–4] and the determination of environmental contaminants [5].

Box-Behnken experimental design was described for the first time in 1960 [6]. Considering that the efficiency of experimental design can be defined as the number of parameters divided by the number of experiments BBD, together with the Doehlert matrix, is very efficient if compared to the three-level full factorial designs and slightly more efficient than the central composite design. Furthermore, BBD does not contain combinations for which all factors are simultaneously at their highest or lowest levels, avoiding experiments performed under extreme conditions, for which unsatisfactory results might occur. On the other hand, it is not indicated for situations in which it is necessary to know responses in these situations. Multivariate techniques are routinely applied to optimize the different working conditions in various extraction processes to improve their performance. Several reviews and research papers have been published on this subject [2], nevertheless, to date, for the determination of polychlorobiphenyls (PCBs) the advantages of the BBD approach have not been fully exploited. Indeed, among the exponentially increasing number of literature works, only a limited number of applications have been reported on the development of optimized analytical methods by Box Behnken design for the analysis of PCBs in food, biological and environmental samples [7–10].

PCBs are a group of anthropogenic chemicals, in the past widely used as dielectric and coolant fluids in electrical apparatus, carbonless copy paper and in heat transfer fluids. Commercial production of PCBs ended in 1977 because of health effects associated

with exposure, but as a consequence of their bioaccumulative character and resistance to metabolic degradation [11,12], PCBs are still extensively widespread along the food chain [13]. In the European Community, Maximum Residue Levels (MRLs) have been established for PCBs in food products (European Commission Regulation 1259/2011/EC). In raw milk and dairy products, an MRL of 40 ng $g^{-1}$ fat has been set for the sum of the six non-dioxin-like polychlorinated biphenyls (NDL-PCBs, congeners # 28, 52, 101, 138, 153, and 180), typically used as indicators to monitor the contamination levels in foodstuffs, due to their higher abundance respect to other congeners [14]. For the determination of PCBs in food samples, screening analytical methods based on gas chromatography (GC) and electron capture detection (ECD) have been proposed [15–18] for high throughput applications in monitoring and risk-assessment studies, whereas for confirmatory analyses, GC coupled with mass spectrometry (MS) is currently used [19–23], as recommended by the European Directives (European Commission Decision 657/2002/EC). The high number of papers dealing with the determination of PCBs confirms that this topic still deserves great attention by the scientific community: furthermore, NDL-PCBs extraction from food samples with high fat content, such as milk, is still a challenging step. Very often, long and tedious extraction procedures are required and the risk of low recoveries and/or chromatograms full of interfering peaks is still high. In particular, for the NDL-PCBs, recognized as food contamination indicators, the development of a suitable analytical method specific for their determination, that can assure high recoveries and performances with an efficient, rapid and easy-to-use extraction procedure is a fundamental issue.

In this work, an analytical method is specifically proposed for the selective determination of six NDL-PCBs (# 28, 52, 101, 153, 138 and 180) in milk samples. A multivariate process by a three-level Box Behnken experimental design (consisting of 26 experiments, carried out in duplicate and with a randomized order) is proposed for the optimization of the sample extraction procedure, followed by detection and quantification by GC-ECD and mass spectrometry. Finally, the method was validated in terms of linearity, selectivity, detection and quantification limits, recovery, precision and ruggedness, following the European directives.

# 4.2Materials and Methods

## 4.2.1Chemicals

High purity (≥ 97%) NDL-PCB mix standards (IUPAC congeners 28, 52, 101, 153, 138, 180) at a concentration of 10 mg $L^{-1}$ in isooctane for each NDL-PCB were provided by Dr Ehrenstorfer (Augsburg, Germany). Working standard solutions were prepared just before injection by dilution in isooctane. NDL-PCB #209 (Dr Ehrenstorfer, > 99.0%, 10

mg $L^{-1}$ in isooctane) was used as internal standard (IS) and added to NDL-PCB standard calibration solutions to a final concentration of 100 µg $L^{-1}$. Solid-phase extraction Bond Elut-PCB cartridges (1 mg, 3 mL) were supplied by Agilent Technologies (Inc. Folsom, CA, 95630). Glassware was treated with a sulphochromic mixture (Carlo Erba Reagenti, Milano, Italy) for organic and inorganic residues removal, followed by a washing step with different solvents (water, acetone, and n-hexane of HPLC grade) to eliminate cross-contamination.

### 4.2.2 Sample collection

Milk samples of different brands were bought in local markets. Other (pasteurized) milk samples were collected from local farms regularly inspected by veterinary services. Samples with different fat content were analyzed to test matrix interferences. For each sample, the fat content determination was performed as described in the following paragraphs.

### 4.2.3 Sample preparation

For the extraction-cleanup process, 100 mL of a hexane/acetone mixture (70/30, v:v) were added to a whole milk aliquot of 11 g. The extraction was carried out for 45 minutes by a magnetic stirrer; after sonication for 30 min, the clear supernatant was evaporated to 4 mL under a nitrogen stream at 45ºC in a Turbovap system (Caliper Mod. LV, Hopkinton, MA, USA). Then, 4 mL of sulphuric acid (98%) was added and the mixture was kept overnight at room temperature. After centrifugation at 4ºC for 20 min at 3500 rpm, the upper clear phase was transferred into a tube and evaporated to dryness under a nitrogen stream at 45ºC. Then, the residue was dissolved in 2 mL of n-hexane and loaded into a Bond Elut-PCB cartridge (1 mg, 3 mL), previously conditioned with 3 mL of n-hexane. The elution was obtained using 10 mL of n-hexane. Finally, after evaporation to dryness under a stream of nitrogen at 45 °C, the residue was dissolved in 0.5 mL of isooctane containing 100 µg $L^{-1}$ of NDL-PCB #209, used as an internal standard, before GC-ECD analysis.

### 4.2.4 Fat content determination and extraction

The Thermo Scientific™ Dionex™ ASE™ 350 Accelerated Solvent Extraction (ASE) system (Thermo Fisher Scientific, Waltham, MA USA) was used for the fat content determination and extraction. The ASE process was performed at an oven temperature of 120°C and pressure of 1500 psi; Other ASE conditions: three 10-min cycles, 6 min and 3 min of heat and static time, respectively; flush volume 100%; 60 s purge time; carrier gas nitrogen; stainless steel extraction cells 10 mL; collection vials 60 mL. An amount of 1 g of milk was mixed with Extrelut® (Merck, Darmstadt, Germany) in a ratio of 1:2, and air-dried in the oven at 100°C for 15 min. An extraction mixture of petroleum

ether/isopropanol (2:1) was used (30 mL $g^{-1}$ of the sample); finally, the extracts were evaporated to dryness at 40 °C by a rotavapor system.

### 4.2.5 Gas Chromatography/Electron Capture Detection

Chromatographic separations were performed by an AutoSystem XL GC (Perkin Elmer, Waltham, MA, USA) coupled with an Electron Capture Detector (ECD). The glass liner Siltek deactivated (Perkin Elmer) was used in splitless mode at 250°C. The chromatographic separations were carried out using an analytical column TG-5SILMS (VF-5 ms, 30 x 0.25 mm inner diameter, 5% diphenyl-95% dimethylsiloxane liquid phase, film thickness: 0.25 µm; Thermo Fisher Scientific, Waltham, MA USA) coupled to the corresponding 5 m safeguard (Thermo Fisher Scientific). A volume of 1.0 µL of the final extract was injected into the chromatographic system. The flow rate of the carrier gas (Helium, 99.999%, pressure-pulse mode: 30 psi for 1 min) was 1.0 mL $min^{-1}$. The ECD temperature was 375°C. The oven temperature was initially set at 130°C, then increased to 190°C in 3 min at a rate of 20°C $min^{-1}$ and to 280°C in 9 min at 10°C $min^{-1}$. The final temperature of 280°C was kept for 15 min, with a total run time of 27.0 min. Acquisition and data processing were performed by the TotalChrom Workstation (Perkin Elmer).

### 4.2.6 Box–Behnken experimental design

The Box Behnken design was used for the evaluation of the effects of the extraction variables on the recovery of NDL-PCBs from milk samples, before GC-ECD determination. Three most influential factors including the acetone percentage in the extraction mixture ($X_1$), the sample/solvent ratio ($X_2$) and the extraction time ($X_3$) were selected as independent variables for optimization at three levels: $X_1$: 5% (-1), 17.5% (0), 30% (+1); $X_2$: 0.075 g $mL^{-1}$ (-1), 0.100 g $mL^{-1}$ (0), 0.125 g $mL^{-1}$ (+1); $X_3$: 45 min (-1), 90 min (0), 135 min (+1). The responses were: the recovery percentage ($Y_1$) and its standard deviation ($Y_2$). A total of 26 experiments were performed in random order with two replicates at the centre point to estimate the pure error. A multi-response optimization was accomplished using a desirability function provided by XLSTAT (Statistical Software for Excel, option Quality). Experimental data were fitted to the quadratic model using a second-order polynomial model. Coefficients (linear, quadratic, and interaction) were determined by the least square regression. Analysis of variance (ANOVA) was used to determine the significance and interactions of the factors ($p < 0.05$). Desirability analysis was employed to assess if a combination of variables satisfies the goal that was defined for the response, using a scale ranging from 0.0 (undesirable) to 1.0 (highly desirable).

### 4.2.7 Confirmatory analyses by GC-MS

GC-MS analyses were performed on a Thermo Scientific TSQ EVO 8000 GC system equipped with a triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA USA). Gas chromatographic analysis was carried out in the monitoring reaction mode. The presence of at least two significant MS/MS transitions was used to identify the analytes. For each PCB, the m/z values for the ms/ms transitions have been fixed based on what was reported in the official European documents (SANTE 2017/11813/EC and Dec 2002/657/EC). The ion selection was performed choosing characteristic isotopic ions, especially Cl and Br clusters, not exclusively originating from the same part of the analyte molecule. The selected diagnostic ions were: 186.1, 256.0 and 258.0 for PCB#28; 220.0, 255.0 and 290.0 for PCB#52; 323.9 and 326.0 for PCB#101; 360.0, 362.0 and 290.0 for PCB#153; 360.0, 362.0 and 290.0 for PCB#138; 393.9 and 395.9 for PCB#180.

The chromatographic separations were performed using the capillary column Rxl (30 m x 0.25 mm x 0.25 µm) from RESTEK Pure Chromatography (Bellefonte, PA, USA). A sample volume of 1.5 µL was injected by Programmed Temperature Vaporizing (PTV) in splitless mode. The injector temperature started at 100°C and ramped to 260 °C in 1 min; finally, a cleaning time of 5 min at 320°C was applied. The oven temperature was initially set at 70°C for 1.0 min and then increased to 150 °C at a rate of 30°C $min^{-1}$ and 260°C at 6°C $min^{-1}$; a final temperature of 290°C was kept for 5.5 min with a total run time of 28.5 min. Acquisition and data processing were performed by the Trace Finder and Xcalibur workstations (Thermo Fisher Scientific).

## 4.3 Results and Discussion

### 4.3.1 PCB extraction and sample clean-up

The sample extraction process is an essential and critical step in the pesticide analysis, representing the base for the determination of residues at the trace level. The major drawbacks are the complexity of food matrices and the low analyte levels to be quantified, therefore the process could be tedious, time-consuming, and labour-intensive. Recently, innovative sample extraction processes based on hollow-fiber liquid phase microextraction [24], QuEChERS [25] or functionalized sol–gel aluminium strip microextraction [26] have been proposed. The extraction efficiency is dependent on several physical and chemical parameters that, whatever is the adopted extraction method, should be further refined to obtain efficient recoveries and reproducible results. The use of the experimental design in the optimization process of the extraction procedure proves to be effective with a minimum of experiments, time, and costs [2].

Preliminary experiments on spiked milk samples at a fortification level of 40 ng $g^{-1}$ fat were carried out to evaluate existing extraction procedures for the determination of pesticides and other persistent organic pollutants in milk, based on QuEChERS [21] and SPE [27]: unfortunately, NDL-PCBs analyses were compromised by the presence of matrix interfering peaks in the relevant retention time-window. In our previous work, high sample throughput and low solvent consumption extraction/clean-up method has been proposed and successfully applied for the detection of NDL-PCBs analysis in eggs [28]. Starting from these results, a new procedure has been set up and optimized by BBD for the analysis of NDL-PCBs in milk samples by GC-ECD.

#### *4.3.1.1 Sample extraction optimization by Box-Behnken design*

On the basis of preliminary results (both performed on egg samples, as reported in our previous work, [28] and further exploratory investigations on the milk matrix), three significant factors (namely, the acetone percentage in the extraction mixture based on acetone/hexane, the sample/solvent ratio, and the extraction time) were considered as the most influencing input variables. Then, their effect on the recovery and the reproducibility of the extraction process was studied by BBD at three levels. The experimental domain for each experimental variable was chosen, according to preliminary results, as follows: (i) acetone percentage in the mixture acetone/hexane between 5 and 30% (v:v); (ii) sample/solvent ratio from 0.075 to 0.125 g $mL^{-1}$; (iii) extraction time from 45 to 135 min. The three-factor BBD consisted of 26 experiments (13 different experimental sets: 12 at factorial points and 1 at the centre) that were performed in duplicate and in a randomized manner to minimize the bias effects of uncontrolled variables. Each of the 26 extracts was injected two times. All the analyses were performed on milk samples fortified with PCBs at a spiking level (as the sum of the 6 NDL-PCBs) of 40 ng $g^{-1}$ fat, corresponding to 1.5 ng $g^{-1}$ fresh sample. The results were evaluated using the extraction recovery percentage (calculated from the ratio between the concentration measured in spiked samples and the nominal fortification level) and the standard deviation of the replicate analyses (associated with each of the 26 BBD experiments), as shown in the bar-chart of Fig. 4.1.

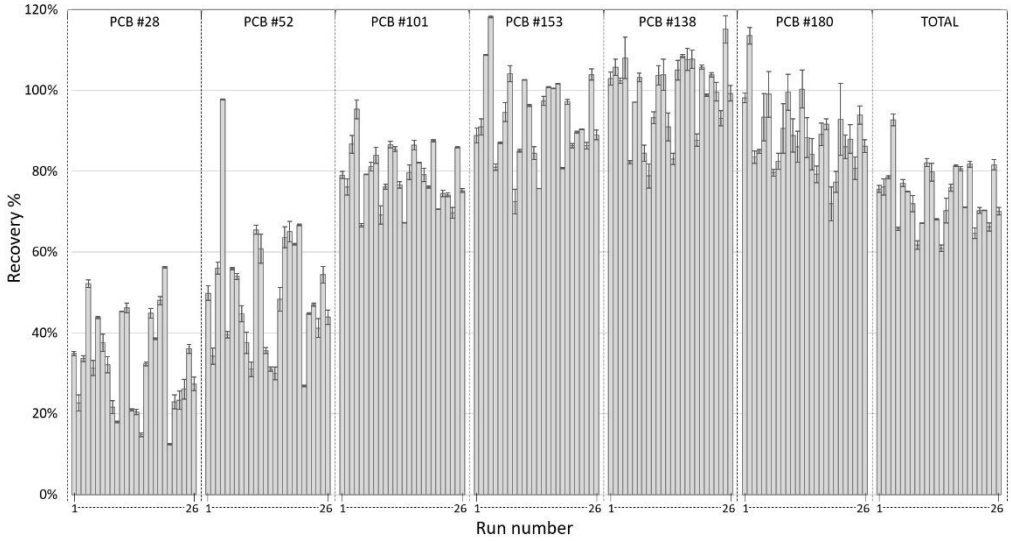



*Fig. 4.1 Recovery values obtained for the sum of NDL-PCBs by BBD. The error bars represent the standard deviations associated with the replicate analyses of the 26 BBD experiments.*

The influence of each variable and the possible effects on the responses (recovery % and relative standard deviation) were studied through response surface methodology. Hypersurfaces were constructed from each response ($Y_i$) as a function of the variable factors ($X_i$) using a quadratic polynomial model as shown below [29]:

$$Y= \beta_0+ \sum_{i=1}^{3} \beta_i X_i+ \sum_{i=1}^{3} \beta_{ii} X_i^2+ \sum_{i=1}^{2} \sum_{J=i+1}^{3} \beta_{ij} X_i X_j \qquad \text{(Eq.1)}$$

where Y is the response variable; $\beta_0$, $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are the regression coefficients for intercept, linearity, square, and interaction, respectively; and $X_i$ and $X_j$ represent the independent variables, coded according to the equation [30]:

$$X_i = \frac{(X_1 - X_0)}{\Delta X_i} \qquad \text{(Eq.2)}$$

where $x_i$ is the coded value of the variable $X_i$; $X_0$ is the real value of $X_i$ at the center point; $\Delta X_i$ is the change in the real value of the variable corresponding to a variation of a unit for the dimensionless value of the variable.

Response surface analysis was carried out using three-dimensional response surface plots, which graphically explained the presence of interactions among the independent variables and their influence on the response variables. In Fig. 4.2, the response surfaces were drawn as 3D plots of two factors while the other factor was kept constant at the central point. In Fig.2A the regions in red correspond to maximum values for the total recovery, where the percentages were close to 85-90% while the regions in blue correspond to minimum values when level factors are not suitable to be chosen. On the contrary, for the RSD% response surface, the ideal conditions associated with minimum values were the blue/green zones (Fig. 2B). ANOVA was used to determine the significance and interactions of the independent variables; the three factors (both the linear and the quadratic terms) were found to have a statistically significant impact on the recovery percentage (p=0.05). In particular, the acetone content was the more influencing effect on the extraction efficiency, reaching maximum recovery and low RSD values at high acetone percentages, while extraction time showed the lower impact factor.

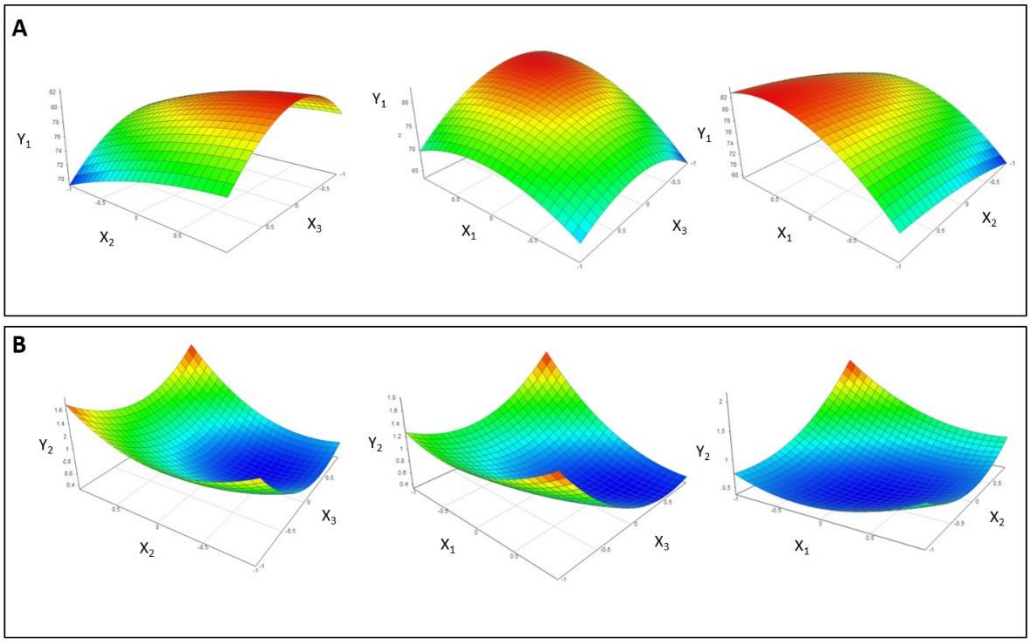



*Fig. 4.2 Response surface plots of the total recovery (response Y1, panel A) and relative standard deviation for the sum of the 6 NDL-PCBs (response Y2, panel B), showing the effect of % acetone in the solvent extraction ($X_1$), sample/solvent ratio ($X_2$) and extraction time ($X_3$) Each response surface refers to couples of factors while the other factor was kept constant at the central point.*

### *4.3.1.2 Partial and global desirability functions*

To obtain a global response that could find the best solution in terms of extraction efficiency and analysis precision, the optimum conditions were determined by Derringer's desirability function [31]. Two desirability functions (total recovery calculated for the sum of each PCB and relative standard deviation) were built assigning a specific desirability value in the 0–1 range, with the following targets: maximization for the total recovery (response $Y_1$) and minimization for RSD% (response $Y_2$). The partial desirability function $d_1$, for the response $Y_1$, was calculated according to Eq. 3, taking into consideration that an expected recovery range of 60-140% is officially reported in the European Commission document SANTE 2017/11813/EC for pesticide residues analysis.

$$d_1 = \begin{cases} 0 & \text{if } Y_i < 60\% \\ \left(\frac{Y_i - 60}{85 - 60}\right) & \text{if } 60\% \leq Y_i < 85\% \\ 1 & \text{if } 85\% \leq Y_i \leq 95\% \\ \left(\frac{120 - Y_i}{120 - 95}\right) & \text{if } 95\% < Y_i \leq 140\% \\ 0 & \text{if } Y_i > 140\% \end{cases} \qquad \text{(Eq. 3)}$$

For the response $Y_2$ the partial desirability function $d_2$ was calculated according to Eq.4, to obtain RSD values lower than 5%:

$$d_2 = \begin{cases} 0 & \text{if } Y_i > 5\% \\ \left(\frac{5 - Y_i}{5 - 1}\right) & \text{if } 1\% < Y_i \leq 5\% \\ 1 & \text{if } Y_i < 1\% \end{cases} \qquad \text{(Eq. 4)}$$

Then, the overall desirability function was obtained by the geometric mean of specific desirability assigned to each factor, to simultaneously optimize the recovery percentage and improve repeatability data. A key aspect of a screening method is the overall analysis time, which should assure high throughput applications in monitoring and risk-assessment studies. Taking into account this aspect and considering the relatively small dependence of the response surfaces from extraction times, the model parameters of the global desirability function together with the 3D-plot have been obtained by fixing the factor $x_3$ at -1 (45 min). At this extraction time, the ideal extraction conditions are associated with $x_1=x_2=1$ (i.e., acetone percentage of 30% and the sample-to-solvent ratio of 0.125 g $mL^{-1}$). Nevertheless, a deeper investigation was necessary to verify if these conditions were also acceptable for the individual recovery of PCB #28 and #52, which are critical congeners during the extraction process. Indeed, PCB #28 and PCB#52 were characterized by very low recoveries from milk samples (see Table 4.1), with a high risk of a complete loss of these compounds. The desirability functions were then determined for PCB #28 and #52. A negative impact on the recovery of PCB #28 was observed for $x_2=1$, being also sub-optimal for PCB #52. To find the optimal conditions taking into account this critical issue on PCB #28 and #52, a new desirability function was obtained as a single composite function, by considering the individual recovery percentage for PCB #28 and PCB #52 and the total recovery for the sum of all PCBs. The 3D-plot of the desirability function (at $x_3=-1$) showed maximum values around $x_1=1$ and $x_2= 0.44$. As a result, the ideal conditions were: acetone percentage of 30%, the sample-to-solvent ratio of 0.11 g $mL^{-1}$ and extraction time of 45 min. With these optimized extraction conditions, a high throughput can be obtained if multiple extracts are prepared in parallel and progressively injected into the chromatographic system With this procedure is then possible to perform a total of 9-10 runs for a working day session, considering a total analysis time of ca. 50 min.

*Tab. 4.1 Box–Behnken design with recovery percentage and standard deviation for each NDL-PCB at a concentration level equal to 0.25 ng $g^{-1}$ fresh sample. The standard deviations are associated with the replicate analyses of the 26 BBD experiments. *$X_1$: % acetone in the solvent extraction; $X_2$: sample/solvent ratio; $X_3$ extraction time*

| Run | Independent Variables* | | | Measured Responses: % Recovery ($Y_1$) ± SD ($Y_2$) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $X_1$ (%) | $X_2$ (g $mL^{-1}$) | $X_3$ (min) | #28 | #52 | #101 | #153 | #138 | #180 | Total |
| 1 | 5 | 0.075 | 90 | 34.94 ± 0.54 | 49.80 ± 1.81 | 79.04 ± 0.85 | 88.84 ± 1.87 | 102.94 ± | 98.14 ± 1.16 | **75.61 ±0.91** |
| 2 | 5 | 0.075 | 90 | 22.64 ± 2.00 | 34.20 ± 2.00 | 76.04 ± 2.00 | 90.92 ± 2.00 | 105.76 ± | 113.48 ± | **76.03 ± 2.00** |
| 3 | 30 | 0.075 | 90 | 33.66 ± 0.71 | 55.98 ± 1.44 | 86.64 ± 2.15 | 108.80 ± | 102.36 ± | 83.48 ± 1.53 | **78.49 ± 0.36** |
| 4 | 30 | 0.075 | 90 | 52.20 ± 0.91 | 97.72 ± 0.17 | 95.30 ± 2.29 | 118.16 ± | 108.00 ± | 84.92 ± 0.51 | **92.72 ± 1.48** |
| 5 | 5 | 0.125 | 90 | 31.26 ± 1.84 | 39.50 ± 0.82 | 66.60 ± 0.40 | 80.98 ± 0.76 | 82.24 ± 0.40 | 93.36 ± 5.83 | **65.65 ± 0.40** |
| 6 | 5 | 0.125 | 90 | 43.76 ± 0.28 | 55.90 ± 0.31 | 79.26 ± 0.03 | 87.04 ± 0.17 | 97.08 ± 0.01 | 99.00 ± 5.66 | **77.03 ± 0.88** |
| 7 | 30 | 0.125 | 90 | 37.56 ± 2.09 | 53.98 ± 0.71 | 81.16 ± 1.07 | 94.60 ± 2.38 | 103.18 ± | 79.64 ± 0.85 | **75.03 ± 0.03** |
| 8 | 30 | 0.125 | 90 | 32.04 ± 2.00 | 44.72 ± 2.00 | 83.88 ± 2.00 | 104.08 ± | 84.40 ± 2.00 | 82.48 ± 2.00 | **71.93 ± 2.00** |
| 9 | 5 | 0.100 | 45 | 21.62 ± 1.61 | 37.56 ± 2.66 | 69.20 ± 2.26 | 72.50 ± 3.03 | 78.84 ± 3.00 | 90.58 ± 6.08 | **61.72 ± 1.09** |
| 10 | 5 | 0.100 | 45 | 17.92 ± 0.17 | 31.00 ± 1.81 | 76.14 ± 0.59 | 85.04 ± 0.28 | 93.24 ± 1.47 | 99.58 ± 4.44 | **67.16 ± 0.07** |
| 11 | 30 | 0.100 | 45 | 45.34 ± 0.03 | 65.50 ± 1.16 | 86.54 ± 0.82 | 102.62 ± | 103.72 ± | 88.88 ± 4.13 | **82.10 ± 1.03** |
| 12 | 30 | 0.100 | 45 | 46.16 ± 1.19 | 60.74 ± 3.59 | 85.42 ± 0.59 | 96.28 ± 0.28 | 103.86 ± | 86.06 ± 3.82 | **79.75 ± 2.21** |
| 13 | 5 | 0.100 | 135 | 20.96 ± 0.28 | 35.58 ± 0.71 | 76.56 ± 0.79 | 84.44 ± 1.64 | 90.98 ± 3.42 | 100.30 ± | **68.13 ± 0.11** |
| 14 | 5 | 0.100 | 135 | 20.40 ± 0.68 | 31.00 ± 0.51 | 67.22 ± 0.03 | 75.64 ± 0.01 | 83.02 ± 1.39 | 88.26 ± 4.89 | **60.92 ± 0.78** |
| 15 | 30 | 0.100 | 135 | 14.76 ± 0.51 | 30.02 ± 1.61 | 79.72 ± 1.75 | 97.36 ± 1.13 | 105.00 ± | 84.14 ± 3.87 | **70.23 ± 3.05** |
| 16 | 30 | 0.100 | 135 | 32.33 ± 0.47 | 48.30 ± 2.91 | 86.48 ± 1.19 | 100.80 ± | 108.50 ± | 79.20 ± 2.09 | **75.93 ± 0.87** |
| 17 | 17.5 | 0.075 | 45 | 44.80 ± 1.19 | 63.60 ± 2.60 | 82.14 ± 0.08 | 100.50 ± | 107.62 ± | 89.08 ± 2.77 | **81.29 ± 0.20** |
| 18 | 17.5 | 0.075 | 45 | 38.56 ± 0.23 | 65.02 ± 2.52 | 79.08 ± 1.70 | 101.72 ± | 107.66 ± | 91.60 ± 1.30 | **80.61 ± 0.50** |
| 19 | 17.5 | 0.125 | 45 | 47.98 ± 0.99 | 61.96 ± 0.17 | 76.12 ± 0.28 | 80.74 ± 0.08 | 87.64 ± 1.53 | 71.90 ± 4.16 | **71.14 ± 0.06** |
| 20 | 17.5 | 0.125 | 45 | 56.22 ± 0.14 | 66.70 ± 0.25 | 87.48 ± 0.28 | 97.14 ± 0.59 | 105.70 ± | 77.32 ± 2.55 | **81.76 ± 0.73** |
| 21 | 17.5 | 0.075 | 135 | 12.48 ± 0.11 | 26.86 ± 0.20 | 70.54 ± 0.03 | 86.30 ± 0.48 | 98.80 ± 0.28 | 92.84 ± 8.94 | **64.64 ± 1.38** |
| 22 | 17.5 | 0.075 | 135 | 22.98 ± 1.73 | 44.74 ± 0.20 | 74.50 ± 0.76 | 89.64 ± 0.23 | 103.88 ± | 86.00 ± 2.94 | **70.29 ± 0.72** |
| 23 | 17.5 | 0.125 | 135 | 23.30 ± 2.23 | 46.92 ± 0.40 | 74.14 ± 48 | 90.38 ± 0.03 | 99.64 ± 2.32 | 87.92 ± 3.51 | **70.38 ± 0.02** |
| 24 | 17.5 | 0.125 | 135 | 26.08 ± 2.49 | 41.16 ± 2.32 | 69.74 ± 1.33 | 86.30 ± 0.76 | 93.12 ± 1.87 | 80.74 ± 2.80 | **66.20 ± 0.99** |
| 25 | 17.5 | 0.100 | 90 | 36.02 ± 1.10 | 54.36 ± 2.04 | 85.94 ± 0.14 | 103.90 ± | 115.12 ± | 93.90 ± 2.29 | **81.54 ± 1.25** |
| 26 | 17.5 | 0.100 | 90 | 27.38 ± 1.73 | 43.86 ± 1.84 | 75.16 ± 0.45 | 88.98 ± 1.16 | 99.20 ± 1.92 | 86.14 ± 1.61 | **70.11 ± 0.92** |

### 4.3.2 Optimization of the fat content determination process

To express the NDL-PCB contents in the samples as ng $g^{-1}$ of fat, as specified by European Commission Regulation 1259/2011/EC, the ASE procedure was used to automate the fat extraction process from milk samples, starting from the optimized procedure recently published in our previous work for the PCB analysis in chicken eggs [32]. Several factors affecting the efficiency of the ASE process (sample weight, extraction solvent volume and composition, temperature, and the number of extraction cycles) were tested on lyophilized milk samples. The fat recovery was evaluated by comparison with the labelled values.

The oven temperature for the drying process was explored in the range 100–125°C (5°C steps) and the optimal value of 120°C was set for 10 minutes. Then, binary and ternary mixtures of apolar solvents, generally used for the fat extraction from food samples (hexane, petroleum ether, isopropanol, chloroform and methanol) at different combination percentages were compared for the liquid-liquid extraction. The best results in terms of recovery (84–113%) were obtained by using the binary mixture petroleum ether/isopropanol (2:1). Three extraction cycles of 10 min were sufficient to recover the fat amount reported in the label (RSD% lower than 9.4%, n = 10, under reproducibility conditions in different working days, with different operators and reagent lots).

### 4.3.3 Optimization of the chromatographic conditions by GC-ECD

In the development of a multi-residue method, the optimization of the chromatographic conditions is a critical stage, in particular when ECD is used for screening analysis. Indeed, ECD could suffer from sample matrix interferences, due to potential co-eluting compounds and then particular attention has been devoted to the optimization of the experimental chromatographic conditions, aimed at ensuring a high-throughput analysis and good peak resolutions. Details of the optimized temperature gradient program are summarized in the experimental section. A satisfactory separation with symmetrical and narrow peaks was obtained in a time window from 12 to 23 min, with a total run time of 27 min.

### 4.3.4 Method validation

As recommended by the European regulations (European Commission SANTE 2017/11813/EC, European Commission Regulation 2017/644/EC and European Commission Decision 657/2002/EC), the validation of the analytical methods is essential to provide reliable results in risk-assessment studies, as well as in official controls for pesticide determinations. Therefore, the screening GC-ECD analytical

method was extensively validated through the evaluation of selectivity, linearity, detection and quantification limits, precision, recovery, and ruggedness.

### *4.3.4.1 Calibration curves and limits of detection and quantification*

The linearity test was performed by three series of analyses on three different days, by injecting six mixed standard solutions of NDL-PCBs, at concentrations of 1.0, 2.5, 5, 10 and 20 μg $L^{-1}$, corresponding to 0.05, 0.125, 0.25, 0.50 and 1.00 ng $g^{-1}$ in the matrix. Calibration curves were obtained by plotting the ratio between analyte peak area and IS peak area against the NDL-PCB concentration. Analogously, the identification of the target compounds was accomplished by calculating the relative retention time as the ratio between the analyte and IS retention times. For all NDL-PCBs, a good fitting was observed in the range 1-20 μg $L^{-1}$ with correlation coefficients higher than 0.9990. The signal-to-concentration ratio (y/x) was calculated for each experimental point to evaluate the goodness-of-fit of the data to the calibration curve). Then, the $x_i/y_i$ ratios were checked to ensure that their deviation from the mean value of the signal-to-concentration ratio did not exceed ±10%. The absence of systematic instrumental bias was confirmed by the confidence interval for the intercept including the zero value at 95% confidence level. By Mandel's fitting test [33], the residual variances, resulting from the linear and the quadratic calibration function, were compared by an F-test and the hypothesis $H_0$ (no significant difference between the residual variances) was accepted for all the NDL-PCBs. Therefore, calibration straight-lines rather than over curvilinear or non-linear models well fitted the experimental data. The calibration parameters, evaluated for each analyte, are reported in Table 4.2. The instrumental limits of detection (LOD) and quantification (LOQ) were estimated by the chromatograms of the NDL-PCB standard solutions obtained for the lowest calibration level (1.0 μg $L^{-1}$), at a signal-to-noise ratio of 3 and 10, respectively. The noise level was evaluated as peak-to-peak value, i.e., the difference between the maximum positive and the maximum negative amplitudes of baseline in the time window around the analyte retention time. LODs and LOQs were in the range 0.13-0.39 ng $\mu L^{-1}$ and 0.44-1.3 ng $\mu L^{-1}$, respectively (corresponding to 0.18-0.52 ng $g^{-1}$ fat and 0.59-1.7 ng $g^{-1}$ fat in the matrix). These values are considerably lower than the legal limits of 40 ng $g^{-1}$ fat (established for the sum of the six NDL-PCBs), allowing NDL-PCB determination at trace levels and reducing the risk of false-negative results. LODs and LOQs in the matrix, obtained by chromatograms of spiked milk samples at a concentration level of 20 ng $g^{-1}$ fat, ranged from 0.26 to 1.1 ng $g^{-1}$ fat and 0.85 to 3.6 ng $g^{-1}$ fat, respectively. These values prove that, even in the case of real sample analyses, the proposed method returns LOQs considerably lower than legal limits.

*Tab. 4.2 Performance and chromatographic parameters of PCBs analyzed by the proposed GC-ECD method.*

| Analyte | $t_R$[a] (min) | Linear Range (R) (μg $L^{-1}$) | Sensitivity ($10^{-5}$ μV μg $^{-1}$ L) | Instrumental | | | | Method | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | LOD (μg $L^{-1}$) | LOQ (μg $L^{-1}$) | LOD (ng $g^{-1}$ fat) | LOQ (ng $g^{-1}$ fat) | LOD (ng $g^{-1}$ fat) | LOQ (ng $g^{-1}$ fat) |
| | | | | Solvent[b] | | Matrix[c] | | Bovine milk[d] | |
| PCB#28 | 8.46 | 0.98-20 (0.9990) | 417±11 | 0.29 | 0.98 | 0.39 | 1.30 | 0.86 | 2.86 |
| PCB#52 | 9.02 | 1.3-20 (0.9991) | 260±9 | 0.39 | 1.29 | 0.52 | 1.72 | 1.09 | 3.62 |
| PCB#101 | 10.51 | 0.87-20 (0.9991) | 433±12 | 0.26 | 0.87 | 0.35 | 1.16 | 0.97 | 3.23 |
| PCB#153 | 11.99 | 0.48-20 (0.9992) | 775±18 | 0.14 | 0.48 | 0.19 | 0.64 | 0.26 | 0.85 |
| PCB#138 | 12.52 | 0.56-20 (0.9990) | 745±19 | 0.17 | 0.56 | 0.22 | 0.74 | 0.27 | 0.91 |
| PCB#180 | 13.87 | 0.44-20 (0.9997) | 1169±15 | 0.13 | 0.44 | 0.18 | 0.59 | 0.39 | 1.30 |

### *4.3.4.2Precision and recovery*

As reported in European Commission Decision 657/2002/EC and SANTE 2017/11813/EC, in absence of official and certified reference material (CRM), the trueness of measurements was assessed through the analysis of spiked samples, prepared starting from blank material with a known fat amount. After homogenization, proper known amounts of PCBs were added to obtain the desired spiking level, thus the same PCB concentration was obtained in all the aliquoted sample portions. Precision and recovery data have been previously processed by the Shapiro-Wilk test [34] to verify normal distribution. Afterwards, a one-way ANOVA test was performed to verify the homogeneity of the mean concentration values evaluated among the validation sessions at each fortification level. Results from ANOVA were used to calculate intra-laboratory repeatability relative standard deviations ($RSD_r$) following the Decision 2002/657/EC. Recovery percentages were calculated by comparing the concentration of spiked samples, determined by the external calibration regression line, with the nominal fortification level. For the sum of NDL-PCBs, it was verified that the calculated mean recovery at each spiking level complied with the recovery range of 60-140%, reported in the official documents (European Commission SANTE 2017/11813/EC) dealing with the method validation and quality control procedures for Pesticide Residues Analysis in Food and Feed. Total recovery values of 74.9±1.6%, 75.7±4.4 % and 85.4±3.6 (n=6) were obtained at the fortification level of 20, 40 and 80 ng $g^{-1}$ fat, respectively. The intra-day $RSD_r$ values (ranging from 2.1% to 5.8%) were well below the reference values of 15%, derived by Horwitz equation [35] for a mass fraction ≤ 0.1 mg $kg^{-1}$, under repeatability conditions, demonstrating a good method precision.

### *4.3.4.4 Method Ruggedness*

As described in Decision 2002/657/EC, the method ruggedness (defined as the capacity to reproduce results when the method is applied under small changes in the nominal values of the experimental factors established in the optimization step) for the extraction process of NDL-PCBs from milk was confirmed by using the Youden experimental design. The seven factors chosen as variables that could influence the results were*:* extraction volume, vortex agitation time, extraction time under ultrasonication, centrifugation time, centrifugation speed, the volume of sulphuric acid and final extract evaporation temperature. For the selected seven factors, alternative lower and higher levels than the nominal mean value are denoted with the upper case letters A, B, C, D, E, F, G, and the corresponding lower case letters a, b, c, d, e, f, and g, respectively, as shown in Table 4.3. Then, among the 128 (i.e. $2^7$) different combinations resulting from the Youden design, a subset of eight experiments was chosen as a balance between capital and small letters. Therefore, only eight

determinations are enough to study the influence of the seven Youden factors. The ruggedness test was performed on spiked milk samples at 40 ng $g^{-1}$ fat and the results were determined by the sum of the observed NDL-PCB amounts as ng $g^{-1}$ fat. Then, the standard deviation of the differences ($S_{Di}$) between the averages of the results associated with the capital letter experiments and the averages of their corresponding small letter experiments were calculated. The statistical comparison between the obtained $S_{Di}$ value with the method standard deviation, determined under within-laboratory reproducibility conditions ($S_{Dr}$) at the same fortification level, demonstrated that none of the factors affects the result. Therefore, the proposed method can be considered robust against the chosen modifications in the extraction process.

*Tab. 4.3 Youden experiment design for ruggedness studies (Dec 657/2002/EC).*

| FACTOR | NOMINAL VALUE | DESCRIPTION | | COMBINATION OF DETERMINATIONS NUMBER | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| **Extraction Volume** | 100 mL | A/a | 105/95 mL | A | A | A | A | a | a | a | a |
| **Vortex Agitation Time** | 45 min | B/b | 50/40 min | B | B | b | b | B | B | b | b |
| **Sonication Time** | 30 min | C/c | 40/20 min | C | c | C | c | C | c | C | c |
| **Centrifugation Time** | 15 min | D/d | 20/10 min | D | D | d | d | d | d | D | D |
| **Centrifugation Speed** | 3000 rpm | E/e | 3500/2800 rpm | E | e | E | e | e | E | e | E |
| **Volume of Sulphuric Acid** | 4 mL | F/f | 4.5/3.5 mL | F | f | f | F | F | f | f | F |
| **Evaporation Temperature** | 45°C | G/g | 50/40°C | G | g | g | G | g | G | G | g |
| **OBSERVED RESULTS: SUM of PCBs (ng $g^{-1}$ fat)** | | | | **30.4** | **30.7** | **30.9** | **28.0** | **30.7** | **31.5** | **27.5** | **29.6** |
| **Recovery %** | | | | **75.8** | **76.5** | **77.0** | **70.2** | **76.6** | **78.8** | **68.7** | **73.9** |

### 4.3.5 Analyses of milk samples by GC coupled with ECD and Mass Spectrometry

Method feasibility has been demonstrated by the GC-ECD analyses of several milk samples of different animal origins. In each batch of milk samples, a matrix blank was also analyzed to reduce the risk of false-positive results due to the potential chemical contamination in the extraction process. Therefore, a reagent blank was processed according to the complete analytical procedure using an equivalent amount of suitable solvent in place of the test portion, and a sample blank (i.e. a compliant control sample) was prepared from a test portion taken from a sample from which the analyte is absent. For every batch of 10 samples, a reagent blank and a sample blank were included in the sample list to be analyzed. Finally, the optimized extraction and chromatographic conditions were also applied to the NDL-PCB analytical determination by mass spectrometry, confirming the method potential in confirmatory analyses. Then, the same extraction procedure optimized by Box Behnken design for the PCB determination by GC-ECD has been applied also to the confirmatory analysis by GC-MS. Indeed, when in official check analyses doubtful or non-compliant results are obtained by the first, screening evaluations by GC-ECD, a confirmatory analysis has to be carried out by using an independent instrumental line based on GC-MS. As an example, in Fig. 4.3 the GC-MS profile obtained for a spiked bovine milk sample (with a fat content of 3.7%) at a fortification level of 40 ng $g^{-1}$ fat is displayed.

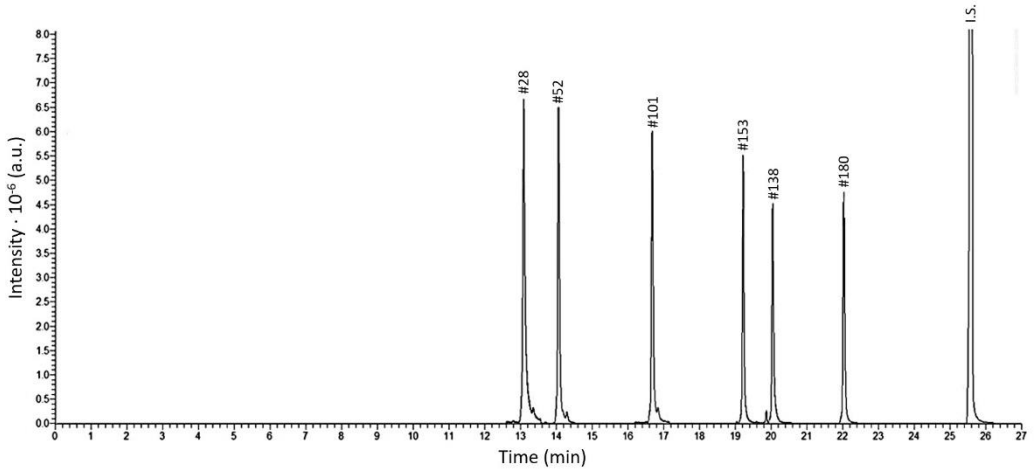



*Fig. 4.3 Total Ion Current chromatogram of a spiked bovine milk sample at 40 ng $g^{-1}$ fat by GC-MS. IS: internal standard (NDL-PCB 209).*

## 4.4 Conclusions

The Box–Behnken experimental design and the global desirability functions were successfully applied for the first time to determine the optimal extraction conditions for NDL-PCBs determination in milk by GC-ECD and confirmation analysis by MS. The effect of three dependent variables (acetone percentage in the extraction mixture, the sample-to-solvent ratio, and the extraction time) was studied at three different levels on the recovery percentage and its standard deviation. The optimized sample extraction/clean-up procedure allowed to perform the simultaneous extraction and clean-up of more than 9 samples in an 8-hour single day working session. Finally, the method was validated through the evaluation of linearity, detection and quantification limits, selectivity, recovery, precision, and ruggedness, demonstrating its conformity with provisions of the European directives for the accurate screening of NDL-PCBs in complex food matrices.

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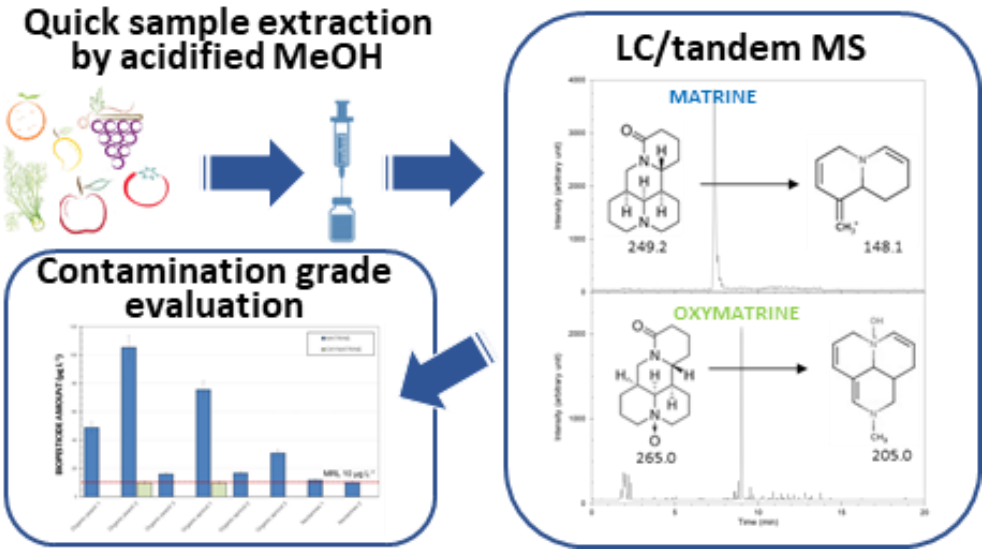
# Chapter 5 - Quick and reliable determination of matrine and oxymatrine in vegetable products by Liquid Chromatography and Mass Spectrometry



# 5.1 Introduction

In recent years, biopesticides have been introduced in modern agriculture to increase the production and enhance the quality of crops, by controlling harmful effects caused by target organisms, as a result of the growing consumer awareness and pressure for safer and healthier products. Biopesticides include naturally occurring substances (biochemical pesticides), microorganisms (microbial pesticides), and pesticide substances produced by plants containing added genetic material (plant-incorporated protectants). Matrine-based biopesticides, belonging to the class of botanical-insecticides [1,2], are extracted from *Sophora flavescens Aiton*, a perennial herbaceous plant widely distributed in China. *Sophora f*. extracts contain tetracyclo-quinolizidine matrine-like alkaloids whose main constituents are oxymatrine and its active metabolite matrine, which are known as bioactive materials against various insect pests, pathogenic fungi, bacteria, and nematodes. For years, the use of these plant extract alkaloids has represented a new trend, in response to the continuous demand for alternatives to synthetic pesticides. Matrine and its analogs exhibit a lot of biological properties [3], including hepatoprotective effects, anticancer [4], anti-inflammatory, analgesic, and antiviral activity [5], and can be also used to treat a variety of itching diseases [6]. However, these compounds may paralyze the respiratory system and cause kidney toxicity in overdose amounts, due to the O=C=N-C-C-C-N sequence, which is toxic to humans and livestock [7,8], and, for this reason, they can be considered emerging contaminants. In the United States and China, the FDA (Food and Drug Administration) and CFDA (China Food and Drug Administration), respectively, have authorized the use of matrine-type alkaloids in the dermatological sector, as well as in the agri-food field and to treat various kinds of cancers. On the contrary, in Europe, due to their neurotoxicity and to the lack of scientific evidence on their safety, matrine and oxymatrine are considered contaminants and they have been included in the list of non-approved substances, for which the legal Maximum Residue Limit (MRL) is set by default at 0.01 mg $kg^{-1}$, according to Regulation (EU) No 396/2005. Nevertheless, plant extract quinolizidine alkaloids are fraudulently used in agriculture, often labeled as fertilizers or as corroborant plant extracts, to avoid the complex legislative process of plant protection products. These agronomic practices have caused a widespread distribution of matrine-like alkaloids in soil, water, and air, therefore the presence of matrine-based pesticides in food products could present a potential health hazard. Recently, the problems and risks associated with biopesticide residues have promoted the development of analytical methods for the determination of plant alkaloid residues. Nevertheless, most of the works deal with the analysis of biological samples [9–11] or are focused on pharmacokinetics studies performed on medicinal preparations [12] or plant extracts, used as drugs or fertilizers [13–19]. Only a limited number of papers have been reported for the determination of matrine–based

biopesticides in food samples by Liquid Chromatography and Mass Spectrometry (LC-MS) [7,20]. Moreover, to the best of our knowledge, in the literature there is no mention of proper analytical methods, specifically proposed and dedicated to the evaluation of the contamination grade by matrine like alkaloids, according to the European guidelines.

In this work, a rapid and reliable analytical method for the determination of matrine and oxymatrine in fruit and vegetable samples by LC and tandem MS, under Multiple Reaction Monitoring mode has been optimized in terms of sample extraction processes, chromatographic separation, MS parameters, and validated according to the European directives through the evaluation of selectivity, linearity, detection and quantification limits, recovery, and precision. Finally, the validated method was successfully applied to the analysis of market samples of peaches, apples, apricots, grapes, and nectarines.

# 5.2 Materials and Methods

## 5.2.1 Chemicals

High purity standards of matrine and oxymatrine (≥ 99%) were obtained from Sigma-Aldrich (Sant Louis, USA). HPLC-grade methanol, acetonitrile, and ultrapure water were supplied by Honeywell (Charlotte, USA); analytical grade formic acid and ammonium formate were provided by Sigma - Aldrich (Sant Louis, USA). For each analyte, individual stock solutions at a concentration of 10 mg $L^{-1}$ were prepared in methanol and stored under refrigeration at 4°C. Working standard solutions were prepared in methanol and stored in amber glass vials at -20°C. Glassware was treated with a sodium hydroxide mixture (Sigma – Aldrich, Sant Louis, USA) and washed with different solvents (water, acetonitrile, and methanol) of HPLC grade to remove contamination residues.

## 5.2.2 Samples and sample preparation

Fruit (oranges and apples) and vegetable samples (fennel**s** and tomatoes) were collected from local farms. A representative portion of each food product (the whole part, without washing) was thoroughly blended using a food processor. An aliquot of 10 g of sample was placed in a 50 mL falcon tube, then 10 mL of acidified methanol by 1% formic acid were added for the alkaloid extraction from the matrix. After manually shaking for 1 min, the extraction mixture was placed in a horizontal shaker for 30 min at 200 rpm. After centrifugation for 5 min at 3000 rpm, the supernatant was collected and filtered on 0.2 µm PTFE membranes (Phenex™ Teflon®, Phenomenex). Finally, the extract was transferred to an amber glass vial and stored at -20°C. Before LC-MS analysis, the extract was diluted 1:5 in acidified methanol. Analyses were performed in triplicate.

### 5.2.3 Liquid Chromatography and Mass Spectrometry analyses

Chromatographic separations were performed using an Agilent 1290 Infinity LC system which consisted of a binary pump for gradient elution, equipped with a micro vacuum degasser, a thermostated autosampler connected to an injection valve with a loop of 10 µL, a column compartment, and a UV–Vis detector. All the separations were performed using an Obelisc R C18 column (2.1 x 150 mm, 5 µm, 100 Å by SIELC Technologies, Wheeling, IL USA) coupled with corresponding precolumn (2.1 x 10 mm, 5 µm, 100 Å) eluted in gradient mode at a flow rate of 0.3 mL $min^{-1}$. The column was kept at 40 °C to decrease the mobile phase viscosity. The mobile phase consisted of 20 mM ammonium formate at pH 3 (A) and acetonitrile (B). The solvent gradient program started with an isocratic step at 10% A for 4 min followed by gradient steps to 80% A in 3 min and then to 20% A in 5 min. Finally, the system was re-equilibrated for 8 min at 10% A with a total run time of 20 min. The injection volume was 10 µL.

LC-MS analyses were performed on an Agilent 6490 Triple QqQ mass spectrometer equipped with an AJS ESI source operating in the positive ionization mode. MS analyses were carried out in the Multi Reaction Monitoring (MRM) mode. The presence of at least two significant MS/MS transitions was used to identify the analytes. The precursor ions were 249.2 and 265.0 for matrine and oxymatrine, respectively. Diagnostic ions at m/z of 148.1 and 110.1 for matrine, and 148.1 and 205.0 for oxymatrine were selected. Acquisition and data processing were performed by Agilent MassHunter LC/SQ Chemstation Integration software.

## 5.3 Results and Discussion

### 5.3.1 Optimization of the chromatographic and mass spectrometry conditions

The analysis of matrine and oxymatrine was carried out by hydrophilic interaction chromatography (HILIC), using hydrophilic C18 stationary phases and reversed-phase type eluents. Indeed, for highly polar basic compounds, like matrine-based biopesticides, it is difficult to obtain satisfactory retention on conventional C18 columns [21]. The presence of an additional mechanism of interaction in the HILIC columns (commercially available in different formats based on silica, urea, amide, zwitterionic groups, polyethyleneimine bonding) provided great selectivity for the separation of closely related compounds, also enhancing the retention of extremely polar compounds. Then, a reversed-phase cation-exchange mixed-mode column was chosen for the analysis of matrine and oxymatrine. Due to the presence of ionic groups and a long hydrophobic chain in the selected HILIC column, additional retention and tuning that is not available with traditional reversed-phase columns were obtained. Peak tailing effect due to the interaction of basic groups with anionic silanol functional

groups, which usually occurs for alkaloid analysis, was successfully faced using a gradient elution program by ammonium formate buffered at pH = 3.0 and acetonitrile, ensuring both a good chromatographic separation in less than 20 minutes and an appropriate ionization for MS characterization.

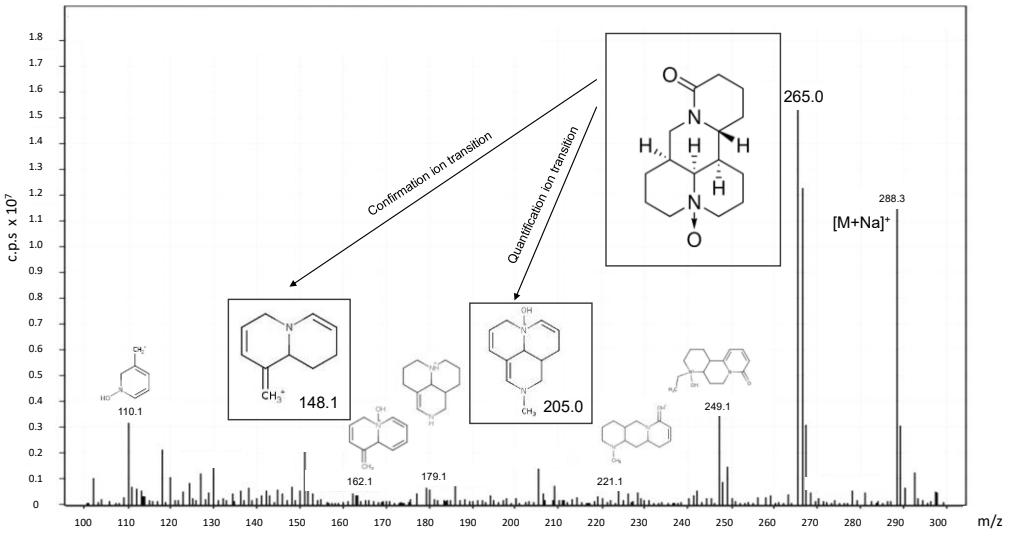



*Fig. 5.1 Clear mass spectra of matrine mixed standard solution at a concentration level of 100 µg $L^{-1}$.*

The MS parameter optimization was performed by analyzing matrine and oxymatrine mixed standard solutions at a concentration level of 100 µg $L^{-1}$. The MS analyses were performed in positive ion mode, which guarantees high sensitivities for both matrine and oxymatrine and clear mass spectra, as shown in Figures 5.1 and 5.2; then, the most intense ions from the MS spectrum were selected and fragmented by Collision-Induced Dissociation (CID). MS/MS analysis was performed on the quasi-molecular ions $[M+H]^+$.

*Table 5.1 Diagnostic ions and mass spectrometry parameters for the determination of matrine and oxymatrine by LC-MS/MS.*

| **Compound (Molecular Formula)** | **Molecular Weight (amu)** | **Precursor ion (amu)** | **Quantification ion transition (amu)** | **Collision Energy (eV)** | **Confirmation ion transition (amu)** | **Collision Energy (eV)** | **Cell Accelerator Voltage (kV)** | **Dwell Time (ms)** |
|---|---|---|---|---|---|---|---|---|
| Matrine ($C_{15}H_{24}N_2O$) | 248.36 | 249.2 | 249.2 → 148.1 | 30 | 249.2 → 110.1 | 30 | 7 | 200 |
| Oxymatrine ($C_{15}H_{24}N_2O_2$) | 264.37 | 265.0 | 265.0→ 205.0 | 20 | 265.0→ 148.1 | 30 | 5 | 50 |

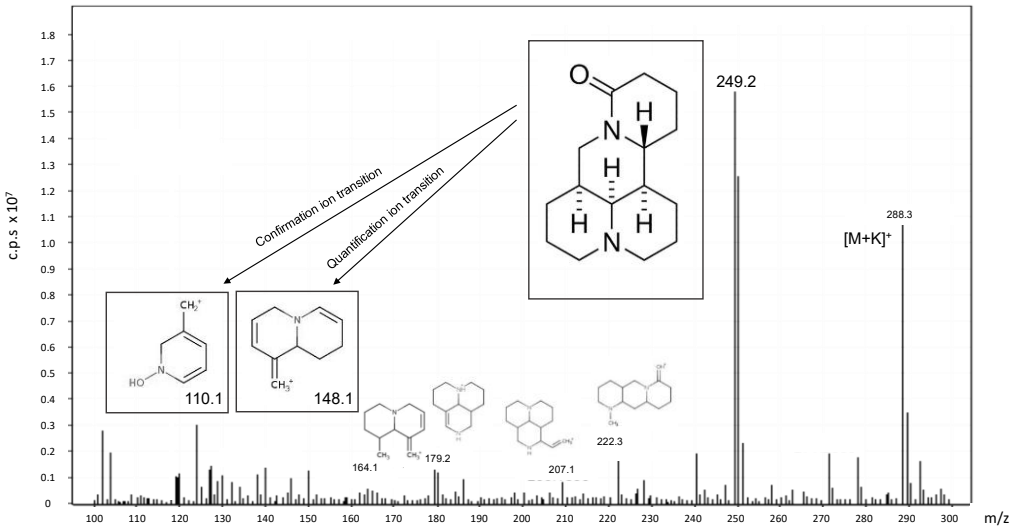



*Fig. 5.2 Clear mass spectra of oxymatrine mixed standard solution at a concentration level of 100 µg $L^{-1}$.*

Each compound was investigated individually to achieve optimal collisional energy for MS/MS experiments and select the quantification and confirmation transitions. Systematic MS/MS experiments were performed by changing the collision energy from 15% to 30% and monitoring the intensities of the fragment ions to achieve the maximum S/N ratio. The cell accelerator voltage was optimized from 5 to 7 kV for the individual molecular species. Dwell time was varied in the range 50-200 ms to examine any possible gain of signal intensity without ion space-charge effect. Finally, the optimized MS parameters were determined, as reported in Table 5.1. Since the multiple reaction monitoring (MRM) mode represents the most promising technique for the determination of pesticide residues at trace levels, the quantification and confirmation ion transitions were chosen for each of the two analytes by using one precursor ion and two transitions, with the relevant two different product ions. The most intense transitions, based on a signal-to-noise ratio, were selected to provide selective detection. Other MRM transitions were used to confirm the presence of matrine and oxymatrine in fruit and vegetable samples.

## 5.3.2 Optimization of the sample preparation procedure

The extraction process from food samples is often the most critical step during the analytical method development, strongly affecting the accuracy and precision of the results. This aspect is particularly important in the analysis of non-approved substances, which request very low detection limits. Methanol and acetone, pure or in combination with acetonitrile, dichloromethane, and ethyl acetate, are the most common extraction solvents used for the determination of pesticide residues in food

products [22,23]. In the present work, acidified methanol by formic acid was proposed as an extraction solvent of matrine alkaloids from fruits and vegetables, as described in the QuPPe method for the analysis of polar pesticides in vegetable origin samples [24]. The experimental extraction parameters have been optimized on spiked samples of orange, chosen as a reference matrix, then, the optimal conditions have been applied to the analysis of all fruit and vegetable samples. Ruggedness tests were performed by varying the extraction time from 15 to 60 min. Five replicates at each extraction time were performed on fortified orange samples with matrine alkaloids at a spiking level of 0.1 mg $kg^{-1}$ each. Recovery percentages of 113±17 and 116±14 were obtained at 15 and 60 min, respectively, demonstrating that the stirring time did not impact the extraction efficiency (F test: 0.95;1;8). Therefore, an extraction time of 15 min was chosen for all the subsequent analyses, guaranteeing high recovery percentages in short analysis times.

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### 5.3.4 Method validation

Analytical method validation is a key step to provide accurate results with a high within- and inter-laboratory reproducibility in official check analyses and risk-assessment studies. Therefore the proposed method for the determination of matrine-based

pesticides was validated through the evaluation of selectivity, linearity, detection and quantification limits, recovery, and precision, in agreement with Decision 657/2002/EC and Regulation 882/2004/EC, which describe the analytical parameters to be tested to assure the method reliability.

### *5.3.4.1 Method selectivity towards interferences*

The method selectivity was tested by the analysis of 20 independent blank samples of fruit and vegetables (2 oranges, 2 apples, 6 fennel extracts, and 10 tomatoes) which were processed under the optimized experimental conditions. The absence of interfering peaks in the retention time window of each analyte, with a maximum tolerance range of ±2.5%, confirmed that the proposed method can distinguish the analytes from possible endogenous matrix interfering components.

### *5.3.4.2 Method linearity*

Linearity tests were performed by three series of analyses on three different days, by injecting five standard solutions of matrine alkaloids, each at a concentration level of 5, 10, 50, 100, and 200 μg $L^{-1}$. The calibration parameters are reported in Table 5.2, where standard deviations of slope and intercept are estimated at the 95% confidence level. Good linearity was found for both target analytes with correlation coefficients higher than 0.9984. The goodness-of-fit of the data to the calibration curve was evaluated through the response factor distribution, by calculating the signal-to-concentration ratio (y/x) for each experimental point. Then the $x_i/y_i$ ratios were checked to ensure that their deviation from the mean value of the signal-to-concentration ratio did not exceed ±10%. Moreover, the confidence interval for the intercept, including the zero value (α = 95%, ν=5), indicates the absence of systematic instrumental bias.

*Table 5.2 Calibration parameters of matrine and oxymatrine by LC-MS/MS. [a]Instrumental LOD and LOQ calculated by analysis of standard solutions. [b]Method detection and quantification limits calculated on spiked samples at 10 μg kg$^{-1}$.*

| Analyte | Linear Range (R) (μg L$^{-1}$) | Instrumental [a] (μg L$^{-1}$) | | Method [b] (μg kg$^{-1}$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Tomato | | Fennel | | Apple | | Orange | |
| | | LOD | LOQ | LOD | LOQ | LOD | LOQ | LOD | LOQ | LOD | LOQ |
| Matrine | 0.11-200 (0.9987) | 0.032 | 0.11 | 0.13 | 0.44 | 0.16 | 0.53 | 0.27 | 0.92 | 1.5 | 5.0 |
| Oxymatrine | 0.48-200 (0.9984) | 0.033 | 0.11 | 0.30 | 1.0 | 0.17 | 0.55 | 0.17 | 0.57 | 0.26 | 0.88 |

### *5.3.4.3 Detection and quantification limits*

By the chromatograms of the matrine-based biopesticide standard solutions at the lowest calibration level (5 μg $L^{-1}$), the instrumental limits of detection (LOD) and quantification (LOQ) were estimated at a signal-to-noise ratio of 3 and 10, respectively. The noise levels were estimated as peak-to-peak value, that is, the difference between the maximum positive and the maximum negative amplitudes of baseline in the time window around the analyte retention time.

LODs of 0.032 μg $L^{-1}$ and 0.033 μg $L^{-1}$ were observed for matrine and oxymatrine, respectively, which are considerably lower than the legal limits of 10 μg $kg^{-1}$, set by default for non-approved substances. These results suggested that the proposed method provides comparable or higher sensitivity than other methods reported in the literature for biopesticide analysis [7,19,25], as shown in Table 5.3, allowing their determination at trace levels and reducing the risk of false-negative results.

*Table 5.3 Comparison of analytical methods for the determination of matrine and oxymatrine*

| **Analyte** | **Sample** | **LOD (μg $kg^{-1}$)** | **Recovery%** | **Reference** |
|---|---|---|---|---|
| matrine, oxymatrine | Fertilizers | 1000 | 97-104 | Sabatino et al. (2015) |
| Matrine | fruit, vegetables, soil | 0.34-1.07 | 83-109 | Liu et al. (2014) |
| oxymatrine, matrine | Fish | 0.29-0.37 | 89-96 | Yang et al. (2011) |
| matrine, oxymatrine | fruit, vegetable | 0.13-1.5 | 81-114 | present study |

Predictable matrix effects in real samples, due to loss or enrichment of analyte during extraction and cleanup steps, have been evaluated by analyzing spiked samples of tomato, fennel, apple, and orange at a fortification level of 10 μg $kg^{-1}$ (three replicates in the two different working sessions). As expected, the slopes obtained by standard solutions and the ones obtained by spiked tomato samples (from 5 to 200 μg $kg^{-1}$) were significantly different at the 95% confidence level, confirming the presence of matrix effects in these analyses (data not shown). Matrix effects and changes in baseline noise can also have a strong impact on LOD and LOQ values: for this reason, their evaluation

under these conditions is mandatory. LODs ranging from 0.13 to 1.5 μg $kg^{-1}$ and from 0.17 to 0.30 μg $kg^{-1}$ were obtained for matrine and oxymatrine, respectively, in fruit and vegetable products. Analogously, for matrine LOQs ranged from 0.44 to 5.0 μg $kg^{-1}$, and for oxymatrine from 0.55 μg $kg^{-1}$ to 1.0 μg $kg^{-1}$. These results demonstrated that, even in the case of real food sample analysis, the proposed method returns LODs and LOQs considerably lower than official limits established for non-approved substances.

### *5.3.4.4 Precision and Recovery*

As reported in Dec 657/2002/EC, the trueness of measurements was assessed through the recovery of additions of known amounts of the analytes to a blank matrix. Spiked samples with matrine and oxymatrine were prepared by adding proper amounts of mixed alkaloid standard solutions to the vegetable samples before the extraction process. Then, after homogenization, proper known amounts of biopesticides were added to obtain the desired spiking level, thus the same alkaloid concentration was obtained in all the aliquoted sample portions. Precision and recovery were determined by performing tests on two sets of blank tomato, fennel extracts, and orange, fortified by adding proper amounts of matrine and oxymatrine. The experiments were carried out on different days with the same instruments, but different operators to ensure the greater variability of results. Precision and recovery data have been previously processed by the Shapiro-Wilk test [26] to verify normal distribution. Afterward, a one-way ANOVA test was performed to verify the homogeneity of the mean concentration values evaluated among the validation sessions at each fortification level. Results from ANOVA were used to calculate intra-laboratory repeatability relative standard deviations (RSDr) following the Decision 2002/657/EC. In Table 5.4 the results of recovery and intraday assay within-laboratory repeatability values evaluated for each alkaloid in spiked vegetable samples are summarized. As can be noted from the table, the obtained intraday RSDr values are well below the reference values of 15%, derived by the Horwitz equation for a mass fraction ≤0.1 mg $kg^{-1}$, under repeatability conditions [27]. Recovery percentages were evaluated by comparing the concentration of spiked samples, determined by the external calibration regression line, with the nominal fortification level. For each analyte, it was verified that the calculated mean recovery complied with the recovery range of 60-140%, reported in the official European Commission document SANTE 2017/11813/EC, dealing with the method validation and quality control procedures for Pesticide Residues Analysis in Food and Feed. Recoveries ranging from 82% to 114% were obtained, demonstrating a good accuracy of the method, which can be considered a useful tool for the official check analysis of biopesticides in vegetable samples.

*Table 5.4 Recovery and reproducibility data for the determination of matrine and oxymatrine by LC-MS/MS in vegetable samples [a] Intraday assay: Ten parallel samples at each concentration level were prepared on the same day. [b]Interday assay: twelve replicate determinations were carried out on 2 different days at the fortification level of 10 µg $L^{-1}$ corresponding to MRL.*

| | | **Intraday ($n$ = 10)[a]** | | | | **Interday ($n$ = 24)[b]** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Matrine** | | **Oxymatrine** | | **Matrine** | | **Oxymatrine** | |
| **Sample** | **Spiking Level (µg $kg^{-1}$)** | **Recovery (%)** | **RSD (%)** | **Recovery (%)** | **RSD %** | **Recovery (%)** | **RSD (%)** | **Recovery (%)** | **RSD (%)** |
| tomato | 10 | 103±12 | 12 | 114.4±4.5 | 3.9 | 102±12 | 12 | 114±14 | 13 |
| | 100 | 108.7±7.8 | 7.2 | 108.7±5.5 | 5.1 | - | - | - | - |
| | 350 | 100.4±9.9 | 9.9 | 91.9±9.2 | 10 | - | - | - | - |
| fennel | 10 | 88.8±8.8 | 9.9 | 82±11 | 13 | 88.2±7.2 | 8.2 | 81.2±7.5 | 9.2 |
| | 100 | 109.8±5.6 | 5.1 | 92.2±8.0 | 8.7 | - | - | - | - |
| | 350 | 106.4±8.1 | 7.6 | 87.5±7.5 | 8.6 | - | - | - | - |
| orange | 10 | 92.3±5.1 | 5.5 | 95.1±3.9 | 4.1 | 94±20 | 22 | 95±12 | 13 |
| | 100 | 104.5±5.5 | 5.3 | 105.4±5.3 | 5.0 | - | - | - | - |
| | 350 | 101.5+6.8 | 6.7 | 102.6±5.3 | 5.2 | - | - | - | - |

### *5.3.4.5 Quantitative analysis of fruit and vegetable samples*

Method feasibility has been demonstrated by the analysis of several vegetable-origin samples. The occurrence of matrine-like pesticides was examined in 102 commercially available samples of fruit and vegetables, including fruit branded as organic products. In each batch of samples, a matrix blank was analyzed to reduce the risk of false-positive results due to the potential chemical contamination in the extraction process. Therefore, a reagent blank was processed according to the complete analytical procedure using an equivalent amount of suitable solvent in place of the test portion, and a sample blank (i.e., a compliant control sample) was prepared from a test portion taken from a sample from which matrine and oxymatrine are absent. For every batch of 10 samples, a reagent blank and a sample blank were included in the sample list to be analyzed. A high sampling rate process (including the pesticide extraction step and the following LC-MS/MS analysis) was obtained considering that in an 8-hour working session, more than 15 samples can be processed and analyzed. Despite the relatively high complexity of the analyzed food and agricultural matrices, a good selectivity was obtained. As an example, in Figure 5.3 the MRM chromatographic separation of a peach sample, contaminated by matrine and oxymatrine, is shown. Peak identity was confirmed by standard additions.

The presence of both biopesticides (above the corresponding limit of detection) was observed only in five samples of peach and apricot coming from organic farming, whereas in a total of 16 samples (peaches, apricots, grapes, and nectarines) only matrine was present. Among them, in 10 fruit samples, the matrine amount was higher than the quantification limit. High contamination levels, above the maximum residue limit of 10 µg $kg^{-1}$, set by the European Community, were observed in 8 fruit samples, with a concentration level up to 105 µg $kg^{-1}$, as shown in Figure 5.4. Overall, a contamination grade of 7.8% was obtained with high matrine amounts in peaches and apricots, while no trace of biopesticides was observed in the analyzed samples of oranges and apples.

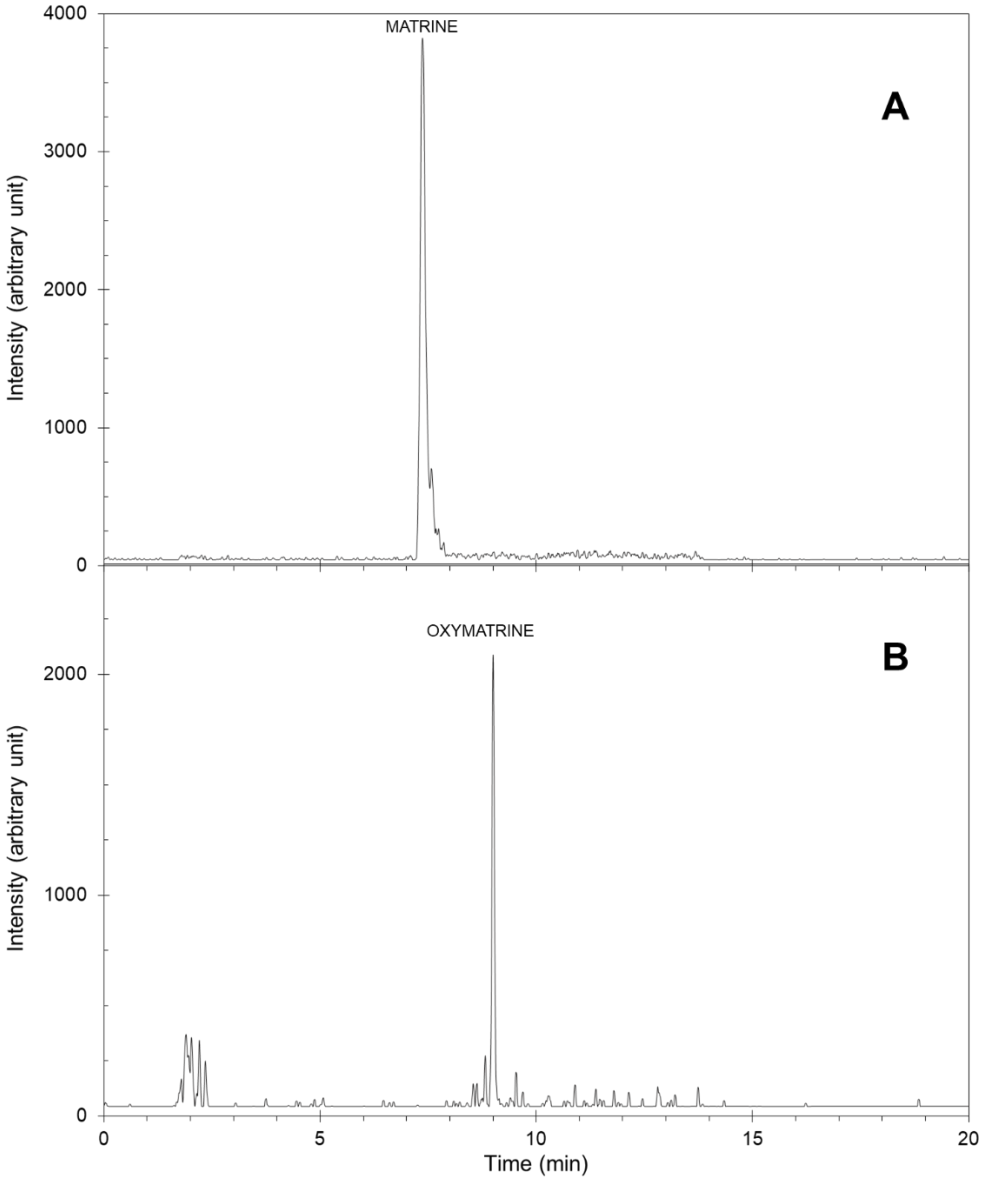


*Fig. 5.3 MRM chromatographic separation of a peach sample contaminated by matrine and oxymatrine.*

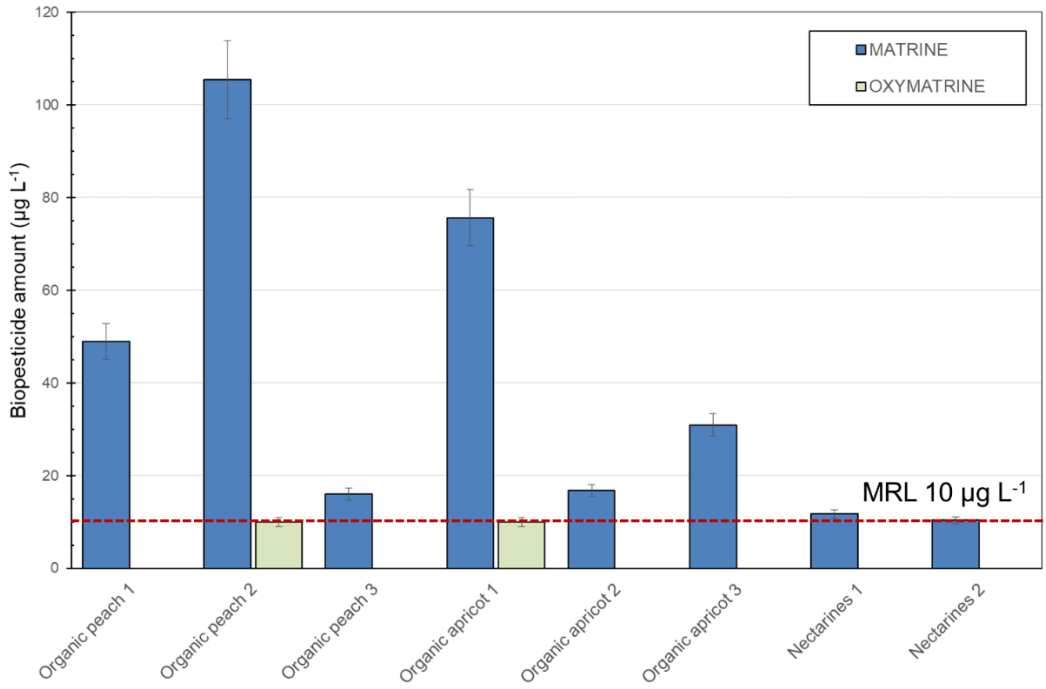


*Fig. 5.4 Contamination levels of both biopesticides examined in 102 commercially available samples of fruit and vegetables, including fruit branded as organic products.*

## 5.4 Conclusions

A reliable and reproducible analytical method is proposed for the analysis of matrine and oxymatrine in vegetable-origin samples by LC-MS/MS. Optimized chromatographic and MS conditions ensure good results in terms of response sensitivity, rapidity, and separation efficiency. The optimized quick sample extraction procedure allowed to perform the simultaneous extraction and clean-up of more than 15 samples in an 8-h single-day working session. The results of the method validation, performed according to the European Commission directives, demonstrated that the proposed method is well suited to satisfy the demand for the accurate determination of quinolizidine alkaloids in fruit and vegetable samples. The contamination grade estimated through the analysis of a high number of vegetable samples emphasizes the need for reliable analytical methods for the determination of matrine-based pesticides in official check analyses and monitoring studies.

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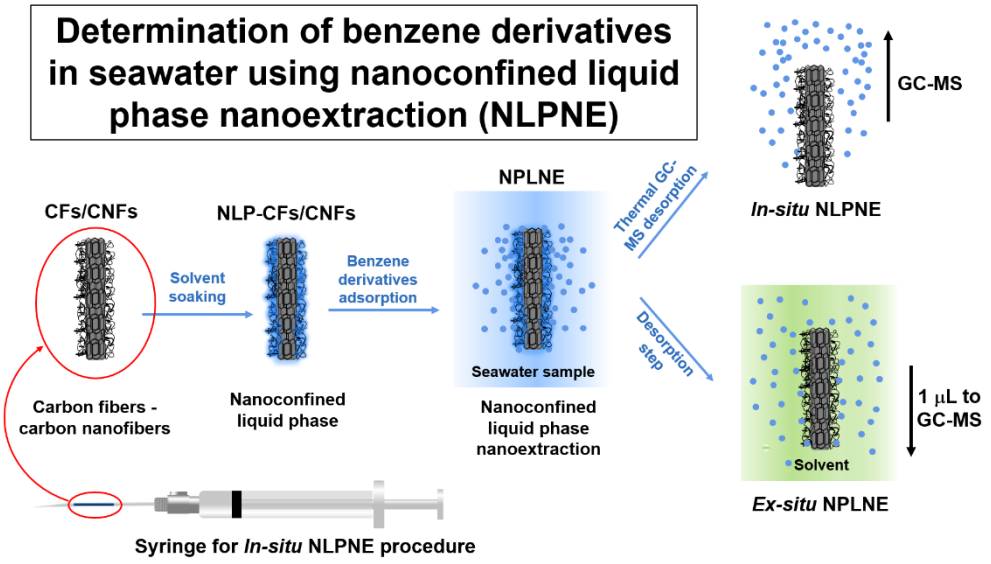
# Chapter 6 - *Ex-situ* and *in-situ* rapid and quantitative determination of benzene derivatives in seawater using nanoconfined liquid phase nanoextraction



# 6.1 Introduction

The innovative characteristics and different benefits of microextraction techniques (METs) allow to sample and analyze compounds through single-step sampling and solvent-free preparation with the potential for automation and reduced analysis times often permitting to meet the requirements of green analytical methods replacing conventional sample preparation and extraction methods. [1] Generally, the efficiency of METs mainly depends on the choice of a suitable extraction media, and many sorbents and solvents are used to enhance this characteristic. In recent years, carbon-based nanomaterials have been investigated such as sorbent materials, resulting in the improvement of existing analytical procedures and the development of new procedures. [2–4] Among these innovative materials, carbon nanofibers (CNFs) have been largely used, also in a modified version, in many scientific research studies for their thermal stability and excellent mechanical/electrical properties. [5] Furthermore, the distinct novel properties, including high mechanical strength, chemical stability, and high flexibility, of CNFs synthesized on the carbon fiber surface (CNFs/CFs) have raised the attention of the scientific community. [6,7] The characteristic of nanoconfined liquids in the nanopores of CNFs/CFs that interact with these materials as nanoconfined solvents (NCS) allows the modification of thermodynamic and kinetic characteristics of CNF/CFs. Indeed, the first proof of concept of their use as a powerful substrate for nanoconfined liquid phase nanoextraction (NLPNE) showed that the high versatility and efficiency of its microextraction capabilities are based on the choice of the NCS. [8] Among the class of volatile organic compounds (VOCs), benzene derivatives (BDs) are polluting materials of anthropogenic origin for the most part occurring through fuel extraction and combustion for many purposes like industrial one. Their association through chronic and acute exposure with human health diseases is well known since the late 1800s. [9] The regulation about these compounds remains uncertain in some cases, like in the Mexican law, or for established levels for VOCs in the Code of Federal Regulations in the USA allowable only for the beverages. [10,11] For the extended definition of emerging contaminants in which belong *"compounds whose legislation is being revised to include new information that had not previously been taken into account or those whose tested methodologies have improved"*, BDs could be considered as belonging to the list of compounds of emerging concerns. [12] To obtain an adequate method for real-time VOCs monitoring, laboratory (*ex-situ*) and needle-tip (*in-situ*) procedures have been developed for the analysis of BDs in seawater based on a new extraction technique introduced in 2020. [8]

## 6.2 Materials and Methods

### 6.2.1 Chemicals

Analytical standards (purity grade >99%) of toluene, chlorobenzene, m-xylene, p-xylene, o-xylene, p-dichlorobenzene, m-dichlorobenzene, o-dichlorobenzene, nitrobenzene, o-methylnitrobenzene, m-methylnitrobenzene, p-methylnitrobenzene, 1,2,4-trichlorobenzene, 1,2,3-trichlorobenzene, and 1,2,3,4-tetrachlorobenzene were purchased from Merck (Darmstadt, Germany). Chlorobenzene-d5 was purchased from AccuStandard (New Haven, CT, USA) and used as a surrogate standard solution at a concentration of 1 mg $L^{-1}$ in acetone. Mixed stock standard solutions of BDs were prepared in acetone at a concentration of 25 mg $L^{-1}$ each. Working standard solutions were prepared from the stock solutions, and kept in the dark at 4 °C. Commercially available T700S carbon fibers (CFs, 12 K, average diameter 7 µm) were purchased from Toray (Tokyo, Japan) and Li Shuo Composite Materials Technology Co. LTD (Shanghai, China). Tetraethyl orthosilicate (TEOS) and surfactant (Pluronic P123, $EO_{20}PO_{70}EO_{20}$, Mav = 5800) were purchased from Sigma- Aldrich (Bellefonte, PA, USA). HPLC grade n-hexane, dichloromethane, acetone, and ethyl acetate were purchased from Thermo Fisher (New Jersey, USA), as well as HPLC-grade ethanol and methanol. Distilled water was prepared using a Milli-Q purification system (Millipore, Billerica, MA). Anhydrous sodium sulfate (≥ 99% purity, baked at 400 °C for 12 h and stored in a desiccator before use) was purchased from Shanghai Ling Feng Chemical Reagent Co. Ltd (Shanghai, China).

### 6.2.2 NLPNE synthesis, solvent soaking, and extraction procedures

Synthesis of CNFs/CFs was performed according to Zou *et al.* procedure. The *ex-situ* NPLNE procedure consists of four steps, as shown in Fig. 6.1: starting with the conditioning of CNFs/CFs (3000 units with a length of 1 cm) through ultrasonication for 5 min in dichloromethane, used as nanoconfined solvent (NCS), the CNFs/CFs were soaked in 100 µL of NCS for 5 sec. Later, the CNFs/CFs were dipped in 1 mL of sample for 90 sec under stirring conditions (400 rpm) for extraction purposes and ultrasonicated in 1 mL of NCS for 30 sec. Before the injection of 1 µL of sample in GC-MS instrument, anhydrous sodium sulfate was added into eluates to remove water. The needle tip device was prepared by inserting 2.0 ± 0.3 mg of CNFs/CFs inside the needle of a Hamilton syringe (250 µL, model 81165, Hamilton Company Inc., Nevada 89502 USA).

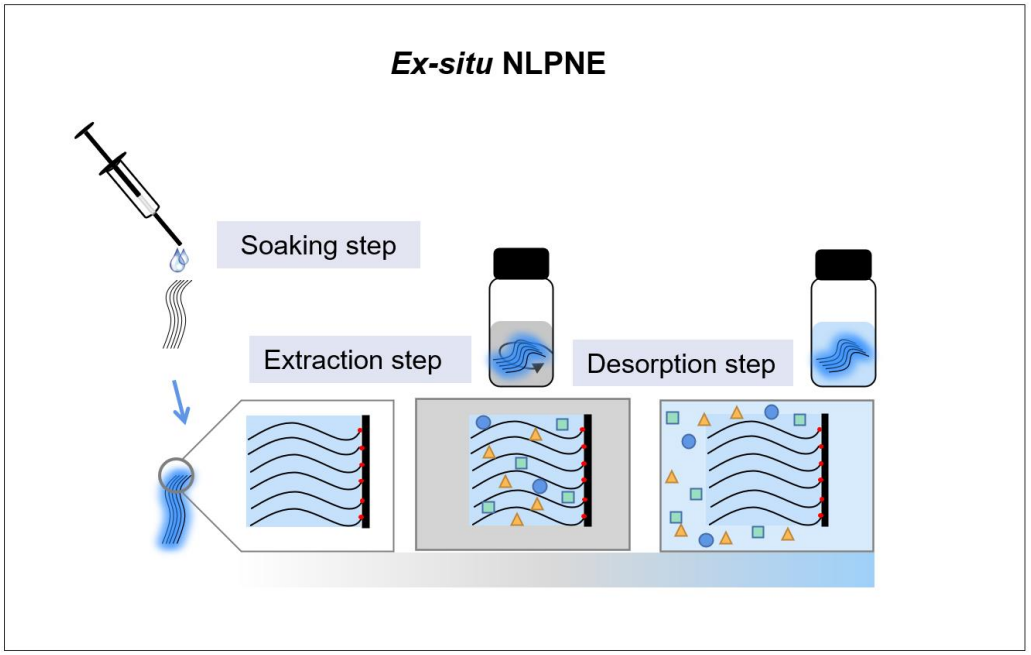



*Fig. 6.1 Schematic representation of ex-situ NLPNE mode for BD analysis.*

The *in-situ* mode NLPNE was realized following three key steps: 10 µL of NCS were used to nanoconfine the CNFs/CFs needle tip with a flow rate of 0.3 $\mu L^{-1}$. After, a ranging volume from 18 to 72 µL was drawn at a rate of 0.3 $\mu L^{-1}$ through the insertion of the needle into the sample solution (standard solution or seawater sample). Finally, after the sample desorption through the needle insertion into the GC inlet at 250° for 0.5 min, the analysis was performed directly in the GC-MS instrument (Fig. 6.2).

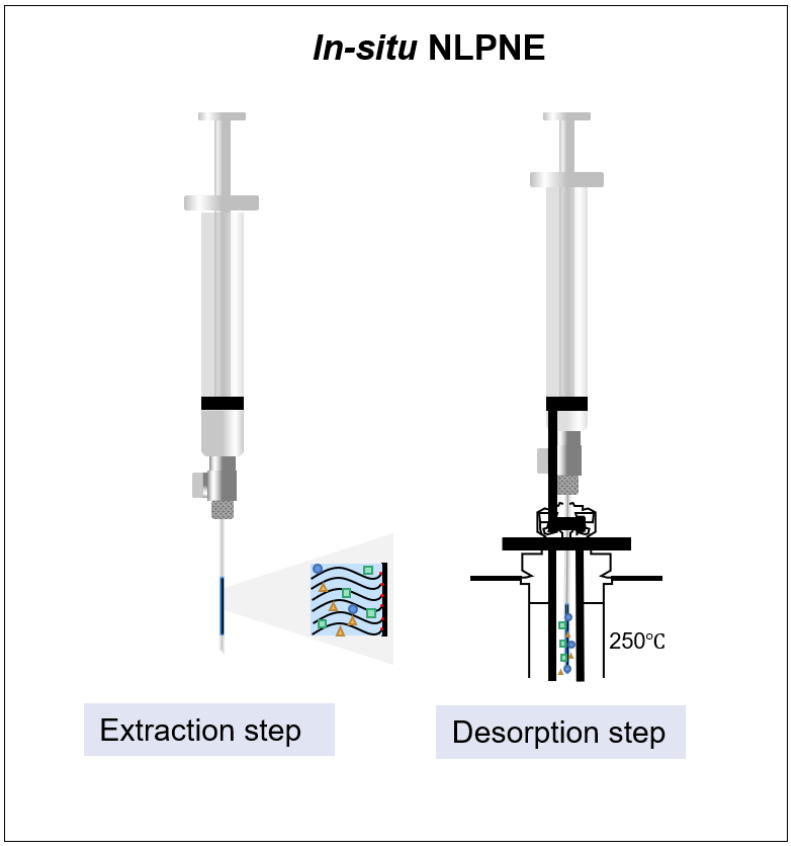



*Fig. 6.2 Schematic representation of in-situ needle-tip NLPNE mode for BDs analysis.*

## 6.2.3 GC and MS Analyses

The Shimadzu GC 2010 (Shimadzu Corporation, Kyoto, Japan) equipped with the Shimadzu QPMS 2010 quadrupole mass spectrometer was used for the GC-MS analyses. The chromatographic separations were performed with the support of a DB5-MS fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm, Agilent Technologies, Santa Clara, CA, USA) under the following instrumental conditions: initial oven temperature 40° C for 5 min; to 220° C at a rate of 5° C $min^{-1}$ and held for 1 min; to 280° C at 20° C $min^{-1}$ and held constant for 1 min with a total run of 50 min. the injection was performed in splitless mode at 280° C. The flow rate of the carrier gas (Helium, 99.999%) was 1.0 mL $min^{-1}$. The GC-MS interface temperature was set at 280° C. The ion source temperature and ionization energy were set at 200°C and 70 eV, respectively. The solvent cut time was 3.5 min. With the *ex-situ* NLPNE procedure, the injection volume was 1 µL. Due to their peak co-elution, m- and p-xylene have not been considered individually. For the in-situ needle-tip NLPNE mode, the thermal desorption

was performed by placing the needle in the injector for 0.5 min. All target analytes were analyzed in selected ion monitoring (SIM) mode.

### 6.2.4 Real sample analysis

Changxing Island (Dalian, China) seawater samples, after NaCl addition to reach the optimum NaCl% concentration of *ca.* 20% (w/v), were directly analyzed by *in-situ* NLPNE mode and GC-MS. The normalization of the analyte peak area with deuterated internal standard (IS) was carried out for all the calibration curves. The examination of BDs volatilization through the monitoring of the residual BD content in water samples was carried out by maintaining sample vials opened in a clean atmosphere at 25° C, at time intervals of 1, 5, 10, 20 and 30 min.

### 6.2.5 Liquid-Liquid Extraction (LLE) and SPME experimental conditions

The Liquid-Liquid Extraction (LLE) was repeated four times. 250 µL of hexane were added and vortexed for 1 min in a sample amount of 1 mL. The addition of a proper amount of anhydrous sodium sulfate made it easier to discard the aqueous phase to collect the organic solvent. 1 µL of the sample extract was injected into GC-MS after filtration through nylon filter membranes (pore size 0.22 µm). For the Solid Phase MicroExtraction (SPME) the SPME fiber (SUPELCO, Bellafonte, PA, USA) was inserted into a glass vial of 1.5 mL with a sample volume and stirred at 400 rpm for an incubation time of 20 min. After extraction, the fiber was withdrawn into the needle, pulled out from the vial, and injected into the GC system by thermal desorption at 250° C for 1 min.

## 6.3 Results and Discussion

The *ex-situ* NLPNE method was the most focused procedure for the overall experimental parameters optimization. The choice of solvent, with its strong influence on the partition coefficients, is the most important factor in the extraction capability of NLPNE. Hexane, acetone, dichloromethane, and ethyl acetate were considered as apolar nanosolvents for NPLNE. As shown in Fig. 6.3, hexane resulted in the best nanosolvent for the 15 BDs extraction in the experiments with standard solutions at 200 ng $mL^{-1}$, most probably for similar polarity with the analytes. The salting-out effect on the extraction procedure was investigated on samples with different NaCl concentrations ranging between 10 and 25% (w/v).

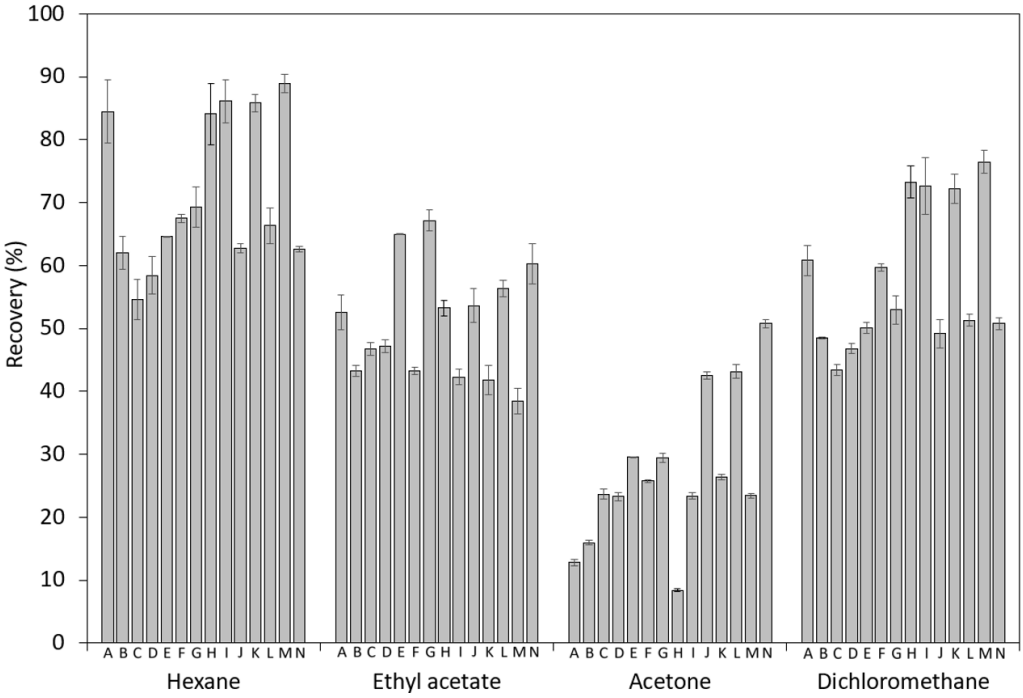



*Fig. 6.3 Effect of different nanosolvents on the extraction efficiency by ex-situ NLPNE. A: toluene; B: chlorobenzene; C: m- and p-xylene; D: o-xylene; E: p-dichlorobenzene; F: m-dichlorobenzene; G: o-dichlorobenzene; H: nitrobenzene; I: o-methylnitrobenzene; J: 1,2,4-trichlorobenzene; K: m-methylnitrobenzene; L: 1,2,3-trichlorobenzene; M: p-methylnitrobenzene; N: 1,2,3,4-tetrachlorobenzene. Experimental conditions: extraction time: 90 s; desorption time: 30 s; %NaCl = 20% w/v.*

The concentration of 20% NaCl was chosen for further experiments for the increased recovery results on all BDs in an aqueous solution, as shown in Fig. 6.4. The optimization of parameters like stirring conditions, adsorption and desorption times were investigated in order to enhance *ex-situ* NLPNE extraction efficiency. The analyte diffusion in the sample solution is the rate determining step of the extraction process as demonstrated also in this case. In fact, for all the analytes in the preliminary experiments, the extraction process in unstirred solution went close to the equilibrium starting from the 3$^{rd}$ minute. A shorter equilibrium time was reached at 90 sec under stirred conditions. Furthermore, nanosolvent loss could be responsible for adsorption times higher than 120 sec led to a decrease in extraction recoveries. No significant changes were noticed for desorption times higher than 30 sec. Ex-situ NLPNE adsorption and desorption times were set at 90 and 30 sec, respectively: the whole ex-situ NLPNE sample pretreatment was then completed in less than 2 minutes with acceptable recoveries (60.1 - 88.7%) and relative standard deviations (1.1 - 6.1%). The sample volume is an important parameter to consider for the *in-situ* NLPNE procedure due to the possible low recovery values with too low sample volumes or too high extraction times with unnecessary sample volume. After further experiments to reach

the best parameter between 18, 32, 54, and 72 µl of sample volume for the *in-situ* NLPNE procedure, the 54 µl volume was chosen to carry out the subsequent analyses.

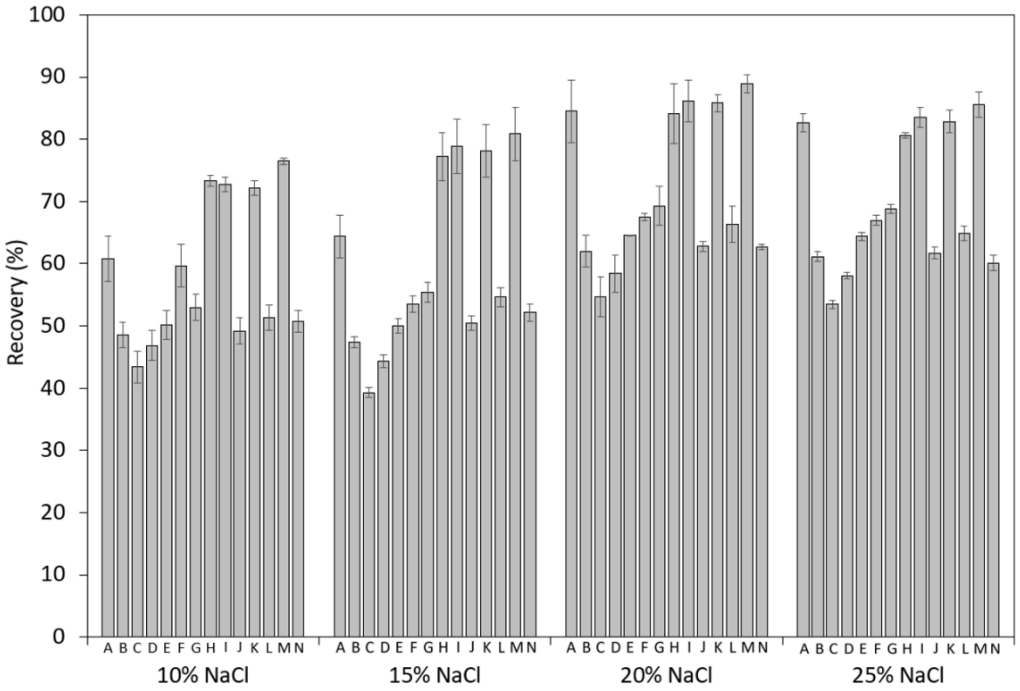



*Fig. 6.4 Effect of the ionic strength on the extraction efficiency by ex-situ NLPNE. A: toluene; B: chlorobenzene; C: m- and p-xylene; D: o-xylene; E: p-dichlorobenzene; F: m-dichlorobenzene; G: o-dichlorobenzene; H: nitrobenzene; I: o-methylnitrobenzene; J: 1,2,4-trichlorobenzene; K: m-methylnitrobenzene; L: 1,2,3-trichlorobenzene; M: p-methylnitrobenzene; N: 1,2,3,4-tetrachlorobenzene. Experimental conditions: nanosolvent: hexane; extraction time: 90 s; desorption time: 30 s; loading sample volume: 54 µL.*

## 6.3.1 NLPNE *ex-situ* and *in-situ* analytical performances

Both NLPNE procedures performances have been evaluated for all the BDs analyzed by means of recovery, reproducibility, linearity, limit of detection and quantification, enrichment factor, and matrix effect.

### *6.3.1.1 Recovery*

After the first extraction step under equilibrium conditions, considering the initial sample volume ($V_0$) and the relevant analyte concentration ($C_0$) we obtain:

$$C_0 \cdot V_0 = C_1 \cdot V_0 + C_F \cdot V_F \qquad \text{(Eq. 1)}$$

Where $C_F$ and $V_F$ are the analyte concentration and the nanoconfined solvent volume, respectively, and $C_1$ is the final concentration in the sample volume. The repartition constant can be calculated, in equilibrium conditions, as follow:

$$K_F = \frac{C_F}{C_1} \quad \text{(Eq. 2)}$$

In the desorption step, considering the nanoconfined solvent volume negligible (in this case, the volume entrapped in the CNFs/CFs is 10 ± 2 µL), we obtain:

$$C_S \cdot V_S = C_F \cdot V_F \quad \textit{(Eq. 3)}$$

Where $C_S$ and $V_S$ are the fial concentration and the solvent used in the desorption step. Combining all the showed equations:

$$C_S \cdot V_S = C_0 \cdot V_0 \left( \frac{K_F^{'}}{1+K_F^{'}} \right) \quad \text{(Eq. 4)}$$

$$K_F^{'} = K_F \cdot \frac{V_F}{V_0} \quad \text{(Eq. 5)}$$

In this case $V_S = V_0 = 1$ mL, $C_s$ tends to $C_0$ as the $K_F^{'}$ increases, and $K_F \approx 100\ K_F^{'}$. The enrichment factor (EF) is strictly dependent on $V_S$ *vs*. $V_0$ ratio and is calculated as $C_S/C_0$ value, but also as the nanoconfined solvent, the salinity of the solution, and the kinetic process parameters (adsorption and desorption times). [13] For the recovery calculation of the *in-situ* NLPNE mode should be considered that the needle is directly inserted into the GC inlet. Then, it is necessary to normalize the chromatographic NLPNE and direct-injected related peak areas to the sampled and the injected volume, respectively:

$$\%_{\text{Recovery}} = \frac{A_{NLPNE} \ / \ V_{NLPNE}}{A_{inject} \ / \ V_{inject}} \cdot 100 \quad \text{(Eq. 6)}$$

Where $V_{NLPNE}$ (equal to 54 µL) and $V_{inject}$ are the loaded sample volume used in NLPNE and the injected volume by standard procedure, respectively; while $A_{NLPNE}$ and $A_{inject}$ are the peak areas obtained by NLPNE needle analyte direct desorption and from the standard analyte injection, respectively. With these values, it has been possible to

estimate the repartition constant $K_F$, defined as the ratio between the concentration of the analyte in the nanoconfined solvent and the sample solution, in equilibrium conditions. Recoveries and $K_F$ with a standard error < 50% are reported in Tab.6.1.

*Tab. 6.1 Ex-situ NLPNE recoveries at 50, 500 and 2000 ng mL$^{-1}$, partition constant, calculated by Eq.4 and Eq.5, and computational octanol-water partition coefficient. Experimental conditions: nanoconfined solvent: hexane; extraction time: 90 s; desorption time: 30 s; %NaCl = 20% w/v.*

| Analyte | Recovery % (n=3) | | | $K_F$ | XLogP3 |
|---|---|---|---|---|---|
| | 50 ng mL$^{-1}$ | 500 ng mL$^{-1}$ | 2000 ng mL$^{-1}$ | | |
| toluene | 63.2 | 76.1 | 73.8 | 260 ± 80 | 2.7 |
| chlorobenzene | 75.1 | 83.4 | 77.4 | 380 ± 100 | 2.9 |
| *m*,*p*-xylene | 57.6 | 81.5 | 87.9 | 730 ± 300 | 3.2 |
| *o*-xylene | 60.2 | 86.3 | 73.1 | - | 3.1 |
| *p*-dichlorobenzene | 84.8 | 89.7 | 80.7 | 620 ± 230 | 3.4 |
| *m*-dichlorobenzene | 61.8 | 88.4 | 76.5 | - | 3.4 |
| *o*-dichlorobenzene | 59.3 | 65.8 | 56.9 | 160 ± 30 | 3.4 |
| nitrobenzene | 78.9 | 101.9 | 94.2 | - | 1.9 |
| *o*-methylnitrobenzene | 96.3 | 107.1 | 82.1 | - | 2.3 |
| 1,2,4-trichlorobenzene | 58.0 | 99.5 | 75.8 | 230 ± 120 | 4.0 |
| *m*-methylnitrobenzene | 70.2 | 107.1 | 83.9 | 380 ± 200 | 2.3 |
| 1,2,3-trichlorobenzene | 63.7 | 100.9 | 70.8 | - | 4.0 |
| *p*-methylnitrobenzene | 77.1 | 75.4 | 69.5 | 290 ± 60 | 2.3 |
| 1,2,3,4-tetrachlorobenzene | 76.5 | 91.6 | 81.5 | 620 ± 410 | 4.6 |

Some interesting correlations emerged from the comparison with XlogP3, the computational octanol-water partition coefficient. [14] In general, calculated $K_F$ values are of the same order of magnitude of computational octanol-water partition coefficient.

### *6.3.1.2 Reproducibility*

The evaluation of *ex-situ* and *in-situ* NLPNE modes has been carried out through the measurements of intra-day and inter-day relative standard deviations (RSDs). For standard solutions, intra-day RSDs ranged from 1.8% to 4.8% and from 6.7% to 17%, while inter-day values ranged from 7.4% to 20% and from 4.6% to 22% for *ex-situ* and *in-situ* NLPNE, respectively (Tab. 6.2). The good reproducibility showed with the

standard solution was also confirmed with 1.0 µg $mL^{-1}$ seawater spiked samples: in this case, the inter-day RSDs fluctuated from 0.8% to 16%.

*Tab. 6.2 Ex-situ and in-situ NLPNE intra-day and inter-day Relative Standard Deviations (RSD) on standard solutions and 1.0 µg $mL^{-1}$ spiked seawater samples. Spiked sample extractions were performed by ex-situ procedure. Experimental conditions are described in the text.*

| | **Precision (RSD %)** | | | | |
|---|---|---|---|---|---|
| | ***ex-situ* NLPNE** | | ***in-situ* NLPNE** | | **Spiked samples** |
| **Analyte** | **Intra-day (n=3)** | **Inter-day (n=7)** | **Intra-day (n=3)** | **Inter-day (n=7)** | **Inter-day (n=7)** |
| toluene | 4.1 | 12 | 17 | 9.7 | 11 |
| chlorobenzene | 3.4 | 13 | 11 | 22 | 12 |
| *m*,*p*-xylene | 3.6 | 19 | 9.4 | 6.2 | 12 |
| *o*-xylene | 2.9 | 16 | 9.7 | 4.6 | 15 |
| *p*-dichlorobenzene | 3.6 | 11 | 16 | 9.6 | 13 |
| *m*-dichlorobenzene | 2.4 | 20 | 14 | 12 | 16 |
| *o*-dichlorobenzene | 2.8 | 20 | 8.6 | 14 | 15 |
| nitrobenzene | 2.2 | 15 | 11 | 17 | 2.7 |
| *o*-methylnitrobenzene | 2.9 | 7.6 | 15 | 22 | 3.8 |
| 1,2,4-trichlorobenzene | 2.9 | 7.4 | 6.7 | 9.3 | 0.8 |
| *m*-methylnitrobenzene | 2.3 | 15 | 14 | 21 | 4.2 |
| 1,2,3-trichlorobenzene | 3.4 | 8.8 | 13 | 18 | 2.7 |
| *p*-methylnitrobenzene | 1.8 | 19 | 13 | 22 | 9.2 |
| 1,2,3,4-tetrachlorobenzene | 4.8 | 16 | 11 | 8.7 | 7.9 |

### *6.3.1.3 Linearity, LOD and LOQ, and EF*

The method linearity was evaluated in the ranges 0.10 – 500 ng $mL^{-1}$ and 5.00 – 500 ng $mL^{-1}$ for all BDs and both procedures. Good linearity was generally found (determination coefficients ranging from 0.9929 to 0.9997). *Ex-situ* NLPNE limit of detection (LOD) and limit of quantification (LOQ), defined as the BDs content that gave a signal to noise (S/N) ratio of 3:1 and 10:1, respectively, ranged from 0.2 to 7.6 ng $mL^{-1}$ and from 0.7 to 25 ng $mL^{-1}$ (Tab. 6.3). For *in-situ* NLPNE, LODs ranged from 0.04 to 1.00 ng $mL^{-1}$ and LOQs from 0.14 to 3.35 ng $mL^{-1}$ (Tab. 6.4).

*Tab. 6.3 Ex-situ NLPNE regression parameters, LOD, LOQ for all BDs calculated in the range 0.1 – 500 ng $L^{-1}$. Experimental conditions are described in the text. In-situ NLPNE regression parameters, LOD, LOQ and EF, calculated in the range 5 - 500 ng $L^{-1}$.*

| **Analyte** | **Linear Regression Equation** | **$R^2$** | **LOD (ng $mL^{-1}$)** | **LOQ (ng $mL^{-1}$)** |
|---|---|---|---|---|
| toluene | y = 3.12x + 0.72 | 0.9985 | 0.2 | 0.7 |
| chlorobenzene | y = 1.99x - 0.19 | 0.9986 | 0.3 | 1.0 |
| *m*,*p*-xylene | y = 5.30x - 0.34 | 0.9984 | 1.0 | 3.4 |
| *o*-xylene | y = 2.83x - 0.23 | 0.9983 | 1.3 | 4.3 |
| *p*-dichlorobenzene | y= 1.55x - 0.14 | 0.9983 | 1.2 | 3.9 |
| *m*-dichlorobenzene | y = 1.58x - 0.14 | 0.9982 | 0.4 | 1.4 |
| *o*-dichlorobenzene | y = 1.49x- 0.14 | 0.9982 | 1.2 | 4.1 |
| nitrobenzene | y = 1.24x - 0.09 | 0.9995 | 0.6 | 1.8 |
| *o*-methylnitrobenzene | y = 0.75x - 0.03 | 0.9997 | 1.2 | 4.1 |
| 1,2,4-trichlorobenzene | y = 0.80x - 0.08 | 0.9981 | 7.6 | 25 |
| *m*-methylnitrobenzene | y = 1.29x - 0.15 | 0.9988 | 1.6 | 5.4 |
| 1,2,3-trichlorobenzene | y = 0.81x - 0.10 | 0.9981 | 6.7 | 12 |
| p-methylnitrobenzene | y = 0.95x - 0.11 | 0.9988 | 0.8 | 2.6 |
| 1,2,3,4-tetrachlorobenzene | y = 0.77x - 0.07 | 0.9982 | 1.5 | 5.1 |

The enrichment factor (EF) obtained using *in-situ* NLPNE, calculated as $A_{NLPNE}$ *vs*. $A_{inject}$ of a spiked sample at 500 ng $mL^{-1}$, ranged from 0.04 to 1.00 ng $mL^{-1}$ and LOQs from 0.14 to 3.35 ng $mL^{-1}$ (Tab. 6.4).

*Tab. 6.4 In-situ NLPNE regression parameters, LOD, LOQ and EF, calculated in the range 5 - 500 ng $L^{-1}$.*

| Analyte | Linear Regression Equation | $R^2$ | LOD (ng $mL^{-1}$) | LOQ (ng $mL^{-1}$) | EF |
|---|---|---|---|---|---|
| toluene | y = 6541x+108174 | 0.9959 | 0.06 | 0.19 | 27.4 |
| chlorobenzene | y = 12057x + 57640 | 0.9964 | 0.04 | 0.14 | 29.4 |
| *m*,*p*-xylene | y = 10223x + 53889 | 0.9973 | 1.00 | 3.35 | 30.7 |
| *o*-xylene | y = 6311x + 13320 | 0.9990 | 0.09 | 0.31 | 32.1 |
| *p*-dichlorobenzene | y = 7793x – 8018 | 0.9993 | 0.39 | 1.29 | 30.6 |
| *m*-dichlorobenzene | y = 7876x – 6813 | 0.9985 | 0.14 | 0.48 | 32.0 |
| *o*-dichlorobenzene | y = 8837x - 24583 | 0.9981 | 0.78 | 2.61 | 21.9 |
| nitrobenzene | y = 30976x + 68446 | 0.9964 | 0.08 | 0.26 | 36.0 |
| *o*-methylnitrobenzene | y = 5428x + 71516 | 0.9959 | 0.11 | 0.37 | 37.6 |
| 1,2,4-trichlorobenzene | y = 2223x + 31740 | 0.9944 | 0.16 | 0.54 | 40.3 |
| *m*-methylnitrobenzene | y = 9986x + 107571 | 0.9929 | 0.24 | 0.80 | 36.5 |
| 1,2,3-trichlorobenzene | y = 2655x + 21203 | 0.9972 | 0.19 | 0.62 | 33.7 |
| p-methylnitrobenzene | y = 15072x + 160941 | 0.9975 | 0.28 | 0.96 | 29.2 |
| 1,2,3,4-tetrachlorobenzene | y = 410x + 6573 | 0.9981 | 0.24 | 0.79 | 33.1 |

### *6.3.1.4 Matrix effect*

The formula ME%=[1-(A-B)/C]·100% was used to evaluate the matrix effect (ME). A, B, C, were the analyte peak areas normalized by the deuterated internal standard (IS) in the spiked sample, in the unspiked sample and the standard solution, respectively. NaCl was added to the real sample to simulate the best ionic strength conditions, considering a native NaCl% equal to 3.5% and increasing this value to 20%, approximatively. A negligible matrix effect was shown for all the chlorobenzene derivatives (< 7%), followed by *o*-, *m*- and *p*-xylene (≈10%). The strongest matrix effect was noticed for toluene and nitrobenzene derivates (30-39%): the standard addition method is necessary for the quantitative analysis of these compounds and has been used for further compound quantification.

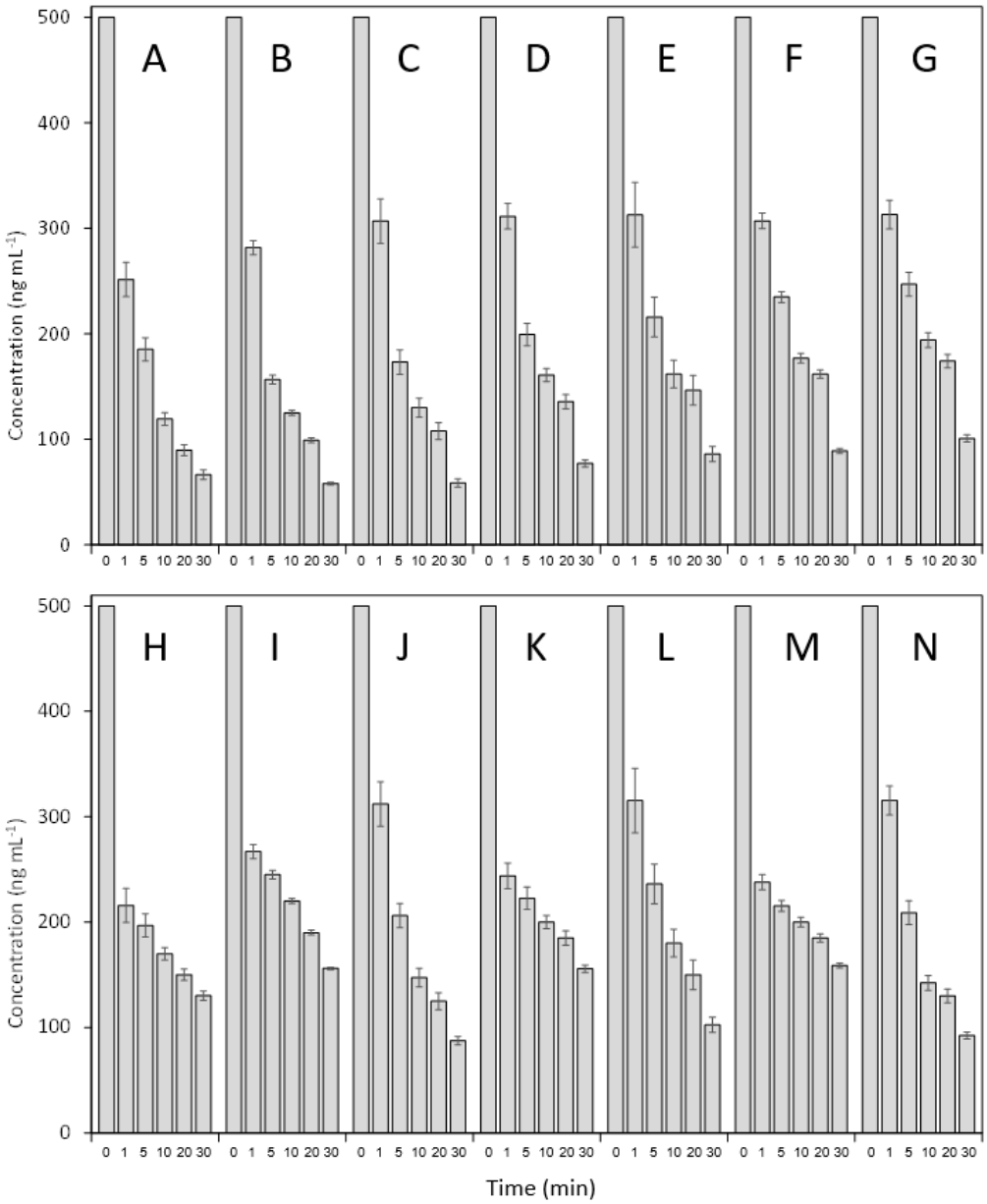



*Fig. 6.5 BDs volatilization as a function of time (0, 1, 5, 10, 20, and 30 min) obtained by in-situ NLPNE GC-MS. A: toluene; B: chlorobenzene; C: m and p-xylene; D: o-xylene; E: p-dichlorobenzene; F: m-dichlorobenzene; G: o-dichlorobenzene; H: nitrobenzene; I: o-methylnitrobenzene; J: 1,2,4-trichlorobenzene; K: m-methylnitrobenzene; L: 1,2,3-trichlorobenzene; M: p-methylnitrobenzene; N: 1,2,3,4-tetrachlorobenzene. Experimental conditions: extraction time: 90 s; desorption time: 30 s; %NaCl = 20% w/v.*

### 6.3.2 Real sample analysis and comparison with other extraction methods

The quantitative analysis of seawater samples from the Dalian Sea (China) was successfully applied through *in-situ* NLPNE coupled with GC-MS. In general, the BD content was a function of the distance of the sampling point from the industrial area, in agreement with the diffusion pattern of pollutants in water. The study of the volatilization rate of the BDs for the monitoring of the residual in water samples was carried out by maintaining sample vials opened in a clean atmosphere at 25° C for the monitoring of the residual BD content in water samples. The BDs concentrations in the function of time, starting from 500 ng $mL^{-1}$ are presented in Fig. 6.5 showing that this application can be conveniently used to obtain kinetic and thermodynamic information, through an easy and fast extraction procedure. The evaluation of NLPNE performances was carried out through the comparison with the existing and well-assessed procedures. The LLE and SPME methods already described in the literature for BDs analysis in seawater samples were used to compare extraction time, amount of used solvent, extraction efficiency, and LOQs (Tab. 6.5). The use of a very low solvent amount with short extraction times offers comparable results in terms of recovery, enrichment factor, and LOQs with other green techniques.

*Tab. 6. 5 Comparison of NLPNE with LLE and SPME for the determination of BDs in seawater matrix. NLPNE, LLE and SPME experimental conditions are described in the text [1].*

| | LLE | SPME | NLPNE | |
|---|---|---|---|---|
| | | | ***Ex-situ*** | ***In-situ*** |
| **Extraction time** | 10 min | 30 min | 1.5 min | 3 min |
| **Solvent volume** | 1000 µL | - | 1000 µL | 54 µL |
| **EF** | 0.8-1.1 | 3.5-94.9 | 0.8 - 1.2 | 21.9 - 40.3 |
| **RSD** | 6.1-30 | 5.9-29 | 1.8-9.6 | 6.8-17 |
| **LOQ (ng $mL^{-1}$)** | 8.4 | 12.2 | 0.7 – 15.4 | 0.2 – 3.3 |

## 6.4 Conclusions

The field of emerging contaminants analyses in terms of qualification and quantification is very challenging and even more substances appear and are incorporated into this class. The disadvantages of LLE and SPE led researchers to develop more suitable analytical techniques that match as well as possible the physicochemical characteristics of some of these contaminants like BDs with nanoconfined liquid phase nanoextraction (NLPNE) technique coupled with GC-MS.

Indeed, the two different NLPNE approaches, ex-situ, and in-situ modes have been proposed as efficient customized extraction techniques that meet good requirements in terms of extraction capabilities, analysis time, precision, and accuracy. Furthermore, these techniques provided a low solvent consumption and improved automation degree showing to meet the goals of green analytical chemistry. The great potential of the in-situ NLPNE technique in high-throughput analyses of emerging contaminants at trace levels has been demonstrated through the results of the method validation in terms of evaluation recovery, reproducibility, linearity, the limit of detection and quantification, enrichment factor, and matrix effect.

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# Chapter 7 Conclusions

The aim of this PhD thesis consisted in the development and optimization of analytical methods for the determination of some emerging contaminants in food and environmental matrices coupling liquid and gas chromatography with mass spectrometry. Innovative extraction methods suitable in terms of simplicity, efficiency, rapidity, sensitivity and reproducibility were designed and applied for trace-level determinations of pesticides, PCBs, benzene derivatives and matrine-based alkaloids. Part of the research activity was performed in a food inspection laboratory (BonassisaLab SRL) located in Foggia, in the south of Italy. Moreover, a work period was spent in a foreign university (Yanbian University, Yanji, China).

The wide use of pesticides, chlorinated compounds, pharmaceuticals, etc. has resulted in a widespread diffusion of residues in the environment (air, ground, and surface water) and along the food chain. Contaminants of emerging concern may have an impact on humans and animals, even if not always there are sufficient large-scale evidence to prove the association with serious and adverse effects on the human body. Environmental and health adverse impacts cannot be ignored, and in-depth studies are required to clarify these aspects and standardize the legislative provisions in Europe and the world. Indeed, shared international regulatory systems and specific classification criteria are not always available. Therefore, validated and robust analytical methods to measure low-level concentrations of known and lesser-known emerging contaminants are necessary for human health and research purposes.

In the present PhD research project, specific and reliable analytical methods for the sensitive determination of pyrethroids, PCBs, and benzene derivatives have been developed by GC-ECD and GC-MS in food and environmental samples. Optimized chromatographic conditions, combined with minimal sample preparation and cleanup, ensured good results in terms of rapidity, response sensitivity, and separation efficiency. The Box-Behnken experimental design and the global desirability functions were successfully applied to determine the optimal extraction conditions. The results of the method validation, performed according to the European Commission directives, demonstrated that the proposed methods are well suited to satisfy the demand of the accurate check analyses of complex food matrices. Finally, an accurate and reproducible analytical method has been proposed for the analysis of matrine-based biopesticides by LC-MS/MS. The optimized quick sample extraction procedure allowed to perform the simultaneous extraction and clean-up of more than 15 samples in an 8-h single-day working session. The results of the method validation, performed according to the European Commission directives, demonstrated that the proposed method is well suited to satisfy the demand for the accurate determination of quinolizidine alkaloids in fruit and vegetable samples. The contamination grade

estimated through the analysis of a high number of vegetable samples emphasizes the need for reliable analytical methods for the determination of matrine-based pesticides in official check analyses and monitoring studies.

In conclusion, the research activity of the present project, through the development of proper analytical tools for the determination of emerging pollutants should actively contribute to a better understanding of the impact of these substances on health and the environment; evaluate the presence at trace levels in food matrices; to clarify the fate of these compounds along the food chain; to minimize the exposure levels, overall improving the quality of life of human beings.

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