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„Pesticides in essential oils"

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Abstract

The use of essential oils worldwide is constantly rising and they have become commercially available in the form of many „over the counter" products (OTC) in recent years. Essential oils already have an established use in folk and western medicine as remedies against inflammation, respiratory diseases, infections, for aromatherapy, as insect repellents, food preservatives and in perfume and cosmetics industries among other things. However, higher demand leads to more production and higher yields, which in turn requires increased pest management. But, pesticide residues are already present in the environment and thus, also in aromatic plants used for EO production. The control of pesticides in EOs is therefore essential especially with the recent trends in EO consumption and ever-growing use of medicinal plants as a primary medication for minor health issues. This paper is an overview on possible methods used for determination of pesticide residues in essential oils, covering the work of the scientific community in the last 20 – 25 years, with the scope on sample preparation, chromatographic separation and instrumental detection and quantification.

Zusammenfassung

Die weltweite Anwendung von ätherischen Ölen ist ständig im Aufstieg und die sind in letzter Zeit gewerblich als „over the counter" (OTC) Produkte frei vorhanden geworden. Ätherische Öle haben bereits bestehende Anwendung in Volks- und westlichen Medizin als Abhilfe gegen Entzündung, respiratorischen Krankheiten, Infektionen, als Aromatherapeutika, Insektenabwehrmittel, Konservierungsmittel für Lebensmittel, als auch in Parfüm- und Kosmetikindustrie. Jedoch, höherer Bedarf fördert vermehrte Produktion und Ausbeuten, die weiter gesteigerte Schädlingsbekämpfung bedürfen. Doch, Pestizidrückstände sind schon in der Umgebung vorhanden, und folglich auch in aromatischen Pflanzen, die als Urpflanzen zur Gewinnung von ÄÖ dienen. Daher ist die Pestizidkontrolle in ÄÖs wesentlich, besonders mit den letzten Trends des immer wachsenden Verbrauchs von ÄÖs und medizinischen Heilpflanzen als primäre Heilmittel gegen leichtere Gesundheitsprobleme. Diese Arbeit ist ein Überblick möglich anwendbarer Methoden zur Bestimmung von Pestizidrückständen in ätherischen Ölen und schliesst die Arbeit der wissenschaftlichen Gemeinschaft in den letzten 20 – 25 Jahren ein, mit dem Umfang von Probenvorbereitung, chromatografischer Trennung und instrumenteller Nachweis und Quantifizierung.

ABREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
4,4'-DCBP	4,4'-Dichlorobenzophenone
BCH	Benzene-hexachloride
ChE	Choline-esterase
D	Dalton
DAD	Diode array detector
DER	Drug/extract ratio
DDT	Dichloro-diphenyl-trichloroethane
DMSO	Dimethyl sulfoxide
ECD	Electron-capture detector
ECG	Electrocardiogram
EDC	Endocrine-disrupting chemical
EI	Electron ionization
ELISA	Enzyme-linked immunosorbent assay
HRGC	High-resolution gas chromatography
FID	Flame-ionisation detector
FPD	Flame-photometric detector
GCB	Graphitized carbon black
LAK	Lymphokine-activated killer
LDH	Low density cholesterol
LOD	Limit of detection
LOQ	Limit of quantification

MDD	Maximal daily dose
MRL	Maximum residue level
MRM	Multiple reaction monitoring
MS	Mass-spectrometry
OC	Organochlorides
OP	Organophosphates
Ph. Eur.	European Pharmacopoeia
ppm	Parts per million
PSA	Primary-secondary amine
QTOF	Quadrupole-time-of-flight
QqLIT	Quadrupole-quadrupole-linear ion trap
QqQ	Triple-quadrupole
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
rcf	Relative centrifugal force
RL	Reporting level
RSD	Relative standard deviation
RT	Retention time
SIM	Selected ion monitoring
UPLC	Ultra-pressure-liquid-chromatography

CONTENTS

1. Introduction

1.1 Pesticides

Pesticides have been used for a long time; for example, ancient Mesopotamians dusted their crops with sulphur [1] and Pliny the Elder (AD 23–79) postulated that arsenic could be fatal to insects [2]. Moreover, arsenic sulphide was used by the Chinese in the late sixteenth century and their use has continued until the early decades of the twentieth century [2].

Pests such as fungi, bacteria, rodents, weeds, insects, mites, or viruses often cause crop diseases, feed or live parasitically on them, or compete for nutrients. Before synthetic-organic pesticides were developed, inorganic compounds were used as pesticides which contained e.g. mercury, selenium, antimony, fluorine etc. as their active ingredients [2]. However, these inorganic compounds were usually not effective nor selective in the control of pests.

In the 1930ies and 1940ies, the first synthetic organochlorides (OCs) were discovered as effective pesticides. The first two OCs that were discovered were benzene-hexachloride (BHC) and the widely known insecticide dichloro-diphenyl-trichloroethane (DDT) [2]. The success of these first OCs resulted in the further discovery of thousands of new pesticidal compounds. Moreover, with increased

Table 1.1 Classification of major pesticides according to the target organism

Pesticide type	Target
Insecticide	Insects
Fungicide	Fungi
Herbicide	Plants
Molluscicide	Slugs, snails
Rodenticide	Rodents
Acaricide	Mites
Nematicide	Nematode worms

Table 1.1 Classification of pesticides according to the target organism [3]

understanding of metabolic pathways of plants the first selective herbicide was introduced, 2,4-dichlorophenoxyacetic acid (2,4-D), which inhibits the growth of dicotyledonous, sparing monocotyledons plants [2].

Due to enormous number of substances used as pesticides, and lack of exact scientific classification thereof, they are typically categorised by the type of pest they control or kill (Table 1.1), chemical composition (organochlorines, organophosphates, carbamates, pyrethroids, etc.), or mode of action [2], [3].

1.2   Pesticides, humans and wildlife

By some estimates, less than 0.1% of the pesticide used actually reaches the target organism; the rest enters the environment, contaminating soil, water, air and non-target organisms [4]. Pesticides are applied using spray and granulate formulations [4] by which they contaminate the soil and air, after which pesticides can migrate via rainfall, irrigation runoff or wind over a distance of a few meters to several hundred kilometres [5]. Pesticides especially lipophilic ones, are slow-degrading and therefore persistent in the environment [6]. Because of this, it is possible that even neighbouring crops are contaminated with pesticides, even if not directly applied locally (e.g. organic crops). This makes medicinal plants collected in the wild, as well as those purposely cultivated, eligible for contamination [2].

As a direct consequence of the contaminated environment, pesticide enters the food chain and accumulates in the fatty tissues of plants and animals. Their lipophilic properties hamper excretion of the pesticides from the organism leading to increasingly higher concentrations in the food chain, as is referred to as biomagnification. Consequently, even though the use of DDT was banned in the U.S.A. in 1972, human biomonitoring studies indicate that most people in the United States had detectable levels of DDT in their bodies in 2005 [7].

Due to the intensive use of pesticides worldwide, the entire human population is likely exposed to pesticides [5]. Exposure to pesticides can occur after ingestion (including dietary supplements and medicinal products containing preparations of herbal origin), inhalation, skin contact and can even be passed on through the placenta and breast milk [3], [8], [9]. Since pesticides present a diverse group of compounds,

there is a wide variety of possible interactions with cellular structures and biological molecules *in vivo* [3]. Therefore, pesticides pose a health risk, especially upon continued exposure. Thus, the quantities of pesticides found in final products must be as low as possible and threshold quantities are strictly regulated by the so-called maximum residue levels (MRLs).

For many pesticides, there is now a more detailed understanding of the potential biological effects *in vivo*. For example, organophosphates can affect immune response *in vivo* and *in vitro,* by inhibiting NK cells, lymphokine-activated killer (LAK) cell, and cytotoxic T-lymphocytes (CTL) activity, along with effects on antibody and IL-2 production, T-cell proliferation, as well as decreased CD-5 and increased CD-26 cells and antibodies [10]. Further on, severe exposures to organophosphates (OPs) cause acetylcholine-related neurotoxic disorders in humans [6], [11], such as the cholinergic syndrome, the organophosphate-induced delayed polyneuropathy (OPIDP), chronic organophosphate induced neuropsychiatric disorder (COPIND) [11].

The OC residues are linked to insulin resistance, and therefore the development of type 2 diabetes [2] as well as the vitamin D deficiency in humans [12]. Organochlorines such as atrazine and vinclozolin act like endocrine-disrupting chemicals (EDCs), and have been implicated in thyroid function alteration and decreased estradiol and testosterone levels, possibly leading to menstrual irregularities, gestational diabetes, infertility, and even fetal death related to congenital birth defects [9], [12]. Further on, occupational exposure to dieldrin and heptachlor is suspected to be partially responsible for increased risk of Parkinson's disease. Additionally, a Chinese study showed that a prenatal exposure to OCs leads to decrease in birth weight of newborns [12].

Professional exposure to methomyl, a carbamate insecticide, affects cholinesterase (ChE) activities, changes in the ECG, and the serum LDH levels, thus leading to cardiovascular diseases [5]. Research also implicates that pesticide exposure plays a role in the development of different tumours and cancers [9], while other studies show, that occupational exposure to pesticides, can be associated with numerical aberrations in chromosomes of the sperm cells [13].

The extensive negative effects of pesticide formulations befall wildlife as well and therefore have a negative effect on the ecosystems. Worms take up pesticides released into the soil and are then ingested by birds, increasing mortality and reduced hatchability [12]. „*The range of chemical effects on adult birds covers acute mortality, sub-lethal stress, reduced fertility, suppression of egg formation, eggshell thinning and impaired incubation and chick rearing behaviors*" [12]. Exposure of DDT and its metabolites is one of the major causes for eggshell thinning in the bald eagle population in the US. In addition to killing birds, pesticides are toxic to fish, beneficial insects and plants. They also affect microorganisms in the soil, which further leads to reduced soil fertility [6].

Thiamethoxam   *Imidacloprid*
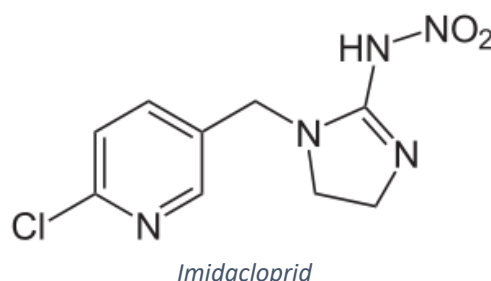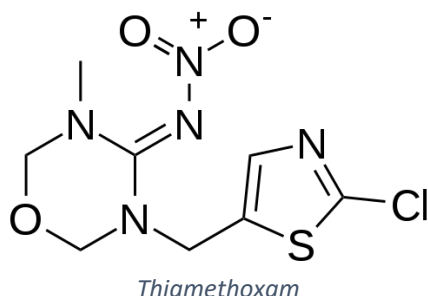
Another significant non-target organism is the honey bee (*Apis mellifera*). Bees are important pollinators of agricultural crops and wild flora and essential providers of ecosystem services. However, they are not spared from pesticide exposure, in fact, bee populations around the world are in decline in recent years. As far as pesticide effects are of concern, bees are primarily affected by neonicotinoid insecticides, which are widely used in agriculture. Nicotinoids' mode of action is based on constant excitation of nicotinic acetylcholine receptors in insects, eventually leading to death. Studies implicate that pesticides are influencing bee behaviour, reduce locomotion and time spent at a food source, limit the movement to light and increase worker mortality [14], [15].

1.3 Essential oils

Essential oils are heterogenic mixtures of up to 200 different volatile lipophilic compounds and can be obtained from raw plant material by a few methods of extraction, namely water vapour distillation and cold pressing. On the other hand, organic solvent extraction and innovative methods such as supercritical fluid extraction, ultrasound-

assisted extraction or microwave assisted extraction and their improvements and modifications may offer shorter extraction time or higher extraction efficiency [16], [17] and produce aromatic oils but not essential oils.

Throughout history, natural EOs have served as food flavours and additives, medicines, aphrodisiacs, or even during cult rituals. Nowadays, they also find application in food preservation, aromatherapy, as insect repellents, cosmetics, perfumes, as well as possible new green pesticides in agriculture [18]. Their medicinal value and a variety of other possible applications have led to an increasing market demand for essential-oil-based phytopharmaceuticals as an alternative to western medicine in recent years [18].

2. Pesticide analysis in Essential oils

2.1 Introduction

The yields of some EOs can be lower than 1 %, whereas other EOs produce up to 15% or more [19]. But, for most commercially used essential oils, like anise, peppermint, eucalyptus or citrus oil, the average yields do not usually exceed 5 % [20], [21]. Although relatively small amounts of essential oils are extracted from medicinal herbs, the fact that both EOs and pesticides are lipophilic in nature indicates that transfer of pesticides into the oil during the extraction process is not only likely [22], [23] but also leads to an intensification of pesticide concentrations.

A successful detection and quantification of pesticides and their residues depend on various factors. Before the pesticide finally arrives at its final destination, i.e. drinking water, food or an essential oil, it has possibly undergone chemical modifications. Pesticide chemistry can be changed by plants, animals, microorganisms, and environmental factors like soil composition or sunlight, but the extraction process as well [2], [24].

Pesticides are often applied in the form of aerial sprays and get into living plants via absorption by stems and foliage or adsorb on their surface. Another path of entry is root-uptake. Inside a plant, pesticides undergo translocation by means of xylem and phloem and are metabolised in different parts of the plant [25]. „*Metabolism of*

pesticides may involve a three-phase process" [24]. In phase 1, pesticides are oxidised, reduced or hydrolysed, giving more hydrophilic and less toxic compounds. In phase 2, a further increase in hydrophilicity and decrease in toxicity is achieved when these compounds are conjugated with an amino acid, sugar or glutathione and may be stored in organelles of the cell. Finally, the third phase leads to secondary conjugation and/or incorporation of pesticides and their metabolites into biopolymer molecules [24] or possibly, even into pathways of essential oil biosynthesis thus, influencing the essential oil composition [26].

When produced by water distillation, pesticides from the entire plant material (leaf, flower herb, etc.) and not only oil-containing cells and tissues are affected by the steam and are eventually co-extracted into the essential oil. Here, pesticide lipophilicity, volatility, and thermostability are of importance [23]. In case of citrus oils, often the entire fruit is destroyed to release the juice, thus releasing pesticides found in all fruit parts. The oil is then separated from the watery emulsion usually by means of centrifugation [27]. In this case, only lipophilicity is influential for co-extraction [23]. Therefore, pesticide transfer and their concentration in phytopharmaceuticals, i.e. essential oils are influenced by the extraction conditions [28]. In contrast to cold-pressing, steam distillation is conducted under high temperatures which may lead to degradation of pesticides, provided they are thermolabile. All these modifications can be problematic, making the analytical process more demanding and resulting in transformation products potentially more toxic than the parent compounds [2].

The global use of herbal medicines is constantly rising and they have become commercially available in the form of many „over the counter" products (OTC) in recent years [2], [22]. Therefore, any amount of pesticide residues, but also biological contaminants, heavy metals or mycotoxins essential oils may contain, comes in direct contact with the end consumer and raises questions about their quality and safety.

European Pharmacopoeia (Ph. Eur.) general monograph *Herbal drugs* suggests general and supplementary tests on essential oil quality, including tests for pesticide residues. However, in a Ph. Eur. general monograph *Essential oils* there is no such requirement. In the general chapter *Pesticide residues,* the concentration of pesticide residues that can be found in herbal drugs is in Table 2.8.13-1, or Regulation EC 396/2005, (containing MRLs for various pesticides), and is expressed in mg/kg

referenced to the fresh or dry herb. A general default value of 0.01 ppm applies where a pesticide is not specifically mentioned [29]. In case of essential oils, MRLs should be calculated in consideration to acceptable daily intake for each pesticide, maximal daily dosage for the essential oil, drug/extract ratio, and the body mass of the patient, using the following equation:

when DER > 10; $\qquad (ADI \times M)/(MDD \times 100)$; and

when DER ≤ 10; $\qquad MRL \times DER$;

with DER being the drug/extract ratio; ADI the acceptable daily intake, in mg/kg body mass; M the body mass in kilograms (60 kg); and MDD the daily dose of the herbal drug preparation, in kilograms [23], [29].

2.2 General analytical approach

Over time different methods have been implemented into the analysis of pesticides in essential oils. In general, the first step in the analysis of any sample is the clean-up, required to eliminate compounds that would interfere with the determination of target analytes. The second step often involves separation either by a liquid or gas chromatography (LC or GC), making each analyte accessible to the detector, i.e. the third step.

2.2.1 Sample preparation

For sample preparation, different methods have been used, mostly based on liquid-phase or solid-phase extraction, where matrix components are dissolved usually in an aqueous phase, and the analytes remain in a water-immiscible liquid or a solid phase, after which they are washed out. The next step involves solvent removal and analyte enrichment, making low concentrated residues available to the detector [2]. The purpose of sample preparation is to remove interfering compounds, improve the detection of residues and achieve lower limits of detection and quantification [30].

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) and its modifications have become a broadly used method since it requires small amounts of used chemicals and is relatively fast and effective. It involves liquid extraction with

acetonitrile and NaCl, MgSO₄ and buffering salts for phase-separation and pH-adjustment to obtain a raw extract, and solid phase extraction step, often dispersive solid-phase extraction, by mixing it with MgSO₄ and a sorbent to remove water and unwanted co-extracted compounds. The final extract can be analysed directly by GC-and/or LC-techniques [22], [31].

Since essential oils share physicochemical similarities to pesticides, matrix effects can also be significantly eliminated by evaporation and resolution, or by diluting the essential oil sample, as seen in some papers.

2.2.2 Chromatography

For pesticide separation, two most used methods in the essential oil analysis are liquid and gas chromatography. As any chromatographic method, they are based on the analytes' affinity between a stationary and a mobile phase. In LC a hydrophilic liquid is used as the mobile phase to carry the compounds along the lipophilic column and is typically implemented for separation of less volatile compounds. To speed up the elution process, a lipophilic solvent is often gradually introduced into the elution liquid. On the other hand, a GC depends on an inert gas as a mobile phase and is more suitable for analysis of volatiles. A temperature increase during a GC separation is implemented, to change the analyte affinities in favour of the gaseous phase, thus making them more mobile. Both methods can be coupled with a variety of detectors, with different sensitivity and selectivity against different compounds [32].

2.2.3 Detection methods

An electron capture detector (ECD) is sensitive to halogenated compounds and is therefore suitable for detection of organochlorine pesticide residues. Conventionally, it uses a radioactive source (^{63}Ni) to emit electrons, which then collide with and excite the molecules of a so-called makeup gas (usually nitrogen) releasing even more free electrons. As they move toward a positive anode, they generate a current which can be measured. Molecules of analytes capture some of the electrons on their way to the anode, thus reducing the signal of the current [2], [33].

The flame ionisation detector (FID) is commonly used with GC/LC. The flame is usually generated by hydrogen-air mixture, which is previously mixed with the eluting sample. When the analytes are burned, electrically charged products are formed and collected at an electrode (collector plate) above the flame. The ions hitting the plate induce an electrical current, whose response is measured and proportional to the amounts of ions present. A flame photometric detector (FPD), functions in a similar way, whereupon being excited by the flame, atoms emit light, characteristic for each species. The emitted spectra are then filtered for the desired wavelength and monitored by a photomultiplier tube. Virtually all hydrocarbons are detectable by the FID, whereas FPD is most sensitive to sulphur and phosphor containing compounds, making it a good choice for detection of organophosphorus pesticide residues [32], [34], [35].

Another method that enables pesticide residue detection is mass spectrometry (MS). In a usual MS measurement, the analytes are ionised and/or fragmented in the ion source and are then separated according to their mass-to-charge ratio (m/z), commonly by subjecting them to an electric or magnetic field and thus, accelerating the, towards the detector, ordinarily an electron multiplier.

For particle ionisation, various methods have been implemented so far, but in pesticide residue detection in essential oils, electron ionisation (EI) or electrospray ionisation (ESI) have been often used. In EI technique, the electrons are generated by a thin metallic ribbon (cathode or filament), by heating and channelling an electric current through it. The electrons are cast in the direction of the anode and collide with the sample molecules, generating ions and or ion fragments. The usual voltage difference between the filament and the anode is 70 V, generating electrons of 70eV. ESI involves passing the sample solution through a needle (cathode) subjected to high voltage (4-5kV) creating charged, fine droplets. The solvent then evaporates from the sample, leaving particles of the same charge tightly packed and finally they disperse toward the analyser due to electrostatic effects [32], [36].

After ions are formed, they are separated in the analyser by their m/z under the influence of electric or magnetic fields. In a time-of-flight (TOF) MS, however, no such fields are used. Instead, accelerated ions move through the flight tube at velocities governed by their mass, making those with lower m/z arrive at a detector before those with a higher m/z. In a magnetic MS, ion separation occurs on a deviated flight path

under the influence of a magnetic field. Influenced by their m/z ratio, ions' trajectories will bend according to the strength of the magnetic field. In a quadrupole MS four metallic rods are arranged parallelly to one another and are electrically connected. Forces of direct current (DC) and radio frequency (RF) between the opposing rods make the ions oscillate in between based on their m/z and reach the detector, while unwanted ions collide with the rods. A further improvement of a quadrupole MS is a quadrupole ion trap functioning on the same basic principle as the quadrupole. However, instead of rods, the trap is constructed of two conical electrodes and one "donut-shaped" ring lens, where ions are trapped, held for a few milliseconds and then released into the detector [32], [36].

As far as detectors are concerned, often it is an instrument that amplifies and records currents produced when an ion encounters the detector. Typically, an electron multiplier is used, where a single ion hits the detector plate thereby causing an electron cascade which amplifies the signal strength. Also used are array detectors, where a scintillator plate converts the ion energy into photons that are afterwards amplified and detected [32], [36].

These separation and detection methods are often combined and coupled forming GC and LC -ECD, -FID, -FPD, -MS, as well as -MS/MS, -QTOF and other in pursuit of higher selectivity and sensitivity.

2.3 Cold-pressed EOs

2.3.1 Lemon oils

Cold-pressed citrus oils are widely used in many fields, including food, flavours, cosmetics and pharmaceutical industries [27], [37], [38]. Dellacassa et al. (1999) [39] investigated the presence of organophosphorus and organochlorine pesticide residues in Uruguayan lemon essential oils produced industrially by FCM extractors in 1995. Twenty-two samples obtained from the northern part of the country and nine from the South. Standard solutions of organophosphorus and organochlorine pesticides were prepared in n-hexane. Pesticides were separated by HRGC and quantified against bromophos-methyl as an internal standard. Detection and

quantification were achieved with an FPD for OPs and ECD for OCs against pesticide standard solutions (30 OPs and 10 OCs).

The organophosphorus pesticide separation was performed using Dani GC coupled with Mega 68 silica stationary phase column (25 m x 0.25 mm, 0.45 µm thickness), by direct injection, with no previous sample preparation. The injector was set for split mode (1:1) and programmed temperature vaporisation (PTV) from 65 to 240 °C at 999 °C/min. Oven temperature was 75 °C for 5 min after which it was heated to 100 °C at the rate of 7.5 °C/min, then up to 170 °C at 2 °C/min (held for 5 min) and then to 250 °C at 10 °C/min. Helium served as a mobile phase at 2 ml/l. Pesticide detection was achieved at 250 °C.

Before organochlorine pesticides could be analysed, a clean-up step was performed. The clean-up was achieved on a silica gel (8g, activated for 3h at 550 °C) filled column with 30ml dichloromethane; after the first 12 ml were discarded, the rest was collected and evaporated to 0.5 ml, after which 0.2 ml internal standard (bromophos-methyl) was added. This approach yielded very good pesticide recoveries from 94.2 ± 2.1 % (endrin) to 105.6 ± 2.3 % (dicofol).

Organochlorines were analysed on Carlo Erba 5300 Mega Series GC coupled with an ECD and a Shimadzu data processor C-R3A. The injection was done in split mode (1:10) at 230 °C. Silica fused capillary column (30 m x 0.22 mm, SPB-5, 0.25 µm film thickness), heated from 150 °C to 230 °C at 2 °C/min, then to 280 °C at 10 °C/min, was used, with hydrogen as a carrier at 2.2ml/min. Detector temperature was 280 °C.

No organochlorine residues were detected, or they were present in concentrations lower than their detection limits (14-45 µg/l). However, all lemon oil samples were contaminated by OPs, i.e. chlorpyrifos and methidathion. Northern Uruguayan oils had average amounts of 0.32 mg/l and 0.52 mg/l of chlorpyrifos and methidathion, respectively. Southern oils were more heavily polluted, with an average of 1.09mg/l for chlorpyrifos and 18.82 mg/l for methidathion, which reflects on different agricultural in northern and southern regions of Uruguay.

Barrek et al. (2003) [40] developed simple and rapid liquid/solid extraction procedure for extraction and analysis of pesticides. Lemon oil (0.5ml) was spiked with 20 pesticides (2 to 30 ppm) and fused with 0.5 ml pentane. After homogenisation in an ultrasonic bath (15min), 400µl of this solution was transferred on a previously equilibrated (5ml pentane at 1 ml/min) Florisil cartridge with an HPLC syringe at 200µl/min. Most of essential oil matrix components were then eluted with 5ml pentane at 1ml/min, leaving pesticides on the solid phase. Pesticides were then washed out with 5 ml dichloromethane at the same flow rate. Before analysis, the sample was concentrated under a stream of nitrogen at 30 °C. The method was repeated 3 times for recovery evaluation.

Separation of twelve pesticides was achieved on a BPX5 column (50m × 0.25 mm 0.25 µm thick film) installed on HP 6890 gas chromatograph. 1µl sample was injected at a temperature of 250 °C in splitless mode; initial oven temperature was 50 °C for 1 min, heated to 150 °C at 30 °C/min (expulsion of EO components), then to 300 °C at 4 °C/min (pesticide separation) and held for 10 min. Helium flow rate was 1.2 ml/min and for ionisation, EI was used (70eV). Transfer line was maintained at 280°C and the quadrupole at 150°C. For detection, selected ion monitoring (SIM) mode was used.

HPLC-MS was conducted on an HP 1100-MSD setup with C_{18} column (150 mm × 2.1 mm, 3.5µm diameter particle), fitted with a pre-column (10mm × 2.1mm) as a stationary phase. Before sample was eluted, it was filtered through a 0.45 µm pore Teflon filter. Ten pesticides were separated by gradient elution. Water (pH=10, NH_4HCO_2 and NH_3) and methanol were used at a constant rate of 0.3 ml/min, starting with 12 % methanol in the first 9.5 min, then 53.9 % for 1 min, 63.4 % for 13 min and 100% methanol in the last 16.5 min. A UV diode array detector (DAD) set to λ=210 nm was used to detect light absorbing compounds. High pH values of the mobile phase are troublesome for positive electrospray ionisation (ESI). Therefore, a methanolic solution of formic acid (1 % v/v) was added at the UV detector. Nebulizer pressure and temperature were held at 60 psi and 60 °C, and ionisation voltage was kept at 60 V for 15 min, 80 V for 5 min, 60 V for 3.5 min, 80 V for 1.5 and 60 V for the last 10 min.

HPLC-MS and GC-MS methods were both successful in detecting pesticides after the extraction. Moreover, these methods were eligible for detection of not only organophosphates and organochlorines, but carbamates, pyrethroids, neonicotinoids benzimidazoles and benzoylureas. The extraction recoveries calculated after GC-MS analysis were in the range of 67 and 95 %, while HPLC-MS yielded slightly lower ones, i.e. 50 and 94 %. In addition, chlorpyriphos and bifenthrin were analysed by both GC–MS and HPLC–MS with recoveries of 79.7 % and 86.5 % and 58.2% and 50.2 %, respectively, thus indicating more notable matrix interferences in HPLC–MS. However, limits of detection obtained after HPLC-MS were somewhat better (from 0.02 - 0.06 mg/l), in comparison to GC-MS method (from 0.03 - 0.50 mg/l).


Chlorpyrifos
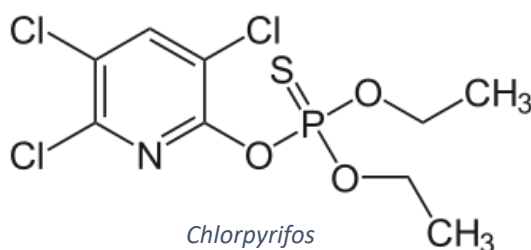
2.3.2 Lemon, orange, mandarin, and bergamot oils

Saitta. et al. (2000) [41] reported the presence of organochlorines in cold-pressed lemon, orange, mandarin, and bergamot essential oils from fruit grown between 1991 and 1996 in Italy, using similar instrumental techniques. Altogether a total of 539 EO samples were screened. Clean-up was executed in the same way as in the aforementioned study and yielded almost identical recoveries [39]. For recovery calculations, ten pesticides were added to a distilled lemon oil in a concentration of 1 ppm (mg/l); after clean-up, bromophos-methyl was added as internal standard and analysed by GC-ECD.

The analysis was performed on a double-channel Shimadzu GC-17A system with 2 columns (Restek RTX-5 and RTX-1701, both 30 m × 0.32 mm, 0.25 μm film thickness), each coupled with an ECD. Injection temperature was held at 230 ℃ and set for the splitless mode. After an initial 2 min hold at 50 ℃, the temperature increased to 150 ℃ at 25 ℃/min, then to 270 at 4 ℃/min and held for 20 min. Helium served as a carrier at a constant rate of 36cm/s. ECD temperature was 280 ℃.

For result confirmation a Finnigan GCQ system fitted with DB-5 column (30 m × 0.25 mm, 0.25 µm film thickness) mass spectrometer was used, with a helium flow of 40 cm/s; splitless mode. The injector temperature was 250 ℃, oven 60 ℃ to 250 ℃ at 15 ℃/min, with 14 min hold, and the transfer line was held at 275 ℃. Particles were activated via electron ionization (EI) and scanned in the mass range of 40 to 500 Dalton (D).

Dicofol   *Tetradifon*
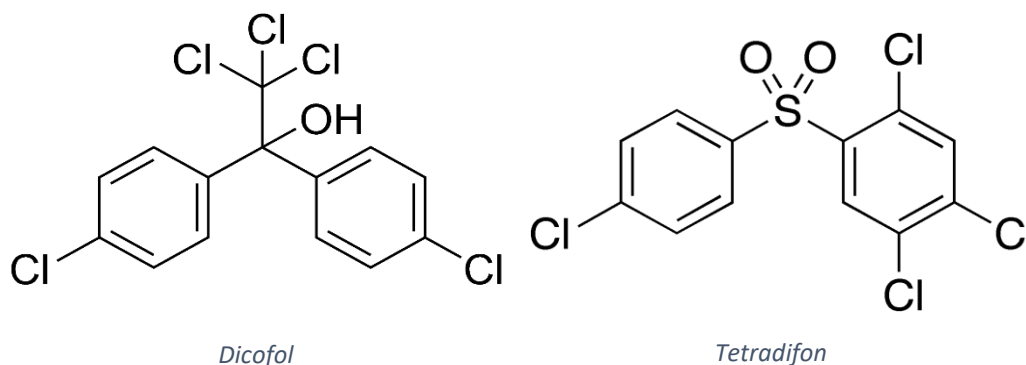
The analysis of these essential oils showed the presence of tetradifon, dicofol and 4,4'-dichlorobenzophenone (4,4′-DCBP), a dicofol decomposition product. These were found in more than 90% of the samples produced in 1991, save for bergamot oil (50%). „*Over the course of the study dicofol and tetradifon residues steadily decreased; the percentage of contaminated samples reflects this course and decreases considerably from 1991 to 1996*" [41]. The mean concentrations of dicofol and tetradifon also diminished significantly across all samples over the same period with lowest being bergamot EO samples (dicofol 0.2→0.05 mg/l and tetradifon 0.08→0.03).

2.3.3 Bergamot oil

Contamination of bergamot essential oils produced in 1999-2000 was further explored by Di Bella et al. (2004) [42]. Fifty-five bergamot oil samples produced in Calabria (Italy), in the years 1999 and 2000 were selected for screening. Standard solutions of OPs (diazinon, methyl chlorpyriphos, methyl parathion, ethyl chlorpyriphos, ethyl parathion, quinalphos, methidathion, ethion, methyl azinphos, ethyl azinphos) and OCs (aldrin, dieldrin, p,p'-DDE, o,p-DDD, endrin, p,p'-DDD, o,p-DDT, p,p'-DDT, dicofol and tetradifon) were prepared in n-hexane and stored at 5 ℃. Organophosphate determination was carried out with an HRGC-FPD, by direct delivery

of samples to the column. Prior to HRGC-ECD analysis, organochlorines were subjected to a clean-up procedure, previously used by Dellacassa et al. (1999) [39]. Also, the same instrumental setup and analysis conditions as before were applied for GC-FPD analysis [39] and for GC-ECD and GC-MS [41]. Analysis showed no traces of organophosphates and presence of 4,4'-DCBP, dicofol, tetradifon in mean concentrations of 0.16, 0.26 and 0.06 mg/l in 1999 and 0.09, 0.20 and 0.06 mg/l in 2000, respectively. „*The results reviewed here provide evidence that the levels of contamination in bergamot essential oils from the crop year 1999 and 2000 are low and do not represent a risk from the toxicological point of view"* [42].

## 2.3.4 Conventional and organic lemon and sweet orange oils

In a study published by Verzera et al. (2004) [26], ten samples of each lemon and sweet orange oils, produced from fruit grown in 2002 by traditional and biological methods, were analysed. These oils were evaluated for OC and OP residues, as well as for differences in EO composition. They aimed to determine if pesticides altered EO composition. Identification of EO components was carried out on an HRGC-MS apparatus and for quantification purposes, a GC equipped with flame ionisation detector (FID) was used.

The determination of OPs was conducted with GC-FPD under same conditions as in [39], and organochlorines were quantified using the GC-ECD setup as described by Saitta. et al. (2000) [41]. OPs contained in traditional sweet orange oils were methidathion, methyl-parathion, ethyl-parathion and methyl-chlorpyriphos in a total amount of 2.55–2.86 mg/l. In traditional lemon essential oils two additional organophosphorus pesticides were found i.e. ethyl-azinphos and quinalphos with total concentrations of 3.52–3.85 mg/l. Biological oils were also contaminated, however in considerably lower degree. Only methidathion and ethyl-chlorpyriphos were detected in orange oil samples, at a total of 0.27 mg/l, combined. Among lemon oils, methidathion methyl- and ethyl-parathion were discovered with a sum of 0.74 mg/l. „*Among organochlorine pesticides, only dicofol was detected and only in lemon and sweet orange oils obtained from fruits grown under traditional agricultural methods, with maximum values of 1.20 ppm (orange oils) and 1.0 ppm (lemon oils)"* [26].

Concerning the influence of pesticides on EO quality, the same constituents were detected in traditional and *bio* orange oils, and the individual components were within the range set for genuine orange oil. Even so, relevant variations of the average quantity of aliphatic, terpene aldehydes and carbonyl compounds were reported. In case of lemon oils, similar observations were noted, mainly concerning terpene aldehydes, but to a lesser extent. „*Pesticides could act in two ways: influencing biochemical processes that govern the formation of aldehydes compounds, or reacting with them, in both cases reducing, as a consequence, the essential oil quality*" [26]. Still, further research is required to confirm what effects pesticides may have on the biosynthesis of essential oil if any.

2.3.5 Organic lemon, orange, clementine and mandarin oils

Di Bella et al., (2006) [37] also investigated the presence of organochlorines, organophosphates and plasticizer residues in citrus EOs of organic origin in 2005. Sicilian EOs were produced industrially by FMC extractor (fruit juice extractor), 40 samples from 2003 crops and 10 from 2004, 16 (13+3) of which were lemon oil, 26 (21+5) orange, 3 (2+1) clementine and 5 (4+1) mandarin oil samples. Pesticides were detected using HRGC coupled with FPD for OPs and ECD for OCs, using already described instrumental settings [39], [41]. Plasticizers were quantified by MS analysis.

Methidathion *Methyl azinphos*
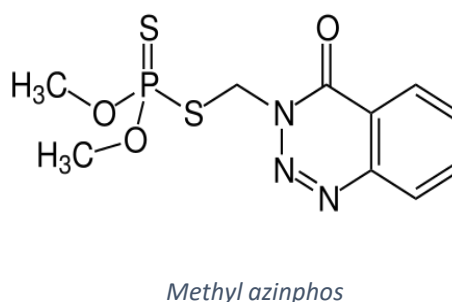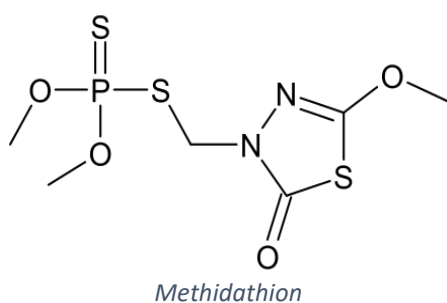
In 2003 lemon oil 5 pesticides were detected. Most commonly detected was methidathion, in 12 samples, followed by ethyl-parathion (6 samples) and methyl azinphos (4 samples), with mean concentrations of 0.6 mg/l, 0.06 mg/l and 0.23 mg/l, respectively. No pesticides could be detected in 2004 samples.

2003 orange oil samples were contaminated with 8 pesticides, methyl-azinphos was found in 18 samples, ethyl-chlorpyriphos in 17 and methidathion in 10, with

average concentrations of 0.09 mg/l, 0.53 mg/l and 1.26 mg/l, respectively. Three pesticides were present in 2004 samples: 2 contaminated with methidathion (0.36 mg/l) and ethyl chlorpyriphos (0.34 mg/l) and one sample with ethyl parathion (0.10 mg/l).

Clementine essential oils were also contaminated. Methidathion, ethyl parathion and methyl parathion were found in a 2004 sample (0.34, 0.25 and 0.09 mg/l). Both 2003 clementine oils had methyl-azinphos (0.04mg/l) and one sample contained ethyl chlorpyriphos (0.02mg/l).

The mandarin sample produced in 2004 was free of organophosphates, whereas all four 2003 samples were contaminated with 5 pesticides. Methidathion and ethyl-chlorpyriphos were found in all EOs with mean concentrations of 0.07 and 0.3 mg/l.

In lemon essential oils of 2003, tetradifon was encountered in 10 and dicofol in 1 instances, at 0.16 mg/l and 2.14 mg/l. In 2004 samples, dicofol was not detected and only one sample was positive for tetradifon (0.01 mg/l). Orange oil samples were most contaminated, with dicofol present in 5 cases and tetradifon in 11. Their average concentrations were 4.77 mg/l and 0.41 mg/l in 2003 and 0.84 mg/l and 0.03 mg/l in 2004 samples, respectively.

Dicofol was not detected in clementine oils, however, like lemon oils, tetradifon was present only in the 2004 sample, at 0.09 mg/l level. Mandarin samples of 2003 and 2004 were also free of dicofol, but tetradifon was encountered in all 2003 samples, at 0.04 mg/l. Even though tetradifon was encountered in most essential oils, it was present in significantly lower averages than those of dicofol.

Despite all these essential oils being produced from organically grown citrus fruit, regrettably, they were not free of pesticide residues. Apart from pesticides' environmental persistence, using the same equipment for processing of conventionally and organically grown fruit may have contributed to further contamination of the *bio* essential oils.

2.3.6 Orange oils

Nichkova et al. (2009) [38] proposed an enzyme-linked immunosorbent assay (ELISA) study for the determination of pesticides in orange EOs. Two pesticides were

selected for testing, simazine, a triazine herbicide and cypermethrin, a pyrethroid insecticide. Two orange EOs samples were available, a conventional cold-pressed oil and a 10-fold concentrated orange oil. Firstly, a direct approach was applied with no previous pesticide extraction, which resulted in high interference with ELISA. To eliminate the interfering matrix effects, adequate extraction methods had to be developed for both pesticides.

After extracting ^{14}C-simazine with water (and/or PBS), 50% MeOH/PBS, 70% MeOH/PBS, and 90% MeOH/PBS, recoveries of 45%, 77%, 88% and 91% were observed, respectively. A 1:1 oil-hexane mixture was extracted (LLE) with 50% MeOH/PBS to limit the amounts of matrix components extracted and keep the LOQ after the dilution as low as possible. The dilution with PBS after the extraction was necessary to lessen the amount of methanol to 10%, which is tolerated by the simazine ELISA but also reduce matrix effects. For the concentrated EO poor recoveries were obtained. 10 different spiking concentrations of simazine (20-400 µg/l) served for calibration purposes and ELISA showed a good correlation with LC-MS/MS, with recoveries of 93.7 % for ELISA and 101 % for LC-MS/MS. The lower recovery of ELISA is justified by the complex sample preparation.

Simazine
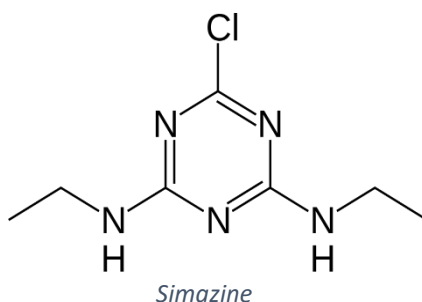
Similar steps were followed in the analysis of cypermethrin. Performing ELISA with an emulsion of the oil in 40% MeOH/PBS and 20% DMSO/PBS (stabilized by Tween 20, Tween 80, and casein) proved inadequate (only 0.1-0.2% EO content tolerable). A liquid-liquid extraction (LLE) of ^{14}C-cypermethrin isotope was than performed with MeOH and DMSO (dimethyl sulfoxide) to evaluate extraction recovery and yielded a success rate of 8 % and 80 %, respectively. However, these also proved unsatisfactory due to high EO amounts, especially in DMSO fraction. Therefore, more

detailed sample preparation method was developed combining LLE and solid phase extraction (SPE).

The EO was mixed with hexane and washed (water), after which extraction with acetonitrile was performed. Acetonitrile evaporation was then followed by re-solvation of the oily residue in MeOH and diluted with PBS to 40% MeOH/PBS. Further on, SPE clean-up was required for more extensive EO elimination. EO (0.3 ml oil + 1.5 ml
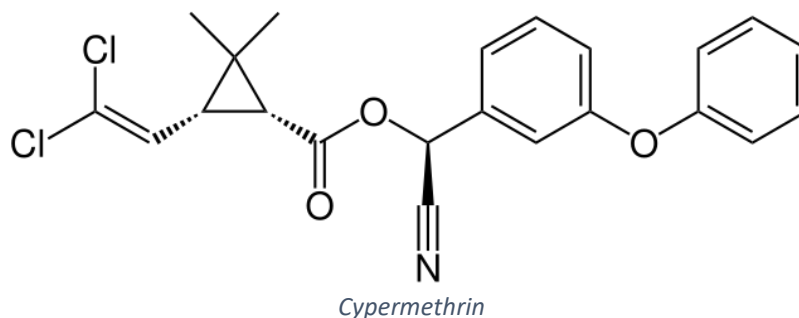
Cypermethrin

hexane) was loaded onto the prewashed (5ml hexane) Sep-Pak Plus silica column, washed again (5ml hexane) and eluted with 4 ml of 5% ethyl-acetate/hexane for elution of cypermethrin. Following solvent evaporation 0.3ml 0.01 % TritonX-100 was added and dissolved in 0.45 ml PBS.

A competitive immunoassay was performed with XIV-OVA-antigen and antibody 2282 for simazine and 4-BSA-antigen and antibody 735 for cypermethrin. The principle was based on competition between pesticides and labelled antibodies for a binding location with the antigen. ELISA analyses were executed in high-binding 96-well microtiter plates and SpectramaxPlus microplate reader was used for absorbance measurements.

For correlation studies both simazine and cypermethrin spiked EO samples were separated on Agilent 1200 LC Luna C18 (150 x 2.0 mm, 3 μm) column, using eluents A (0.1% HCOOH in water) and B (5 mM $HCOONH_4$ in acetonitrile). The elution started with 5% B for 1 min, then increased from 5% to 95% B in 2 min, held at 95% B for 3 min, back to 5% B in 2 min and equilibrated for 2 min (Flow rate: 250 µl/min). Simazine was detected in API 2000 MS/MS-system in multiple reaction monitoring (MRM), 202 m/z for precursor and 132 m/z and 104 m/z for fragmented ions.

Despite LC-MS/MS analysis showing no presence of simazine (LOQ 20µg/l) and cypermethrin (LOQ 40 µg/l) in these oils, a procedure was developed to verify the sensitivity and reliability of ELISA for pesticide detection and quantification. Pesticides could not be analysed by ELISA by direct application of EOs to the plates. Extraction and clean-up were necessary and time-consuming and had to be done according to physicochemical properties of the pesticides. ELISA itself is a highly selective method and is not applicable for testing of multiple pesticides at once. To test the presence of more than one pesticides, an extraction method would have to be developed for each one, and a specific antigen/antibody combination would first have to be validated in the presence of target pesticide. „*The immunoanalysis of both pesticides in 10-fold orange oil had unsatisfactory accuracy and precision due to the formation of unstable emulsions in the methanolic PBS extracts*" [38]. On the other hand, implementation of ELISA analysis of pesticides whose presence is suspected (e.g. an illegal pesticide) could be considered.

Fillâtre et. al. (2017) [43] recently used UPLC-QTOF-MS and GC-MS/MS methods to determine pesticide residues in 49 orange EOs, where 75 pesticides were tested as contaminants. The 49 essential oil samples were derived from oranges grown between 2013 and 2015, (most originating from South America - 25), 12 of which were reported to originate from organic production. One orange EO was rectified and another concentrated. Validation of the procedure was conducted by SANCO guidelines, where an organic EO was spiked at concentrations of 10, 25, 50 ,100 and 500 µg/l establishing limits of detection (LODs) for each pesticide (10 µg/l for 57, 25 µg/l for 13, 50 µg/l for 4 and 100 µg/l for 1). The 75 pesticides and/or metabolites used as reference were set to 10 mg/l and were used as a working standard mixture, for spiking the samples for standard addition analysis, and validation purposes. Samples were prepared with 100µl essential oil diluted in 900µl Ethyl-acetate (EA) for GC and 1% acetic acid in Methanol (MeOH) for UPLC-analysis. For the UPLC-analysis a freeze-out step was performed to eliminate possible wax presence. The EO samples were analysed using standard addition method against EOs samples spiked with 10, 100 and 500 µg/l. Adjustment of standard addition or dilution were performed in cases where the detected pesticide concentration exceeded 1000 µg/l, for confirmation analysis.

Separation of pesticides in an „ACQUITY UPLC" column (ACQUITY HSS T3) was achieved with a gradient elution of MeOH (eluent B) and water (eluent A), both containing 0.1% of acetic acid and 5 mM of ammonium acetate and a flow rate of 0.45 ml/min (t = 0, 30% B; t = 1 min, 30% B; t = 11 min, 100% B; t = 15 min, 100% B; t =16 min, 30% B, until 20 min.). Injected sample amount was 2 µl for all samples. To limit pesticide degradation, autosampler temperature was held at 4 °C, and oven temperature at 45°C.

Chromatographic system was coupled with „Xevo G2"-QTOF mass spectrometer equipped with ESI system. Nitrogen was used both as desolvation and nebuliser gas. For protonated molecular ions and adducts low energy ionisation was applied and high energy ionisation for molecular fragmentation. QTOF-MS data were collected in the scan range of 50 to 650 m/z with resolution around 30 000 and analysed with MassLynx software, which enabled targeted quantitative analysis, and a POSI±IVE algorithm-assisted non-target qualitative analysis.

An „Agilent 7890A" gas chromatograph, coupled with an „Agilent 7000" triple-quadrupole MS for detection, was used. The sample injection volume was equal to 1 µl. Two capillary columns (l = 15 m, ∅ = 0,25 mm and 25µm film thickness) „ Rxi-5ms" were used for separation while helium served as a carrier gas with a constant flow rate. The inlet temperature was 60 °C (10s), then heated to 280 °C at a rate of 700 °C/min, after which the inlet was cooled with N_2 to reduce the cycle time. The oven temperature was programmed to rise from 60 °C to 150 °C, then 200 °C ending on 280 °C with a total run of 45 min. The pressure was held at 50 psi throughout the entire process.

Ionisation was achieved through EI at -70eV, with He as a quenching gas (to eliminate non-target ions) and nitrogen as a collision gas (for fragmentation). MS transfer line and source were kept at 280 °C for sample stability. Detection of fragmented ions was performed after 2 or 3 MS/MS transitions, optimized for sensitivity and selectivity for every analyte. The MassHunter software was used for quantitative data analysis.

In the 49 EO samples, a sweeping 701 pesticide detections were observed. 57 different pesticides were found, 7 of which were not on the list of 75 tested compounds.

They were detected and confirmed via algorithm-assisted non-target qualitative analysis using a pre-existing pesticide library, comprised of pesticides' retention times (RT), adducts and fragments.

In conventionally produced EOs, 56 different pesticides were detected 650 times, and 72% of detections could be quantified being above the reporting limits (RLs). Most of the EO samples contained chlorpyrifos, diphenylamine and biphenyl found in 37, 28 and 26 samples, respectively. Single pesticide concentration above 1000 µg/l was detected 44 times, 4 of which were above 10 000 µg/l, the highest one being pyrimethanil (19 345 µg/l). An average concentration of pesticide residues in conventional EOs was 5.1 mg/l, with 17 contaminants per EO. Interestingly, with only 2 pesticides detected and sum concentration of 40 µg/l in the rectified EO sample, indications were made that a rectification could partly eliminate pesticides from the EO. However, this is speculative, considering an unmodified conventional EO sample also contained only 2 pesticides, but even lower sum concentration of 20 µg/l. On the other hand, the highest pesticide count (39) and concentration sum (51.1 mg/l)[1] were found in the concentrated EO sample, implying that EO concentration also concentrates pesticides present after extraction. Nevertheless, both statements instigate further research of these observations.

In comparison to conventional EOs, 51 detections were noted, and 24 detections quantified for a total of 18 pesticides. Still, most detections per sample were made for diphenylamine, biphenyl, chlorpyrifos and atrazine, i.e. 9, 7, 6, 6, respectively. Only 4 of the quantified pesticides exceeded concentrations higher than 100µg/l, with the topmost being piperonil-butoxide (278µg/l), a plant growth regulator. Total pesticide concentrations were in scope of 0.02 mg/l and 0.355 mg/l, averaging at 0.087 mg/l, and 4 pesticides per EO.

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[1] This concentration is a sum of quantified and non-quantified pesticides for which 1/3 of LOQ was used for summation

In summation, conventional and organic orange EOs were all contaminated by pesticide residues, though significantly lower levels were detected in organic EO samples. The presence of pesticides in organic samples could be explained as an aftereffect of their persistence in the environment.
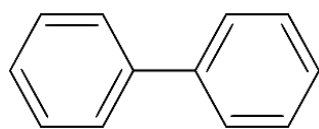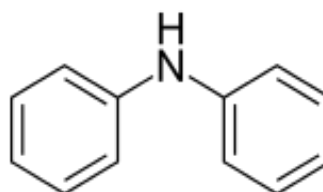


Biphenyl   Diphenylamine

2.4 Distilled oils

2.4.1 Peppermint oil

Garland et al. (1999) [44] examined the dissipation of tebuconazole and propiconazole fungicides after application to peppermint crops, and their presence and co-distillation in peppermint essential oil. Fungicides were applied in concentrations of 125 and 250g per hectare three times in two-week intervals at two crop sites. The essential oil was produced from the last harvest, 64 days after the last fungicide application and yielded 0.21 and 0.3 %. About 20 mg of oil from each site was dissolved in 1 ml methanol and spiked with 25 µl of a 222 µg/ml solution of octadecane as an internal standard.
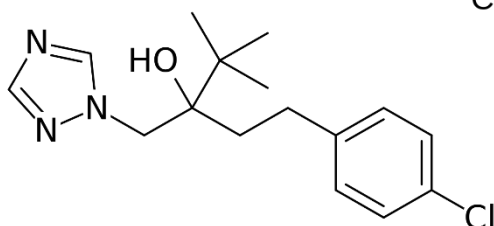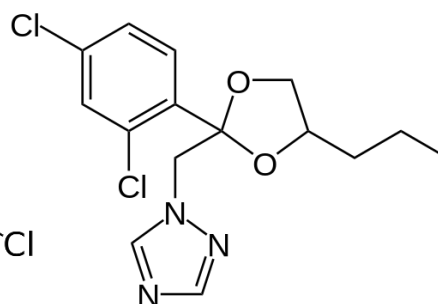
Tebuconazole   Propiconazole

HP 5890 gas chromatograph equipped with a BP1 fused silica capillary column (25 m x 0.22 mm, 0.25 µm film thickness) and coupled with Kratos Concept ISQ mass spectrometer was used for analysis. The injector temperature was held at 260 °C and

the oven 60 °C to 290 °C at 20 °C/min. Samples were injected in the GC in splitless mode, using a carrier gas flow of 30 psi/min from 25 to 40psi (held for 6 s), then at 30 psi/min to 25 psi, and at 1 psi/min to 35 psi. Mass spectrometry was conducted in high resolution selected ion monitoring (HRSIM) mode. For propiconazole, ions monitored by SIM were m/z 259.0291 and m/z 261.0262, and for tebuconazole m/z 252.0714 and m/z 250.0743, as well as m/z 254.2973 for octadecane as an internal standard. The source temperature was 210 °C, for electron ionisation energy of 70 eV was used on all ions, with 5.3 kV for ion acceleration.

In peppermint oil, propiconazole was found in concentrations of 0.017 (125g/ha) and 0.039 (250 g/ha) mg/kg from site 1 and 0.029 (125 g/ha) mg/kg at site 2; tebuconazole at 0.011 and 0.53 mg/kg (site 1) and 0.021 mg/kg (site 2). The amounts of co-distilled pesticides were calculated in comparison to levels detected in peppermint before distillation. It was found that propiconazole co-distilled in higher rates than tebuconazole, i.e. 0.7 % and 0.09 % respectively, primarily due to its higher vapour pressure.

2.4.2 Boronia oil

Additionally, Garland et al. (2004) [45] conducted a similar experiment on the dissipation and presence of tebuconazole and propiconazole in black boronia (*Boronia megastigma)*. Comparable field trials were conducted to those of peppermint, where crops were again sprayed with 125 and 250g per hectare, two times, the second time after 14 days at site 1 and after 10 days at the second site. At harvest, flowers were combed and extracted on a shaker for two hours at 20 °C in petroleum ether. The solvent was decanted, and the extraction repeated. After combining the washes, the solvent was removed by evaporation at 40 °C and reduced pressure; at the end, the temperature was increased to 60 °C to remove solvent residues. 10-20 mg of the EOs were than dissolved in 1ml methanol and 5µl of a 192 µg/mL solution of endosulfan as an internal standard was added. Solvent extracted essential oil from site 1 and 2 were 0.39% and 0.38%.

The analysis was performed on HP 5890 GC fitted with BPX5 fused silica capillary column (25 m, 0.22 mm, 0.25 µm film thickness), and coupled to a quadrupole HP 5970B MSD or Kratos Concept ISQ mass spectrometers. Parameters of the Kratos system were previously described [44]. In Quadrupole GC-MS, helium gas served as a

carrier at a pressure of 27 psi. The oven temperature was held at 50 °C for a minute, followed by an increase to 290 °C at 10 °C/min and held for 5 minutes. The injector temperature was held at 250 °C and transfer line at 290 °C. Ions monitored for detection in SIM were m/z 195 for the internal standard, m/z 250 for tebuconazole, and m/z 259 for propiconazole.

Flowers used to produce the essential oil had tebuconazole levels of approximately 0.3 and 0.5 µg/l for dusting concentrations of 125 and 250 g/ha from site 1 and 0.7 µg/l for dusting concentration of 125 g/ha at site 2, compared to wet weight. However, tebuconazole concentrations found in these oils were 60, 100 and 200 µg/l, respectively. This concentration build-up of tebuconazole in the oil can be explained by the solvent extraction process, where volatility and thermolability are irrelevant factors, in relation to previously discussed steam distillation. „*Potentially all of the residues that are present in the flowers at harvest and that are soluble in petroleum ether may coextract*" [45].

2.4.3 Lavandin oil

Fillâtre et. al. (2011) [46] developed a method for the detection of 70 pesticides in lavandin essential oil. The sample was of organic origin was obtained from a cosmetics company. Sample preparation involved a ten-fold dilution of the sample in methanol acidified with 1% acetic acid.

An Ultimate 3000 high-pressure liquid chromatograph equipped with C18 Synergy Hydro-RP (150 mm × 4.6 mm, 4 µm particle size) column was used. This column was chosen because it is amenable to substances exhibiting a wide scope of both polarity and acidity. The temperature of the column oven was set at 35 °C and the pump at a flow rate of 1 ml/min. Elution was conducted with eluent A and B, consisting of 0.1% acetic acid and 5mM ammonium acetate, respectively, in water and methanol. Acetic acid was added to suppress the ionization of residual silanols in the stationary phase and improve peak shapes. Ammonium acetate served for controlled formation of NH_4^+ adducts needed for some pesticides. The injection volume was 20 µl.

MS spectra were obtained on API 4000 QTrap fitted with an ESI source operating in positive and negative modes, and trough scheduled reaction monitoring

mode (SRM). Scheduled means that precursor ions selected for analysis are chosen by their retention times in the LC, thus implementing SRM only when necessary. A QTrap is a triple-quadrupole system where the third quadrupole operates as a classic quadrupole (QqQ), or as a linear ion trap (QqLIT). In the first quadrupole (Q1) a precursor ion is selected by its m/z, is then fragmented and accelerated in Q2 and product finally analysed in Q3, in either scan or SIM mode. For each pesticide, two reaction transitions were monitored, for quantification and confirmation, except in three cases, where pesticides produced only one fragmentation product. In positive mode, ESI voltage was 5500 V and in negative -4500 V. The source temperature was set to 550 °C, with curtain, nebulizer and heating gas pressures of 35, 40 and 50 psi, respectively. Nitrogen served as collision and curtain gas and compressed air for heating and nebulisation. The sSRM was conducted with a detection window of 60 seconds (± 30s) for each analyte's retention time.

To validate the method and examine matrix effects, solvent-based and matrix-matched calibration curves for each analyte were established and compared. Lavandin EO was spiked with every pesticide at concentration of 200 µg/l, after which it was diluted to create 12 samples concentrated at 0.1, 0.25, 0.5, 0.75, 1, 2, 2.5, 5, 7.5, 10, 12.5, and 15 µg/l. Each sample was analysed two times, for positive and negative ESI mode; the relative standard deviation (RSD) was calculated over the five replicates for five concentrations and was under 17% for all analytes. The original lavandin oil sample was also analysed and showed no pesticide residues. „*The analytical method has been validated for 67 of the 70 pesticides and meets the following LOQs: ≤ 1 µg/L for 9 pesticides, ≤ 5 µg/L for 44, ≤ 10 µg/L for 9, and ≤ 20 µg/L for 5*" [46]. Only three pesticides failed validation due to linearity problem.

In comparison to earlier studies [40], [41], a superior method, with no previous clean-up and higher LOQs is presented. This detection method is independent of the analytes' class and pH values, requires lower sample quantities and thus applicable to a higher number of pesticides [46].

Fillâtre et al. (2014) [47] also tested evaporation under nitrogen as a possibility to further concentrate pesticide residues in the EO, thus improving the limits of quantification. Additionally, instead of 70 pesticides, the list of analytes was expanded to 256.

Before being analysed, lavandin essential oil was spiked at concentrations of 1, 5, 10, 25, and 50 µg/l. In addition to diluting the sample tenfold, 500 µl of lavandin EO was also set under a gentle nitrogen stream and evaporated to dryness for 40 minutes at 80 °C. 500 µl of 1% acetic acid in methanol was then used for resolvation of the dried residues. LC/ESI/sSRM was performed on the same equipment and under same conditions as depicted previously. However, due to a large number of analytes in positive ESI mode, only one SRM transition was observed per analyte. For each analyte, the collision energy (CE), declustering potential (DP) and cell exit potential (CEP) were optimized.

The dilution method gave the LOQs below or equal to 1 µg/l for 28 analytes, 5 µg/l for 71, 10 µg/l for 90, 25 µg/l for 41 and 17 for 50 µg/l. Lastly, LOQs for eight pesticides could not be determined, because they were not detected in the range tested or were not included in the calibration mixture.

LOQs of the sampling by evaporation was observed in concentrations of 10 and 50 µg/l demonstrated following limits of quantification: below or equal to 1 µg/l for 49 pesticides, 5 µg/l for 83, 10 µg/l, for 80, 25 µg/l for 19, and 50 µg/l for 12. Also, evaporation leads to good recoveries of pesticides, with 82 % of them between 70 and 120%, and a relative standard deviation under 20% for more than 95% of pesticides and under 5 % for 72 %. On the other hand, 10 pesticides were not detected, and 9 % of the residues had recoveries lower than 50%, most likely due to loss during evaporation, caused either by thermal instability or high volatility.

A significant betterment could be observed in the general LOQ values under the evaporation procedure i.e. an improvement of LOQs for 92 pesticides was noticed. „*Here, 82.8 % of the pesticides (i.e., 212 pesticides) have LOQs below or equal to 10 µgL^{-1} compared with 73.8 % (i.e., 189 pesticides)*" [47]. Further, compared to dilution method, LOQ of 1 µg/l or below had almost doubled under nitrogen evaporation.

In summation, sampling by dilution provides a good method for determination of 247 of 256 pesticide residues under 50 µg/l (189 under 10 µg/l), nonetheless, evaporation under nitrogen boosts LOQs of some pesticides, even though few others are lost through this process. Because of this, the best way to utilize both methods is to use them as complementary to one another [46], [47].

2.4.4 Rose absolute

Tascone et al. (2015) [48] used rose petals spiked with 54 multiclass pesticides to track their concentrations during the transformation processes from rose petals to rose concrete and rose absolute. The differences between the rose essential oil and absolute are in the extraction method and content. The oil mainly contains monoterpene alcohol (citronellol, linalool, nerol, geraniol), while the compounds such as phenyl ethyl alcohol, citronellol, geraniol, nerol, eugenol, nonadecane, nonadecene, etc. are concentrated in the absolute.

The samples used for this study were obtained from organic and non-organic production. One part of organic rose petals (*Rosa centifolia*) was used as a blank control, and the other was spiked at a concentration of 0.1 mg/kg. Non-organic samples were of *Rosa damascena* originating from 4 crop fields in Turkey. The rose petals were macerated into the concrete with n-hexane, followed by filtration and evaporation of the solvent. The concrete was then extracted with ethanol, frozen, filtered and evaporated to obtain the absolute.

The analysis of pesticides was carried out at every point of the production process. For sample preparation, a modified QuEChERS approach was used. 5g of flowers and 2,5g of concretes and absolutes were added to 15 ml of acetonitrile and stored in a 50 ml tube. After being cooled at 4 °C for four hours $MgSO_4$, sodium citrate, NaCl, and sodium sesquihydrate were added to remove any remaining water and help the pesticides' extraction. The tube was then shaken on a vortex mixer for 1 minute and centrifuged for 10 min at relative centrifugal force (rcf) of 2630. 8 ml of the supernatant was transferred on a d-SPE (desorptive solid phase extraction) tube containing $MgSO_4$ (900 mg), PSA (150 mg), octadecane (150 mg), and graphitized carbon black (GCB) (150 mg), after which 3 ml of toluene was added. Subsequently, shaking and centrifugation steps were repeated, a second d-SPE clean-up was performed and shaken and centrifugated for the third time. Finally, 4 ml of this solution was evaporated under nitrogen at 35 °C to approximately 200 μl. This was then transferred to a GC column.

The analysis was performed on an Agilent 7890A GC system equipped with a fused silica (95% dimethylpolysiloxane 5% phenyl) capillary column (30 m × 0.25 mm, with 0.25 μm film thickness) and interfaced with an Agilent 7000 triple-quadrupole

MS. Helium had a flow rate of 1ml/min. The injection was done in pulsed splitless mode at a temperature of 280 °C, with 1 µl sample. The initial oven temperature was 90 °C, increased to 180 °C at 30 °C/min, then to 280 at 3°C/min and held for 5 min. Transfer line was held at 280 °C and ionisation was done by EI at 70 eV. An SRM mode divided into 20 time segments was selected for the analysis, with two transitions per pesticide for identification and quantification.

The analysis of the unspiked flowers, concrete and absolute produced from them showed no pesticide presence. In the spiked flowers, however, 52 pesticides were quantified, 19 of which in concentrations lower than 0.05 mg/kg and 2 pesticides were undetected, before the extraction. After the extraction 3 were undetected, 17 unquantifiable and 34 were quantified in the range of 0.01 and 0.08 mg/kg. The concrete produced from these flowers showed the presence of all 54 target pesticides. Twenty-two pesticides were detected below the LOQ, and six pesticides were quantified at 0.01 mg in the n-hexane after the concrete extraction. However, significant amounts of pesticides remained in the spent flowers. The absolute extracted from spiked samples was also positive for all the target pesticides and their recoveries were in the range of 69-88 %. In ethanol, only traces of some pesticides were found after the extraction. The study showed that all the targeted pesticides exhibited average concentration by a factor of 100-300 from rose petals to absolute, with three exceptions. The Turkish samples showed similar effects of concentration. In 4 samples 17 pesticides were detected in at least one of the samples. In all these samples, the concentration was observed to be between 87 and 545-fold. Methidathion and chlorpyrifos were detected and quantified in every sample, i.e. in rose flowers at $0.01 - 0.05$ mg/kg and 0.01 mg/kg, respectively, and in the complementary absolutes at concentrations of $0.65 - 27.25$ mg/kg for methidathion and 4.7 mg/kg.

2.4.5 Patchouli oil

Yang et al. (2015) [49] performed a study involving the simultaneous determination of 44 organophosphorus pesticide residues in patchouli (*Pogostemon cablin*), patchouli powder and patchouli essential oil. For pesticide analysis ten samples of patchouli essential oil produced in Germany, Indonesia, Portugal, India and China and were purchased at different markets in China.

Firstly, a trial and error study of the clean-up step was performed, where different amounts of chemicals used for QuEChERS method were evaluated for recoveries from spiked patchouli oil samples. Namely, 250 mg of the EO was mixed with 5 ml of a 9:1 (v/v) mixture of acetonitrile and water solution of 1 % acetic acid in a centrifuge tube. Subsequently, the solution was shaken by hand for 30s and by a vortex mixer for 1 min, followed by addition of 3.5g of $MgSO_4$ and 1g of NaCl, was then shook again for 30s and centrifuged at 10,000 rpm for 6 min. 1,5 ml of the supernatant was transferred into a tube containing 60 mg of PSA, 20mg GCB and 120mg $MgSO_4$. The tube was then shaken for 30s and centrifugated for 2 min at 12,000 rpm.

1 ml of this solution was used for GC-FPD analysis. An Agilent 6890N gas chromatograph fitted with DB-1701 (30 m × 0.25 mm with 0.25 µm film thickness) capillary column was used for OP residue separation and FPD for detection. The injector temperature was held at 250 ℃, initial oven temperature 70 ℃ for 1 min, then raised to 210 ℃ at 15 ℃/min, held for 6 min, then to 220 ℃ at 1.5 ℃/min, 2 min hold, and to 260 ℃ at 20 ℃/min and held for 8 min. The carrier gas was nitrogen at 1.3 ml/min. Confirmation of the target analytes in positive samples was achieved on a Shimadzu GCMS-TQ8030 equipped with a Rxi-5Sil capillary column (30 m × 0.25 mm, 0.25 µm thickness). 1µl of the sample was injected at 260 ℃. The oven temperature started at 60 ℃, held for 3 min, ramped up to 200 ℃ at 20 ℃/min, then to 220 ℃ at the rate of 5 ℃/min. Helium was used as a carrier, EI performed with the standard energy of 70 eV and the scan in the range of m/z 50-500.

Pesticide-free samples were spiked at 0.05, 0.1, and 1 mg/kg for the evaluation of the recoveries and yielded very good results. Namely, all the pesticides' recoveries were in the range of 70- 120 %, with an average recovery of 99.34 %. Relative standard deviation (RSD) was done in triplicate and varied between 0.08 to 9.71% (3.71 on average). Limits of detection and quantification were estimated by a signal-to-noise ratio of 3 and 10 and were in the range of 0.004–0.02 and 0.01–0.04 µg/ml, respectively. Out of 10 *P. cablin* oil samples, only one showed pesticide presence where chlorpyrifos was detected at 0.024 mg/kg.

Even though the described methods used for pesticide determination in *P. cablin* oil showed good recoveries and relative standard deviation values, it is important to

note that this method is developed for organophosphate pesticides only. The behaviour of other pesticide classes during sample preparation and column separation would prompt additional investigation and detection methods since FPD is not suitable for phosphorus-free compounds.

2.4.6 Cypress and lemon oils

Fillâtre et al. (2016) [50] have made further developments related to previous studies on lavandin oil [46], [47]. Samples used for these experiments were a biological lemon and cypress essential oils supplied by a private company. Again, samples were prepared by already mentioned dilution and nitrogen evaporation protocols. However, to minimise thermal degradation and pesticide loss, nitrogen evaporation was conducted at the temperature of 45 °C. Before sampling procedures, EOs were also spiked at concentrations of 1, 5, 10, 25 and 50 µg/l as before; to evaluate the recovery and repeatability of the evaporation performance, 10 and 50 µg/l concentration levels were used. For lemon EO, a freeze-out step was performed to reduce solubility issues with methanol by eliminating the waxes present. The diluted oil was set in liquid nitrogen for 4 minutes, followed by cold centrifugation for 5 minutes at 4000 rpm. Finally, at least 400µl of the supernatant was precipitated.

Once again, liquid chromatography and mass spectrometry were performed on the same instruments and under same conditions (LC–ESI/sSRM). Furthermore, a confirmation of the residues was carried out using the second analysis, taking additional fragmentations into account for pesticides ionised in positive ESI mode. Namely, for the confirmation, information-dependent acquisition (IDA) mode was used in consideration of positive results acquired by SRM survey scan and pesticides' retention times. When an SRM transition matched the IDA criteria, enhanced product ion (EPI) mode switched on to generate the mass spectrum. In LC–ESI/SRM–EPI mode, the third quadrupole functions as a linear ion trap. The following parameters were set for EPI mode: DP of 40 V, CE spread between +40 and -20 V and LIT acquisition speed 1000 Da/s.

Set side by side with the previous study with lavandin EO, the comparison of the two sampling methods gave somewhat better results regarding the LOQs: in lemon essential oil, 112 gave better LOQs after being evaporated. About two times more

pesticides could be quantified in concentrations lower than 1 µg/l (36.3%) and 91.7 % of analytes under 10 µg/l with the evaporation, compared to 18% (1 µg/l) and 81.6 % (10 µg/l) with dilution. In cypress oil similar results were obtained; after evaporation 91 % of pesticides gave LOQs ≤ 10 µg/l and 30.5 % were quantified with LOQs ≤ 1 µg/l, compared to dilution, after which 78.6% of pesticides had LOQs ≤ 10 µg/l and

Buprofezin

Prochloraz
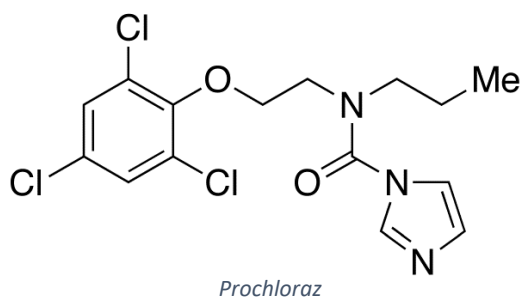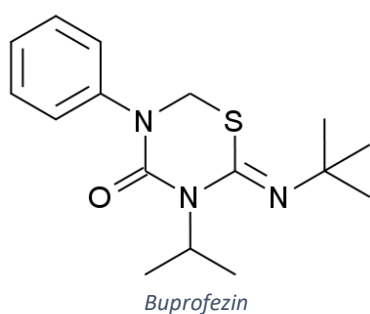
14.1% had LOQs ≤ 1 µg/l. Improvement of LOQs was observed for 119 pesticides. Moreover, pesticide recoveries in lemon and cypress oils were in the range of 70 to 120 % for 97.6 % and 94.8 %, respectively. Also, RSDs were below 5 % for 90.4 % of pesticides in lemon and 91.3 % in cypress oil.

Blank essential oil samples were also analysed for pesticide residues. In correlation to pesticides' SRM transitions and retention times, significant responses were detected. It appeared that lemon oil was contaminated with 16 pesticides and cypress oil with 7. Furthermore, two lemon EOs of organic and conventional origins were analysed as well. In negative ESI mode, LC–ESI/sSRM detected two pesticides in conventional oil and none in organic oil. In positive ESI, 30 and 20 transitions were detected in conventional and biological oils, respectively. To avoid false positives, the second analysis performed by LC–ESI/SRM–EPI method, revealed the presence of 18 residues in conventional and 2 residues (concentrations lower than LOQ) in biological
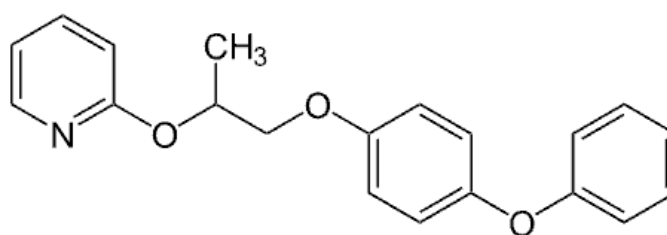
Pyriproxyfen

lemon EOs. Three pesticides were present at concentrations higher than 1 mg/l: buprofezin (1.3 mg/l), pyriproxyfen (2.3 mg/l) and prochloraz (11 mg/l).

Finally, based on these results and techniques, the authors proposed a workflow for multi-residue pesticide determination in essential oils. Considering the essential oil's flash point and evaporation temperature, either dilution ($\geq$ 45 °C) or evaporation ($\leq$ 45 °C) sample preparation protocols should be conducted as the primary choice, with regard that evaporation should also be considered as a complementary method to dilution in case the EO has the evaporation temperature higher than 45 °C, so long as it leads to a betterment in the sensitivity of a considerable number of compounds. Provided that the solubility problems with methanol are observed, which often happens with cold-pressed citrus oils, a freeze-out step should be performed. The intensity of interfering matrix effects can vary not only between different oils but also between samples of the same oil type. Thus, an accurate quantification demands a systematic standard addition of a pesticide mixture at 5 µg/l, for dilution, or 50 µg/l, for evaporation. In the end, pesticide determination is conducted on an LC-MS/MS, using both detection methods, i.e. LC–ESI/sSRM and LC–ESI/SRM–EPI for proper confirmation. Additionally, for the determination of residues which are not amenable to liquid chromatography, the utilization of the gas chromatography is proposed.

3. Conclusion

The increase of world population in the twentieth century has led to increased agricultural production and thus, increased pesticide use worldwide. Sadly, in pursuit of higher yields and profits, mankind has neglected its environment, allowing pesticides to spread around the globe, into the waters, soil and air, hence infiltrating the food chain and affecting every living being. This profit-induced environmental poisoning has consumed the production of valued essential oils as well. Surely, integrated pest management has been in use for a long time, and the use of many highly toxic pesticides has been made illegal over the years, however, they still circulate and persist in the environment. As an alternative, biological-farming has been implemented in both EO and crop production and it strictly prohibits the chemical use, but still, as seen in this paper, even biologically produced oils showed some traces of pesticide residues. These residues are most likely derived from soil or rainfall contaminated with the persisting

pesticidal compounds, or simply from contaminated extraction equipment. In any way, despite the most likely intensification of pesticide concentrations in the EOs after the extraction, and considering that the recommended daily dosage of an essential oil is often expressed in a number of drops, the possibility of pesticide intoxication by EO use is relatively low. „*Recent evaluation of a database containing 127 517 sets of data compiled over 8 years, showed positive results in 1150 cases (0.90 %), and the limits of Ph. Eur. general chapter 2.8.13 Pesticide residues or Regulation (EC) 396/2005, both applicable to herbal drugs, were exceeded in 392 cases (0.31 %, equivalent to 34.1 % of the positive results), particularly in cases of oils produced by cold pressing.*" [23].

As a possible alternative to pesticide use, with the aim of reducing environmental and human contamination with pesticides, essential oils have gained some momentum as possible green pesticides in recent years, because many of the EOs or their constituents exert negative effects on insects, fungi or microorganisms. Additionally, they are environmentally safe and non-persistent. However, their general efficacy is not as high as that of chemical pesticides, though there are some pest examples where this is not the case [51].

Over the years EO pesticide analysis has evolved drastically. At first OCs and OPs were in the focus of researchers, mostly because they were easily detectable by ECD and FPD detectors, but with the improvement and development of new instrumental MS techniques, methods for multiclass and multiresidue pesticide determination were developed including simple preparation techniques. Even though pesticide analysis has evolved for the better in recent decades, still, further control and research on this topic may be required, especially because of the recent trends in EO use, market demand and products arriving from around the globe, where, in many cases, pesticide use lacks serious regulation and does not conform to EU regulations.
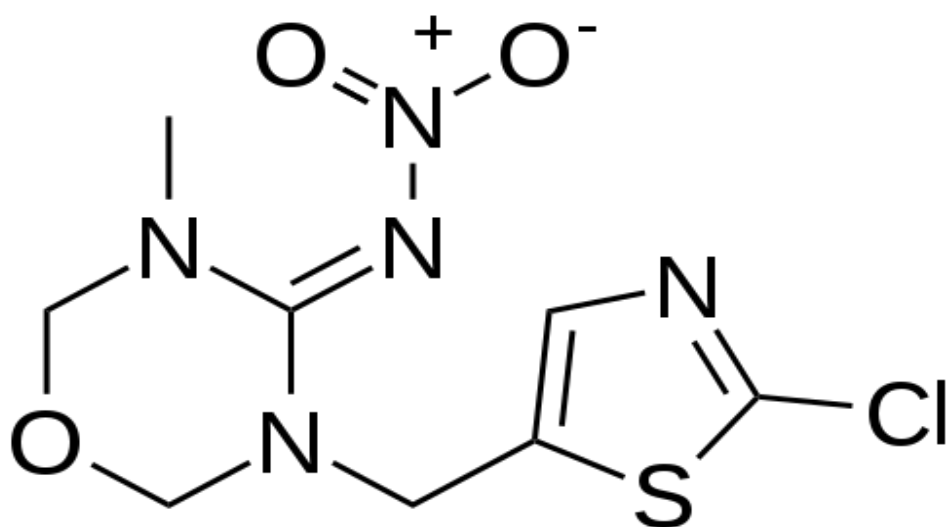
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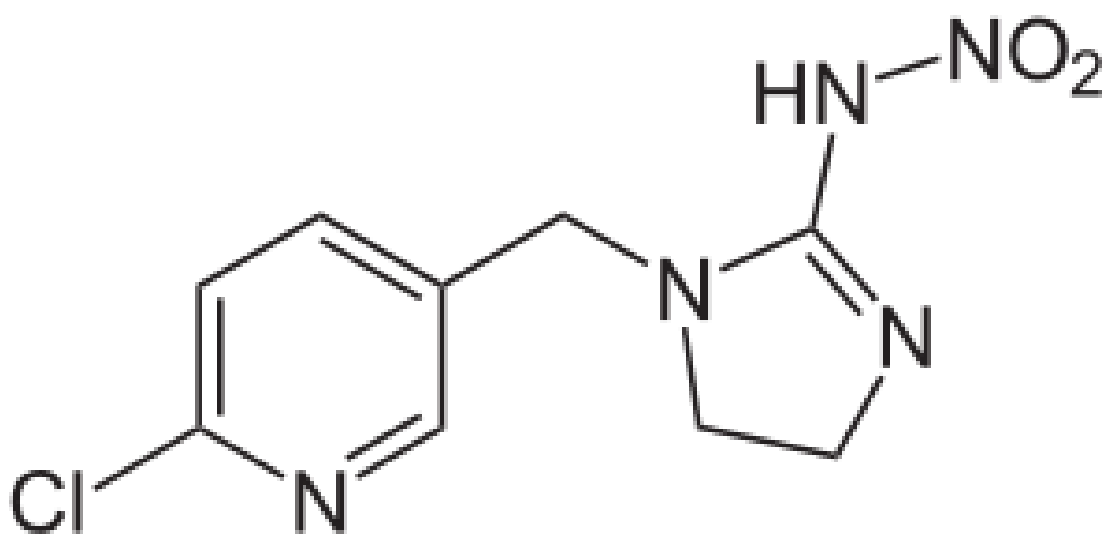
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Thiamethoxam

Imidacloprid