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MULTI-PESTICIDE RESIDUE DETERMINATION OF AVOCADO SAMPLES FROM DIFFERENT ORIGINS, COMPARISON OF DEFATTING STEPS

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Multi-pesticide residue determination of avocado simples from different origins, comparison of defatting steps

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ABRREVIATIONS AND ACRONYMS

ACN:	Acetonitrile
C1:	Clean up by freezing out of co-extracted fat, wax, sugars.
C18:	Octadecyl silane
C2:	Clean-up by dispersive solid phase extraction with PSA
C4:	Clean-up by dispersive solid phase extraction with a mixture primary secondary amine and octadecyl silica.
C8:	Octyl silane
CAC:	Codex Alimentarius Commission
COLEACP:	Europe-Africa-Caribbean-Pacific Liaison Committee
DIGESA:	Dirección General de Salud Ambiental (Peru Ministry of Health Directorate-General for Environmental Health)
dMRM:	Dynamic Multiple Reaction Monitoring
DNA:	Deoxyribonucleic acid
D-SPE:	Dispersive Solid-phase extraction
EC:	European Commission
EFSA:	European Food Safety Authority have
EN:	European Standards
EPA:	US Environmental Protection Agency
EU:	The European Union
FAOSTAT:	Food and Agriculture Organization Corporate Statistical Database
FCM:	False codling moth
FDA:	U.S. Food and Drug Administration
GCB:	Graphitized carbon black
GC-MS:	Gas chromatography-mass spectrometry
GPC:	Gel permeation chromatography
HCOOH:	Formic acid
HPLC-MS/MS:	High Performance Liquid Chromatography coupled with Tandem Mass Spectrometry
IPM:	Integrated Pest Management

KEPHIS:	Kenya Plant Health Inspectorate Service
LC-MS:	Liquid chromatography-mass spectrometry
LLE:	Liquid-liquid extraction
MRLs:	Maximum residue levels
NCBI:	National Center for Biotechnology Information
ODS:	Octadecyl silica
PCPB:	Pest Control Products Board (Kenya)
PIP:	Plant-incorporated protectants
ppb:	Parts per billion
ppm:	Parts per million
PSA:	Primary secondary amine sorbent
PTFE:	Polytetrafluoroethylene
QQQ:	Triple quadruple
QuEChERS:	Quick, Easy, Cheap, Effective, Rugged, and Safe
RP-HPLC:	Reverse Phase High Performance Liquid Chromatography
RPM:	Revolutions per minute
SENASA:	Servicio Nacional de Sanidad Agraria (Peru Ministry of Agriculture National Food Safety and Quality Service)
SIL-IS:	Stable isotopically labeled internal standard.
SPE:	Solid-phase extraction
TBS:	Tanzania Bureau of Standards
TPP:	Triphenyl phosphate
TPRI:	Tropical Pesticides Research Institute (Tanzania)
UHPLC-MS/MS:	Ultra performance liquid chromatography - tandem mass spectrometer

CHAPTER 1

INTRODUCTION

1.1. Background of the study

Avocado (*Persea americana)* is a popular fruit with high nutritional value, and it is cultivated in many countries worldwide, according to Hurtado-Fernandez et al. (2018). Pesticides used in avocado cultivation has been reported to cause adverse health effects, environmental pollution, and decreased marketability due to strict regulations on pesticide residues (Shahbaz et al., 2022).

Pesticide use varies by country due to a difference in range of factors such as pest and disease pressures, regulatory frameworks, and cultural traditions. Pesticides are generally used to manage pests and diseases that damage or destroy crops. Avocado trees are vulnerable to a range of pests and diseases, including mites, thrips, whiteflies, and fruit flies. In addition, fungal diseases such as anthracnose and root rot can also affect avocado trees. Pesticides can help to prevent and control these pests and diseases, thereby reducing crop losses and increasing yields (Bennett et al., 2010).

However, the use of pesticides raises concerns about the likely risks to human health and the environment. Pesticides can pose a danger to the people handling them and the consumers of the fruits. They can also contaminate soil, water, and other natural resources, leading to environmental pollution and biodiversity loss (Shahbaz et al., 2022).

Consequently, it is critical to follow good agricultural practices that decrease pesticide use and the dangers associated with it. Integrated Pest Management (IPM) is a sustainable approach to pest management that stresses the use of multiple control strategies, such as biological control, cultural practices, and chemical control, to minimize the use of pesticides while maintaining high crop yields and quality (Muñoz et al., 2021).

Accurate detection of pesticide residues is crucial for reducing consumer exposure to potentially dangerous amounts of pesticides and ensuring that products conform to regulatory standards (Lehotay et al., 2005). The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) technique is one of the most often used methods for determining pesticide residues in avocados. It is a simple and low-cost method involving sample extraction, clean-up and analysis using gas or liquid chromatography. The method has been widely used by the food industry and regulatory

bodies for the determination of pesticide residues in a wide range of matrices, including fruits and vegetables. (Chamkasem et al., 2013).

It is essential to establish regulatory limits for pesticide residues in avocados based on scientific risk assessments that consider aspects such as pesticide toxicity, exposure risk, and the sensitivity of diverse groups. Pesticide residue levels (MRLs) in numerous food products, including avocados, have been defined by regulatory bodies such as the US Environmental Protection Agency (EPA) and the European Food Safety Authority (EFSA). Compliance with these MRLs is crucial for ensuring consumer safety and preventing market access restrictions.

HPLC-MS/MS (High Performance Liquid Chromatography combined with Tandem Mass Spectrometry) with dynamic Multiple Reaction Monitoring (dMRM) is a sophisticated analytical technique commonly used for pesticide residue analysis in avocados. It has several advantages over other analytical methods, such as improved sensitivity and specificity, reduced sample preparation requirements, and the capability to analyze numerous compounds simultaneously (Belarbi et al., 2021). However, the technique also requires careful sample preparation, particularly the defatting step to ensure accurate and reliable results when analyzing complex matrices such as avocados.

1.2. Goal of the Thesis Work

The goal of the thesis work is to investigate the pesticide residues in avocado produced in different countries and compare the results to regulatory limits and guidelines in addition to assessing potential health risks associated with the consumption of avocados contaminated with pesticide residues and identifying possible strategies to mitigate these risks.

The thesis also aims at comparing the defatting steps suggested by EC for extraction of multiple pesticide residues in avocado samples from different origins. The study aims to identify the optimal defatting method that provides the most efficient extraction of pesticide residues while maintaining the quality of the avocado samples.

These goals enhance the relevance and impact of the study, and provide valuable insights and recommendations for researchers, regulators, and the food industry.

CHAPTER 2

LITERATURE OVERVIEW

2.1. Avocado

Avocado is a popular and nutritious fruit that is consumed widely across the world(Hurtado-Fernández et al., 2018). The global demand for avocados has increased significantly in recent years due to their unique taste, nutritional value, and health benefits (Bhore et al., 2021). According to FAOSTAT (2022), avocado production has steadily increased from 2015 to 2021 (Fig. 1). The most common varieties are Fuerte and Hass. Fuerte is mainly for processing while Hass is for export (Wasilwa et al., 2018).

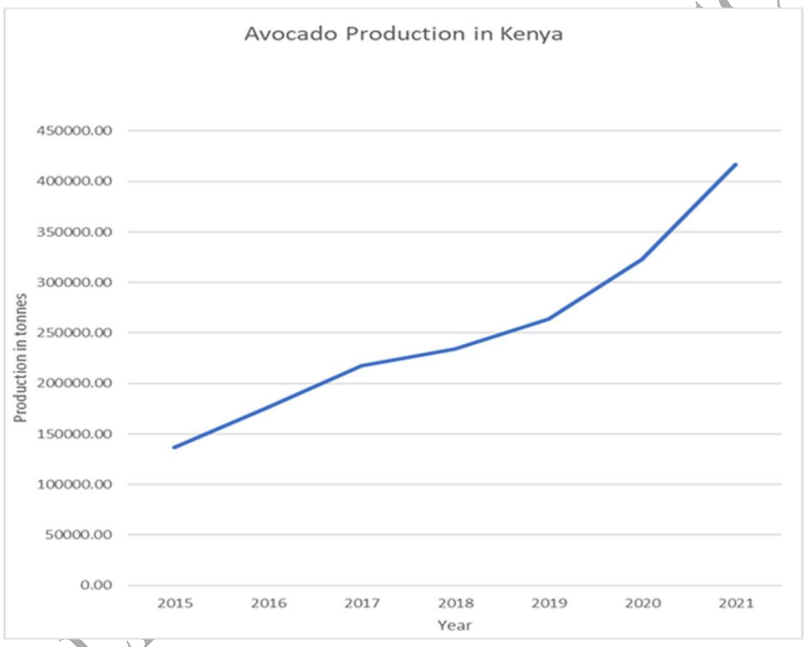

Figure 1. Avocado Production in Kenya from 2015 to 2021 (FAOSTAT)

Figure 2. Hass Variety

2.2. Pesticides used in avocado cultivation.

Pesticides are used by farmers to protect avocados from a variety of pests and diseases that can damage or destroy the fruit. Avocado trees are particularly susceptible to several pests, such as the avocado thrips, avocado fruit fly, and avocado mite, which can cause significant damage to the fruit if not controlled (Humeres et al., 2009).

Figure 3. Avocado tree

Pesticides are also used to control diseases such as Phytophthora root rot, which can kill avocado trees if not treated. Avocado root rot is a worldwide disease which might be caused due to poor phytosanitary conditions. Farmers prefer to use chemical products against this disease (Ramírez-Gil et al., 2017).

An increase in avocado productivity can be attributed to the effective control of production costs. Therefore, the use of herbicides to selectively control weeds is an appropriate method because it is labor and energy saving, because it requires less manpower, and allows control throughout the crop cycle (Silva et al., 2022).

However, use of pesticides has some drawbacks, such as the possible threat to human health and the ecosystem, as well as the development of pesticide resistance in pests, which can lead to the need for more frequent and higher doses of pesticides (Mac Loughlin et al., 2018). As a result, it is critical to use pesticides with caution and to follow the manufacturer's instructions and safety precautions. In the following chapter some often used pesticides are introduced during avocado production.

2.2.1. Thiabendazole

Thiabendazole is a fungicide and anthelmintic used for controlling a wide range of fungal and nematode pests. It is a member of the benzimidazole class of fungicides, which work by inhibiting the growth of fungal cells (NCBI, 2023a).

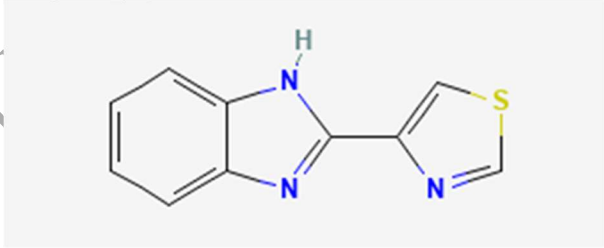

Figure 4. Thiabendazole (PubChem)

Thiabendazole is used as a fungicide in avocado after harvesting. The mode of application is through waxing, spraying, or dipping. The dosage rate varies according to the type of product. The dosage for avocado is 0.25–0.45 g L^{-1} (Perruchon et al., 2017).

Thiabendazole is quite stable because of its benzimidazole ring. According to Dong et al. (2017)

thiabendazole has a half-life of 933 days in soil and is stable in frozen crops for 12-28 months. Thiabendazole residues can enter the food chain and water bodies via various environmental sources. Eventually this might lead to negative health implications including teratogenicity hepatotoxicity, carcinogenicity, and nephrotoxicity (Séïde et al., 2016). In 2017, the approval of thiabendazole was renewed in accordance with Regulation (EC) No 1107/2009 (Alvarez et al., 2022).

2.2.3. Fenpyroximate

Fenpyroximate ($C_{24}H_{27}N_3O_4$) belongs to the family of acaricides, which are used to control mites and ticks in agricultural crops. It works by inhibiting the electron transport chain in the mitochondria of the target pests, leading to their death (Canada, 2016).

The use of fenpyroximate in avocado farming has raised some concerns regarding its potential impact on human health and the environment. A study by Graillot et al. (2012) has shown that fenpyroximate causes DNA damage in human cell lines, most probably by oxidative stress. Fenpyroximate has been permitted for use as an acaricide since May 2009 by the EU (EFSA, 2013).

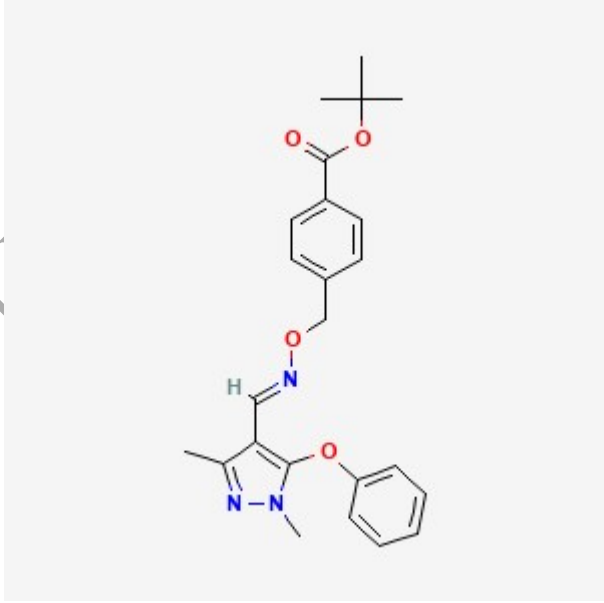

Figure 5. Fenpyroximate (PubChem)

2.2.4. Methoxfenozide

Methoxfenozide ($C_{22}H_{28}N_2O_3$) is a diacylhydrazine insecticide that binds to the ecdysone receptor complex with high affinity and acts as a powerful agonist, or mimic, of the insect molting hormone, 20-hydroxyecdysone (20E). It is highly effective against a wide range of caterpillar pests, including several kinds of lepidopteran insects such as navel orange worm, peach twig borer, leafrollers, loopers, armyworms, and citrus leaf miners (NCBI, 2023b). In avocado production, methoxyfenozide was found to be effective in COLEACP/PIP trials against FCM (Leone, 2020).

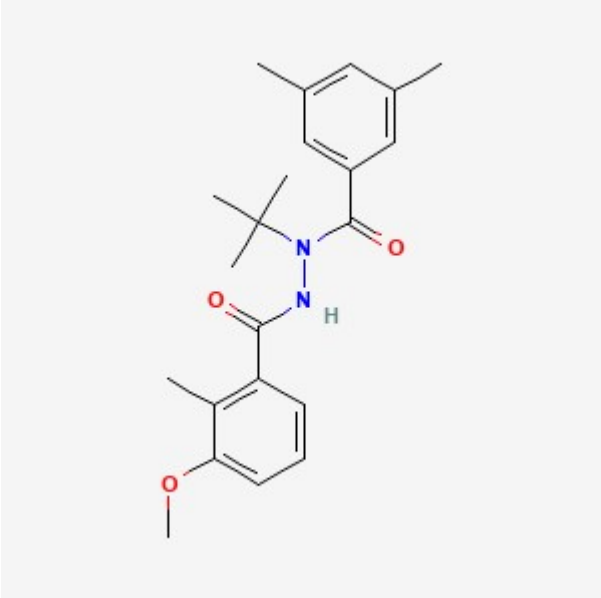

Figure 6. Methoxfenozide (PubChem)

Methoxfenozide has been found to be less toxic as compared to other pesticides and can thus be deemed the most appropriate chemical for use in an integrated pest control program (Saad et al., 2012).

2.3. Pesticides residues

The increase in demand for avocados has resulted in a surge in avocado production, which necessitated the use of several pesticides(Wangithi et al., 2022).These pesticides are used in boost the crop yield and protect crops from pests and diseases. However, pesticides application has raised concerns about potential health risks to consumers and the environment(Shahbaz et al., 2022). Pesticide residues can persist in the environment, leading to contamination of soil, water, and air. In addition, the consumption of avocados containing pesticide residues can have adverse health effects, including birth defects, cancer, and neurological disorders (Abong'o et al., 2014).

2.4. Sample preparation

The extraction of the target analytes from the sample matrix is a vital step in the study of pesticide residues in avocados. For the simultaneous assessment of various pesticide residues in avocados, sample preparation procedures have been established. Since multiple pesticides can be analyzed simultaneously in a single analysis, these methods offer cost, time, and labor advantages. However, the methods have limitations in terms of sensitivity and selectivity because the accuracy might be affected by interference from matrix components (Kruve et al., 2008).

2.4.1. Liquid-liquid extraction

Liquid-liquid extraction (LLE) is a conventional sample preparation method used for the extraction of pesticide residues from avocados (Fuentes et al., 2009). In LLE, the avocado sample is mixed with an organic solvent and then shaken vigorously to extract the pesticide residues. The organic phase containing the pesticide residues is then separated from the sample matrix and analyzed by an appropriate analytical method. Despite its effectiveness, LLE has some limitations, such as its time-consuming nature, the need for large volumes of organic solvents, and the potential for co-extraction of interfering compounds. When employing GC or LC apparatus for determination of pesticide at levels required by current standards, this method for samples cleaning is far from acceptable (Gilbert-López et al., 2009).

2.4.2. Solid-phase extraction

Solid-phase extraction (SPE) method is also used for the extraction of pesticide residues (Villaverde et al., 2016). In this extraction method, the avocado sample is loaded onto a solid-phase cartridge, and the target analytes are retained while the unwanted matrix components are washed away. The retained analytes are then eluted from the cartridge and analyzed by an appropriate analytical method. Compared to LLE, SPE involves analyte dispersion between a liquid (sample medium) and a solid (adsorbent) phase, allowing for analyte enrichment and purification on a solid adsorbent via adsorption (Keçili et al., 2020).

2.4.3. Quick, Easy, Cheap, Effective, Rugged, and Safe method

Recently, the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method has gained popularity for the extraction of pesticide residues in various food matrices, including avocados (Chamkasem et al., 2013). QuEChERS method combines the benefits of LLE and SPE while minimizing their limitations. In QuEChERS, the avocado sample is first homogenized and then extracted with an organic solvent and a buffer salt. The mixture is then centrifuged, and the organic

phase is mixed with a dispersive solid-phase extraction (dSPE) sorbent, such as C18, to remove interfering matrix components. The final extract is then analyzed by an appropriate analytical method. QuEChERS is a fast, simple, and cost-effective method that has provided high recoveries and selectivity for the extraction of pesticide residues in avocados (Villaverde et al., 2016).

2.4.4. Matrix Effect

Pesticides analysis in avocado is often complicated by matrix effects, which can lead to inaccurate results. Matrix effects are brought about by the presence of matrix components in the sample that interfere with the analysis of the target analyte. This interference can occur either during sample preparation, chromatographic separation, and/or detection. Matrix effects can result in either enhancement or suppression of the signal, leading to inaccurate results (Qin et al., 2021). Several methods have been established to reduce the matrix effects in avocado extracts.

2.4.4.1. Dilution

Diluting the avocado sample before analysis can reduce the matrix effect by reducing the concentration of interfering compounds thus improving the performance of the analytical method. However, it is crucial to ensure that the dilution does not result in the loss of analytes or affect the detection limit of the method. According to a study conducted by Stahnke et al. (2012), dilution of extracts by a factor of 25-40 decreases ion suppression to less than 20% if the original suppression is 80%. Higher dilution factors, on the other hand, were necessary for greater matrix effects or total suppression eradication.

2.4.4.2. Matrix-matched calibration

This involves preparing calibration standards that are spiked into a matrix that is similar to the avocado sample. This approach can account for the matrix effect by mimicking the sample matrix during analysis. A study by Pano-Farias et al. (2017) evaluated the matrix effect and observed a difference in the signal detection between pesticides (standards) and pesticides extracted from avocado matrices. The study recommended using matrix matched calibration to curb this matrix effect and ensure reliable findings. Further studies by Qin et al. (2021) found out that matrix-matched monitoring ion selection technique of typical pesticides minimized of the matrix effect interference and enhanced the detection accuracy.

2.4.4.3. Internal standards

Internal standards can be used to correct the matrix effects by compensating for variations in sample matrix composition. The internal standard should be structurally similar to the analytes of

interest and added at a known concentration. Different coeluting substances may be encountered due to differences in retention periods of target analytes and ISs, resulting in poorly adjusted findings. This issue can be solved by using a stable isotopically labeled internal standard (SIL-IS) that coelutes with the analyte, making it a suitable internal standard (Niessen et al., 2006).

2.4.4.4. Defatting Methods

Avocado samples contain 15% fat (Lehotay et al., 2005), which can interfere with some analytical techniques used to detect pesticides. To improve the analytical method's accuracy and sensitivity, defatting is carried out. However, defatting may cause loss of some analytes leading to higher detection limits and contamination of LC and GC systems (Theurillat et al., 2021). Low temperature precipitation (freezing-out), gel permeation chromatography (GPC), and adsorption (dispersive solid-phase extraction, solid-phase extraction) are the most often used defatting procedures.

Freezing-out is the simplest method involving freezing the sample at a low temperature to solidify the fats, which can then be easily removed by filtration or centrifugation. Unfortunately, this method is time-consuming and does not eliminate all the fat so usually an additional clean-up step is required (Santana-Mayor et al., 2019).

Gel permeation chromatography aids in separation of low molecular mass compounds, such as pesticides, from high molecular mass compounds, for instance lipids. An aliquot form the extract is injected in the GPC system which comprises of an LC pump, a fraction collector and sometimes a detector. This principle enables separation of pesticide from the high molecular weight triglycerides. The method can be automated giving it an advantage over the other manual methods. In addition, a study by Guardia-Rubio et al. (2006) achieved good recoveries when an extraction procedure was used with GPC clean-up. The main challenge is the presence of pesticides with high molecular mass which cannot be separated from triglycerides (Gilbert-López et al., 2009).

SPE technique can be used instead of GPC due to less solvent consumption and less waste generation. It also gives good recovery including high polar compounds (Hakme et al., 2018). Examples SPE sorbents used include C18, Florisil (magnesium silicate) and GCB (graphitized carbon black) (Madej et al., 2018).

D-SPE is an alternative to column-based SPE in which a sorbent material is inserted directly in the analytical solution. Subsequently, sorbent separation is done by filtration or centrifugation. This technique is simple and time saving. According to Islas et al. (2017), this method is selective, robust, and versatile.

The preferred clean up module of avocado samples according to EN 15662 (2018) is combination of modules C1 and C2. Module C1 involves freezing out of the fat from the extract followed by centrifugation where necessary. In module C2, the extract is cleaned-up by dSPE using PSA for removing organic acids. An alternative to this clean-up is module C4 which involves dSPE using a mixture of PSA and ODS. The latter aims to remove lipids from the extract.

2.5. Analytical methods for the determination of multi-pesticide residues in avocado

There is a growing need to develop proficient and dependable methods for the determination of pesticide residues in avocados (Gilbert-López et al., 2009). These methods can help safeguard the safety of consumers and protect the ecosystem. Gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) are some of the analyticl methods used. (Gilbert-López et al., 2009).

2.5.1. GC-MS

GC-MS is commonly used for the analysis of pesticide residues in avocados (Pano-Farias et al., 2017). It offers high sensitivity, selectivity, and accuracy, making it suitable for the analysis of multi-pesticides. However, it has limitations in the determination of thermally unstable and high-molecular-weight pesticides, which can break down during the analysis. In addition, the method might require derivatization of nonvolatile compounds with higher polarity which can increase the cost and complexity of the analysis (Raina Renata, 2012).

2.5.2. LC-MS

Because of its great sensitivity and specificity, LC-MS has been used to determine pesticide residues in avocados (Brutti et al., 2010). The method offers good separation of pesticides and can analyze a broad range of polar and nonpolar analytes. However, the method has limitations in the determination of low-molecular-weight pesticides and the analysis of complex samples due to interference from matrix components (Kowalski et al., 2014). The most often utilized LC separation technology for pesticides is RP-HPLC with nonpolar stationary phase modified with

either C18 (octadecyl silane) or C8 (octyl silane) (Rejczak & Tuzimski, 2015). The use of dynamic multiple reaction monitoring (dMRM) in HPLC-MS/MS has been found to be effective in the detection and quantification of multiple pesticide residues (Belarbi et al., 2021).

2.6. Identification of pesticides in LC MS/MS system

To conclusively identify an analyte during LC MS/MS analysis, several requirements must be met to avoid false positive results (Peng et al., 2003). This task is particularly important for multicomponent analysis, in which several hundreds of MRM mass transitions are monitored during one chromatographic run to identify and quantify all the targeted components. For each analyte, two MRM transitions are recorded: one for quantification, the other for identification. The identification requirements might be based on mass spectrometry as well as chromatography. First, the signal of both MRM-transitions must be equal to or greater than the detection limit of the analyte. In addition, the retention time must be greater than the dead time of chromatography. Moreover, the retention time of both MRM-transitions in the sample must be the same as the retention time of the standard. Lastly, the ionic ratio (the peak area ratio of the two MRM-transitions) of the product ions measured in the sample must be within 30% of the ionic ratio measured in the standard.

2.7. Regulations and guidelines for pesticide residues in avocados

Regulatory frameworks for pesticide residues in avocados vary in different regions/countries. The comparison of maximum residue limits (MRLs) and tolerances for pesticide residues in avocados across different regions/countries is an important aspect of food safety. MRLs and tolerances for pesticide residues in avocados across different regions/countries are generally consistent and based on internationally recognized standards to ensure that the levels of pesticide residues in foods do not pose a risk to human health. However, there may be some variations in the specific MRLs established by each country, based on factors such as local agricultural practices, climate, and consumer preferences (Handford et al., 2015a). The differences in MRLs can have implications for international trade, as exporters may need to comply with the MRLs of the importing country (Schiffers, 2006).

2.7.1. Kenya

In Kenya, legislation of pesticides residues is governed by the Pest Control Products Act, Cap 346 of the Laws of Kenya (Pest Control Products, 2012). The Act regulates the import, export, manufacture, distribution, and use of products applied for the control of pests and of the organic

functions of plants and animals. This Act dictates that any pest control product must be registered, packed and labelled accordingly as per the standards as per the regulations in order to be manufactured, imported or sold.

MRLs for pesticide residues in avocados are set by the Pest Control Products Board (PCPB) which is a regulatory agency under the Ministry of Agriculture, Livestock and Fisheries. The PCPB sets MRLs and tolerances for pesticide residues in avocados based on the recommendations of the Codex Alimentarius Commission standards, which are internationally recognized and the World Health Organization (Lengai et al., 2022). These standards are regularly reviewed and updated to reflect the latest scientific knowledge and safety concerns. The PCPB provides guidelines on the safe use of pesticides in avocado production, which cover topics such as pesticide selection, application rates and timing, and safety precautions. The guidelines are aimed at promoting sustainable agricultural practices that protect human health and the environment (PCPB, 2023).

Apart from setting MRLs and providing guidelines, the PCPB also conducts regular monitoring of pesticide residues in food products, including avocados, to ensure compliance with the established standards. If a violation of the MRLs is detected, the PCPB may take regulatory action, such as prohibiting the sale of the affected product or imposing fines on the responsible parties. The PCPB's regulations and guidelines for pesticide residues in avocado are aimed at promoting sustainable agricultural practices that safeguard human health and the environment (PCPB, 2023).

KEPHIS (Kenya Plant Health Inspectorate Service), a regulatory agency in Kenya is in charge of inspection and certification of agricultural products, including avocados, for export. It ensures that avocado exports meet the established phytosanitary standards. KEPHIS also conducts monitoring of pesticide residues in avocados, which is aimed at ensuring compliance with the established MRLs and other standards for pesticide residues in avocados (KEPHIS, 2023). KEPHIS works closely with Pest Control Products Board (PCPB), to ensure that the avocado production and export value chain is safe and sustainable, while also meeting both domestic and international market requirements (Fulano et al., 2021).

2.7.2. Tanzania

The Tropical Pesticides Research Institute (TPRI) Act No 18,1979 (TPRI, 1979) governs laws in Tanzania. Part (V) of this Act requires any pesticide made or imported in the United Republic of

Tanzania to have a name, a minimum quality appropriate for use, and other standards. The institute also ensures the establishment and maintenance of the registration of these pesticides.

The Tanzania Bureau of Standards (TBS) is responsible for setting MRLs and tolerances for pesticide residues in various food products, including avocados. The TBS establishes MRLs based on international standards and guidelines, including the Codex Alimentarius Commission (CAC) guidelines. The authority also considers the local conditions and practices of pesticide use in Tanzania, as well as the latest scientific data and risk assessments. Once the MRLs and tolerances for pesticides in avocados are established, the TBS conducts monitoring to ensure compliance with the established limits (TBS, 2023).

2.7.3. European Union

Czech Republic imports and exports avocados because growing conditions do not favor avocado cultivation. We suspected that the avocado from Czech Republic might have been imported from another EU country such Spain. Spain is the leading producer of avocados in the European Union due to the favorable subtropical Mediterranean climate (Moreno-Ortega et al., 2019).

In the European Union, the control of pesticide residues in food is regulated by Regulation (EC) No. 396/2005, which sets MRLs for pesticides in different food products, including avocados. Another regulation (EC 284/2013), specifies the data requirements for plant protection goods in compliance with Regulation (EC) of the European Parliament and Council on the marketing of plant protection products.

2.7.4. Peru

Peru is found in South America. Regulations and guidelines for pesticide residues in avocados are based on the U.S. Food and Drug Administration and the Animal and Plant Health Inspection Services and Food Safety. The regulatory agencies for food safety in Peru are the Ministry of Health Directorate-General for Environmental Health (Dirección General de Salud Ambiental, DIGESA) and the Ministry of Agriculture National Food Safety and Quality Service (Servicio Nacional de Sanidad Agraria, SENASA (Ramirez-Hernandez et al., 2020).

The National Agrarian Health Service (SENASA) establishes MRLs for pesticide residues in avocados. The MRLs are based on the CAC standards and are regularly reviewed and updated. SENASA conducts regular monitoring of pesticide residues in food products, including avocados, to ensure compliance with established MRLs (SENASA, 2023).

2.8. Codex Alimentarius Commission MRLS

In 1963, the World Health Organization and the Food and Agriculture Organization of the United Nations established the Codex Alimentarius Commission. To protect consumer health and encourage ethical business practices, the Codex committee on pesticide residues (CCPR) develops non-binding consensus based MRLs and other food standards. (Neff et al., 2012). These form the basis of globally accepted standards, although some countries or unions set their own independent standards.

The table below shows the MRLs for some selected pesticide residues (as examples) as per the CAC online database (CAC, 2021) and the European Union online database (EC, 2023).

Table 1. MRLs of some pesticides for Avocado

Pesticide	**Functional Class**	**Codex Alimentarius**		**European Union**	
		MRL	**Year of Adoption**	**MRL**	**Year of Adoption**
Thiabendazole	Fungicide	15mg/Kg	2003	20mg/Kg	2022
Pyridate	Herbicide	Undefined		0.05mg/Kg	2014
Fenpyroximate	Acaricide	0.2mg/Kg	2018	0.2mg/Kg	2020
Methoxyfenozide	Insecticide	0.7mg/Kg	2010	0.7mg/Kg	2022

2.9. Challenges and limitations of the current regulatory frameworks

Some of the challenges and limitations of the current regulatory framework include lack of harmonization in MRLs and regulations among countries can create confusion and difficulties for importers and exporters, which can negatively impact trade according to Yeung et al., (2018). Handford et al., (2015b) opines that even when regulations and MRLs exist, inadequate monitoring and enforcement may result in residues exceeding the set limits. Many existing regulations and MRLs only cover a subset of pesticides, leaving gaps in coverage for other potentially harmful compounds (Arena et al., 2018).

According to Dalmas & Eleftherohorinos (2011), there may be insufficient data on the effects of certain pesticides or combinations of pesticides on human health and the environment, which can make it difficult to establish appropriate MRLs. The rapid introduction of new pesticides and changes in the way pesticides are used can challenge regulatory frameworks that may not be able to keep up with these changes. This is due to the continuous and rapid changes of the pesticide market, making manufacturers one step ahead of regulatory bodies, which lag because of lack of

current pesticide policy and inadequacy of financial resources (Storck et al., 2017). Lastly, the general public may not be aware of the potential risks associated with pesticide residues in avocados and other foods, which can lead to a lack of demand for stricter regulations.

CHAPTER 3

MATERIALS AND METHOD

3.1. Sampling

Four pieces of avocado each from Kenya, Tanzania, Peru, and Czech Republic were purchased randomly in supermarkets in Budapest. Since temperate climate in Czech Republic is not suitable for avocado farming, we suspected the avocado were originally from Spain. The avocados were stored in deep freezer at -18°C.

3.2. Chemicals and reagents

Magnesium sulphate was supplied by WWR International bv Geldenaaksebaan, Belgium. Primary Secondary Amine (PSA) and Octadecyl silica (ODS)were sourced from Sigma – Aldrich, USA Acetonitrile of 99.9% was bought from Honeywell, Germany. Sodium chloride, trisodium citrate, disodium hydrogen citrate, triphenyl phosphate (TPP), and formic acid were provided by the Department of Food Chemistry and Analytics. Other chemicals used in this study was the same as in the diplome thesis of Nándor Majercsik (Majercsik, 2020).

3.3. Multi-pesticide screening

To qualify and quantify the multi-pesticide residues in the avocado samples, sample extraction and clean up was done according to EN 15662:2018. The method used for analysis was developed at the Department of Food Chemistry and Analytics, Hungarian University of Agriculture and Life Sciences.

3.3.1. Sample extraction using Module E6 (EN 15662:2018)

Sample preparation was done based on the EN 15662:2018 (Module E6). The samples were removed from the deep freezer and allowed to defrost. They were then cut into small pieces using a stainless-steel knife (without washing). The cut pieces were grounded and homogenized using an electric grinder.

5g of the sample was weighed into a 50ml PTFE centrifuge tube and 6ml of water was added to it. 100µl of 50µg/ml TPP ("surrogate standard") were also added to the mixture followed by 10.0 ml of acetonitrile. The resulting mixture was shaken intensively for 1 minute. 4.0g of magnesium sulphate and 2.5g buffer salt were then added to the mixture. The buffer salt was prepared by mixing 1g of sodium chloride, 1g of trisodium citrate and 0.5g disodium hydrogen citrate. The mixture was shaken intensively followed by centrifugation for 5 minutes at 6000rpm.

3.3.2. Sample clean up using Module C4 (QuEChERS EN 15662:2018)

6ml of the supernatant from Module E6 was carefully transferred into a 15ml PTFE sample container pre-containing 900 mg of magnesium sulphate, 150 mg of PSA and 150 mg of ODS. The resulting mixture was shaken intensively for 0.5 minutes then centrifuged for 5 minutes at 6000 rpm. 4.0ml of supernatant were transferred into vial and acidified with 40µl of 5% HCOOH. Three replicates of each sample were prepared. The extracts were put in the deep freezer until the time of analyzing it.

3.3.3. Dilution of Extracts

The extracts were removed from the deep-freezer and left to warm up to room temperature. They were then diluted by taking 200µl of extracts and 300µl of ACN and 500µl water. They were well vortexed and filtered using 0.22µm filter.

3.3.4. Multi-standards preparation

1000ul of 1ppm multi-standard calibration MIX was prepared in a vial as shown in table 2. The MIX calibration stock solutions used contained high concentration, with MIX 1 up to MIX 8 containing 100mg/L, while the unique MIX contained 50mg/L).

Table 2. Preparation of 1ppm multi-standard calibration mix

Stock	Pipetted into a vial	ACN
MIX1 (100ppm)	10µl	900µl
MIX2 (100ppm)	10µl	
MIX3 (100ppm)	10µl	
MIX4 (100ppm)	10µl	
MIX5 (100ppm)	10µl	
MIX6 (100ppm)	10µl	
MIX7 (100ppm)	10µl	
MIX8 (100ppm)	10µl	
Unique (50ppm)	20µl	

It was vortexed well and used to prepare the matrix matched multi standard calibration solution as shown in table 3 below. Since the pesticides present in the samples were unknown, 200µl of extract from Peru samples was used for matrix matched calibration.

Table 3. Preparation of matrix-matched multi-standard calibration solutions

Standard MIX concentration (ppb=ng/ml)	Matrix (µl)	ACN (µl)	Water (µl)	1ppm STD MIX (µl)
0	200	300	500	0
5	200	295	500	5
10	200	290	500	10
25	200	275	500	25
50	200	250	500	50
150	200	150	500	150
250	200	50	500	250

3.3.5. Pesticides analysis using UHPLC – MS/MS

The samples were suspected to contain multiple pesticides. In addition, the exact identity of the pesticides in the samples was unknown. Therefore, a broad scaled method was preferred. In this study, the 250-analytes-screening method was used for multi-pesticide residue determination in avocado samples. This method was previously developed at the Department of Food Chemistry and Analytics. The method was validated for high water-content fruit samples (Majercsik, 2020).

3.3.5.1. Preparation of Eluent

For multipesticide determination the eluent A and B were 5mM ammonium formate, 0.1%HCOOH in water/methanol, respectively.

For the preparation of eluent A, 63.06mg ammonium formate were dissolved in about 20ml of water. It was then filtered through a 0.22µm filter into a 200 ml volumetric flask. The flask was filled nearly to the mark. 200µl of HCOOH were added and then filled to the mark. It was mixed well and transferred to an eluent bottle and labelled.

For the preparation of eluent B, 63.06mg ammonium formate were dissolved in about 20ml of methanol. An ultrasound was used to help in dissolving. It was then filtered through a 0.22µm filter into a 200 ml volumetric flask. The flask was filled nearly to the mark with methanol. 200µl of HCOOH were added and then filled to the mark. It was mixed well and transferred to an eluent bottle and labelled.

3.3.5.2. UHPLC-MS/MS instrumental parameter

Multipesticide residue analysis was performed using a UHPLC instrument composed of a pump, autosampler complete with a temperature control module. Eluent A and eluent B were used as the

mobile phase. A C18 column (Agilent Zorbax Eclipse Plus C18 2.1 x 150 mm) was used for chromatic separations. The injection volume was 5 µL. The flow rates were set at 0.4 mL/min. Detection was done by mass spectrometer using triple quadruple (QQQ) in dynamic multiple reaction monitoring (dMRM) mode.

3.3.5.3. Qualification of compounds

The chromatograms were screened with eyes and the samples with distinct peaks were recorded. The detected peaks were then subjected to the following criteria to identify them:

i) the retention time of the compound must be greater than the dead time of chromatography.
ii) the retention time of both MRM-transitions of the compound in the sample must be the same as in the standard (max. 0.3min variation is allowed)
iii) the signal of both MRM-transitions of the compound must be equal to or greater than the detection limit.
iv) the peak area ratio of the two MRM-transitions (so called "ionic ratio") measured in the sample must be within 30% of the ionic ratio measured in the standard.

Only the peaks that fulfilled all the four criteria were quantified, afterwards.

3.3.5.4. Quantification of compounds

Matrix matched calibration was used to quantify the compounds identified. A graph of area under the curve versus the concentration was plotted for each detected pesticide. Extract of sample from Peru was used as "matrix". Therefore, in the case of the calibration graphs that were not passing through the zero-mark, only the slope of the graph was used for quantification.

3.4. Comparison of different defatting methods and the matrix effect

Two different defatting methods recommended by EN 15662:2018 were compared in this study. The first method was a combination of Module C1 and C2 which involves freezing out followed by clean-up by dSPE with PSA. The second method was Module C4 where the extracts were cleaned-up by dSPE with a mixture of PSA and silica ODS. To compare the two defatting methods, the extractions were carried out on the sample from EU origin. Three replicates were made for each method by the following steps.

3.4.1. Sample extraction

Sample extraction was done based on EN 15662:2018 (Module E6) and the same way mentioned in chapter 3.3.1.

3.4.2. Sample clean-up

The sample was cleaned using two different defatting steps. To compare the two defatting methods based on their matrix effects, the slopes of the matrix-matched calibration curves were calculated separately, in three parallels. The average slopes were calculated and compared. This method was done in the case of three pesticides, separately.

3.4.2.1. Module C1 and Module C2 (QuEChERS EN 15662:2018)

6ml of the supernatant from Module E6 was carefully transferred into a 15ml PTFE sample container pre-containing 900 mg of magnesium sulphate and 150 mg of PSA. The resulting mixture was shaken intensively for 0.5 minutes then centrifuged for 5 minutes at 6000 rpm. 4.0ml of supernatant were transferred into vial. Three replicates were prepared. The extracts were put in the deep freezer until the time of analyzing it.

3.4.2.2 Module C4 (QuEChERS EN 15662:2018)

6ml of the supernatant from Module E6 was carefully transferred into a 15ml PTFE sample container pre-containing 900 mg of magnesium sulphate, 150 mg of PSA and 150 mg of ODS. The resulting mixture was shaken intensively for 0.5 minutes then centrifuged for 5 minutes at 6000 rpm. 4.0ml of supernatant were transferred into vial. Three replicates were prepared. The extracts were put in the deep freezer until the time of analyzing it.

3.4.3. Dilution of extracts

Different concentrations were made by mixing 200µl of the matrix with ACN, water and 1ppm standard mix to make 1000µl.

Table 4. Matrix dilution

Standard MIX concentration (ppb=ng/ml)	Matrix (µl)	ACN (µl)	Water (µl)	1ppm STD MIX (µl)
0 (a,b,c)	200	300	500	0
5 (a,b,c)	200	295	500	5
10 (a,b,c)	200	290	500	10
25 (a,b,c)	200	275	500	25
100 (a,b,c)	200	100	500	100
250 (a,b,c)	200	50	500	250

They were well vortexed and filtered using 0.22µm filter.

3.4.4. Multi-standard preparation

A multi standard (Submix 5) was used containing all the three pesticides (thiabendazole, methoxyfenozide and fenpyroximate) together with the TPP stock solution. To prepare 4000µl of 1ppm STD-all-MIX, 40µl of submix 5 and 80µl of unique (TPP stock solution) were added to 3880µl ACN in a 4ml vial. It was well mixed by vortexing and used to prepare three matrix matched multi standard calibration solutions in parallels of three as shown in table 5 below.

Table 5. Multi-standard preparation in three paralles (a. b and c)

Standard MIX concentration (ppb=ng/ml)	ACN (µl)	Water (µl)	1ppm STD MIX (µl)
0 (a,b,c)	500	500	0
5 (a,b,c)	495	500	5
10 (a,b,c)	490	500	10
25 (a,b,c)	475	500	25
100 (a,b,c)	300	500	100
250 (a,b,c)	250	500	250

They were well vortexed.

3.4.5. Pesticides analysis using UHPLC-MS/MS

The Eluent, the MS parameters, the Qualitative and quantitative analyses was done with the same method as was used in the case of multi-standard monitoring (chapter 3.35), except the column, which was much shorter to measure and focus only the 3 pesticides and TPP in the samples. A C18 column (Agilent Zorbax Eclipse Plus C18 2.1 x 50 mm) was used for chromatic separations. Detection was done in multiple reaction monitoring (MRM) mode. Table 6 below shows the MRM transitions of the compounds.

Table 6. MRM transitions of the detected compounds

Pesticide	Precursor mass (m/z)	Product mass (m/z)	Dwell time (ms)	Fragmentor (V)	Collision Energy (V)	Polarity
Fenpyroximate	422.10	366.2	20.00	135.00	15.00	Positive
Fenpyroximate	422.10	135.1	20.00	135.00	30.00	Positive
Methoxyfenozide	369.20	313.2	20.00	85.00	0.00	Positive
Methoxyfenozide	369.20	149.1	20.00	85.00	10.00	Positive
Pyridate	378.90	350.8	20.00	100.00	4.00	Positive
Pyridate	378.90	207.1	20.00	100.00	10.00	Positive
Thiabendazole	202.00	175.1	20.00	130.00	25.00	Positive
Thiabendazole	202.00	131.1	20.00	130.00	35.00	Positive
TPP	327.10	215.0	20.00	90.00	15.00	Positive
TPP	327.10	152.1	20.00	90.00	15.00	Positive

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Pesticides residues measurement

Avocado from different regions might contain different pesticides residues depending on the country of origin. Different countries have different legislation on pesticide usage for avocado farming, leading to the difference in pesticide residues among the samples from different regions. Module C4 was used for the multi-residue determination of the avocado samples from Kenya, Tanzania, EU, and Peru.

4.1.1. Quality analysis

Four distinct peaks were observed on the quantitative (first) transition of the analytes, after multipesticide screening of the samples. The distinct peaks belonged to thiabendazole, methoxyfenozide, fenpyroximate and pyridate. Figure 7 below shows compounds at a glance.

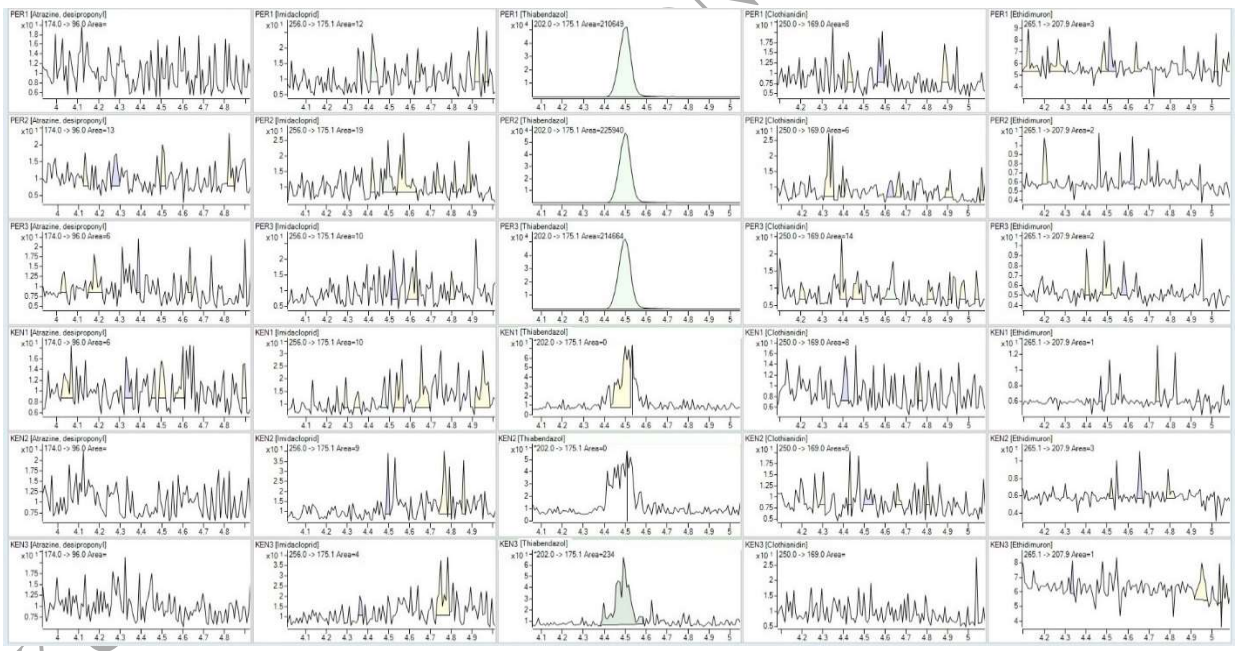

Figure 7. "Compounds at a glance" visualization method of compounds

We used a special software tool, called "compounds at a glance" in order to check the compounds detected in the samples (figure 7.). In this method, only the first (quantitative) MRM transition of each compound is visualized. In the figure 7., one can see that thiabendazol is detected in all the

three samples from Peru, however, is not detected in the samples from Kenya. The detected compounds are presented in table 7.

Table 7 Compounds detected in samples from different origin

Country	Pesticide
Kenya	Pyridate
Tanzania	Thiabendazole
	Pyridate
EU	Thiabendazole
	Methoxyfenozide
	Fenpyroxmat
	Pyridate
Peru	Thiabendazole
	Pyridate

In order to identify the pesticides detected, the criteria were used to investigate each of the four MRM-pairs, mentioned in Chapter 4.1.1 and the findings presented in table 8.

Table 8. Criteria for identifying analytes.

	$\Delta t_R <$ 0.3min	$t_R > t_0$	Signal intensity of the 2 MRMs	Ionic ratio ±30%
Thiabendazole	Yes	Yes	≥LOD	Yes
Methoxyfenozide	Yes	Yes	≥LOD	Yes
Fenpyroximate	Yes	Yes	≥LOD	Yes
Pyridate	Yes	Yes	1st MRM≥LOD 2nd MRM <LOD	No

Using the above criteria, the retention time of the MRMs were investigated first. The differences in retention time for all the four detected peaks was smaller than 0.3 minutes. It was also observed that retention time for all the four peak-pairs was greater than the dead time of chromatography (1.5min). Then signal intensity of the 2 MRMs and their ratio were investigated. The signal intensity was greater than the limit of detection for the three detected peaks. However, in the case of pyridate, only the first MRM signal intensity was greater than the limit of detection. The second

MRM was undetectable. A similar case was observed for ionic ratio where three detected peaks' ionic ratio was within the limit of ±30% of the ionic ratio in the standard. The pyridate peak's ionic ratio was well outside of the range. The characteristics of the peak of pyridate in a sample from Peru, which did not meet the criteria are illustrated in figures 8. and 9. below.

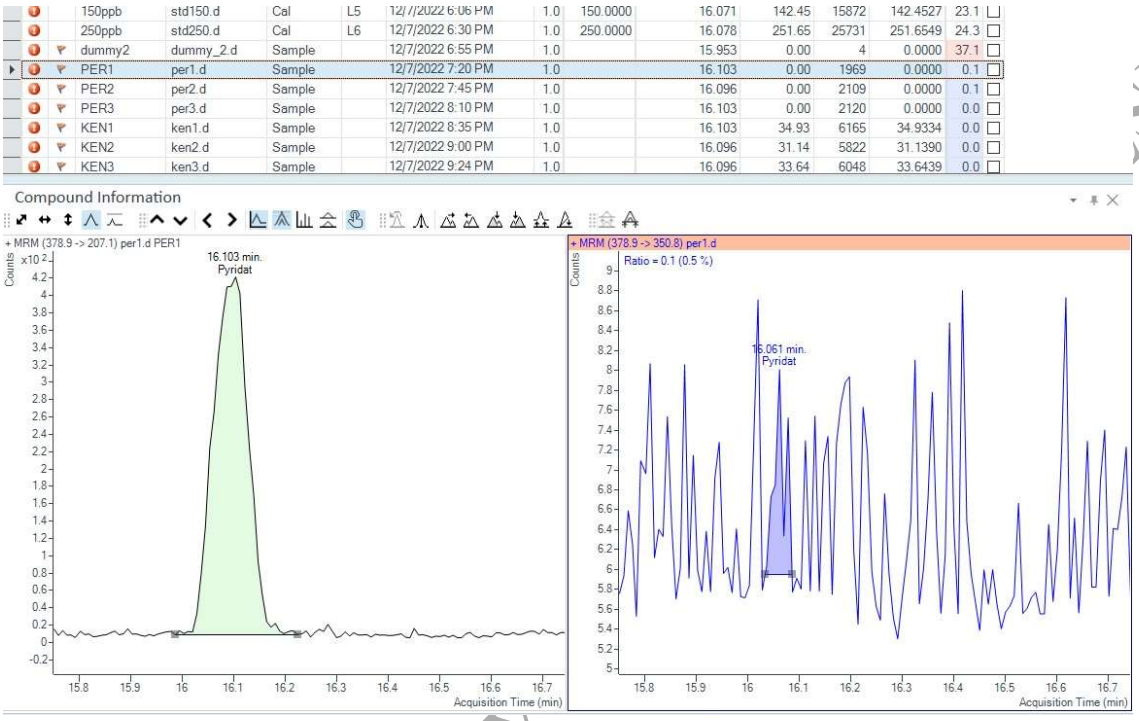

Figure 8. Pyridate ionic ratio in a sample from Peru

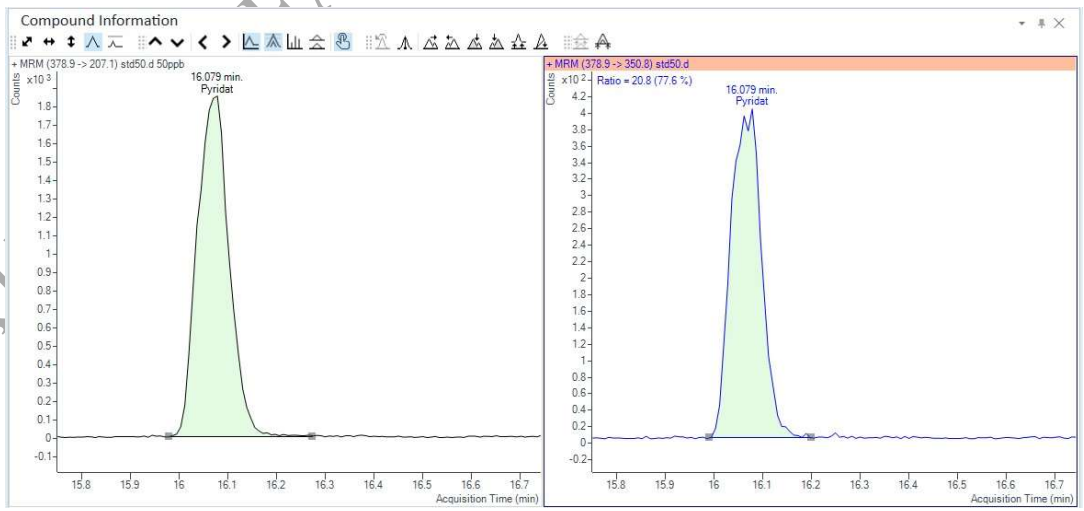

Figure 9. Pyridate ionic ratio of pyridate in standard solution

Based on the above criteria, the three compounds, namely thiabendazole, methoxyfenozide and fenpyroximate were identified in the samples and were subject to further quantification.

4.1.2. Quantitative analyses

To quantify the above identified compounds, a matrix-matched calibration method was used. For matrix, one of the avocado samples was applied because it provides the best matrix-matching. Matrix of sample from Peru was used for this purpose. The slope data was used for quantification as highlighted in chapter 3.

Figure 10 shows the matrix-matched calibration curve for fenpyroximate. From the calibration graphs, the pesticide concentrations of the analytical samples in ng/ml were calculated using the slope of the line.

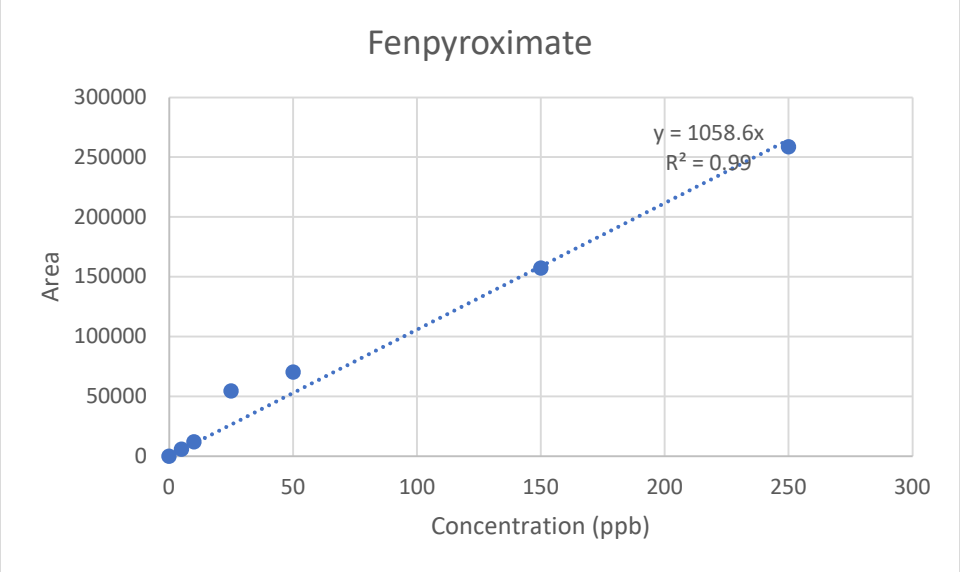

Figure 10.Matrix matched calibration curve for fenpyroximate

A summary of the slopes of the calibration curves for the different pesticides is shown in table 8 below.

Table 9.Summary of calibration curve slopes

Pesticide	**Slope of matrix-matched calibration curves**
Thiabendazole	1386.8
Methoxyfenozide	1729.2
Fenpyroximate	1058.6

The original concentration (wet weight) was calculated by multiplying the concentration by the dilution factor of 10 and converted to mg/Kg. A summary of pesticide residues in the four samples is listed in table 10 below. All the samples were measured in three parallels and the average value is presented.

Table 10. Pesticide residues

Country	Pesticide	Average concentration of pesticide in the analytical samples (ng/ml)	Average concentration of pesticides in samples (wet weight) (mg/Kg)	Standard deviation (mg/Kg)	Relative standard deviation
Kenya	None	Not detected	-	-	-
Tanzania	Thiabendazole	3.55	0.04	<DL	3.70
EU sample	Thiabendazole	0.61	0.01	<DL	46.62
	Methoxyfenozide	7.52	0.08	0.04	52.92
	Fenpyroxmate	0.43	<DL	<DL	37.54
Peru	Thiabendazole	156.54	1.57	0.06	3.65

Thiabendazole is a postharvest fungicide used for prevention of avocado spoilage and to prolong shelf life. It was detected in the samples from Tanzania, EU and Peru. The Peru sample recorded the highest concentration of 1.57mg/Kg, followed by Tanzania with 0.04mg/Kg. Peru might be focusing more on post-harvest treatment to improve shelf life leading to higher residues as compared to other regions. The EU sample posted the lowest concentration of 0.01mg/Kg which might be attributed to the EU strict regulation of plant protection practices as well as use of modern and effective pesticides next to thiabendazole, by which it requires lower dose of application. Thiabendazole was not detectable in the Kenyan sample probably because of alternative methods of post-harvest preservation such as cold chain regime. This might be due to the high cost of thiabendazole.

Methoxyfenozide is used as an insecticide which is very effective against FCM. It was only detected in the EU sample at low concentrations. It is a comparatively expensive and modern pesticide and might be avoided by other regions where plant protection practices are less strict.

Fenpyroximate is an acaricides used to control mites and ticks in agricultural crops. It was also found in the EU sample only and might be attributed to the EU strict regulation of plant protection practices.

From the results, samples from all the four Peru, Tanzania and EU contained pesticide residues. However, all the detected residues are within the limits set by the EU. It can be concluded that all the countries of origin obey the regulations of pesticides usage for avocado production; hence the fruits are safe for human consumption.

4.2. Comparison of defatting steps

Avocado contains about 15% fat which interferes with pesticides during the multi-residue determination. To improve on extraction, a defatting step was necessary to mitigate the matrix effect. Two methods were used and compared according to the EU standard on multi-pesticide residues analysis (EN 15662:2018). A combination of module C1 and C2 was compared with module C4 using matrix matched calibration solutions separately for each. The slope of these calibration curves was compared to evaluate the matrix-effect of each defatting method. Additionally, solvent calibration curves were also prepared to quantify the matrix effect of the two different cleaning methods.

4.2.1. Comparison of cleaning methods

The accuracy of the analytical method can change if different defatting steps are used because the defatting step can eliminate the analyte to a different extent. In addition, the defatting steps can alter the matrix effect and consequently, which is manifested in the slope of calibration curves. The slope of each parallel matrix matched calibration solution for C1C2 was compared to those of C4. The slopes for the three parallels of thiabendazole are represented below from figure 11 and figure 12.

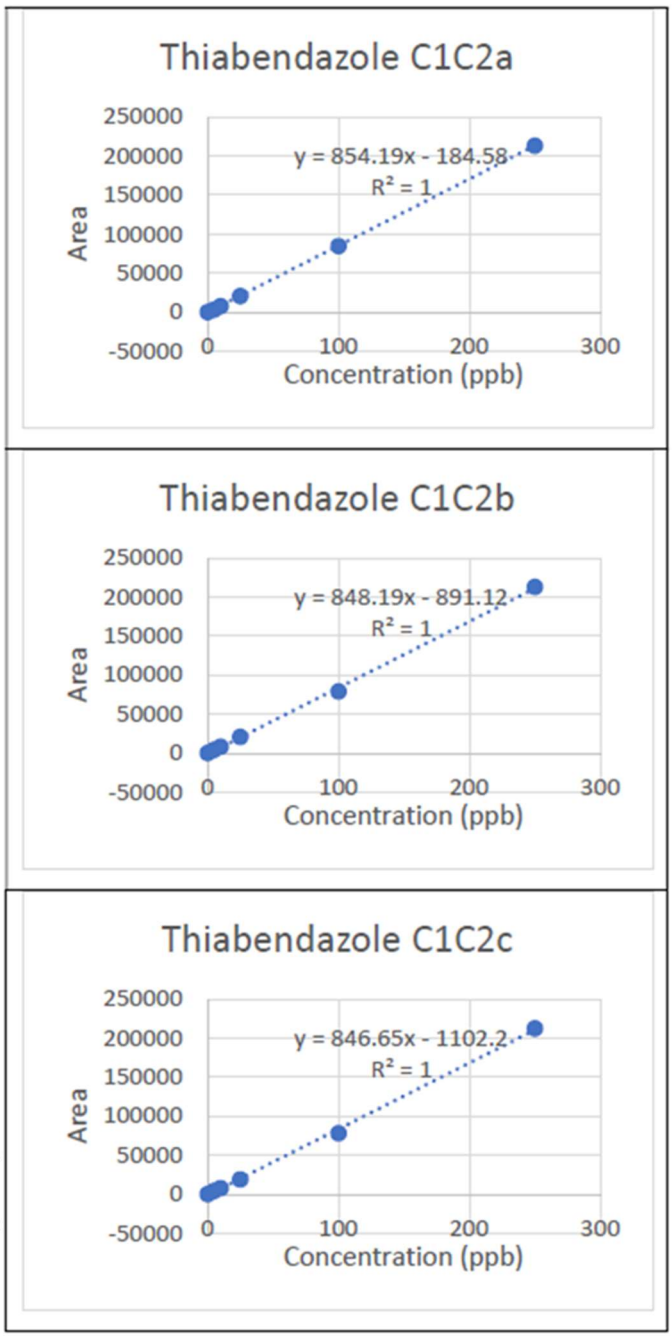

Figure 11. CIC2 slopes for three parallels (a, b and c)

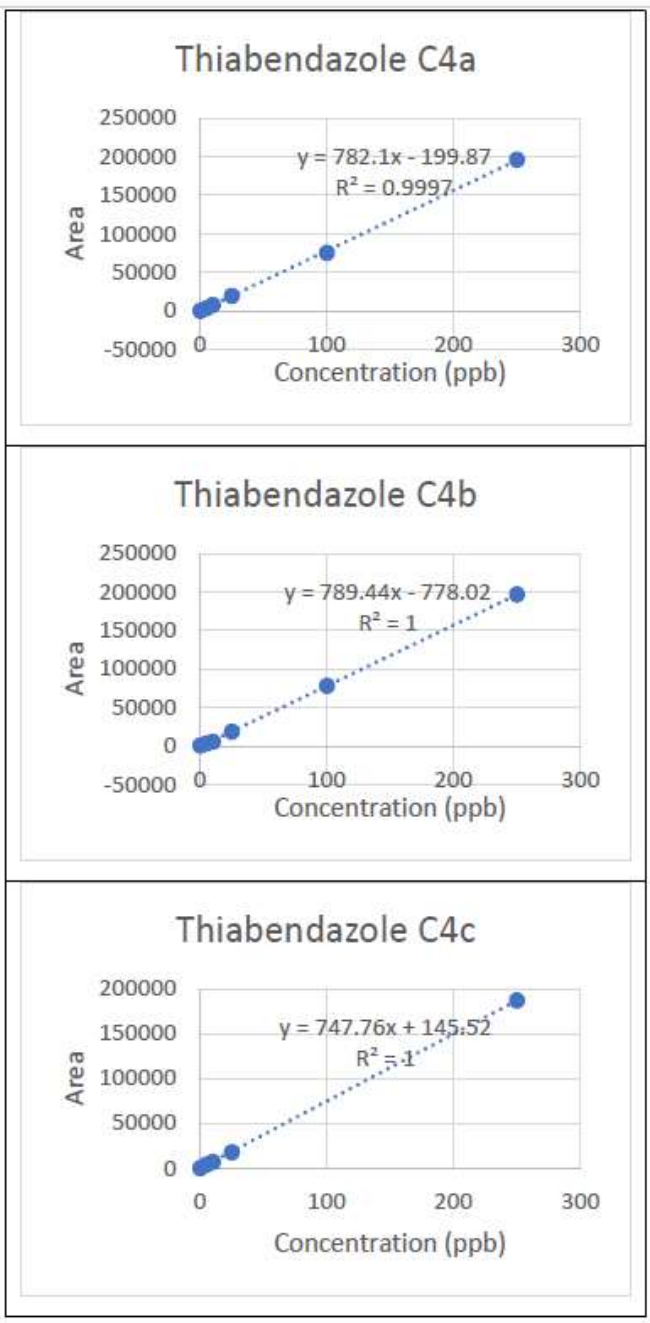

Figure 12. C4 slopes for three parallels (a, b and c)

A summary of the average and standard deviations of the slopes of the compounds were presented in table 11 below.

Table 11. Summary of the slopes

	Average of the slopes	Standard Deviation of the slopes	Significance test
Thiabendazole C1C2	849.68	3.98	P=0.051
Thiabendazole C4	776.43	24.87	
Methoxyfenozide C1C2	2188.23	5.47	
Methoxyfenozide C4	2010.83	18.95	
Fenpyroximate C1C2	1774.53	7.78	
Fenpyroximate C4	1640.53	31.01	

T test was used to compare the slopes of the three pairs. The p value of the three pairs was above 0.05. Therefore, there was no significant difference in the matrix effect of the samples prepared by C1C2 and C4. These two methods can be used interchangeably depending on the circumstances. Module C1C2 is a cheaper defatting step because it uses less reagents, for instance, it does not use ODS which is used in module C4. However, the freeze out step takes 12 hours and therefore increases the time of analysis. On the other hand, module C4 takes a shorter time for analysis but is expensive because an additional reagent (ODS) is used. The choice of the defatting step therefore depends on the cost and time of analysis.

In conclusion, the 250-analytes-screening method used for multi-pesticide residue determination in avocado samples was able to detect three pesticide residues in avocado samples from Tanzania, EU, and Peru. The pesticides detected were within the set limits by the EU. Therefore, the fruits from the sampled regions meet the set requirements hence are safe for human consumption. Comparison of the two defatting methods showed that there was no significant difference between the two methods.

This study was done on samples from only four regions. Further studies can focus on several regions to get more representative data. More research can also be done on samples collected randomly from the farms across the different regions before the fruits are exported to the EU.

SUMMARY

Avocado (Persea americana) is a popular fruit with high nutritional value, and it is cultivated in many countries worldwide. There has been an increase in production of the fruit to match the increasing demand. To mitigate production losses, pesticides are used to control pests, weeds and fungal spoilage. However, pesticides usage might lead to human health related issues as well as environmental contamination.

Multi-pesticides determination is a crucial step in checking the quality of avocado presented for human consumption. European Union regulates the maximum permissible level of pesticides in fruits, including avocados imported from abroad. Several methods have been developed and the choice of use depends on selectivity, sensitivity, and cost. HPLC-MS/MS offers good separation of pesticides and can analyze a wide range of polar and nonpolar compounds. However, it has limitations in the determination of low-molecular-weight pesticides and the analysis of complex samples due to interference from matrix components.

Avocado fruits contain around 15% fat which interferes with multipesticide determination. To reduce the matrix effect, two defatting steps are suggested based on EN 15662:2018. In my work, the two methods were compared regarding matrix effect reduction. The first method consisted of freeze-out followed by sample clean -up by dSPE with PSA. The alternative method involved clean-up by dSPE with a mixture PSA and ODS.

Qualification of the detected pair of MRMs was done based on a set criterion that included comparison of the retention times, ionic ratio, and signal strength. Quantification of the analytes was done using matrix-matched multi-standard calibration curve. The study identified 3 pesticides (thiabendazole, fenpyroximate and methoxyfenozide) residues in avocado samples from EU region at very low concentrations which were within the EU limits. Samples from Tanzania and Peru contained thiabendazole residues which were also within the EU limits. The pesticide residues in samples from Kenya were not detectable. All samples were therefore fit for human consumption.

Comparison of clean up steps was evaluated by comparing the slopes of the matrix matched calibration curves of each parallel sample of C1C2 against C4. There was no significant difference between the two clean up steps. Therefore, the methods can be used interchangeably.

This study was done on samples from only four regions. Further studies can focus on several regions to get more representative data. More research can also be done on samples collected randomly from the farms across the different regions before the fruits are exported to the EU.

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DECLARATION

on authenticity and public assess of master's thesis

Student's name:	**Kuloba Leakey Aliong'o**
Student's Neptun ID:	**NFZXGD**
Title of the document:	**Multi-Pesticide Residue Determination of Avocado Samples from Different Origins, Comparison of Defatting Steps**
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Department:	**Food Chemistry and Analytics**

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As a supervisor of KULOBA LEAKEY ALIONG'O (Student's name) NFZXGD (Student's NEPTUN ID), I here declare that the final essay/thesis/master's thesis/portfolio has been reviewed by me, the student was informed about the requirements of literary sources management and its legal and ethical rules.

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REQUEST FOR CONFIDENTIALITY

I, the undersigned KULOBA LEAKEY ALIONG'O (Neptun code: NFZXGD) student at MSc Food Safety and Quality Engineering programme request that my thesis / diploma thesis titled Multi-pesticide residue determination of avocado samples from different origins, comparison of defatting steps (name of supervisor(s): Dr Sörös Csilla Marczika Andrásné be encrypted by applying point c) of Section 95 (5) of the Study and Examination Regulations of the Hungarian University of Agriculture and Life Sciences (hereinafter referred to as 'SER'). I understand that if my request is approved, the encryption of the thesis / diploma thesis will cover 5 years following the successful defense, in accordance with point c) of Section 95 (5) of SER.

Done at: Budapest, 26th April 2023

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I, the undersigned Dr Sörös Csilla Marczika Andrásné, Professor (name and position of company representative), as the representative of Hungarian University of Agriculture and Life Sciences, 1118 Villányi út 29-43 (name and address of the company), request that the diploma thesis titled Multi-pesticide residue determination of avocado samples from different origins, comparison of defatting steps made by KULOBA LEAKEY ALIONG'O (name of the student) (Neptun code: NFZXGD) by using the data provided by Hungarian University of Agriculture and Life Sciences (name of company) be encrypted.

Done at: Budapest, 26th April 2023

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Representative's signature

I APPROVE / REJECT the request for confidentiality.

Reasons for rejection: _____

_____

Done at: _____ _____

_____

Programme leader's signature

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