Developing analytical tools to ensure food safety

Desarrollo de herramientas analíticas para garantizar la inocuidad alimentaria

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This work is dedicated to my dearest father who passed away after spending a life for sustainable development projects and putting ethical and moral values at the top of his beliefs.

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Executive summary

This Ph.D. thesis represents a contribution to enhancing food safety and environmental sustainability, especially targeting minor crops, for which very little analytical testing is implemented and for which there is, in general, scarce information on the food safety aspects. The long-term objective includes the possibility to contribute to the establishment or improvement of current regulatory frameworks (i.e. under regulatory schemes such as the CODEX Alimentarius), while providing for the availability of validated analytical methods together with residue/contaminant baseline data.

The main objective of this work was to further contribute to the establishment and validation of analytical tools that shall be combined in a fit for purpose way with food control processes to ensure food safety for the worldwide consumers. Food control systems are conceptual frameworks within which analytical testing and control processes are interacting with each other to identify, measure and manage food safety challenges, while maintaining and adequate level of protection for the consumers and the trade markets. This thesis contributed to enhancing the important role represented by the analytical laboratory in the farm to fork chain and further highlighted the need of interaction between the analytical side of testing and the monitoring / regulatory aspects, in a tandem process, where both sides are strongly depending on each other, and need to become flexible to adapt to ever changing food safety conditions.

The thesis work was divided in three main blocks, each looking at different aspects for minor and other crops, namely analytical method development and validation, quality of the analytical results, and linkages between analytical testing and monitoring / regulatory processes.

Chapter 1 of this manuscript is providing a general introduction to food safety aspects. A fully functional food control system includes both food control processes and analytical food control tools, which are interrelated. Both are needed to ensure protection for the consumers and environmental sustainability. The food control processes encompass all those managerial activities that combined ensure the strategic objectives of a food control system. The essential pillar of a food control system is the ability to implement “analytical food controls” that demonstrate safety and quality pre-requisites and compliance to regulatory requirements.

The analytical food controls are all necessary tools, actions and activities to implement food controls at analytical level. They are described in detail in Chapter 1.

Chapter 2 is the collection of five papers that were published in peer reviewed journals under this thesis work. Each paper is individually focusing on several aspects of the analytical development work.

Chapter 3 is where the joint analysis of the published work is presented together with the conceptualization of the work performed under this thesis work.

Optimization studies were implemented to take into account the challenges offered by analytical testing of minor crops. Available analytical instruments at the laboratories were optimized in their use to broaden the testing scope to multiresidue, multiclass and multi-contaminants methods, while optimizing extraction and clean-up processes. Different calibration strategies were studied to compensate for unavoidable matrix effects. It was shown that in the case of minor crops each testing laboratory needs to validate the analytes under its own instrumental setup and conditions, and as much as possible using “multiplex” methods. Chromatographic instrumentation such as GG-MS/MS and LC-MS/MS provided the necessary tools to monitor a broad scope of polarities and analytes of pesticides, dyes, persistent organic pollutants and mycotoxins. Thin Layer Chromatography (TLC) and Ion Mobility Spectrometry (IMS) were also used to optimize sample preparation methodologies with excellent performance. The main part of the thesis aimed at the development / validation approaches and data analysis for new testing methods for minor crops such as plant leaves, herbs and spices. Method validation was fully implemented for each of the matrices and performance criteria were fully evaluated and described for each of the developed methods. This thesis work highlighted the importance of analytical validation for each minor crops, and provided evidence that the concept of validating representative matrices within commodity groups cannot be applied in the case of minor crops. Individual full method validation needs to be implemented to be able to provide performance criteria that cover the variability within each analyte / commodity combination.

To provide an additional confidence level to the generated results and methodologies, and to address the quality of the results, robustness / ruggedness testing was studied in two very different commodities: a major and a minor crop. Two different experimental approaches were used: a design of experiments in potato samples, a staple crop; and an interlaboratory comparison in turmeric spices, a minor crop. A specific study on the subsampling step, was also included in this thesis work, as this is essential to provide representativeness and confidence of the analytical results. Uncertainty estimations, collaborative studies and

specific ruggedness / robustness tests were shown to be necessary to provide objective evidence of the goodness and fitness for testing of analytical methods.

In the last part of the thesis the attention was paid to the linkages between analytical testing and the monitoring / regulatory aspects applied to minor crops, such as vine leaves, boldo leaves or turmeric powder. This aimed at a verification of the combination of processes and tools that shall be applied correctly for targeting food safety requirements in a risk-based environment. It was shown that only the combination of tools and methodologies, including legislation, may enable a fully functional food safety system. Without a proper regulatory limit minor crops cannot be shown to comply with trade requirements; at the same time without a defined value of pesticides residue dissipation in vine leaves, for example, it is hard to set adequate regulatory limits for vine leaves used in foods, so commonly consumed in the Middle East and Greece, which has spread throughout the world.

The outcome from this work is a clear demonstration of the new active role of the analytical laboratory in terms of analytical capacity, developing and optimizing testing and validation procedures, as well a proactive approach in setting up a scope of analytes focused on evidence-based risks, while implementing the usage interface for utilization of the data through networking and data sharing for sustainable development.

Chapter 4 provides a short analysis for the follow up work to this thesis and the final conclusions.

Resumen ejecutivo

Este trabajo de tesis representa una contribución para mejorar la inocuidad alimentaria y la sostenibilidad medioambiental, especialmente dirigida a cultivos menores, para los que se conocen y realizan muy pocos ensayos analíticos y para los que existe, en general, escasa información sobre los aspectos de inocuidad alimentaria. El objetivo a largo plazo incluye la posibilidad de contribuir al establecimiento o mejora de los marcos regulatorios actuales (es decir, bajo esquemas regulatorios como el CODEX Alimentarius), al tiempo que se prevé la disponibilidad de métodos analíticos validados junto con datos de referencia de residuos / contaminantes.

El objetivo principal de este trabajo fue contribuir aún más al establecimiento y validación de herramientas analíticas que, combinadas de manera adecuada con los procesos de control de alimentos, para garantizar la inocuidad alimentaria para los consumidores de todo el mundo. Los sistemas de control de alimentos son marcos conceptuales dentro de los cuales las pruebas analíticas y los procesos de control interactúan entre sí para identificar, medir y gestionar los desafíos de la inocuidad de los alimentos, manteniendo al mismo tiempo un nivel adecuado de protección para los consumidores y los mercados comerciales. Esta tesis contribuyó a realzar el importante papel que representa el laboratorio analítico en la cadena de la granja a la mesa y destaca aún más la necesidad de interacción entre el lado analítico y los aspectos de monitoreo / regulación, en un proceso en tándem, donde ambos dependen fuertemente entre sí, y necesitan ser flexibles para adaptarse a condiciones de inocuidad alimentaria en constante cambio.

El trabajo de tesis se dividió en tres bloques principales, cada uno de los cuales analizó diferentes aspectos para cultivos menores y otros cultivos. Los tres grandes capítulos desarrollados fueron: a saber el desarrollo y la validación de métodos analíticos, la calidad de los resultados analíticos y los vínculos entre las pruebas analíticas y los procesos de seguimiento / reglamentación.

El **Capítulo 1** de este manuscrito presenta una introducción general a los aspectos de inocuidad alimentaria. Un sistema de control de alimentos completamente funcional incluye tanto procesos de control de alimentos como herramientas analíticas de control de alimentos, que están interrelacionados. Ambos son necesarios para garantizar la protección de los consumidores y la sostenibilidad del medio ambiente. Los procesos de control de alimentos abarcan todas aquellas actividades de gestión que, combinadas, garantizan los objetivos estratégicos de un sistema de control de alimentos. El pilar esencial de un sistema

de control de alimentos es la capacidad de implementar "controles analíticos " que demuestren los requisitos previos de seguridad y calidad, así como el cumplimiento de los requisitos reglamentarios.

Los controles analíticos de alimentos son todas las herramientas, acciones y actividades necesarias para implementar dichos parámetros a nivel analítico. Se describen en detalle en el Capítulo 1.

El **Capítulo 2** presenta los cinco artículos que fueron publicados en revistas revisadas por pares bajo este trabajo de tesis. Cada artículo se centra individualmente en varios aspectos del trabajo de desarrollo analítico.

El **Capítulo 3** presenta el análisis conjunto de los trabajos publicados junto con la conceptualización global de los resultados en este trabajo de tesis.

Se implementaron estudios de optimización para tener en cuenta los desafíos que ofrecen las pruebas analíticas de cultivos menores. Los instrumentos analíticos disponibles en los laboratorios se optimizaron en su uso con el fin de ampliar el alcance de las pruebas a métodos de residuos múltiples, clases múltiples y contaminantes múltiples, al tiempo que se optimizan los procesos de extracción y limpieza.

Se estudiaron diferentes estrategias de calibración para compensar los inevitables efectos de la matriz. Se demostró que, en el caso de cultivos menores, cada laboratorio necesita validar los analítos bajo su propia configuración y condiciones instrumentales, y en la medida de lo posible utilizando métodos “multiplex”, donde se estudian contaminantes de diverso origen. La instrumentación cromatográfica como GC-MS/MS y LC-MS/MS proporcionó las herramientas necesarias para monitorear una amplia gama de polaridades y analítos de plaguicidas, colorantes, contaminantes orgánicos persistentes y micotoxinas. También se utilizó cromatografía de capa fina (TLC) y espectrometría de movilidad iónica (IMS) para optimizar las metodologías de preparación de muestras con un rendimiento excelente.

La parte principal de la tesis tuvo como objetivo los enfoques de desarrollo / validación y el análisis de datos para nuevos métodos analíticos para cultivos menores como hojas de plantas, hierbas y especias. Se validaron los métodos desarrollados, para cada una de las matrices y se evaluaron y describieron completamente los criterios de desempeño para cada uno de ellos.

Este trabajo de tesis comprobó la importancia de la validación analítica para cada cultivo secundario estudiado y proporcionó evidencia de que el concepto de validar matrices representativas dentro de los grupos de productos no se puede aplicar para estos cultivos. Es necesario implementar la validación del método completo individual para poder

proporcionar criterios de rendimiento que cubran la variabilidad dentro de cada combinación de analíto / producto.

Para proporcionar un nivel de confianza adicional a los resultados y metodologías generados, y para abordar la calidad de los resultados, se estudiaron las pruebas de robustez / solidez en dos productos muy diferentes: un cultivo principal y uno secundario. Se utilizaron dos enfoques experimentales diferentes: un diseño de experimentos en muestras de papa, un cultivo básico; y una comparación entre laboratorios en las especias de cúrcuma, un cultivo secundario.

También se incluyó en este trabajo de tesis un estudio específico sobre el paso de submuestreo, ya que este es fundamental para brindar representatividad y confianza a los resultados analíticos. Se demostró que las estimaciones de incertidumbre, los estudios colaborativos y las pruebas específicas de robustez / robustez son necesarias para proporcionar evidencia objetiva de la bondad y la idoneidad de los métodos analíticos.

En la última parte de la tesis se hizo hincapié a los vínculos entre las pruebas analíticas y los aspectos de seguimiento / regulación aplicados a cultivos menores, como hojas de *Vitis*, hojas de boldo o polvo de cúrcuma. Esta parte del trabajo tenía como objetivo una verificación de la combinación de procesos y herramientas que deben aplicarse correctamente para abordar los requisitos de inocuidad alimentaria en un entorno basado en riesgos. Se demostró que solo la combinación de metodologías analíticas e instrumentación, incluida la legislación, puede permitir un sistema de inocuidad alimentaria completamente funcional. Sin un límite reglamentario adecuado, no se puede demostrar que los cultivos menores cumplen con los requisitos comerciales; al mismo tiempo, sin un valor definido de disipación de residuos de plaguicidas, por ejemplo en las hojas de *Vitis*, es difícil establecer límites reglamentarios adecuados para este alimento tan común en la comida del medio oriente y Grecia, que se ha expandido a todo el mundo.

El resultado de este trabajo es una clara demostración del nuevo papel activo del laboratorio analítico en términos de capacidad analítica, desarrollando y optimizando los procedimientos análisis y validación, así como un enfoque proactivo en el establecimiento de un alcance de analítos centrado en los riesgos basados en la evidencia, mientras se implementa la interfaz de uso para la utilización de los datos a través de redes y el intercambio de datos para el desarrollo sostenible.

El **Capítulo 4** proporciona un breve análisis para el trabajo de seguimiento de esta tesis y las conclusiones finales.

“We need to find new ways of thinking to deal with the problems caused by the old way of thinking”. A. Einstein

I feel as being an ant. Contributing to carrying twig after twig, we were able to contribute to the creation of an anthill that will serve as a new home for the next generation of ants. I believe in the values of humility, mutual support and cooperation. I really enjoy working as a team member.

Chapter 1: Introduction

1.1. Food safety and food integrity

“All life on the planet depends on food (and water)” [1]. An obvious statement that greatly summarizes the benefits of healthy foods for the humankind to survive and that is undoubtedly true. According to the Food and Agriculture Organization (FAO) of the United Nations (UN) “food security exists when all people, at all times, have physical and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life” [2]. Food security is a non-negotiable situation and deals with a primary need of human beings. Access to safe, reliable and nutritious food supplies is a basic need for all people. Governments have an obligation to ensure this need is met. Social, ethical, political, economic, scientific and religious aspects should be analyzed at length to frame food security in the world situation of 2021. A United Nations report from 2017 predicted that the world population will increase to 9.8 billion people in 2050 from the actual 7.6 billion of today [3]. This will require an increased food production to support an expanded consumer demand for food and water, which needs to be safe and of high quality including high nutritional value, especially in emerging economies. A global political agreement to achieve zero hunger, food security and improved nutrition, and to promote sustainable agriculture has been published as “Sustainable Development Goals (SDG)” by the United Nations [4]. No poverty and zero hunger are the first two most important global challenges the humankind is currently faced with. The SDGs represent the blueprint to achieve a better and more sustainable future for all. Food safety is an integral property of food and agrifood products, that must be secured, to reach the goals set in the SDGs. As such, food safety is a basic human right, and all governments need to ensure enough and safe food for all. In general, consumers also rely on government to ensure all food products are sold within a country are as what they claim to be and contain [5]. Since we need to eat to survive, it would be a sad waste of opportunities to eat “badly”. The food might be unsafe due to contamination from heavy metals, microbiological agents, chemical agents, including residues of pesticides and veterinary drugs residues, may contain illegal dyes or allergens or simply the food is not integer according to the definition of food integrity, as “the state of being whole, entire, or undiminished or in perfect condition in its nature, origin, identity, and claims, and to meet expected properties' [6].

Hazards of natural origin are mainly related to the activity and/or presence of microorganisms, fungi or plant alkaloids. Thus, foods may be contaminated directly by the presence of pathogens, which could cause an infection to the consumer, or may be indirectly contaminated by toxins produced by a microorganism, a fungus or a plant. Contamination of

food with pathogens may imply very serious consequences on health and can occur at any point of the food production chain due to inadequate hygiene conditions. On the other hand, the presence of toxin producers, within or near food related products, can be a potential source of contamination. This is the case, for instance, of cereal products contaminated with mycotoxins, or other seeds containing dangerous alkaloids such as the Ergot alkaloids, or for instance shellfish contaminated with microalgal toxins that are bioaccumulated in those filter-feeding animals [7].

The World Health Organization's (WHO) report on “Estimates of the Global Burden of Foodborne Diseases” estimated that in Africa such food safety hazards were responsible for approximately 137,000 deaths and about 91 million cases of acute foodborne illnesses on an annual basis, while on a worldwide and yearly basis unsafe food causes 600 million cases of foodborne diseases and 420 000 deaths [8].

Hazards of chemical origin are well defined, for example in the European Union (EU) legislation. According to the EU regulations, chemical hazards are grouped into regulated food ingredients (food additives, food enzymes, food flavourings, nutrient sources including food supplements and botanicals), food chain residues (feed additives, veterinary medicines, pesticides), contaminants (environmental pollutants, natural contaminants, process contaminants) and food contact materials [9].

Food integrity is the term commonly used to indicate the sum of all the essential characteristics of food and includes the definitions of food security, safety, quality and authenticity aspects. Food Integrity is defined as “the state of being whole, entire, or undiminished or in perfect condition” [6]. Food safety is a fundamental condition to ensure food integrity.

According to FAO food safety refers to all those hazards, whether chronic or acute, that may render food injurious to the health of the consumer [10]. Ensuring that foods are safe must be a priority for any producer, manufacturer, retailer, user and public authority in any country. Consumers are also responsible to ensure that the food they buy and prepare is kept safe. In other words, food safety implies a shared responsibility among all the actors in the food safety chain and a central role played by the analytical laboratory to ensure and carry out analytical testing at different chain points. While food safety is the basis for any food to be traded, distributed, consumed and manufactured, food integrity adds up additional features of quality and authenticity. From this point of view, food integrity implies a coverage of the broadest range of regulatory requirements that can be setup along any type of food product and its handling, distribution and use.

Examples of standard setting bodies are the Codex Alimentarius Commission of the FAO and World Health Organization (WHO) of the UN.

The Codex Alimentarius Commission currently comprises 188 Member Countries and 1 Member Organization (EU) and 229 Observers of which 57 are intergovernmental organizations, 156 non-governmental organizations and 16 United Nations agencies. The Codex Alimentarius, generally abbreviated to Codex, is a “food code”, a collection of standards, guidelines and codes of practice that governments may opt to use to ensure food safety, quality and fair trade [11]. According to Codex, when the standards are followed, consumers can trust the safety and quality of the products they buy, and importers can trust that the food they trade will meet the specifications. This last generic sentence needs to be considered in detail and implies a large range of responsibilities in the farm to fork chain along with many essential and coordinated activities to be implemented to effectively ensure food safety and food integrity.

Another example of a regulatory initiative is the European’s Union (EU) food safety model [12], which is considered a point of reference around the world and, according to the World Health Organization (WHO), “European citizens enjoy one of the highest levels of assurance on the safety of their food in the world” [13]. The strength of the EU food safety model is based on:

- its governance structure, with the division of responsibilities between EU decentralised agencies and the EU Commission, charged mainly with risk management and risk communication tasks, which are separate from risk assessment;
- its goal to assess the safety of chemicals before they are used in the food chain and placed in the market;
- and it's clear allocation of responsibilities between the private sector and public control authorities. In addition, the EU requires non-EU countries to comply with EU standards in order to guarantee that food imported to the EU fulfils the same high standards of safety.

In the EU food integrity is seen as a very strong driving force to ensure a healthy economy for all countries, due to the fact that providing assurance to consumers and other stakeholders about the safety, authenticity and quality of food (integrity) adds value to the agri-food economy [14].

Not only trade and consumers’ safety are important, but also the safety of the environment, where people live and where food is produced, is essential. An integrated approach to food safety and integrity includes ensuring the sustainability of the agricultural areas and

catchments where food is produced and manipulated. Also, a “healthy and pristine” environment ensures a safe food production and a sustainable farm-to-fork system [15].

Food safety implies that processes and tools are in place to ensure that untargeted and / or targeted controls are effective and implemented, while correctly assigning responsibilities along the food chain and facilitating a fully functional food control system [16].

The ultimate goal of food safety is to assure/ensure consumers about the safety and quality of the food supply and to promote and facilitate trade both domestically and externally [17]. Internal food trade would benefit the countries due to value addition to products, from protection of careful and scrupulous producers and processors against unlawful competitors, and from the development of trusted food industry and transparent trade actions. External food trade would be facilitated due to better international market access, to foreign exchange earnings and avoidance of dumping of inferior quality products [18].

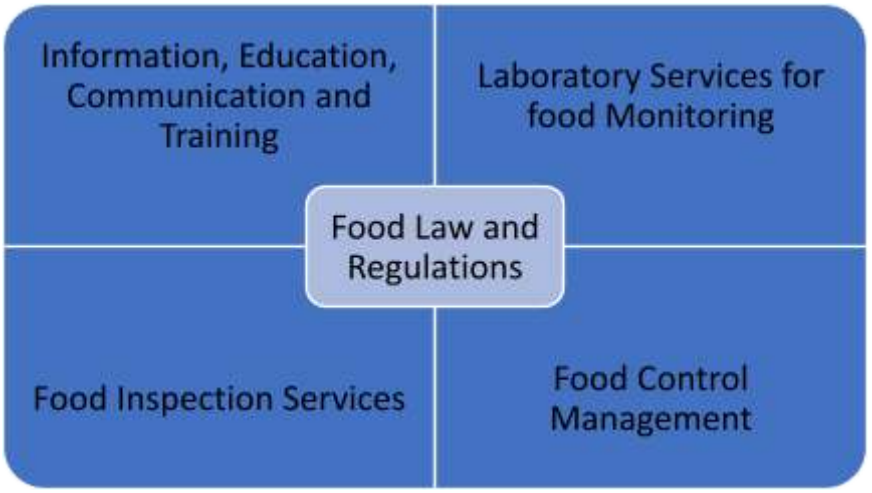
In addition to being an issue for public health, the presence of dangerous substances also provokes undesirable economic effects, because it can limit or reduce the marketing and trade of food products in national or international markets. Therefore, the control of residues and contaminants is one of the most important problems in food safety, and public and private organizations have made huge efforts in order to minimize this risk [19]. Although Good Agricultural Practices (GAPs) [20], Good Manufacturing Practices (GMPs) [21] and Hazard Analysis and Critical Control Point (HACCP) [22] systems are useful tools to prevent, mitigate or control food contamination, the total elimination of contaminants or residues in the food supply is statistically impossible. Therefore, keeping harmful chemical residues and contaminants below certain risk levels could be scientifically reasonable as well as it should be considered a social responsibility [23].

1.2. Food control systems

While acknowledging the broad range of food safety and integrity issues, the focus of this thesis will strictly consider hazards of chemical origin, including residues from pesticides, veterinary drugs, mycotoxins, dyes, persistent organic pollutants and other organic contaminants, in relation to food safety. Therefore, biological, inorganic contaminants and other food safety hazards will not be discussed in the rest of this dissertation.

Food systems in developing countries are not always as well organised and structured as in the most industrialised countries. Current challenges relate to a growing population, increased urbanisation, lack of resources to deal with pre- and post- harvest losses in food,

emerging and increasing environmental contamination and, in some countries, challenging food hygiene conditions. All these aspects may adversely affect the quality and the safety of food supplies [24]. Government response should be in terms of establishing proper food control systems that include all necessary components to assure the quality and safety of food. Since every country has a specific challenge with regards to current or emerging food safety and quality issues, a national strategy to enhance the food control system requires a specific knowledge of the current situation in the context of the national targets [25]. However, while each country will have a specific combination of elements and priorities, most food control systems will typically comprise the components as shown in **Text box 1** [26].



Text box 1: Typical elements of a food control system

Robust food control systems are essential to adequately support the application of food safety and/or sanitary and phytosanitary (SPS) measures [27] at national, regional and continental levels [28]. Food safety competent authorities must be capable of cooperating and coordinating efforts with each other according to guidance outlined in the Codex Alimentarius Commission's Principles and Guidelines on National Food Control Systems (CAC/GL 82–2013) [29].

Generic guidelines on assessing the status of national food control systems are available by the Food and Agriculture Organization (FAO) [30] and are a useful reference to deepen the understanding of the possible structures of national food control systems and their efficacy on a worldwide basis.

A recent study commissioned by the EFSA indicated that, when asked about a restricted number of issues in relation to food, EU citizens perceived the use of pesticides, antibiotics and additives in food production as the issue that worries them the most and that 86 % of respondents were very or fairly worried about the use of such substances in food production (see **Figure 1**) [31].

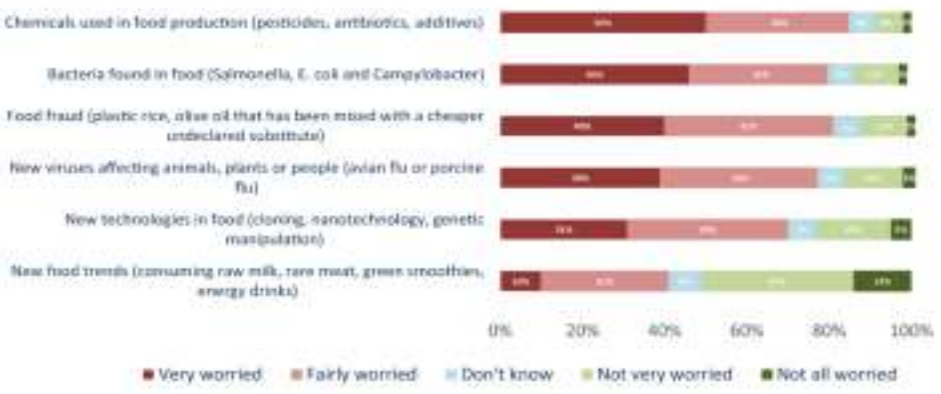


Figure 1: Perceptions of risk associated with different food safety and food fraud challenges by European consumers (extracted from [31])

An intrinsic function of food control systems is to constantly maintain an appropriate degree of security for consumers, and this is the basic challenge that every national government is challenged with.

A fully functional food control system includes both food control processes and analytical food control tools [26]. Food control processes and analytical tools are interrelated. Without any of those components, food safety cannot be ensured. Both are needed to ensure protection for the consumers and environmental sustainability.

Text box 2: The framework within which food control processes and tools operate

In order to manage current and emerging challenges, it is important for a food control system to be based on evidence and science, incorporate risk analysis principles and keep abreast of new scientific developments and innovations to continuously improve the effectiveness and efficiency of the food control activities both for the processes and the tools. Therefore, the framework, depicted in **Text box 2** as a blue frame, within which a food control system operates is of paramount importance and can have serious impacts on the effectiveness of the control processes and tools.

1.3. The processes to ensure food safety

The food control processes encompass all those managerial activities that combined ensure the strategic objectives of a food control system.

The food control processes include, but are not limited to:

- The regulatory processes
- Roles and responsibilities of food safety stakeholders
- Food safety management (strategic thinking and resource allocation)
    - monitoring and surveillance programs
    - risk assessments
    - modelling approaches

- Food safety communication
- Capacity building for human resources

1.3.1. The regulatory processes: food safety standards and guidelines

Food legislation and regulations should meet the following requirements [32]:

- Offer a high level of sanitary protection.
- Include clear definitions to achieve greater coherence and legal certainty.
- Be based on risk analysis through independent, transparent and quality scientific advice for evaluation, conduct inclusive, transparent and systematic risk management and communication. The Codex Alimentarius standards for risk management are based on solid scientific evidence, these can be used as a reference by countries.
- Include provisions regarding the right of consumers to have access to accurate and sufficient information.
- Allow tracing of food products and their recall in case of problems.
- Contain clear provisions stating that the primary responsibility for food safety and quality lies with the producers and processors.
- Collect the obligation to guarantee that only safe and properly presented food is placed on the market.
- Also recognize the country's international obligations, particularly in relation to trade.
- Guarantee transparency in the development of food legislation and access to information.
- Have established mechanisms and procedures that allow their modification in an easy and timely manner.

Food legislation is paramount to define official food control activities. A proper regulatory framework gives credibility and has a positive impact on the effectiveness of all food control activities [33]. In many countries, food regulations are shared by different ministries, i.e. the ministry of health and the ministry of agriculture. It is fundamental that full cooperation exists among these ministries in the way food regulations are interpreted and applied [34].

In addition to generic laws and regulations, governments need updated food standards, that are applicable to the multiple food products within a country. In recent years the tendency has been to move to horizontal standards that address the broad challenges involved in

achieving food safety objectives rather than addressing each food commodity specifically and with a highly prescriptive piece of legislation [35]. While horizontal standards are a viable approach to delivering food safety goals, they require a farm to fork food chain that is highly controlled (the processes) and managed by the support of reliable and quality data on food safety (the tools), including the provision of food safety risk. Such systems may not be easily implemented in many developing countries.

Examples of standards and guidelines for food safety are given by Codex Alimentarius Commission [36], the European Commission (EU) [37], the United States Environmental Protection Agency (US EPA) [38], the Mercosur [39], Japan [40], Canada [41], among others.

The EU has adopted numerous pieces of legislation, including directives, regulations, decisions and agreements, for each specific area (food additives, flavourings, feed additives, pesticides, etc.). Overall, this legal framework regulates around 8, 000 chemical substances [42]. Although this enormous regulatory framework, taken as a model in many countries worldwide, there is still a lack of regulation for some food commodity-residue/contaminant combinations [43]. In this situation, a “zero tolerance” policy is applied in the EU. In such cases, the verification of regulatory compliance becomes a more complex question and, therefore, more difficult to answer.

In the case of the pesticide residues regulations in the EU, when a pesticide Maximum Residue Limit (MRL) is not available for a particular commodity, from an analytical point of view, the regulatory limit is set at the limit of determination for that pesticide using the available infrastructure at the laboratory. In practice the EU zero tolerance level is set at 0.01 mg/kg.

The MRL is defined as the acceptable concentration of a substance found in foods that are consumed by humans for long periods of time and should not constitute any health risk when the chemical is applied following good agricultural practices (GAPs). This level is known as a tolerance in the USA and as maximum residue limits (MRLs) in the EU and other countries.

In the case of pesticide residues regulations in the USA, as to the absence of a tolerance value, the Food and Drug administration (FDA) may establish an “action level” for such unavoidable pesticide chemical residues. An action level is a recommended level of a contaminant not to be exceeded. An action level is not legally binding and FDA may take enforcement action on a case-by-case basis whether a contaminant is below, at, or above an action level [44].

MRLs are still widely inappropriately perceived only as levels of safety concern. Violative residues are rarely of health significance as acute events. Safety factors of 100 to 1000 are

included to set the MRL well below the toxicological threshold, called no-observed-adverse-effect-level (NOAEL) and acceptable daily intake (ADI) of the substances (see **Figure 2**).

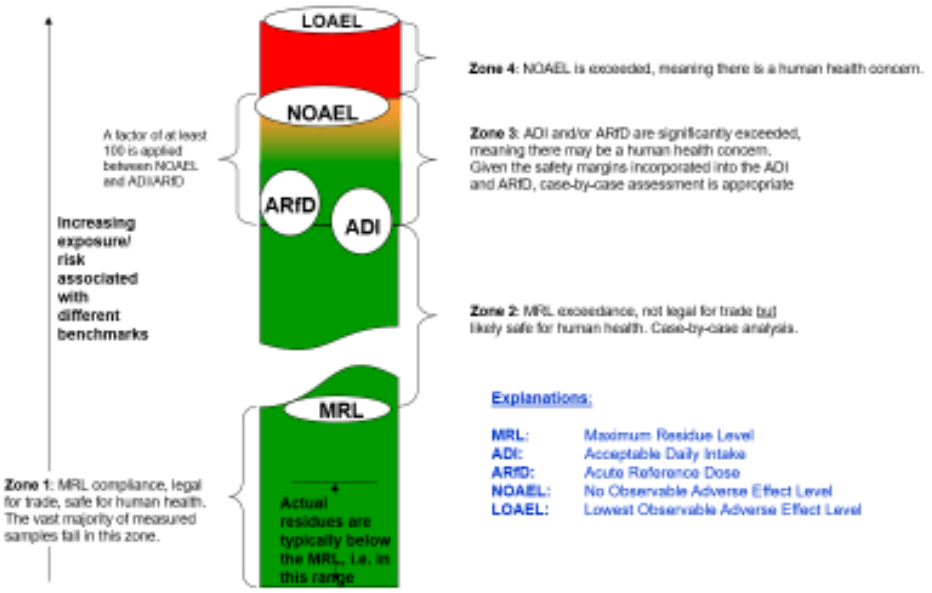


Figure 2: Simplified scheme showing the safety margins and the toxicological endpoints for MRL setting (adapted from Grossgut, AGES)

Pesticides are applied to the crops following GAPs to control pests and plagues; the residues that may be left behind do not impose an unacceptable risk for consumers.

In the United States, the Food and Drug Administration (FDA) [45] is responsible for the safety and security of food supply, dietary supplements, etc., while the Environmental Protection Agency (EPA) [46] regulates pesticide applications used by growers to protect crops.

In Canada, Health Canada's Food Directorate [47] is the federal health authority responsible for establishing policies, setting standards, and providing advice and information on the safety and nutritional value of foods available for sale. An important part of the Food Directorate's mission is to ensure that human exposure to chemical contaminants and residues in food, whether from natural or man-made sources is kept to a minimum and that it is not detrimental to Canadians' health [48].

Under the Agreement on the Application of Sanitary and Phytosanitary Measures (the 'SPS agreement' [49] members of the World Trade Organization (WTO) consider to develop their health standards on risk-based criteria. Under WTO rules, importing countries cannot use a simple hazard-based criterion alone as a basis for excluding potential imports. Article 5 of the SPS agreement rather requires countries to implement a complete risk assessment study and demonstrate a significant risk. The Codex is part of a committee on sanitary and phytosanitary measures, established to provide a regular forum for consultations for members of the WTO [16]. However, in the EU legal framework two types of criteria are existing: risk based (in most cases) and hazard-based 'cut-off' criteria, specifically in the legislation governing the marketing and use of pesticides. Risk based criteria mean that a specific substance has to go through the entire risk assessment process to determine its safety limits; while hazard-based criteria bans certain substances purely on the basis that it considers them potentially hazardous (e.g. carcinogenic), without the need for a full risk assessment [50].

The WTO helps ensuring that adherence to food safety regulations ultimately results in equivalence of food safety systems to protect all consumers worldwide. This is particularly true when dealing with food trade, and the assurance of the highest protection for the consumers. To this end, private standards, such as global GAP, were born as a mean to compensate where there was a lack in the standards and to resolve complicated trade situations [51].

The topic of pesticide residues in foods still represents a controversial and complicated issue of interest to consumers, food producers and processors, regulators, legislators, and scientists throughout the world [52]. Consumer concerns regarding pesticide residues has led, in part, to the steady growth of the organic food industry [53].

1.3.2. Roles and responsibilities of food safety stakeholders

Food may be exposed to chemical residues and contaminants via several pathways, including agricultural practices, industrial processes, inappropriate storage, environmental contamination, wrong doings by consumers and natural toxins. Chemical hazards may occur at any point in the farm-to fork food chain. Therefore, the responsibilities to ensure food safety shall be shared among all stakeholders in the farm-to-fork chain. The EU has set this principle at the base of its food law. According to EU food law, responsibility for ensuring compliance with legislation – and in particular the safety of food – lies primarily with food (or

feed) business operators [54]. World consumers have the right to healthy and safe food and are also responsible for certain aspects related to food safety, such as observing good hygiene practices during handling and storing food properly according to the manufacturer's recommendations listed on the labels. National authorities are responsible for protecting public health by reducing the risks of contracting foodborne illness and for educating and informing consumers and the food industry on all aspects of food safety. Ultimately, responsibility for food safety stays with producers, processors, retailers, and those responsible for preparing or serving food. Although every individual or every company has the right to produce, elaborate, prepare, serve, import or export food, this right carries with it an inseparable obligation to ensure that it is healthy and safe food, and that said individuals or companies comply with all legislation in force, even with the rules that protect consumers from possible fraud. Producers and processors, as well as retailers and food preparers, are part of the chain and their work is essential to fulfil their role within effective food control systems and, in turn, have the capacity to use them in their work. As well as a legal obligation, private food business operators have a strong branding reputation and economic interest in ensuring that the food they sell is safe. Private businesses involved in the food chain have primary responsibility for food safety and frequently have assurance systems that extend to the point of supply and comply also to private regulatory schemes that are stricter than official regulations. An example of this type of private scheme is the Global GAP [55].

1.3.3. Food safety management

Another important process for food control is food management. This implies issuing rules and regulations that quickly address food safety challenges and emerging issues. Core responsibilities include establishing regulatory measures, monitoring system operation, promoting constant improvements, and general advice on policy formulation.

In the EU, for example, the European Commission (EC) is the responsible body for food safety management [56]. Its work is based on scientific opinions provided by the European Food Safety Authority (EFSA) [57], which is an independent body responsible for conducting scientific risk assessments for food safety according to the EU General Food Law [58].

In the EU, the DG Health and Food Safety carries out audits, inspections and related non-audit activities aimed at ensuring that EU legislation on food and feed safety, is properly implemented and enforced [59]. As part of food safety management is the possibility to apply decision rules, for example when an infringement is detected during official inspections. The

“precautionary principle” is another example of a food safety risk management tool available to decision makers [60]. It provides a basis for action when science is unable to give a clear answer. In the EU it allows the risk manager to take provisional measures while waiting for further scientific information needed in order to conduct a comprehensive risk assessment.
Additional enforcement measures taken to ensure that unsafe food does not make it onto the market include destroying a particular product or withdrawing it from the market, suspending or shutting an activity down. In general, it is recommended that governments lay down their own rules on sanctions, which should be effective, proportionate and dissuasive [9]. If laboratory tests show that a sample exceeds a limit set in the legal regulations, governments may follow up the infringement and carry out a safety assessment and, in the meantime, may apply a warning or an increased check. Where the safety assessment shows a risk to health, governments may apply fines [61]. It is essential to indicate in the regulatory framework what are the rules and the possible sanctions, to discourage fraudulent operators from committing or perpetrating “food crimes” and food frauds [62].

1.3.4. Monitoring and surveillance programs

Food control systems aim to protect the health of the population and to promote trade. They require at a minimum a risk-based monitoring program that is appropriate and properly designed for the purpose of monitoring food safety and quality.
The activities involved in a system that provides knowledge about the current situation and trends regarding the appearance and spread of pathogenic microorganisms and chemical and natural contaminants in the food production chain are grouped under the terms "surveillance" and "monitoring." Monitoring can be defined as: "the performance and analysis of routine measurements, aimed at detecting the presence or absence of hazards or contaminants in food for the implementation of immediate corrective actions. While surveillance can be defined as "the continuous systematic collection, collation, analysis and interpretation of data, followed by the dissemination of information to all those involved so that targeted actions can be taken" [63].
Effective monitoring /testing schemes depend on the co-existence of sound sampling plans, valid analytical methods and regulatory limits at a minimum [64]. For example, in the EU the Official Food and Feed Controls Regulation (EC) No 882/2004 forms the basis for the testing carried out in the EU. The regulation aims at an integrated and uniform approach to official controls along the agri-food chain on legal provisions for the frequency and nature of checks.

It provides the framework for competent authorities to verify compliance with food and feed law and to prevent, eliminate and reduce to acceptable levels risks to human beings and animals [65]. In the EU, Member States are not obliged to test for all regulated substances, but they are obliged to make sure that their monitoring plans are designed based on risk, which may lead to a different depth and frequencies of controls for different groups of substances. The principle of -proportion for sampling purpose- highlights that only mostly consumed foods are subject to heavy routine monitoring. The minor commodities or crops are left out and the MRLs set at 0.01 mg/kg level.

The approach of the Codex Alimentarius Commission is different from the EU. No default MRL limit is set by the Codex.

Codex MRLs are established only where there is supporting evidence concerning the safety to humans of the resulting residues as determined by the Joint FAO/WHO Meeting on Pesticide Residues and this means that Codex Maximum Residue Limits represent residue levels which are toxicologically acceptable [66].

For the purpose of monitoring residues/contaminants, there is a classification into major and minor crops. Minor crops are those for which the daily dietary intake contribution < 1.5 g (i.e. 1.5 g mean daily consumption over the population for a 60 kg person) and/or the cultivation area < 600 ha (less than 0.0035 % of the total cultivation area) [67].

The monitoring programmes also apply to imported products. The EU checks on imports aim at ensuring that imports are compliant with EU legislation in the same way that food produced in the EU are. The underlying principle is that all food products on EU markets must be safe, irrespective of their origin. In general, the EU control system for higher-risk imports requires stricter conditions for the entry into the EU and therefore a higher level of controls than lower-risk imports. The EU's approach considers that food of animal origin involves a higher degree of risk than food of non-animal origin [68]. Food of non-animal origin posing a known or emerging risk are also subject to increased controls. This applies for mycotoxins, pesticide residues and food additives.

The measures taken by Member States, i.e. to reject the consignment and invoice laboratory costs and storage fees, are not "cost-free" and act as a deterrent or de facto penalty. They can also have potential contractual implications between the operators concerned [9].

The fundamental principle of monitoring and surveillance is that it must be designed and implemented to provide valid (true) information that can be evaluated and analyzed by decision makers in a timely manner at the lowest possible cost and thus ensure public health.

Food safety surveillance is a system that collects data on all types of contaminants in food. This has to be conducted in a structured and planned manner in order to understand the entire known spectrum of food safety and detect information on new hazards, identify hazards in a timely manner and provide relevant information for food safety monitoring, risk assessment. and standard setting [69].

Monitoring is based on a sound sampling plan performed by trained sampling inspectors [70]. Sampling is an event performed outside the laboratory; however, it is fully integrated into the analytical process. An example of a transversal guideline for sampling is the Codex General Guidelines on Sampling [71]. Inevitably, food sampling is firmly regulated as it assures that the analytical result is representative for the tested commodity. The sampling plan should describe the sampling procedures necessary to obtain representative samples for collection from animals / crops / food products or from the food production environment, at a specific point in the food chain. Clear definitions shall be provided to define the "laboratory sample" and practical instructions shall be given on how to obtain a representative laboratory sample [72]. The ISO/IEC 17025:2017 standard contains some provisions for the accreditation of the sampling step [73].

1.3.5. Food safety communication and capacity building

An increasingly important role for food control systems is the delivery of information, education and advice to stakeholders of the farm-to-fork chain. These activities include the provision of balanced factual information to consumers (risk communication); the provision of information packages and educational programmes for key officials and workers in the food industry; development of train-the-trainers' programmes; and provision of reference literature to extension workers in the agriculture and health sectors [16].

Risk communication is defined as the exchange of information and opinions concerning risk and risk-related factors among risk assessors, risk managers, consumers and other interested parties [74]. Risk communication is an essential part of the risk analysis process. The main goal of food safety risk communication is to increase understanding among various food safety stakeholders regarding the rationale behind the decisions taken to assess hazards and manage food safety risks, and to help people to make more informed judgements about the food safety hazards and risks they face in their lives [75].

The Rapid Alert System for Food and Feed (RASFF) is an example of an EU communication tool, [76] to quickly exchange information between national authorities on health risks related

to food and feed. The RASFF is widely used as a source of information for planning laboratory tests as it provides important information on risks.

1.4. The analytical tools to ensure food safety

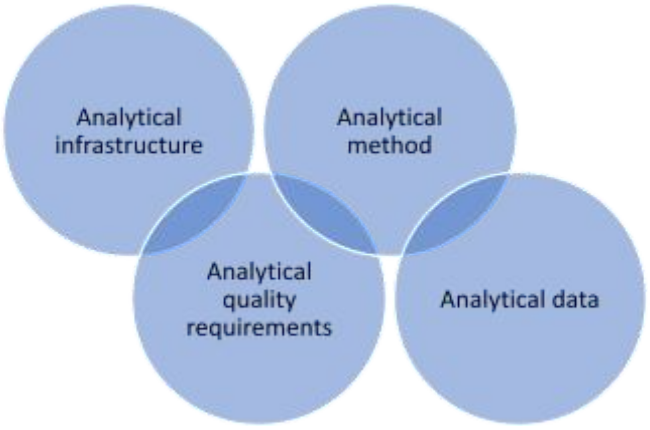
The essential pillar of a food control system is the ability to implement “analytical food controls” that demonstrate safety and quality pre-requisites and compliance to regulatory requirements.

The analytical food controls, also called food control analytical tools, or simply analytical tools, are all necessary tools, actions and activities to implement food controls at analytical level [70].

The food control analytical tools include, but are not limited to:

- The analytical infrastructure
- The analytical method
- The analytical data
- The analytical quality requirement

Those components are interrelated, see **Text box 3**. Without any of the components, food controls cannot be ensured, implemented and used to guarantee food safety.



Text box 3: Food control tools, summary of the main requirements

1.4.1. The analytical infrastructure

The analytical laboratory plays a fundamental role in the national/regional food control system. Representative testing is fundamental to ensure that food safety is always maintained and ensured by all stakeholders along the farm to fork chain. In a utopic food control system, preventative approaches would suffice to maintain confidence in the food safety chain, however the food safety scandals of the last years ([77], [78], [79]), are a proof that end control testing is necessary and needs to be even stricter and smarter. The “fight” against “food fraud” is paved by a series of international R&D programs and alliances such as the food integrity project [80], the global food safety initiative [81], the IAEA coordinated research programme [82], among many others. However due to the complexity of the food system, such programs are far away from covering the whole possible food safety and fraud scenarios. The assurance of food safety is paramount to maintain public health, and governments need to implement the best possible strategies to maintain consumers in the food they eat.

To date it is difficult to provide a standardized guidance on the setup for the best analytical strategy.

Ideally the analytical laboratory should be included in a functional network of national/regional/interregional laboratories of acknowledged competence (i.e .official control laboratories or designated laboratory), either public or private, that interact and communicate with each other, sharing analytical capacities, infrastructure when possible, experiences, data and provide testing to ensure access to the safest foods from the domestic and international traded products. The Red Analitica de Laboratorios de America Latina y el Caribe (RALACA) is an example of such an analytical network of laboratories [83].

The scope of food safety testing can be so wide, and it requires broad imagination by food managers to be able to cover all possible natural and anthropogenic emerging challenges.

A first classification of food safety infrastructure is whether the scope of testing is targeted or untargeted and this brings the linkages to the food safety processes aspects. De facto “to be able to manage it is essential to measure”.

Progress of the analytical technology have made it possible to extend the scope of testing to untargeted testing. The targeted approach consists in confirming the presence of a specific target list of residues/contaminants, while the untargeted approach aims at identifying any residue/contaminant that could occur in food products, without any previous knowledge of its potential presence [84].

The essential laboratory infrastructure [85] consists of:

- A physical laboratory including gas systems, ventilation, optimal space allocation and separation of chemical/physical activities
- Essential equipment and instrumentation
- Information technology (IT) solutions for modelling, and data transfer and storage
- A cash flow and a business plan to maintain the activities and keep fully functional instrumentation and their upgrade
- Access to representative samples, acquired through a standardized sampling and a harmonized official monitoring program
- Trained and motivated laboratory analysts that work towards ensuring quality of all laboratory processes.

Food control bodies should also address the specific training needs, in principle, of all stakeholders in the farm-to fork chain, but at least of their food inspectors and laboratory analysts as a high priority. These activities provide an important means of building food control expertise and skills to ensure food safety. In the EU, the EFSA has been working on capacity building training, such as the "Better Training for Safer Food initiative" [86]. At an international level the FAO and the IAEA, for example, are targeting capacity building needs through seminars, workshops and meetings, to enable members state country food safety stakeholders tackle their own food safety challenges [87].

1.4.2. Description of essential laboratory instrumentation

Laboratories deal daily with the determination of a wide range of chemical compounds in a high variety of complex matrices, from either vegetal or animal origin, which supposes a real challenge in terms of concentration sensitivity and selectivity. In this scenario, liquid chromatography (LC) and gas chromatography (GC) coupled to mass spectrometry (LC-MS and GC-MS) instruments have become the main tools for analysts because they allow testing for residues and contaminants reaching the selectivity, sensitivity and specificity required by current food safety legislation in targeted mode ([88], [89], [90], [91]).

Modern high-performance liquid chromatography (HPLC) uses high pressure to force a liquid mobile phase and an analyte through a closed column packed with micron-sized particles, which constitute the stationary phase [92]. In gas chromatography (GC), the sample is vaporized and injected onto the head of a chromatographic column. Elution is brought about by the flow of an inert gaseous mobile phase such as helium and the analytes are separated

into individual peaks or individual components. In GC, the mobile phase does not interact with molecules of the analyte, and it only transports the analyte through the column [93]. LC is commonly used for detection of analytes that are difficult to vaporize; GC is commonly applied for the separation of volatile and semi-volatile compounds. Derivatization reactions can also be used prior to analysis directly or indirectly making a large number of non-volatile and more polar compounds amenable to GC separation. The main disadvantage of using GC over LC, to analyse moderate polar substances is the derivatization step needed to obtain symmetric peaks, to ensure satisfactory precision, and to improve the separation and detection of these compounds [94]. In this case the analyst, if given the choice, should opt for an LC determination. In 2006 Lutz Alder ran a study and compared between GC-MS and LC-MS for 500 high priority pesticides. The result showed that the LC-based techniques are more advantageous in terms of wider scope, better selectivity, and increased sensitivity, without the need for derivatization [95].

HPLC instrumentation is made up typically of the following basic components: mobile phase/solvent reservoir, solvent delivery system, sample introduction device, an analytical column, post-column apparatus, detector, data collection and output system, post-detector eluent processing, and connective tubing and fittings.

A typical gas chromatograph (GC) consists of an autosampler, an injection port, an analytical column, carrier gas flow control equipment, ovens and heaters for maintaining temperatures of the injection port and the column, and a detector.

In general, the chromatography process occurs in four sequential steps: injection of the sample, separation of individual compounds in distinct elution bands in the analytical column, detection of each eluted band and registration of a “chromatogram “by means of a detector [96].

A universal detector for both liquid and gas chromatography is the mass spectrometry detector (MS). It is said that LCs and GCs are coupled to the MS.

The mass spectrometry detector consists of:

- an ionization source, where the molecules arriving from the analytical column are transformed from chemically neutral species into ions, and referred to as fragments, usually positive cations, breaking various bonds along the process;
- an ion analyzer, where the ions are collected and separated according to their mass to charge (m/z) ratio for each of the individual fragments
- a detector, where the ions are transferred to a data registration system to provide an ion chromatogram.

The MS process allows the possible identification of the chemical structure of the analyte and therefore suggests identity [97]. The more popular mass spectrometer detectors are those equipped with quadrupoles or quadrupoles in tandem as mass analyzers. The analysis is often performed in targeted mode, that is to look for a desired compound by comparing the analyte(s) in the sample to that of a known (reference) compound. The reference is used to identify the unknown compound by matching retention time (from chromatography) and ion fragmentation patterns from mass spectral libraries (for mass spectrometry). In mass spectrometry, the combination of compound separation and ion fragment identification yields an extremely powerful analysis that is said to be confirmatory. Criteria for confirmation of analytes will be discussed in other sections. Tentative identification without the use of a reference standard (if a reference compound is not available or is too expensive for example) is also possible using mass spectral libraries that contain mass spectra for numerous chemicals [98]. In the last two decades, specific innovations in the field of liquid chromatography brought improved analytical possibilities for food safety testing [99] and liquid chromatography in combination with atmospheric pressure ionization tandem mass spectrometry (LC/MS/MS) became the mainstream strategy for quantitative analysis of chemical contaminants [100]. At the very base of these innovations is the Van Deemter equation [101]. This is the basic equation that describes an analytical column efficiency, and provides an insight into the factors that lead to the broadening of the analyte band as it travels along a chromatographic column. It relates separation efficiency in terms of the Height equivalent of the theoretical plate (HETP) of the column to the linear velocity of the mobile phase, which is dependent, among other factors, on the size of the stationary phase particles. According to the Van Deemter equation, there is a significant improvement in efficiency when using sub-2-mm particle size for the stationary phase of the analytical column.

Ultra-high performance liquid chromatography (UHPLC) was developed when columns started to be packed with smaller sized particles, sub-2-mm (for example porous hybrid organic–inorganic silicon-based particles with a narrow size distribution in the range of 1.7 um) [102] and concomitantly new technological innovations allowed pumping and injecting liquids at pressures of 1000 bar and above [103]. A measure of this performance is the generation of peak widths (at half-height) of less than 1 s. This, at the same time, it required enhanced features for quadrupole mass spectrometers (MS), to be able to define a LC peak for accurate and reliable quantitation. Instrument manufacturers developed fast scanning speed acquisition for simple MS, allowing acquisition of enough data points even with few milliseconds of dwell times and also improved the resolution through deconvolution and

smoothing software that allowed defining a peak with a high certainty even if with fewer data points [104].

When combined with the high specificity of tandem mass spectrometry, UPLC/MS/MS has been demonstrated to be a powerful platform, which improves assay sensitivity, selectivity and throughput over traditional LC/MS/MS. It was shown that the UPLC/MS/MS method can provide up to a 3-fold reduction in retention time, one order of magnitude increase in detected peak height and a 2-fold decrease in peak width compared to traditional HPLC-MS/MS method [105]. A major consideration in selecting UHPLC in place of conventional HPLC is that the higher throughput obtained from using UHPLC coupled to MS/MS increases the requirement to perform regular preventative maintenance on the instrument to ensure continued optimal performance. This is especially important when working with more complex matrices; the orifice of the MS/MS must be routinely cleaned, to avoid accumulation of contamination that could potentially enter the mass analyzers and in turn lead to a reduction in sensitivity or to the release of less-intense analytes [106]. Current MS analyzers have ideal characteristics to be coupled to UHPLC, with high full scan acquisition rates, dwell times of 1 ms and polarity switching of 30 ms or less. As to GC instrumentation technological innovations are directly linked to high-speed capillary GC. Most high-speed GC applications have been carried out by means of reduced internal diameter (I.D.) analytical columns, called microbore, and shorter/different geometry columns. The narrower I.D. leads to the shorter analysis time at constant resolution. Thus, the increase of analysis speed does not compromise the separation efficiency. The decrease of column diameter results in a proportionally decreased value of minimum plate height. Therefore, the column length can be decreased by the same factor in order to yield the same plate number in a shorter time [107]. The drawback is that the column features provide lower sample capacity, which can result in higher limits of detection (LODs) and quantification (LOQs). Additionally, columns can deteriorate more easily, and this is seen when peaks start broadening and tailing [108]. Another innovation in GC analysis is represented by low-pressure gas chromatography (LPGC), especially when coupled to mass spectrometry. The use of low-pressure gas chromatography–mass spectrometry was recently revamped as a proven solution for fast, sensitive, and robust GC–MS analysis [109], [110]. Low-pressure gas chromatography (LPGC) has been known to be advantageous compared to standard GC since Giddings first described the concept in 1962 [111]. A practical solution for its use was identified by de Zeeuw [112] who simply used a guard column restrictor concept to maintain positive inlet pressure for a wide- (i.e. 0.53 mm id_{int}) analytical column under vacuum connected to an MS detector [113]. LPGC–MS is the most practical and beneficial

fast-GC technique available to achieve a short analysis time (i.e. less than 10 minutes) in applications that typically take 20–40 minutes. Sample capacity and column robustness are increased greatly using LPGC to permit large-volume injection with standard inlets without column maintenance, and, because vacuum conditions generate taller and narrower peaks that are still suitable for standard MS data acquisition rates, sensitivity is also increased. Another innovation in GC is an improvement in the resolution power of the GC instrument by the development of comprehensive two-dimensional GC (GCxGC) [114].

Another innovation is the development of applications of ion chromatography (IC), for the determination of polar ionic analytes, such as anions, cations or small polar analytes (metabolites), and sugars. When combined with mass spectrometry, namely triple quadrupole MS/MS systems, IC-MS/MS offers very low detection limits and high detection selectivity. Using the ion chromatography tandem mass spectrometry (IC-MS/MS) approach for direct analysis of quick polar pesticide method extracts (QuPPe, see section 1.4.5.8.5), low limits of quantification (typically < 5 ng/g), and associated repeatability (typically < 20 %) have been achieved for glyphosate (and metabolites AMPA (aminomethylphosphonic acid) and N-Acetyl AMPA), glufosinate (and metabolites, chlorate, perchlorate, glufosinate, N-acetyl glufosinate, 3-MPPA (3-methylphosphinicopropionic acid), Fosetyl-Al, phosphonic acid, ethephon and more, in a single analysis [115].

1.4.2.1. Mass spectrometric detectors

While ionization methods determine the classes of substances available for measurement, it is a combination of the characteristics of the mass analyzer with the detector that ultimately determines the quality and reliability of analysis [116]. As described earlier, mass spectrometry (MS) offers a highly sensitive detection technique that ionizes the sample components, under vacuum separates the resulting ions based on their mass-to-charge ratios (m/z) and measures the intensity of each ion. A mass spectrum is a plot of the relative ion intensities against the m/z values, and a series of mass spectra are generated at each retention time, see **Figure 3.** This information indicates the “concentration” of ions having a defined mass to charge ratio. This is extremely valuable for the unique identification of molecules, also known as qualitative analysis. Moreover, MS provides added specificity and sensitivity, and the convenience of simultaneous multicomponent analysis [117].

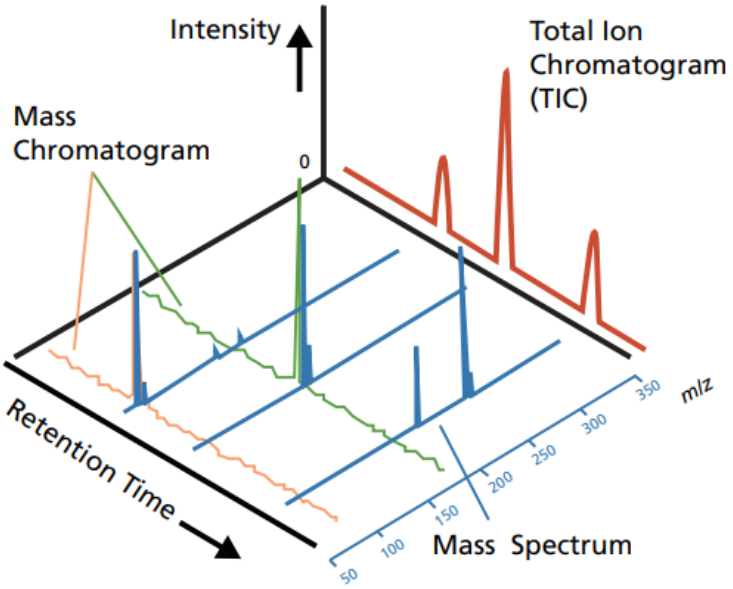


Figure 3: A typical total ion chromatogram, TIC, (red) and a mass chromatogram (green and orange) and mass spectrum (blue) (Courtesy of Shimadzu).

There are several types of mass analyzers that can be used for the separation of ions in a mass spectrometry: quadrupole mass analyzer, time of flight mass analyzer (TOF), magnetic sector mass analyzer, ion trap mass analyzer.

Both LC and GC instrumentation are compatible with most of the mass spectrometers such as quadrupole MS, tandem MS, orbitraps, TOF and QTOF-MS.

There are two fundamentally different approaches to tandem mass spectrometry (MS/MS): tandem in space and tandem in time.

Triple quadrupole (QqQ) mass spectrometers are among the most common MS/MS systems operating as tandem in space analyzers.

Tandem-in time instruments are typically ion-trapping mass spectrometers, which comprise three-dimensional (3-D) quadrupole ion trap (QIT), linear ion trap (LIT), and Fourier transform ion cyclotron resonance (FTICR) instruments [118].

The Orbitrap analyzer is an ion trap variant that has been available for several years in LC-MS platforms, but has only recently become available in the GC-MS configuration [119]. Orbitrap analyzers exhibit high mass resolution (> 150,000) with 1–5 ppm mass accuracy [120]. The use of high-resolution mass spectrometry (HRMS), such as TOF and Orbitrap, in the field of food safety is showing an increase of use [121]. One of the advantages of using HRMS is the possibility of constructing databases for the target compounds, when operating

under targeted approaches. The use of these databases together with parallel reaction monitoring using a Q-Orbitrap analyzer has been shown for example to be effective for the appropriate screening and quantification of 157 residues of different nature in honey [128]. Similar approaches have involved an expansion on the studied compounds to more than 600 different contaminants, including pesticides, veterinary drug residues, contaminants, perfluoroalkyl substances, mycotoxins and nitrosamines [122]. In the Orbitrap mass analyzer the mass resolution is dependent on the acquisition time, more specifically on the number of harmonic oscillations, and so higher mass resolutions are acquired over longer periods of time. Mass resolutions of 200,000 at m/z 400 can be achieved, which are significantly higher than for most TOF instruments [123]. One major advantage of HRMS technology is based on the easy adaption for non-targeted analysis, which opens up possibilities for unexpected findings. In addition, retrospective analysis enables the identification of residues and contaminants not targeted in the first data processing and reading of the results [124]. It can be expected that the number of HRMS instrumentations applied in routine analysis will strongly increase in the next decade. However, in order to fully compete with MS/MS instruments, two significant adjustments are necessary: (i) expand the linear working range which is important in the applicability of these instruments in routine analysis and (ii) make the devices affordable for a wide range of applications [132]. When comparing mass analysers, it is important to note that no single mass analyser is perfect for all analyses, it really depends on the purpose of testing. Therefore, it is important to understand the different principles, features and characteristics of the different mass analysers and choose what is appropriate for the objective of testing, or in other words, fit for purpose.

Recent studies are available to compare the use of tandem MS versus HRMS. For example, it was demonstrated that GC-HRMS may fit better for monitoring purposes for the quantification of polychlorinated dioxins and biphenyls in foods as it was shown to produce less false positives than using GC-MS/MS, although both technologies allow meeting the EU legislation requirements [133]. An LC-full-scan HRMS method has been suggested as an alternative for triple quadrupole MS-based methods. A fully non-targeted approach for data acquisition combining full-scan and fragmentation was presented for the quantitative validation of the methodology using a mixture of 184 pesticides in two food matrices. This approach was suitable for ca. 93 % of the assayed pesticide/matrix/concentration combinations studied in agreement with EU regulatory guidelines [128]. The most interesting aspect related to the non-targeted methodology is based on the possibility of detecting substances not previously pre-selected, even at a later stage (reprocessing features), thus, increasing the chance for the proper detection of unknown and unexpected compounds. It is

generally recognized that for targeted testing, low resolution mass spectrometry (LRMS) using triple quadrupole (QqQ) is the preferred analyser due to its intrinsic characteristics such as high scan speed, that permits the monitoring of a high number of tandem MS transitions within the time segments; due to the increased selectivity offered by selected reaction monitoring (SRM/MRM) mode; and due to the different acquisition modes that can be used with this analyser, allowing qualitative and quantitative analysis, although this analyser is mainly used for quantification purposes ([1], [129], [130]).

The legislation requirements in the EU pose an example of a dual challenge with respect to extreme low limits of quantification (LOQ), e.g., 0.025 µg/kg for aflatoxin M1 in infant formulae, and additionally a broad working range, e.g. in case of mycotoxins and veterinary drugs, which requires high instrumental performance in both ultra-low and high concentration levels.

Therefore, LRMS-QqQ is the first choice for the development of multiresidue/multi-class quantitative methods at trace and ultra-trace levels and with a broad working range for the analytes of interest.

Table 1 contains an indication of the identification requirements for different LRMS analysers according to Codex CAC/GL 90-2017 [131] and EU SANTE/12682/2019 guidelines [132].

Table 1: Identification requirements for different LRMS analysers (according to CAC/GL 90-2017 and EU SANTE/12682/2019)

MS analyzer	Acquisition mode	Requirement for minimum number of ions	Identification requirement
Single MS quadrupole	full scan, SIM	3 ions	S/N ≥ 3. The extracted ions chromatograms from analyte peaks must overlap. The ion ratio from sample extracts should be within ±30 % (relative) of average of calibration standards from same sequence
Triple quadrupole MS/MS, ion trap, Q-trap,	SRM or MRM,	2 product ions	S/N ≥ 3. Analyte peaks from both product ions in the extracted ion chromatograms must fully overlap. Ion ratio from sample extracts should be within ±30 % (relative) of average of calibration standards from same sequence

1.4.2.2. Ion mobility spectrometry (IMS)

Ion mobility spectrometry is an additional technique that residue/contaminant laboratories may use for their work, and which has been re-discovered recently for chemical testing. IMS is founded on the discovery that ions can be created at ambient pressure from radioactive materials (i.e. tritium source) and that these gas-phase ions can be characterized rapidly for mobility in comparatively weak electric fields [133]. In IMS, soft chemical ionisation of the volatile molecules is achieved at atmospheric pressure using a β-radiator tritium (3H) source. Ionized compounds are then separated based on their mobility and shape in a carrier buffer gas under an electric field at atmospheric pressure or near to atmospheric pressure [134]. The separation is based on the specific 'drift times' that ionized compounds pass over a fixed distance in a defined electric field, in the so-called 'drift tube'. The mobility of the ions is related to their mass to charge ratio (m/z) and their shape, as retarding collisions with the drift gas molecules are more frequent for sterically hindered compounds. Consequently, IMS can even separate different molecules with the same mass/charge ratio (isobaric molecules). [135]. Ions will move faster or slower through a drift tube, depending on the cross-sectional area of each compound, also known as collision cross-section (CCS) and will reach the detector at different times. Compact molecules exhibit lower CCS values, migrate faster than larger molecules and lead to lower drift times. Detection of the ion current is achieved using an electrometer as a function of time. The measured CCS provides an added value to the analysis because it depends on the molecular structure of the compound and contributes to the unequivocal identification of the analyzed substances.

Unlike other techniques such as time-of-flight/mass spectrometry, which operates under vacuum, the ions in the IMS drift tube travel at atmospheric pressure versus a flow of inert nitrogen gas. IMS appears as a powerful technique that reduces the background noise and allows the separation of isomers and isobaric compounds. As a result, cleaner MS spectra are obtained, and the identification process is facilitated [135]. IMS is relatively sensitive compared to other techniques with detection limits typically in the low ppb-range for volatile organic compounds (VOCs). Ion mobility spectrometry is an analytical technology used to separately detect compounds of interest in a mixture of gaseous analytes, such as the volatile compounds associated with the flavour and aroma of foods. An example of IMS instrumentation is the Flavourspec Instrument by Gas Dortmund [136]. This instrument includes a GC column that allows a chromatographic separation of the analytes in the system, prior to IMS separation and detection. Chromatographically separated compounds do not compete for reactant ions when they reach the IMS source and this yields better

sensitivity for the time-resolved components of complex mixtures of volatile organic compounds. This provides a two-dimensional separation for complex volatile profiles which are often encountered in herbs and spices.

1.4.2.3. Chromatographic instruments operation modes

A single quadrupole mass analyser can operate in two different modes: scan and selected ion monitoring (SIM) mode. In the scan mode, mass spectral data are acquired in sequence at specific intervals. The voltages of the quadrupoles are configured in a way that the entire mass range specified in the software is scanned sequentially with an appropriate dwell time for each m/z, as shown in **Figure 4**. The blue, the red and the yellow ions pass sequentially through the mass analyser and the result is a record of the ion abundance in the specified range of the mass spectrum. In SIM mode the MS is programmed to acquire only specific masses. Only the selected m/z (red ion in **Figure 4**) is passing through the quadrupole and is reaching the detector.

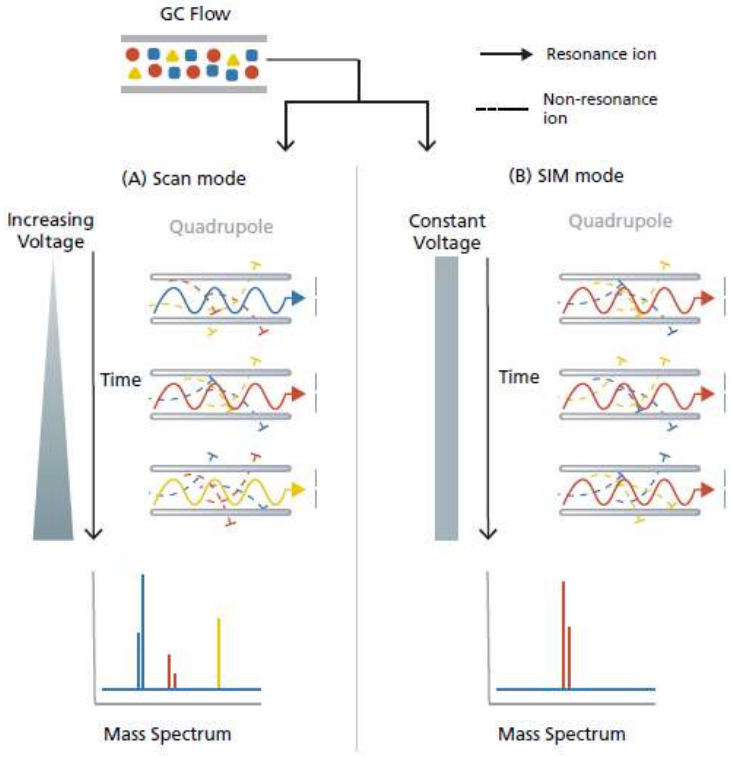


Figure 4: Scan and Selected Ion Monitoring (SIM) mode in single quadrupole MS (Courtesy of Shimadzu)

As previously discussed, the most frequent analytical approach to determine residues and contaminants in foods relies on the use of tandem MS detection for both LC and GC-based methods. This detection procedure allows the quantification of known compounds with greater selectivity and sensitivity compared to single quadrupoles. Typically, triple quadrupole analysers have been widely used for this aim, run under selected reaction monitoring (SRM), also called multiple reaction monitoring (MRM) mode, as shown in **Figure 5**. The ions formed in the ion source of the MS, called the parent ions, are further fragmented by collision-induced dissociation (CID) in the collision cell and the most-intense product ions (or daughter ions), at least two, are detected in the analyser. The most intense product ion is used for quantification purposes, whereas the second ion is employed for qualification purposes. This detection procedure allows complying with European legislation on banned and controlled substances in foods [137]. In SRM or MRM mode the precursor ion is selected in Q1, fragmentation occurs in Q2 and the product ion is selected by Q3. Sensitivity is achieved by a two-stage mass selectivity and very little interference from the background matrix [138].

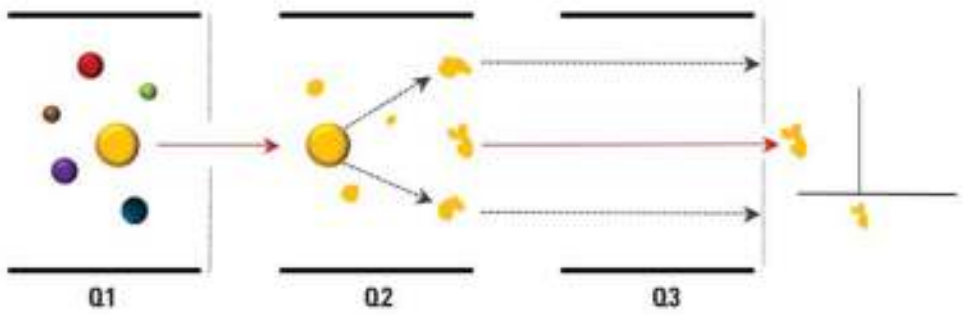


Figure 5: Selected reaction monitoring (SRM or MRM) (Courtesy of Agilent technologies)

The limitations of targeted approaches using LC-MS/MS or GC-MS/MMS are mainly related to the challenges and complexities in the determination of unknown compounds as well as the need of reference standards for quantification purposes. Untargeted screening (e.g., suspected-target) is emerging as an attractive tool to investigate the occurrence of contaminants in food. Untargeted testing is taking a larger role in food safety testing and entirely profits from the capabilities of HRMS modern analyzers [139], [128].

1.4.3. Optimization of the chromatographic separation:

The instrumental configuration and the settings of operational conditions greatly predetermine the performance characteristics of the analytical methods. The optimization step is fundamental to achieve reliable and significant results.

At the base of optimization, the optimization of the chromatographic conditions is based on the Van Deemter equation [140]. The efficiency of a column can be constantly optimized by maintaining the Height Equivalent to a Theoretical Plate or simply plate height (H) at its minimum value in the Van Deemter curve. This optimization is accomplished by systematically altering the solvent composition in HPLC or the column temperature in GC. In reverse-phase HPLC, analytes with more polarity will travel fastest and less polar analytes will begin to move as the polarity of the mobile phase is decreased. In GC analytes in the chromatographic column spend their time either “dissolved” in the stationary phase or vaporized in the mobile phase. When analytes are in the stationary phase they are not moving through the system and are present in a narrow band in the length of the column coating. As the oven temperature is increased, each unique analyte has a point where it enters the mobile phase and starts to move down the column. In GC, analytes with low boiling points will move down the column at lower temperatures, exit the system, and be quantified. As the temperature is slowly increased, more and more analytes (with higher boiling points) exit the system in a similar manner. Thus, the true power of chromatographic separation is achieved in LC by the gradient programming (composition of the mobile phase) and in GC by increasing the oven/column temperature (referred to as ramping). This is “the general elution problem” that is solved by optimizing the linear velocity, the mobile phase, and the type of stationary phase [141].

Thin layer chromatography (TLC) is one of the simplest and inexpensive techniques that can be used to screen for the presence of matrix compounds and chemical contaminants in food, especially in developing countries. Thin-layer chromatography is performed on a sheet of an inert substrate such as glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase. After the sample is applied to the plate as a spot, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, chromatographic separation is achieved. In recent years, there have been various developments in the quality of plate coating and in detection systems, as well as in

extraction and cleanup methods, that made it possible to apply TLC according to current international quality standards as an alternative technique for screening for known pesticide residues, for confirmation of tentatively identified compounds or to simply separate and screen for sample matrix components [142].

TLC is a time-consuming technique and requires more manual skill and attention than modern instruments equipped with auto-samplers. Applications of TLC are related to monitoring of reaction progress, identification of compounds present in a given mixture, and determining the purity of a substance. Specific examples include: analysis of ceramides and fatty acids, detection of pesticides or insecticides in food and water, or identification of medicinal plants and their constituents [143].

1.4.3.1. Optimization of the MS conditions

Optimization of the MS conditions is the second most important step for adjustment in analytical determinations.

The sensitivity of a triple quadrupole (QqQ) MS method is related to the selection of the correct selected reaction monitoring (SRM) transitions and optimization of the transmission and fragmentation parameters for the compounds of interest.

Robust optimization of fragmentation and collision energies, through individual experiments, is very important for MS optimization. In addition, the acquisition rate in SIM (selected ion monitoring) and SRM (selected reaction monitoring) modes is determined by the switching time and the dwell time per SIM or SRM channel.

The dwell time of a mass spectrometer describes the amount of time the instrument is collecting data for a specific SRM mass transition (in milliseconds). This value should also be optimized during method development. Increasing the dwell time will increase the sensitivity and precision of the method, but it will reduce the number of SRM mass transitions that can be monitored in a given assay. Dwell time may be decreased to improve peak definition and allow the MS to acquire more data points. It is best practice to optimize the dwell time so that 15–20 data points are collected across a chromatographic peak. The minimum number of points required to adequately define a peak is 10 data points.

In recent years, the number of multiclass approaches covering up to 1000 compounds have steadily increased. High sample throughput can be achieved by UHPLC-based systems as they ensure a significant reduction in analysis time. However, the broad applicability of such systems might be limited with the increasing number of target compounds, since a sufficient achievement of data points per peak and dwell times is not feasible [144].

1.4.4. Matrix Effects (ME)

Another important analytical parameter to be considered in both LC and GC based methods deals with matrix effects (ME). Matrix components, which are unavoidably present in analyzed samples (even after a thorough clean-up step), are directly linked to the
of data generated by the analytical laboratories and their influence needs to be known and characterized. In general, the wider the range of physico-chemical properties of target analytes of multiresidue methods, the more complicated is the efficient removal of co-extracted matrix components from a particular crude extract [145]. Depending on their nature (molecular size, polarity, thermal stability, volatility etc.), these substances may interfere in various stages of the chromatographic process. The phenomena governing ME in LC and GLC are different [146].

1.4.4.1. ME in LC-MS

In the LC case, co-extracted matrix compounds are ionized together with the analytes in the ion source creating ion suppression (decreased ion formation) or ion enhancement (increased ion formation). LC–MS instruments employing atmospheric pressure ionisation (API) are probably the most common used instruments in trace analysis. API sources include the electrospray ionisation (ESI) and the atmospheric pressure chemical ionisation (APCI) sources [147]. The performance of an API ion source is considerably influenced by the composition of liquid entering the detector, i.e., not only the type and amount of organic mobile phase modifiers and volatile buffers, but also the type and amount of sample matrix components play an influential role. Co-extracted substances present in the injected sample can cause serious quantitation problems when co-eluted with the analyte of interest; either suppression or enhancement of the analyte signal are typical symptoms in LC-MS. Matrix components may influence the effectivity of the ion formation in the ionisation process by altering the surface tension of the droplets of column eluent entering the ion source, and by building adduct ions or ion pairs with the analytes. As a result of matrix suppression or enhancement phenomena in LC-MS the response of an analyte in pure solvent standard differs significantly from that in matrix sample ([148], [149]). When UHPLC-MS is used, resolving power is increased, and ion suppression can be minimized because the coelution

of matrix interferences can be avoided, as well as peak shape can be improved, allowing better peak definition, and more reproducible and accurate peak integration compared to HPLC-MS [106].

1.4.4.2. ME in GC-MS

In GC-MS effects that can be attributed to ME are co-injection of non-volatile matrix components such as lipids (waxes, triacylglycerols, phospholipids etc), various pigments (chlorophylls, carotenoids, etc.) and other higher molecular mass components (plant resins) that are soluble in the solvent used for extraction and represent the typical bulk co-extracts, part of which can be contained even in well purified samples. Depending on the employed injection technique, the building up of deposits of dirt in a GC inlet and often also in the front part of the analytical column may lead to successive, in most cases adverse changes in performance of the chromatographic system. Consequences are loss of analytes, tailing analyte peaks and integration problems, that in turn increases the limits of detection and determination (LOD) [145]. Matrix-induced chromatographic response enhancement is a typical ME effect in GC determinations ([150], [151], [152]). The number of molecules of analyte introduced into a GC column is lower when injected in a solvent compared to the injection realised in the presence of matrix components. The impurities from the matrix compete with the analytes for the active sites of the GC inlet liners (although these are sold as deactivated), and thereby reduce the analyte interactions with active sites compared to the solvent case. At the same concentration of analytes, the result is an increase of the analyte signal. The same happens in the analytical column, where volatile impurities compete with the analytes and lead to an overestimation of the analyte concentration compared to solvent standards. In the case of matrix-induced chromatographic response enhancement effects the impurities are either thermolabile or rather polar and they are typically capable of hydrogen bonding [153]. Among the pesticides, compounds mainly affected by ME are organophosphates (–P=O); carbamates (–O–CO–NH–); hydroxy compounds (–OH); amino compounds (R–NH–); imidazoles, benzimidazoles (–N=); urea derivatives (–NH–CO–NH–) [154]. However, depending on the matrix, analytes can also show a significant suppression effect. This effect can be explained by some degradation processes for some of the analytes in the matrix when injected in the GC-MS/MS ([155], [156], [17]).

1.4.4.3. ME compensation strategies

MEs are the major bottleneck in multiclass method development and compensation and reduction strategies are rather limited. Strategies to compensate for ME are:

Matrix-matched calibration is commonly used to compensate for matrix effects. Extracts of blank matrix, preferably of the same type as the sample, should be used for calibration [158]. The applicability of matrix matching for multiclass methods covering several hundred compounds is limited due to the lack of matrix reference materials which are entirely blank for all target analytes. For GC, efforts were made to study the use of one "universal" matrix-matched standard matrix that represents a feasible means of compensating for the matrix effects of many other vegetable and fruit simples [159], [160]. An alternative practical approach to compensate for matrix effects in GC-analyses is the use of analyte protectants that are added to both the sample extracts and the calibration standard solutions in order to equalise the response of pesticides in solvent calibrants and sample extracts ([161], [162], 163]).

An alternative practical approach is the standard addition technique (see paragraph 1.4.6.2.). According to the SANTE guideline this procedure is designed to compensate for matrix effects and recovery losses [164], [132].

The most effective way to compensate for matrix effects is the use of isotopically labelled analogues as internal standards for each of the target analytes. The so-called stable isotope dilution assays (SIDA) are very common in routine analysis of confirmatory assays. This approach is based on the use of small amounts of isotopically labelled internal standards (ILSTD) which are simultaneously injected with sample extracts. See **Figure 6**. Despite the powerful compensation of matrix effects using SIDA, its wide application is limited since only a small number of ILSTD is commercially available [165]. Furthermore, the procedure matched (applying the internal standard to the raw material prior extraction) use for ISTDs also pose an economic challenge, since certified internal standard solutions are at higher price level and thus not affordable for many research groups [126]. Although in another field of science, it was shown that the analyte to internal standard peak area ratio changed with two specific lots of commercial samples. For the first time, it was demonstrated that a slight difference in retention time between the analyte and its deuterated internal standard, probably caused by deuterium isotope effect, has resulted in a different degree of ion suppression between the two analogues, questioning the assumption that a stable isotopically labelled analogue is believed to be the most appropriate internal standard in a quantitative liquid chromatography/tandem mass spectrometry (LC/MS/MS) assay [166].

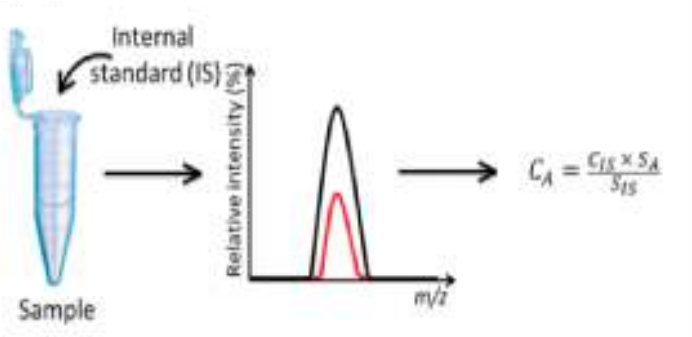


Figure 6: Visual representation of the stable isotope dilution technique

Dilution of crude sample extracts can also be considered an analytical strategy to significantly reduce unwanted ME, however only in those cases where an increase of the estimated detection limits (LODs) is not compromising the compliance to MRLs [167]. Dilution approaches can be useful when last generation MS instruments are available and demonstrate extreme sensitivity for the analytes of interest. (See paragraph 1.4.5.8.4.). In contrast to HRMS, MS/MS devices are able to tackle high dilution factors and have become state-of-the-art instruments for ultra-trace analysis due to strong improvements in terms of sensitivity [126]. Recently, nanoflow LC-HRMS (nano-LC-HRMS) has been proposed as an alternative in order to reduce ME problems [168]. In this approach, nano-C18 column is used, resulting in reduced dead volumes and increased ionization efficiency. The impressively low LODs (ng/kg level) obtained, made high dilution factors feasible, thus minimizing the MEs.

As discussed in paragraph 1.4.2.2, IMS integrated to LC and GC systems is a promising alternative to reduce ME by providing improved selectivity, lower LODs and additional information to mass spectra and retention time due to an additional principle of separation [169], [170], [171].

1.4.5. The analytical method

At the core of the food safety tools is the availability of an analytical method. This comprises a set of steps that allow to know the qualitative and / or quantitative presence or concentration of a specific analyte (s) in a specific matrix. The analytical steps required for

the analysis of trace and ultra-trace residues and contaminants in food typically involve sample preparation, which consists in the extraction of the analytes from the matrix, including a clean-up and/or a pre-concentration step, analytical separation and detection of the compounds.

The analytical method needs to be fit for purpose, thereby meeting changing needs, emerging challenges, robust enough to be able to generate reliable results under changing conditions and at the right scale of operation. The latter is very important in the development of an analytical method. Potential limitations accrue to the amount of sample available for the analysis, the expected concentration of the analyte in the samples, and the minimum amount of analyte that will produce a measurable signal [172].

1.4.5.1. The power of multiresidue and multiclass methods

As discussed in the previous paragraphs, instrumentation such as liquid and gas chromatography coupled to mass spectrometry has provided the ability to detect chemical contaminants at trace levels (mg/kg or µg/kg) or ultra-trace levels (ng/kg) concentrations, improving confidence in the safety of food supply. However, to improve the quality of monitoring and surveillance for food safety, it was important to swift to multiresidue methods, wherein multiple substances can be analyzed simultaneously at concentrations equal to or lower than their MRLs. The concept of multiresidue methods is very important from an analytical perspective as it enables the screening of a larger number of analytes, and in several cases, covering several classes and several contaminants in a single sample preparation step [173], [174], [175], [176]. In addition, it represents a way to improve the cost-effectiveness of the analytical procedures, as it maximizes the number of analytes that may be determined by a single procedure or from a single portion of the test material. This implies the reduction of the number of analyses per sample (less time and money). Nevertheless, the development of multi-class methods is a challenging task due to the different characteristics of the compounds from each group (chemical structure, polarity, stability, etc.). Furthermore, typical difficulties such as matrix composition and low concentration of compounds also complicate the simultaneous analysis of several classes of compounds [170].

In the last few years many efforts have been focused on the improvement of the speed, simplicity, reliability and low-cost of sample treatment, as well as the extraction of as many residues as possible and as low coextractives as possible. Several extraction techniques

such as pressurized liquid extraction, microwave extraction and matrix solid phase extraction among others have been applied (see paragraph 1.4.5.5.). As a result, sample preparation has evolved towards the use of smaller sample sizes, reducing or eliminating the use of organic solvents and performing automated extraction procedures, in the so-called total analysis systems, which integrate in the same instrument both sample pre-treatment and analytical separation [1]. Nevertheless, specific methods should be developed for problematic compounds. For instance, polar compounds such as glyphosate, or some families of veterinary drugs, such as tetracyclines, are not usually extracted using generic methods, and a method protocol was developed in order to quantitatively extract these compounds (see paragraph 1.4.5.8.5.). Among the most investigated organic contaminants/ residues are natural toxins (e.g., mycotoxins and plant toxins) in nuts and cereals [177], pesticides residues in fruits and vegetables [178] and veterinary drugs residues in meat and animal products [179].

However, the number of analytical approaches combining several classes of contaminants within one analytical run is still comparatively scarce [180]. The majority of multi-target publications are either focusing on one single substance class, or the substance class is segmented into subcategories, for example in the case of veterinary drugs, e.g., sulfonamides, tetracyclines, or penicillins in order to obtain a multiclass scope [126]. Numerous methods in the area of mycotoxin [181] veterinary drug analysis [182] and pesticide residue analysis [183], [184], [185], [186] were developed using LC-MS/MS.

The first comprehensive method combining several compounds and classes within one analytical procedure was designed in the year 2008 [2159]. Since then, the number of so-called multiclass methods has increased considerably, as a comprehensive overview has recently revealed [187]. **Table 2** provides an indication of recent methods published for multiresidue, multi-toxin and multiclass methods for determination of organic chemical residues and contaminants.

Table 2: bibliographical research of methods published in the years 2014-2020, for concomitant multiresidue, multi-toxin, "multiplex" methods covering pesticides (PS), veterinary drugs (VD), mycotoxins (MYC), plant toxins, dyes, environmental pollutants and other chemical substances, (adapted from [180]).

Author	Matrix analyzed	Analytes	MS analyzer	Solvent of extraction	Extraction procedure	LOQ (µg/kg)	Nr analytes
Mol et al. 2008 [159]	Several	VD, PS, MYC, Pltox	LRMS, HRMS	Water/Acetonitrile/1 % formica cid 5;15, v/v	Dilute and shoot	10-250	258
Gómez-Pérez et al. ,2014 [188]	Matrices of animal origin	VD, PS	HRMS	Acetonitrile			Above 350
Han et al., 2014 [189]	Shrimps	PS, PCBs, PAHs, environmental contaminants	LRMS	Acetonitrile	QuEChERS		59
Jia et al.,2014 [190]	Baby foods	VD, PS	HRMS	Acetonitrile	QuEChERS		333
Gómez-Pérez et al. ,2015 [191]	Feed	VD, PS	HRMS	Acetonitrile			Above 350
Dzuman et al., 2015 [192]	Leek, wheat, tea	PS, MYC, plant toxins	HRMS	Acetonitrile	QuEChERS	0.2-5000	389
Gómez-Pérez et al. ,2015 [193]	Food and various matrices	VD, PS	HRMS	Acetonitrile			
Xie et al.,2015 [194]	Dairy products	VD, PS, MYC	LRMS	Acidified ACN + ethyl acetate	Low temperature partitioning, SPE		40

Danezis et al, 2016 [195]	Various matrices	VD, PS, MYC, plant growth regulators	LRMS	Acetonitrile	Low temperature partitioning		28
León et al.,2016 [196]	Feed	VD, PS, Pltox	HRMS	Acetonitrile	QuEChERS		77
Munaretto et al.,2016 [197]	Fish	VD, PS, Personal care products	HRMS	Acetonitrile	QuEChERS	5-25	182
Souza et al.,2016 [198]	Bovine tissue	VD, PS	LRMS	Ethyl Acetate	Modified d-SPE		55
Niladri et al., 2016 [199]	Fatty fish matrix	PS,PAHs	LRMS	Acetonitrile	d-SPE		119
Piatkowska et al, 2016 [200]	Egg	VD, PS, Dyes	LRMS	Acetonitrile	LLE-SPE	CCβ 2.04–1316	121
Pérez Ortega et al.,2017 [201]	Tomato orange baby food	PS,VD, MYC	HRMS	Acetonitrile	QuEChERS	1-10	Above 600
Al-Alam et al., 2017 [202]	Honey	PS, PCB, PAH	LRMS	Acetonitrile	QuEChERS	0.16-168	128
Sapozhnikova, 2018 [203]	Poultry tissue	PS, PCBs, PAHs, PBDEs, other environmental contaminant	LRMS	Acetonitrile	QuEChERS		265

Zhou et al.,2018 [204]	Egg, milk	VD, MYC	LRMS	Acetonitrile	QuEChERS	0.01-31	104
Aparicio et al., 2018 [205]	Chard, spinach, lettuce, carrot, turnip and potato	pollutants	LRMS	Acetonitrile	UAE+SPE		35
Reichert et al., 2018 [206]	Raw coffee	PS, MYC	LRMS	Acetonitrile	QuEChERS no clean-up	5-1000	147
Kong et al., 2018 [207]	Carp, shrimp, crab, eel, and mussel	VD, PS	HRMS	acetonitrile and ethyl acetate.	Simple extraction with solvents	Not reported	206
De Paepe et al.,2019 [208]	Edible insects	PS,VD, MYC	HRMS	Acetonitrile	SPE		77
Jadhav et al.,2019 [209]	Milk	PS,VD	LRMS	Acetonitrile	SPE and QuEChERS		316
Mijangos et al.,2019 [210]	Mussels,fish	PS,VD, Artificial sweeteners, PCPs, phytoestrogens, human drugs	LRMS	Methanol	Forced ultrasonic solid liquid (FUSLE) w/ MeOH		41
Turnipseed et al. ,2019 [211]	Shellfish	VD, PS , Disinfectants, human drugs	HRMS	Acetonitrile	SPE		128
Xu et al.,2019 [212]	Eggs	PS,VD, MYC	LRMS	Acetonitrile	QuEChERS with multiwall		77

					carbon nanotubes		
Monteiro et al., 2020 [213]	Beef	PS,VD, Environm. Cont.	LRMS	Acetonitrile	QuEChERS	10-500	262
Zhang et al. ,2020 [214]	Infant formula	VD, PS, Human drugs	HRMS	Acetonitrile	d-SPE		49
Panseri et al. [215]	Honey	PS, POPs (PAHs, PCBs, PBDEs, OCs, OPs,	LR-GC-MS/MS and LC-HRMS	Methanol	Accelerated Solvent Extraction (ASE)		
Steiner et al, 2020 [153]	Feed matrices	VD, PS, biotoxins	LRMS	acetonitrile/ water/- formic acid 79:20:1, v/v/v	Dilute and shoot	0.1-900	1467
Sulyok et al., 2020 [216]	Wheat, maize, figs, dried grapes, nuts	Myc, plant toxins, bacterial metabolites	LRMS	acetonitrile/ water/- acetic acid 79:20:1, v/v/v	Dilute and shoot	0.02-1900	Above 500

From the above table of mixed multiresidue, multi-toxin and multiclass methods of organic chemical residues and contaminants it is possible to observe that in terms of:

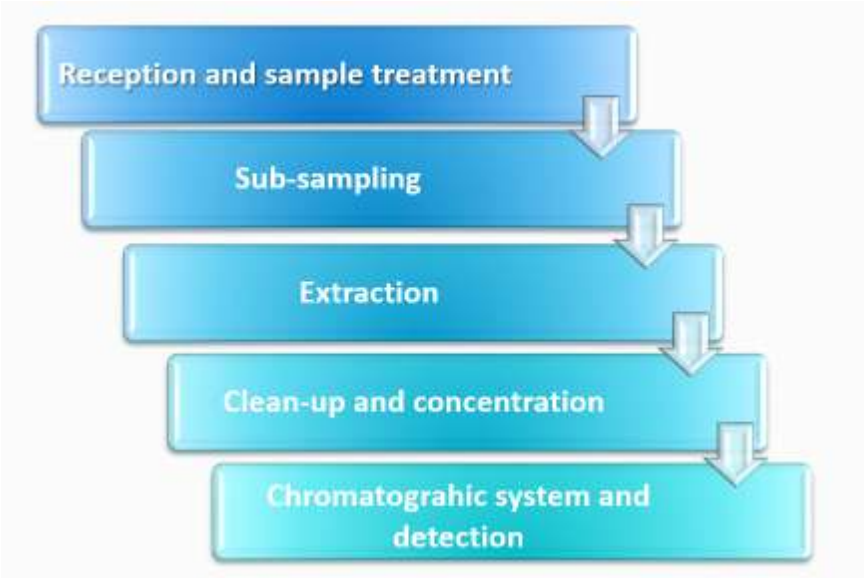
- number of publications: published methods have increased since the year 2014, due to the increased availability of mass spectrometric instrumentation at affordable prices and due to “economies of scale” generated by recent multiclass methods that are significantly less complicated compared to single analytical approaches, which require specific sample preparation techniques and instrumentation.
- validated matrices: very few studies targeted baby foods, which, from a legal limit perspective, require very low LOQs in order to comply with very low maximum levels.
- instrumentation (mass analyzers): there is a clear trend towards the use of MS/MS and HRMS in combination with an electrospray ionization (ESI) interface.
- extraction solvent: most methods utilize acetonitrile, a solvent compatible with LC approaches.

- extraction procedure: most methods adopt the QuEChERS protocol (see paragraph 1.4.5.8.1.)
- reported limits of quantification (LOQs): current multiclass methods, using MS analyzers, have sufficient sensitivity (LOQs) which help comply with the legislative maximum permitted levels of contaminants and residues, i.e. maximum levels (ML) for mycotoxins and maximum residue levels (MRLs) for pesticides and veterinary drug residues.
- method scope: existing multiclass approaches have a generic scope of target compounds in the range of 28-1467 analytes.

1.4.5.2. The analytical method in a nutshell

Text box 4 provides a generic indication of the steps involved in the implementation of an analytical method. In practice, multiresidue methods consist of the following basic steps:

- Reception at the laboratory and sample treatment/manipulation for analysis
- Sample homogenization and subsampling
- Isolation of residues from a representative sample (extraction);
- Separation of co-extracted matrix components (clean-up);
- Identification and quantification of target analytes (quantitative step);
- Confirmation of result



Text box 4: The generic laboratory steps of an analytical method

1.4.5.3. Sampling, sample reception and sample treatment

Food control systems aim to protect the health of the population and to promote trade. An appropriate and properly designed sampling plan for the purpose of monitoring food safety and quality is required for this purpose. The objective of sampling is to provide the laboratory with samples for analysis (and results), called the laboratory sample, that represent the entire population being monitored (e.g., batch, lot, orchard, farm, and shipment). The analytical laboratory can play an active role in relation to the following aspects concerning sampling:

1. The design of the sampling plan (mainly for research purposes)
2. The sampling itself—e.g., by ensuring that the samples are collected according to an established protocol and transported to the laboratory under conditions that prevent their integrity being compromised (mainly for compliance monitoring)
3. Ensuring that adequate information about the sampling is recorded and conveyed to enable the correct interpretation of the analytical data (this is mostly the case for risk assessment studies). As mentioned previously (see paragraph 1.4.5.3.) sampling protocols are available describing recommended procedures for the sampling of many types of materials and chemical components. These protocols are sometimes specified in national regulations or international agreements ([217], [64], [218]). Upon arrival at the laboratory, the laboratory sample must be received, and all information recorded for traceability issues. The first step is the laboratory sample treatment/manipulation to convert it into the analytical sample by removal of parts not to be analyzed, if any. Examples of this step are removal of adhering soil, plant stems, withered leaves, bones etc.

1.4.5.4. The subsampling step

Subsampling or sample manipulation is the procedure (e.g. cutting, grinding, mixing, etc.) used to make the analytical sample homogeneous with respect to the analyte distribution and ready for extraction, prior to removal of the analytical portion. The analytical portion is the test portion that is solvent-extracted and analyzed using an approved and validated analytical procedure to quantify/qualify the analyte(s) of interest [219]. The ultimate goal is to ensure that the analytical portion (aliquot/sub-sample taken for extraction, cleanup and

analysis) is representative of the entire population of the batch/lot sampled. To ensure an unbiased and representative sample, each laboratory must create and strictly follow scientifically sound and management-approved documented procedures such as Standard Operating Procedures (SOPs) - for sample manipulation and processing. As a requirement for compliance with ISO/IEC 17025 guidelines, the uncertainty resulting from the sample processing step must be evaluated and factored into the overall uncertainty in the measurement resulting from the entire analytical procedure [85].

1.4.5.5. The sample preparation step

In analytical chemistry, sample preparation is the general term used to describe the ways in which a sample is treated prior to its analysis. The sample preparation is a fundamental component of the analytical method. It is particularly important in trace analysis, as it can account for a significant amount of the variability of a particular method [220]. Some of the compounds naturally present in the foods will have an effect on the analysis of the targeted analytes, and thus, different sample preparation techniques have been applied to extract and/or concentrate those analytes. The generic term sample preparation incorporates many steps as it needs to convert a complex matrix into a sample extract in a format that is suitable for final instrumental analysis.

It may involve solvent extraction, reaction with some chemical species, filtering, dilution, and/or many other techniques. The aim is to prepare and process the sample, extract the analytes from the matrix, bring the analyte(s) to a suitable concentration level, remove possible interferences (clean up step) and, when is required, to convert the analytes into a more suitable form for detection or separation [221].

Some of the traditional sample extraction techniques for organic contaminants residues analysis extraction, isolation, clean-up, and preconcentration of analytes have been carried out by Soxhlet, ultrasound assisted extraction (UAE), liquid-liquid extraction (LLE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), solid-phase microextraction (SPME), matrix solid phase dispersion (MSPD), supercritical fluid extraction (SFE), solid phase extraction (SPE), and, in recent years, by dispersive solid phase extraction (d-SPE) [222].

1.4.5.5.1. Soxhlet extraction

It is based on solvent extraction of solid samples, commonly known as solid–liquid extraction. A porous thimble loaded with a solid sample is placed inside the main chamber of the Soxhlet extractor (1) and gradually filled with condensed fresh solvent from a distillation flask (2), see **Figure 7**. When the liquid reaches an overflow level (2-3), a siphon aspirates the whole contents of the thimble-holder and unloads it back into the distillation flask, carrying the extracted analytes in the bulk liquid. This operation is repeated until complete extraction is achieved (4). The extraction cycle is typically repeated many times. Soxhlet extraction is a rugged, well-established technique and permits unattended extraction. However, it requires a long extraction time, the consumption of a large amount of solvent and most important it cannot be used to extract thermolabile or volatile compounds [223].

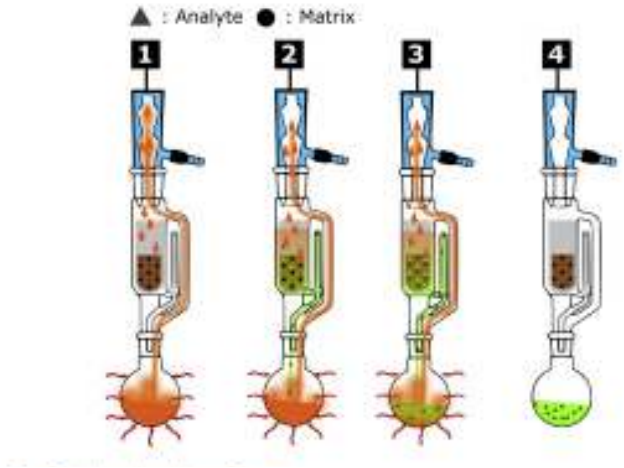


Figure 7: Schematic illustration of the Soxhlet extraction process [extracted from 223]

1.4.5.5.2. Ultrasound assisted extraction (UAE)

This technique is based on the use of ultrasonic waves for the extraction of numerous analytes from a diversity of matrices. The propagation of ultrasonic waves causes the implosion of bubbles that induces macroturbulence and perturbation in the microporous particles of the sample. As a result, the solute quickly diffuses from the solid phase to the solvent. The ultrasonic bath is the most commonly known type of ultrasonic device; it usually consists of a stainless steel tank with one or more ultrasonic transducers. Ultrasonic baths usually operate at a frequency of around 40 kHz and can be equipped with temperature control. They are readily cheap, available and large numbers of samples can be

simultaneously treated. Nevertheless, the application of UAE is limited to very few matrices and there are many compounds such as acidic herbicides that cannot be extracted as they degrade [224].

1.4.5.5.3. Liquid-liquid extraction (LLE)

Liquid-liquid extraction is based on the solubility of the analyte in two immiscible solvents and is governed by the equilibrium distribution coefficient. The homogenized liquid samples are extracted, commonly three times, with an immiscible organic solvent and the extracts are then centrifuged, concentrated and purified before the final analysis. L-L extraction is one of the preferred methods in organic contaminant residue analysis especially for environmental water analysis, which requires concentration of the extracts and still need to ensure sufficient LOQs. However, LLE may use considerable amounts of toxic solvent (i.e. chloroform), may be affected by the formation of emulsions, which are difficult to break up; in addition, it is difficult to automate the process, which is considered tedious, time-consuming, and also costly [225].

1.4.5.5.4. Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is based on the principle that microwaves activate the rotational energy levels of dipolar and charged molecules, thus allowing for rapid extraction of a sample contained in a closed vessel in the presence of an extraction solvent. The final extract is separated from the matrix by centrifugation or filtration. MAE has substantial advantages compared to other sample preparation techniques, as it requires a much lower solvent volume, reduces extraction time and allows the processing of a large number of samples simultaneously. However, it requires costly equipment, and very few applications have been developed so far for food residue/contaminant work due to the thermolability of the analytes [226].

1.4.5.5.5. Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE**)**, also known as accelerated solvent extraction (ASE), pressurized fluid extraction (PFE), enhanced solvent extraction (ESE), and high pressure solvent extraction (HSPE), is based on the use of solvents at high pressure and/or high temperature without reaching the critical point. The high temperature (usually 50- 200ºC) and pressure (500-3000 psi) enhance the solubilization and desorption of analytes from the matrix, accelerate the speed of the extraction process, and provide good recoveries of analytes at this stage of the procedure. PLE has many advantages over traditional techniques such as Soxhlet extraction and ultrasonication extraction, e.g. a short extraction time, low solvent consumption and additional extract filtration, which is done by adding the inert material to the extraction cell. The advantage of PLE, over techniques such as MAE, is that the extraction solvents available for MAE are limited to those that do not absorb microwaves such as chloroform and dichloromethane [227]. The main limitation of PLE is the low selectivity towards the analytes due to fact, depending on the sample, of the co-extraction, many interferents, such as lipids, pigments, cholesterols and others [228].

1.4.5.5.6. Matrix solid phase dispersion (MSPD)

Matrix solid phase dispersion (MSPD) can be defined as a solid-solid extraction procedure, in which one phase is the investigated (semi-) solid matrix and the other an appropriate sorbent. In practice, the technique is applicable to liquid, viscous and solid samples. In a typical MSPD experiment with a (semi-) solid matrix, the tested sample structure is completely disrupted by abrasion with the selected extraction sorbent(s). During this mixing process, sample components are homogeneously dispersed on the sorbent surface. The resulting homogeneous, dried sample-sorbent(s) mixture shall subsequently be extracted with a suitable solvent. Reversed-phase bonded materials such as C18 have been widely used as MSPD sorbents for selective retention of medium-polar and nonpolar matrix components. Normal-phase inorganic materials (e.g., bare silica, alumina or Florisil) or celite, sand or diatomaceous earth have also been used for MSPD, although with less extensive retention power than reversed-phase bonded materials [229]. The field of application recently extended to the determination of emerging pollutants, such as parabens, plasticizers or fragrance allergens.

Novel dispersant materials are special sorbents that improved selectivity and/ or selectivity during MSPD, in particular molecularly-imprinted polymers (MIPs) [230]. The main limitation of MSPD is the format of the technique, which requires extensive manipulation and additional extraction for example by packing the sample-sorbent mixture in an SPE-like format and elution with a solvent.

1.4.5.5.7. Solid phase extraction (SPE)

Solid phase extraction (SPE) is based on the dispersion of the analyte between a sample extract and a solid adsorbent phase and the partitioning of the analyte between two phases. To be able to be effective, the analyte must show greater affinity for the adsorbent phase than for the sample matrix. The analyte(s) retained on the solid phase are removed by eluting with a solvent having a greater affinity for the analytes. In a modern SPE system, the adsorbent is packed between two fitted disks in a polypropylene cartridge and liquid phases are passed through the cartridge either by suction or by positive pressure. Many different types of adsorbents are available. [231]. Among traditional sample preparation techniques, SPE has probably been the most extensively used despite its long procedural time for multiple steps, such as cartridge conditioning, extract loading, washing, and analytes elution. However, SPE is only suitable for compounds with similar physicochemical properties, and so is less applicable when using multi-residue methods. The current trend is on the development of more generic extraction procedures, and dispersive SPE (d-SPE) emerged as an alternative, offering analytical convenience, simplicity and good matrix elimination, as discussed in the following section.

1.4.5.5.8. Dispersive solid phase extraction (d-SPE)

Dispersive solid phase extraction (d-SPE) can be described as the clean-up step of the new concept of sample preparation performed for pesticide residue analysis, the QuEChERS (see section 1.4.5.8.1.), and it is based on the addition of a sorbent directly into the analytical sample extract followed by mechanical dispersion that favours the contact between the sorbent and analytes / matrix components. When the dispersion process is completed, the sorbent is separated by a mechanical process, for example filtration or centrifugation [232].

Common sorbents for d-SPE are the primary-secondary amine (PSA), used to remove co-extracted matrix components such as organic acids, sugars, amino acids among other components of the matrix. Reversed phase (RP) sorbents, such as C18, provide good results for the purification of samples with significant fat, wax content and also terpenes or other volatile compounds. Graphitized carbon black (GCB) is another example of sorbent that helps eliminate chlorophyll and carotenoids or other pigments from plants; zirconia-based sorbents help remove high levels of lipids. Sorbents can be applied independently or in combination.

Under a quality-controlled system, potential losses of target analytes should be controlled when using d-SPE sorbents through a quantitative estimation of the (recoveries of the targeted analytes and internal standards (ISs) and matrix matched calibration curves have to be used to ensure high recovery rates [233]. Care should also be exercised to verify the applicability of d-SPE since some target analytes might be lost during this step, e.g., fumonisins are lost after a PSA clean up [233].

d-SPE together with SLE, LLE and SPE are, probably, the sample preparation methods most-employed for organic contaminant residue analysis [7].

1.4.5.5.9. Solid-phase microextraction (SPME)

Solid-phase microextraction (SPME) is a technique that works on the principle of adsorption/absorption and desorption and uses a silica fiber, coated with an extractive phase, generally organic polymers (i.e. DB1, DB1701) to concentrate analytes in a sample. SPME combines the sampling, isolation, and enrichment of an analyte to one step. In SPME, the extraction phase can be exposed directly to the sample media (direct immersion, DI) or to its headspace (HS). The amount of analyte extracted onto the SPME coating is linearly proportional to the analyte concentration in the sample, which is the analytical basis for quantitative analysis using SPME [235]. The analytes absorbed to the fiber are then typically desorbed, for example, using thermal desorption during GC analysis. The major advantage is that it allows rapid and solvent-free extraction of the analytes. Very good extraction has been achieved for water samples; the drawback is that SPME has been shown to be less precise with more complex matrices [236].

1.4.5.5.10. SFE Supercritical fluid extraction (SFE)

SFE Supercritical fluid extraction (SFE) is a relatively recent extraction technique based on the enhanced solvent power of fluids above their critical point. Its usefulness in extractions is due to the combination of gas-like mass transfer properties and liquid-like solvating characteristics with diffusion coefficients, which are higher than those of a liquid. The majority of SFE studies for organic contaminants have focused on the use of CO_2 because it is non-toxic, non-flammable, cheap, easily eliminated after extraction and possessing a high solvating capacity for non-polar molecules. The major advantages of SFE include pre-concentration effects, cleanness and safety, relative simplicity and fast applications. The drawbacks of SFE are the need for expensive equipment and the difficulty of extracting polar molecules, unless using modifiers to add to CO_2 [237].

1.4.5.5.11. Gel permeation chromatography (GPC)

Gel Permeation Chromatography (GPC), also known as Size Exclusion Chromatography (SEC), is a very robust technique used to separate molecules of different sizes using porous materials and sorbents. Small molecules can enter the entire intra-particular pore space and hence elute last, whereas large molecules are excluded from all pores and hence elute first. Gel permeation chromatography (GPC) is known to be well suited for the separation of fatty matrix components from target analytes. It is a well-established technique but has the disadvantage of being time consuming and requiring intensive optimization before its routine use due to the calibration of the different fractions of eluate and very difficult to automate [238].

A summary and a comparison of sample preparation techniques is presented in **Table 3**.

Table 3: Comparison of different extraction techniques for food matrices (adapted from [239]).

Extraction technique	Advantages	Disadvantages
Soxhlet	High T increase the solubility; no need for filtration, extraction efficiency is high	Decomposition of thermolabile analytes, not a green techniques, high solvent consumption, long procedure.
USE	Short extraction time; cheap equipment, useful for thermolabile analytes.	Need for filtration; high matrix content in the extract.
LLE	An option that exists	Formation of emulsions, tedious, time-consuming, and costly technique
MAE	Rapid technique, low solvent use, high T are possible, for both solid and liquid samples	Limited solvent choice, expensive lab equipment, filtration need

PLE	Rapid technique,	Low selectivity
MSPD	Increased number of sorbents, expanded field of applications	Time-consuming
SPME	Coating neds to match analyte of interest	Amount of analyte extracted is directly proportional to coating affinity, most suited to qualitative work.
SFE	Rapid technique, short extraction time, low volume of organic solvents	expensive lab equipment, requires the use of modifiers
d-SPE	Fast, a number of sorbents can be used	Unspecific clean-up, needs quality control procedures
GPC	Very robust	Very time consuming and requiring intensive optimization; difficult to automate

1.4.5.6. Choosing the right method

To be able to choose the right analytical method it is important that the scientist/chemist is aware of the following issues at a minimum: the scope of testing, type of method required screening vs confirmatory or routine testing/research method, the type of sample to be analyzed, the target analyte(s), the need for a single analyte or a multi-analyte method, the available instrumentation, the availability of certified reference materials and/or analytical standards, the availability of current validated methods from standards/literature, what criteria to be met for trueness, precision, sensitivity, selectivity, robustness and, ruggedness, what are the current national/regional/international legislative requirement(s) in terms of method performance criteria, where and when will the analytical results be used, how quickly do the results need to be reported, how much can be spent for each sample, how much analytical uncertainty can be tolerated. With the above information at hand the analyst should conduct a proper bibliographical review and identify options for suitable methods. These criteria are not mutually independent, and it is often necessary to find an acceptable balance between them. In cases where a method does not exist, the analytical scientist should add an additional component for method development to the total investment plan. It is important to note that any method to be adapted for use in the laboratory should undergo a complete method validation meeting internationally acceptable standards (as suggested by regulatory authorities or reputable bodies such as the Codex Alimentarius Commission) prior to routine use of the method for sample analysis. The European approach, also more recently adopted by Codex Alimentarius, requires methods to comply with certain performance criteria, and in general is more accommodating in terms of the methods used, provided these deliver results according to the criteria established by the European Commission. The US approach is more defined in terms of the methods that can be used, e.g. the tolerance (MRL) enforcement program imposes certain requirements and

restrictions on the method to be used. Only collaboratively studied methods and stringent guidelines specifying the analytical procedure are permissible [240].

1.4.5.7. Implementation of the method

The implementation of an analytical method requires that a method has been chosen, that all necessary conditions in terms of necessary equipment, consumables and instrumentation are met and that the method has been adapted to the laboratory conditions, optimized, validated and characterized for fitness for purpose, including the application of QA/QC measures, before it can be implemented under routine conditions (see **Text box 5**).

Text box 5: Different phases of analytical method implementation

1.4.5.8. Main modern methods for multi-residues/multi-contaminants analysis.

Three modern multiresidue methods, initially developed for the monitoring and control of pesticide residues, but nowadays used for other food residues and contaminants areas, are presented in this paragraph. One difference between these methods is the organic solvent used for extraction, ranging from acetonitrile for the QuEChERS method [176], ethyl acetate for the SweEt method [241], [242], and acetone with partitioning with n-hexane/dichloromethane, for the Dutch Mini Luke method [243], [244].

In all the three methods, analytes of interest are partitioned between an aqueous phase and an organic phase.

1.4.5.8.1. The QuEChERS method

One of the most developed approach for modern sample preparation has been achieved by the QuEChERS method, which was initially developed for the extraction of pesticides from vegetables, and it is widely known because of its simplicity and effectiveness well described by its acronymic name: quick, easy, cheap, effective, robust and safe. This methodology is a simplified and miniaturized solid liquid extraction (SLE) technique, which mainly reduces solvent consumption and analytical steps and efforts. The original procedure was based on an extraction of 10 g samples with acetonitrile from samples with high water content (mainly fruits and vegetables), addition of internal standard, followed by partitioning of the analytes between water and organic phase, salting out by adding salts (sodium chloride and magnesium sulphate). After centrifugation both phases are separated, and a clean-up step based on dispersive solid phase extraction (d-SPE) is used. An aliquot of the organic phase is subjected to a clean-up step by using a small amount of the sorbent. Originally, a primary secondary amine (PSA) was used; to increase the efficiency of the clean-up step additional sorbents started to be used. Several modifications were included in order to improve the extraction of difficult pesticides, such as the use of acidified acetonitrile, combination of several sorbents for d-SPE. An improved version of the QuEChERS method includes the use of buffers (citrate or acetate buffer) during extraction [245]. This allows to attain sufficiently high recoveries for the majority of pH-dependent pesticides. The two buffered versions of QuEChERS have been extensively evaluated and adopted as the official method in the EU and the United States for the analysis of pesticide residues in fruits and vegetables: EN 15662 [246] and AOAC 2007.01 [247].

QuEChERS-based methods are widely used in routine testing for several contaminants and residues (pesticides, mycotoxins, veterinary drugs, dyes, phytohormones, polycyclic aromatic hydrocarbons) covering different matrices (food and environmental). The main advantages of this procedure are the high number of compounds that are simultaneously extracted from a small amount of the matrix (10 g), using a reduced volume of the organic solvent (10 mL) in a short period of time (<15 min). Moreover, several samples can be extracted simultaneously [233], [248], [118].

1.4.5.8.2. The SweEt method

A multiresidue method based on extraction with ethyl acetate has been developed at the Swedish National Food Administration and used since 1989 to monitor pesticide residues in fruit and vegetables. The method has been continuously adjusted, resulting in simple and quick analyses of pesticide residues [249]. Ethyl acetate has proven to be an almost universal solvent and its ability to extract many different classes of pesticides from various commodities has been proven. The initial method had been validated for 309 analytes, of which 187 were detected by LC-MS/MS using positive and negative modes and 122 by GC-MS/MS. With regard to extraction efficiency, ethyl acetate has been shown to be suitable for products with a high fat content—because of the solubility of fat in ethyl acetate, pesticides are released and extracted efficiently. In addition, ethyl acetate is very suitable either for GC or for LC analysis. It has good wettability in GC (pre)columns; this is of benefit for solvent trapping of the most volatile analytes, which is required for refocusing after injection and it is compatible with all GC detectors. The original ethyl acetate-based multi-residue method for pesticides in food produce, SweEt method, has been recently revisited, re-validated and modified for gas chromatographic (GC) analysis by implementation of dispersive solid-phase extraction (using primary–secondary amine and graphitized carbon black) and large-volume (20 µL) injection. The same extract, before clean-up and after a change of solvent, was also analyzed by LC–MS–MS [250].

1.4.5.8.3. Dutch Mini-Luke method

The original multiresidue method was introduced in 1975 as the Luke method: originally 100 g of the sample were extracted with 200 mL of acetone and a mixture of petroleum ether and dichloromethane (1: 1, v/v), 100 mL each, were used in a partitioning step [251]. Many optimizations were later performed on the original method, and in the 1980s, researchers from The Food and Consumer Product Safety Authority (VWA, Amsterdam, the Netherlands) reduced the solvent amounts significantly, and the method was renamed the Dutch mini-Luke method as mentioned above. The so-called ‘Dutch mini-Luke’ method, which uses a combination of acetone/petroleum ether/dichloromethane (v/v 1/1/1), has been successfully validated for a wide range of LC and GC amenable pesticides. It is preferred by some laboratories because liquid/liquid partitioning provides relatively ‘clean’ extracts without the need for additional clean-up. The lower concentration of coextractives compared to acetonitrile and ethyl acetate methods results in less contamination of the instrument systems [250]. Wider adoption of the method has possibly been hindered by the need for slightly higher volumes of solvent compared to other methods including due to the use of

dichloromethane, a solvent to be avoided according to green chemistry principles. However, experiments to further reduce the volume of solvents required (dichloromethane reduced to 10 mL) and validate the improved 'NL method' were recently reported and the method renamed the New-Dutch Mini Luke method [252]

The major drawback of this method is that laboratories must be able to homogenize the sample in the presence of solvent using high-performance dispersing equipment, such as an ultraturrax® [253]. Such a device is not always available in analytical laboratories.

1.4.5.8.4. Dilute and shoot" technique

Another approach currently used in advanced laboratories is the "dilute and shoot" technique, which was proposed for multi-class methods, in liquid matrices, strictly based on the availability of analytical instrumentation that is providing the required LOQs and improved instrument performance [254]. This approach requires less stringent sample preparation, and it involves the dilution of samples with an internal standard and then the direct injection into the chromatographic system coupled to tandem MS, operated in multiple reaction monitoring (MRM) mode. The performance of the separation system and the mass spectrometer are essential considerations; thus, the cost of sample preparation must be balanced with maintenance costs of advanced analytical instrumentation.

Dilution of the extracts can reduce matrix effects to a certain degree, but it can also lead to an increase of the estimated quantitation limits (LOQs) as some problems can be observed at low concentration levels for several analytes. This strategy was developed for the simultaneous extraction of a wide variety of residues and contaminants (firstly pesticides, and then mycotoxins, plant toxins and veterinary drugs) from different food (meat, milk, honey and eggs) and feed matrices allowing the extraction of more than 300 compounds [7].

Table 4 provides a comparison of some analytical features peculiar to a Dilute and Shoot approach in comparison to the SLE and SPE approaches.

Table 4: comparison of some features of dilute and shoot approach with an SLE and SPE

	Dilute and Shoot	SLE	SPE
Sample preparation time	fast	fast	slow
Process difficulty	easy	easy	difficult
Consumables required	fewer	more	more

Sensitivity achieved	low	high	higher
Chromatographic column life	high	low	high

1.4.5.8.5. The QuPPE method

The Quick Polar Pesticides (QuPPe) Method was first introduced by the European Reference Laboratory for single residue methods (EURL-SRM) and has enabled more laboratories to conduct analysis for at least some of the polar pesticides. QuPPe allows the analysis of a number of highly polar pesticides non-amenable to common multiresidue methods (e.g. QuEChERS). The method involves extraction with acidified methanol and LC-MS/MS measurement. Isotope labelled analogues of the compounds are used as internal standards (ILISs) to correct for volumetric variations, matrix effects and other errors. Various LC-MS/MS methods were validated ([255], [256], [257]) each covering a different scope of pesticides. This provides laboratories a range of options, depending on the pesticide scope they would like to cover. The method is dynamic and is periodically being updated as more pesticides or separation possibilities are being introduced [258].

1.4.5.9. Method validation

Method validation is a requirement of national, regional and international standards that help directly ensure the quality of produced data and the quality of the analytical capabilities needed to ensure compliance to the legislation [259], [260]. The validation of a methodology must be performed for all the analytical methods prior to its use in the lab and after its development and adjustment.

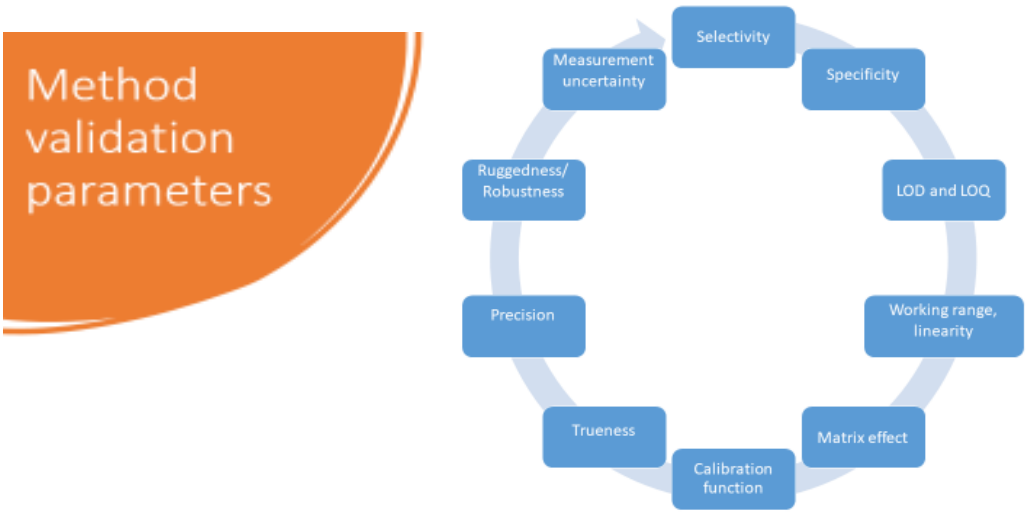
The ISO/IEC 17025:2017 quality standard requires that “the laboratory shall validate non-standard-methods, laboratory -developed methods and standard methods used outside their intended scope or otherwise modified. The validation shall be as extensive as is necessary to meet the needs of the given application or field of application” [261]. The purpose of method validation is to show that the method of analysis chosen is capable of producing accurate, precise and reproducible results for the analytes of interest in the specified matrices. The data collected (and retained) to prove the validity of the method provide the basic evidence to support the validity of the results subsequently generated using the method for sample analysis. In other words, a method must be fit for the purpose of analysis and should provide reliable results.

Essential information for the characterisation of a method may be gathered during the development or adaptation of an analytical procedure, the establishment of acceptable performance (in house validation), the regular performance verification of methods applied in the laboratory, the demonstration of acceptable performance in a second or third laboratory (AOAC Peer-Verified Method) [262] and participation in inter-laboratory collaborative studies [263].

As mentioned above, it is essential that method validation be carried out following completion of the method development and before introducing the method for routine analysis. Additional validation is essential when the method is transferred to another laboratory or whenever the conditions or method parameters for which the method was initially validated have changed.

To date no regulatory standard or guideline is currently available that regulates all classes of residues and contaminants simultaneously. In the case a residue/contaminant is being detected, the analysts are obliged to comply with the requirements of the specific legislation for the class of interest. Those requirements also apply to method validation procedures.

In general, across all legislation requirements, the most important parameters to be studied and characterized during method validation are (see **Text box 6)**:



Text box 6: Common validation parameters

1.4.5.9.1. Selectivity

Selectivity Is the ability of an analytical procedure to assess the analyte in the presence of interferences such as matrix components, impurities, degradation products etc. Some regulatory authorities use the term specificity to refer to selectivity. (see **Text box 7)**:

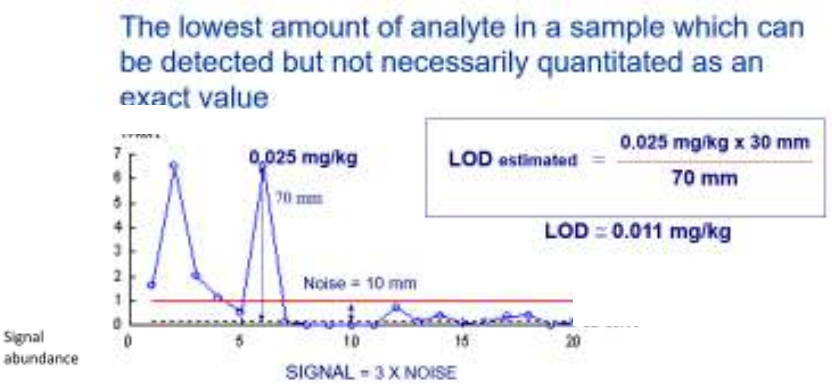
Text box 7: Visual representation of the selectivity concept: only the red target fits the yellow shape

1.4.5.9.2. Specificity

Specificity of analyte detection means that the detection system response, used for calibration, must be demonstrated as being completely attributable to the analyte, preferably by mass spectrometry in full-scan or multiple reaction monitoring or exact-mass/high resolution mass spectrometry. In other words, that the signal is due to the analyte presence only.

1.4.5.9.3. Limit of detection and quantitation

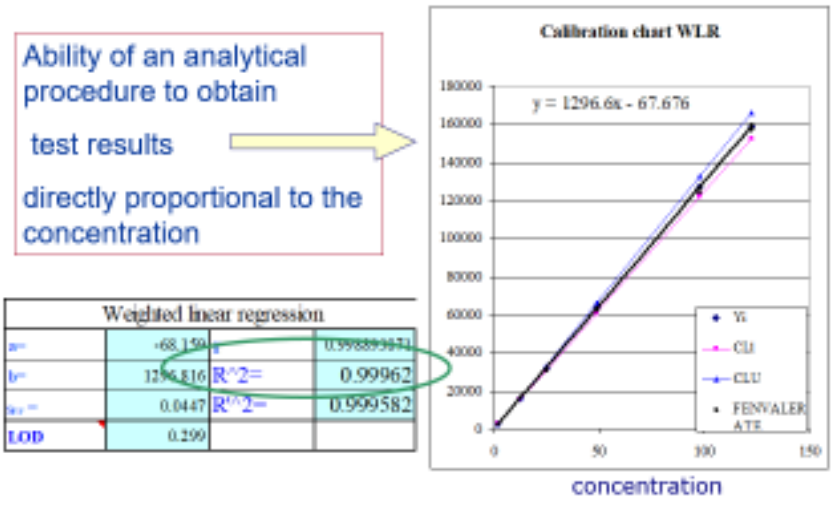
Limit of detection (LOD) is the smallest amount or concentration of the analyte in the test sample that can be reliably distinguished from zero (see **Text box 8**). This information is necessary to characterize the analytical method in terms of its ability to detect low levels of analytes and compare it to other methods, laboratories or standards. The limit of quantitation (LOQ) is the lowest spike level meeting the identification and method performance criteria for accuracy and precision.



Text box 8: The manual estimation of the LOD by measuring the noise and the analyte peak heights in relation to the injected analyte concentration.

1.4.5.9.4. Linearity, working range

Linearity is the power of a method to elicit test results that are directly proportional to the concentration of an analyte in the sample within a specific working range (see **Text box 9**). Linearity is important for the confirmation of the method's sensitivity for the analysis of the analyte's concentration within a defined range. Linearity of a given response must be evaluated using at least a minimum of 5 concentrations of the analyte (multi-point calibration) and the data must be statistically analyzed, e.g. by performing regression analysis using the method of the least squares. Linearity studies are important because they define the range of the method within which the results are obtained accurately and precisely.



Text box 9: Visual representation of the linearity concept using the calibration regression [132]

1.4.5.9.5. Calibration function

The calibration function is the relationship between the observed signal from the target analyte in the sample extract and the known quantities of the analyte prepared as calibrant. This function must be defined for its linearity, analytical range, limit of detection and limit of determination and response range [132].

1.4.5.9.6. Matrix effects

Matrix effects: these are described as the possible impacts of sample matrix components on the measurement of analyte concentration (analyte signal). If present, matrix effects may need to be compensated for, for example through the use of matrix-matched calibration.

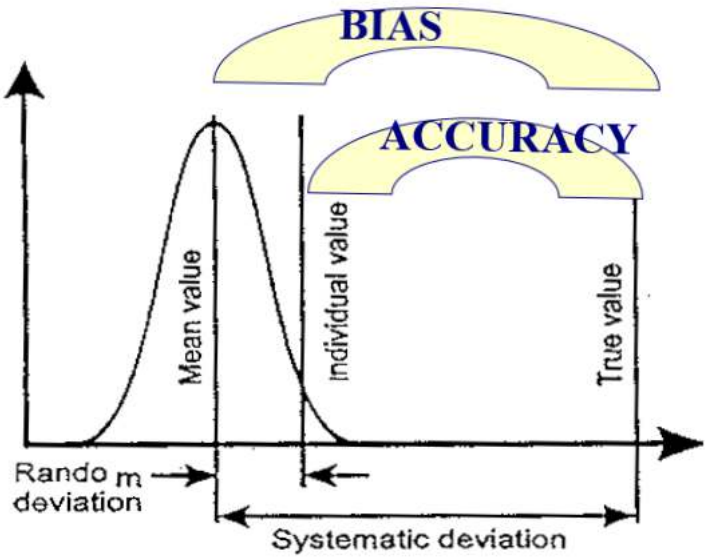
1.4.5.9.7. Precision and reproducibility

Precision is the degree of agreement among individual test results, or in other words the extent to which the individual test results of multiple injections of a series of standards agree. The measured standard deviation can be expressed as repeatability, within laboratory reproducibility and reproducibility. Repeatability refers to the closeness of agreement between mutually independent test results obtained with the same method on identical test material, in the same laboratory by the same operator using the same equipment within short intervals of time. The repeatability (within-run effect) includes contributions from any part of the procedure that varies within a run, including contributions from normal gravimetric and volumetric errors, heterogeneity of the test material, and other procedural errors during the analysis. It is expressed as repeatability relative standard deviation (RSD_r). Reproducibility refers to the closeness of agreement between independent results obtained with the same method on identical test material obtained but under different conditions. It is expressed as reproducibility relative standard deviation (RSD_R). Within-laboratory or intra-laboratory reproducibility contributes to day-to-day variations in the analytical system due to changes of analyst, batches of reagents, recalibration of instruments and laboratory

environment (e.g. temperature changes). Between-laboratory or interlaboratory or multiple-laboratory reproducibility (laboratory effect) contributes to additional variations such as variations in calibration standards, differences between local interpretations of a protocol, differences in equipment or reagent source, or environmental factors, such as differences in average climatic conditions.

1.4.5.9.8. Trueness

Trueness is defined as the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value [262]. A measure of trueness is the determination of the method bias, which is defined as the deviation of the mean value from a reference value (true value). The accuracy on the other hand is the measure of the deviation of an individual value from the reference value (true value) (see **Text box 10**). Recovery is the amount measured as a percentage of the number of analyte(s) (active substance and relevant metabolites) originally added to a sample of the appropriate matrix, which contains either no detectable level of the analyte or a known detectable level. Recovery experiments provide information on both precision and trueness (bias), and thereby the accuracy of the method.



Text box 10: Visual representation of the trueness concept, extracted from [264]

1.4.5.9.9. Uncertainty

Uncertainty of measurement results is the range around the reported result within which the true value is expected to lie with a specified probability. It is well known that replicate analyses of the same sample, analyzed either within- or between-laboratories, will generate different results, especially at trace (0.001–10 mg/kg) levels. The estimation of uncertainty is approached either by a “top-down” or “holistic” approach or by an alternative “bottom-up,” “uncertainty budget, “component-by-component”, metrological approach. The estimation of the uncertainty of the results is further addressed in paragraph 1.4.6.4.

1.4.5.9.10. Robustness/ruggedness

Robustness/ruggedness is the capacity of a method to withstand and remain unaffected by any deliberate variation to the parameters.

The ruggedness/robustness of the analytical method is further addressed in paragraph 1.4.6.5.

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to unknown samples to be analyzed routinely. The preparation and execution of a method validation study should follow a validation protocol, preferably written in a step-by-step instruction format. The objective of analytical method validation is to ensure that valid analytical data are generated both during initial use of the method and also during its entire lifetime of application. However, appropriate quality control checks should be included during routine sample analysis to verify that the performance of the method and the system has not changed from the initial method validation.

The performance parameters that are related to the ability of the method to detect, identify and quantify low analyte levels in samples are the LOD and the LOQ.

A full method validation is a laborious and a challenging task.

Regulatory documents and guidelines that provide information and guidance on method validation are available from the AOAC, Codex Alimentarius, European Medicines Agency, the EU, the Eurachem, the FDA, the ISO among many others They are indicated in **Table 5** and **Table 6** as either vertical or horizontal standards depending on whether they are applicable to one specific chemical area or if they are more of a general competence for more chemical residues/contaminant areas.

Table 5. Examples of vertical validation guidelines or standards that provide requirements for method validation for specific compounds/ matrices.

Validation guidelines/standard	Application area

Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs [**265**]	Mycotoxins method validation guidelines
CEN TR 16059. Food analysis - performance criteria for single laboratory validated methods of analysis for the determination of mycotoxins [**266**]	Mycotoxins method validation guidelines
Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results [**267**]	Performance of analytical methods and the interpretation of results, in the area of certain substances and residues thereof in live animals and animal products.
CAC/GL 71-2009: Guidelines for the design and implementation of national regulatory food safety assurance programme associated with the use of veterinary drugs in food producing animals [**268**]	Use of veterinary drugs in food producing animals
Guidance on bioanalytical method validation, European Medicines Agency, 2011 [**269**]	Measurement of drug concentrations in biological matrices

Table 6. Examples of horizontal validation guidelines or standards that provide requirements for method validation

Horizontal validation guidelines / standard
CXG 49-2003 Harmonized IUPAC Guidelines for Single-Laboratory Validation of Methods of Analysis [**270**]
CAC/GL 40-1993 Guidelines on Good Laboratory Practice in Pesticide Residue Analysis [**271**]
SANTE/12682/2019 Analytical quality control and method validation procedures for pesticide residue analysis in food and feed [**132**]
FDA Regulations and specific guidelines for the validation of analytical methods and procedures. Guidelines for the Validation of Chemical Methods for the FDA FVM Program [**272**]
AOAC international guidelines for validation of qualitative binary chemistry method [**273**]
Eurachem Guide: The Fitness for Purpose of Analytical Methods - A Laboratory Guide to Method Validation and Related Topics [**274**]

1.4.5.9.11. Practical steps at the laboratory

The implementation of method validation includes several steps, such as:

- Planning and preparation of a validation plan by specifying the method requirements in terms of accuracy, precision, selectivity, specificity, sensitivity, LOQ, linearity,

ruggedness, among others. **Table 7** provides the information about the "how to" implement, and is a good starting point for the practical implementation at the laboratory.

- Selection of matrices and analytes and planning of fortification levels
- Confirmation of the stability of analytes, efficiency of extraction and homogeneity of distribution of the analyte in the processed sample, as evaluated during method development and optimization
- Establishment of the experimental design
- Running of the experiments
- Statistical analysis of the generated data
- Comparison of the results with requirements of guidelines/standards
- Documentation of method validation performance criteria

1.4.5.10. Evaluation of the method

Once method validation experiments are analytically implemented, method performance parameters must be established to characterise the method. An example of minimum performance criteria for an analytical method is given in **Table 7**.

Table 7: minimum performance criteria for an analytical method, based on CXG 90-2017 [131] and SANTE/12682 /2019 [132].

Evaluated parameter	Reference guideline	What / How to	Min. criterion required
Selectivity (rate of false positives and negatives)	CXG 90-2017	To estimate rates of false positives and negatives during method validation, an adequate number of blanks per matrix [not from the same source] should be analyzed along with spiked matrices at the analyte reporting level.	No interferences shall occur which significantly affect the analysis.
Specificity	SANTE/12682 /2019	Response in reagent blank and blank control samples	<30 % of the reporting limit
Reporting limit (RL)	SANTE/12682 /2019	The lowest level at which residues will be reported as absolute numbers.	It is equal to or higher than the LOQ.
Linearity	CXG 90-2017	Examination of a plot of residuals produced by linear regression of the responses on the concentrations in an appropriate calibration set. Any curved pattern another function such as quadratic should be tested and applied, using at least five concentration levels. The use of weighted-linear regression or weighted-quadratic function is recommended rather than linear regression for low part per	Residuals of the calibration curve deviate by more than ±20 – 30 % (30% for calibration concentrations near the instrument LOQ), statistical consideration of outliers should be made, possibly leading to re-analysis of the sequence if quality control criteria are not met. The coefficient of determination (R2) used with an appropriate weighting factor such as 1/x or 1/x2 to

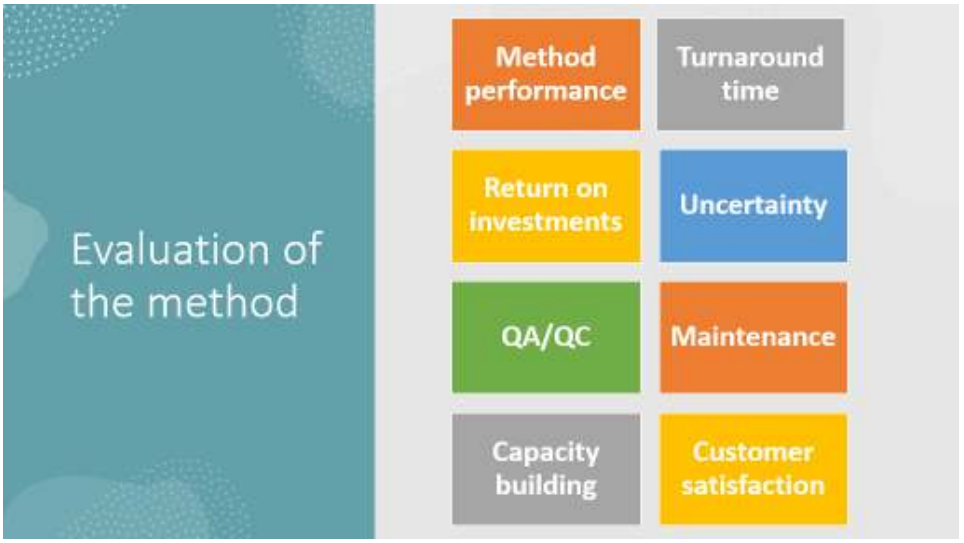
		billion (µg/kg) concentration determination	minimize the potential impact of the relative concentration range.
Linearity	SANTE/12682 /2019	Linearity check from five levels	Deviation of back calculated concentration from true concentration <20 %
Matrix Effect-	CXG 90-2017 and SANTE/12682 /2019	Comparison of response from solvent standards and matrix matched standards	Estimation of the ME and use of compensation approaches in calibration (matrix matched, analyte protectants, ILISTD, etc.)
Limit of quantitation (quantification)-LOQ-	SANTE/12682 /2019	Lowest spike level meeting the identification and method performance criteria for recovery and precision. It is equivalent to the limit of determination (LOD), which means the validated lowest residue concentration which can be quantified and reported by routine monitoring with validated control methods;	
Lowest validated level	(LVL)- CXG 90-2017	The S/N at the lowest calibrated level (LCL) must be ≥10 (conc. ≥ LOQ), which can be set as a system suitability check required for each analytical sequence.	Spiking experiments at the Lowest Validated Level (LVL).
Recovery	SANTE/12682 /2019	Average recovery for each spike level tested	70- 120 %
Trueness	CXG 90-2017	The trueness of a method may be determined by analysis of a certified reference material, by comparison of results with those obtained using another method for which the performance criteria have previously been rigorously established (typically a collaboratively studied method), or by determination of the recovery of analyte fortified into known blank sample material.	70- 120 %
Precision (RSDr)	SANTE/12682 /2019	Repeatability RSD r for each spike level tested	≤ 20 %
Precision (RSDwR)	SANTE/12682 /2019	Within laboratory reproducibility, derived from on going method validation/verification	≤ 20 %
Precision-	CXG 90-2017	Degree of variability of a measurement around a mean	RSD ≤ 20%. For very low concentrations (e.g. <0.01 mg/kg) RSD < 30 %.
Ion ratio -	CXG 90-2017 and SANTE/12682 /2019	Check compliance with identification requirements for MS techniques	See **Table 1**-paragraph 1.4.2.1
Retention time	SANTE/12682 /2019	The retention time of the analyte in the extract should correspond to that of the calibration standard	Matching tolerance of ±0.±0.1 min.
Retention time-	CXG 90-2017	The retention time of the analyte in the extract should correspond to that of the reference value.	Matching tolerance of ±0.2 min or 0.2 % relative retention time, for both gas and liquid chromatography (preferably + 0.1 min if possible).
Robustness	SANTE/12682 /2019	Average recovery and RSDwR, derived from on going method validation/verification	

Ruggedness	CXG 90-2017	Ruggedness (often synonymous with robustness) of an analytical method is the resistance to change in the results produced by the analytical method when deviations are made from the experimental conditions described in the procedure.	

Table 7 indicates that according to both guidelines, CXG 90-2017 and SANTE/12682 /2019, a quantitative analytical method should be capable of providing acceptable mean recovery values at each fortification level and for at least one representative commodity from each of the relevant commodity groups. Mean recoveries from initial validation should be within the ranges as specified in the Table 7 with an associated repeatability RSD_{wr} and a within-laboratory reproducibility (RSD_{wR}), for all analytes within the scope of a method. Method performance criteria must be fully documented. In this thesis, the definition adopted for the LOQ is the lowest fortified level of the validation meeting the method performance acceptability criteria.

Next step, is setting the parameters for verification of method performance during its routine application. Generally quality control charts are very useful for monitoring such performance [275]. Any deviation from the validation criteria shall be investigated. In addition, ISO/IEC 17025:2017 states that when changes are made to a validated method, the influence of such changes shall be evaluated and if found to affect the original method, a new validation shall be performed. In addition to ensuring that the method meets the needs and field of application for the intended purpose, including the estimation of uncertainty (see paragraph 1.4.6.4.), it is important to verify that there is a return on research and development efforts and investments, and that the method is fast enough to comply with “customer needs”. (see **Text box 11**).

The required regular maintenance also needs to be documented in the standard operating procedures or method protocol together with the quality assurance and quality control measures associated with the analytical method. Such documentation is the fundamental document according to which capacity building shall be provided to the technical staff at the laboratory.



Text box 11: Aspects that are important for the evaluation of the fitness for purpose of an analytical method

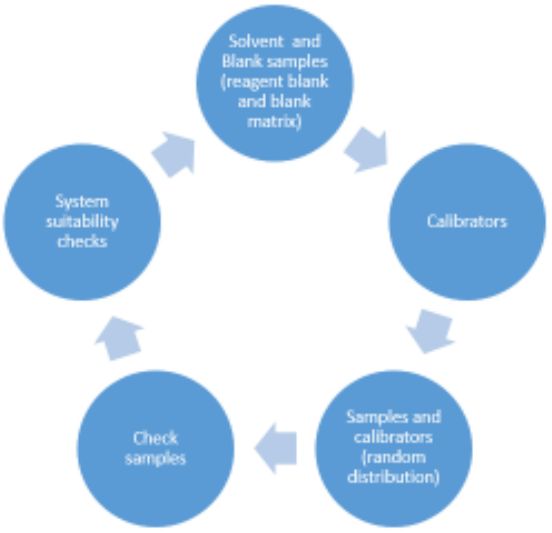
1.4.6. The analytical data

The analytical data is the result deriving or associated with an analytical method. Ultimately data (analytical result) need to be univocally related to a residue/contaminant concentration in a sample, which is processed and prepared in such a way that the information contained in the original population is preserved during all the steps including sampling, transportation to the laboratory, storage and analysis. One can imagine the number of traps and pitfalls that exist throughout this “representative” data trip. ISO 17025:2017 focuses the attention on the issue of representativeness of the sampling step. In the end the analytical data is valid only if it represents the original sample, and this chain of representativeness is maintained intact by all possible means. When the analytical determination can avail of HRMS instruments, data can be generated and reprocessed a posteriori, using a retrospective evaluation of compounds based on their isotopic profile and accurate mass.

1.4.6.1. Processing of data files

Data processing typically commences on completion of the entire sample sequence from the final determinative step. The instrument software allows to manually or automatically

process the data files. In the case of targeted approaches, which are currently the most common analytical choice for food residue/contaminant detection, a specified list of analytes is investigated using a QqQ detector operated in selected reaction monitoring mode (SRM/MRM). Specific instrument software is used to identify the compounds and run a quick screening of the samples. In GC-MS/MS, reverse-search methods, based on the National Institute for Standards and Testing (NIST) (electron impact) libraries, are available [276]. In LC-MS/MS there is very little availability of commercial libraries which allow a rapid screening of the samples. The reason for this is the scarce reproducibility of in-source collision-induced dissociation spectra and the difficulty of interchanging spectra acquired with instruments from different manufacturers. Through comparison of retention time information, MRM quantification and qualification transitions, analyte peaks are integrated, and quantified using calibration approaches. According to the SANTE/12682 /2019 guideline at least two product ions are necessary for a compound identification while the ion ratio from sample extracts should be within ±30 % of calibration standards from the same sequence. In multi-residue analysis an increased number of analytes means that a higher number of necessary ion transitions have to be recorded. This can result in an increased chance of common or overlapped transitions affecting the method LOQs [277]. In general, the batch sequence will contain files about reagent blanks, blank samples, calibrators and samples, including ongoing validation samples and QA/QC samples, (see **Text box 12**)



Text box 12: Samples included in a typical batch sequence

Reagent blanks are important as they provide information about possible laboratory contamination from solvents, reagents and process related, and represent a first quality control measure.

Blank samples are processed to ensure that the matrix used to prepare matrix matched standards is free from any residue/contaminant at the retention time of interest for each analyte.

Calibrators are essential to predict the unknown concentration of a target analyte through the establishment of a calibration curve. This is achieved by measuring reference standards, called the calibrators, and plotting area responses as a function of their concentration to establish a relationship in the form of a linear regression, preferably weighted regression to take into consideration inconsistency in the errors. Unknowns in the samples can then be predicted using the established calibration curve.

Validation samples and/or on-going validation samples (i.e. a type of check samples) include sample extracts fortified at a known concentration of the analytes used for the purpose of validation studies and or ongoing validation. The information from these samples is usually included in the quality control charts, to provide regular information about the performance of the analytical method. Additional check samples are the QA/QC samples. For example, blind fortified samples, that are randomly included in the analytical batch, and the results of which are managed by the quality manager of the laboratory as part of the regular laboratory quality control program.

All obtained chromatograms must be examined by the analyst and the baseline fit must be checked and adjusted, as is necessary. Where interfering or tailing peaks are present, a consistent approach must be adopted for the positioning of the baseline. Peak area or peak height, whichever yields the more accurate results, may be used as described for example in the SANTE/12682/2019 guideline. Identification of peaks includes appropriate peak integration (area threshold for peak start), peak identification (expected analyte retention time window, m/z and MRM transitions), and calibration parameters (weight and concentration of samples and reference standards) [278].

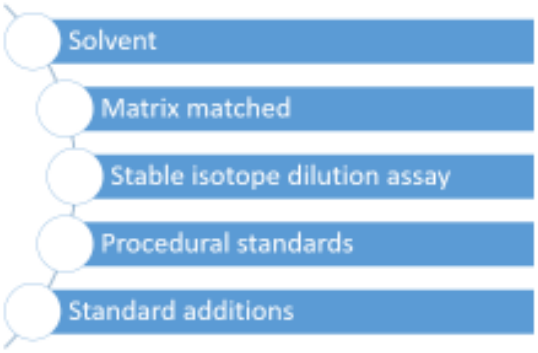
1.4.6.2. Analytical calibration options

When it comes to analytical calibration, chemists are given several options (see **Text box 13**)

- solvent calibration

- matrix-matched calibration
- stable isotope dilution assay with use of isotopically labelled internal standards
- standard additions
- procedural standards calibration

The choice of calibration to adopt depends on several factors, but primarily on the target matrix-analyte combination.



Text box 13: Analytical calibration options

Solvent calibration is very useful when matrix effects have been proven to be negligible. However, as discussed in section 1.4.5.9.6., in the analysis of fresh fruits and vegetables, extracts to be analysed by chromatography coupled to mass spectrometry are often prone to "matrix effects" in the system (GC or LC has signal enhancement or suppression). The calibration option should be as close as possible to the real sample situation. The calibration standards should preferably be in an environment that is similar to the sample, to reduce the effect from the sample matrix.

A reasonable option could, therefore, be to opt for a matrix matched standard calibration, in which extracts of blank matrix, preferably of the same type as the sample, are used for calibration. However, it may not be that easy to find a suitable blank matrix. The SANTE/12682/2019 guideline indicates that in GC, representative matrix calibration, using a single representative matrix or a mixture of matrices, can be used to calibrate a batch of samples containing different commodities. Although this is preferable to the use of calibration standards in solvent, compared to exact matrix matching, it is likely that the calibration will be less accurate. The SANTE/12682/2019 guideline recommends that the relative matrix effects are assessed and the approach is modified accordingly.

Another analytical calibration option is the use of isotopically labelled internal standards (ILSTD) in the SIDA format (see paragraph 1.4.4.3.). The ILSTD matches completely with the analytes in terms of chemical behaviour, being essentially the same compound; however, this option is expensive, especially when the screening is for multiple analytes in the sample. The use of ILSTD calibration is the preferred option, since ILSTD should behave exactly like the target analyte, thus compensating for any matrix effect on the peak response. For multiresidue methods (10+ analytes) one can adopt the option of calibration by standard addition. In this case a sample is divided into several test portions. One portion is analysed directly and increasing amounts of the analyte are added to the other test portions immediately prior to extraction. The “unknown” concentration of the analyte is derived by extrapolation, from a calibration curve prepared from the relative responses of the analyte in the sample and the spiked samples extracts. According to the SANTE/12682/2019 guideline in this approach it is a must to demonstrate a linear response for the analytes of interest in the appropriate concentration range, to achieve accurate results.

The standard addition is designed to compensate for matrix effects and recovery losses, but not for extraction efficiency (unlike procedural calibration) or chromatographic interferences caused by overlapping/unresolved peaks from co-extracted analytes. The standard addition technique assumes some knowledge of the likely residue level of the analyte in the sample, so that the amount of added analyte is similar to that already present in the sample. In addition, the SANTE/12682/2019 guideline recommends that the standard addition calibration is used for confirmatory quantitative analyses in cases of MRL exceedances and/or when no suitable blank material is available for the preparation of matrix-matched standard solutions.

The use of procedural standards is an alternative type of calibration. Procedural standards are prepared by fortification of a series of blank test portions with different amounts of analyte, prior to extraction. The procedural standards are then analyzed in exactly the same way as the samples, undergoing the same sample preparation procedure and analysis. This approach can compensate for matrix effects and low extraction recoveries associated with certain pesticide/commodity combinations, especially where isotopically labelled standards are not available or are too costly. According to the SANTE/12682/2019 guideline, this analytical calibration approach is only applicable when a series of samples of the same type are to be processed within the same batch of analysis.

As discussed in paragraph 1.4.2.1 mass spectrometry coupled to a chromatographic separation system is a very powerful combination for identification of an analyte in the sample extract. It simultaneously provides retention time, mass/charge ratios and relative

abundance (intensity) data. Data files for samples are processed to provide identity and confirmation of residues/contaminants and analytical requirements are indicated in **Table 1**.

1.4.6.3. Data analysis and statistical evaluation

Statistical evaluation of data is of utmost importance in residue/contaminant analysis to establish performance criteria from method validation or to interpret residue/contaminant data from sample analysis. Calculation of the mean (or average), standard deviation, relative standard deviation, confidence intervals, regression analysis, are usually performed using statistical software packages or simply excel spreadsheets. The simplest tools are F-test, t-test, regression and correlation analysis. However, the optimization step of the analytical methods has often been performed utilizing multivariate techniques, including the testing for robustness/ruggedness of an analytical method. Design of Experiment (DoE) is a chemometric approach used to design experimental runs, identifies significant factors, and estimates the main and interaction effect of various factors under study [279].

DoE requires few experimental runs that are carried out in an orderly manner, saving thus analysis time and improving sample throughput [280].

Additionally, multivariate data analysis is required for non-targeted screening approaches using HRMS. Many different software exists for the extraction of peaks from the large amount of data, the deconvolution of such data, and the subsequent analysis in both multivariate and univariate ways. Data processing is generally carried out using specific statistical software, such as SIMCA [281] or the statistical software R [282] that allows alignment, pre-processing, outliers search and modelling of MS profile data. Recently principal component analysis (PCA) was employed to help establish the processing factors for pesticide residues from apple to juice [283].

1.4.6.4. Uncertainty estimation

The main purpose of making measurements is to use the results to help in making decisions. The reliability of the decisions made depends, therefore, on the uncertainty of the results, and their fitness for purpose. Analytical laboratories are mostly concerned with estimating

the uncertainty of measurements for the results they produce for a given sample. It is important to note that estimation and reporting of the uncertainty of measurement is a requirement under ISO/IEC 17025:2017. It is therefore essential that effective procedures are established for estimating the uncertainties arising from all parts of the process that lead to a result. These include sampling, sample preparation and processing, extraction, clean-up and analysis. There are several approaches to estimating the measurement uncertainty [284]. One approach is described in the Guide to the Expression of Uncertainty in Measurement (GUM) published by ISO [285]. Also referred to as the "bottom-up" approach, and shared by EURACHEM [274] this consists of identifying and quantifying the contribution of each step of the analysis separately and combining all contributions into an overall uncertainty estimate. An alternative approach, described in the SANTE/12682/2019 guideline and referred as the "top-down approach", attempts to estimate the all-inclusive variability of the measurement using information from proficiency testing, collaborative studies or from in-house validation data.

The "top-down" approach is probably simpler but provides little information as to how uncertainty could be improved. In contrast, the investigation and estimation of the contributions made in the "bottom-up" approach is more complicated and time consuming but is the more useful approach for any laboratory aiming to improve its methodology. Both approaches are acceptable within the ISO/IEC 17025 standard.

If the uncertainty of measurements is underestimated, for example because the sampling variability is not taken into account, then erroneous decisions may be made that can have important consequences for consumer health or trade.

ISO/IEC 17025:2017 now requires that laboratories shall identify the contributions to measurement uncertainty, including those arising from sampling. [see paragraph 7.6.1. of ISO/IEC 17025:2017]. The standard discusses the laboratory responsibility for sampling, the need for having a sampling plan and a sampling method available at the site where sampling is taking place [see paragraph 7.3.1. of ISO/IEC 17025:2017]. While many texts and guidelines exist for measuring the uncertainty component of an analytical measurement, only one guideline exists to date for estimating the sampling uncertainty. Eurachem has published a guideline entitled, "Measurement uncertainty arising from sampling – a guide to methods and approaches" [286].

1.4.6.5. Ruggeddness / Robusteness estimation

The data collected during method validation provide the basic evidence to support the validity of the results subsequently generated using the method for sample analysis. Ruggedness, sometimes termed robustness, is one of the performance criteria that need to be assessed as part of method validation. There is currently no harmonization in the definitions of the terms robustness and ruggedness as applied to analytical methods. A review of current international guidelines shows that the terms are used either as synonyms or as complementary terms. **Table 8** summarizes the definitions and the requirements of some international bodies.

Table 8: Definitions and requirements adopted by some international bodies for robustness/ruggeddness.

Guideline	Term Used/ Defined	Definition/Criteria	Comments
European Commission SANTE/12682/2019 [132]	Robustness	Average recovery and RSD_{WR}, derived from on-going method validation/verification	Focus on application of quality control
Codex Alimentarius CAC-GL 90-2017 [131]	Ruggedness	The ruggedness of an analytical method is the resistance to change in the results produced by an analytical method when minor deviations are made from the experimental condition described in the procedure. The limits for experimental parameters should be prescribed in the method protocol, and such permissible deviations, separately or in any combination, should produce no meaningful change in the results produced. The aspects of the method that are likely to affect results should be identified, and their influence on method performance evaluated by using ruggedness tests.	Changes in the instrument, operator, brand of reagent; concentration of a reagent; pH of a solution; temperature of a reaction; time allowed for completion of a process, and/or other pertinent factors.
Document prepared for IUPAC [287], [288]	Robustness and Ruggedness	The relative robustness of an analytical method is defined as the ratio of the ideal signal for an uninfluenced method compared to the signal for a method subject to known and unknown operational parameters as studied in an intralaboratory experiment. The relative ruggedness of an analytical method is defined as the ratio of the ideal signal for an uninfluenced method compared to the signal for a method subject to known and unknown operational parameters as studied in an interlaboratory experiment."	
Eurachem [260]	Ruggedness (robustness)	The 'ruggedness' ('robustness') of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. Ruggedness provides an indication of the method's reliability during normal usage. The ruggedness of a procedure must be established for in-house developed methods, methods adapted from the scientific literature and methods published by standardisation bodies used outside the scope specified in the standard method.	Most effectively evaluated using experimental designs. E.g. 7 parameters can be studied in 8 experiments using a Plackett-Burman experimental design.

International Accreditation Service [289]	Ruggedness or Robustness	Ruggedness or robustness: The ability of a method to resist changes in test results when subjected to minor deviations in experimental conditions of the procedure. Ruggedness testing examines the behaviour of an analytical process when subtle small changes in the environment and/or operating conditions are made, similar to those likely to arise in different test environments.	Subtle small changes in the environment and/or operating conditions

A shown in **Table 8** robustness can also be termed ruggedness and it needs to be assessed as part of intra-laboratory method validation. Generally, this is done by evaluating the intra-laboratory reproducibility of the method with small variations in the conditions of the test, similar to those that may arise in different test environments. If it can be verified that the changes introduced do not cause any significant effects on the results, the analyst can have confidence that the method will most probably perform within its specified operating criteria when applied routinely. It is important to perform the robustness testing as early as possible during optimization and validation of the method, to avoid spending effort and money on a method that is not suitable. The traditional approach is the one suggested by Youden and Steiner [290] and is based on the Plackett-Burman experimental design [291]. Other approaches using DoE are applicable [292], see paragraph 1.4.6.3.

1.4.6.6. Compliance assessment

The result of an analysis is frequently used to decide about compliance or non-compliance with a regulatory limit. Once the information about uncertainty has been generated, the next step is to use it for compliance assessment. Assuming that the objective is to assess whether or not a sample contains a compound above a set (or permissible) upper limit, four possible scenarios are possible when comparing the result with the upper limit (see Figure 8):

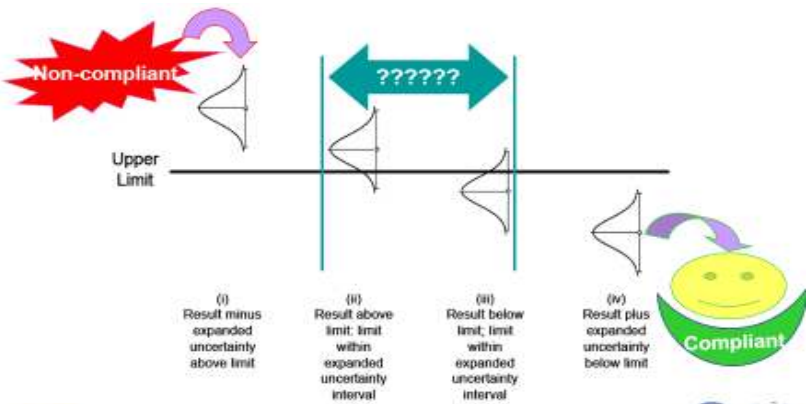


Figure 8: Uncertainty and compliance limits

(i)The result of the measurement exceeds the upper limit by more than the measurement uncertainty.

(ii)The result of measurement exceeds the upper limit but the upper limit is within the measurement uncertainty.

(iii)The result of the measurement is below the limit but the upper limit is within the measurement uncertainty.

(iv) The result of measurement and the measurement uncertainty are below the upper limit.

It is easy to conclude that case (i) is truly a “non-compliant sample” and case (iv) is truly a “compliant sample”. However, in cases (ii) and (iii), rules are needed to decide whether or not a sample is compliant or non-compliant. The Eurachem-Citac guide describes the need for decision rules and the setting of acceptability levels with acceptance zones (for the product to be declared compliant) and rejection zones (for the product to be declared non-compliant). The Eurachem guide suggests that decision rule to be applied and method of calculating the critical value shall need to be fully specified and agreed by the regulating body or competent authority and the laboratory and /or set by national legislation.

1.4.7. The analytical quality requirements

Method validation and analytical quality control/assurance measures are required to ensure the validity of data reported within the framework of analytical testing. A harmonized, cost effective, mutually accepted quality assurance and quality control system is the aim of guidelines such as the CODEX CXG 65-1997 [293], EU SANTE/12682/2019 [132] and ISO/IEC 17025:2017 [261]. Through strict adherence to these guidelines laboratories ensure that analytical results are meaningful, valid, reliable, accurate, in time, within budget, comparable and that false positives or false negatives results are minimized. To ensure the validity of results, the ISO/IEC 17025:2017 mentions that it is the responsibility of the laboratory to set up a procedure for monitoring and recording results, to be able to detect trends and to apply statistical techniques to review the data. The monitoring shall include for example the use of reference materials or quality control materials, functional checks of measuring equipment, replicate tests or calibrations, intralaboratory comparisons, testing of blind samples, participation in proficiency testing and/or in interlaboratory comparisons.

1.4.7.1. Quality assurance/Quality control measures (QA/QC)

Laboratories can improve their performance and ensure reliability of their test results by implementing quality assurance and quality control procedures. Quality assurance (QA) is defined by ISO as that component of quality management, focused on providing confidence that quality requirements are fulfilled [294]. The Citac/Eurachem Guide to Quality in Analytical Chemistry refers to quality assurance as the overall measures that a laboratory uses to ensure the quality of its operations [295]. Quality control refers to the operational techniques and activities that are used to fulfil requirements for quality. For example, to ensure the quality of a specific batch of samples one can make use of reference materials to check for recoveries of fortified samples and monitor those in control charts. The purpose of quality assurance is to provide confidence in the results produced and that all data generated in the laboratory are fit for their intended purpose. This demonstrates that the laboratory has adequate facilities/equipment and competent staff; the work is carried out in a controlled manner; the methods are validated, and the process is well documented. The Citac/Eurachem guide also mentions that QA should focus on the key issues which determine quality results, costs and timeliness and avoid diversion of energies into less important issues. In general laboratories may opt to design their own QA system; however, it is recommended to follow an established QA system to be able to claim compliance according to that system and to obtain an independent assessment or endorsement by a qualified body (accreditation or certification). Current regulations indirectly determine the features of analytical instrumentation and procedures for validation of the analytical methods and the application of quality control (QC) measures for the analysis. Therefore, the quality of the analytical data is directly proportional to the investments and efforts that laboratories can afford [296]. For example, the European Union Commission Decision 2002/657/EC establishes the requirements that an analytical method must meet for an unequivocal identification and quantification of a controlled substance in a food sample, which means to gain, at least, four identification points. The EU legislation specifies that one identification point is gained by retention time confirmation with a commercial analytical standard, whereas additional 1.5 identification points are gained for each ion MRM transition successfully confirmed. It implicitly requires the use of tandem mass spectrometry instruments. **Table 9** indicates some exemplary QA/QC requirements relative to the implementation of an analytical method according to the EU SANTE/12682/2019 guideline.

Similar requirements can be identified in the Codex guideline for good laboratory practice CAC/GL 90-2017.

Table 9: QA/QC requirements relative to the implementation of an analytical method according to the Codex guideline CAC/GL 90-2017 and the EU SANTE/12682/2019 guidelines.

Analytical step	QA/QC measures	How to
Sampling	Food samples should be taken in accordance with respective legislative framework (i.e. CAC/GL 33 [297] or EU 2002/63/EC [298])	Read from the guideline the minimum nr of primary samples to be taken from a lot, and the minimum size of laboratory samples. Also include contribution of the sampling variability to the total variability of residue analytical results
Transport	Ensure transport is done under appropriate conditions to the laboratory in clean containers and robust packaging. Samples must be identified clearly and indelibly, in a way to ensure traceability.	Polythene or polypropylene bags are acceptable for most samples. Frozen samples must be transported without thawing. Perishable products may have to be frozen to avoid spoilage and then transported in “dry ice” or similar. Samples that may be damaged by chilling must be protected from both high and low temperatures.
Storage	On receipt, each laboratory sample must be allocated a unique code by the laboratory. If not analyzed immediately, it should be stored under conditions that minimise decay.	Use refrigerators for fresh products, cold rooms or dark rooms. Freezers are used after sub-sampling for long storage periods.
Subsampling	All sample preparation, processing, subsampling procedures should be undertaken within the shortest time practicable to minimise sample decay and analyte losses and implemented according to the validated procedures. Homogeneity of sampling needs to be acceptable, alternatively larger test portions or replicate portions should be analysed in order to be able to obtain a better estimate of the true value.	The parts of the commodity that should be analyzed are stipulated in the legislation (i.e. CAC/GL 41-1993 [299]; EC/396/2005 [300]). Use of dry-ice or liquid nitrogen is recommended for processing of samples to avoid degradation of analytes and to improve the homogeneity for subsampling and increase efficiency of disintegration to obtain the smallest particle size as possible (1 mm).
Extraction	The recovery of incurred residues can be lower than the percentage recovery obtained from the analysis of fortified samples. Where practicable, samples containing incurred residues can be analyzed using varying extraction conditions and an optimization can be sought.	Water needs to be added to low moisture commodities to improve the extraction efficiency.
Clean-up	A clean-up, or dilution step, may be necessary to reduce matrix interferences and reduce	Using less selective extraction and clean-up procedures is likely to result in greater

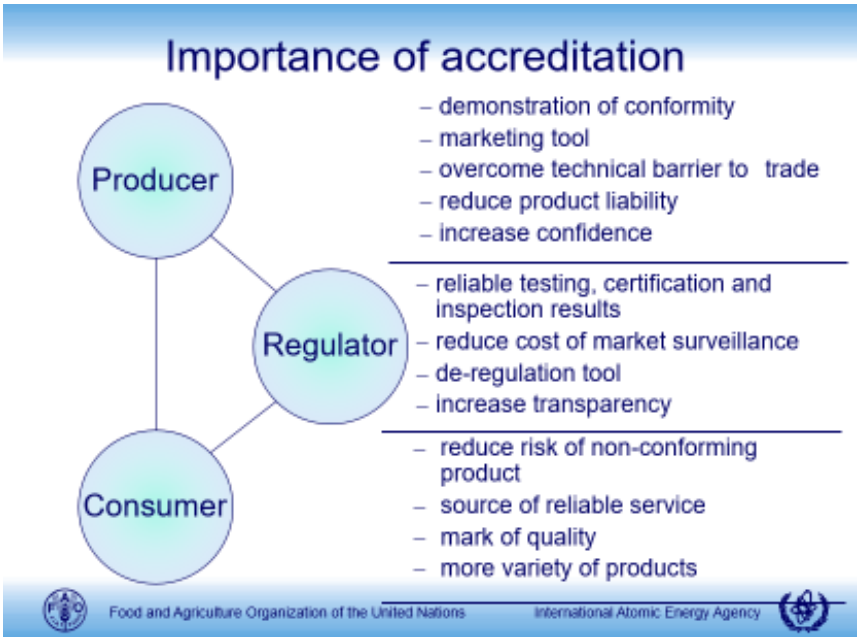
	contamination of the chromatographic instrument.	co-extracted matrix material in the final extract. An optimization is required, and minimization of ME shall be implemented.
Calibration	Bracketing mode, the use of more than 3 calibration standards, linear or quadratic fit, weighted non weighted calibration approach can be implemented.	Determinations at five or more concentrations should be performed; the calibrators should be evenly spaced over the concentration range of interest and the calibration range should encompass the entire concentration range likely to be encountered; According to CAC/GL 90-2017 the fit of the calibration function must be plotted and inspected visually and/or by calculation of the residuals. According to SANTE/12682/2019 linearity must be checked by back calculated concentration from true concentration.
Determination	Ongoing method performance verification during routine analysis shall be applied. Where practicable, recoveries of all analytes in the scope should be measured within each batch of analyses. The recovery of an analyte should normally be determined by fortification within a range corresponding to the reporting level and 2-10 times, or at the MRL, or at a level of particular relevance to the samples being analyzed.	At least 10 % of the analytes (with a minimum of 5) should be included in each batch for detection (SANTE/12682/2019). Suggested sample sequence: Blanks > Calibrators > Solvent Blanks > Unknown Samples (with interspersed Solvent Blanks) > Solvent Blanks > Calibrators > Solvent Blanks
Identification and Confirmation	As to chromatography: the minimum acceptable Rt for the analyte(s) under examination should be at least twice the retention time corresponding to the void volume of the column. The Rt of the analyte in the extract should correspond to that of the calibrator with a tolerance of ±0. 1 min, for both GC and LC. As to MS, this simultaneously provides retention time, mass spectra, mass /charge ratios and relative abundance (intensity). Selected ions should not exclusively originate from the same part of the analyte molecule; however, the choice of ions may change depending on background interferences. Different types and modes of mass spectrometric detectors provide different degrees of selectivity, which relates to the confidence in identification. The requirements for identification are given in **Table 1**.	For identification using MS spectra it is recommended that reference spectra for the analyte should be generated using the same instruments and conditions used for analysis of the samples. For identification using selected ions, they must be sufficiently selective for the analyte in the matrix being analyzed and in the relevant concentration range (high m/z ions are more selective than low m/z ions). Extracted ion chromatograms of sample extracts should have peaks of similar retention time, peak shape and response ratio to those obtained from calibrators at comparable concentrations in the same batch. For a higher degree of confidence in identification, further evidence may be gained from additional mass spectrometric information. For example, evaluation of full scan spectra, isotope pattern, adduct ions, additional accurate mass fragment ions, additional product ions (in MS/MS), or accurate mass product ions.

1.4.7.2. Proficiency testing and interlaboratory studies

Proficiency testing is the determination of laboratory testing performance by means of interlaboratory test comparisons. Interlaboratory comparison is the organization, performance and evaluation of tests on the same or similar test items by two or more laboratories in accordance with predetermined conditions [301]. Participation in interlaboratory studies allows independent verification of laboratory competence. It is an important way of meeting the requirements of ISO/IEC 17025 in the area of quality assurance of laboratory results.

1.4.7.3. Quality systems and accreditation

The EU SANTE guideline clearly states that laboratories designated for official control of pesticide residues must be accredited to ISO/IEC 17025. Accreditation is a procedure carried out by an authoritative body (usually a national accreditation body) which gives a formal recognition that the laboratory is competent to carry out specific tests or calibrations. Accreditations are usually given for specific combinations of analyte, matrix and method [302]. **Text box 14** provides some useful insights on why accreditation helps consumers, regulators and producers.



Text box 14: The importance of accreditation for different stakeholders

1.4.8. Data sharing

Current challenges highlight the extreme need for transparent information, including the provision of data generated worldwide. Several researchers mentioned that it will be important that the regulatory framework opens new possibilities for comprehensive data analysis in order to enable a simultaneous monitoring of hundreds of contaminants and residues in as many food and feed commodities as possible [126]. Pioneer in this area is the EFSA that promoted the principles of the open data charter [303]. EFSA has several mechanisms in place to allow data collection from all EU member states [304]. These data collections are the basis upon which the EFSA carries out the risk assessment processes.

As one of the initiatives from economies in transition, the Analytical Network of Latin America and the Caribbean (RALACA) has started focusing its work on the concept of data sharing based on the EFSA model [305]. Within this framework, a Data Sharing Committee (DSC) of RALACA is being established in Latin America and the Caribbean to collect and manage a repository of data related to food safety. The database will allow food safety authorities and decision makers to institute preventive or proactive measures to ensure that food is safe for consumption. The long-term objective of this initiative is to pave the way to the establishment and deployment of early warning systems for food safety in Latin America and the Caribbean, enabling countries to take preventative approaches, including the establishment of monitoring programs and management decisions based on risk-based evidence and assessments [304]. Sharing of food safety data among the countries in the region of LAC bears several benefits. It opens the possibility for carrying out secondary analysis (i.e. gap analysis) of data, enhances the efficiency and effectiveness in the management of food safety and strengthens south-south cooperation in the region. The scoping, recompilation and cataloguing of different types of data and information generated by different national institutions leads to optimized allocation of resources and ultimately improved data quality. Basic assumptions in the establishment of the regional database are that the data are timely, accurate, reliable and secure, and that rights, integrity and confidentiality are protected. Data should be shared in an aggregated manner, and in accordance with the institutional policies and international standards, e.g. ISO 17025:2017. In this context, the following benefits for data sharing for food safety are to be highlighted [307]:

- Maximization of the utility of datasets;
- Improvement of the data quality;

- Minimization of inequity in data access and reuse for secondary analysis;
- Aggregated data visualization as a basis for evidence-based decision making and the formulation of policies in food, agriculture and health;
- Improvement of the transparency of food safety information at the regional level;
- Increase of research and development (R&D) in the area of food safety;
- Improvement of the institutional position in the application for donor funding for food safety;
- Strengthened collaboration with like minded institutions;
- Increased visibility of work amongst regional food safety stakeholders.

The role of the RALACA-DSC is to facilitate the work of national institutions on scientific and practical matters related to the collection, analysis and reporting of data on arising from the results of chemical monitoring in food. Ultimately the national authorities analyse the data, and assess the results of monitoring programmes conducted by the countries and perform exposure assessment.

Ultimately data sharing increases data circulation and use within the scientific community by encouraging better transparency, enabling reproducibility of results, and informing the larger scientific community. This, in turn, can greatly benefit the public as better and more widely disseminated information can lead to informed decision making for food safety and planning and policy [308].

1.5. Work objectives of the thesis

The main objective of the thesis was to work towards the establishment and validation of processes and tools that combined in an innovative way help contribute to food safety and environmental sustainability. This thesis had the specific goal of focusing on minor crops, such as herbs and spices, for which very little analytical testing is implemented and for which there is scarce information. Ultimately the availability of analytical methods together with residue/contaminant baseline data shall contribute to the establishment of regulatory frameworks, under regulatory schemes such as CODEX.

Minor crops are typically of potential high-value according to trade markets. Challenges for this category of commodities is that crop protection technologies are lacking from application authorizations, good agricultural practices are less developed and most often regulations, if any, are country-specific and addressed at a relatively local level. Specifically, the generation

of data to obtain and maintain pesticide registrations, for example, for minor uses is costly and may not be justified by the economic returns on investments by pesticide manufacturers [309].

Therefore, the first specific objective was set for the development of the analytical tools, as described in this document, to ensure and help the verification of food safety for minor crops. The second specific objective was set for the provision of confidence in the generated results and methodologies that can be summarized in a “toolbox” and applied in analytical laboratories worldwide as a means of demonstrating confidence in the quality of analytical testing processes for minor crops. The specific work in this case was the setting up of design of experiment approaches to study the variability of the performance of the analytical methods once subjected to changes in the experimental conditions. This included a specific study on the subsampling step, which is essential to provide representativeness and confidence of the analytical results, and most often this is a neglected aspect of method validation studies.

The third specific objective was the verification that processes and tools are fit for purpose and correctly targeting food safety requirements in a risk-based environment, providing the necessary significance and confidence in the generated analytical data. Regulatory requirements for analyte/matrix combinations are scarce for minor crops. In the EU if a safe MRL cannot be recommended, or when an MRL has not been set, the default MRL is usually established at a general default MRL of 0.01 mg/kg. This brings additional challenges for the analytical method and for the testing laboratory. Therefore, a fit for purpose approach to target food safety needs to be verified in the case of minor crops.

1.6. Research questions

Research questions were targeted to different aspects of the processes and tools, specifically to the development, optimization, validation and verification of analytical methods as tools, but also considering processes for food safety. The need to investigate those aspects derived from a literature review that generally indicated that relatively few published papers related to monitoring of residues/contaminants in minor crops, and that legislation for this type of commodities is often scarce. In addition, analytical methods have not been sufficiently validated and, while robustness testing is generally a requirement for accreditation, laboratories have difficulties in providing objective evidence of robustness and ruggedness.

1.6.1. Optimization

Optimization work was considered as the first analytical challenge in this thesis in relation to instruments performance and sample preparation options. Will it be possible to apply optimization approaches to complicated matrices such as herbs and spices and prove acceptable analytical performance?

1.6.1.1. Instrumental optimization

To increase the scope of analytical methods it is necessary to adjust acquisition algorithms, since MRM-based targeted data acquisition is limited to the number of analytes that can be detected within one run. The research work targeted optimization of the number of contemporary transitions to define reasonable, detectable and confirmatory chromatographic peaks close to the LOQ of the method, while minimizing the overall measurement error.

1.6.1.2. Compensation of matrix effects

Minor crops have been analytically characterized much less than other matrices. This leads to unknown challenges in the analytical methods used for compliance testing. Co-extraction of matrix inherent components, especially secondary metabolites, some carbohydrates, proteins, and lipids may negatively contribute to the accurate quantitative analysis, reduce the lifetime of analytical columns, and increase maintenance of mass spectrometric instrumentation. These sample-dependent effects may cause a suppression or enhancement of the analyte response within MS systems. In order to reduce or compensate for these effects, research targeted novel combinations of sample clean-up, and calibration strategies.

1.6.1.3. Sample preparation development and optimization

Greening the analytical process is a current trend in analytical chemistry. In this thesis the selection of solvents and materials was carefully designed depending primarily on the nature

of the analytes, and the complexity of the extract, but also on the selectivity and the sensitivity of the analytical technique used for final instrumental determination of the target analytes. Undoubtedly, sample preparation is one of the most crucial steps to achieve low LOQs and a selective analyte detection. Optimization of the sample preparation, including the subsampling step, was fundamental to avoid the bottleneck represented by co-extraction of matrix components, thus affecting method detectability. Work was carried out to develop an affordable and rapid detection tool to verify the homogeneity of laboratory samples, using IMS.

1.6.2. Validation of analytical methods for minor crops

Routine food safety testing is carried out according to legislative requirements to detect chemical residues and contaminants that can occur naturally or accidentally during the food production process. Food safety testing is based on scientific knowledge of the critical points during the food production process combined with an understanding of the likelihood of natural and accidental contaminating agents in that food chain, for example through the use of HACCP (Hazard Analysis and Critical Control Points) principles [22]. Minor crops have been investigated very little. For the management of pests and diseases, farmers are dependent on available chemical pesticides that are registered to protect the target crop. Therefore, residues of pesticides, or other contaminants, are likely to exist. In some instances, pesticides that are not approved for use, are unscrupulously applied to the crops. Additional challenges accrue to the potential of detecting other organic contaminants that could potentially originate from the farm to fork chain, i.e. toxic dyes in spices, or persistent organic pollutants present in soil and translocated to edible parts of the crops by the plant itself. If undetected, those chemicals could affect the food safety and the health of consumers, export and domestic trade, and the ecosystem. Therefore, the analytical challenge was to increase the scope of testing to several classes of residues/contaminants likely to be applied on the crops.

The aim of this research objective was to develop and validate an easy, fast, and efficient method for the extraction and analysis of residues and contaminants in vine leaves, boldo and turmeric using liquid and gas chromatography coupled to tandem mass spectrometry. The methods were applied to the analysis of residues and contaminants in market samples.

1.6.3. Verification of fitness for purpose

To measure fitness for purpose it was important to address the challenge of ruggedness and robustness of the developed and validated methods. Essentially it was important to demonstrate that the measurement approaches were providing meaningful data under different measurement conditions. Research targeted two different commodities: a major and a minor crop.

1.6.4. Co-optimization of quality, safety and legislation

The research question was how to find the right balance between quality, safety and regulatory requirements. For example, controlled applications of chemicals (i.e. pesticides) will make crops uninteresting from the point of view of pests, and provide intact food crops at harvest, however potential residues of pesticide and/or their metabolites may compromise food safety in terms of cocktails of organic chemicals offered to the consumers. There is some contradiction in all this process and a balance need to be sought between social, economic and safety aspects. The research question focussed on the need to understand the dependence of compliance of residues of analytes to regulatory MRLs (safety aspects) as a function of the legislative framework chosen (economical aspects) in the best interest of the consumers (social aspects). In this sense, it was important to challenge the role of the analytical laboratory in the interpretation of chemical residues and contaminants, and its advisory role for the improvement of good agricultural practices (GAPs) towards better agricultural practices and improved food produce for the consumers.

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Chapter 2: Published papers in peer-reviewed journals

The following peer reviewed papers were published in the period 2018-2021

1. Maestroni, B., Vazquez, A.R., Avossa, V., P.Goos, V. Cesio, H. Heinzen, J. Riener, A. Cannavan, (2018). Ruggedness testing of an analytical method for pesticide residues in potato. Accreditation and Quality Assurance, 23, 303–316. Doi: 10.1007/s00769-018-1335-7

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5. Maestroni, B, Besil, N., Rezende, S., Liang, Y., Gerez, N., Karunarathna, N., Islam, M., Heinzen, H., Cannavan, A. and Cesio, M.V. (2021). Method optimization and validation for multi-class residue analysis in turmeric. Food Control, 121, 107579. Doi: 10.1016/j.foodcont.2020.107579

2.1 Ruggedness testing of an analytical method for pesticide residues in potato

This paper studied an often-neglected aspect of method validation being the initial ruggedness of the method. Lack of harmonization in current international guidelines for robustness or ruggedness of analytical methods lead to setup this study, to be able to provide analytical laboratories with options for carrying out an initial demonstration of the intra-laboratory ruggedness of the method.

The study focused on a validated multi residue method (24 pesticides) for a worldwide traded crop such as potato to identify design of experiments (DoE) that properly addressed "changing operation conditions" of the method.

Two DoE were applied: a Plackett Burman design (PBD) and an augmented definitive screening design (DSD). Both designs are useful and can be recommended for use in analytical laboratories worldwide. While the PBD is cheaper in terms of the number of replicates needed, the DSD provides more information to the analytical chemist, mainly in the identification of the quadratic effects. In practical/analytical terms, quadratic effects are a tool that helps identify which analytical parameters need to be under a strict control through adherence to prescriptions such as standard operating procedures. An example of this is demonstrated in this paper for diflufenicam and isofenphos, two analytes included in the scope of the method.

The main conclusion of this work was that DoE are important to be able to detect the "critical points" of a multiresidue method especially during initial method validation. The quality assurance and quality control program needs to incorporate such information, and be strict around the analytical implementation of the method under routine conditions.

Using either of these DoE the method was considered rugged, and 13 out of 24 analytes could "pass the test". These initial results hinted at the idea that if ruggedness can be proved for a minimum number of compounds, it means that the method itself is analytically rugged. Therefore, it was important to conduct an investigation to understand the reasons behind lack of ruggedness for certain analytes (cypermethyn, fenarimol, kresoxym methyl, pendimethalin, pyrimethanil, trifluralin) in defined matrices. In turn, this led to further studies on method validation procedures.

Accreditation and Quality Assurance
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PRACTITIONER'S REPORT

Ruggedness testing of an analytical method for pesticide residues in potato

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Abstract
The best demonstration of the ruggedness of a method is monitoring its performance on an ongoing basis as part of the analytical quality control applied in the laboratory. However, an initial demonstration of the ruggedness is often performed as one aspect of the method validation, to give confidence that the method should perform well under normal variations in conditions in routine application. This initial ruggedness testing is typically performed using either multiple replicate analyses or application of design of experiments (DoEs) which minimizes the number of analyses, time and effort required to detect influences on the measurement results. Two DoEs were applied for ruggedness testing for a modified QuEChERS multiresidue method for the detection of pesticide residues in potato by GC–MS/MS. Seven experimental factors were studied using an eight-run Plackett–Burman design replicated three times and an augmented definitive screening design with 34 runs. The relative effectiveness of the two approaches is discussed, in terms of their statistical significance, their cost-effectiveness and the richness of information they provide on the effects of the parameters investigated and the actual robustness of the method being tested.

Keywords Design of experiments · Plackett–Burman design · Definitive screening design · Method validation · Ruggedness and robustness testing

Introduction

Analytical laboratories need to demonstrate, through method validation, that a testing method is fit for the intended purpose. The validation shows that the method of analysis is capable of producing accurate, precise and reproducible results for the analytes of interest in the specified matrices. The data collected during method validation provide the basic evidence to support the validity of the results subsequently generated using the method for sample analysis. Ruggedness, sometimes termed robustness, is one of the performance criteria that need to be assessed as part of method validation.

There is currently no harmonization in the definitions of the terms robustness and ruggedness as applied to analytical methods. A review of current international guidelines shows that the terms are used either as synonyms or as complementary terms. Table 1 summarizes the definitions and requirements of some international bodies.

The Codex Alimentarius [1] defines the term ruggedness as given in Table 1, and it states that a test for ruggedness should be carried out by evaluating the intra-laboratory reproducibility of the method with small variations in the test conditions. If the changes introduced do not cause any significant effects on the results, the analyst can be confident that the method is "stable" and is expected to perform

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Table 1 Definitions and requirements adopted by some international bodies

Guideline	Term used/ defined	Definition/criteria	Comments
European Commission Guidance document SANTE/11813/2017 [2]	Robustness	Average recovery and RSDWR, derived from ongoing method validation/verification	Focus on application of quality control
Codex Alimentarius-Guideline document CAC-GL 90-2017 [26]	Ruggedness	The ruggedness of an analytical method is the resistance to change in the results produced by an analytical method when minor deviations are made from the experimental condition described in the procedure. The limits for experimental parameters should be prescribed in the method protocol, and such permissible deviations, separately or in any combination, should produce no meaningful change in the results produced. The aspects of the method that are likely to affect results should be identified, and their influence on method performance evaluated by using ruggedness tests	Changes in the instrument, operator, brand of reagent; concentration of a reagent; pH of a solution; temperature of a reaction; time allowed for completion of a process, and/or other pertinent factors
Document prepared for IUPAC [34], [35]	Robustness and Ruggedness	The relative robustness of an analytical method is defined as the ratio of the ideal signal for an uninfluenced method compared to the signal for a method subject to known and unknown operational parameters as studied in an intralaboratory experiment. The relative ruggedness of an analytical method is defined as the ratio of the ideal signal for an uninfluenced method compared to the signal for a method subject to known and unknown operational parameters as studied in an interlaboratory experiment"	
Eurachem document [4]	Ruggedness (robustness)	The 'ruggedness' ('robustness') of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. Ruggedness provides an indication of the method's reliability during normal usage. The ruggedness of a procedure must be established for in-house developed methods, methods adapted from the scientific literature and methods published by standardisation bodies used outside the scope specified in the standard method	Most effectively evaluated using experimental designs. E.g. 7 parameters can be studied in 8 experiments using a Plackett–Burman experimental design
International Accreditation Service guideline [3]	Ruggedness or Robustness	Ruggedness or robustness: The ability of a method to resist changes in test results when subjected to minor deviations in experimental conditions of the procedure. Ruggedness testing examines the behaviour of an analytical process when subtle small changes in the environment and/or operating conditions are made, similar to those likely to arise in different test environments	Subtle small changes in the environment and/or operating conditions

within its specified operating criteria almost all the time. With regard to the "small variations" introduced in the analytical method as part of robustness/ruggedness testing, the Codex refers to the concept of "permissible deviations" that should produce no meaningful change in the results. The Codex states that those deviation limits for experimental parameters should then be prescribed in the protocol of the method.

The SANTE guidance document on analytical quality control and method validation procedures for pesticide residues analysis in food and feed was conceived as a guidance document of the Commission Services that is

reviewed every 2 years by the EU reference laboratories [2]. In this document, the term robustness is mentioned without a clear definition. According to SANTE, the best demonstration of the ruggedness of a method is monitoring its performance on an ongoing basis as part of the analytical quality control applied in the laboratory.

This monitoring ensures that the performance remains within the parameters established during method validation and does not require a separate test for robustness, since the concept is intrinsic to the notion of quality control.

Both the International Accreditation Service [3] and the Eurachem laboratory guide to method validation [4] make reference to the terms robustness and ruggedness as if they are synonymous. The Eurachem guide, as given in Table 1, recommends testing for ruggedness using a Plackett–Burman experimental design and provides an indication of how to identify the variables that have a significant effect on method performance. In this paper, the concept of robustness and ruggedness as in the Eurachem laboratory guide was adopted, and the term "ruggedness" was used throughout. Ruggedness is defined as the intra-laboratory verification that the method applied will not generate any significant changes to the measurement results when slight modifications are made to the analytical method such as different instrumental and environmental conditions.

Ruggedness evaluation is considered as a part of the method validation process, prior to method transfer for further interlaboratory comparisons. Ruggedness verification is best based on the statistical evaluation using design of experiments (DoEs) approaches rather than the one-variable-at-a-time (OVAT) procedure. The latter involves varying individual factors one at a time, while keeping the rest of the factors fixed. The OVAT approach, even if logical and pragmatic, would involve too many experiments when the number of factors is large and is generally inefficient and ineffective [5]. On the other hand, DoE approaches vary all factors at the same time, under repeatability conditions. DoE has gradually gained importance as it maximizes the efficiency of scientific studies while minimizing costs [6]. A structured experimental design gives scientists the capability to model the measured outputs using empirical functions that can include linear, quadratic and interaction effects, of the experimental factors.

The traditional DoE approach is the one suggested by Youden and Steiner [7], and it is based on Plackett–Burman designs (PBDs; [8]). PBDs are screening designs that permit the study of many factors using a small number of testing combinations (runs). More specifically, PBDs are two-level designs in which each of the factors is set either at a low or at a high level. For this reason, PBDs are attractive options to identify the linear (main) effects of the factors as well as some two-factor interactions [9]. Alternatives to PBDs include two-level full factorial designs and two-level fractional factorial designs: These designs are reported in [9] and [10]. Other common DoE approaches are based on response surface designs. Response surface designs have three or more levels per factor and offer information on the quadratic effects of the factors, in addition to their linear effects and interactions. Response surface designs include the central composite designs and the Box–Behnken designs, which are reported in [11].

Several authors [6, 7, 12–16] provide detailed reviews on DoE approaches applied to ruggedness and robustness validation. These authors strongly recommend PBDs for robustness studies where a small deviation from method conditions is required and only linear effects are considered. Table 2 includes additional contributions for testing robustness and ruggedness based on DoE approaches. In general, much attention has been given to this issue within the analytical area for clinical and pharmacological studies while limited contributions are found in the area of contaminants testing. The study reported in this paper was developed to further expand the application of DoE to testing the intra-laboratory ruggedness of a multiresidue method for pesticide analysis in potato, a commodity consumed worldwide.

Recently, Jones and Nachtsheim [17] developed a new experimental design, called the definitive screening design (DSD), to study the effects of many quantitative factors using an economical number of observations. More specifically, the original DSD in [17] has three levels per factor and a number of runs that is one more than twice the number of factors. Therefore, a DSD offers information on the quadratic effects of the factors using a smaller number of runs than a central composite design and a Box–Behnken design. An attractive feature of DSDs is that they provide an efficient estimation of the factors' linear effects, independent of quadratic effects and two-factor interactions (referred to as second-order effects). Moreover, second-order effects are never fully correlated in a DSD. Recent applications of DSDs can be found in [18–25].

In this study, the ruggedness of an analytical method for pesticides in potato was tested by assessing the degree of intra-laboratory reproducibility of the method under deliberately introduced variations in the conditions of the test. To this end, a PBD and a DSD were chosen as they are relatively cheap to implement and provide substantial information to the analytical chemist on the sources of variability of an analytical procedure. The study was performed at the Food and Environmental Protection Laboratory (FEPL) to provide a step forward toward developing a "template" for a specific test to verify intra-laboratory ruggedness for an analytical method according to the Codex Alimentarius [26]. The comparison between the

Table 2 Specific contributions of design of experiments for testing robustness and ruggedness

References	Terms used	Comment/guideline	Example of DoE
[36]	Robustness	The first step in robustness analysis is to find the normal variation in the performance of the process equipment and to determine the **normal operating range**, and the **deviation** from the normal operating point for each process parameter	**Box–Behnken** design
[37]	Robustness/ ruggedness	The robustness/ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage (ICH 1994)	**Plackett–Burman** experimental design
[38]	Robustness/ ruggedness	Robustness of a procedure is determinated in order to estimate the influence of small fluctuations in analytical condition on the final result. Its value has bearing on the measuring technique regime for a given analytical procedure. As the influence of small changes in parameters on the final analytical result increases, it becomes mandatory to maintain those parameters at the constant level	**Plackett–Burman** experimental design
[39]	Robustness	Robustness is tested by introducing variations in experimental conditions and examining the effects on the results	**Plackett–Burman** experimental design
[40]	Ruggedness and Robustness	Robustness of a procedure is determinated in order to estimate the influence of small fluctuations in analytical condition on the final result. Its value has bearing on the measuring technique regime for a given analytical procedure. As the influence of small changes in parameters on the final analytical result increases, it becomes mandatory to maintain those parameters at the constant level. Ruggedness, on the other hands, is a parameter that defines the suitability of a given analytical procedure under varying conditions; it can be estimated from the reproducibility value	The robustness and ruggedness of analytical procedure are determinated via **interlaboratory comparisons**

PBD and the DSD showed that the DSD is an effective DoE approach for demonstrating ruggedness, if this is required to comply with quality requirements of analytical laboratories and by accreditation bodies.

Experimental

Materials

Solid powders of pesticide reference standards with the minimum purity of 96 % were provided by Sigma–Aldrich.

Solvents such as acetonitrile and ethyl acetate were of residue analysis grade and were obtained from VWR (Germany).

QuEChERS kits with sodium chloride (1.0 g), magnesium sulfate (4.0 g), sodium citrate (1.0 g), sodium hydrogen citrate sesquihydrate (0.5 g) and dispersive solid-phase extraction (SPE) tubes (15 mL), containing PSA (150 mg) and magnesium sulfate (900 mg), were obtained from Agilent Technologies (Santa Clara, USA).

Equipment

A gas chromatograph coupled to a mass spectrometer system equipped with an Agilent 7693 autosampler, 7890 B GC system and an Agilent 7000 C GC–MS/MS triple quadrupole system (Agilent Technologies, Santa Clara, USA) was used to analyze pesticide residues. Data acquisition and processing were implemented using Agilent Mass Hunter Quantitative Analysis B07.00 software.

Two 15.0 m×0.25 mm ID×0.25 μm HP-5 ms ultra-inert columns from Agilent Technologies were connected by an electronic pressure controller to enable a 5-min post-run backflush. The samples were injected using a multimode injector inlet in splitless mode through an ultra-inert inlet liner with a glass wool from Agilent. The injection volume was 3 μL. The injector port was programmed so that the initial injection temperature was kept at 70 °C for 0.02 min and then ramped up to 320 °C at 850 °C min^{-1}, hold for 5 min and then ramped down to 280 °C at 20 °C min^{-1}. Helium (99.999 % purity from Linde) was used as the carrier gas and nitrogen (99.999 % purity from Linde) as the collision gas. The oven temperature program consisted of a ramp from 70 °C (1 min hold) to 150 °C (at a rate of 50 °C min^{-1}), increased to 200 °C at 6 C min^{-1} followed by a final ramp to 280 °C at 16 °C min^{-1}. The total run time was 24 min. The transfer line was maintained at 280 °C and the ion source at 300 °C. The retention time lock setting (RTL) used chlorpyrifos-methyl as the locking compound at retention time of 10.83 min. The instrument worked at a constant flow of carrier gas (1.25 mL/min in

Column 1 and 1.45 mL/min in Column 2). Chlorpyrifos-methyl was injected daily for relocking the instrument method at the desired retention time and also for quality control purposes to check that the method was adequately performing during the analytical determination.

Cryogenic milling was carried out using liquid nitrogen (from Linde) using a Stephan UM12 food processor (Stephan Machinery GmbH, Germany).

A TurboVap LV concentration evaporator was obtained from Zymark (Biotage, Sweden). A centrifuge model 3–30 KS was from Sigma (Sigma Laborzentrifugen GmbH, Germany). An analytical balance model MC 410 S was from Sartorius (Sartorius Mikrowaagen, Germany). A pulsing mixer model vortex genie plus was from Scientific Industries (VWR, Germany). A − 20 °C freezer was from Liebherr (Germany).

Analytical conditions

A sample of potatoes was obtained from an organic farm in Austria. Adhering soil was carefully removed from each potato. Approximately 2 kg of potatoes was homogenized using cryogenic milling in a Stephan UM5 food processor. The bulk sample was stored in a freezer at a temperature of −20 °C until analysis. Analytical portions of 10 g were placed in a 50-mL Falcon tube. They were individually fortified with a mixture of pesticides with 200 µL of a mixture in ethyl acetate of 5 ng/µL and then immediately subjected to the analytical procedure.

For the selected pesticides, individual stock solutions were prepared at 1 mg/mL by weighing appropriate amounts of active ingredients into brown vials and diluting with pesticide residue grade ethyl acetate. Appropriate aliquots of stock solution were subsequently mixed into a 50-mL volumetric flask which was made up to volume with ethyl acetate to achieve a concentration of 5 ng/µL. This pesticide mixture, appropriately diluted, was used for spiking purposes and for the preparation of working calibration solutions.

Sample preparation

The European standard method for pesticide residues analysis is named EN 15662 QuEChERS 15662 (Quick, Easy, Cheap, Effective Robust and Selective). A modified QuEChERS method (see Fig. 1) was applied to analyze pesticide residues in analytical portions of potato [27]. Aliquots (10 g) of potato sample were fortified at 0.1 ng/µL with a pesticide mixture in 50-mL extraction tubes. The samples were left to rest for 20 min in a freezer at − 20 °C. Acetonitrile (10 mL), the triphenyl phosphate (TPP) and polichlor biphenyl 207 (PCB 207) prepared in acetonitrile were added to each sample to give 0.1 ng/µL. The conditions for the experimental factors and the coded

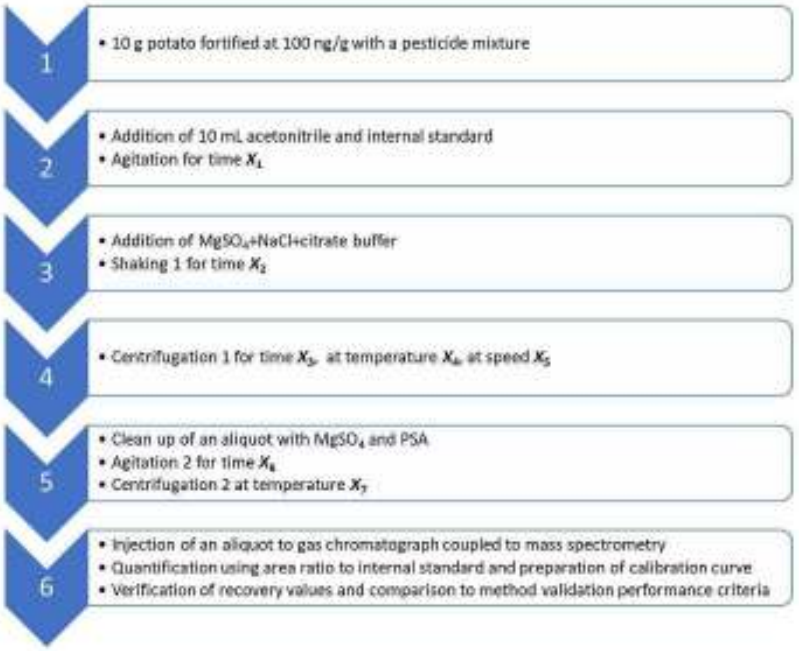

Fig. 1 Flowchart and description of the method factors

Table 3 The experimental factors of the method and the corresponding coded levels chosen for the ruggedness testing

Label	Factor	Coded levels		
		−1	0	+1
X_1	Agitation time (min)	20	30	40
X_2	Shaking time 1 (min)	2	5	8
X_3	Centrifuge temperature 1 (°C)	16	20	24
X_4	Centrifuge speed (*g*)	3743	6654	10397
X_5	Centrifuge time (min)	3	5	7
X_6	Shaking time 2 (min)	2	5	8
X_7	Centrifuge temperature 2 (°C)	16	20	24

levels as applied during the method are indicated in Table 3. Each Falcon tube was agitated for time X_1. The first salt mixture of the QuEChERS kits was added, and the Falcon tubes were shaken vigorously by hand for few seconds and then vortexed for time X_2. The tubes were centrifuged for time X_3, at temperature X_4 and at speed X_5. The cleanup step was a dispersive one with a mixture of different salts. An amount of 6 mL of supernatant was cleaned up using 15-mL Falcon tubes containing the second salt mixture primary and secondary amine (PSA) and magnesium sulfate. The cleanup tubes were shaken for time X_6 and then centrifuged at temperature X_7 for 5 min and at a centrifuge speed of 10397*g*. The clean extracts were then filtered through 0.2-µm microfilters and injected to a gas chromatograph coupled to a tandem mass spectrometry (GC–MS/MS) system.

Methods

Four-step methodology

The methodology applied in this study consisted of four steps. Step one defined the method and the factors to be studied. Step two was to identify an experimental design that was economically viable and easy to apply. Step three was to run the experiments according to the chosen design. Step four included the data analysis and inferring some conclusions about the ruggedness of the method.

Experimental factors

The factors and the limits to be studied for ruggedness were chosen according to their potential influence on the analytical method. Table 3 shows the experimental factors considered for evaluation. We considered the agitation time (X_1), shaking time 1 (X_2), centrifuge temperature 1 (X_3), centrifuge speed (X_4), centrifuge time (X_5), shaking time 2 (X_6) and centrifuge temperature 2 (X_7). The table shows the actual and coded levels used for the experimental designs. The limits were chosen according to the possible variations that may arise in analytical laboratories worldwide.

Experimental designs

For this study, two designs were compared: an eight-run Plackett–Burman design (PBD) replicated three times and a definitive screening design (DSD).

The eight-run PBD design was constructed for seven factors and replicated three times using the statistical software JMP 12 [28]. The final PBD design had 24 runs. Table 4a shows the eight distinct experimental conditions generated for this design.

The initial DSD considered was generated using JMP 12. To study the seven factors, the software generated a 17-run DSDs. A closer inspection of this design revealed that the correlation between the estimates for second-order effects (two-factor interactions and quadratic effects) can be large, which may hinder the detection of second-order effects. For this reason, an augmented DSD was constructed that minimized the correlations between estimates for second-order effects by concatenating two standard DSDs with 17 observations and eight factors. The procedure of Vazquez-Alcocer et al. [29] was used to concatenate the two copies of the DSD so as to minimize the correlations between the estimates of the second-order effects. The procedure to concatenate the designs was implemented in the software MATLAB [30]. The augmented DSD studied the seven experimental factors using 34 observations. The augmented DSD (from now on simply called DSD) improved the statistical properties of the standard DSD and is still economical when compared to a classical seven-factor central composite design or Box–Behnken design which would require at least 62 observations. Table 4b shows the experimental conditions for the final DSD. A comprehensive comparison between the PBD and the DSD is included in supplementary materials.

Conduct of the design

The experimental conditions given in Table 4 for the PBD and the DSD were applied to the QuEChERS method. The analysis for testing ruggedness was performed on selected representative pesticides given in Table 5. A three-level matrix-matched calibration curve, from 0.05 ng/µL to 0.15 ng/µL bracketing the 0.1 ng/µL spiked pesticide level, was used to quantify the pesticide residues in the potato extracts. Its linearity was evaluated through visual inspection, and the correlation coefficient was at least 0.99. Individual residue values of the pesticides from each

Table 4 Experimental design conditions for PBD (5a) and DSD (5b)

X_1 Agitation 1 time	X_2 Shaking time 1	X_3 Centrifuge 1 temperature	X_4 Centrifuge 1 speed	X_5 Centrifuge 1 time	X_6 Shaking 2 time	X_7 Centrifuge 2 temperature
(5a)						
+ 1	+ 1	+ 1	− 1	+ 1	− 1	− 1
− 1	+ 1	+ 1	+ 1	− 1	+ 1	− 1
− 1	− 1	+ 1	+ 1	+ 1	− 1	+ 1
+ 1	− 1	− 1	+ 1	+ 1	+ 1	− 1
− 1	+ 1	− 1	− 1	+ 1	+ 1	+ 1
+ 1	− 1	+ 1	− 1	− 1	+ 1	+ 1
+ 1	+ 1	− 1	+ 1	− 1	− 1	+ 1
− 1	− 1	− 1	− 1	− 1	− 1	− 1
X_1	X_2	X_3	X_4	X_5	X_6	X_7
Agitation 1 time	Shaking time 1	Centrifuge 1 temperature	Centrifuge 1 speed	Centrifuge 1 time	Shaking 2 time	Centrifuge 2 temperature
(5b)						
0	0	0	0	0	0	0
0	+ 1	+ 1	+ 1	+ 1	+ 1	+ 1
0	+ 1	+ 1	− 1	− 1	+ 1	+ 1
0	− 1	− 1	+ 1	+ 1	− 1	− 1
0	− 1	− 1	− 1	− 1	− 1	− 1
− 1	0	− 1	− 1	− 1	+ 1	+ 1
− 1	0	+ 1	− 1	+ 1	+ 1	− 1
+ 1	0	− 1	+ 1	− 1	− 1	+ 1
+ 1	0	+ 1	+ 1	+ 1	− 1	− 1
− 1	+ 1	0	+ 1	− 1	− 1	+ 1
+ 1	+ 1	0	+ 1	+ 1	+ 1	− 1
+ 1	− 1	0	− 1	+ 1	+ 1	− 1
− 1	− 1	0	− 1	− 1	− 1	+ 1
− 1	+ 1	− 1	0	+ 1	− 1	− 1
− 1	− 1	− 1	0	− 1	+ 1	− 1
+ 1	− 1	+ 1	0	− 1	+ 1	+ 1
+ 1	+ 1	+ 1	0	+ 1	− 1	+ 1
− 1	+ 1	+ 1	− 1	0	+ 1	− 1
− 1	+ 1	− 1	+ 1	0	+ 1	+ 1
+ 1	− 1	− 1	+ 1	0	− 1	+ 1
+ 1	− 1	+ 1	− 1	0	− 1	− 1
− 1	− 1	+ 1	+ 1	− 1	0	− 1
+ 1	+ 1	− 1	− 1	− 1	0	− 1
+ 1	+ 1	− 1	− 1	+ 1	0	+ 1
− 1	− 1	+ 1	+ 1	+ 1	0	+ 1
− 1	− 1	− 1	+ 1	+ 1	+ 1	0
+ 1	− 1	+ 1	+ 1	− 1	+ 1	0
+ 1	+ 1	+ 1	− 1	− 1	− 1	0
− 1	+ 1	− 1	− 1	+ 1	− 1	0
− 1	− 1	+ 1	− 1	+ 1	− 1	+ 1
+ 1	− 1	− 1	− 1	+ 1	+ 1	+ 1
+ 1	+ 1	− 1	+ 1	− 1	+ 1	− 1

Table 4 (continued)

X_1	X_2	X_3	X_4	X_5	X_6	X_7
− 1	+ 1	+ 1	+ 1	− 1	− 1	− 1
0	0	0	0	0	0	0

Level + 1 and − 1 are indicated in Table 3

experimental run were converted into recovery values and compared to the accepted Codex recovery range of 60 %–120 %. This ensured that each individual experimental data point was reliable and obtained under a quality controlled system.

Analysis of the experimental data

The experimental data were analyzed by finding the statistical models that best explained the results obtained from the analytical methodology. These results consisted of area values standardized through the internal standard. The statistical models identified contained the significant factor effects. For the PBD, models were built considering the intercept, seven linear effects and the 21 two-factor interactions. For the DSD, the intercept, the seven linear effects, the seven quadratic effects and the 21 two-factor interactions were considered. Higher-order interactions, like three-way interactions and cubic effects, are typically not relevant in practice and thus were assumed to be negligible. If the final model includes a two-factor interaction, then the average change in measurement results of a factor depends on the settings used for another factor.

Note that the PBD cannot estimate the model including the intercept, seven linear effects and the 21 two-factor interactions of the seven factors. This is because the number of distinct test combinations for the PBD is only 8 while the total number of effects in this model equals 29. The augmented DSD is also not able to estimate the model including the intercept, seven linear effects, the seven quadratic effects and the 21 two-factor interactions of the seven factors (a total of 36 effects) because it involves only 34 observations. However, in robustness and ruggedness studies, it is reasonable to assume that at most a few factor effects affect the measurement results significantly. That is, most likely, most of the effects will not be significant, making the true model estimable by the experimental design. This assumption is known as the effect sparsity principle (see Sect. 3.5 in [9]). As a result, to analyze the data from the two experimental designs, model selection techniques available in JMP 12 were used under the assumption that the effect sparsity principle would hold.

A suitable model for each pesticide was identified using the forward selection technique available in JMP 12. More specifically, the Bayesian information criterion (BIC) was used as the model selection criterion [29]. It is based, in part, on the likelihood function. The model with the lowest BIC is preferred. When fitting models, it is possible to increase the likelihood by adding parameters, but doing so may result in overfitting. The BIC ranks models according to their fitness to the data and the number of effects included, and so it chooses parsimonious models that explain the observed responses well. The search for the models containing the significant effects was restricted to only those that that satisfy the effect heredity principle (see section 3.5 in [9]) using the "Combine" rule in forward selection in JMP 12. The effect heredity principle stipulates that two-factor interactions can only be included in the model if the linear effects of the factors involved are already included. For quadratic effects, this principle implies that a factor's quadratic effect is eligible to enter to the model only when the linear effect of that factor is already included in the model. The effect heredity principle, for which there is empirical support as shown by the meta-analyses of [31], [32] and [33] to a large number of two-level factorial experiments and response surface experiments, respectively, helped to reduce the number of possible models under consideration substantially.

Results and discussion

The pesticides considered for this study belong to different chemical classes as given in Table 5. The results obtained using the PBD and the DSD provided substantial information on the sources of variability of the analytical procedure, as described earlier. Tables 6 and 7 present the effects appearing in the best model obtained for each pesticide when analyzing the data produced by the PBD and DSD, respectively.

Overall, for the subset of the 24 pesticides considered in this analysis, only vinclozolin did not show any significant effect with either the PBD or DSD method. PBD indicated no effects for 29 % and DSD for 33 % of the pesticides selected for this study. All other selected pesticides showed different significant effects or no effects at all (see Tables 6, 7). According to Table 6, the main effects identified through PBD were for the factors centrifuge speed (X_4) and shaking time 2 (X_6). A closer look at the most representative quadratic effects by DSD, as presented

Table 5 Pesticides selected for the robustness testing and their physicochemical characteristics

Pesticide	Chemical class	Solubility in water	Log Kow	Vapor pressure	Method validation recovery (%), n = 18	Within-laboratory reproducibility relative standard deviation (%)	CAS number
Aldrin	Organochlorine	0.027 mg/L at 27 °C	6.5	89.32 mPa at 20 °C	85	12	309-00-2
Benfluralin	2,6-Dinitroaniline	0.1 mg/L at 25 °C	5.29	8.7 mPa at 25 °C	96	20	1861-40-1
Boscalid	Anilide	4.6 mg/L at 20 °C	2.96	0.0072 mPa at 20 °C	78	9	188425-85-6
Cadusafos	Organophosphorus	245 mg/L at 25 °C	3.9	120 mPa at 25 °C	103	17	95465-99-9
Carbetamide	Amide	3500 mg/L at 20 °C	-1.6	8.4 mPa at 25 °C	79	15	16118-49-3
Chlorpropham	Carbamate	89 mg/L at 25 °C	3.51	24 mPa at 20 °C	95	20	101-21-3
Cyflufenamid	Amide	0.52 mg/L at 20 °C	1.7	0.035 mPa at 20 °C	85	15	180409-60-3
Cyfluthrin	Pyrethroid	0.003 mg/L at 20 °C	5.95	0.00002 mPa at 20 °C	58	13	68359-37-5
Cypermethrin	Pyrethroid	0.004 mg/L at 20 °C	6.6	0.00022 mPa at 20 °C	77	18	52315-07-8
Deltamethrin	Pyrethroid	0.002 mg/L at 25 °C	6.2	0.000012 mPa at 25 °C	117	8	52918-63-5
Diflufenican	Anilide	<0.05 mg/L at 25 °C	4.9	0.0043 mPa at 25 °C	82	12	83164-33-4
Endosulfan sulfate	Organochlorine	0.32 mg/L at 22 °C	3.83	0.023 mPa at 25 °C	69	18	1031-07-8
Ethalfluralin	2,6-Dinitroaniline	0.3 mg/L at 25 °C	5.11	11.73 mPa at 25 °C	95	20	55283-68-6
Fenarimol	Pyrimidine	13.7 mg/L at 25 °C (pH = 7)	3.69	0.065 mPa at 25 °C	77	7	60168-88-9
Isodrin	Organochlorine	0.014 mg/L at 25 °C	6.75	5.9 mPa at 25 °C	91	19	465-73-6
Isofenphos	Organophosphorus	22.1 mg/l at 20 °C	4.12	0.40 mPa at 25 °C	91	15	25311-71-1
Kresoxim-methyl	Strobin	2 mg/L at 20 °C	3.4	0.0023 mPa at 20 °C	83	15	143390-89-0
Metazachlor	Chloroacetanilide	430 mg/L at 20 °C	2.13	0.093 mPa at 20 °C	82	14	67129-08-2
Pendimethalin	2,6-Dinitroaniline	0.33 mg/L at 20 °C	5.2	1.25 mPa at 25 °C	80	12	40487-42-1
Pirimiphos-ethyl	Organophosphorus	2.3 mg/L at 25 °C	5	38.7 mPa at 25 °C	85	14	23505-41-1
Propyzamide	Amide	15 mg/L at 25 °C	3.1	< 0.001 hPa at 25 °C	80	19	23950-58-5
Pyrimethanil	Pyrimidine	0.121 g/l at 25 °C	2.84	2.2 mPa (25 °C);	79	15	53112-28-0
Trifluralin	2,6-Dinitroaniline	0.3 mg/L at 25 °C	5.34	13.7 mPa (25 °C);	92	20	1582-09-8
Vinclozolin	Dicarboximide	2.6 mg/L at 20 °C	3.1	0.13 mPa (20 °C);	81	17	50471-44-8

Table 6 Effects detected with PBD

Pesticide	Effects detected
Aldrin	None
Benfluralin	Centrifuge.Speed
Boscalid	Shaking.Time.1, Centrifuge.Temp.1, Shaking.Time.2, Shaking.Time.1:Centrifuge.Temp.1
Cadusafos	Centrifuge.Speed
Carbetamide	Centrifuge.Speed, Centrifuge.Time
Chlorpropham	Centrifuge.Speed
Cyflufenamid	Shaking.Time.2
Cyfluthrin	Agitation.Time, Agitation.Time, Centrifuge.Temp.1, Centrifuge.Time,Agitation.Time*Centrifuge.Time
Cypermethrin	Agitation.Time, Centrifuge.Temp.1, Agitation.Time*Centrifuge.Temp.1
Deltamethrin	Agitation.Time, Shaking.Time.1,Centrifuge.Temp.1,Centrifuge.Time, Shaking.Time1*Centrifuge.Temp.1
Diflufenican	None
Endosulfan sulfate	Agitation.Time, Shaking.Time.1, Centrifuge.Temp.1, Centrifuge.Time, Agitation.Time*Centrifuge.Time
Ethalfluralin	Centrifuge.Speed
Fenarimol	Shaking.Time.2
Isodrin	None
Isofenphos	None
Kresoxim-methyl	Shaking.Time.2
Metazachlor	Shaking.Time.2
Pendimethalin	Shaking.Time.2
Pirimiphos-ethyl	None
Propyzamide	None
Pyrimethanil	Centrifuge.Speed
Trifluralin	Centrifuge.Speed
Vinclozolin	None

The intercept is included in all models. Labels like Agitation.Time*Centrifuge.Time denote interactions

in Table 7, indicates that the effect for the factor centrifuge T 1 (X_3) was significant for five pesticides and the effects for the factors centrifuge time (X_5) and shaking time 1 (X_2) were significant for four pesticides each. The most frequent significant second-order effect in both PBD and DSD was the two-factor interaction between the factors agitation time and centrifuge time (denoted as X_1*X_5). The results in Tables 6 and 7 show that the factors chosen for the ruggedness testing were important as they affected the analytical method. In addition, for those pesticides having a log Kow lower than 4, such as boscalid, cyflufenamid, fenarimol, kresoxim-methyl and metazachlor, the effects identified by the PBD and the DSD are mainly related to the "shaking" time. This effect can be explained by the chemical partitioning taking place between the water phase and the organic phase in the extraction phase during shaking. The statistical approach is able to identify shaking time as a critical factor that needs to be accounted for during method development. On the other hand, for those compounds having a log Kow higher than 4 the effects for the PBD and the DSD are mainly related to the centrifugation conditions, indicating that evaporation of solvent and concentration effects could play a major role for those pesticides.

Table 6 shows that aldrin, diflufenican, isodrin, isofenphos, pirimiphos-ethyl and propyzamide were six pesticides that did not show any significant effects using the PBD. However, Table 7 indicates that these pesticides showed significant quadratic effects using the DSD. To illustrate this issue, consider the pesticides diflufenican and isofenphos. Figure 2 shows the area values for these pesticides versus the settings of factor X_5, centrifuge time. Due to the DSD, there are three levels (settings) for this factor, a middle (5 min), upper (7 min) and lower (3 min) level. The figure shows the average change (curve line) in the area values of diflufenican and isofenphos as a change in the settings of centrifuge time. The figure suggests a quadratic relationship between this factor and both pesticides. Tables 8 and 9 show the t tests for the effects identified by the DSD for diflufenican and isofenphos, respectively. t tests for the other pesticides are included in supplementary materials. The tables show that the quadratic effect of the centrifuge time is significant at a level of $\alpha = 0.05$ for both pesticides. It is interesting to mention that Table 8 shows that the quadratic

Table 7 Effects detected with DSD

Pesticide	Effects detected
Aldrin	Agitation.Time, Centrifuge.Temp.1, Shaking.Time.2, **Centrifuge.Temp.1*Centrifuge.Temp.1**, Agitation.Time*Shaking.Time.2
Benfluralin	Agitation.Time
Boscalid	None
Cadusafos	None
Carbetamide	Centrifugue.Temp.1
Chlorpropham	None
Cyflufenamid	Shaking.Time.1, **Shaking.Time.1*Shaking.Time.1**
Cyfluthrin	None
Cypermethrin	Centrifuge.Temp.1, Centrifuge.Speed, **Centrifuge.Temp.1*Centrifuge.Temp.1**, Centrifuge.Temp.1*Centrifuge.Speed
Deltamethrin	None
Diflufenican	Agitation.Time, Shaking.Time.1, Centrifugue.Time, Shaking.Time.2, Centrifuge.Temp.2, Agitation.Time.Shaking.Time.1, Agitation.Time*Centrifuge.Time, Shaking.Time.1*Centrifuge.Time, **Centrifuge.Time*Centrifuge.Time**, Shaking.Time.1*Centrifuge.Temp.2
Endosulfan sulfate	None
Ethalfluralin	None
Fenarimol	Agitation.Time, Shaking.Time.1, Centrifuge.Time, Agitation.Time*Centrifuge.Time, Shaking.Time.1*Centrifuge.Time
Isodrin	Agitation.Time, Shaking.Time.1, Centrifuge.Temp.1, Centrifuge.Time, Shaking.Time.2, Centrifuge.Temp.2, **Shaking.Time.1*Shaking.Time.1**, **Centrifuge.Temp.1*Centrifuge.Temp.1**, Agitation.Time*Centrifuge.Time, Shaking.Time.1*Centrifuge.Time, Centrifuge.Temp.1*Centrifuge.Time, Agitation.Time*Shaking.Time.2, Shaking.Time.1*Centrifuge.Temp.2
Isofenphos	Agitation.Time, Centrifuge.Temp.1, Centrifuge.Speed, Centrifuge.Time, Shaking.Time.2, Agitation.Time*Centrifuge.Temp.1, **Centrifuge.Temp.1*Centrifuge.Temp.1**, **Centrifuge.Speed*Centrifuge.Speed**, Centrifuge.Speed*Centrifuge.Time, **Centrifuge.Time*Centrifuge.Time**, Centrifuge.Temp.1*Shaking.Time.2, **Shaking.Time.2*Shaking.Time.2**
Kresoxim-methyl	Agitation.Time, Shaking.Time.1, Centrifuge.Temp.1, Centrifuge.Time, Centrifuge.Temp.2, **Centrifuge.Temp.1*Centrifuge.Temp.1**, Agitation.Time*Centrifuge.Time, Shaking.Time.1*Centrifuge.Time, **Centrifuge.Time*Centrifuge.Time**
Metazachlor	Agitation.Time, Shaking.Time.1, Centrifuge.Temp.1, **Shaking.Time.1*Shaking.Time.1**, Agitation.Time*Centrifuge.Temp.1, Shaking.Time.1*Centrifuge.Temp.1, **Centrifuge.Temp.1*Centrifuge.Temp.1**
Pendimethalin	Shaking.Time.1, Centrifuge.Temp.1, Shaking.Time.2, **Shaking.Time.1*Shaking.Time.1**, Shaking.Time.1*Centrifuge.Temp.1, Shaking.Time.1*Centrifuge.Speed, Centrifuge.Temp.1*Centrifuge.Speed, **Shaking.Time.2*Shaking.Time.2**
Pirimiphos-ethyl	Agitation.Time, Shaking.Time.1, Centrifuge.Temp.1, Centrifuge.Time, Shaking.Time.2, Centrifuge.Temp.2, **Agitation.Time*Agitation.Time**, Agitation.Time*Centrifuge.Time, Shaking.Time.1*Centrifuge.Time, Shaking.Time.1*Shaking.Time.2, Centrifuge.Time*Shaking.Time.2, Centrifuge.Temp.1*Centrifuge.Temp.2
Propyzamide	Centrifuge.Time, **Centrifuge.Time*Centrifuge.Time**
Pyrimethanil	Agitation.Time
Trifluralin	Agitation.Time
Vinclozolin	No

The intercept is included in all models. Labels like **Agitation.Time*Agitation.Time** denote quadratic effects, and labels like Agitation.Time*Shaking.Time.2 denote interactions

effects of factors X_3 (centrifuge temperature 1) and X_6 (shaking time 2) are significant and borderline significant at a level of $\alpha = 0.05$, respectively. In contrast to the DSD, the PBD did not allow to detect quadratic effects as it only involves two levels per factor. Thus, the information of each factor at its middle level is missed with the PBD. Based on the models identified by the DSD, it is very important in the practical work in the laboratory to be aware of those quadratic effects and make sure that quality assurance and quality control (QA/QC) measures are applied. This is an example of how a statistical result can indicate the necessity to implement a strict control of the method, for example,

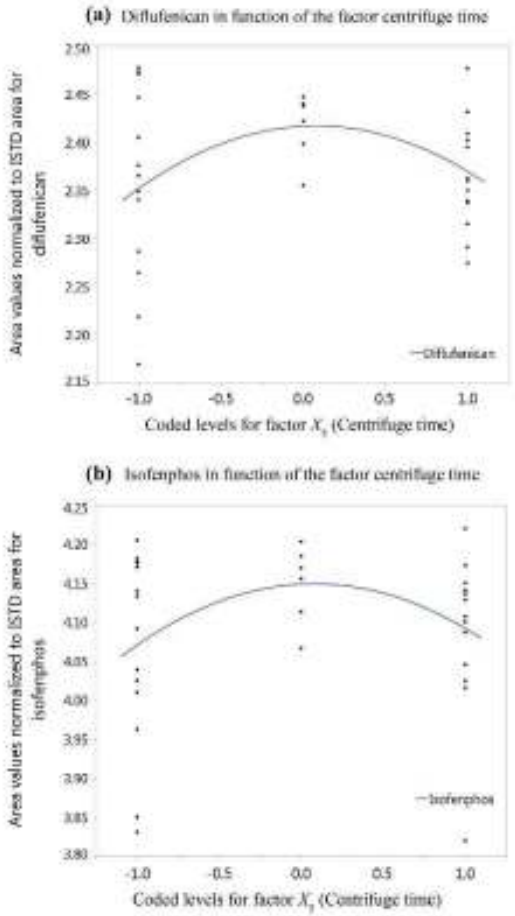

Fig. 2 Quadratic effect of centrifuge time in diflufenican and isofenphos using DSD. The standardized to ISTD area values are shown at different centrifuge times corresponding to 3 min (level − 1) and 7 min (level + 1). Coded level 0 for centrifuge time 1 corresponds to 5 min. Curve line: average change in the area values as a change in the centrifuge time. **a** Diflufenican in function of the factor centrifuge time. **b** Isofenphos in function of the factor centrifuge time

through adherence to a standard operating procedure where the limits of the parameters are clearly indicated.

The difference between the effects highlighted by the PBD and DSD can be explained by: (1) the type of second-order effects these designs can account for and (2) properties of the designs. If only the linear effects of the factors had been present, both designs would have identified the same effects because they are highly efficient for detecting main effects (see supplementary materials). However, some pesticides reported significant second-order effects. Since the PBD involves two levels per factor, it allows to explain the data using linear effects and two-factor interactions only. The DSD involves three levels per factor and permits to study quadratic effects, which increases the possible ways in which the data can be interpreted.

Supplementary materials show that the DSD and the PBD provide pairs of effects that are correlated. The correlation, or multicollinearity, in the PBD is more severe than in the DSD. For instance, in the PBD, some linear effects are fully correlated (confounded) with the two-factor interactions. Therefore, the PBD cannot estimate both effects at the same time. The practical implication of this perfect correlation is that a two-factor interaction reported in Table 6 may be significant because it is fully confounded with a significant linear effect. Since the PBD cannot disentangle the two effects involved, it is unknown whether either the linear effect or the two-factor interaction is significant. In contrast to the PBD, the information for the linear effects is much richer in the DSD since these effects are not correlated with the two-factor interactions nor with the quadratic effects. This feature of the DSD permitted a more reliable identification of the significant linear effects in Table 7. A comprehensive comparison between the properties of the PBD and the DSD is included in supplementary materials.

Conclusions

Ruggedness testing yields useful information concerning the performance of an analytical method under changing operating conditions. The best demonstration of the ruggedness of a method is monitoring its performance on an ongoing basis as part of the analytical quality control applied in the laboratory. This ensures that the performance remains within the parameters established during method validation under natural variations that can occur on a day-to-day basis like different operators, different batches of reagents, slight variations in working practices, temperature, etc. However, if a laboratory needs an additional test to verify the ruggedness of the method, then design of experiments is an attractive statistical approach which is relatively cheap and easy to apply.

Using either of the design of experiment approaches, as shown in this paper, the analytical method was considered statistically robust for 13 out of the 24 representative pesticides tested. Two experimental designs were applied, a replicated Plackett–Burman design (PBD) and an augmented definitive screening design (DSD), to compare the effects of seven experimental factors of a pesticide residue method for potato. The DSD was more expensive as it required 34 experimental runs compared to 24 runs in the replicated PBD. However, the PBD provided fully

Table 8 *t* tests for the effects detected with DSD for diflufenican

Term	Estimate	SE	t ratio	Prob > |t|
Intercept	2.429	0.021	113.27	<.0001*
Agitation.Time	0.003	0.009	0.35	0.7287
Shaking.Time.1	0.028	0.009	3.02	0.0061*
Centrifuge.Time	0.009	0.009	0.94	0.3548
Shaking.Time.2	− 0.018	0.009	− 1.94	0.0651
Centrifuge.Temp.2	− 0.007	0.009	− 0.79	0.4366
Agitation.Time*Shaking.Time.1	0.019	0.011	1.73	0.0965
Agitation.Time*Centrifuge.Time	0.055	0.011	5.13	<.0001*
Shaking.Time.1*Centrifuge.Time	− 0.027	0.010	− 2.58	0.0168*
Centrifuge.Time*Centrifuge.Time	− 0.071	0.024	− 2.97	0.0069*
Shaking.Time.1*Centrifuge.Temp.2	− 0.025	0.011	− 2.30	0.0306*

Labels like **Centrifuge.Time*Centrifuge.Time** denote quadratic effects, and labels like Shaking.Time.1*Centrifuge.Temp.2 denote interactions. Significant effects at a level of $\alpha = 0.05$ are marked with an asterisk. Nonsignificant linear effects are included because of the restriction to models following effect heredity

Table 9 *t* tests for the effects detected with DSD for Isofenphos

Term	Estimate	SE	t ratio	Prob > |t|
Intercept	4.180	0.031	134.25	<.0001*
Agitation.Time	0.027	0.010	2.82	0.0103*
Centrifuge.Temp.1	− 0.017	0.010	− 1.73	0.0980
Centrifuge.Speed	− 0.008	0.010	− 0.80	0.4355
Centrifuge.Time	0.011	0.010	1.12	0.2773
Shaking.Time.2	0.017	0.010	1.82	0.0823
Agitation.Time*Centrifuge.Temp.1	− 0.082	0.014	− 5.75	<.0001*
Centrifuge.Temp.1*Centrifuge.Temp.1	− 0.047	0.024	− 2.00	0.0589
Centrifuge.Speed*Centrifuge.Speed	− 0.081	0.028	− 2.94	0.0077*
Centrifuge.Speed*Centrifuge.Time	0.088	0.011	7.70	<.0001*
Centrifuge.Time*Centrifuge.Time	− 0.058	0.024	− 2.46	0.0224*
Centrifuge.Temp.1*Shaking.Time.2	− 0.062	0.011	− 5.83	<.0001*
Shaking.Time.2*Shaking.Time.2	0.082	0.028	2.97	0.0074*

Labels like **Shaking.Time.2*Shaking.Time.2** denote quadratic effects, and labels like Centrifuge.Temp.1*Shaking.Time.2 denote interactions. Significant effects at a level of $\alpha = 0.05$ are marked with an asterisk. Nonsignificant linear effects are included because of the restriction to models following effect heredity

confounded effects; in other words, the variables being studied were affected so that the results did not reflect the actual relationship and that made the data analysis cumbersome compared to the DSD. In addition to main and two-factor interaction, the DSD also provided an indication of quadratic effects that are important to be aware of, especially in the context of method development. The DSD was slightly more expensive. It is interesting to mention that alternative DoE approaches to detect quadratic effects such as the Box–Behnken and central composite designs would have required at least 62 experimental runs.

Knowing the type of effect caused by a variation in conditions is very important in order to adequately control the analytical procedure. To this end, design of experiments can provide different information and it is a responsibility of the analytical practitioner to know which design fits the purpose. The results in this study suggest that the DSD is an attractive option to test for ruggedness. Finally, to comply with specific requirements of quality requirements, this study can be considered as an example that can be modified according to the purpose, of a specific test to verify intra-laboratory ruggedness for an analytical method according to the Codex Alimentarius guidelines and can be easily applied in analytical laboratories worldwide.

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2.2 Validation of an Analytical Method for the Determination of Pesticide Residues in Vine Leaves by GC-MS/MS

This paper addresses the validation of a residue method at trace levels for vine leaves, which are considered a minor crop. The availability of a validated and published method represents an important step towards the protection of local consumers, if sufficient monitoring programs are in place. In addition, it serves the purpose of providing a tool to the local farmers through the local analytical laboratory, to help gain market access for export of their certified produce, if local trade is consistent with food safety principles.

Vine leaves are a secondary product of grapevine cultivation, grapes being the main crop. In terms of circular economy, this is a great example of how everything is being used up and nothing goes unused, under the condition that, in conventional agriculture, the pesticides applied to grapevines are those strictly registered for use on grapes, and that the withholding periods for vine leaves are applied, so that “good agriculture practice” can be claimed for vine leaves too.

The method was validated for 54-59 residues of pesticides in the range 0.01-0.1 mg/kg in fresh vine leaves using GC-MS/MS. From an analytical point of view, it is important to recognize that matrix effects in vine leaves are very strong and need to be compensated for, requiring the use of matrix matching strategies. This is a very important aspect to be aware of as it could lead to very biased analytical results, resulting in enhanced or reduced results, depending on the compound.

The paper also emphasizes the need to carry out validation of the method, possibly according to the Codex Alimentarius Guidelines, which provides a very good idea of the method performance under repeatability and within laboratory reproducibility conditions.

Proficiency testing for vine leaves is unfortunately not available to date; however regional collaborations in the Middle East are starting to take place and this represents a positive outcome of FAO/IAEA and GACT studies on minor crops.

Having a validated multi residue method for pesticides in vine leaves was an important step and essential tool towards the comprehension of pesticide residues decay in vine leaves matrix.

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Validation of an Analytical Method for the Determination of Pesticide Residues in Vine Leaves by GC-MS/MS

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Supporting Information

ABSTRACT: A modified and miniaturized SweEt/QuEChERS method for pesticide residue analysis applied to vine leaves is presented. The deep-frozen plant material was cryogenically processed. A 2 g analytical portion was hydrated for 30 min and extracted with acidified ethyl acetate after buffering with $NaHCO_3$ and adding Na_2SO_4. A dispersive solid-phase (d-SPE) cleanup step with primary−secondary amine (PSA) was performed. The pesticide residues were determined using GC-MS/MS. The whole procedure was validated for 54−59 pesticides at 0.01, 0.02, 0.1, and 0.2 mg/kg in fresh vine leaves (*Vitis vinifera*). The key method performance parameters investigated were specificity, linearity, trueness, within laboratory repeatability and reproducibility, limit of detection, limit of quantitation, and matrix effects. Recoveries for the 59 pesticides tested ranged from 60 to 110%, and the RSDs were lower than 20% for the majority of the pesticides studied.

KEYWORDS: *pesticide residues, method validation, vine leaves, GC-MS/MS analysis*

INTRODUCTION

Grape (*Vitis vinifera*) is the most widely cultivated fruit crop in the world, with an estimated cultivation surface area of 7.5 million hectares in 2014.[1] It is not only grapes that are of economic importance; vine leaves have also been used as a nutritious food in Greece and the Middle East for centuries and their popularity as a healthy food is increasing globally. As an example, the United States of America's Department of Health and Human Services lists vine leaves in the dietary guidelines for healthy food.[2] It has been reported that extract of red vine leaves can improve the symptoms of chronic venous insufficiency (CVI) and may prevent CVI deterioration; moreover, it might also be effective in the treatment of impaired microcirculation related to other diseases such as diabetes mellitus and Raynaud's Syndrome.[3] The analysis of vine leaves for pesticide residues is often part of monitoring and surveillance projects in order to verify good agricultural practices related to specific regulatory requirements. Therefore, it is important to set up, optimize, and validate methods of analysis for vine leaves as a separate commodity in addition to grapes.

Grapevines are susceptible to infection by fungi such as gray rot (*Botrytis cinerea*), powdery mildew (*Uncinula necator*), downy mildew (*Plasmopora viticola*), and black mold (*Aspergillus niger*) and to attack by insects such as grape fruit worm (*Eudemis botrana*), vine mealybug (*Planococcus citri*), and European grapevine moth (*Lobesia botrana*).[4] To protect the vines, farmers apply a range of regulated pesticide formulations, especially fungicides and insecticides, which can sometimes leave residues on or in the crop.[5−7] Grimalt et al.[7] presents an extensive overview of analytical methods for the determination of pesticide residues in grapes. However, there is very limited published literature available on validated multiresidue methods for pesticide residues on vine leaves. Pihlström et al.[8] reported the analysis of pesticide residues in food using ethyl acetate extraction and detection with gas or liquid chromatography coupled to tandem mass spectrometry (SweEt method) and gave an example of the EU import control of vine leaves from Turkey. The SweET method included 145 GC amenable analytes. Residues of 40 pesticides were found, 17 of which exceeded the maximum residue limit for EU enforcement control. The SweEt method was employed for the analysis of pesticide residues in vine leaves and gave good results in an interlaboratory comparison with European institutes.[8] Arora et al.[9] present a single residue method for residues of imidacloprid in vine leaves using acetonitrile extraction and HPLC determination. Dissipation curves for azoxystrobin, fenhexamid, and lufenuron in vine leaves and berries using separate single residue methods for the pesticides were evaluated.[9] Single residue methods have been quite extensively used, mainly by crop protection companies in their evaluations for formulation approvals.[10]

Guidance for laboratories involved in the control of pesticide residues analysis in food and feed has been set out, inter alia, by the Codex Alimentarius and the EU (SANTE/11813/

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Table 1. List of Pesticides, Their Retention Times, Transitions Used for Quantitation and Confirmation, Together with the Optimized Collision Energies (CE) and the Corresponding Retention Time Windows in the Multiple Reaction Monitoring (MRM) Acquisition Method

pesticides	MRM time segment	RT (min)	quantifier transition 1	qualifier transition 2	CE 1 (V)	CE 2 (V)
amphos-methyl	21	16.2	160.0 → 77.1	132.0 → 77.1	20	15
azoxystrobin	25	20.9	344.1 → 329.0	344.1 → 171.9	15	40
benalaxyl	18	14.9	148.0 → 77.0	148.0 → 105.1	20	20
bifenthrin	20	15.7	181.2 → 165.2	181.2 → 166.2	25	10
boscalid	22	18.1	140.0 → 112.0	140.0 → 76.0	10	25
bromopropylate	18	15.8	338.8 → 182.9	340.8 → 184.9	15	20
cadusafos	4	8.0	158.8 → 97.0	158.8 → 131.0	15	5
chlorpyrifos	11	12.0	196.9 → 169.0	198.9 → 171.0	15	15
chlorpyrifos-methy.	8	10.8	285.9 → 92.9	287.9 → 92.9	25	25
cyfluthrin beta	22	17.9	162.9 → 127.0	162.9 → 90.9	25	15
cypermethrin fam.	22	18.0	163.0 → 127.0	164.9 → 91.0	10	10
cyprodinil	12	12.6	225.2 → 224.3	224.2 → 208.2	10	20
deltamethrin	24	20.4	252.9 → 93.0	181.0 → 152.1	15	35
diazinon	6	9.5	137.1 → 84.0	137.1 → 54.0	10	20
dichlofluanid	10	11.7	123.0 → 77.1	223.9 → 123.1	20	10
dimethoate	4	8.6	86.9 → 46.0	92.9 → 63.0	15	10
dimethomorph	25	21.1	300.9 → 165.0	302.9 → 164.9	20	10
endosulfan alpha	15	13.4	194.9 → 160.0	194.9 → 125.0	5	20
endosulfan beta	17	14.5	206.9 → 172.0	194.9 → 158.9	15	10
ethoxyquin	4	8.6	202.1 → 174.1	202.1 → 145.1	15	30
etrimfos	7	10.0	181.0 → 153.1	291.9 → 181.0	5	5
fenamidone	20	15.9	238.0 → 237.2	268.0 → 77.1	10	35
fenarimol	21	16.6	139.0 → 75.0	219.0 → 107.1	30	10
fenhexamid	18	15.0	177.1 → 78.0	177.1 → 115.0	10	15
fenpropathrin	20	15.9	264.9 → 210.0	181.1 → 152.1	10	25
fenchlon	8	9.9	124.9 → 47.0	124.9 → 79.0	10	5
fludioxonil	16	13.8	248.0 → 127.1	248.0 → 154.1	30	20
flusilazole	17	14.0	233.0 → 165.1	233.0 → 91.0	15	20
hexachlorobenzene	6	9.2	248.9 → 214.0	248.9 → 179.0	15	30
hexaconazole	16	13.6	231.0 → 175.0	231.0 → 111.0	10	30
indoxacarb	24	20.3	202.9 → 77.9	218.0 → 202.9	10	10
iprodione	19	15.6	314.0 → 56.0	314.0 → 245.0	20	10
iprovalicarb	17	13.8	116.0 → 98.1	119.1 → 77.1	15	20
isocarbophos	11	12.2	135.9 → 108.0	121.0 → 65.1	10	15
kresoxim-methyl	18	14.1	116.0 → 89.0	116.0 → 63.0	15	30
malathion	10	11.8	126.9 → 99.0	172.9 → 99.0	5	15
mepanipyrim	15	13.4	223.2 → 222.2	222.2 → 207.2	10	15
metalaxyl	9	11.2	234.0 → 146.1	206.0 → 132.0	20	20
methidathion	14	13.2	144.9 → 85.0	144.9 → 58.1	5	15
myclobutanil	17	14.0	179.0 → 125.1	179.0 → 90.0	10	30
omethoate	2	7.1	155.9 → 79.0	155.9 → 110.0	5	5
parathion-methyl	8	10.8	125.0 → 47.0	125.0 → 79.0	10	5
phosalone	21	16.2	182.0 → 111.0	182.0 → 75.1	15	30
phthalimide	1	5.6	147.0 → 76.1	147.0 → 103.1	25	5
procymidone	14	13.1	282.8 → 96.0	284.8 → 96.0	10	10
profenofos	16	13.8	207.9 → 63.0	338.8 → 268.7	30	15
pyraclostrobin	23	19.5	132.0 → 77.1	164.0 → 132.1	20	10
pyrimethanil	6	9.5	198.0 → 118.1	198.0 → 183.1	35	15
quinalphos	14	12.9	157.0 → 102.0	146.0 → 118.0	15	10
quintozen	6	9.2	248.8 → 214.0	294.8 → 237.0	15	20
spiroxamine	8	10.8	198.0 → 126.1	100.0 → 58.1	10	10
tebuconazole	18	15.2	125.0 → 99.0	250.0 → 125.0	20	20
tefluthrin, cis-	7	9.8	177.1 → 127.1	177.1 → 87.0	15	30
terbufos	6	9.2	230.9 → 175.0	230.9 → 129.0	20	20
tetradifon	20	16.1	158.9 → 111.0	226.9 → 199.0	10	15
thiabendazole	13	13.0	201.0 → 174.0	128.9 → 102.0	10	10
triadimefon	11	12.1	208.0 → 181.1	208.0 → 111.0	5	20
trifloxystrobin	16	14.1	116.0 → 89.0	116.0 → 63.0	30	30
zoxamide	16	13.9	257.9 → 187.1	187.0 → 159.1	15	15

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Table 1. continued

pesticides	MRM time segment	RT (min)	quantifier transition 1	qualifier transition 2	CE 1 (V)	CE 2 (V)
Internal Standards						
sulfotep	3	8.0	201.8 → 145.9	201.8 → 81.9	10	25
triphenyl phosphate	19	15.3	326.0 → 325.0	214.9 → 168.1	5	15

2017).[11,12] A major requirement of the guidelines in the frame of an ISO 17.025 quality assurance environment is that an analytical method for a given matrix and specified pesticide residues must be validated. The aim of this study was to validate a multiresidue method for selected pesticide residues in vine leaves according to the Codex Alimentarius Guidelines on Good Laboratory Practice in Pesticide Residue Analysis.[11]

MATERIALS AND METHODS

Chemicals. All water used in this study was obtained from an in-house Milli Q purification system (Merck Chemicals and Life Science, Vienna, Austria). Certified pesticide analytical standards were purchased from Sigma-Aldrich, (Vienna, Austria). Stock solutions of individual standards were prepared at 10 mg/mL and used in the preparation of a mixed stock solution at 25 ng/μL. The individual stock solution and the stock mixture were stored in amber screw-capped vials with septa in the dark at −20 °C. Working standard solutions were prepared from the mixed stock solution according to requirements. Residue grade glacial acetic acid and ethyl acetate were purchased from Merck (Vienna, Austria), and ascorbic acid was purchased from Sigma-Aldrich (Vienna, Austria).

Acidified ethyl acetate was prepared by dissolving ascorbic acid (0.5 g) in Milli Q water (10 mL), to which glacial acetic acid (10 mL) was added and the volume made up to 1 L with ethyl acetate. The solution was stored in a screw-capped bottle with septum, wrapped with aluminum foil, and kept in the dark at 4 °C.

Residue grade sodium hydrogen carbonate ($NaHCO_3$), anhydrous sodium sulfate (Na_2SO_4), and anhydrous magnesium sulfate ($MgSO_4$) were purchased from Sigma-Aldrich (Austria). Residue grade primary-secondary amine (PSA) sorbent was purchased from Varian.

Sample Extraction. Fresh vine leaves were obtained from a vineyard in Syria and stored at −20 °C until analysis. The leaves were homogenized cryogenically with liquid nitrogen in a UM 5 Stephan Chopper (Stephan, Hameln, Germany). Analytical portions of 2 g of homogenized vine leaves were weighed into 50 mL labeled PTFE centrifuge tubes. The analytical portions were individually fortified with the required amount of pesticide mixture (refer to section on Validation Procedure) and kept in a refrigerator for about 30 min. Thereafter they were subject to extraction as described in the following. Milli Q water (4 mL) was added, and the samples were left to soak for 30 min. Acidified ethyl acetate (4 mL) and 200 μL of the surrogate standard triphenyl phosphate (TPP) at 2 ng/μL concentration were added to each sample. The tubes were shaken vigorously by hand for 1 min and then for 30 min on a horizontal shaker. After this step, the first salt mixture made up of 0.3334 g of $NaHCO_3$ and 2 g of Na_2SO_4 was added, the tubes were hand-shaken vigorously and the contents were thoroughly homogenized using an ultra turrax IKA-T25 homogenizer (IKA, Staufen, Germany) at maximum speed for 1 min. The homogenates were centrifuged for 5 min at 12 581*g* at 20 °C. A volume of 2 mL of the organic supernatant was cleaned up in a test tube by adding a second salt mixture, made up of 50 mg of PSA and 300 mg of $MgSO_4$ and vortexed for 30 s. The test tubes were then more intensively agitated on an orbital vortex mixer at maximum speed for 5 min. The tubes were then centrifuged for 5 min at approximately 12 581*g* at 20 °C. The extracts were filtered using a 0.2 μm syringe filter yielding a 0.5 g/mL sample extract. Finally, 100 μL of internal standard sulfotep, at a concentration of 1 ng/μL, was added to 900 μL of each extract, including calibration standards, to reach a final volume of 1 mL and injected into the GC-MS/MS triple quadrupole system.

Apparatus and Analytical Conditions. The gas chromatography system used to analyze the pesticide residues consisted of a 7693 autosampler, 7890 B GC, and 7000 C GC-MS/MS triple quadrupole system (Agilent Technologies, Santa Clara, CA). Data acquisition and processing were implemented using Agilent Mass Hunter Quantitative Analysis B07.00 software.[12]

Two ultra inert columns were used, they were 15.0 m × 0.25 mm i.d., 0.25 μm, HP-5 ms (Agilent J&W, Santa Clara, CA). They were connected by an electronic pressure controller to enable a 5 min postrun backflush. The samples were injected using a multimode injector inlet in splitless mode through an ultrainert inlet liner with a glass wool plug (Agilent Technologies, Santa Clara, CA). The injection volume was 3 μL. The injector port was programmed so that the initial injection temperature was 70 °C for 0.02 min and then ramped up, at the rate of 850 °C/min, to 320 °C, kept at this temperature for 5 min and then cooled down at a rate of 20 °C/min to 280 °C. Helium (99.999% purity) was used as the carrier gas and nitrogen (99.999% purity) as the collision gas. The oven temperature was held at 70 °C for 1 min, then ramped to 150 °C at a rate of 50 °C/min, increased to 200 °C at 6 °C/min, followed by a final ramp to 280 °C at 16 °C/min. The total run time was 24 min. The transfer line was maintained at 280 °C. The retention time lock setting (RTL) used chlorpyrifos-methyl as the locking compound at retention time of 10.83 min. The instrument worked at a constant flow (1.25 mL/min in column 1 and 1.45 mL/min in column 2).

The ion source and the quadrupole analyzer temperatures were fixed at 300 °C/min and 150 °C/min, respectively. The pesticide and environmental pollutants MRM database was used to select the best transitions for each of the pesticides.[13] The collision energy for each transition was optimized in the range 5−50 eV. The list of the studied pesticides, the transitions observed as well as the collision energies chosen are shown in Table 1. Twenty-five MRM time-segments were created to obtain adequate sensitivity, and in each time segment dwell times were optimized to collect at least 12 points across a peak (cycles between 3 and 4). The solvent delay was 4 min.

Validation Procedure. Uncontaminated vine leaves were obtained from Syria for use as blank materials. A blank matrix and a reagent blank were prepared and injected in each batch of samples to demonstrate that there was no cross-contamination and interference during the analysis. The analytes, to be included in the method validation, were selected from those GC-MS/MS amenable pesticides that are included in grapevine cultivation. They are listed in Table 1.

The parameters tested for validation included analytical range, linearity, limits of detection and quantification, trueness, within laboratory repeatability and reproducibility, and matrix effects.

The lowest calibrated level (LCL) is the lowest concentration of analyte detected by GC-MS/MS and measured in the analytical calibration; it may be expressed as a solution concentration in the test sample.[11] The analytical calibration was carried out in each batch of analysis using matrix-matched standards, covering the range of 0.005−0.240 μg/g (2.5−120 μg/L) and using the bracketing calibration modality.

Linearity was evaluated using matrix-matched calibration curves. Six concentrations in the range 2.5−120 μg/L were injected in duplicate and the entire calibration curve was injected at the beginning and at the end of the batch (bracketing calibration), with additional calibration injections distributed evenly among the samples. The analytical calibration curve was considered acceptable when the bracketing drift was lower than 30%, the deviation of the individual residuals was lower than 20%, and the coefficient of determination (R^2) was at least 0.99.

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Table 2. Limit of Detection (LOD), Lowest Calibrated Level (LCL), Method Limit of Quantitation (LOQ), Matrix Effects (ME), and Maximum Residue Limit (MRL) for Grape Leaves Set by the European Union (EU)

pesticide	LOD (mg/kg)	LCL (mg/kg)	LOQ (mg/kg)	ME (%)	MRL-EU (mg/kg)
azinphos-methyl	0.0014	0.010	0.02	90	0.02
azoxystrobin	0.0012	0.005	0.01	75	0.01
benalaxyl	0.0013	0.005	0.01	6	0.05
bifenthrin	0.0007	0.005	0.01	9	0.01
boscalid	0.0006	0.005	0.01	33	0.01
bromopropylate	0.0005	0.005	0.01	12	0.01
carbaxfos	0.0003	0.005	0.01	7	0.01
chlorpyrifos	0.0008	0.005	0.01	−4	0.05
chlorpyrifos-methyl	0.0005	0.005	0.01	2	0.05
cyfluthrin beta	0.0004	0.005	0.01	49	0.01
cypermethrin fam.	0.0004	0.005	0.01	41	0.05
cyprodinil	0.0002	0.005	0.01	4	0.02
deltamethrin	0.0007	0.005	0.01	89	2
diazinon	0.0005	0.005	0.01	1	0.01
dichlofluanid	0.0015	0.100	0.1	−15	0.01
dimethoate	0.0005	0.005	0.01	43	0.01
dimethomorph	0.0007	0.005	0.01	66	0.01
endosulfin I&II					0.05
endosulfin I (alpha isomer)	0.0019	0.010	0.01	−15	0.05
endosulfin II (beta isomer)	0.0033	0.010	0.02	−17	0.05
ethoxyquin	0.0005	0.005	0.01	−15	0.05
etrimfos	0.0016	0.005	0.01	8	0.01
fenamidone	0.0006	0.005	0.01	8	0.01
fenarimol	0.0004	0.005	0.01	16	0.02
fenhexamid	0.0005	0.005	0.01	33	0.01
fenpropathrin	0.0007	0.010	0.01	6	0.01
fenthion	0.0005	0.005	0.01	13	0.01
fludioxonil	0.0047	0.005	0.01	−12	0.01
flusilazole	0.0008	0.005	0.01	4	0.01
hexachlorobenzene	0.0006	0.005	0.01	2	0.01
hexaconazole	0.0067	0.005	0.01	−5	0.01
indoxacarb	0.0023	0.005	0.01	−5	0.02
iprodione	0.0013	0.010	0.02	−15	0.01
iprovalicarb	0.0005	0.010	0.01	17	0.01
isocarbophos	0.0008	0.005	0.01	17	0.01
kresoxim-methyl	0.0007	0.005	0.01	−4	15
malathion	0.0007	0.005	0.01	14	0.02
mepanipyrim	0.0009	0.005	0.01	30	0.01
metalaxyl	0.0012	0.010	0.02	5	0.05
methidathion	0.0006	0.005	0.01	−3	0.02
myclobutanil	0.0005	0.005	0.01	−3	0.02
omethoate	0.0009	0.005	0.01	83	included in residue definition of dimethoate
parathion-methyl	0.0004	0.005	0.01	24	0.01
phosalone	0.0006	0.005	0.01	47	0.01
pirimiphos	0.0005	0.005	0.01	25	0.01
procymidone	0.0015	0.005	0.01	−7	0.01
profenofos	0.0008	0.005	0.01	−16	0.01
pyraclostrobin	0.0007	0.010	0.02	>100	0.02
pyrimethanil	0.0006	0.005	0.01	14	0.01
quinalphos	0.0013	0.005	0.01	16	0.01
quinoxyfen	0.0008	0.005	0.01	4	0.02
spiroxamine	0.0009	0.010	0.01	12	0.01
tebuconazole	0.0009	0.005	0.01	10	0.02
tetrachlorvinphos, cis-	0.0005	0.005	0.01	3	0.05
terbufos	0.0003	0.005	0.01	−35	0.01
tetradifon	0.0007	0.005	0.01	−5	0.01
thiabendazole	0.0014	0.005	0.01	30	0.05
triadimefon	0.0006	0.005	0.01	9	0.01
trifloxystrobin	0.0007	0.005	0.01	−4	0.01
zoxamide	0.0029	0.010	0.01	>100	0.02

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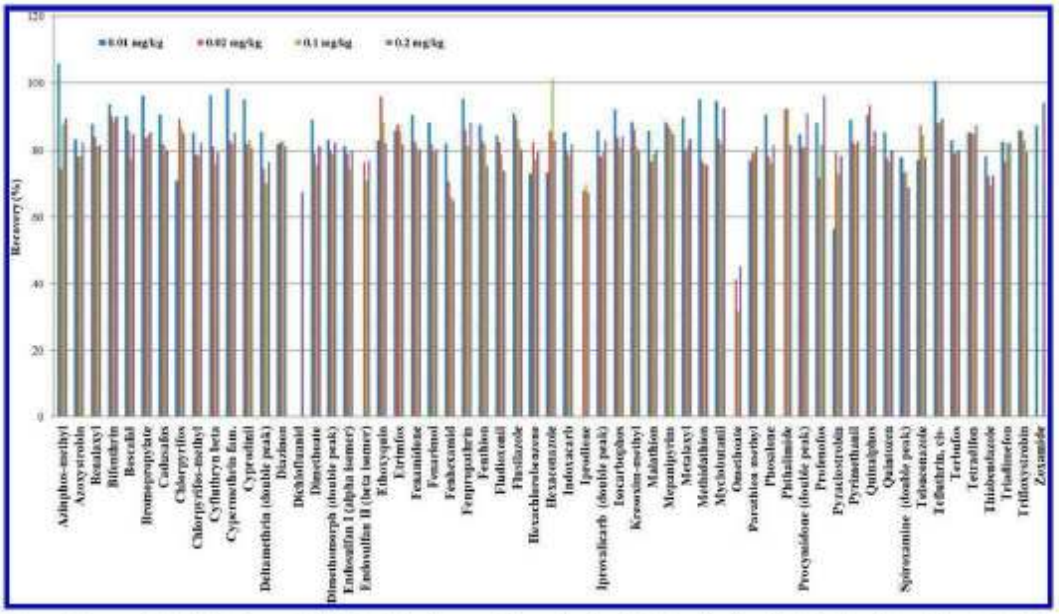

Figure 1. Recovery data (%) for the pesticides included in the scope of the method at the different fortification levels, from left to right at 0.01, 0.02, 0.1, 0.2 mg/kg (n = 15 at each level).

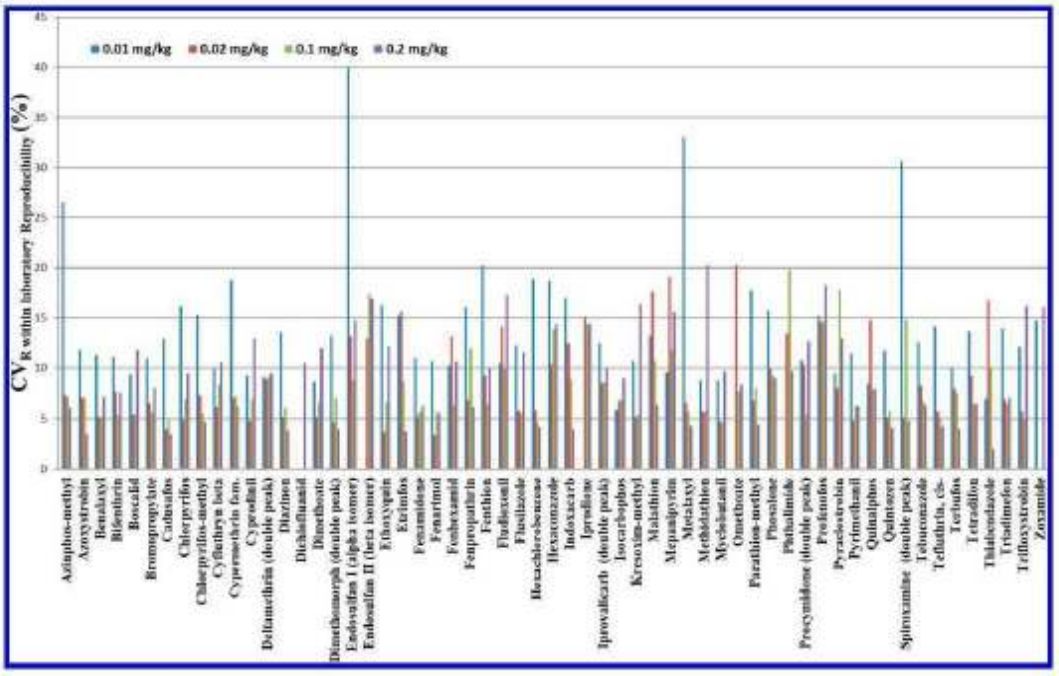

Figure 2. Coefficient of variation for within laboratory reproducibility for the validated compounds at the different fortification levels from left to right at 0.01, 0.02, 0.1, 0.2 mg/kg (n = 15 at each level).

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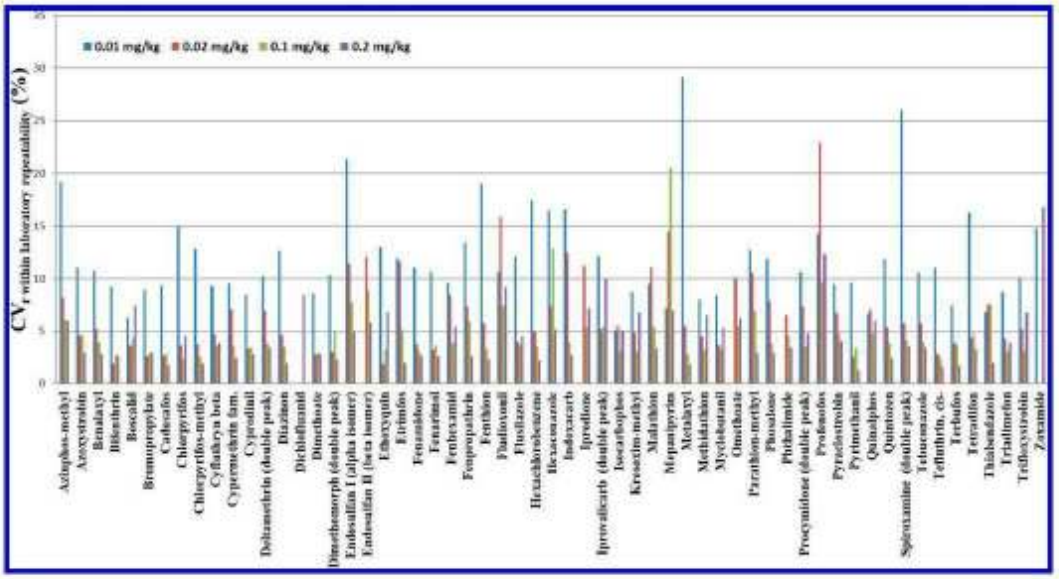

Figure 3. Coefficient of variation for within laboratory repeatability for the validated compounds at the different fortification levels, from left to right at 0.01, 0.02, 0.1, 0.2 mg/kg (n = 15 at each level).

The limit of detection (LOD) was calculated from the matrix-matched calibration curve, assuming a 5% error and using the standard deviation from the LCL according to the method described by Miller et al.[15] and confirmed experimentally. The limit of quantitation (LOQ) of the method was the lowest fortification level of the validation meeting the method performance acceptability criteria. As described by Ucles et al.,[16] this refers to the lowest recovery level for which a significant signal could be obtained in both confirmatory transitions and with the correct ion ratio (<30%). The LOQ for each pesticide was obtained from the recovery studies.

Precision was established as the within laboratory percentage of relative standard deviation for repeatability and reproducibility. Five replicate measurements at three different fortification levels over three different days were analyzed. For the repeatability within laboratory relative standard deviation (RSD_r%), the standard deviations at each fortification levels were pooled together using eq 1:

$$SP = \mathrm{sqrt}((\mathrm{var}\,1 \times \mathrm{df}1 + \mathrm{var}\,2 \times \mathrm{df}2 + \mathrm{var}\,3 \times \mathrm{df}3) / \mathrm{sum}(\mathrm{df}1, \mathrm{df}2, \mathrm{df}3)) \quad (1)$$

where var 1 is the variance obtained on day 1, var 2 on day 2, and var 3 on day 3, and df is the number of degrees of freedom on each measurement day.

For the reproducibility, within laboratory relative standard deviation (RSD_R%) from all the values obtained over several days and by several operators were considered as deriving from a single population and computed for overall average recovery at each fortification level and variability (CV_R%).

Accuracy, evaluated in terms of % recovery, was established at four fortification levels: 0.01, 0.02, 0.1, and 0.2 mg/kg. Replicate analytical portions of 2 g of homogenized matrix were individually spiked with 100 μL of 0.2, 0.4, 2, and 4 ng/μL standard solution in ethyl acetate in order to reach 0.01, 0.02, 0.1, and 0.2 mg/kg, respectively. The samples were kept in a refrigerator for about 30 min and then rehomogenized before extraction with the ethyl acetate method, as described previously.

Matrix effects were estimated through the comparison of the response of the analytes when the standards were prepared and injected in pure ethyl acetate and matrix-matched. Two sets of calibration curves were prepared covering the range of 2.5−120 μg/L concentration for all analytes. Weighted linear regression was applied to estimate the slope of the curves (w). Equation 2 was used to establish a standardized value for the matrix effect (ME):

$$ME = (\text{slope of curve in matrix} - \text{slope of curve in solvent}) / \text{slope of curve in solvent} \quad (2)$$

Chlorpyrifos methyl was injected daily for relocking the method at the desired retention time and also for quality control purposes to check that the method performance was adequate during the determination. A quality control chart was constructed for the surrogate standard triphenyl phosphate (TPP) to identify the need for maintenance operations. The area for TPP over a period of 19 days was used to calculate the average TPP value and the warning and action limits were established using 2 and 3 times the standard deviation of the 19 measurements.

RESULTS AND DISCUSSION

A study on the effect of simulated rain on folpet and mancozeb residues on grapes and vine leaves was conducted by Cabras et al.[17] and they concluded that the portion of mancozeb washed

Table 3. Minimum and the Maximum Concentration, Expressed in mg/kg, from the Analysis of 27 Samples of Vine Leaves Originating from Syria

pesticide	minimum concentration (mg/kg)	maximum concentration (mg/kg)
chlorpyrifos	0.012 ± 0.001	487.9 ± 31.0
chlorpyrifos methyl	<LOQ	<LOQ
diazinon	0.066 ± 0.005	22.8 ± 4.0
dimethoate[a]	0.032 ± 0.001	43.3 ± 3.1

[a]Sum of dimethoate and omethoate.

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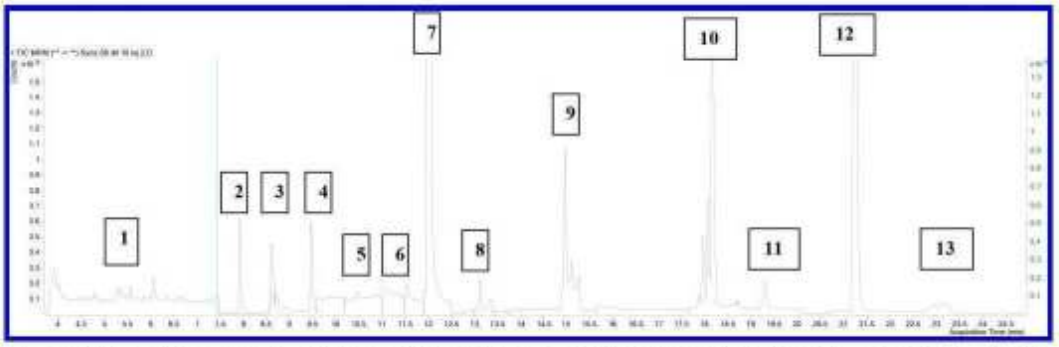

Figure 4. Total ion chromatogram of a vine leave sample from Syria analyzed with the validated method. Peaks in 1 are from solvent, peak in 2 is from sulfotep, peak in 3 is from dimethoate, peak in 4 is from diazinon, peak in 5 is from chlorpyrifos methyl, peaks in 6 are from matrix, peak in 7 is from chlorpyrifos, peak in 8 is from matrix, peak in 9 is from triphenyl phosphate, peaks in 10 are from matrix, peaks in 11 are from column bleed, peaks in 12 are from matrix, peaks in 14 are from column bleed.

off by a light rainfall was higher for the grapes than for the leaves, probably due to a different composition of the epicuticular wax of the fruit compared to that of the leaves. These results suggest that, depending on the physicochemical properties of the pesticides, the residues level on vine leaves can be different from that of the grapes. Furthermore, the different composition of grapes and vine leaves indicates that, for the analysis of pesticide residues in vine leaves, a different analytical method may be required from that used for grapes.[18,19]

Single quadrupole GC-MS using electron ionization (EI) mode is the most conventional method used for pesticide residues analysis in grapes.[7,20] Chemical ionization (CI) is less commonly used for the analysis of pesticide residues in grapes.[21] Negative CI mode provides better selectivity for most typical pesticides since they contain electronegative groups in their structures which stabilize the molecules in the negatively charged state. An analytical method based on GC-MS with negative CI for the analysis of 82 pesticide residues in cabbage and apple was developed and applied to grape samples as well.[21] In general, the acquisition modes selected in published methods are mainly single ion monitoring (SIM) and full scan m/z covering the range 50−600 m/z and with at least 2−3 ions selected in SIM mode in order to achieve a reliable identification and quantitation of the analyte.[12] Recently, quadrupole GC-MS/MS analysis has been introduced into routine analysis of pesticide residues in grapes which resulted in an improvement of the sensitivity and selectivity of the analytical methods.[22] Tandem mass detectors using multiple reactions monitoring (MRM) can minimize, or sometime eliminate, potential matrix effects due to isobaric compounds and can also reduce chromatographic noise caused by matrix coextractives, thereby allowing lower limits of detection. The SweEt method adopted by the Swedish National Food Administration takes advantage of the capability of MS/MS to reduce matrix effects to eliminate the cleanup step in the analysis of pesticides in nonfatty sample matrixes.[23] The SweEt method uses ethyl acetate as extraction solvent and $NaHCO_3$ for pH adjustment, and no further clean up or sample concentration is performed for pesticide residue analysis in fruits and vegetables, cereals, and other matrixes with low fat content. The extract is directly injected into the chromatographic system coupled to tandem mass (MS/MS) detectors.

The analytical method used in this study was adopted from that reported by Aysal et al.[24] This method combines aspects of the SweET method and the "Quick, Easy, Cheap, Effective, Rugged, and Safe" (QuEChERS) method but miniaturizing it. Whereas the original and official procedures for fresh produce employs 10 g of sample, the present method requires only 2 g of sample. The initial extract stage is performed with acidified ethyl acetate (EtOAc), which is more effective for a wider range of compounds than the acetone extraction, which was widely used previously, but which gave low recovery for polar pesticides. Cleanup is performed using a modified QuEChERS procedure on the ethyl acetate extract, rather than the acetonitrile extract in the original QuEChERS procedure. The use of ethyl acetate as extractant and the inclusion of the simple cleanup procedure allow the possibility of analysis using conventional GC detectors if MS/MS is not available and also further reduces matrix effects in the MS/MS method. Previous work at the FAO/IAEA laboratory demonstrated that the EtOAc extraction procedure produced a final extract that was suitable for analyses using conventional GC detectors such as nitrogen−phosphorus and electron capture detectors as well as MS. Also, as shown previously,[25,26] with the injection of uncleaned extracts several problems were experienced such as contamination of the GC inlet and rapid deterioration of system performance because of accumulation of nonvolatile material in the inlet. This made the system less robust and frequent exchanging of the liner (in every batch) and cutting the GC-precolumn (in every sequence) was required. As shown by Mol et al.,[27] a shift in the retention times of pesticides was observed for some of the sample extracts and that made detection and identification complicated to implement. They detected retention time shifts in the range 0.05−0.20 min and these were most abundant for the "azole" pesticides. The method presented here used reduced volumes compared to that published by Aysal et al.[24] and was expanded to include a wider scope of pesticides in vine leaves, using an MS/MS detector. The optimization of the analytical method also included a verification of the homogeneity of the analytical portions at the 2 g level.

Figure 1 in the Supporting Information shows the total ion chromatogram (TIC) for solvent, reagent blank, blank matrix, the TIC and the extracted ion chromatogram for a matrix-

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matched calibrator at 0.01 μg/g, and the TIC for a matrix-matched calibrator at 0.24 μg/g.

Method Validation. The process of method validation is intended to demonstrate that a method is fit for-purpose.[11,12] The validation should demonstrate the identity and concentration of the analyte taking into account matrix effects, providing a statistical characterization of recovery results and other method performance criteria. To ensure that validation of the method remains appropriate over time, the method should be continuously assessed by participation in proficiency testing and appropriate quality control measures.[12] Performance parameters for analytical methods include scope, analytical range, sensitivity, limits of detection and quantification, trueness (accuracy), within laboratory repeatability and reproducibility. The Codex guidelines on good laboratory practice is a very detailed document which includes some other parameters, such as testing for robustness.[11] The SANTE/11813/2017 guideline considers that these requirements are fulfilled through continuous QA/QC and ongoing verification/validation of the method in routine use.[12]

Limits of Detection and Quantitation. The analytes covered by the method, their retention times, the multiple reaction monitoring transitions used for quantitation and qualification, and the selected collision energies are presented in Table 1.

Table 2 gives the LOD, the LCL, and the LOQ for each compound and the EU maximum residue limits (MRL). There are no Codex MRLs set for grape leaves.

The LOD and LCL were obtained from the calibration curve, as explained above. 58 of the 59 pesticides had LODs lower than 5 μg/kg; hexaconazole had a LOD of about 7 μg/kg. For 49 of the 59 pesticides included in the method, the LCL was 5 μg/kg and 10 pesticides had LCL at 10 μg/kg. This result is in line with previously reported LCL for the same pesticides in similar matrixes.[16] 53 of the 59 pesticides included in the analytical scope, had an LOQ of 0.01 mg/kg; 5 pesticides had an LOQ of 0.02 mg/kg; and 1 pesticide had an LOQ at of 0.1 mg/kg.

For all the studied compounds, with defined EU-MRLs for vine leaves, except iprodione, the LOQs were below or at least equal to the EU-MRLs, demonstrating the fitness-for-purpose of the method. Additionally, five pesticides, phthalimide, fenpropathryn, etrimfos, isocarbophos, and dichlofluanid, that do not have a listed MRL within the EU commodity group for "grape leaves and similar species", were studied and their LOQs were set according to the precautionary principle below or equal to 0.01 mg/kg.

Calibration and Matrix Effects. The linearity was checked using a matrix-matched calibration curve in the 5−120 ng/mL concentration range for all pesticides. Linearity was tested by examination of the plot of residuals produced by weighted regression. Good linearity was achieved for almost all pesticides with a coefficient of determination (R^2) better than 0.99 and relative residuals lower than 20%, as measured by the accuracy value in the Mass Hunter software.[13] Weighted linear regression did not give a good fit for fludioxinil and profenofos, so a weighted quadratic function was used to calibrate the detection system for these two compounds.

Details of the estimated matrix effects for all pesticides under study are presented in Table 2. The matrix effect was evaluated by comparing the slope of the calibration curve in matrix to that in solvent. Matrix effects (ME) above 20% (significant effects) were observed for 18 of the 59 pesticides included in the method. A signal enhancement in the chromatographic response was obtained for 17 pesticides. This is in accordance with the results reported by Poole et al.[14] Significant suppression effect, when the slope in the matrix was lower than the slope in pure solvent, was observed only for terbufos. According to Ucles et al.,[16] this effect can be explained by some degradation processes for some of the pesticides in the vine leave matrix. There was no correlation between retention time and matrix effect. Most of the compounds showing significant matrix effects belong to the organophosphorus and pyrethroids classes.

In general, matrix effects cannot be eliminated;[29−32] however, compensation can be achieved with matrix-matched calibration, even though this process may be laborious and time-consuming.[12,29,30,33,34] For that reason all the studies were performed using matrix-matched calibration curves.

Recovery Studies and Precision Data. Recovery studies were performed to determine the trueness or bias of the method. Figure 1 shows the recoveries obtained at the four different spiking levels. According to the Codex Alimentarius Guidelines, the acceptable trueness range at concentration values at ≤0.01 mg/kg is 60−120%; at >0.01 mg/kg, the acceptable range is 70−120%; at concentrations >0.1 mg/kg, the acceptable recovery range is 70−110%. As can be seen from Figure 1, in general the recoveries are within the range 70−120%, which complies with both the Codex criteria and the SANTE document with a few exceptions, for dichlofluanid, zoxamide, fenhexamid, iprodione, omethoate, and spiroxamine. Dichlofluanid could only be quantified with acceptable precision at the highest spiking level. Zoxamide could be quantified with acceptable precision at both the lowest and highest spiking level. Dichlofluanid, iprodione, omethoate, pyraclostrobin, and spiroxamine presented lower recoveries than Codex acceptable levels; however, the precision data indicated that the within laboratory repeatability and reproducibility is good. Pyraclostrobin had unacceptable recovery only at the lowest spiking level. Folpet was included in the original spiking mixture, and phthalimide was monitored as the degradation metabolite with very good recoveries. This is in line with previous studies from Ucles et al.[16] which could not detect the parent compound but only via the degradation products. Phthalimide could not be quantified at the lowest spiking level.

Values for the reproducibility and repeatability relative standard deviation (RSD_R% and RSD_r%) at the different spiking levels for the pesticides included in the method scope are presented in Figures 2 and 3.

The majority of the pesticides had within laboratory precision data below 20% at all spiking levels both for repeatability and reproducibility. The Codex and the SANTE/11813/2017 precision criteria are indicated in the figures with corresponding color lines.[11,12] At the 0.02 mg/kg spike level, only profenofos (CV_r% = 22.9) had a value for repeatability slightly outside the criteria accepted by the regulations; nevertheless, the value for reproducibility is within the acceptable range as well as the recovery value. At the 0.1 mg/kg spike level mepanipyrim (CV_r% = 20.5) also had a value for repeatability slightly outside the acceptable criteria. In both cases, ongoing routine use of the method will be useful to define the precision values corresponding to these pesticides and additional work may be required to redefine the validation parameters for these two pesticides. The repeatability of zoxamide (CV_r% = 16.8) at the high spike level was also not

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compliant with Codex criteria but would be acceptable according to the EU SANTE guidelines.[12] Zoxamide suffers from a high degree of matrix effects, as shown in Table 2. With regard to repeatability only fludioxinil (CV,% = 17) at the high spike level is not compliant according with Codex or EU acceptance criteria.

Omethoate, that is included in the residue definition of dimethoate, despite the low recovery values, presented acceptable values for precision both for repeatability and within laboratory reproducibility, except at 0.01 mg/kg level; omethoate could be included in the scope of the method because of its consistent behavior. However, the results would need to be corrected for recovery and indicated in the laboratory result report.

Chlorothalonil, imazalil, captan, and its degradation metabolite tetrahydrophthalimide could not be included in the scope of the method at any of the studied levels as the peak shapes were unacceptable for all of the acquired ions; the ion ratios were not acceptable and the recoveries and/or precision parameters could not be established.

Those pesticides not complying with both recovery acceptable ranges and precision criteria were excluded from the method scope at the concentration level considered in this study. Therefore, the method reported here for vine leaves was validated for 51 pesticides at the 0.01 mg/kg concentration level. The method scope was increased to 57 pesticides at the 0.02 and 0.1 mg/kg concentration level, to 59 pesticides at 0.2 mg/kg concentration.

Internal Quality Control. A quality control chart for the surrogate standard triphenyl phosphate injected as a matrix-matched standard was constructed. When data were found to be outside the action limits, maintenance operations were carried out on the instrument, such as change of liner, septum, or trimming of the guard column. For example, on one occasion the chart indicated values outside the action limits; the tune of the instrument also showed increased electron multiplier voltage (EMV) compared to previous tunes. Therefore, the instrument ion source was thoroughly cleaned. In general, the liner and septum were changed after every batch of analysis to keep the ion source and the first 15 m column as clean as possible and to prevent increased matrix effects. Instrument backflush also helped to keep the system clean to avoid the need to trim the guard columns too much and avoid cleaning the ion source.[35]

The validated method was applied to the analysis of 27 real samples of vine leaves from Syria, which were found to be contaminated with chlorpyrifos, dimethoate, diazinon, and omethoate in most cases at levels above their corresponding EU MRL. Chlorpyrifos methyl was also detected but at levels below the LOQ of the method. Table 3 summarizes the minimum and the maximum value of the residues found in the samples. Figure 4 shows an example of a total ion chromatogram of one of the real samples analyzed using the validated method.

In summary, a protocol based on a previous method developed at the FAO/IAEA Laboratories but with reduced sample mass and solvent volumes that combines features from the QuEChERS and SweEt methods for pesticide residues analysis in vine leaves was presented together with its validation. The method reported here for vine leaves was validated for 51 pesticides at the 0.01 mg/kg concentration level. The method scope was increased to 57 pesticides at the 0.02 and 0.1 mg/kg concentration level, to 59 pesticides at 0.2 mg/kg concentration as discussed above. The method is simple, cheap and straightforward, with no solvent exchange or prior concentration and proved to be suitable for the routine determination of pesticide residues in vine leaves. The validation criteria discussed in this paper were met. The LOQs for the studied pesticides fulfilled the MRL requirements in the EU. Additionally, the scope of the validated method included pesticides that are not listed in the EU for grape leaves, accomplishing the EU precautionary criteria that unlisted pesticides have a tolerance level of 0.01 mg/kg. Finally, to prove the fitness-for-purpose of the method, it was successfully applied to real samples analysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b00453.

TIC for solvent, reagent blank, blank matrix, the TIC and the extracted ion chromatogram for a matrix-matched calibrator at 0.01 μg/g, and the TIC for a matrix-matched calibrator at 0.24 μg/g; structures of the pesticides from this study (PDF)

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Notes

The authors declare no competing financial interest.

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2.3 Required Withholding Period for Vine Leaves Following Spraying with Pesticide

This paper addresses the important point of withholding periods in vine leaves, as an essential requirement to protect the consumers from consumption of conventional crops contaminated with excessive residues of pesticides. Every applied pesticide has a withholding period (WHP) or pre-harvest interval (PHI), which is defined as the number of days required to lapse, between the date of final pesticide application and harvest, for residues to fall below the legal level established for that crop or for a similar food type. Food products become “fit for consumption and trade” only after the withholding period has lapsed. The WHP differs from pesticide to pesticide and crop to crop, and it is important to note that they depend on the legal limits established for the pesticides in the legislation. The maximum residue level (MRL) is a conventional measure that needs to reflect the conditions of use under specific environmental conditions, hence the need to generate sufficient support data that can help set and harmonize MRLs in international meetings and bodies such as the Codex Alimentarius Commission.

In this paper a collaboration within FAO/IAEA, GACT and the Syrian Atomic Energy Commission made it possible to run a field study to establish the dissipation rates of applied organophosphorus pesticides (OPs) on vine leaves, a minor crop amply consumed in the Middle East region. The validated method for pesticides in vine leaves was applied to study the decay of OPs residues in vine leaves collected in a 21 days’ period after pesticide spraying. Analysis of the data provided an indication of the dissipation rates, the half-lives and the WHPs. Ultimately these are essential information to guarantee good agricultural practices (GAPs) and therefore provide for control of unnecessary consumer exposure to residues of pesticides, if the legal framework is properly functioning and requiring monitoring of crops for consumer food safety.

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Required Withholding Period for Vine Leaves Following Spraying with Pesticide

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Abstract: Vine leaves are consumed in many countries but little attention is paid to the residues left on them after the application of pesticides that help prevent pests and protect the grapes, the economically important target. Therefore, it is of outmost importance to study the dissipation of the pesticides applied to this crop to protect the consumers that also eat vine leaves. Dissipation kinetics of chlorpyrifos, chlorpyrifos-methyl, diazinon and dimethoate residues were studied in vine leaves grown under sunny conditions in Syria, using an ethyl acetate based sample preparation followed by GC-MS/MS determination. The dissipation rate for all doses applied followed first-order kinetics, with half-lives in grape leaves in the range of 2.9 – 3.9 days. At the recommended application dose, a withholding period of 8.9-37.1 days before consumption should be applied to meet current MRLs and minimise risks to consumers. The effectiveness in the reduction of pesticide loads in vine leaves through washing with either cold or hot water was dependant on the physicochemical properties of the studied pesticides. Hot water washing was very effective for dimethoate, a polar and water-soluble pesticide, with an effective reduction of 92% of the residue level, but no significant effect was observed for chlorpyrifos, the most apolar compound in this study.

Keywords: Vine Leaves, Dissipation Kinetics, Half-Life, Withholding Period

1. Introduction

Grape (*Vitis vinifera*) was estimated by Food and Agriculture Organisation of the United Nations to be the most widely cultivated fruit crop in the world [1]. The Mediterranean countries constitute the main area of cultivation of grapevines. While the main product are grapes, with most of the production destined for wine making, vine leaves are also important for cultural and nutritional reasons. Vine leaves have been used as a nutritious food in Greece and the Middle East for centuries and their popularity as a healthy food is increasing globally [2]. In countries such as Syria, where vine leaves are widely consumed, it is important to assess human dietary exposure to residues of pesticides applied to vine leaves. As vine leaves may represent an important contribution to the Mediterranean diet, data on the fate of pesticides in vine leaves after application are essential for both the calculation of the theoretical maximum daily intake and the establishment of the Maximum Residues Limits (MRLs) [3]. Most studies on pesticide residues deal with the analysis of grapes or the transformation from vine to wine, as discussed in the review by Grimalt et al. [4]. To date only a few studies have been dedicated to residues in vine leaves and foliage in general [5-10]. Insecticides and

fungicides are applied to be effective against a wide range of insect pests, such as grape moth (*Lobesia botrana*), and fungal diseases such as downy mildew (*Plasmopora viticola*), powdery mildew (*Uncinula necator*) and gray mold (*Botrytis cinerea*), but their use has to be strictly regulated. The legal parameter with respect to pesticide residues, which determines whether a food product may be placed on the market, is the MRL expressed in mg/kg of product. This value is calculated from toxicological data such as Acceptable Daily Intake (ADI in mg/kg/day) and Food Daily Intake (FDI in kg/day), and agronomic data such as active dose and dissipation curves in the field. FAO and the World Health Organization (WHO), through their Joint Meeting on Pesticide Residues (JMPR) meetings evaluate pesticides and their residues in food to set MRLs for food commodities [11-12]. The occurrence of pesticides in the environment, as well as in vine leaves, remains an important challenge to address in Syria and elsewhere. The agricultural practices, including pesticide management practices, must be continually improved to meet it. It is important to generate analytical data on the actual levels of crop protection chemicals on crops and in the environment, and to consider these data with respect to the agricultural practices applied under local conditions to be able to optimize pesticide management. In assessing the impact of dietary exposure to pesticides, a number of parameters have to be taken into account and carefully evaluated, including MRLs, withholding times and dissipation rates and post-harvest management, amongst others. These parameters are obtained either experimentally or through modelling and they vary according to the pesticide, the type of crop and the prevailing environmental conditions. A combination of effects results in a reduction, over time, from the initial amount of active ingredient on crops after application. The dissipation rate (K_{diss}) is an important kinetic parameter used to calculate residual concentrations of pesticides in crops harvested for human or animal consumption [13]. The term dissipation is defined as an integrated process where several effects play a role, including volatilization, photodegradation, wash-off, leaching, hydrolysis, chemical and biological degradation, among others [13]. Dissipation rates are usually expressed as the pesticide half-life; the half-life being the time required for the pesticide residue level to fall to half of the initial concentration directly after application. Because of the large combinations of pesticides and crops, it is very important to estimate dissipation half-lives to contribute to risk assessments, for example when establishing MRLs for pesticides in various commodities. The dissipation rate for pesticides applied on a specific crop depends on several factors: the chemical formulation and application method, climatic conditions - especially rainfall and temperature, vapour pressure of the pesticides, and the potential for photodegradation and other chemical degradation [14]. This means that dissipation curves are very specific to local conditions in each growing area [15-20], and therefore generation of precise knowledge of pesticide degradation kinetics is very important to produce reliable data for international bodies, such as Codex Alimentarius Commission, that set MRLs [21]. Because of the agronomic behaviour and differences among regions in the world, the same active ingredients may have different MRLs depending on the country and the climatic conditions [22]. Fantke et al. [13] systematically reviewed 811 scientific literature sources, and analysed 4513 dissipation half-lives of 346 pesticides measured in 183 plants. The authors emphasized that further experiments are needed to analyse pesticide-plant species combinations that so far have not been covered and to allow the use of prediction models. The fundamental reason is because modelling dissipation in plants is highly uncertain and the estimation of dissipation half-lives strongly relies on experimental field data. A study was initiated in Syria on the behaviour of specific organophosphate pesticides (OP) in the field under the current agricultural practices for grape vines. The compounds included in the study were chlorpyriphos, chlorpyriphos methyl, diazinon and dimethoate. OPs are extensively used due to their high insecticidal activity and relatively low persistence [5]. The dissipation rate of OPs is very fast [23-25]. The objective of the study was to establish the withholding period (WHP) for these specific OP pesticides used in vineyards in Syria. The WHP is the minimum period of time that must be allowed after pesticide application before the treated area or crop can be grazed, cut for fodder or harvested. Withholding periods vary for different pesticide/crop combinations. They help to ensure that residues in the treated crop will not exceed the maximum residue limits when the crop is placed on the market [26]. When being prepared as a food in the home (or by industry), vine leaves are first washed. The fate of pesticide residues on raw agricultural crops has been well studied and recently well reviewed by Amvrazi [27], Kaushic [28], Holland [29]. According to these studies the rinsability of a pesticide is not always correlated with its water solubility and different pesticides may be rinsed off commodities using different washing procedures and washing agents [27]. In the present study, after sampling, vine leaves were analysed unwashed, washed with cold water, and washed with boiling tap water to assess whether domestic washing can offer a practical method for decreasing the intake of pesticides for consumers.

2. Materials and Methods

2.1. Field Trials

2.1.1. Site Descriptions

The experimental trials were carried out during the summer of 2016 in two different vineyards located outside the city of Damascus, Syria. A local cultivar of table grape was used for testing. The Al Soujah (Yafour) area, located 23 km west of Damascus, is an arid zone where the vineyard is surrounded by olive trees and is an experimental station belonging to the Atomic Energy Commission of Syria (AECS). The Al-Hamah area is a private vineyard in a greener zone located 14 km north west of Damascus and close to a river (*see Figures A1 and A2 in the Appendix*).

Meteorological data were collected by an agrometeorological station in Damascus. During the field trials the average daily temperature was 26°C, the maximum temperature was 36°C, the average humidity was 27%, the average solar radiation was 209 w/m^2, the average wind speed was 11.1 km/h and there was no rain. Each growing area was divided into two zones. One zone was treated and the other one was left untreated to be used as a control. The vines did not receive any irrigation, or pesticide or fertilizer treatment throughout the growing season. Four different formulations of organophosphate pesticides (OPs) were purchased from a local supplier and applied at the concentrations indicated on the labels, using a 20 L hand-pump pesticide sprayer (in Al Soujah area) and an electronic sprayer (in Al-Hamah area). Care was taken to ensure that the vines were well covered with the spraying mixture. The formulations applied to the vine areas, their label concentrations and the target application rate for each pesticide are presented in Table 1. Formulated forms of the pesticides dimethoate (400 mg/mL) and chlorpyrifos ethyl (480 mg/mL) were from AgriPest, diazinon 600 mg/mL was from Orient for Veterinary Pesticides and chlorpyrifos methyl (20%, 200 mg/mL) was from OvaGreen. At the Al Soujah site, 50 m^2 of vines trees were treated with 7 L of the pesticide formulation mixture, at the Al-Hamah site 100 m^2 of vines were sprayed with 100 L of the formulation mixture. In both cases the total volume of prepared pesticides was sprayed on the vines to deliver the amount of kg/Ha as shown in Table 1. Only one pesticide application was carried out at both locations at the end of the flowering stage.

Table 1. OP insecticides, their concentration and the amount sprayed on vine leaves in Al-Hamah and Al Soujah area.

Insecticide	Label concentration (mg/mL)	Amount of OP used for the mixture in Al Hamah (mL)	Amount sprayed in Al Hamah (kg/Ha)	Amount of OP used for the mixture in Al Soujah (mL)	Amount sprayed in Al Soujah (kg/Ha)
Dimethoate	400	50	2	7	0.56
Diazinon	600	50	3	7	0.84
Chlorpyrifos-methyl	200	200	4	280	11.2
Chlorpyrifos-ethyl	480	50	2.4	7	0.672

2.1.2. Sampling

For studying the dissipation of the four evaluated OP pesticides, vine leaves were collected at time 0 (one hour after spraying, when the spraying mixture had dried) and then after 1, 7, 14 and 21 days. Sufficient leaves were randomly collected from at least 5 vine trees to provide an overall sample weight of 500 g of vine leaves. The samples were labelled and immediately dispatched to the laboratory, where they were frozen and kept at -18°C until analysis.

2.2. Chemicals

All chemical analyses were performed at the Joint FAO/IAEA laboratories in Seibersdorf, Austria. All water used in this study was obtained from an in-house purification system (Milli Q, Millipore, USA). Certified pesticide analytical standards and triphenylphosphate (TPP, certified analytical standard) were purchased from Sigma Aldrich, Austria. Stock solutions of individual standards were prepared at 10 mg/mL and used in the preparation of a mixed stock solution at 25 ng/µL. The individual stock solution and the stock mixture were stored in amber screw-capped vials with septa in the dark at –20°C. Working standard solutions were prepared from the mixed stock solution according to requirements. Residue grade glacial acetic acid and ethyl acetate were purchased from Merck (Austria) and ascorbic acid was purchased from Sigma Aldrich (Austria). Acidified ethyl acetate was prepared by dissolving ascorbic acid (0.5 g) in milliQ water (10 mL), to which glacial acetic acid (10 mL) was added and the volume made up to 1L with ethyl acetate. The solution was stored in a screw-capped bottle with septum, wrapped with aluminium foil and kept in the dark at 4°C. Residue grade sodium hydrogen carbonate ($NaHCO_3$), anhydrous sodium sulphate ($NaSO_4$) and anhydrous magnesium sulphate ($MgSO_4$) were purchased from Sigma Aldrich (Austria). Residue grade primary-secondary amine (PSA) sorbent was purchased from Varian (USA).

2.3. Sample Preparation

Upon arrival at the laboratory each sample was divided into three parts. One part was directly stored at -20°C pending analysis. The other two parts were washed by immersion for 5 seconds into either boiling water or tap water, air dried in the dark and stored -20°C pending analysis. Before analysis, the vine leaves were prepared by removing the stems and crushing the leaves to small particles using liquid nitrogen in a mortar and pestle. To ensure effective homogenization, the samples were further homogenized, while still frozen, using a common food chopper (Moulinex, 1000 W) for 1 minute.

2.3.1. Analytical Method

A validated method for the determination of chlorpyrifos, chlorpyrifos-methyl, diazinon, and dimethoate was applied for the analysis of the samples [30]. Analytical portions of 2 g of homogenised vine leaves were weighed into 50 mL labelled PTFE centrifuge tubes. Milli Q water (4 mL) was added and the samples left to soak for 30 min. Acidified ethyl acetate (4 mL) and the surrogate standard, TPP (200 µL of a 2 ng/µL solution) were added to each sample. The tubes were shaken vigorously by hand for 1 minute and then for 30 min on a horizontal shaker. After this step the first salt mixture (0.3334 g $NaHCO_3$ + 2 g $NaSO_4$) was added, the tubes were hand-shaken vigorously and the contents were thoroughly homogenized using an ultra-turrax homogenizer (IKA-T25, IKA, Germany) at maximum speed for 1 min. The homogenates were centrifuged for 5 min at approximately 12,600 g at 20°C. Two mL of the organic supernatant were

cleaned up in an Eppendorf tube of 15 mL by adding a second salt mixture (50 mg PSA + 300 mg $MgSO_4$), and vortexed for 30 seconds. They were then more intensively agitated on an orbital vortex mixer (Scientific industries) at maximum speed for 5 min. The tubes were then centrifuged for 5 min at approximately 12,600 g at 20°C. The extracts were filtered using a 0.2 µm syringe filter yielding a 0.5 g /mL sample extract. Finally, 100 µL of internal standard (sulfotep, 1 ng/µL) was added to 900 µL of each extract, including calibration standards, to reach a final volume of 1 mL and injected into the GC-MS/MS triple quadrupole system.

2.3.2. Apparatus and Analytical Conditions

The gas chromatography system used to analyse the pesticide residues consisted of an Agilent 7693 autosampler, 7890 B GC and 7000C GC-MS/MS triple quadrupole system (Agilent Technologies, Santa Clara, USA). Data acquisition and processing were implemented using Agilent Mass Hunter Quantitative Analysis B07.00 software. Two 15.0 m x 0.25 mm ID x 0.25 µm HP-5 ms ultra-inert columns (Agilent J&W, USA) were connected by an electronic pressure controller to enable a 5-minute post-run backflush. The samples were injected using a multimode injector inlet in splitless mode through an ultra-inert inlet liner with a glass wool plug (Agilent Technologies, Santa Clara, USA). The injection volume was 3 µL. The temperature programmable injector port was set up to an initial injection temperature of 70°C for 0.02 minutes and then ramped up to 320°C (at 850°C/min) for 5 minutes, then cooled down to 280°C at 20°C/min. The oven temperature was held at 70°C for 1 minute, then ramped to 150°C (at a rate of 50°C/min), increased to 200°C at 6°C/min followed by a final ramp to 280°C at 16°C/min. The total run time was 24 min. Helium (99.999% purity) was used as the carrier gas and nitrogen (99.999% purity) as the collision gas. The transfer line was maintained at 280°C. The retention time lock setting (RTL) used chlorpyrifos-methyl as the locking compound at retention time of 10.83 min. The instrument worked at a constant flow (1.25 mL/min in column 1 and 1.45 mL/min in column 2). The ion source and the quadrupole analyser temperatures were fixed at 300°C/min and 150°C/min, respectively. The transitions and the collision energies used for detection are shown in the Appendix (*Table A1 of the Appendix*). A twenty-five time-segment method was created to obtain adequate sensitivity, and in each time segment dwell times were optimised to collect at least 12 points across a peak (cycles between 3 and 4). The solvent delay was 4 minutes.

2.3.3. Residues Analytical Determination

The analytical calibration was carried out using matrix matched standards calibration curves, covering the range 0.005-0.240 µg/g (2.5-120 µg/L) and using the bracketing calibration modality. When needed, the samples and the corresponding blanks, used for the preparation of the matrix matched calibration curves, were diluted with acidified ethyl acetate to meet the analytical range of 0.005-0.240 µg/g. Where more than a single estimate of the residue level was available (based on different dilutions required for assessment) an average estimate was used for that sample. The limit of quantitation (LOQ) and the precision data obtained during method validation are presented in the Appendix (*Table A2 of the Appendix*). Current EU and Codex MRLs for chlorpyrifos, chlorpyrifos-methyl, diazinon and dimethoate are given in Table 2. Values below the minimum reporting level or lowest calibrated level (LCL) were not included in the data set.

Table 2. *European Union MRL for grape leaves and current Codex MRL for commodities similar to vine leaves, and selected properties of the three OP pesticides (from Lewis et al. [31]).*

Pesticide	MRL-EU (mg/kg)	MRL-Codex Alimentarius (mg/kg) for similar commodities	Koc	Kow	Half-life on plants (days)	Photolysis half-life (days)
Chlorpyrifos	0.05	0.5 (grapes)	8151	50100	3.3	29.6
Chlorpyrifos-methyl	0.05	0.5 (grapes)	4645	10000	2.4	1.74
Diazinon	0.01	0.5 (lettuce leaf)	609	4900	2.4	50
Dimethoate	0.01	0.5 (peppers)	8	5.06	3.7	175

2.4. Statistical Methods

In most of the cases pesticide residue dissipation curves can be described mathematically by a first order decay model [32- 38]. Occasionally other mathematical models have to be applied to describe the decay [12]. Results obtained from the analysis of sampled vine leaves were plotted and statistically analysed using the statistical software R (R Core Team, 2017) [39]. In this study, where possible a first order decay model was used to describe the data according to Equation 1:

$$C_t = C_0 \times \exp(-k_{diss} \times t) \quad (1)$$

Where C_t is the residual pesticide concentration in the vine leaves (mg/kg) at time t, C_0 is the initial pesticide concentration, in mg/kg on the harvested plant material immediately after pesticide application, t the time since spraying and k_{diss} is the dissipation constant, which is related to the rate of pesticide breakdown. Equation 1 is non-linear and implicitly assumes a constant variance along time. A logarithmic transformation is therefore applied resulting in Equation 2, which is linear:

$$\text{Ln}(C_t) = \ln(C_0) - k_{diss} \times t \quad (2)$$

Equation 2, was applied to fit the residue data obtained for all the pesticides. An alternative approach was required for diazinon. In the case of diazinon, a biphasic model was required to obtain homoscedasticity, i.e. homogeneity of variances. This was achieved by minimising the sum of

squares of the residuals on the logarithmically transformed data. Rearranging Equation 2 and solving for k_{diss} yields Equation 3:

$$k_{diss} = (\ln (C_0) - \ln (C_t)) / (t) \quad (3)$$

k_{diss} represents the slope of the regression line. From this, one can finally obtain the half-life ($t_{1/2}$), defined as the time needed to reduce the initial pesticide residue level to half of its initial value [33], and calculated from Equation 4:

$$t_{1/2} = \ln (2) / k_{diss} \quad (4)$$

For each pesticide there were two data series, based on the different study areas. Where these two series had different starting residue levels at the time of the first sampling, a convenient method for estimating the initial application residue level was to use the intercepts of the regressions models for each data series separately. The maximum intercept value was used further to estimate the half-life and the required WHP for that pesticide. The WHP was estimated as the time needed for the residues to dissipate to values equivalent to the MRL after pesticide application (time 0) using the established regression model. The Equation used was:

$$WHP = [(intercept - \ln (MRL\ value)) / k_{diss}] \quad (5)$$

Paramasivam et al. [40] used Equation 5 to calculate the pre-harvest interval (PHI) defined as the maximum time (days) required for the residues to fall below the MRL. Confidence limits (CL) for the regression curves were obtained from Equation 6:

$$CL = X \pm t\ SE \quad (6)$$

Where t is the Student's t value and SE is the standard error. The statistical model used to describe the data included additional terms to take into account the effect of the study areas, the effect of washing and their interactions. Where the results indicated that these terms were not significant, then the model was simplified and those additional terms deleted. Confidence limits for the WHP were obtained from the confidence limits of the regression slope, using a stepwise approach, at the point of intersection between the regression line and the MRL. The first step in estimating the confidence limits for the WHP was to estimate the standard error of the fitted line at the intersection point of the regression line with the MRL value (as shown in Figure 1). Finally, the standard error value (SE) was divided by the slope (with its sign) of the regression line to obtain the SE of the WHP. The confidence interval for the WHP was then obtained from Equation 6. The appraisal of the withholding period required to achieve the MRL value for diazinon was estimated using a two-compartment model following a Newton-Raphson technique [41]. Confidence limits of the required withholding period were estimated for the two-compartment model using a bootstrap technique [42], which was used to obtain 1000 estimates. The quantiles of the estimates obtained were used for the estimation of the confidence limits of the withholding period.

3. Results and Discussion

For each of the organophosphate pesticides in this investigation, the residues data obtained from the analytical method were mathematically transformed according to Equation 2 and, where possible, a first order decay model was fitted to the data using the statistical software R and following the equations above. Table 3 presents a summary of the WHP for the studied pesticides.

Table 3. Comparative summary of the withholding period (WHP) for chlorpyrifos, diazinon and dimethoate according to the Codex and EU MRL. Estimated confidence intervals (in days) are for unwashed leaves. Lower confidence level (LCL) and upper confidence level (UCL).

Pesticide	Estimate WHP (Codex)	LCL-UCL	Estimate WHP (EU)	LCL-UCL
Chlorpyrifos	26.0	22.2-29.5;	37.0	31.6-42.1
Diazinon	8.9	6.0-10.6	37.1	n/a
Dimethoate	19	18-20	33	31-34

3.1. Chlorpyrifos Methyl

No detectable residues were found for chlorpyrifos methyl (CPM). None of the samples analysed showed CPM residues above the limit of detection. This behaviour can be explained by photolysis, as shown by the photolysis half-life data in Table 2, and a fast dissipation rate of CPM under the local conditions in Syria. This is in line with previous findings about this pesticide for which the half-life ranged from 0.97 to 3.27 days in Italy [17]) or 0.9-2.6 days on different foliage [7]. The concentration of chlorpyrifos methyl applied at the Al-Hamah site was very small (200 mL of 20% pesticide formulation dissolved in 50 L of water) compared to that used at the Al Soujah site (280 mL of 20% pesticide dissolved in 7 L of water). However, there is no information about the authenticity of the pesticide formulation. No statistical analysis could be carried out on the data relating to CPM.

3.2. Chlorpyrifos

The details of the regression model of chlorpyrifos presented in Table 4 indicates that there is an effect for the variables time and study area (Al-Hamah and Al Soujah), but there is no interaction between the variables.

Table 4. Details of the regression model of chlorpyrifos residue data (in natural logarithm scale) for dissipation time and study area.

Parameter	Statistical estimate	Standard Error	t value	Pr (>|t|)	Lower 95%	Upper 95%
Time 0 (intercept value)	4.73	0.19	24.76	<< 0.001	4.34	5.12
Dissipation time (slope value)	-0.209	0.016	-13.00	<< 0.001	-0.243	-0.1176
Study area (slope value)	-1.589	0.233	-6.83	<< 0.001	-2.069	-1.1096

That means it is possible to construct separate regression models for each of the study areas. However, it is acceptable to build a regression model with an average slope from both series [42] as shown in Figure 1A.

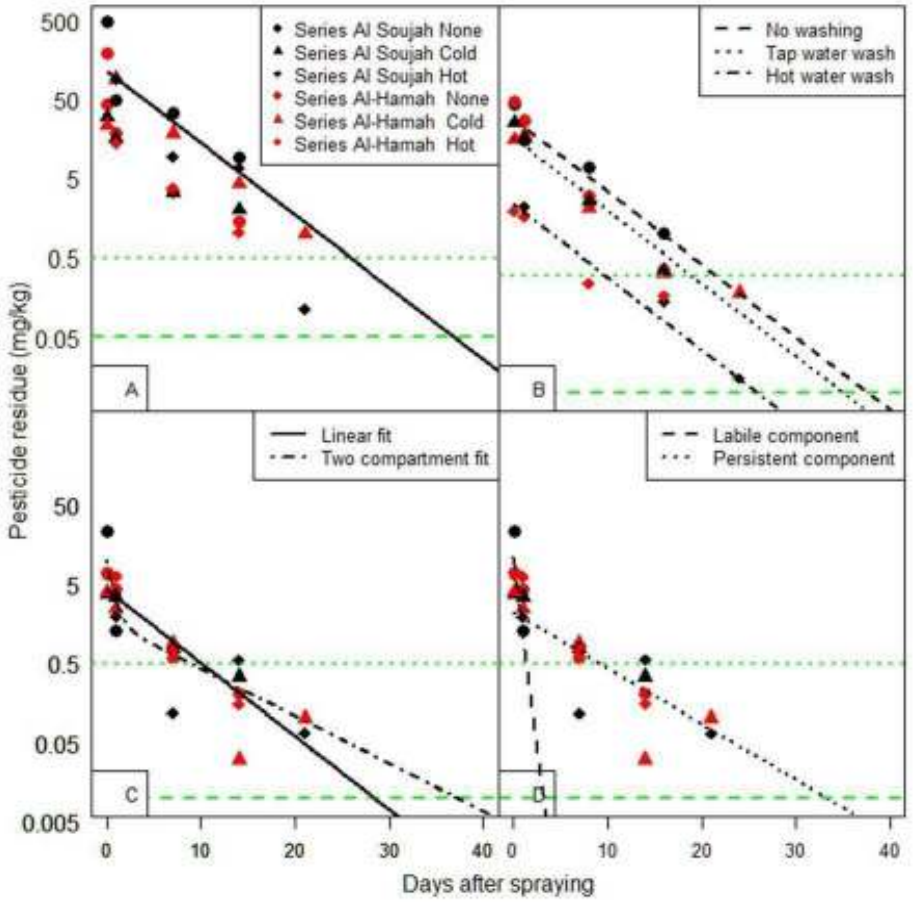

Figure 1. A) logarithmic decay of chlorpyrifos for the estimation of dissipation of chlorpyrifos; the fitted line was based on Al Soujah data; B) logarithmic decay of dimethoate for the estimation of dissipation of dimethoate and showing the effect of washing; C) logarithmic decay of diazinon for the estimation of dissipation of diazinon and showing linear and two compartment models; D) showing the modelled dissipation of the labile and persistent compartments for diazinon.

In this study the first sample of vine leaves was collected only after 60 minutes from pesticide application, due to manpower constraints and other operational factors. The volatile losses from leaves that may have occurred immediately after application can in fact be substantial [43]. Therefore, the initial application residue data, at time 0 hours, was estimated from the intercept of the regression model from the highest residue data set (Al Soujah). This conservative approach was taken to avoid overestimating the half-lives. The intercept (i.e. time zero) was estimated from the constructed models. The initial pesticide residue loads for the two study locations were 114 mg/kg (Al Soujah series) (back transformed data for 4.73) and 23.22 mg/kg (back transformed data for 3.14) (Al Hamah series), see Table 4.

Although the initial loads seemed different between the study sites, from a statistical point of view there was no significant difference. The estimated half-life for chlorpyrifos was calculated using Equation 4, which gave an estimate of

3.3 days. Using the standard error of the slope (0.016), 95% confidence limits for the half-life were established at 2.9 – 3.9 days. The estimated half-life was similar for both data sets from each study area. These results for half-life values for chlorpyrifos are in line with previous ranges of 2.9-4 days as reviewed by Willies et al. [7] and Lu [10] in various foliage crops. The suggested withholding period (WHP) for chlorpyrifos was estimated using Equation 5. A key component of the estimate of the withholding period was the initial load. For chlorpyrifos this varied widely between the two study areas – each estimate having its own confidence limits. Several estimates of the initial load were, therefore, considered, including the average of the estimates, the maximum of the two estimates and the upper confidence limit of the upper estimate. The latter was chosen as a worst-case scenario, providing a safety margin when estimating the WHP. The initial load corresponded to 168.9 mg/kg. Depending on which MRL was used for the calculation, the WHP for chlorpyrifos, i.e. the time required to reduce the initial load of 168.9 mg/kg to 0.5 mg/kg if using the Codex MRL, or to 0.05 mg/kg if using the EU MRL for vine leaves as shown in Table 2, ranged from 26 to 37 days respectively. The WHP for chlorpyrifos is shown in Figure 1A as the time corresponding to the intersection point between the regression line and the MRL. This difference in WHP is significant and implicitly influences the agricultural practices in a relevant way. It also implies that vine leaves should not be used for human or animal consumption for the entire duration of the WHP. Approximate confidence ranges for the withholding period (based on the initial load of 168.9 mg/kg) were then 22.2-29.5 days (using Codex MRL) and 31.6-42.1 days (using EU MRL). These confidence limits can only be considered as theoretical extrapolations assuming that the dissipation behaviour of chlorpyrifos approximated a linear function, as samples of vine leaves were not collected at those times. Each collected sample of vine leaves was also washed using tap water and boiling water. In on the case of chlorpyrifos, there was no statistically significant effect of washing the vine leaves with tap water or hot water. There was also no interaction between washing and the effect of dissipation in time. That means that washing the leaves did not decrease the chlorpyrifos load at any of the residue levels studied.

3.3. Dimethoate

In the case of dimethoate there was no statistically significant difference between the data sets from the two study areas but there was a large difference between the washing treatments. There was no interaction between the washing treatments and the holding time. Washing with water removed the same fraction of the pesticide over time. Details of the initial estimated loads are given in Table 5.

Table 5. *Details of regression of dimethoate against washing type and time.*

Washing	Estimate (ln)	Std.Error	Estimated initial residue level at t0 (mg/kg)	t value	Pr (>|t|)
No washing	3.34	0.18	28.2	18.13	<< 0.001
Tap water	2.79	0.18	16.30	15.15	<< 0.001
Hot water	0.85	0.18	2.3	4.62	<< 0.001
Slope	-0.242	0.013		-18.9	<< 0.001

There was no significant difference between the two study areas. One of the results from the hot water wash series in area 1 (Al Soujah) had an initial value comparable to that of the samples with no washing. This is most probably an outlier but was included in the analysis as there was no valid reason for rejecting it. An estimate of the half-life was obtained using Equation 4 which gave an estimate of 2.9 days. By using the standard error of the slope, 95% confidence limits for the half-life were 2.6 – 3.2 days. These estimated values are in line with previous findings for which the half-lives for dimethoate were in the range of 0.8-7.2 days depending on the crop [43]. The time required to reduce the initial load of 28.2 mg/kg (back-transformed value of 3.34) to the MRL value was calculated according to Equation 5 and shown in Figure 1B. The WHP for unwashed leaves was estimated as 19 days to accomplish the Codex MRL and 33 days to reach the EU MRLs, with approximate confidence intervals for the WHP as shown in Table 6.

Table 6. *Estimated confidence intervals (in days) for withholding period for dimethoate for washed and unwashed leaves. Lower confidence level (LCL) and upper confidence level (UCL).*

	With EU-MRL of 0.01 mg/kg			With Codex Alimentarius MRL of 0.2 mg/kg		
Washing	LCL	Estimate (days)	UCL	LCL	Estimate (days)	UCL
None	31	33	34	18	19	20
Tap water	29	31	32	16	17	17
Hot water	22	23	24	8	8	9

These differences in WHP demonstrate the need to adopt the agricultural practices that best protect consumers and the environment. The MRL is a conventional measure that needs to reflect the conditions of use under specific environmental conditions, hence the need to generate sufficient support data, from all regions in the world, that can help set and harmonize MRLs in international meetings and bodies such as the Codex Alimentarius Commission. Cabras et al. [44] provided data on the dissipation of dimethoate on grapes, showing that after 8 days from treatment 80% of dimethoate was dissipated in the field. The final residue then remained constant in the following 3 weeks. As to the effect of

washing, details of the regression of dimethoate against the washing regimes and holding time (average slope) and the series is given in Table 6. Figure 1 B shows the effect of washing on the dissipation of dimethoate. There was a very significant effect of hot water washing with an effective reduction of 92% of the residue level by washing the leaves with hot water. There was also a reduction of the residue level by washing with tap water but that effect was not statistically significant. This result is in line with results presented in an extensive review by Kaushik et al. [28], which demonstrates that washing and cooking help eliminate most of the pesticides in different food commodities.

3.4. Diazinon

In the case of diazinon the linear regression model according to Equation 2 fitted the data very poorly (correlation r^2= 0.78). Diazinon showed a rapid decomposition phase in the first day (s) followed by a slower decomposition phase (see Figure 1C). This observation and the difference in dissipation kinetics may be explained by the fact that pesticides applied to the vine leaves may be adsorbed, absorbed, altered, volatilized, or removed by water and the integrated results of these processes may be seen as an initial rapid decline in surface residues followed by a slower, asymptotic decrease [36]. Environmental factors, especially sunlight through photolysis, may cause a very rapid dissipation of diazinon within days from pesticide application. In addition, some of the diazinon may be absorbed by the plant tissues, reaching dynamic equilibrium and degrading thereafter very slowly [45].

An alternative two compartment model was therefore used for diazinon, as discussed by Torabi et al. [45]. The model is described by Equation 7, and graphically shown in Figure 1D.

$$C_t = C_A \times \exp(-k_1 \times \text{time}) + C_B \times \exp(-k_2 \times \text{time}) \quad (7)$$

Where C_A was the initial amount of rapid phase (estimated at t=0) and k_1 the dissipation rate in the rapid dissipation phase and C_B the amount in the slow phase (estimated at time t= change of slope) and k_2 the dissipation rate in the slow dissipation (persistent) phase. The parameter estimates for Equation 7 are shown in Table 7.

Table 7. Details of linear regression of diazinon residue data (in natural logarithm scale) against dissipation time for fast and slow phases.

	Intercept		Slope	
Phase	Estimate	Std. Error	Estimate	Std. Error
Fast	2.109	0.311	-1.043	0.420
Slow	0.296	0.533	-0.135	0.039

The slope of the regression line is derived by taking the differential from Equation 7 and estimated as shown by Equation 8:

$$K_{diff}(\text{Slope}) = -CA \times k_1 \times \exp(-k_1 \times t) - CB \times k2 \times \exp(-k_2 \times t) \quad (8)$$

The estimated half-life of diazinon varies with time since the initial application of pesticides as shown in Figure 2.

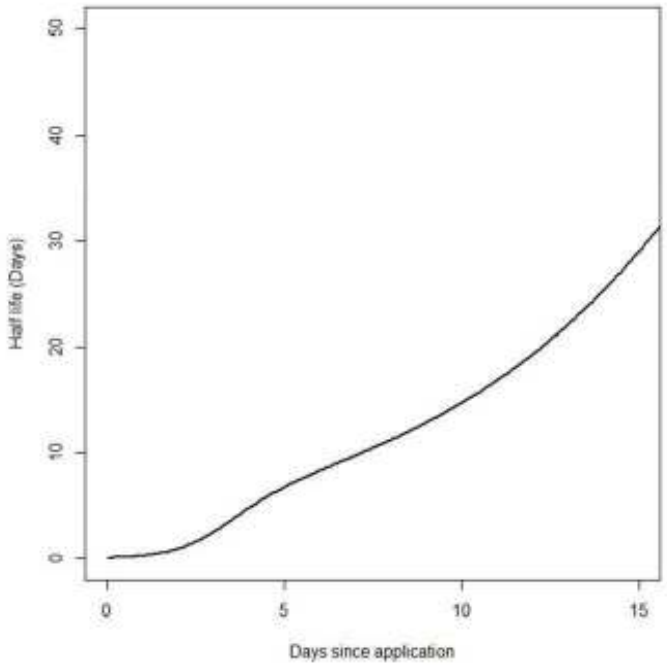

Figure 2. Changes in estimated half-life for diazinon with time since pesticide application.

These results are in line with previous findings by Torabi et al. [45], Willis et al. [43], where half-lives data for diazinon were in the range of 0.4-5.3 days depending on the crop. The estimation of the initial load of diazinon at time 0 (application time) was estimated in two different ways. A direct method was to sum the C_A and C_B terms from Equation 7 (Table 8) and this gave an estimate of 10.06 mg/kg.

Table 8. *Details of two compartmental model for diazinon residue data. Indication of the regression estimates for the slow component.*

Parameter	Estimate (mg/kg)	Std. Error
CA	8.34	2.99
CB	1.72	2.61
k1	1.53	1.01
k2	0.139	0.16

An alternative method was to use the linear approximation, using the sum of the back-transformed intercepts, as shown in Table 7, which gave an estimation of 9.58 mg/kg. The two values are not significantly different. The WHP was then estimated based on the two-compartment model using the slow component. This gave an estimate, using the Codex MRL, of 8.9 days with confidence intervals of 6.0-10.6 days. The estimated time to achieve the EU's MRL was 37.1 days. The latter value is outside the range of the sampling time and is therefore considered as an approximation only, and no confidence limits can be provided. It is important that estimates of WHP are based on residue data from a sampling regime that includes the estimated time as one cannot be sure that the behaviour of the pesticides in the field follows a linear trend. In relation to the washing of the leaves for diazinon, there was no statistically significant effect of washing the vine leaves with tap water or hot water. There was also no interaction between washing and the effect of time. Although the difference was not statistically significant, the no wash treatment had more pesticide, about 30% more than the cold wash. Kaushik et al. [28] reviewed food processing treatments such as washing and cooking, among others, and demonstrated that in most cases washing and cooking particularly lead to large reductions in pesticide residue levels. Angioni et al. [46] demonstrated that washing with tap water reduced the residues of azoxystrobyn and fenhexamid but not pyrimethanil. Similarly, in relation to washing, the three pesticides for which data were collected in this study behaved in very different ways. For better comprehension of the observed behaviour, Table 2 presents selected properties of the three studied pesticides. Chlorpyrifos is not a systemic insecticide [1], however it has a high Kow/Koc and would have been strongly bound to the cuticles of the leaves, so there would have been very little wash-off. This is in line with studies by Teixeira et al. [47] and Buschhaus et al. [48] on the composition of the epicuticular composition of leaves and their water barrier action as well as the mobility of pesticides in the plant where lipophilicity and concentration of the active ingredient are the driving forces in the transfer. Adjuvants in commercial formulations may also regulate the transfer processes. Ling et al. [49] came to similar conclusions for this pesticide. Dimethoate has a low Kow/Koc and hence the wash-off was effective – especially when hot water was used. The dissipation of dimethoate in time, and especially the persistent portion, may be explained by translocation of the residues into other plant compartments, since it is classified as a systemic pesticide [47]. This is also in line with studies by Cabras et al. [50], where he showed that dimethoate degraded rapidly during the first week after pesticide application but remained constant in the following two weeks. The intermediate Kow/Koc values for diazinon may have resulted in a fraction of the pesticide being gradually absorbed into a protected layer, i.e. the epicuticular wax of the leaves, which progressively increases in terms of yield during the surface development of the vine leaves as suggested by Buschhaus et al. [48] and previously by Baker et al. [51], Torabi et al. [45], Willis et al. [7], Lu et al [10]. The results from the present study agree with previous data for OPs. Willis et al. [7] reported average values of half-life for OPs of 3.0 ± 2.7 days for foliage. Cabras et al. [50] reported half-lives for OPs in grapes, wine and their processing products ranging between 0.97 and 3.84 days. Angioni et al. [22] showed decline curves for boscalid that do not follow a linear decay curve and stressed the idea that field agricultural practices critically influence the decrease or the disappearance of pesticide residues. Fantke et al. [13] provided impressive information by reviewing 811 publications in the scientific literature. Comparative assessment showed that that 95% of all half-lives, for all pesticide-plant species combinations, fall within the range of 0.6 and 29 days. Marin et al. [14] found that dissipation rates for selected pesticides (especially cyprodinil) were higher in the field at ambient temperature and with natural light than in cold conditions and in darkness. Chemical degradation caused by high temperatures and solar radiation is an important factor in pesticide dissipation kinetics. In the study presented here, environmental conditions possibly favoured chemical degradation in the field, which would have contributed to the short half-life of the studied OP pesticides.

4. Conclusions

Vine leave samples were collected over a 21-day period and analysed to assess the decay of four OPs, chlorpyriphos-methyl, chlorpyriphos, diazinon and dimethoate. Analysis of the data provided an indication of the dissipation rates, the half-lives and the WHPs. The three pesticides that could be evaluated behaved in very different ways. For diazinon, regression analysis could not fit the dissipation data to a first order decay model. The estimate of the half-life and withholding time for diazinon depended on the model used. The results from the present study are in line with previous data for organophosphorus pesticides. As part of the study

vine leaves were also washed with cold and boiling tap water. The data indicate that washing with hot water removed approximately 92% of the dimethoate residues. This offers a practical method of decreasing the intake of that pesticide and directly protects consumers of, for example, stuffed leaves. To conclude, the reported study can be replicated and applied in field monitoring, with the objective of gaining information on the results of current agricultural practices, thus contributing to set MRLs under the conditions of use in countries worldwide.

Acknowledgements

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Appendix

The study areas are presented in Figures A1 and A2. They are Al Soujah (Yafour) area, located 23 km west of Damascus, and the Al-Hamah area located 14 km north west of Damascus respectively.

Figure A1. Al Soujah area. Vineyard surrounded by olive trees. Located west of Yafour.

Figure A2. Al Hamah area, a river crosses the area.

Table A1 gives the list of the pesticides, their retention times, the transitions used for quantitation and confirmation together with the optimised collision energies (CE) and the corresponding retention time windows in the multiple reaction monitoring (MRM) acquisition method obtained using a gas chromatograph coupled to tandem mass spectrometry (GC-MS/MS).

Table A1. *List of pesticides, their retention times, the transitions used for quantitation and confirmation together with the optimized collision energies (CE) and the corresponding retention time windows in the multiple reaction monitoring (MRM) acquisition method.*

Pesticides	MRM Time segment	RT (min)	Quantifier transition 1	CE 1 (V)	Qualifier transition 2	CE2 (V)
Chlorpyrifos	11	12.0	196.9 -> 169.0	15	198.9 -> 171.0	15
Chlorpyrifos-methyl	8	10.8	285.9 -> 92.9	25	287.9 -> 92.9	25
Diazinon	6	9.5	137.1 -> 84.0	10	137.1 -> 54.0	20
Dimethoate	4	8.6	86.9 -> 46.0	15	92.9 -> 63.0	10
Internal standards						
Sulfotep	3	8.0	201.8 -> 145.9	10	201.8 -> 81.9	25
Triphenyl phosphate	19	15.3	326.0 -> 325.0	5	214.9 -> 168.1	15

Method validation information for each pesticide including method limit of quantitation (LOQ), and the precision data obtained during method validation are presented in Table A2. The limit of quantitation (LOQ) of the method was the lowest fortification level of the validation meeting the method performance acceptability criteria and it was obtained from recovery studies.

Table A2. *Method validation information for each pesticide including method limit of quantitation (LOQ), average recovery and intermediate precision data (repeatability and reproducibility relative standard deviation) at 0.01 mg/kg level.*

Pesticide	LOQ (mg/kg)	Average recovery (%)	Repeatability RSD (%) at 0.01 mg/kg	Reproducibility RSD (%) at 0.01 mg/kg
Chlorpyrifos	0.01	83	15	16
Chlorpyrifos-methyl	0.01	81	13	15
Diazinon	0.01	82	13	14
Dimethoate	0.01	81	9	9

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2.4 Optimization and validation of a single method for the determination of pesticide residues in *Peumus boldus Molina* leaves using GC-MSD, GC-MS/MS and LC-MS/MS

This paper addresses the analytical optimization and the validation of a trace residue method for *Peumus boldus Molina* leaves (Boldo), a South American herb, and a minor crop of the region. Boldo is a medicinal herb, and as such, a very complicated matrix from an analytical point of view: it contains terpenes, alkaloids, phenolic acids at a concentration that are 100 times higher than the trace pesticide residues, which were the analytical targets of this study. The novelty of this study was the use of thin layer chromatography (TLC) to address the optimization of the analytical conditions for clean-up. TLC was used as a quick evaluation tool for the identification of the combination of sorbents that provided the "cleanest extracts" for chromatographic determination. The challenge was to "reduce" the amount of matrix coextractives as much as possible, without reducing the recovery of analytes, to be able to determine traces of pesticide residues with a reasonable analytical performance. A trade-off is fundamental in the analysis of herbs and spices, provided method performance is demonstrated to be within acceptable ranges.

The optimized method was validated for more than 50 pesticides covering the analytical range of 0.01-0.02 mg/kg.

As mentioned, the trade-off was that matrix effects were still present and highly significant for the majority of analytes. Therefore, analytical strategies to compensate for matrix effects were implemented as an additional tool and discussed in the paper.

The collaboration between FAO/IAEA and GACT was very efficient and helped validating the method using all available instruments, at both laboratories, ranging from LC-MS/MS to GC-MSD and GC-MS/MS, thus providing an option to apply the methodology in countries where tandem mass spectrometry is still not available. While this study was conducted around a local South American matrix to contribute to regional R&D efforts, the following study targeted a spice, such as turmeric, traded globally.

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Optimization and validation of a single method for the determination of pesticide residues in *Peumus boldus* Molina leaves using GC-MSD, GC-MS/MS and LC-MS/MS

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ABSTRACT

The QuEChERS methods, CEN 15662 and AOAC 2007.01, and different clean-up variations were applied to the determination of pesticide residues in *Peumus boldus* Molina leaves, using LC and GC coupled to single and tandem mass spectrometry. Boldo belongs to the class of medicinal and aromatic plants. Because of its complex chemical composition including terpene peroxides, alkaloids and phenolic acids at concentrations typically 100-fold higher than that of a possible contaminant, it is a very challenging matrix for pesticide residue determination. Thin layer chromatography was used to evaluate the efficacy of removal of matrix co-extractives using different clean-up sorbents, including neutral alumina, graphitized carbon black (GCB), octadecyl reverse phase silica gel (RP-C_{18}) and pH modification in the extraction and clean-up steps. The most promising method was a combination of GCB, RP-C_{18} and primary-secondary amine (PSA) within the QuEChERS template. The final optimized method was validated according to the SANTE guidelines using LC-MS/MS and GC-MS/MS. The method scope includes 84 representative pesticide residues with recoveries ranging from 70 to 119% and within laboratory relative standard deviations below 15%. The method was initially validated for 25 representative pesticides using GC-MSD, making it applicable in those laboratories equipped only with a single mass detector. It is important to emphasize that the method complies with the requirements of international pharmacopoeias and food regulatory agencies, since herbal teas are found in both categories, as herbal remedies and foods.

1. Introduction

According to the World Health Organization, 70% of the world population uses non-conventional medicines, including medicinal plant treatments, at some point in their lives (Vogel et al., 2011; WHO, 2015). The market for medicinal and aromatic plants (MAPs) is expanding steadily, with a growing demand from consumers' worlwide. The global market for botanical and plant-derived drugs is expected to grow from USD 29.4 billion in 2017 to around USD 39.6 billion by 2022 with an annual growth rate of 6.1% for the period 2017–2022 (BCC Research, 2017). While MAPs were originally naturally growing in the environment and collected there, nowadays, due to their marketability, they are cultivated on a relatively extensive basis. This more extensive cultivation requires agricultural inputs, which depend on many factors, including the prevalence of insects and diseases in the field and during storage. As in any other crops, pesticides are used to ensure highe yields and adequate quality (Abhilash and Singh, 2008).

Much attention is paid to the development of analytical methodologies to assure the safe use of aromatic plants as medicines and food. The complex chemical composition of medicinal plants poses a challenge to the selective detection of contaminants present at trace levels. Some secondary metabolites have similar physicochemical properties to

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the pesticides under study, interfering with their determination. These metabolites are frequently present at higher concentrations than the pesticide residues that should be determined, forcing the analyst to implement complex purification processes. Such is the case with *Peumus boldus*, commonly known as boldo, a medicinal plant widely used in Latin America, the use of which is expanding to other parts of the world. The leaves of this Chilean tree are used for digestive and hepatobiliary disorders (Speisky and Cassels, 1994). The concentrations of terpene peroxides, alkaloids and phenolic acids in boldo leaves are higher than those of the suspected contaminants. The content of essential oils is approximately 2% w/w, mainly comprising ascaridol, a terpene peroxide. Boldo also typically contains alkaloids, mainly boldine, at approximately 0.5%, and polyphenols such as chlorogenic acid and shiquimates. This complex chemical composition makes analytical method development and validation rather challenging.

Different procedures such as matrix solid phase dispersion (MSPD), solid-liquid extraction (SLE) and solid phase extraction (SPE) have been applied for pesticide residue analysis in MAPs (Zuin et al., 2003; Pérez-Parada et al., 2011; Lozano et al., 2012; Pareja et al., 2015). With the advent of tandem mass spectrometry detectors, the sample preparation requirements have been reduced and are more straightforward. Nowadays, trends in pesticide residues analysis tend towards reduced sample size, solvent consumption and minimal clean-up steps. The QuEChERS method, introduced by Annastasiades & Lehotay (Anastassiades et al., 2003), is among the most widely employed methods for pesticide residues analysis due to its versatility. Variations of QuEChERS have been used for pesticide residue analysis in herbs, teas and spices, complex matrices that have in common high contents of pigments and secondary metabolites (Rajski et al., 2013). Modifications of this methodology have also been used for the determination of pesticide residues in camomile, medicinal plants from China and for a number of botanicals (Cajka et al., 2012; Lozano et al., 2012; Dzuman et al., 2015; Nie et al., 2015; Tripathy et al., 2017). The original QuEChERS method has been validated for the analysis of 24 pesticides in *Calendula officinalis* inflorescences (Besil et al., 2017). A citrate buffered version of the QuEChERS method was applied for the determination of pesticide residues in *Cannabis sativa* by LC–MS/MS (Pérez-Parada et al., 2016). Hayward et al. (2013) added a solid phase extraction cleanup step using PSA and GCB cartridges in tandem for the purification of acetonitrile extracts and applied this protocol to the analysis of various MAPs. Recently another unbuffered QuECHERS-based method was presented for the analysis of carbamate residues in different herbs without using PSA in the clean-up step (Nantia et al., 2017).

Many pharmacopoeias from all over the world include a special chapter for the determination of pesticide residues in MAPs. Within this context, the World Health Organization (WHO) advised that every country should have at least one official laboratory for pesticide residue analysis in MAPs and for quality control of those medicinal plants used as raw materials for phytopharmaceuticals (WHO, 2007). Pesticide residue analysis in MAPs according to pharmacopoeias such as the European (EU, 2019) or the MERCOSUR (GMC, 2016) Pharmacopoeias, are not performed following a fixed protocol. Instead, they require that the proposed methodology for the analysis of medicinal plants should be "fit for purpose" and validated according to the SANTE guidelines (DG-SANTE, 2015). Consequently, individual methods should be developed for the analysis of pesticide residues in MAPs due to their varied and different compositions. The presence of co-extractives in the extracts for pesticide residue analysis should be minimized to avoid excessive matrix effects and contamination of the analytical instrumentation. This is the case for boldo, an herb widely used for consumption as an infusion, and for which a pesticide residue analysis method is needed. As already described, it is an unusually complex analytical matrix because it contains high amounts of secondary metabolites from different chemical families. Variable amounts of chlorophylls and their degradation products can also be found in the primary extract for pesticide residue analysis. Boldo leaves are widely used and traded. In the nineteen-nineties more than 2000 tons per year of dried boldo leaves were collected from native trees and exported. Nowadays, due to its high consumption, cultivation systems have been developed and applied to domesticate boldo trees, which are native to South America but are now also cultivated in Africa, India and Europe. The agricultural practices and the technological package used depends on the region, therefore the pesticide residues can vary according to the geographical origin of the product (Bruneton, 1999). Due to the global trade of MAPs through complex networks, wide scope analytical methods for the determination of pesticide residues are required to cover the possible pesticide combinations used in different regions and ascertain the quality and safety of the herb. To the best of the authors' knowledge there is, to date, no specific multiresidue method for the monitoring of pesticide residues in boldo leaves that can cover the broad scope of agrochemicals applied in the different regions of the world were the crop is grown.

Using the advantages of the flexibility of the QuEChERS sample preparation technique and the sensitivity of triple quadrupole detectors coupled to gas or liquid chromatography, the development of a multiresidue method for pesticide residues in boldo was explored. The aim of the work was to find a suitable method for routine analysis. The final selected methodology was fully validated.

2. Material and methods

2.1. *Chemicals, materials, and standards*

Acetonitrile (MeCN) and ethyl acetate (EtOAc), both HPLC grade, were supplied by J.T. Baker (USA). MeCN (ACS grade) was provided by Dorwil (Argentina). Deionized water was obtained using a Thermo Scientific (Marietta, OH, USA) EASY0 pure RoDi Ultrapure water purification system. Anhydrous magnesium sulphate extra pure ($MgSO_4$), sodium citrate dibasic sesquihydrate, primary secondary amine (PSA), graphitized carbon black (GCB) and octadecyl reverse phase silica gel (RP-C_{18}) were obtained from Sharlau (Barcelona, Spain). Sodium chloride (NaCl) and methanol (MeOH) were purchased from Carlo Erba (France), and trisodium citrate dihydrate ($Na_3Cit{\cdot}2H_2O$) and alumina were from Merck (Germany). Dichloromethane was provided by Droguería Industrial Uruguaya (Montevideo, Uruguay). The precoated silica gel plates (Polygram® Sil G/UV_{254}) used for the TLC studies were from Macherey-Nagel (Hamburg, Germany).

High purity reference pesticides standards (see Supplementary Material S1) were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and HPC Standards GmbH (Cunnersdorf, Germany). Stock solutions (solution of 2000 mg L^{-1} aproximately) from each pesticide standard in MeCN or EtOAc were prepared. Working solutions were prepared at 1 mg L^{-1}, by taking appropriate aliquots of the stock solutions.

2.2. *Chromatographic equipment*

2.2.1. *GC-MSD*

The gas chromatograph (GC- 2010 Plus) coupled to a QP2010 Ultra mass spectrometer (Shimadzu, Japan), was equipped with a TR-5MS Thermo (30 m x 0.25 mm x0.25 μm) column. Electron impact (EI) mass spectra were obtained at 70 eV. The injector temperature was 230 °C; the interface temperature was 280 °C; the carrier gas was He with a flow of 1 mL min^{-1}. The oven conditions were: initial temperature 120 °C (5 min), increased to 190 °C at 10 °C min^{-1} (held for 1 min), increased to 250 °C at 5 °C min^{-1} (held for 5 min), increased to 280 °C at 5 °C min^{-1} (held for 5 min) and finally increased to 320 °C at 20 °C min^{-1} and held for 2 min (45 min total). Split injection mode was used with a split ratio of 12:1 and an injection volume of 1.0 μL. The MS system was programmed in the selected-ion monitoring (SIM) mode. Mass spectrometric parameters of each pesticide are presented in

Supplementary Material S1. LabSolutions GC–MS solution Version 4.11 SU2 from Shimadzu was used for instrument control and data processing.

2.2.2. GC–MS/MS

An Agilent 7890 B gas chromatograph coupled to 7000 MS/MS triple quadrupole system (Agilent Technologies, Santa Clara, CA) was equipped with two ultra inert HP-5 ms (Agilent J and W, Santa Clara, CA) columns (15.0 m x 0.25 mm i.d., 0.25 μm). The system was operated with a 5 min post-run backflush. The multimode injector was programmed so that it started at 70 °C for 0.02 min and then ramped up, at the rate of 850 °C min^{-1}, to 320 °C, kept at this temperature for 5 min and then cooled down at a rate of 20 °C min^{-1} to 280 °C. The interface temperature was 280 °C; the carrier gas was helium (high purity, 99.999%) with a flow of 1.25 mL min^{-1} in column 1, and 1.45 mL min^{-1} in column 2. The oven conditions were 70 °C (initial (1 min), increased to 150 °C at 50 °C min^{-1}, increased to 200 °C at 6 °C min^{-1} and increased to 280 °C at 20 °C min^{-1} (24 min total). Splitless injection mode was used, with an injection volume of 2.0 μL into a liner with a glass wool plug. The retention-time lock setting (RTL) used chlorpyrifos-methyl as the locking compound at a retention time of 18.51 min. The ion source and the quadrupole analyser temperatures were fixed at 230 °C min^{-1} and 150 °C min^{-1}, respectively. High purity nitrogen (99.999%) was used as the collision gas. Multiple reactions monitoring (MRM) acquisition mode was applied for pesticide analysis and the spectrometric conditions are presented in Supplementary Material S1. Instrument preliminary setup included the optimization of collision energies for each MRM transition in the range 5–50 eV. The solvent delay was 4 min. Agilent Mass Hunter Quantitative Analysis B07.00 software was used for acquisition and data processing.

2.2.3. LC–MS/MS

The analysis was performed with an Agilent 1200 LC system coupled to a 4000 QTRAP LC–MS/MS System from AB SCIEX in the scheduled MS-MS mode. The chromatographic separation was performed on a ZORBAX Eclipse XDB-RP-C18 (150 mm × 4.6 mm, 5 μm) column setting thecolumn oven temperature at 20 °C. The mobile phases were (A) 0.1% formic acid in water and (B) MeCN. The gradient elution program started with 10% B for 3 min, then increasing to 100% B over 17 min and holding at 100% B for 5 min. Finally, the system returned to the original conditions over 3 min and equilibrated for 5 min (33 min total run). The injection volume was 5 μL and the flow rate 0.6 mL min^{-1}. The source temperature was 500 °C, the ionization voltage was 5000 V, the curtain gas was nitrogen at 20 psi and the nebulizer gas was air at 50 psi. The MRM mode was performed for tandem MS detection. The optimal MRM conditions for each analyte were optimized using direct infusion in the ESI + mode. The MS/MS settings (transitions, collision energy and declustering potential) used in this study are presented in Supplementary Material S1. Scheduled MRM was used with a 90 s detection window covering the expected retention time of each analyte and the target scan time was 2 s for all pesticides. To acquire and process the data, Analyst software version 1.5.1 from AB SCIEX was used.

2.3. Boldo samples

Entire Chilean boldo leaves were purchased from a local company (Botica del Señor, Montevideo, Uruguay). All *Peumus boldus* leaves were purchased as dry leaves, 8–10% moisture w/w, as they are usually marketed. Each entire batch was processed in an herb grinder and milled in an IKA®-WERKE Model M20 (USA) with a 2 mm in diameter sieve. The blank samples were from a declared organic store. This samples were screened for pesticide residues before method validation.

2.4. Sample preparation

Two grams of ground and homogenised boldo leaves were weighed into a Falcon tube and 10 mL of Milli Q water was added to hydrate for 30 min. The boldo samples were then extracted using different modifications of the QuEChERS CEN 15662 and AOAC 2007.01 methods as described below. Different combinations and different amounts of the salts/clean-up materials PSA; GCB; neutral alumina; $CaCl_2$, $MgSO_4$ florisil, and RP-C_{18} were tested. For each evaluated procedure 3 replicates were perfomed.

2.5. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was applied to study the co-extractive profiles obtained from different sample preparation procedures. Ten microliters of each studied extract were applied to the TLC plate. The mobile phase was dichloromethane – methanol (9:1). The plates were developed to a distance of 10 cm. After air drying, the plate was visualised under UV at λ = 254 nm and the purple spots marked on the plate. The TLC plate was then sprayed with the visualizing reagent (aq. H_3PO_4 10%- $CuSO_4$ 20%) and charred at 110 °C for approximately 10 min until purple and dark spots appeared.

2.6. Co-extractive evaluation

The tested methods were the acetate buffered QuEChERS (Method 1) (Lehotay et al., 2007) and the citrate buffered (Method 2) (Anastassiades et al., 2007) and 3 modifications, listed below as methods 2, 4 and 5 (The conditions changed in each consecutive method are marked in bold font):

Method 1: AOAC 2007.01 extract; 50 mg of PSA, 50 mg of GCB, 50 mg of RP-C_{18} and 150 mg of $MgSO_4$ per mL of extract.

Method 2: AOAC 2007.01 extract, 50 mg of PSA, 50 mg of GCB, 50 mg of RP-C_{18}, **150 mg of $CaCl_2$** per mL of extract (Lozano et al., 2012).

Method 3: CEN 15662 extract, 50 mg of PSA, 50 mg of GCB, 50 mg of RP-C_{18} and 150 mg of $MgSO_4$ per mL of extract.

Method 4: CEN 15662 extract, **100 mg of alumina**, 50 mg of GCB, 50 mg of RP-C_{18} and 150 mg $MgSO_4$ per mL of extract.

Method 5: CEN 15662 extract, **100 mg of florisil**, 50 mg of GCB, 50 mg of RP-C_{18} and 150 mg of $MgSO_4$ per mL of extract.

All the extracts obtained using the procedures described above were spotted on TLC plates where a mixture of ethyl acetate, hexane and formic acid. (8:2:0.1) was used as mobile phase to evaluate the presence of co-extractives after visualization with a solution of 5 g of $CuSO_4$ in 10% H_3PO_4.

2.7. Final method selected for validation

The final sample preparation procedure selected was method 4. The final method was as follows:

Homogenised boldo leaves (2.0 g) were placed in a Falcon tube and 10 mL of distilled H_2O were added. The suspension was vortexed and allowed to stand for 10 min to hydrate the leaves. Then, 10 mL of MeCN was added and the tube was shaken for 1 min by hand. Immediately, 4 g of $MgSO_4$ with 1 g NaCl, 1 g $Na_3Cit{\cdot}2H_2O$ and 0.5 g $Na_2Cit{\cdot}1.5H_2O$ were added and the tube shaken for 5 min by hand. The extract was centrifuged for 5 min at 2066 g. Five mL of supernant were taken and placed in a clean-up tube containing 250 mg of PSA, 250 mg of GCB, 250 mg of RP-C_{18} and 750 mg of $MgSO_4$. The suspension was vortexed for 1 min and centrifuged for 3 min at 2066 g. From the upper layer, 2.7 mL were taken and placed in a Turbovap tube and dried under a N_2 stream. The residue was finally redissolved in 1 mL of ethyl acetate containing 1 mg mL^{-1} of TPP, the internal standard, and subjected to GC–MS/MS or GC-MSD analysis. From the same final extract 1 mL was taken for direct analysis in the LC–MS/MS.

Table 1
Percentage of recovery (Rec%), relative standard deviation (RSD%) for each analyte in method 2 and 4 by GC-MS and LC-MS/MS.

Pesticide	GC-MS				LC-MS/MS			
	Method 2		Method 4		Method 2		Method 4	
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)
Acephate	–		–		Only analyzed by GC-MS			
Acetamiprid	Only analyzed by LC-MS/MS				96.8	1.4	88.1	2.0
Azoxystrobin	83.2	4.0	82.2	10.9	101.0	3.8	94.5	4.0
Carbaryl	92.6	8.0	88.7	11.5	100.1	2.0	80.9	0.7
Carbendazim	Only analyzed by LC-MS/MS				47.0	4.4	50.2	9.9
Cypermethrin	86.1	6.1	83.3	9.8	Only analyzed by GC-MS			
Diazinon	98.3	9.2	90.3	5.3	99.1	0.9	92.3	1.9
DDT	109.7	10.9	72.9	11.7	Only analyzed by GC-MS			
Difenoconazole	71.2	7.5	74.4	9.7	86.3	2.5	81.3	0.3
Dimethoate	98.8	10.8	78.4	6.4	98.5	2.3	91.0	0.4
Endosulfan α+β	113.5	6.1	109.4	7.8	Only analyzed by GC-MS			
Imidacloprid	Only analyzed by LC-MS/MS				96.0	2.8	86.3	1.0
Lambda -cyalothrin	80.2	7.0	78.6	3.6	Only analyzed by GC-MS			
Malathion	122.9	7.8	119.7	10.1	96.8	1.5	90.1	4.5
Methalaxyl	Can not be evaluated		76.9	3.0	99.1	0.9	92.3	1.9
Methamidophos	–		–		49.9	6.2	73.7	7.1
Methidathion	87.3	4.3	90.1	11.3	103.4	2.9	90.9	2.9
Metolachlor	84.4	3.5	89.2	2.3	102.2	1.7	92.0	3.6
Pyraclostrobin	Only analyzed by LC-MS/MS				78.9	5.6	83.3	1.5
Tebuconazole	68.7	6.1	73.8	4.9	94.8	1.7	84.3	0.4
Thiamethoxam	76.9	22.4	56.2	16.1	93.2	1.6	96.6	2.5

2.8. Compound identification and confirmation

In all cases, the identification of analytes and confirmation of the results was performed according to SANTE (DG-SANTE, 2017) guidelines for each analytical instrument:

i) same retention time as the standard (±0.1 min);
ii) for GC-MSD, 3 fragment ions, and for GC–MS/MS and LC–MS/MS, 2 product ions (analyte peaks in the extracted ion chromatograms must fully overlap).
iii) in all cases: "Ion ratio from sample extracts should be within ±30% (relative) of average of calibration standards from same sequence" (DG-SANTE, 2017)

2.9. Method validation

The method was validated according to the SANTE document (DG-SANTE, 2017). Linearity, trueness, precision, limits of quantitation (LOQ) and matrix effects (ME) were evaluated. Linearity was evaluated in solvent and in matrix. Matrix-matched calibration, using pesticide free boldo leaves was performed to compensate for matrix effects and minimise quantification errors. The range evaluated was from 1 to 250 μg L^{-1} for GC–MS/MS and 10–800 μg L^{-1} for LC–MS/MS.

The matrix effects were calculated using the slopes of the curves prepared in solvent and in matrix.

Trueness, expressed as percentage of recovery, was evaluated at 50, 100 and 300 μg kg^{-1} for GC–MS/MS amenable pesticides and 10, 50 and 200 μg kg^{-1} for the LC–MS/MS amenables. For each level five genuine replicates were performed.

The intermediate RSDr (intra-day) was studied at the defined levels of concentration by quintuplicate analyses for each instrument.

The method-LOQ was defined as the lowest spiking level of the validation meeting the method performance acceptability criteria.

3. Results and discussion

3.1. Method selection

For the selection and evaluation of the analytical method to use on boldo leaves, a representative group of GC and LC amenable insecticides, herbicides and fungicides, belonging to different chemical families (organophosphates, carbamates, pyrethroids, neonicotinoids; chloroacetanilide, phenylamides; triazoles, strobilurins, and benzimidazoles), were chosen. The strategy was to test the QuEChERS CEN 15662 and AOAC 2007.01 methods and variations in the clean-up step using salts/materials with potential capability to eliminate most of the coextracted metabolites from the matrix. The coextractive profiles of each tested method were studied using TLC. Chen et al. suggests some variations to the clean-up salts that were used (Chen et al., 2012). Alumina, PSA, and florisil were used aiming at the removal of organic acids; the latter two also show high specificity towards some polyphenols and other glycosides. GCB was used to reduce the content of chlorophylls and pigments and RP-C_{18} was added to minimize the lipophilic non-polar compounds from the essential oils (Lehotay et al., 2007). Surprisingly, neither alumina, nor florisil were able to efficiently remove the coextrated compounds. $CaCl_2$ was used by Lozano et al. (2012), to avoid the presence in the extract of catecholic, phenolic and fatty acids that precipitate as calcium soaps.

It was found that boldo extracts treated with RP-C_{18} and GCB yielded cleaner extracts. This was observed on the TLC plate, where spot size is directly dependant on the concentration. The spots are less intense at higher R_f, due to the absortion by the RP-C_{18} sorbent during sample preparation of the non polar essential oils compounds.

The TLC objectively indicated the sample preparation procedure that gave the cleanest extract. The two best sample preparation procedures were selected and initially compared for their performance (recovery and RSD) using GC-MSD. The best methods selected using TLC were method 2 and method 4.

A comparision of both methods by GC-MSD and LC–MS/MS is presented in Table 1.

The study was first designed to be performed for the GC-MSD determination of pesticide residues in boldo leaves. The conventional acetate and the citrate QuEChERS extraction protocols using higher amounts of clean-up sorbents and salts yielded the most effective ways to eliminate the high co-extractives concentration. The multiresidue sample preparation method that can best clean the boldo leaves extracts, was also applied when using the tandem mass spectrometry detectors.

The extracts were then evaluated using LC–MS/MS. Table 1 indicates the pesticides that could be detected with method 2 and method 4, and the recovery and RSD obtained for each pesticide using both GC-MSD and LC–MS/MS.

Of the 18 pesticides tested by GC-MSD, 13 were also LC amenable pesticides. Only pyrethroids were exclusively GC amenable. On the other hand, neonicotinoids could be only analyzed by LC. Acephate, due to strong matrix interferences probably because of the coelution of ascaridol, gave unacceptable results in GC-MSD. However, acephate, as well as methamidophos, were successfully determined by LC–MS/MS. As expected, in a complex matrix such as boldo, the RSD range was broader in GC-MSD than LC coupled to tandem MS for both methods (Table 1). Method 4 showed consistently better recoveries than method 2 in GC-MSD). Metalaxyl could be only determined through method 4. Malathion yielded good recoveries in GC-MSD and better values by LC–MS/MS. Both methods showed similar precision (2.6 and 2.8%) but only method 4 successfully determined difficult compounds such as methamidophos or acephate. Therefore, method 4, a citrate buffered QuEChERS variation where the absorbents are used at higher levels than in the original CEN 15662, was chosen for validation using GC–MS/MS and LC–MS/MS systems.

3.2. Method validation

For the final validation, the scope of the method was expanded to a total of 84 compounds: 56 analytes were validated for analysis by LC–MS/MS and 65 by GC–MS/MS; 37 of the compounds could be analysed using both instrumental systems. Percentages of recovery and RSD at three levels and LOQs for each pesticide under study are shown in Table 2. The three instrumental systems yielded an average RSD for all the analytes of 10–11%, showing the precision of the method. Fenitrothion could not be detected at 10 μg kg^{-1}, and boscalid, carbofuran, clorpyrifos and kresoxim methyl had recoveries and RSD beyond the SANTE accepted boundaries at this level, so their LOQs were fixed at 50 μg kg^{-1}. For GC analysis, parathion methyl did not show a linear response. BupROfezin, iprodione, permethrin, pyriproxifen and trifloxystrobin were not detected at 50 μg kg^{-1} in GC-MSD (See Supplementary Material S2). The higher selectivity of the GC–MS/MS improved the method's scope compared to GC-MSD and also permitted the attainment oflower LOQs for iprodione, permethtyn and pyriproxyfen. It is noteworthly that difficult pesticides seldom addressed in multiresidue methods, such as captan, can be analyzed using this method.

3.3. Matrix effects

For GC–MS analysis 49% of the analytes presented high matrix effects (>50%), while the remaining 49% showed negligible matrix effect (<20%) (Fig. 1).

For the LC–MS/MS analysis, from a total of 58 analytes, 38% showed medium matrix effects (from 20 to 50%) while 31% suffered strong signal suppression (ME > 50%).

It is reported that, even if a sample extract has gone through extensive clean-up, there are still enough coextracted compounds to potentially cause signal suppression or signal enhancement, affecting quantitation adversely (Kryniitsky et al., 2017). For the method presented here, the co-extractives remaining in the final extract caused a matrix effect, typically suppressing the signal in LC analysis and suppressing or enhancing the signal in GC analysis. In GC and LC coupled to mass spectrometry, the causes of the matrix effects are different (Trufelli et al., 2011; Kwon et al., 2012; Silvestro et al., 2013). Signal supression occurs in GC when active surfaces in the system (injector, column, or detector) cause retention and/or degradation of analytes. However, the co-extractives can also act as analyte protectants and signal enhancement is observed in comparison with the analytes injected in solvent.

In all cases, the highest values observed in GC correspond to signal enhancemenet, as expected. Analytes that could not be detected by GC–MS such as the organophosphates, (malathion, parathion, acephate, methidathion), pyrimiphos or vinclozolin were analyzed by GC–MS/MS with good to very good precision. Some others, such as iprodione, and acephate could be analyzed at lower levels by GC–MS/MS. Imazalil could not be detected with GC-MSand yielded recoveries only between 50–60% with GC–MS/MS. Acceptable results were achieved for this compound only using LC–MS/MS.

The extent of the matrix effects in GC–MS/MS could not be determined due to the low instrumental response obtained for analytes in solvent. In addition, peak widening and tailing caused pesticides to elute outside the acquisition windows, and correct integration was impossible (Fig. 2).

This phenomenon was compensated for when a matrix matched calibration curve was used, due to the presence of co-extractives than act as analyte protectants in the calibrators. Nevertheless, despite the greater selectivity of GC–MS/MS compared to GC–MS, some interferences were observed for methamidophos, and omethoate, either masking the signals behind the matrix co-extractives or affecting the ratios of the ions so that the ion-ratio requirements were not achieved at low concentrations, thereby raising the LOQs.

In LC–MS/MS analysis, matrix effects are mainly produced during atmospheric pressure ionization and are an inherent aspect of electrospray ionization (ESI). Matrix components can affect the properties of the droplets (e.g. viscosity changes) and analyte ionization efficiency (Kostiainen and Kauppila, 2009).

The modifications to the methods and the amounts of salts/sorbents used in the clean-up step were optimized in order to cover the widest scope of compounds whilst trying to lower the co-extractives load. The use of the recommended amount of PSA, RP-C_{18} and GCB in the official QuEChERS methods, CEN 15662 and AOAC 2007.01 yielded extracts that were rather "dirty" and therefore fewer analytes could be detected. On the other hand, the use of higher amounts of these adsorbents to exhaustively remove coextractives also caused the loss of some pesticides, either due to their lipophilicity or their planar to semi planar configurations, by the RP-C_{18} and GCB respectively. The final method selected was a trade off between residue determination and high matrix effects.

3.4. LOQs and linearity ranges

The lowest concentration levels validated in GC–MS/MS and LC–MS/MS determinations using matrix-matched calibrations were 50 and 10 μg kg^{-1}, respectively. The LOQ for each analyte in each instrumental system was the lowest concentration at which the identification criteria were met, according to the DG SANTE guidance document (DG-SANTE, 2017). Thirty-seven analytes yielded recoveries between 70–120% by both instrumental methods. For such cases, the variations between the methods were within the assumed default expanded uncertaintiy (50%) of pesticide residue analysis, except for carbofuran and pendimethalin. From those 37 analytes, 32 could be analyzed through LC–MS/MS at 10 μg kg^{-1}. Exceptions, such as fenitrothion, showed the same LOQ in both instruments.

For most of the analytes studied the linearity range (R^2>0.99) was 12-600 μg kg^{-1} and 10-600 μg kg^{-1} for GC and LC–MS/MS, respectively.

4. Real samples analysis

Commercial samples were analyzed in order to investigate the applicability of the method. Twentysix commercial samples of *Peumus boldus*, purchased from local markets, were analysed. Four samples yielded positive results for carbendazim (230 μg kg^{-1}, EU MRL 100 μg kg^{-1}), acetamiprid (12 μg kg^{-1}, EU MRL 3 mg kg^{-1}), azoxystrobin (21 μg kg^{-1}, Codex and EU MRL 70 mg kg^{-1}) and pyraclostrobin (130 μg kg^{-1}, EU MRL 2 mg kg^{-1}). The MRLs showed correspond to the categories "Herbs" in *Codex* and the EU "Leaf vegetables, herbs and

Table 2
Percentage of recovery (Rec%), relative standard deviation (RSD%) for the validated method for each analyte by LC–MS/MS and GC–MS/MS systems.

Pesticide	LC-MS/MS Validation							GC-MS/MS Validation						
	Concentration level (µg kg^{-1})						LOQ (Limit of Quantification) (µg kg^{-1})	Concentration level (µg kg^{-1})						LOQ (µg kg^{-1})
	10		50		200			50		100		300		
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)		Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
2-phenylphenol								86.6	9.4	73.8	5.9	75.9	3.8	50
Acephate	107	17	75	4	75	1	10	< LD		135.3	11.8	83.4	3.3	100
Acetamiprid	105	7	79	3	81	2	10	69.5	17.1	62.2	15.5	75.4	6.8	
Aldicarb	93	18	79	17	87	9	10							
Azinphos methyl	109	13	61	9	103	9	10							
Azoxystrobin	120	4	96	5	98	5	10	105.3	7.1	112.3	3.0	111.4	4.1	50
Benalaxyl								89.7	7.7	81.4	3.7	86.5	4.1	50
Bifenthrin								64.3	8.6	57.9	4.0	57.8	6.3	
Boscalid	73	24	84	9	79	9	50	85.4	20.7	89.2	3.3	89.9	6.0	50
Bromopropylate								78.0	8.5	71.3	3.9	73.4	3.1	50
Buprofezin								< LD		95.9	11.6	65.4	4.3	
Captan								79.2	13.1	78.0	4.1	84.0	8.6	50
Carbaryl	71	2	91	5	92	2	10	91.7	8.7	86.6	4.8	88.1	2.4	50
Carbendazim	76	5	71	10	70	3	10							
Carbofuran	< LD		95	0	99	4	50	126.5	12.2	94.7	3.6	85.3	10.1	50
Chlorfenvinphos	78	18	72	5	88	4	10	86.6	8.6	84.1	4.1	88.5	3.5	50
Chlorothalonil								55.4	6.6	30.9	12.3	33.1	11.6	
Chlorpyrifos	42	28	84	15	92	13	50	67.1	12.5	63.8	4.6	66.3	4.3	
Chlorpyrifos methyl	76	17	73	4	80	11	10	74.0	9.1	67.9	4.6	69.3	6.6	50
Cyproconazole	110	5	81	5	79	5	10	93.1	8.3	85.0	3.0	89.3	3.5	50
Cloticlothin	104.9	8.7	88	6	89	4	10							
Cyfluthrin								84.0	7.4	77.1	3.8	73.2	3.0	50
Cypermethrin								76.7	12.1	75.8	4.2	69.9	6.2	50
Deltamethrin								79.0	7.3	74.4	4.0	67.6	4.6	50
Diazinon	116.5	13.8	69.3	5.9	86.3	2	10	74.3	11.2	75.4	5.5	81.1	5.8	50
Difenoconazole	85.4	6.9	77.7	4.8	77.5	2.5	10	86.2	8.5	92.0	4.8	91.7	4.6	50
Dimethoate	106.9	5.7	91.1	4.8	87.3	2.5	10	90.7	7.0	88.9	2.4	92.4	3.7	50
Endosulfan alpha								70.7	9.2	62.6	7.9	66.6	6.3	50
Endosulfan beta								91.2	7.5	88.4	6.8	90.2	3.1	50
Endosulfan sulfate								81.3	8.3	79.6	4.4	83.9	4.4	50
Epoxiconazole	76.1	9.4	81.6	6.4	78.7	2.8	10	77.9	9.6	76.1	4.3	83.2	4.4	50
Ethion	98.3	11.2	71.7	9.5	106.5	4	10	81.2	8.4	73.7	4.0	76.6	3.9	50
Ethoprophos								93.5	9.3	79.9	3.1	78.5	3.5	50
Fenhexamid								78.0	7.8	75.9	3.9	79.0	3.3	50
Fenpropathrin	–	–	111.7	8.6	94.7	5.6	50	85.6	12.1	83.7	4.2	91.7	3.6	50
Fenvalerate								80.1	7.8	75.2	2.9	78.1	4.3	50
Fipronil								105.8	7.5	100.5	5.4	107.1	3.4	50
Fludioxonil								87.4	8.1	81.0	3.4	83.6	4.6	50
Flufenoxuron	83	19.6	90.7	9.6	89.4	2.8	10							
Fluopicolide	86.6	9.8	85.8	4.9	91.7	2.9	10							
Flutriafol	72	1.1	54	3	75.8	1.6	10	90.2	8.1	84.3	3.6	90.6	3.2	50
Fluvalinate								84.8	6.8	77.4	4.8	71.9	4.5	50
Hexaconazole	95.2	10.4	91.2	4.4	74.6	1.1	10							
Hexythiazox	70.7	9.4	94.4	6.1	92.2	5.9	10							
Imazalil	86.1	12.7	100.8	11.3	71	6.4	10	63.6	12.7	58.4	2.2	66.4	5.5	
Imidacloprid	84.7	13.9	83.8	15.6	85.2	3	10							
Iprodione	112.9	17.1	78.9	7.4	81.1	3.8	10	113.2	7.2	105.6	4.1	107.3	4.7	50
Isoprothiolane	117.4	3.7	90.5	4.9	99.6	2.2	10							
Kresoxim methyl	40.9	21.5	78.9	7.4	81.1	3.8	50	91.7	8.7	86.6	4.8	88.1	2.4	50
Lambda -cyhalothrin														
Linuron	102.8	19.2	100.4	5.9	84	3.6	10							
Lufenuron	73.6	14.8	87.8	11.4	88.8	6.8	10							
Mikanozin	110.8	4.4	61.2	2.1	93.3	0.8	10	88.2	15.2	87.2	4.5	89.8	2.7	50
Mikanthon	118.4	12.2	71.6	4.9	90	4.2	10	82.3	28.1	86.5	4.3	94.3	4.8	50
Mepanipyrim	84.1	18	84.4	4.9	94.1	4.2	10	110.3	9.2	93.3	3.9	87.9	3.4	50
Mepronanide	96.3	7	76.7	4.4	77.9	2.5	10	81.0	8.5	78.1	3.2	81.6	3.5	50
Methamidophos	79.1	5.9	75.3	1.5	70.1	2	10	< LD		75.8	7.9	60.2	7.5	100
Methidathion	96.6	7.9	73	6.4	86.3	4.3	10	87.7	5.4	88.6	5.0	91.6	4.7	50
Methiocarb	103.3	13.5	77.6	9.2	84.6	2.6	10	56.9	9.3	53.2	6.2	56.2	5.5	
Methomyl	109.3	4.1	86.6	3.5	82.5	3.9	10							
Methoxyfenozide	75.3	13.4	97.2	6.4	98.6	9.9	10							
Omethoate								< LD		65.3	6.6	83.5	7.1	300
Oxadixyl	95.4	5.9	94.2	6	102.6	5.4	10	99.4	11.1	94.8	2.4	104.0	4.2	50
Paraoxon								87.0	14.7	81.7	2.8	93.3	3.9	50
Paraoxon methyl								83.1	7.8	81.2	5.0	87.8	4.5	50
Penflufenadin	108.7	18	119.5	7	99.3	3.4	10	76.1	9.0	73.2	7.1	80.1	5.1	50
Permethrin								64.1	8.7	62.0	6.6	59.3	6.7	

(continued on next page)

Table 2 (continued)

LC-MS/MS Validation								GC-MS/MS Validation						
Pesticide	Concentration level (μg kg^{-1})						LOQ (Limit of Quantification) (μg kg^{-1})	Concentration level (μg kg^{-1})						LOQ (μg kg^{-1})
	10		50		200			50		100		200		
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)		Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
Fluxapyroxad	81.3	7.3	77.5	8.9	93.5	2.5	10	85.2	6.2	86.6	4.3	88.4	6.2	50
Fenpiclonil	96	4.8	76.8	4.7	90.8	1.9	10	76.1	7.0	72.6	3.1	76.9	5.4	50
Fenpiclonil-ethyl								77.6	7.1	75.7	4.4	81.0	5.4	50
Fludioxonil	92	6.2	73.8	5.2	72.4	3.5	10	75.7	48.7	88.9	23.6	175.1	16.6	50
Procymidone								84.9	10.2	79.1	2.9	84.9	3.3	50
Propamidp (Propyzamide)								83.8	7.8	81.1	4.5	88.9	3.3	50
Propiconazole	76.2	11	81.6	4.9	81.4	2.8	10	81.8	8.6	75.7	4.1	82.3	4.3	50
Pyraclostrobin	90.5	4.3	86.1	5.1	85.2	2.3	10	61.4	37.2	71.6	8.5	74.4	8.1	100
Pyrimethanil	100.6	15.9	71	5.8	70.9	2.9	10	67.3	9.1	54.9	5.9	51.0	5.4	
Pyriproxifen								66.1	10.3	62.1	5.2	63.9	4.8	
Tebuconazole	105.4	8.2	83	4.7	78.1	1	10	84.8	7.4	79.1	1.9	82.5	3.5	50
Tolylfluanide	115.9	2.8	85.6	7.2	80.2	9.3	10							
Trifloxystrobin	94.1	11	86.7	6.3	73.9	7.4	10							
Triticonazole	100.4	5	82.4	7.2	86.1	4.6	10							
Tetradifon								71.8	9.6	66.8	5.5	69.0	3.8	50
Thiacloprid	114.1	8.3	79.1	4.5	80.3	2.3	10							
Thiamethoxam	108.7	14.5	88.6	12.6	81.5	8.9	10							
Thiabendazole	79.6	8.1	79.7	5.1	73.7	3.2	10							
Triticonazole	96.2	3.2	74.3	5.8	75	2.5	10	83.4	11.6	73.3	6.7	88.0	6.7	50
Trifloxystrobin	110.8	4.6	99.3	4.9	99	3.4	10							
Trifloxystrobin								84.1	8.7	75.9	5.3	75.4	5.1	50
Vinclozolin								90.9	7.6	77.5	3.7	76.9	3.1	50

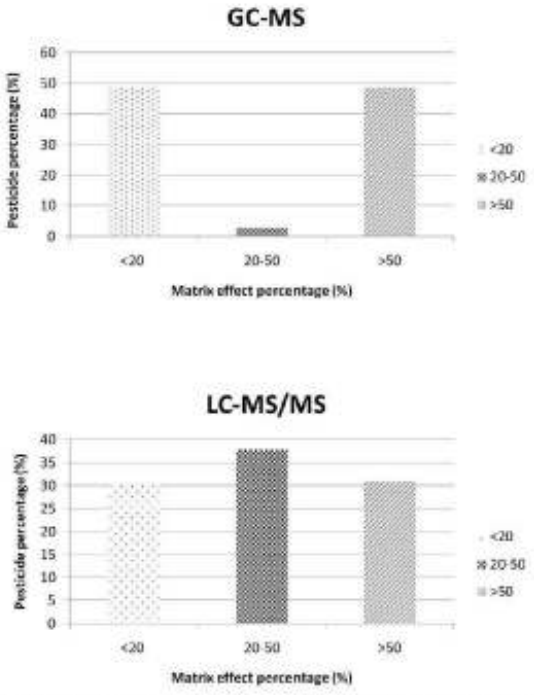

Fig. 1. Percentage of compounds clustered according to matrix effect (ME): lower than 20%, from 20 to 50% and higher than 50% for GC-MS and LC-MS/MS analysis.

edible flowers' (European Commission, 2020).

The *Codex Alimentarius* has a very short list of MRLs for "herbs" and "dry herbs", and almost no MRLs for specific herbs. The MRLs in Codex are, in general, at much higher levels than the LOQs of the presented method (FAO, 2020).

5. Conclusions

A multiresidue method was developed for the determination of pesticide residues in boldo leaves, used as a representative matrix of a herb. The method was collaboratively developed and validated in two different laboratories, GACT-Uruguay and FEPL-Austria. The procedure showed satisfactory recoveries and precision (70–120%, RSD below 20%) at 50 μg kg^{-1} for 65 GC and 56 LC amenable pesticides. In addition, 51 of the pesticides could be analyzed at 10 μg kg^{-1}. The final validated method, based on the CEN 15662 procedure, can be used for the quality control of this widely used medicinal herb, as it complies with the guidelines of the European and the Mercosur Pharmacopoeia where boldo is included, as well as the requirements of *Codex Alimentarius* and EU for the category "herbs and dry herbs". The method is useful for control purposes because the European regulations have assigned a default safety level of 50 μg kg^{-1} for pesticides not included in Good Agricultural Practices schemes, in most herbs used as infusions and teas. The protocol presented was able to minimize the influence of co-extractives in the pesticide residue determination in boldo leaves. Given the unusual combination of chemical classes and families of natural substances present in boldo, with wide physicochemical properties, it is likely that the method would be effective in other matrices. It presents a possible global solution for the minimization of the influence of these different chemical classes of co-extractives either when they are present together or alone in an herbal extract. Additionally, the data for a small list of GC amenable pesticides were studied by GC-MSD, giving the opportunity to apply this methodology in countries where tandem mass spectrometers are not available. Therefore, the method could be considered as a potential harmonized protocol for the control of pesticide residues in herbs.

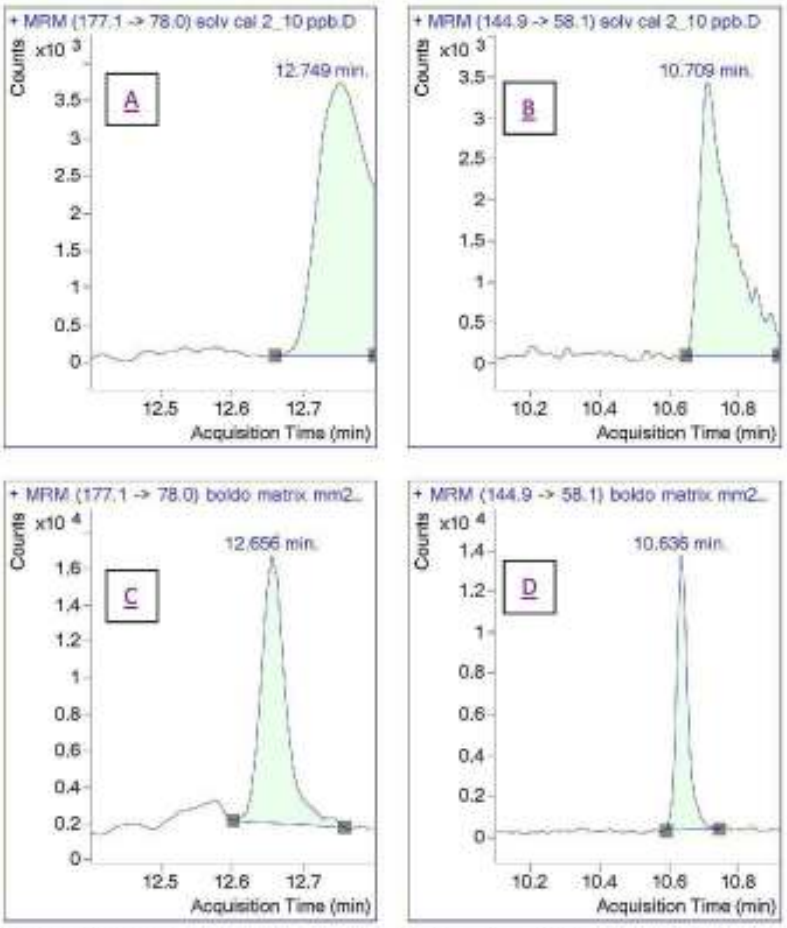

Fig. 2. Solvent and matrix matched chromatogram for fenhexamid (A and C) and methidathion (B and D) at 10 μg kg^{-1} concentration level.

Authors' contributions

BM contributed to the design of the study, participated in the laboratory work in both labs, performed the treatment of the data recovered and drafted the paper. NB ran the laboratory work, adjusted the instrumental conditions, performed the data treatments and collaborated in the preparation of the manuscript. AB adjusted the sample preparation, TLC analysis. NG processed the real samples for analysis and helped in laboratory work and design the graphical abstract. VC coordinated the practical work, participating particularly in the GC–MS/MS analysis with BM and NB at the FEPL. AC and HH gave the conceptual frame of the manuscript, adjusting and editing its final version. All the authors have read the final manuscript and approved the submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doihttps://doi.org/10.1016/j.jarmap.2020.100254.

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2.5 Method optimization and validation for multi-class residue analysis in turmeric

This paper addresses the analytical optimization and the validation of a trace residue method for different classes of analytes in turmeric, a valued root spice present on the international market and a minor crop in relation to regulatory limits.

Similar to *Peumus boldus Molina*, turmeric has a high chemical complexity due to the large number of secondary metabolites that interfere with the analytical determination of traces of residues of pesticides, persistent pollutants, mycotoxins, etc. Method optimization therefore aimed at diminishing the effects of secondary metabolites and enhance the detection of target analytes. Matrix effects were studied using a representative turmeric commodity, which helped in the optimization of the final method chosen for validation.

Representativeness of the turmeric powder, used as the method validation reference matrix, was essential to be able to take into account differences arising from environmental growing conditions as well as possible different cropping technologies. The blank reference matrix was characterized for its volatile fraction using an innovative method, through ion mobility spectrometry (IMS), and the representative homogenization status was identified and used for the validation studies.

The novelty of the method was the validation of multi class analytes such as characteristic persistent soil pollutants, residues of pesticides, dyes and aflatoxins. Those different analytes were chosen based on the chance of being found on turmeric samples. The analytical challenge was to optimize and validate a multi-residue, multi class, multi analyte method with acceptable analytical performance, as the one prescribed by international guidelines. 67 pesticide residues, including 12 persistent organic pollutants, 4 aflatoxins and 3 dyes were validated in turmeric in the analytical range 0.01-0.05 mg/kg under acceptable performance conditions.

The method was cross validated between FAO/IAEA and GACT by an interlaboratory study, this helped proving the method performance and the robustness of the methodology. This study opened the field to further investigations into the authenticity of herbs and spices, for which fraud happen to be very common in trade, especially when traded in powder format.

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Method optimization and validation for multi-class residue analysis in turmeric

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ABSTRACT

Turmeric (*Curcuma longa* L.) is an economically important food and medicinal plant that grows in tropical and subtropical regions consumed worldwide. Turmeric is a difficult matrix for residue/contaminant analysis due to the high content of potentially interfering polyphenols (curcuminoids) and essential oils with physicochemical properties similar to target analytes. To solve this problem, a simple and straightforward sample preparation protocol based on ethyl acetate extraction and dispersive solid phase clean up, followed by gas and liquid chromatography coupled to tandem mass spectrometry analysis, was optimized and validated, after selecting a representative blank material using gas chromatography coupled to ion mobility spectrometry and multivariate data analysis. Method validation was performed according to Codex guidelines for 67 pesticide residues, including 12 persistent organic pollutants, 4 aflatoxins and 3 dyes at 0.01, 0.02 and 0.05 mg kg^{-1}level. Recoveries ranged between 60 and 120%, with relative standard deviations for repeatability and reproducibility below 20% and 32% respectively. Method performance was tested collaboratively between laboratories in Austria and Uruguay which helped proving the robustness of the methodology. The method was applied to the analysis of commercial samples and chlorpyrifos was detected in most of them.

The novelty of the work relies in the optimization and validation of a multiclass method that allows analysis of four types of organic residues/contaminants, of paramount importance for food safety, using a single sample preparation step followed by gas and liquid chromatography coupled to tandem mass spectrometry determination.

1. Introduction

Herbs and spices have been used worldwide throughout human history as ingredients in food, teas and medicines due to their flavors and their pharmacological, biological and antimicrobial properties. Most are commercially produced and generally traded as powders or milled leaves, barks or roots. Due their high value, spices are important to the economy of countries that produce and export them. As a result of the growing demand for spices, agricultural cultivation has become increasingly intensive. To ensure optimum crop yields, agrochemicals are frequently applied, unless the product is declared as organic. The crops, before or after harvest, may be fumigated or treated to prevent insect and fungal infestation (Food and Agriculture Organization of the United Nations (FAO), 2004). The chemicals used may not always be authorized for use on the spice plants or, although allowed, may be misused and the residues found at higher levels than the maximum residue limits.

Turmeric (*Curcuma longa* L.) is an economically important food and medicinal spice plant that grows primarily in tropical and sub-tropical regions. The consumption of turmeric is forecast to increase

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worldwide by more than 10% in the next five years, driven by its associated health benefits (CBI, Ministry of Foreign Affairs, 2019). Globally, India is the largest producer, consumer and exporter of turmeric, accounting for more than 80% of the total production and 60% of the world exports (Karunga, 2016).There is generally little emphasis on the inclusion of spices in national monitoring plans due to their low consumption and, therefore, limited information about exposure (while there are often higher priority food safety issues to contend with), and limited resources in addition to the analytical challenges deriving from the complex nature of spices.

The Ministry for Primary Industries in New Zealand (NZ) analysed chemical contaminants in imported dried spices in 2012 (Ministry for Primary Industries New Zealand, 2012). Out of 115 samples, 15 samples had detectable levels of pesticide residues. In particular, garam masala powder and curry powder contained acetamiprid and ethion, respectively, at levels exceeding the NZ maximum residue level (MRL). Both garam masala and curry powder are blends of different ground spices, and therefore, it was difficult to interpret which spice contributed towards the pesticide residues. Thailand submitted to the Codex Committee on Pesticide Residues (CCPR) residue data obtained from 407 spice samples collected in a targeted monitoring programme carried out during 2005–2008 (FAO, 2010). Detectable residues were found in turmeric for methomyl (Ambrus, 2006). Recently Goon et al., (2017), reported a methodology for the determination of LC amenable pesticides in spices, particularly turmeric, using acetonitrile as extraction solvent and a clean-up step by HLB cartridges, with analysis by LC-Orbitrap mass spectrometer. The method was applied to spice samples from the market and the insecticide anilophos was detected. Ghosh et al. (2020) reported a validated method for multiresidue analysis of 208 pesticides in turmeric powder and rhizome and reported no detection of target pesticide residues from market samples. When turmeric grows in soils with a history of banned organochlorine usage, some of the persistent pollutants in the soil can accumulate in the rhizome. Srivastava et al. reported residues of organochlorine pesticides in several Indian spices including turmeric (Srivastava et al., 2001). Mussiger et al. (2008) reported contamination of turmeric powder in Bahrain with heptachlor at 0.04 mg kg $^{-1}$.

In Europe, there have been several notifications through the rapid alert system for food and feed safety (RASFF) concerning contamination of spices with bacteria, mycotoxins, azo dyes, heavy metals and pesticides (RASFF Portal European Commission,). Due to rural and often uncontrolled production conditions, natural spices can sometimes be also subject to contamination from pathogenic bacteria or mycotoxigenic fungi. Jeswal and Kumar (2015) reported that fungal species responsible for the production of the aflatoxins, ochratoxin A and citrinin were isolated from several spices including turmeric. Jalili (2016) reported that 35% of the samples of commercial turmeric that she analysed in Iran contained aflatoxins B1, B2, G1 and G2 level between 1.5 and 5.7 ng g $^{-1}$. According to a Codex report (Codex Alimentarius Commission, 2017 a), 855 (whole) and 169 (ground) samples of turmeric were analysed, from 2009 to 2015, for total aflatoxins and ochratoxin A, and 4.91% and 0.59% of samples exceeded the maximum concentration levels for total aflatoxins and ochratoxin A, respectively.

Colour intensity is an important attribute of spices, affecting their market value. Spices may, therefore, be targets for adulteration with colorants. Yellow-orange dyes such as butter yellow and the sudan dyes are potential turmeric adulterants, which are banned for use in foods within the EU due to their carcinogenicity. A method was reported for the determination of several dyes in chili and curry powder (Gomes et al., 2006). In Europe, all commodities containing chili, curry and curcuma must be certified free of Sudan dyes (Official Journal of the European Union, 2005). Ferrer Amate et al. (2010) reported for the first time a multi contaminant method for the analysis of pesticides, aflatoxins and dyes in spices through an acetonitrile extraction without clean up in the sample preparation step, followed by liquid chromatography coupled to mass spectrometry (LC-MS/MS) determination at spiking levels of 0.05 and 0.5 mg kg $^{-1}$. The scope of the method comprised 12 dyes, four aflatoxins and 20 pesticides. The method was shown to be fit for purpose and was applied to the analysis of 50 real samples from the Austrian market, with carbendazim detected in turmeric powder. From a regulatory point of view, several pharmacopoeias as well as the Environmental Protection Agency (EPA) of the United States of America have set maximum residue limits (MRLs) for a variety of pesticides in root and tuberous vegetables, and among them, turmeric (United States Environmental Protection Agency). Canada (Health Canada, 2020) has set MRLs for 42 pesticides in turmeric root. The European Union and the Codex Alimentarius Commission (Codex Alimentarius Commission) have also set MRLs for a number of pesticides, aflatoxins and dyes in herbs and spices, including turmeric. The Spices Board India summarized the common mandatory tests required for the export of spices and spice products that are controlled in the EU, Canada, USA, Japan and South America (Spices Board India, 2013).

There have been few validated methods reported for the analysis of contaminants in spices because of the complexity of the matrices and the difficulty in establishing appropriate representative matrices. This is a key issue as the materials to be used for blanks and for spiking experiments, during method validation, should be authentic or at least characterized from a chemo-taxonomic point of view and have the same properties as the actual test matrix. Matrix effects (ME) can only be properly evaluated when this condition is met, to ensure a method that is fit for purpose (Parrilla Vázquez et al., 2019). In addition to the problems raised by the inherent chemical complexity of herbs and spices due to the high number and amounts of secondary metabolites and their dependence on environmental factors and cropping technologies, the multi-national origin of turmeric samples in the market must be considered when selecting an appropriate raw material to validate the method. It has previously been demonstrated that the secondary metabolite profiles vary widely among samples depending on factors such as the location, the climate, the variety of *C. longa* cultured and the type of cultivation (e.g. organic or conventional) (Booker et al., 2014). The main components of turmeric are essential oils, the curcuminoids and related compounds, polyphenols of similar physicochemical properties to common pesticides, mycotoxins and dyes. Carotenoids and essential oils are also present in the plant. These are lipophilic compounds that could interfere in the determination of apolar pesticides such as the organochlorines and others (Li et al., 2011). The variability of the matrix chemical composition is an additional problem for method development and optimization. The changes in coextractives thus obtained in the sample preparation step can change dramatically the matrix effects and the quantification values.

In this work, a straightforward multiclass sample preparation protocol was optimized and validated for the determination of selected representative pesticide residues, aflatoxins and dyes in turmeric, using gas chromatography and liquid chromatography coupled to tandem mass spectrometry (GC-MS/MS and LC-MS/MS). For the selection of an appropriate matrix for method development an innovative approach using gas chromatography coupled to ion mobility spectrometry (GC-IMS) was employed.

2. Materials and methods

2.1. Chemicals and reagents

Pesticide standards, dye standards, ascorbic acid (≥99.7%) and glacial acetic acid (≥99.85%) were from Sigma-Aldrich (Schnelldorf, Germany).

Mycotoxin standards as acetonitrile solutions (0.5–10 μg mL $^{-1}$) were from Romer Labs (Tulln, Austria). Analytical standards were of purity ≥95%. Acetone, dichloromethane, n-hexane, sodium hydrogen carbonate (99.5–100.5%), sodium sulfate (99.0–100.5%) and formic acid (98–100%) were purchased from Merck (Darmstadt, Germany). Magnesium sulfate, sodium chloride, sodium citrate dihydrate, sodium

Table 1
Analytes included in the scope of the method at PEPL, the matrix effects (ME), the recovery values (R%) at each fortification level, the precision data (repeatability, CV_r and reproducibility, CV_R) and the lowest validated level (LVL) are shown.

Analytes by GC/MS/MS	ME %	ME diluted 10 x %	Linear range (mg kg^{-1})	0.01 mg kg^{-1}			0.02 mg kg^{-1}			0.05 mg kg^{-1}			
				R%	CV_r%	CV_R%	R%	CV_r%	CV_R%	R%	CV_r%	CV_R%	LVL (mg kg^{-1})
CAC/GL 40-1993 CRITERIA				60–120	<30	<45	70–120	<20	<32	70–120	<20	<32	
Alachlor	48.1	134	0.004–0.8	80.5	13.6	20.2	91.6	10.9	10.7	96.6	5.1	5.0	0.01
Aldrin	5.6	64	0.004–0.8	67.3	17.8	34.1	85.3	9.6	12.9	94.4	4.4	5.0	0.01
Atrazine	−5.5	122	0.004–0.8	85.8	11.3	34.1	103.4	10.4	23.0	102.2	8.3	24.0	0.01
Azinphos-ethyl	−24	−113	0.004–0.8	86.1	26.0	25.6	86.2	14.5	14.6	83.3	11.7	12.8	0.01
Azoxystrobin	25.5	646	0.004–0.8	87.0	11.1	18.4	73.2	14.0	30.2	94.4	1.5	4.6	0.01
BHC-gamma (Lindane, gamma HCH)	−51.9	1100	0.004–0.8	91.1	10.3	13.4	95.9	17.7	18.2	101.1	9.6	13.7	0.01
Bioralid	−25.0	254	0.004–0.8	84.1	28.3	29.3	86.4	18.3	17.8	95.9	12.1	12.7	0.01
Bromophos	32.3	73	0.004–0.8	60.1	12.7	16.1	83.5	10.2	9.9	90.2	7.3	8.1	0.01
Bromopropylate	86.2	130	0.004–0.8	61.4	13.4	19.5	87.1	9.7	10.0	94.2	6.9	7.8	0.01
Chlorfenvinphos-1	158	158	0.004–0.8	60.46	10.1	11.2	84.7	9.0	9.0	93.0	5.3	6.4	0.01
Chlorfenvinphos-II	158	156	0.004–0.8	60.47	10.1	11.2	84.7	9.0	9.0	93.0	5.3	6.4	0.01
Chlorpyrifos	41.4	71	0.004–0.8	71.2	12.5	22.9	90.7	11.6	12.1	95.4	6.9	6.9	0.01
Chlorpyrifos-methyl	12.7	91	0.004–0.8	77.0	16.6	25.0	89.6	8.3	9.0	91.9	5.6	6.8	0.01
Cyprodinil	69.5	88	0.004–0.8	74.2	11.8	13.0	90.7	8.7	9.0	99.4	6.9	6.7	0.01
DDT-pp'	96.1	−27	0.004–0.8	80.3	19.4	32.3	76.9	12.9	19.3	90.1	10.8	10.6	0.01
Deltamethrin	145	315	0.004–0.8	61.0	20.5	35.1	86.9	7.4	11.7	99.7	8.7	17.4	0.01
Diazinon	44	91	0.004–0.8	78.8	16.6	16.9	96.8	17.7	20.4	103.6	8.6	12.5	0.01
Dichlofluanid	9.4	144	0.004–0.8	67.2	22.2	23.1	89.5	9.5	16.7	93.7	8.4	11.0	0.01
Dichlorvos	−0.5	−1	0.004–0.8	75.1	8.9	12.4	92.2	8.1	8.8	99.2	5.7	6.5	0.01
Dicofol	4.0	171	0.004–0.8							96.9	12.2	13.8	0.05
Dieldrin	11.7	2852	0.004–0.8	73.8	27.8	28.0	85.0	14.2	14.7	96.5	11.2	11.8	0.01
Endosulfan I (alpha isomer)	7.9	0	0.004–0.8	90.6	15.2	17.5	81.6	11.0	14.9	84.7	13.3	15.9	0.01
Endosulfan II (beta isomer)	−24.2	59	0.004–0.8	69.9	25.4	24.8	91.4	11.6	11.7	101.1	9.0	10.4	0.01
Endosulfan sulfate	−41.2	89	0.004–0.8	85.3	21.2	22.1	90.9	10.5	14.9	104.0	6.8	10.8	0.01
Endrin	7.4	200	0.004–0.8	69.6	25.1	26.0	89.1	13.7	13.6	94.4	6.7	6.7	0.01
Epoxiconazole	−32.1	331	0.004–0.8	80.5	15.1	14.5	88.2	8.1	10.7	91.7	7.1	9.4	0.01
Esfenvalerate (Fenvalerate A alpha)	80	121	0.004–0.8	93.8	24.3	24.3	91.5	12.5	12.6	89.8	6.8	9.8	0.01
Ethion	108.2	177	0.004–0.8	68.7	14.7	20.1	82.6	8.6	10.6	88.1	5.8	5.6	0.01
Ethirfos	39.0	72	0.004–0.8	79.7	8.5	9.1	95.2	9.1	9.1	99.4	4.6	4.8	0.01
Fenchlorphos	28.1	66	0.004–0.8	70.2	13.9	15.9	86.8	7.7	7.6	90.0	5.4	5.6	0.01
Fenitrothion	64.2	158	0.004–0.8	83.2	10.9	11.4	96.4	9.3	12.8	105.5	4.2	9.8	0.01
Fenpropathrin	70.3	140	0.004–0.8	74.8	15.9	23.6	88.7	13.0	13.3	96.0	7.1	7.5	0.01
Fenvalerate	122	124	0.004–0.8	98.4	2.8	3.1	80.5	19.3	32.6	85.6	8.8	10.7	0.01
Fipronil	157.3	209	0.004–0.8	71.8	28.8	35.7	94.2	14.9	18.7	101.2	6.8	8.7	0.01
Flusilazp-butyl	66.9	95	0.004–0.8	66.3	14.2	19.8	87.5	9.9	12.0	95.2	5.9	8.6	0.01
Fluvalinate-tau I	192	83	0.004–0.8	93.0	26.5	31.2	84.3	15.9	16.6	98.0	6.3	11.0	0.01
Fluvalinate-tau II	−37.6	346	0.004–0.8	97.1	28.8	36.0	95.3	12.4	13.2	96.8	9.5	9.4	0.01
Halosylp-methyl	40.7	83	0.004–0.8	64.7	14.1	17.1	86.7	9.5	12.6	94.2	7.3	8.9	0.01
Heptachlor endo-epoxide (isomer A)	−3.7	39	0.004–0.8	80.1	19.6	18.9	83.8	12.8	12.6	90.9	7.9	8.6	0.01
Heptachlor exo-epoxide (isomer B)	3.3	370	0.004–0.8	75.5	19.2	29.5	84.4	20.3	21.3	96.3	9.5	10.0	0.01
Hexachlorobenzene	−33.2	3	0.004–0.8	80.0	7.7	11.1	100.0	7.3	10.0	104.3	4.5	4.4	0.01
Malaoxon	95.6	284	0.004–0.8	92.1	12.3	12.1	92.0	10.7	12.5	106.0	5.5	9.4	0.01
Malathion	102.0	180	0.004–0.8	72.1	12.4	13.2	93.5	8.8	11.4	98.2	3.6	8.0	0.01
Mevinphos	117.2	54	0.004–0.8	90.7	12.7	18.7	110.9	8.9	16.8	119.8	8.8	18.9	0.01
Pentachlorobenzene	−2.9	1	0.004–0.8	73.8	8.6	9.5	89.8	7.1	8.3	97.0	5.8	7.5	0.01
Phenthoate	−8.2	206	0.004–0.8	76.5	18.2	18.1	84.7	13.1	14.9	91.1	8.3	8.3	0.01
Pirimicarb	53.2	−19815	0.004–0.8	81.8	10.1	11.3	97.4	6.5	8.1	98.2	4.1	7.3	0.01
Pirimiphos-methyl	32.1	95	0.004–0.8	66.4	15.3	15.1	88.9	7.9	8.2	93.5	4.3	6.3	0.01
Procymidone	18.5	47	0.004–0.8	111.3	9.4	25.9	111.9	9.6	12.9	104.0	5.9	5.9	0.01
Profenofos	64.2	145	0.004–0.8	64.0	25.5	26.5	94.7	7.7	8.0	95.8	7.7	10.1	0.01
Quinalphos	45.7	−122	0.004–0.8	88.1	25.8	28.5	90.3	12.8	12.6	88.6	8.7	8.5	0.01
Quintozene	−19.4	23	0.004–0.8	85.5	11.6	14.2	93.5	7.4	9.9	97.0	6.6	9.1	0.01
Rannel (Fenchlorphos)	28	66	0.004–0.8	70.2	16.6	15.9	86.6	7.7	7.6	90.0	5.4	5.6	0.01
Tebuconazole	69.4	121	0.004–0.8	86.6	22.2	23.2	82.1	12.5	13.3	90.5	6.9	7.9	0.01
Tetradifon	13.8	32	0.004–0.8	67.7	21.9	37.1	89.6	12.5	13.9	92.2	10.9	11.4	0.01
Vinclozolin	7.8	102	0.004–0.8	69.7	15.9	16.4	92.3	10.1	10.5	100.2	5.4	5.6	0.01

Analytes by LC-MS/MS	ME %	ME % DILUTED 10 x	Linear range (mg kg^{-1})	0.01 mg/kg			0.02 mg/kg			0.05 mg/kg			
				R%	CV_r%	CV_R%	R%	CV_r%	CV_R%	R%	CV_r%	CV_R%	LVL (mg kg^{-1})
				60–120	<30	<45	70–120	<20	<32	70–120	<20	<32	

(continued on next page)

Table 1 (continued)

Analytes by LC-MS/MS	ME %	ME % DILUTED 10 x	Linear range (mg kg $^{-1}$)	0.01 mg/kg			0.02 mg/kg			0.05 mg/kg			LVL (mg kg $^{-1}$)
				R%	CV_r%	CV_R%	R%	CV_r%	CV_R%	R%	CV_r%	CV_R%	
GAC-GL 40-1993 CRITERIA				60–120	<30	<45	70–120	<20	<32	70–120	<20	<32	
GAC-GL 40-1993 CRITERIA													
Acephate	−15.4	6.3	0.004–0.8	30.0	15.5	31.6	29.3	13.4	19.0	28.9	12.6	15.4	nn
Acetamiprid	−45.0	1.8	0.004–0.8	98.8	5.8	7.3	93.5	4.6	4.5	88.9	6.8	8.2	0.01
AFB1	−57.2	11.0	0.004–0.8	76.3	10.6	20.6	73.6	12.3	13.5	73.2	10.3	16.0	0.01
AFB2	−58.0	−1.3	0.004–0.8	67.4	16.8	27.7	70.0	12.3	12.7	70.6	9.0	13.7	0.01
AFG1	−41.6	22.0	0.004–0.8	67.4	7.7	27.3	70.2	10.8	13.6	72.6	8.6	17.1	0.01
AFG2	−44.9	10.3	0.004–0.8	60.1	9.3	40.0	70.4	10.8	15.4	70.3	8.8	10.0	0.01
Alachlor	−93.1	−15.0	0.012–0.8							114.1	15.6	17.9	0.05
Atrazine	−83.1	−44.4	0.012–0.8				110.3	13.2	15.2	98.6	7.3	11.4	0.02
Azinphos ethyl	−91.1	−19.2	0.012–0.8							104.4	17.2	24.6	0.05
Azoxystrobyn	−79.0	−45.6	0.004–0.8	113.2	9.5	10.4	104.7	10.6	11.3	101.5	6.7	9.7	0.01
Boscalid	−95.8	−77.3	0.012–0.8				103.8	16.9	17.8	103.2	13.8	13.6	0.02
Butter yellow	−79.3	2.3	0.004–0.8	106.9	6.5	10.3	106.9	6.7	8.9	105.3	4.0	8.4	0.01
Carbaryl	−87.2	−35.2	0.004–0.8	116.7	9.8	34.0	99.6	12.9	17.1	101.8	8.3	8.9	0.01
Carbendazim	−3.7	6.7	0.004–0.8	91.9	6.0	19.6	93.1	9.9	10.4	99.5	7.9	17.4	0.01
Chlorfenvinphos	−81.0	−22.9	0.004–0.8	102.9	11.3	16.3	101.3	11.8	15.2	107.9	8.6	13.5	0.01
Chlorpyrifos	−78.7	0.5	0.004–0.8	100.3	21.4	20.9	105.6	13.3	14.6	104.2	10.4	13.9	0.01
Chlorpyrifos methyl	−85.3	−36.2	0.004–0.8							95.2	20.1	19.9	0.05
Cyprodinyl	−82.4	−5.6	0.012–0.8				115.6	23.6	41.6	93.0	6.4	46.4	nn
Diazinon	−87.1	−12.3	0.004–0.8	98.1	19.2	19.5	98.6	13.0	15.7	100.4	10.9	12.2	0.01
Dichlofluanid	−88.8	−9.1	0.012–0.8				93.3	20.3	30.6	94.52	19.5	48.5	nn
Dimethoate	−30.1	4.8	0.004–0.8	108.0	3.9	7.8	101.7	5.4	6.8	94.4	7.2	11.5	0.01
Epoxyconazole	−88.1	−32.4	0.004–0.8	102.1	14.8	22.9	100.9	14.1	17.1	115.4	11.4	19.5	0.01
Ethion	−87.3	−26.3	0.012–0.8	102.4	18.0	20.3	94.5	15.5	17.9	98.6	11.1	14.6	0.02
Etrimfos	−71.7	−7.9	0.012–0.8	118.9	10.3	10.9	106.1	8.7	8.3	103.9	12.4	16.2	0.02
Fenpropathrin	−82.3	11.3	0.04–0.8							98.9	12.5	18.9	0.05
Fipronil	107.7	81.1	0.004–0.8	97.4	9.5	12.1	101.6	8.4	9.9	104.7	9.8	15.3	0.01
Fluvalinate	−65.2	1.7	0.012–0.8				111.9	19.1	19.4	88.7	13.8	14.3	0.02
Fosthiazate	−81.0	−72.1	0.004–0.8	92.9	20.4	19.7	88.3	11.3	11.0	83.5	10.4	13.5	0.01
Hexythiazox	−87.4	−13.4	0.004–0.8	110.6	16.3	16.0	110.8	13.0	12.5	110.0	7.6	8.1	0.01
Imazalil	−37.9	9.6	0.012–0.8							81.3	11.0	11.0	0.05
Imidacloprid	6.4	−6.4	0.004–0.8	104.7	8.6	23.6	92.2	11.0	14.5	93.6	7.8	10.9	0.01
Malaoxon	−53.5	10.7	0.004–0.8	111.2	3.8	6.0	103.8	3.9	3.8	97.0	5.4	6.5	0.01
Malathion	−93.2	−56.9	0.004–0.8				114.0	15.8	15.3	104.5	12.6	13.2	0.02
Methamidophos	−3.8	0.4	0.004–0.8	21.2	16.7	26.9	27.5	16.0	30.0	30.2	14.1	18.8	nn
Methidathion	−83.8	−29.6	0.004–0.8	110.8	11.9	12.4	107.2	10.9	10.4	100.4	6.3	8.0	0.01
Methomyl	−3.7	2.0	0.004–0.8	101.4	7.3	13.7	93.3	8.3	10.8	90.1	6.3	7.1	0.01
Mevinphos	−31.6	nn	0.004–0.8	106.9	9.1	9.1	100.2	8.3	8.1	95.1	7.2	10.0	0.01
Omethoate	−14.4	6.4	0.012–0.8	50.3	10.7	19.6	44.0	11.5	12.1	39.5	11.7	13.7	nn
Paraoxon methyl	−29.0	28.2	0.02–0.8							84.1	28.2	24.7	0.05
Pendimethalin	−66.0	4.8	0.004–0.8	104.1	12.3	16.8	108.4	8.7	11.6	102.1	18.5	19.3	0.01
Permethrin	−79.9	−11.7	0.004–0.8	117.0	30.3	36.2	99.4	17.0	24.1	100.5	17.5	18.4	0.01
Phorate sulfoxide	−77.7	−20.9	0.004–0.048	198.1	8.1	11.0	175.1	6.8	18.1	159.8	6.9	21.3	nn
Phorlone	−81.3	−43.1	0.004–0.8	117.6	19.0	18.9	105.1	12.2	14.2	103.7	10.3	11.4	0.01
Phosmet	−86.9	−45.7	0.012–0.8				123.3	8.5	14.3	115.0	9.7	10.9	0.02
Pirimicarb	−16.7	0.9	0.004–0.8	112.4	8.0	8.6	110.3	8.0	8.2	107.1	7.4	10.8	0.01
Pirimiphos methyl	−88.6	−11.9	0.012–0.8				93.7	7.2	22.99	111.5	6.7	22.4	0.02
Profenophos	−90.4	−16.5	0.004–0.8	110.1	12.4	12.3	105.3	9.3	9.3	104.9	6.6	6.6	0.01
Pyrazophos	−85.3	−10.8	0.004–0.8	100.8	13.5	19.2	99.2	12.9	13.6	105.5	11.8	15.2	0.01
Quinalphos	−81.6	−16.0	0.004–0.8				97.7	10.1	17.4	97.3	11.0	13.4	0.02
Sudan I	−69.7	−11.3	0.004–0.8	116.9	7.2	22.9	102.9	14.0	14.7	104.9	12.6	12.4	0.01
Sudan IV	−31.7	3.2	0.004–0.8	90.2	7.2	9.8	98.0	8.0	9.1	98.5	7.2	10.8	0.01
Tebuconazole	−77.3	−9.0	0.004–0.8	112.6	13.3	15.9	106.7	11.5	14.6	103.7	8.7	10.0	0.01
Thiacloprid	−13.7	4.9	0.004–0.8	90.5	4.1	5.4	93.2	4.6	5.1	91.0	5.2	9.1	0.01
Thiamethoxam	−30.9	−14.6	0.004–0.8	86.2	6.7	16.3	82.1	9.0	12.5	79.8	6.0	6.6	0.01

hydrogen citrate sesquihydrate were from Agilent Technologies (Santa Clara, USA). Acetonitrile, methanol (≥99.9%), ethyl acetate (≥99.7%) and ammonium formate (≥99.0%) were purchased from Honeywell International (North Carolina, United States). Ultrapure water was produced using a Milli-Q® integral system equipped with a 0.22 μm point-of-use membrane filter cartridge. BEKOlut PSA-Kit-03 (150 mg magnesium sulfate, 25 mg primary secondary amine, 25 mg C_{18}) was from BEKOlut (Haupstuhl, Germany).

Individual pesticide and dye stock solutions (1000 μg mL $^{-1}$) were prepared in ethyl acetate and stored in the dark at - 18 °C. Mixed solutions used for calibration and spiking procedure were prepared from the stock standards at appropriate dilutions.

2.2. Samples

2.2.1. Blank matrix sample

The final turmeric powder blank sample, used for method validation, was a composite sample made up of 6 turmeric powders, bought on the Austrian market, free from any detectable residue of the target analytes. The composite sample was obtained after homogenization by mixing for 5 min in a Retsch ball-mill (Düsseldorf, Germany) followed by mixing using a mortar and pestle for 15 min with the addition of liquid nitrogen.

2.2.2. Sample collection

Twenty-one turmeric powder samples were collected from March to August 2019 from Austrian supermarkets. Nine samples were labelled as organic. The samples were sold in powder format and stored under dry conditions in sealed containers.

2.3. Spiking procedures

A portion of 2 g of turmeric powder was hydrated with 8 mL ultrapure water. The suspension was vortexed and left to stand for 30 min then fortified with 100 μL of the appropriate concentration of spiking mixture solutions to achieve concentrations of 0.01, 0.02 and 0.05 mg kg^{-1} and left to rest at room temperature under a fume hood for 30 min.

2.4. Sample preparation

A 2 g portion of turmeric powder, homogenized using a ball mixer and blended in a mortar, was weighed into a 50 mL centrifuge tube and 8 mL ultrapure water was added. The mixture was vortexed for 30 s and left to hydrate for 30 min. Acidified ethyl acetate (8 mL, 0.05 g L^{-1} ascorbic acid and 0.01% glacial acetic acid in ethyl acetate) was added to the tube with 2 g of sodium sulfate plus 0.333 g of sodium hydrogen carbonate and the mixture was vortexed for 10 min. After centrifuging (5730 g, 25 °C, 5 min) the organic layer was cleaned up using BEKOlut PSA-Kit-03 and then vortexed for 10 min. After centrifugation (12600 g, 25 °C, 5 min), the upper layer of the extract was filtered and 2 μL was injected in the GC-MS/MS. For LC-MS/MS analysis, 2 mL of the filtered cleaned-up organic extract was evaporated under a nitrogen stream. The dry extract was reconstituted with 2 mL of methanol and injected into the LC-MS/MS.

2.4.1. Scope of the method

The analytes included in the scope of the method are indicated in Table 1. All these analytes are currently regulated in turmeric under international standards including the US (United States Environmental Protection Agency) Pharmacopoeia, the EU food regulations (European Commission, 2005; 2006, 2010) and the WHO (2007)/FAO Codex Alimentarius standard, subgroup HS 0193 (spices, roots and rhizomes) (Codex Alimentarius Commission).

2.5. Instruments and analytical determination

2.5.1. Headspace gas chromatography coupled to ion mobility spectrometry (HS-GC-IMS) for matrix studies

The volatile components of the turmeric samples were measured using a FlavourSpec headspace gas chromatography - ion mobility spectrometer (HS-GC-IMS) (G.A.S. GmbH., Dortmund, Germany). Aliquots (0.3 g) of turmeric blank samples were weighed into 20 mL glass vials and sealed with gas tight caps. In the instrument the vials were incubated at 60 °C for 10 min with gentle rotational agitation. Each vial's headspace was sampled (200 μL) and injected, using an autosampler, into the heated injector port, which was kept at 80 °C. Nitrogen was the carrier gas with a flow of 2 mL min^{-1} until 2 min, raised to 150 mL min^{-1} until 10 min and then kept at this flow for 5 min. The oven program was isocratic and maintained at 40 °C. The chromatographic column was a FS-SE-54-CB 15 m × 0.53 mm x 1 μm (Ziemer Chromatographie, Langerwehe, Germany). The drift gas flow was 150 mL min^{-1} and the temperature of the IMS was maintained at 45 °C. The tube length was 98 mm and the drift voltage was 5000 V.

2.5.2. Determination with GC-MS/MS

The gas chromatography system consisted of an Agilent 7693 Autosampler and a 7890 B Ga Chromatographic System coupled to a 7000 C triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, USA).

Data acquisition and processing were implemented using Agilent Technologies, Inc. 2016, Mass Hunter Quantitative software. Two ultra-inner columns (15.0 × 0.25 mm ID x 0.25 μm HP5 ms, Agilent J&W, USA) were connected by an electronic pressure controller to enable a 5 min post run backflush. The samples were injected using a multimode injector inlet in splitless mode through an ultra-inert inlet liner with a glass wool plug (Agilent Technologies, Santa Clara, USA). The injection volume was 2 μL. The injector port was programmed so that the initial injection temperature was 40 °C for 0.05 min, then ramped to 320 °C at 900 °C min^{-1}, maintained at this temperature for 1.39 min, then cooled down to 150 °C at 900 °C min^{-1} until end of the run.

The oven temperature was held at 60 °C for 0.5 min, then ramped to 150 °C at 80 °C min^{-1}, increased to 200 °C at 6 °C min^{-1} followed by a ramp to 300 °C at 16 °C min^{-1} and a final ramp to 320 °C at 3 °C min^{-1} with the temperature maintained at 320 °C for 23 min. The total run time was 45.9 min. The transfer line was maintained at 280 °C. The carrier gas was helium (99.999% purity) and the collision gas was nitrogen (99.999% purity). The instrument was operated in constant flow mode (1.39 mL min^{-1} in column 1, 1.43 mL min^{-1} in column 2). The average velocity in column 1 was 33.0 cm s^{-1} and 61.8 cm s^{-1} in column 2. The ion source was set at 300 °C and the quadrupole analyser temperatures were both set at 150 °C. The pesticide and environmental pollution database from Agilent Technologies was used to optimize the best transitions needed for each evaluated compound. The collision energy for each targeted molecule was optimized between 5 and 50 eV. One hundred and fourteen MRM groups were created to obtain adequate sensitivity, with dwell times between 5.23 msec and 165.69 msec, which allowed collection of 12–20 data points in each peak. The solvent delay was 3 min. The mass spectrometer was operated in positive mode using electron impact (EI) ionization. The electron ionization energy was 70 eV and the flow values of the quench gas and the collision gas were 2.25- and 1.5-mL min^{-1} respectively. The list of the evaluated pesticides, the optimized transitions and the collision energies chosen are presented in Table 1 of the supplementary materials.

2.5.3. Determination with UHPLC-MS/MS

The samples were analysed using ultra high-performance liquid chromatography (Shimadzu Nexera X2 Series, Kyoto, Japan) equipped with LD-30AD solvent delivery unit, SIL-30AC autosampler, CBM-20A communications bus module and CTO-20AC prominence column oven, coupled to Shimadzu 8060 triple quadrupole mass spectrometer (Kyoto, Japan). An Acquity UPLC™ BEH C_{18} (2.1 × 100 mm, particle size 1.7 μm; Waters, Milford, USA) column was used for chromatographic separation. The mobile phases were: A, 0.1% formic acid in water; B, 5 mM ammonium formate with 1% formic acid in methanol. The gradient program was as follows: 2% B to 100% of B over 12 min, held at 100% B until 20 min then decreased to 2% B at 20.01 min. The total run time was 24 min with a flow rate of 0.4 mL min^{-1}. The oven temperature was 40 °C. Injection volume was 1 μL. The MS parameters and the collision energies were optimized to achieve the highest responses for all analytes. The nebulizing gas flow was 3 L min^{-1}, the heating gas flow rate was 10 L min^{-1} and drying gas flow rate was 10 L min^{-1}. The interface temperature was 300 °C, the desolvation line temperature was 250 °C, and the heat block temperature was 400 °C. Electrospray ionization (ESI) was employed in simultaneous positive and negative mode. The detector voltage was 2.3 kV and the interface current was 0.63 μA. The multiple reaction monitoring (MRM) transitions and collision energies were selected and optimized by flow injection analysis. The pesticide database from Shimadzu was used to optimize the best transitions needed for each evaluated compound. The list of the evaluated pesticides, the optimized transitions and the collision energies chosen are presented in Table 1 of the supplementary materials.

2.6. Validation procedure

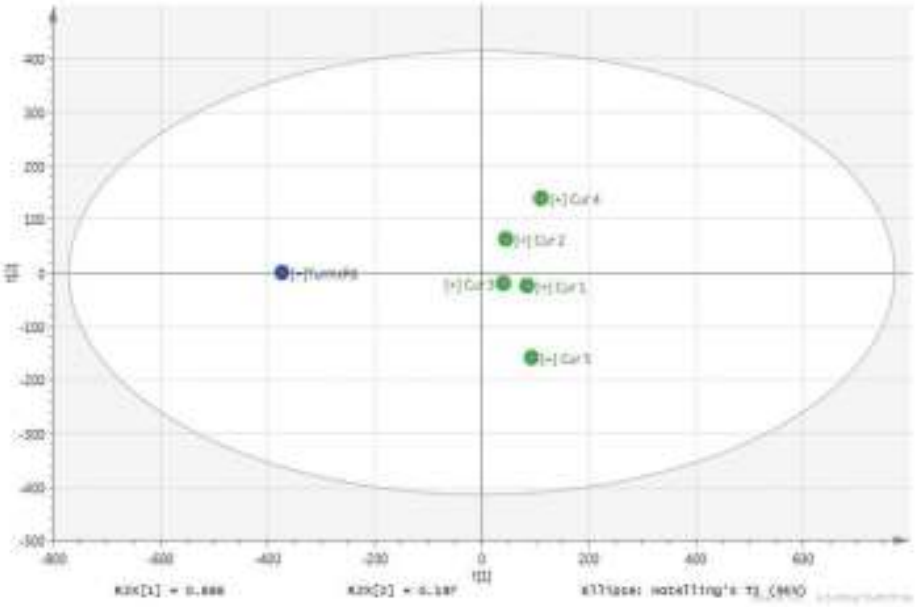
2.6.1. Experimental design

The experimental design for method validation consisted of 3 days of

Table 2
The analytes commonly analysed at FEPL and GACT, the CAS numbers, the Maximum Residue Limit (MRL) for turmeric according to the regulations from European Union, Codex and the United States Environmental Protection Agency (US-EPA), LVLs by GC-MS/MS and LC-MS/MS obtained by FEPL and GACT laboratories.

Analyte	EU MRL (mg kg $^{-1}$)	Codex MRL (mg kg $^{-1}$)	US-EPA MRL (mg kg $^{-1}$)	LVL GC-MS/MS FEPL (mg kg $^{-1}$)	LVL LC-MS/MS FEPL (mg kg $^{-1}$)	LVL LC-MS/MS GACT (mg kg $^{-1}$)	LVL GC-MS/MS GACT (mg kg $^{-1}$)
Acetamiprid	0.05	–	–	-	0.01	0.01	*
Alachlor	0.05	–	0.05	0.01	0.05	0.01	*
Aldrin	0.1	–	0.05	0.01	*	*	0.02
Atrazine	0.1	–	–	0.01	0.02	0.01	*
Azinphos-ethyl	0.05	–	0.1	0.01	0.05	0.01	0.05
Azoxystrobin	0.05	–	–	0.01	0.01	0.01	0.05
BHC-gamma (Lindane, gamma HCH)	–	–	0.6	0.01	*	*	*
Bromide	0.4	–	–	0.01	0.02	0.01	0.05
Bromopropylate	0.05	–	3.00	0.01	*	*	0.01
Carbaryl	0.1	0.1	–	*	0.01	0.01	*
Carbendazim	0.1	0.1	–	*	0.01	0.01	*
Chlorfenvinphos I	0.05	–	0.5	0.01	0.01	0.01	0.01
Chlorfenvinphos II	–	–	–	0.01	*	*	0.01
Chlorpyrifos	1	1.00	0.2	0.01	0.01	0.02	0.01
Chlorpyrifos-methyl	5	5.00	0.1	0.01	0.05	*	0.01
Cyprodinil	1.5	–	–	0.01	*	0.01	0.02
Deltamethrin	0.5	0.5	0.5	0.01	*	*	0.05
Diazinon	0.5	0.5	0.5	0.01	0.01	0.02	0.05
Dichlofluanid	–	–	0.1	0.01	*	0.01	*
Dichlorvos	0.1		1.00	0.01	*	*	0.01
Dicofol	0.1	0.1	0.5	0.05	*	*	0.05
Endrin	0.1	–	0.05	0.01	*	*	0.05
Epoxiconazole	0.1	–	–	0.01	0.01	0.01	0.05
Esfenvalerate (Fenvalerate A-alpha)	–	–	–	0.01	*	*	0.05
Ethion	0.3	0.3	2.00	0.01	0.02	0.01	0.05
Etrimfos	–	–	0.05	0.01	0.02	0.01	*
Fenchlorphos	0.1	–	0.1	0.01	*	0.02	0.02
Fenitrothion	0.05	0.1	0.5	0.01	*	*	0.02
Fenpropathrin	0.02		0.03	0.01	0.05	0.02	*
Fenvalerate	0.1	0.05	1.5	0.01	*	*	0.05
Fipronil	0.005	–	–	0.01	0.01	0.01	0.01
Flonicamid	–	–	–	0.01	*	*	*
Fluvalinate-tau I	0.01	–	0.05	0.01	0.02 sum of isomers	0.01 sum of isomers	0.05
Fluvalinate-tau II	0.01	–	–	0.01	*	*	0.05
Halosylop-methyl	–	–	–	0.01	*	0.01	*
Heptachlor exo-epoxide (isomer B)	0.1	–	–	0.01	*	*	0.05
Hexachlorobenzene	0.02	–	0.1	0.01	*	*	*
Hexythiazox	0.05	–	–	*	0.01	0.01	*
Imazalil	0.1	–	–	*	0.05	0.01	*
Imidacloprid	0.05	–	–	*	0.01	0.01	*
Iprodione	–	–	–	*	*	0.01	*
Malaoxon	0.02	–	1.00	0.01	0.01	*	*
Malathion	0.02	0.5	–	0.01	0.02	0.01	0.01
Methamidophos	0.1	–	–	Ns	ns	ns	*
Methidathion	0.1	–	–	*	0.01	0.01	*
Methomyl	0.05	–	–	*	0.01	0.01	*
Omethoate	0.05	0.05	–	*	ns	ns	*
Pendimethalin	0.05	–	0.1	*	0.01	0.01	*
Pendimethalin	0.05	–	0.1	*	0.01	0.01	*
Permethrin	0.1	–	sum 1	Ns	0.01	0.01	*
Phorate sulfoxide	0.1	–	–	*	ns	0.01	*
Phosalone	2	–	–	0.01	0.01	0.02	*
Pirimicarb	0.05	–	–	0.01	0.01	0.01	*
Pirimiphos-methyl	0.05	–	0.05	0.01	0.02	0.01	0.02
Procyxidone	0.05	–	0.1	0.01	*	*	0.01
Profenophos	0.05	0.05	0.1	0.01	0.01	0.01	0.05
Pyraclostrobin	0.1	–	–	*	0.01	0.01	*
Quinalphos	0.05	–	0.05	0.01	0.02	*	*
Quintozene	2	2	1.00	0.01	*	*	0.02
Tebuconazole	0.05	–	–	0.01	0.01	0.01	*
Thiacloprid	0.05	–	–	*	0.01	0.01	*
Thiamethoxam	0.05	–	–	*	0.01	0.01	*
Tetradifon	0.05	–	0.3	0.01	*	*	0.02
Vinclozolin	0.05	–	0.4	0.01	*	*	0.02

* Not studied/validated pesticides.



(A)

(B)

Fig. 1. The principle component analysis (PCA) in (A) shows the composite sample (TurmaPd) in relation to the individual blank turmeric samples (Cur 1, Cur 2, Cur 3, Cur 4, Cur 5). The PCA analysis in (B) shows a different composite sample (TP mix) in relation to the individual blank turmeric samples (TP 1 b, TP 2 b, TP 3 b, TP 4b, TP 5b, TP 6b). Additional turmeric samples are also shown on the PCA in (B): a commercial turmeric sample (TP7b), laboratory dried samples of turmeric (freeze dried -TP FD- and oven dried -TP CO) and a mustard sample (MP Ind).

experiments. Each day a batch of 15 replicate blank samples, with 5 replicates fortified at each of three different levels, was extracted and analysed in duplicate. In each batch, a reagent blank, a matrix blank and eleven matrix matched calibrators were also prepared and determined by GC-MS/MS and LC-MS/MS. The fortification levels were 0.01, 0.02 and 0.05 mg kg $^{-1}$ to cover concentrations at or below the MRLs of the target analytes. The MRLs for the different analytes are listed in Table 2.

2.6.2. Validation parameters

The validation procedure adopted in this study followed the criteria set in Codex Guidelines CXG 90-2017 (Codex Alimentarius, 2017 b) and CAC-GL/40-1993 (Codex Alimentarius Commission, 2010). The parameters evaluated were selectivity, calibration, linearity, matrix effects, trueness and recovery, precision, lowest validated level (LVL) and measurement uncertainty (MU). Given the high quantity of co-extractives, the recommendations of the Codex guideline CXG 90-2017 for calibration is to use matrix matching to compensate for the matrix effects. The calibration procedure included determinations of matrix matched standards prepared at eleven concentrations, injected in duplicate and applied using the bracketing approach. The entire calibration curve was injected both at the beginning and at the end of the batch, with additional calibration standards injected throughout the sequence. The calibration curve covered the range of 1–200 μg L $^{-1}$, corresponding to 0.004–0.8 mg kg $^{-1}$. The quality control criteria for calibration required that the individual residuals (that is the differences between the actual and calculated concentrations of the calibration standards) were below 20%, or below 30% for calibration concentrations of 1–5 μg L $^{-1}$, close to the lowest calibrated level (LCL). The linearity was considered acceptable if the correlation coefficients were higher than 0.99.

Selectivity, matrix effects, trueness, recovery and precision were estimated as previously discussed in Maestroni et al. (Maestroni, Abu Alnasr et al., 2018, A). The lowest validated level (LVL) is the lowest concentration assessed during validation that meets method performance criteria according to the Codex guideline (CXG 90-2017). It corresponds to the concept of limit of quantification (LOQ) as described in the SANTE document 12682/2019 (European Commission, 2019).

The measurement uncertainty (MU) was estimated according to the Codex guideline CAC/GL 59-2006 (Codex Alimentarius Commission, 2011) and SANTE/12682/2019 document (European Commission, 2019) using bias and precision values obtained from the in house validation study.

2.6.3. Interlaboratory comparison study

The performance of the method was tested in an interlaboratory comparison study between the FEPL, in Austria, and the GACT laboratory in Uruguay. The collaborative study was set up so that both laboratories used the same composite blank turmeric sample to carry out spiking experiments at the same fortification concentrations with the same fortification mixture and sample preparation protocol. Both laboratories determined the analytes by GC-MS/MS and LC-MS/MS. The instruments available at GACT were a 4000 Sciex QTRAP and a TQ8040 Shimadzu GC-MS/MS. The details of these instruments and the operational parameters are described in the supplementary materials.

3. Results

3.1. Blank matrix homogeneity studies

The selection of a representative blank material for method optimization is fundamental to develop a reproducible protocol for multi-class residue analysis, including the preparation of suitable matrix matched calibrators. The initial goal of the study was to develop a suitable sample preparation procedure for multi-class analysis of GC amenable compounds, focusing on the GC-MS/MS determination. For that reason, it was assumed that the comparison of the volatile organic compound profiles of turmeric samples with that of a composite sample would give a good indication of the homogeneity of the composite sample. HS-GC-IMS data in combination with multivariate analysis (PCA) was used to evaluate for the suitability of a pooled blank matrix for method validation purposes. Ion mobility spectrometry is a powerful and sensitive technique based in the differential mobility of charged ions in a buffer carrier gas. When coupled to gas chromatography the two-dimensional analysis can be represented in a contour plot, which permits a highly sensitive detection of the molecular species present in the sample. Eiceman (2002) provides an introduction to GC-IMS and describes the value of mobility measurements. In a first attempt, five different commercial blank turmeric samples, one pooled sample and an authentic (laboratory-dried/finely powdered turmeric rhizome) sample profiles obtained by GC-IMS, were compared.

As shown in Fig. 1A, the HS-GC-IMS data of the pooled sample (TurmxPd) and the individual blank turmeric samples (Cur 1, Cur 2, Cur 3, Cur 4, Cur 5) were compared in terms of their chemical composition using principal component analysis (PCA). Each sample produced a specific chemical fingerprint. The pooled sample, prepared by geometric dilution in the first attempt, was insufficiently homogeneous. Therefore, a new blank composite (pooled) preparation approach was implemented as described in 2.2.1. The final pooled turmeric powder thus obtained (TPmix) was tested for homogeneity and representativeness by GC-IMS. This second pooled sample (TPmix) was considered representative and homogeneous as shown in Fig. 1B. In the PCA diagram TPmix is well centred in comparison to the original individual turmeric blanks.

3.2. Method optimization

The turmeric samples were initially extracted with the reference methods currently used for pesticide residue analysis: the SweEt (Pihlström et al., 2007), the QuEChERS CEN-15662 (CEN, 2018) and the Dutch Mini Luke (Lozano et al., 2016).

In general, each analytical method produces a specific co-extractive profile, which in turn may yield specific matrix effects. It has been reported that the higher the number and concentrations of co-extractives, the higher the chance of generating significant ME (Anastassiades et al., 2003; Lehotay et al., 2010). Therefore, the co-extractive profiles from each tested reference method were evaluated and compared to select the most promising sample preparation protocol for method optimization for turmeric. HS-GC-IMS was used to compare the number of volatile co-extractives from each sample preparation method applied to blank extracts (. Dutch Mini Luke, QuEChERS CEN- 15662, and SweEt procedures) All the methods yielded high amounts of co-extractives, but the ethyl acetate-based method resulted as being the best option. The final decision on the most suitable method for sample preparation was reached after the evaluation of the profiles obtained using GC-MS/MS in scan mode.

Finally, the ethyl acetate extraction procedure was selected for further method optimization of the clean-up step and validation in turmeric. Some advantages of ethyl acetate as extracting solvent over acetonitrile are the minimization of polar interferents in the extract (Parrilla Vázquez et al., 2019) while overcoming the instrumental restrictions of some laboratories have to the direct injection of acetonitrile in the GC systems (Aysal et al., 2007).

The SweEt method was compared to the analytical method reported by Aysal et al. that combines the extraction of the SweEt method and the QuEChERS clean-up (Aysal et al., 2007). Because of the high content of polar and volatile compounds, such as curcuminoids and terpenes (Li et al., 2011), different combination of sorbents, including RP-C_{18} were also evaluated for the clean-up step. The sample preparation method that provided the highest number of recovered analytes during spiking experiments (at 0.05 mg kg $^{-1}$) is described in 2.4.4. This method uses RP-C_{18} to absorb the essential oils and some of the carotenoids present in turmeric as opposed to Ghosh et al. (2020) that uses graphitized carbon black (GCB) for clean-up for determination of pesticides in turmeric.

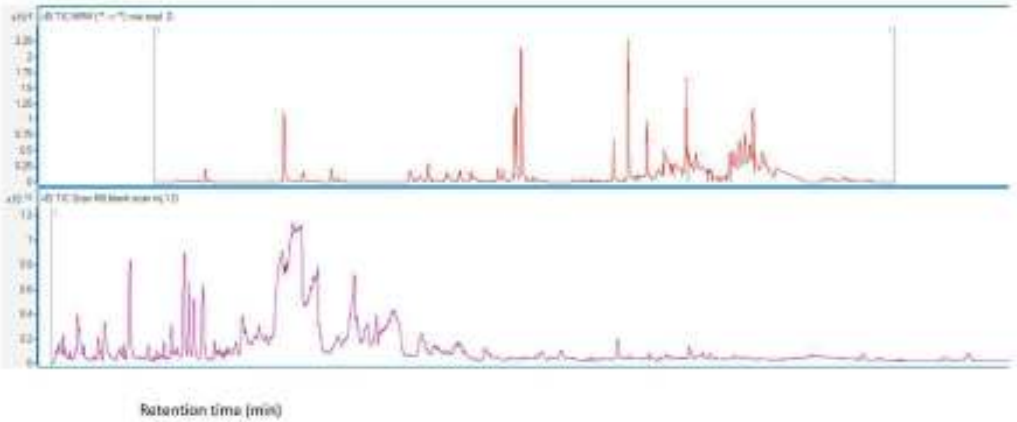


Fig. 2. The total ion chromatograms (TICs) of the MRMs for the pesticide mixture at 0.05 mg/L (upper figure) and a blank matrix obtained by the described sample preparation procedure (lower figure).

Fig. 2 presents the total ion chromatogram (TIC) of the MRMs for the pesticide mixture at 0.05 mg/L (A) and a blank matrix obtained by the final sample preparation procedure (B). It is shown that the blank matrix co-extractives mostly eluted at retention times separate from those of the analytes of interest.

The selected sample preparation procedure, initially intended for pesticide residue analysis, was fine-tuned to allow the multi-class determination of the selected residues and contaminants at trace levels, including the pesticides, organic pollutants (aldrin, dieldrin, DDT and endrin), aflatoxins and dyes as listed in Table 1. To achieve the best resolution, especially for the pyrethroid pesticides (cyfluthrin and cypermethrin), and to avoid over-contamination of the column, the oven temperature program was optimized, resulting in a relatively long run time of 45.9 min.

3.3. Method validation

Although the Codex guidelines refer specifically to pesticide residue analysis, it was decided in this study to adapt these validation criteria to all of the analytes included in the scope regardless of their analytical class. This decision arose from the fact that no regulatory guideline or standard is currently available that regulates all classes of contaminants simultaneously. The advantage of this method is the ability to screen for a broad scope of contaminants. In the case of a contaminating compound being detected, the analyst is obliged to comply with the requirements of the specific legislation for the class of interest.

3.3.1. Selectivity, linearity and calibration

The selectivity of the method was demonstrated by showing that the chromatographic signals obtained originated from the analytes of interest only and not from interferences. The composite sample of blank turmeric was analysed in scan mode in both GC-MS/MS and LC-MS/MS to verify that no interferences were present at the retention times of the analytes of interest. Ion ratios were assessed for each analyte. Sample GC-MS/MS chromatograms for fortified turmeric, reagent and matrix blanks are provided in Fig. 1 of the supplementary material.

The linearity was tested for all the analytes in both the GC and LC instruments, with residuals ≤20% at concentrations between 5 and 200 μg L $^{-1}$ and ≤30% for concentrations between 1 and 5 μg L $^{-1}$. All calibration curves had correlation coefficients (R^2) ≥ 0.99.

The calibration was performed with matrix matched calibrators in both GC-MS/MS and LC-MS/MS systems. Weighted linear regression (1/x) was applied to all analytes in the GC-MS/MS detection system in accordance with Codex guidelines. Ordinary linear regression was applied to the LC-MS/MS detection system. The linear range is presented in Table 1 for each of the analytes determined by GC-MS/MS and LC-MS/MS.

3.3.2. Trueness, recovery and LVL

The codex guideline CXG 90-2017 requires the use of a certified reference material to establish a measure of trueness; however, a reference material for turmeric was not available, and the authors could not find any proficiency testing or interlaboratory trial for residues in turmeric (Codex Alimentarius Commission, 2017). Therefore, an alternative approach was to perform a collaborative trial, on the same turmeric matrix and using the same standard mixture of analytes, between FEPL laboratory in Austria and GACT laboratory in Uruguay. The results of the interlaboratory results are discussed in 3.3.6.

The mean recoveries of all the analytes included in the scope of the method were assessed. Table 1 shows the recovery data for each analyte at the three fortification levels (0.01, 0.02 and 0.05 mg kg $^{-1}$) obtained by GC-MS/MS and LC-MS/MS. In general, the recoveries were between 60 and 120% at 0.01 mg kg $^{-1}$, and 70–120% between 0.01 and 0.05 mg kg $^{-1}$, as recommended by the Codex guideline. Fipronil was validated using either GC-MS/MS or LC-MS/MS, however in LC-MS/MS it required ESI (−).

As shown in Table 1, the method with analysis by GC-MS/MS, was successfully validated for a total of 54 analytes at all three fortification levels. One analyte (dicofol) could be validated only at 0.05 mg kg $^{-1}$, in line to the results reported by Ghosh et al. (2020) for this pesticide, that reported recovery values at 0.01 mg kg $^{-1}$ lower than the guidelines' acceptable ranges.

Consequently, as defined in the Codex guidelines, the LVL of the method was set for 54 analytes by GC-MS/MS at 0.01 mg kg $^{-1}$. Dicofol has an LVL of 0.05 mg kg $^{-1}$.

As shown in Table 1, despite the efforts in optimizing the instrumental method for GC-MS/MS, the pyrethroid pesticides cyhalothrin, cyfluthrin and permethrin could not be quantified because the linear range of the calibration was above the validation range. On the other hand, the recovery of deltamethrin, another pyrethroid pesticide, at the three fortification levels was compliant to the Codex guidelines (Codex Alimentarius Commission, 2010). In general, the LVL and the recovery values obtained by the method are in agreement with those reported recently by Ghosh et al. (2020). The recoveries of dieldrin, endosulfan sulfate, pentachlorobenzene, tetradifon and the planar pesticide hexachlorobenzene, are compliant to the Codex guidelines at all fortification

levels and better than those reported by Ghosh et al. (2020) at 0.01 mg kg $^{-1}$ for these pesticides. An explanation for the low recovery values obtained by Ghosh et al. is possibly due to the use of GCB in the clean-up step of their method as it is a well-known fact that planar compounds are readily adsorbed on to GCB (Niell et al., 2017).

As shown in Table 1, the method with analysis by LC-MS/MS was successfully validated for a total of 33 analytes at all fortification levels (0.01, 0.02 and 0.05 mg kg $^{-1}$). Methamidophos, acephate and omethoate showed consistent and reproducible recoveries but at recovery levels much lower than the Codex criteria, even though repeatability and reproducibility values were within the acceptable ranges. This result was confirmed by the GACT results (see section 3.3.6, "Interlaboratory Study"). The lower recoveries for methamidophos, acephate and omethoate could be explained by their low Kow. This is in line with the poor recoveries for polar or semipolar analytes (logKow <4) reported by Parrilla Vazquez et al. (Parrilla Vázquez et al., 2019). These analytes could eventually be included in the scope of the method provided this consistent behaviour is further validated during the on-going QA/QC of the method. To quantify these compounds, the results should be corrected for recoveries and the correction should be specified in the laboratory results report (European Commission, 2019). Additionally, alachlor, aldrin, azinphos ethyl, chlorpyrifos methyl, fenpropathryn, imazalil and paraoxon methyl met the validation criteria only at the 0.05 mg kg $^{-1}$ fortification level.

In summary, the method using LC-MS/MS, was successfully validated for 48 pesticides at 0.05 mg kg $^{-1}$33 analytes had an LVL of 0.01 mg kg $^{-1}$, 9 analytes had an LVL of 0.02 mg kg $^{-1}$ and 6 analytes had an LVL of 0.05 mg kg $^{-1}$. The reported LVLs are consistent with previously reported values by Ferrer Amate et al. (2010) and Goon et al. (2017).

The other two classes of organic contaminants, the dyes (butter yellow, sudan I, sudan IV), and the aflatoxins (B1, B2, G1 and G2), had acceptable recoveries by LC-MS/MS using the validated sample preparation method. Particularly, the recovery value for Sudan IV at 0.05 mg kg $^{-1}$ was within the acceptable range of the Codex guidelines. Previous reports by Ferrer Amate et al. (2010) showed recoveries, above 120%.

3.3.3. *Precision*

The precision data, expressed relative standard deviation or coefficient of variation (CV%), are presented in Table 1. Most analytes comply with the Codex criteria for within laboratory repeatability and reproducibility (34) (CV_r < 30% at 0.01 mg kg $^{-1}$ and CV_r < 20 at 0.02 and 0.05 mg kg $^{-1}$; CV_R < 45 at 0.01 mg kg $^{-1}$ and CV_R < 32 at 0.02 and 0.05 mg kg $^{-1}$).

3.3.4. *Matrix effects*

Matrix effects (ME) were evaluated by comparing the slope of the calibration curve prepared in solvent with the slope of the matrix-matched calibration curve. Table 1 shows the matrix effects for all the studied analytes for the GC and the LC determination. In general, it has been shown that residual matrix components have different effects in LC and GC systems: while they are shown to reduce the efficiency of ionization in atmospheric pressure ionization techniques, such as in ESI, giving rise to ionization suppression (Ilesil et al., 2017), it has been demonstrated that matrix components can also enhance the signal from analytes in gas chromatography (Poole, 2007). This general trend can be observed in Fig. 2 of the supplementary material which compares the ME for the group of common analytes quantified by both GC-MS/MS and LC-MS/MS. Some exceptions to this general behaviour occur, especially in GC-MS/MS. As discussed by Ueles et al. (2014) signal suppression in GC is not uncommon and can be attributed to degradation of matrix components in the injector port. In the case of the LC-MS/MS determination, only fipronil, showed a significant enhancement effect. This could be explained by either the formation of adducts which make this analyte more volatile or prone to ionize (Ilesil et al., 2017) or by the contribution of the matrix that generates isobaric ions very similar to those of the analyte and not fully resolved in a unit mass resolution instrument (Kauffmann et al., 2010). ME can be divided into 3 categories: high (>50%), moderate (20%–50%) and low (<20%) (Ferrer Amate et al., 2010). Most of the evaluated analytes presented ME. They were high for 40% and 69% of the compounds analysed by GC-MS/MS and LC-MS/MS respectively. Moderate ME were exhibited by 32% and 17% of the analytes in GC-MS/MS and LC-MS/MS determinations respectively. No significant ME (ME < 20%) were observed for 28% and 15% of the analytes in GC-MS/MS and LC-MS/MS determinations, respectively.

It was observed that the ME of the aflatoxins and the dyes evaluated through the LC determination were all significant. ME were high for aflatoxins B1 (57%) and B2 (−58%); for the dyes butter yellow (−79%) and sudan I (−70%).

To compensate for the significant ME observed, matrix matched calibration curves were employed. This was more effective in GC-MS/MS determination than in LC-MS/MS. In LC-MS/MS an alternative approach to compensate for ME is the dilution of extracts to reduce the matrix components. This approach depends on the instrument having the sensitivity to detect and quantify the analytes at a low concentration (ppb/ppt). To investigate this possibility, a 10 times dilution was performed with mobile phase B in LC-MS/MS and acidified ethyl acetate in GC-MS/MS determination. The ME of the original and diluted extracts is shown in Table 1. The ME drastically changed from that observed without dilution. In GC-MS/MS this approach was unsuccessful as the percentage of analytes showing high ME increased from 40% to 82%. On the contrary, in LC-MS/MS the ME decreased, as expected. The percentage of analytes showing high ME decreased from 69% to 6%, and the pattern of ME drastically changed from a majority of analytes presenting high ME (>50%) to a majority of analytes showing no ME (<20%). Table 2 of the supplementary material presents the recovery and repeatability relative standard deviation for the diluted extracts determined by LC-MS/MS for one spiking experiment. For the compounds tested by LC-MS/MS, the method performance at 0.01 mg kg $^{-1}$, improved for 5 compounds (alachlor, atrazine, cyprodinil, malaoxon and pirimiphos methyl), but worsened for 7 compounds (chlorfenvinphos, chlorpyrifos, diazinon, dichlofluanid, pendimethalin, permethrin II and phosmet) in comparison to the undiluted extracts. In the diluted extracts, no matrix effects were observed for the aflatoxins and the dyes.

3.3.5. *Measurement uncertainty (MU)*

The measurement uncertainty was evaluated as described in 2.6.2. The estimated expanded measurement uncertainty for all the analytes within the scope of the method did not exceed 50%, the default value specified in SANTE/12682/2019 document.

Twenty five of the target analytes were analysed by both GC-MS/MS and LC-MS/MS and the absolute recovery values (Table 1) were sometimes different. However, the reported values individually comply with the requirements of the Codex guidelines and, according to the estimated uncertainty, the results were assumed to be comparable.

3.3.6. *Interlaboratory study*

The Codex guideline for method validation also requires the evaluation of the ruggedness or robustness of the method. The robustness can be evaluated in different ways, for example, by applying a specific experimental design where minor changes are introduced in the method for the most significant parameters, as described in detail in Maestroni et al. (Maestroni, Vazquez, et al., 2018, B). For the study presented here, the robustness of the method was demonstrated by the successful comparison of results through an interlaboratory study with the GACT laboratory in Uruguay.

The key method performance parameters investigated in GACT were specificity, linearity, trueness, within laboratory repeatability, lowest validated level and matrix effects. Only pesticides were analysed at GACT. In general, the recovery values were similar in FEPL and GACT falling within the uncertainty of the method, yielding overall

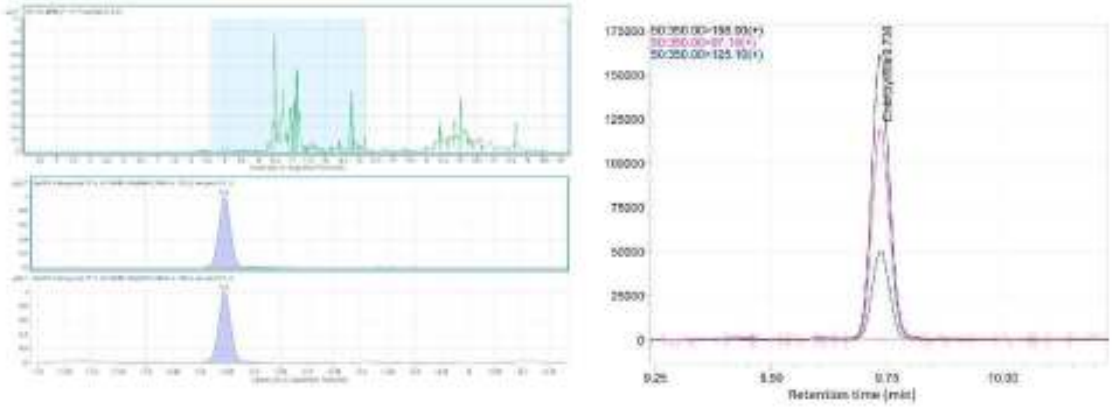


Fig. 3. Chlorpyriphos residue identification and confirmation in an organic commercial sample by GC-MS/MS (A) and LC-MS/MS (B) using the validated method.

satisfactory results and complying with the requirements of the Codex guidelines, as shown in Tables 1 and 2.

A comparison of the results demonstrated that the inter laboratory reproducibility was confirmed for 32 GC and 34 LC amenable compounds in Austria and Uruguay. See Supplementary Materials, for detailed information. Whereas the matrix effects in LC-MS/MS as well as the LVLs for both laboratories were similar, the LVLs in GC-MS/MS at FEPL were lower than those at GACT. The chromatographic conditions were different, and similarly the interferences. Additionally, the instrumental settings of the GCs were different in the two laboratories. The GC-MS/MS at FEPL has a temperature programmable injector and the possibility of performing a back-flush cleaning of the system. The GC-MS/MS at GACT is equipped with a normal splitless injector operated at 280 °C. This could lead to different matrix effects that would greatly influence the determination. Nevertheless, the LVLs in both laboratories are suitable to study the MRL compliance of the pesticides within the scope of the method as shown in Table 2.

These results demonstrate the robustness of the method, indicating that it can be validated and applied in laboratories worldwide equipped with a range of instrumental configurations, what represents an added value to the present work.

3.4. Analysis of commercial samples

The method was also applied to the analysis of 21 commercial samples bought in supermarkets. Residues of between 1 and 3 pesticides (chlorpyrifos, malathion and acetamiprid) were detected in 11 of the samples. Fig. 3 shows chlorpyriphos residue identification and confirmation in a commercial sample, labelled as 'organic', by LC-MS/MS (A) and GC-MS/MS (B) using the validated method. Two of the positive samples were declared and marketed as organic samples. This demonstrates the importance of having a method suitable for the detection of organic residues and contaminants in turmeric powder.

4. Conclusions

A multi chemical class method for the determination of trace contaminants in turmeric was developed using low amounts of solvents for sample preparation. The representativeness of the matrix used for analytical method development was tested using HS-GC-IMS, a screening tool providing rapid comparisons of sample co-extractive profiles.

The method was optimized and validated in compliance with the Codex guidelines for a total of 74 analytes: 67 pesticides residues (insecticides, fungicides, herbicides and persistent organic pollutants), 4 mycotoxins and 3 banned dyes.

The LVLs of the method were lower or equal than the established MRLs. The scope of the method includes the internationally regulated analytes for turmeric. For those analytes not currently regulated, the precautionary principle of 0.01 mg kg^{-1} as tolerance level was applied. The robustness of the method was successfully tested in an interlaboratory study with the GACT laboratory in Uruguay using different brands of instrumentation, laboratory facilities and operators. It was also applied to the analysis of 21 commercial samples from the Austrian supermarkets. Chlorpyrifos was detected in most of them demonstrating the performance of the developed method. Based on these results the method can confidently be used in the routine control of turmeric powders in any laboratory that has instrumental configuration similar to any of those used in this collaborative study. The method can thus help facilitate international trade for multi class contaminants analysis.

It was the first time that a multi-class method was validated by an intercomparison study in turmeric enabling the detection and quantification at low spiking levels and using simultaneously two different detection instruments covering a broad range of polarities.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2020.107579.

Authors' contributions

BM and VC designed the study, participated and coordinated the

laboratory work in both labs (FEPL, Austria and GACT, Uruguay), performed the treatment of the data recovered, discussed the whole results and drafted the paper. NB run the laboratory work, performed the data treatments at GACT, Uruguay, and collaborated in the preparation of the manuscript. SR run the laboratory work, adjusted the instrumental conditions for LC-MS/MS and performed the data treatments at GACT and also performed the instrumental determination by GC and the data treatments at FEPL laboratory and collaborated in the preparation of the manuscript, tables and figures. MI ran the HS-GC-IMS and prepared the PCA plots. YL and NK performed the practical work for the full validation at FEPL. NG performed the instrumental determination and data treatments of the GC-MS/MS analysis at GACT. AC and HH gave the conceptual framework of the manuscript, adjusted and edited the final version. AC, as native speaker, made the final English language revision of the manuscript. All the authors have read the final manuscript and approved the submission.

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Chapter 3: Global discussion of the results

3.1. Optimization of conditions before validation

Optimization of instrumental performance and method's sample preparation conditions was paramount to obtaining reliable data that could be used for proving fitness for the purpose of methods and results of studies. Optimization approaches were applied to complex matrices and, as a result, analytical performance was demonstrated to be acceptable for residues and contaminants in potato, vine leaves, boldo and turmeric. The chromatographic equipment available were LRMSs, and the analytical determinations were applied in targeted mode.

3.1.1 Instrumental optimization

Before any measurement can be made, it is essential to carry out the instrumental optimization. The LC-MS/MS and GC-MS/MS conditions were selectively optimized for the analysis of the method scope which was specific to each type of matrix (potato, vine leaves, boldo and turmeric). The initial mixture of pesticides and contaminants was prepared in solvent (i.e. ethyl acetate) to identify the most suitable MRM transitions and optimize the collision energies (CE) used during compound analysis. The MRM transitions and the MS conditions were established using flow injection analysis mode for the LC-MS/MS methodology and using parent search and product ion scanning mode for identification in the GC-MS/MS. Each of the analyte/commodity combinations proved to be unique in their instrumental optimization.

Matrix effects needed to be identified and assessed to be able to adjust the instrumental strategy to avoid as much as possible coelution of analytes and matrices. In other words, MRM transitions needed to be carefully adapted to avoid peak distortions due to matrix influences, aiming at the identification and qualification of the most selective MRM transition that provided acceptable peak shape, and still an acceptable S/N ratio at the lowest calibration level for quantification in targeted mode.

Table 1: Influence of the studied matrix on the conditions and performance of selected pesticides in GC-MS/MS and LC-MS/MS determinations

		Vine leaves	Boldo	Turmeric
Chlorpyrifos (GC-MS/MS)	**RT**	12	9.6	11.7
	R% at LOQ	70	67	71
	MRMs	196.9->169 (CE 15) 198.9->171(15)	314->258(15) 198.9->171(CE 15)	313.8->257.8 (CE 15) 196.9->107.0 (CE 45)
	ME (%)	-4	-74	41
Tebuconazole (GC-MS/MS)	**RT**	15.2	12.9	14.7
	R%	78	84.8	66.6
	MRMs	125->99(CE 20) 250->125(CE20)	250->125 (CE 30) 226->186 (15)	125->89 (CE 15) 250->125 (CE 20)
	ME (%)	10	334	89
Malathion (GC-MS/MS)	**RT**	11.8	9.4	11.47
	R%	94	82	72
	MRMs	126.9->99 (CE 5) 172.9->99 (CE 15)	173->117(CE15) 143->111(CE 10)	172.9->99.0(CE 15) 157.8->125.0 (CE 20)
	ME (%)	14	121	102
Acetamiprid (LC-MS/MS)	**R%**	na	99	105
	MRMs	na	223->126(CE25) 223->99(CE47)	223.1->126.1 (CE 21) 223.1->73.0 (CE 55)
	ME (%)	na	-35	-45
Thiamethoxam (LC-MS/MS)	**R%**	na	109	86
	MRMs	na	292->211(CE 10) 292->246(CE 10)	292->181.1 (CE 22) 292->211.1 (CE 5)
	ME (%)	na	-73	-31

As shown in **Table 1** for few pesticides analysed across all matrices and taken as an example, the target matrix had a direct influence on the optimization of the instrumental conditions, mainly due to the different residual matrix that elutes isobarically with the target analytes. In this case the peak shape of target analytes was affected and compromised analytical determination and confirmation criteria such as ion ratios. Additionally, it was noted that the type of ME also influenced the retention time of the analytes. Shift in RTs were

observed for the elution of analytes prepared in solvent versus those prepared in matrix. This is a direct consequence of the distribution coefficient of the analytes, that tend to increase when additional “matrix” is present in the chromatographic column. The use of retention time locking as a chromatographic technology for GC-MS/MS proved essential for qualitative and quantitative determinations. In all validated methods performed for vine leaves, boldo, turmeric and potato it was demonstrated that method validation needs to be specific for each analyte/matrix combination in each laboratory and for each instrumentation, as optimization of the instrumental conditions is a specific and not a generic condition. Therefore, it was concluded that literature information and manufacturers’ databases about MRMs and CE can be a good start for the analysts, but in-house validation is a must for reliable quality-controlled results and must be specific for each matrix and analyte combination.

One conclusion from this thesis is that the concept of representative matrices is not applicable to minor crops.

3.1.2 Compensation of matrix effects

Vine leaves, boldo leaves and turmeric powder matrices have been shown to be rich of secondary metabolites, such as organic acids, vitamins, terpenes, alkaloids, phenolics, tannins, carotenoids, polyphenols, etc. Due to the fact that the target analytes are generally present at trace levels, and the secondary metabolites in about 100-1000 times higher concentrations, sample preparation shall eliminate in bulk, as much as possible of the plant secondary metabolites. However, this is not always 100 % possible using cheap, fast, effective, modern methods such as QuEChERS, and an inevitable amount of coextractives will be present in the extracts for chromatographic determination. One could revert to traditional sample preparation methods, but that would represent a pitfall of modern analytical technologies and eliminate all the benefits linked to QuEChERS type of approaches. Coextractives from the matrix are unpredictable and can cause various issues such as: ion suppression (or in some cases enhancement), poor chromatography, false positives due to the presence of isobaric compounds, poor analyte recovery due to partitioning or adsorption processes, and interferences with the ion ratio of the product ions in targeted analysis. The coextractives generally cause moderate to high matrix effects, typically suppressing the signal in LC analysis and suppressing or enhancing the signal in GC analysis.

It was noted that the use of mass selective detectors employing quadrupole and/or ion trap analyzers can, under high matrix effects, fail to provide unbiased confirmation of analyte identity. This lack of selectivity applies especially to compounds yielding non-specific ions of low m/z values by electron impact ionisation process, i.e. dichlorvos. Under these circumstances achieving unbiased identification and accurate quantitation may become unfeasible because of interfering matrix ions that have the same characteristic ions as in the target analyte. The conclusion is that the analytical laboratory must be aware of this challenge, and identify alternative tools to overcome this phenomenon.

The lack of selectivity associated with modern generic sample preparation and clean-up can be compensated by the increased selectivity provided by triple quadrupole (QqQ) mass spectrometers that generally imply high sensitivity when operated in SRM/MRM target mode. In other words, it is up to the laboratory instrumental capabilities to be able to “deviate and account” from significant matrix effects. This conclusion is supported by the performance criteria set by international guidelines and standards. However, analytical technology is developing rapidly, with new, more powerful techniques available all the time [1]. To enable analytical laboratories to fulfil their role in ensuring a safe global food supply, authorities in developing countries, and capacity building organisations working with them, must invest effort in optimising the application of their current analytical capabilities, and in identifying and selecting those emerging technologies that are most appropriate in terms of performance, cost-effectiveness, longevity and sustainability. It is important that the development or revision of analytical performance criteria is based on sound risk analysis rather than instrumental capabilities, and the suitability of the older, proven techniques (i.e. specific detectors for GC) should still be recognised.

It was demonstrated that ME cannot be eliminated and therefore compensatory strategies had to be implemented. Using the LC-MS/MS detection system it was possible to dilute the turmeric extracts using ‘dilute and shoot” strategies. The extracts were diluted 1:5- 1:10 with ethyl acetate before injection into the chromatographic system. In general matrix matching was the preferred strategy adopted for compensating ME in all studies of matrices. In the case of GC-MS/MS, the matrix coextractives acted as analyte protectants in the calibrators and enabled proper quantification and confirmation thereby ensuring low enough LOQs.

A comparison of matrix effects observed in GC-MS/MS for cypermethryn, fenarimol, kresoxym methyl, pendimethalin, pyrimethanil as common analytes included in the scope of vine leaves, boldo and turmeric powder are shown in **Figure 1**. These analytes had shown a lack of ruggedness in potato extracts when using QuEChERS as sample preparation method. Only for the case of vine leaves, opposite matrix effects are shown for pyrimethanil,

with a signal enhancement in vine leaves and signal suppression in boldo matrix by GC-MS/MS.

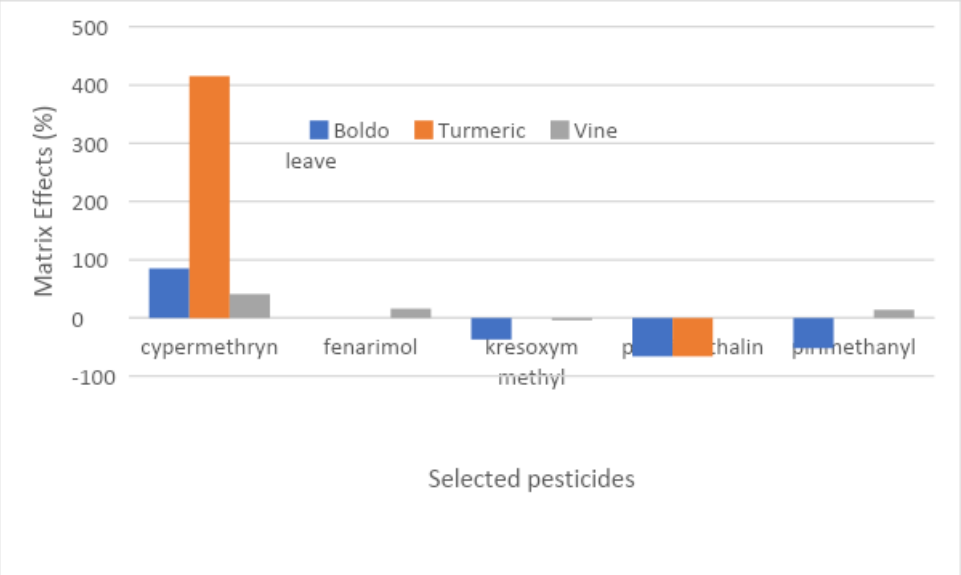

Figure 1: Comparison of matrix effects in vine leaves, boldo and turmeric powder for cypermethyn, fenarimol, kresoxym methyl, pendimethalin and pyrimethanil obtained by GC-MS/MS.

This is a further confirmation that validation parameters require fine tuning for each studied matrix and confirms the generic conclusion that matrix effects cannot be predicted. It is possible to conclude that the main limitation of LC–MS and GC-MS based “multiplex” approaches is the inability to overcome matrix effects; however, compensation approaches are available, although time consuming and with typical efficiency less than 100 %.

In addition, it was shown in the case of vine leaves, boldo and turmeric powder that, with increasing sample matrix complexity, the high intra-matrix variations cannot be compensated by using a “default” blank sample extract for matrix matched calibration. It is therefore essential to identify a composite sample for the purpose of carrying out method validation studies. Most often, finding a proper blank is a very challenging task for the analytical chemist.

3.1.3 Optimization of calibration strategies

Additional work was implemented to compare different calibration strategies for the detection of 50+ pesticides in vine leaves by gas chromatography coupled to mass spectrometry, namely matrix matching, solvent calibration and procedural calibration. The calibration curves were constructed using linear weighted regression, and the slope of the solvent calibration curve was the highest, while the procedural calibration curve was the lowest. Figure 2 shows, as an example, the analyte zoxamide (**Figure 2**).

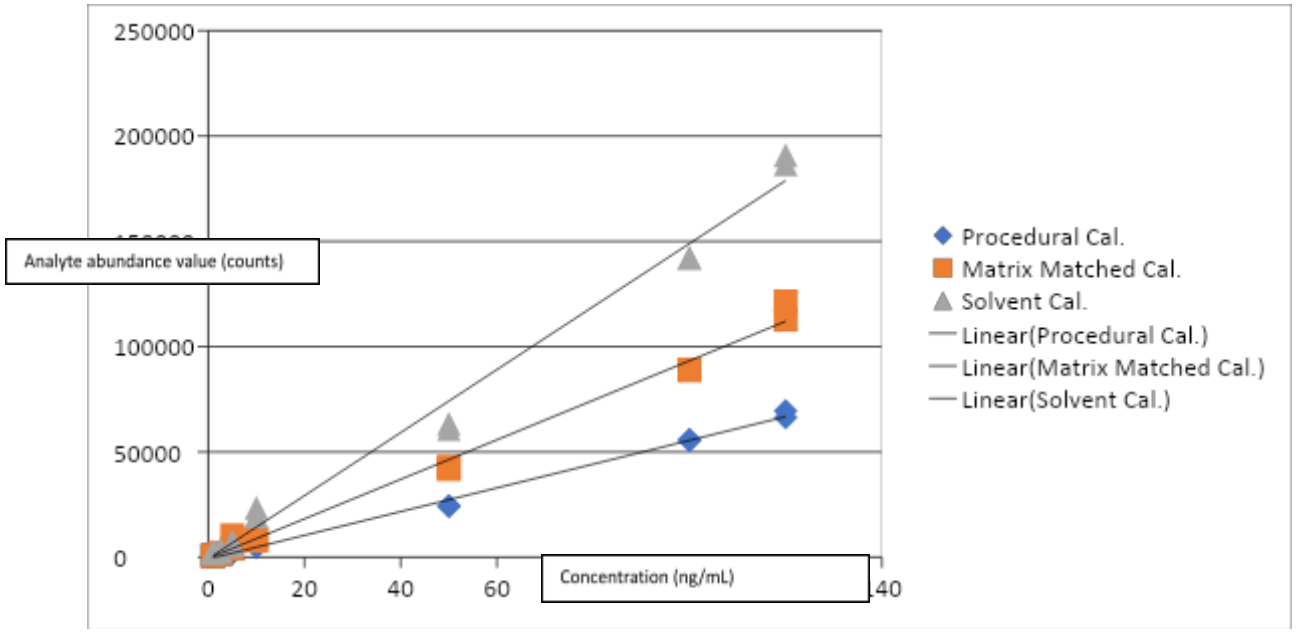

Figure 2: Calibration curves for Zoxamide by GC-MS/MS

The ME of sample extracts injected in GC-MS/MS, could be well compensated by the matrix matched curve approach. On the other hand, procedural calibration helped to compensate for matrix effects and recovery losses, and also for extraction efficiency, if that is known to be an issue previously identified during method validation studies for the particular matrix-analyte combination. It was concluded that individual calibration requirements shall be assessed on a case by case and the calibration approaches modified accordingly and in line with reference guidelines. Investigations on the use of isotopically labelled internal standards (ILSTD) to compensate for matrix effects were not implemented in this thesis. Initial work indicated that ILSTD is quite challenging for GC-MS/MS applications, and LC-MS/MS is the

preferred chromatographic technique for the Stable Isotope Dilution Assay (SIDA) format. The ILSTD was not further implemented in this thesis as the scope was large and the costs associated with the experimental work were outside of the allowed budget.

3.1.4 Subsampling optimization

An often-neglected aspect is the optimization of the subsampling step. This ensures homogeneity of the analytes in the processed commodity using a defined analytical portion amount. Sample treatment is defined as the procedure (e.g. cutting, grinding, mixing) used to make the analytical sample acceptably homogeneous with respect to the analyte distribution, prior to removal of the analytical portion. As part of the method validation studies for vine leaves, this thesis also included the verification of the homogeneity of the analytical portions at 2 g level. This was the analytical portion amount that is solvent-extracted and analyzed using the validated analytical procedure to quantify/qualify the pesticides included in the method scope for vine leaves. Two different approaches were adopted to verify that the analytical portion was homogeneous enough to ensure that the sub-sampling uncertainty was acceptable.

The first approach consisted in analysing in a single batch all analytical portions deriving from a naturally contaminated sample. In this case the variability deriving from the analysis of pesticide residues in the sample can be approximated to the variability of subsampling as all other factors are kept constant.

The second approach consisted in evaluating differences arising from fortification (spiking) experiments using blanks. Spiking was done on the blank vine leaves before sample processing and compared to spiking in individual analytical portions after homogenization, see **Figure 3**. The difference between the two procedures can be approximated to the variability arising from sample homogenization and subsampling as all factors were kept as constant as possible during the analytical procedure. The spiking procedure consisted in placing the entire vine leaves on clean aluminium foil and carefully spiking using a Hamilton syringe with mixture of pesticides at 0.01 mg/kg (see **Figure3**). After waiting for 30 minutes to allow absorption of residues into the surface, the vine leaves were carefully transferred to a mortar, and using a pestel and liquid nitrogen, the leaves were cryogenically processed to a fine powder (See **Figure 4**). The samples were prepared according to the validated method for vine leaves.

Figure 3: Vine leaves are placed on aluminium foil and spiking experiments are carried out using a Hamilton syringe. The solvent applied to the tray for washing the aluminium is also analyzed and subtracted from the calculations applied for mass balance.

Figure 4: Ms B. Maestroni is processing blank samples of vine leaves using liquid nitrogen and a mortar and pestle.

A naturally contaminated sample, found to be positive for chlorpyrifos residues, was used to study the sample processing homogeneity according to the first approach. Chlorpyrifos was therefore selected as the study pesticide for the spiking experiments and included in the spiking mixture according to the second approach.

Codex guideline CAC/GL 59-2006 [2] on estimation of uncertainty of results provides information for the statistical background and the principle of estimating sample processing from spiking experiments, according to approach 2. According to CAC/GL 59-2006, CVL is the relative uncertainty of the laboratory phase of the determination which may derive from the sub-sampling, sample preparation, sample processing and analytical steps. CVSP is the relative uncertainty of the preparation of test portion including sub-sampling, sample preparation and sample processing and CVA relative uncertainty of the analysis including extraction, clean-up, evaporation, derivatization, instrumental determination.

Results of the sample processing experiments are summarized in **Table 2** for the pesticide chlorpyrifos according to the two approaches.

Table 2: Summary results for chlorpyrifos obtained from experiment aiming at verifying the sub sampling (or sample processing) homogeneity according to two approaches, described in the text.

Approach 1			Approach 2	
nr samples	25		nr samples	9
average residue (µg/kg)	93		average residue (µg/kg)	95.7
CV_{SP}%	6		CV_A (%)	2.2
			CV_L (%)	3.4
			CV_{SP} (%)	2.7

According to **Table 2** both approaches account for less than 10 % of variability deriving from the subsampling step, and are in line with estimated values for subsampling in other commodities [3], [4]. This result allowed us to conclude that processing of vine leaves using liquid nitrogen in a mortar and pestle was giving an acceptably homogeneous sample, and that the sub-sampling variability was within acceptable levels. On the contrary if this would have not been the case, the use of larger analytical portions (i.e. > 2 g) should have been considered to obtain a better estimate of the true value.

A quick verification of the subsampling step was also implemented for boldo and turmeric, which were homogenized to fine powders using cryogenic approaches. Although apparently easy to mix, turmeric powders surprisingly showed inhomogeneity at a first verification step after preparation using geometric dilution. The use of IMS quickly allowed confirmation of the subsampling homogeneity using the information provided by the volatile fraction. The subsampling step had to be redesigned to account for mixing for 5 minutes in a ball mill, followed by mixing using a mortar and pestle for 15 minutes with the addition of liquid nitrogen.

3.1.5 Sample preparation development and optimization

Before validation can be demonstrated, it is imperative to develop and/or adapt a sample preparation method. From the more simplistic approach of dilute and shoot, a wide variety of sample preparation approaches can be applied. In this thesis, generic sample preparation protocols such as QuECheRS sample preparation were applied to the minor crops under study, and specific clean-up combinations were implemented on vine leaves, boldo and turmeric matrix. TLC and IMS techniques were used to identify the optimized conditions for the studied analyte/matrix combinations (**Figure 5**).

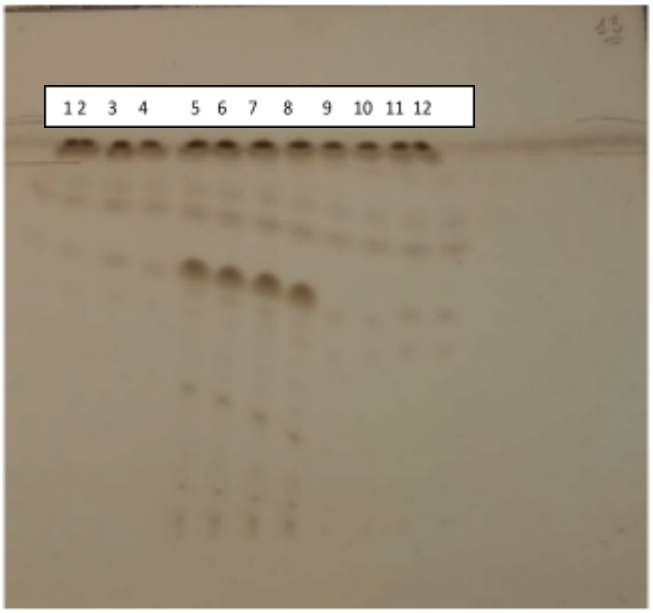

Figure 5: Example of a set of clean-up salt combinations (from 1 to 12, different concentrations of PSA and $MgSO_4$) as applied to blank vine leaves matrix and spotted on thin layer chromatography (TLC) plates.

An important variable in all studies was the addition of water to the food matrices, to help the organic solvent to better extract the analytes. The vegetal material swells with the added water, facilitating the extraction of some of the more polar analytes. In the case of water miscible solvents (acetonitrile, methanol), the analytes are easily dissolved or partitioned when water immiscible solvents (Ethyl Acetate, Dichloromethane) are used.

In general, primary secondary amine (PSA) in combination with reversed phase carbon (RP-C18), graphitized carbon black (GCB) and other sorbents such as florisil, alumina and calcium chloride ($CaCl_2$) were shown to be effective in a certain extent in matrix removal according to the published literature [5], [6]. In all studies, the final selected sample preparation method was a trade-off between residue determination and a certain degree of matrix effects. In this thesis an effort to “greening” the analytical process was also achieved by avoiding the use of very toxic chlorinated solvents such as dichloromethane, and by miniaturizing the method conditions. The optimization included a verification of the method performance parameters, especially recovery, and standard deviation (accuracy & precision) using the lowest possible analytical portion weight, i.e. 2 g of sample, that resulted being sufficiently homogeneous in respect to analyte distribution, and sufficient in respect to the achieved LOQ. This consequently implied lower volumes of solvents used for extraction and lower amounts of sorbents for sample preparation, thereby going in the direction of “green chemistry” principles [7].

3.1.5.1 Homogeneity verification process

A new approach to verify the homogeneity of the turmeric powder samples was implemented using IMS. In practice, the volatile components of the turmeric samples were measured using a headspace gas chromatography - ion mobility spectrometer (HS-GC-IMS). In a first attempt, five different commercial blank turmeric samples, one pooled sample and an authentic (laboratory-dried/finely powdered turmeric rhizome) sample profiles were obtained by GC-IMS and were compared in terms of their chemical composition using principal component analysis (PCA) as shown in literature [8]. Each sample produced a specific chemical fingerprint. The pooled sample, prepared by geometric dilution, was offset in comparison to the original incremental samples and therefore was consider as insufficiently homogeneous. Therefore, a new blank composite (pooled) preparation approach was implemented, and the final pooled turmeric powder thus obtained was sufficiently

homogeneous and representative for method validation studies. The novelty of this approach relies upon the use of IMS as a quick and non-destructive technique for turmeric powder, that carries a lot of valid information and helps verifying the homogeneity of the subsampling step.

3.2 Validation of analytical methods for minor crops

The present work focuses on different aspects of method validation, which is the process of characterising the performance to be expected from a method in terms of its scope, specificity, accuracy sensitivity, repeatability, within laboratory reproducibility, among others, or, in other terms, defined as the process to assess the analytical method's fitness for purpose.

This thesis had the objective to investigate all aspects inherent to the minor crops' analytical method validation. The optimization of subsampling homogeneity, the efficiency of extraction for crops traded as dry matrices, the representativeness of blank matrices used for validation studies, the matrix effects, the achievable LOQ and compliance to existing MRLs, the chromatographic optimization, the stability of retention times, were some of the challenges studied in due course. Precise adjustments of all conditions and complex sample preparation details and optimization demonstrated that each matrix behaves very differently from all others, thus supporting the concept that method validation shall be a "fine-tuning moving mechanism" to allow proof of fitness of complex biological structures. Existing method validation guidelines are helpful to provide the desirable benchmark for performance criteria, but ultimately it is the specific fine tuning that the analyst can apply, based on professional judgement and experience, that provides the best trade-off for all conditions. This was demonstrated for vine leaves, boldo and turmeric. To summarize, mechanization of certain analytical steps can represent a useful help to the analytical chemist, but cannot replace the judgement of a well-trained analyst in delivering the best conditions for testing. The methods were all successfully applied to the analysis of real market samples and proven fit for purpose.

3.3 Verification of ruggedness and robustness

Ruggedness and robustness were studied in two different commodities, potato and turmeric, a major and a minor crop respectively. Different experimental approaches were applied:

design of experiments (DoE) and interlaboratory comparisons. Although those approaches are applied to intra-laboratory and inter-laboratory conditions, they both provided a measure of fitness for purpose for the developed and validated methods. This thesis considered that the necessities of laboratories may vary depending on the availability of commercial proficiency testing schemes, and the availability of valid collaborators that are working on similar matrices and studies. It is not always possible to identify such schemes or collaborations, and the analytical laboratory needs to have tools at hand to demonstrate fitness for purpose, as an objective proof which is often required by accreditation bodies during accreditation processes. The goal to provide laboratories with a methodology to validate the ruggedness or robustness of a method was achieved. The study in potato showed that several DoE can be utilized to generate such information, in a relatively fast and cheap way. A Plackett Burman design (PBD) and an augmented definitive screening design (DSD) were implemented and compared for use in analytical laboratories worldwide. While the PBD is cheaper in terms of the number of replicates needed, the DSD provides more information to the analytical chemist, mainly in the identification of the analytical parameters that need to be under strict control through adherence to prescriptions such as standard operating procedures.

The interlaboratory work was applied to two matrices: boldo and turmeric. In boldo the analytical determinations were carried out using GC-MS/MS, and these data contributed to establishing the method performance parameters for the boldo multiresidue method. In the case of turmeric, a full interlaboratory study was implemented to study key method performance parameters. A comparison of the results demonstrated that the inter laboratory reproducibility was confirmed for 32 GC and 34 LC amenable compounds in Austria and Uruguay, according to the results presented in Annex 1. As a general conclusion of this interlaboratory tool, it was noted that a minimal financial investment is required by the analytical laboratory to be able to implement the DoE and proficiency testing and / or collaborative studies. The required resources need to be available and included in the planning of the analytical processes of the laboratory.

3.4 Provision of inputs for regulatory frameworks

To contribute to the provision of information for food safety as an input to the food control process (regulatory framework), a study on dissipation of residues in vine leaves was implemented in Syria and Austria with contributions from Uruguay. The goal was to highlight

the dependence of compliance of pesticide residues to potential MRLs as a function of the regulatory framework chosen. To be more specific, this study helped generating information for the potential establishment of MRL, as they are practically non-existent for grapevine leaves, apart from only one MRL for one neonicotinoid pesticide (in the EU legislation, only). Information about maximum residue values, withholding time and dissipation rates, amongst others, are important information required to evaluate, and assess the impact of dietary exposure to pesticides from consumption of contaminated food products. These parameters are obtained either experimentally or through modelling, and vary according to the type of pesticide, type of crop and prevailing environmental conditions. In this collaborative work it was shown that the WHP varies for the different pesticides and crop combinations and corroborates previous findings [9], [10], [11], [12]. The WHP is important information that needs to be generated to demonstrate that residues in the treated crop will not exceed the maximum residue limit. The study on dissipation behaviour of pesticides in vine leaves helped address the role of the analytical laboratory in the interpretation of chemical residues and contaminants, and its advisory role for the improvement of good agricultural practices (GAPs) towards improved agricultural practices. From this work it was possible to show that due to different conditions, i.e. climatic conditions, applications, type of pests, etc. each country should make local efforts to protect its market and consumers. Therefore, MRLs setting at CODEX should be considered as a harmonization of MRLs with efforts and contributions from all countries worldwide, to take into account regional and country specific differences. While MRLs are a means to protect the market, toxicological endpoints (ADI, etc,) are directly considered for the protection of consumer health and are indirectly monitored through national monitoring programmes.

3.5 Contributions to the understanding of minor crops organic trace analysis

Another conclusion from this work is that the concept of representative matrices, as discussed in the Codex [13] and the SANCO [14] guidelines, is not applicable to minor crops. According to the above-mentioned guidelines, in the category of “difficult or unique commodities” spices are listed as an example of typical representative commodities within the category. This isn’t exactly a specific indication of what a representative matrix should be. The category of minor crops is so wide and includes very different commodities from a botanical aspect. The attribute minor crop refers to a quantitative (trade) aspect as discussed previously. Specific optimizations of sample processing and analytical sample

determination were shown to be strictly necessary in the case of analytical validation for pesticide and other organic contaminants in minor crops. There is a demonstrated need for specific validation of each minor crop commodity, especially due to the inherent matrix effects that challenge the analytical determination. In addition, the EU SANCO document includes the statement that “difficult commodities should only be fully validated if they are frequently analyzed. If they are only tested occasionally, validation may be reduced to just checking the reporting limits using spiked blank extracts”. It was possible to demonstrate that full validation is a must for each minor crop or difficult commodity. High matrix effects were encountered for vine leaves, boldo and turmeric matrices, requiring a full validation and an extended knowledge of the studied matrices to identify the critical points of the analytical procedure. Just checking the reporting limits using spiked blank extracts may not be sufficient. Subsampling and homogeneity need to be verified, sample preparation needs to be optimized along with the instrumental optimization (MRM transitions) as previously discussed. Eventually minor crops, such herbs and spices, are commodities that within each type, may have such a variety of different commodities that specific and holistic methods for ensuring food safety need to be implemented in each case.

3.6 First insights to multiclass methods

Rather than classical methods that are more single class oriented, modern multiresidue methods were applied to the determination of residues and contaminants in vine leaves, boldo and turmeric samples using LC-MS/MS and GC-MS/MS. The importance of developing, adapting and validating multi residue, multi analyte, multi class or in other words “multiplex analytical methods” to be able to study a broad scope of analytes within the same sample preparation procedure was shown in each single study. To date no regulatory guidelines or standards are available that regulate all classes of contaminants and residues simultaneously. However, in case of a contaminant or residue being detected, the analyst is obliged to comply with the requirements of the specific legislation for the class of interest. “Multiplex” technology is becoming more and more applied due to its analytical screening advantages compared to single classical analytics. The analytical community recognizes the importance of being able to screen for a large number of commodities, however confirmatory methods need to be at least as selective, specific, precise, accurate, rugged, fit for purpose as possible, and eventually also quick, cheap, environmentally friendly and risk based.

3.7 Uncertainty estimations

An uncertainty estimation was carried out in the turmeric study as a quantitative indicator of the confidence in the generated analytical data, and it was shown that the uncertainty of all the analytes did not exceed the default value of 50 % as indicated in the Codex [2] and SANTE guideline [14]. It was shown that a thorough analysis of the method not only identified possible uncertainty budget, but also helped identifying critical points and where needed, helped improve the analytical method. As in the case of ruggedness testing, the estimation of uncertainty is a useful tool to identify pitfalls in the analytical procedure and correct for small possible sources of errors that would otherwise go undetected. **Figure 6** provides a quick summary of the possible sources of uncertainty of the analytical procedure as identified in the turmeric study.

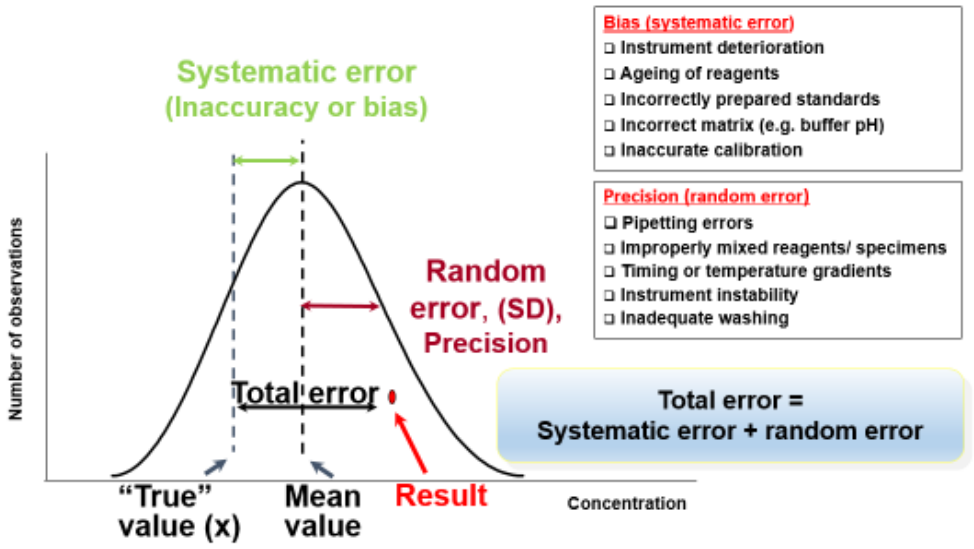

Figure 6: Possible sources of errors in quantitative analysis

Uncertainty evaluation has become a must in organic trace analysis. The accurate evaluation of the uncertainty measurement gives an extra value to the analytical data and provides reliability and analytical confidence in the obtained results.

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Capítulo 3: Discusión global de los resultados

3.1. Optimización de condiciones antes de la validación

La optimización del método instrumental y de las condiciones de preparación de la muestra fueron fundamentales para obtener datos fiables que pudieran utilizarse para obtener resultados precisos y exactos en los estudios. Se aplicaron enfoques de optimización a matrices complejas y, como resultado, se demostró que el rendimiento analítico es aceptable para residuos y contaminantes en papa, hojas de *Vitis*, boldo y cúrcuma. Los equipos cromatográficos empleados fueron equipos de cromatográfia gaseosa y líquida acoplados a espectrometría de masas, tal como es el requerimiento del estado del arte en análisis de trazas orgánicas, empleando la estrategia “target” o dirigidas para las determinaciones analíticas.

3.1.1 Optimización instrumental

Antes de ejecutar cualquier medición, es fundamental realizar la optimización instrumental. Las condiciones de LC-MS / MS y GC-MS / MS se optimizaron selectivamente para el análisis del alcance del método específico para cada tipo de matriz estudiada (papa, hojas de *Vitis*, boldo y cúrcuma). La mezcla inicial de pesticidas y contaminantes se preparó en disolvente (acetato de etilo) para identificar las transiciones más adecuadas utilizando Multi Reaction Mechanisms (MRM) y optimizar las energías de colisión (CE) utilizadas durante el análisis de los compuestos. Las transiciones MRM y las condiciones de MS se establecieron usando el modo de análisis de inyección de flujo para la metodología LC-MS / MS y usando la búsqueda de iones padres y el modo de escaneo de iones productos para la identificación en GC-MS / MS. Cada una de las combinaciones de analito / producto resultó ser única en su optimización instrumental.

Los efectos de la matriz debían ser identificados y evaluados para poder ajustar la estrategia instrumental y así evitar en la mayor medida posible la coelución de analitos y matriz. En otras palabras, las transiciones de MRM debían adaptarse cuidadosamente para evitar distorsiones de los picos debido a las influencias de la matriz, con el objetivo de identificar y calificar la transición de MRM más selectiva que proporcionaba una forma de pico aceptable y una relación S / N aceptable en la calibración más baja a nivel de cuantificación en modo dirigido.

Tabla 1: Influencia de la matriz estudiada sobre las condiciones y desempeño de plaguicidas seleccionados en determinaciones por GC-MS / MS y LC-MS / MS

		Vine leaves	Boldo	Turmeric
Chlorpyrifos (GC-MS/MS)	**RT**	12	9.6	11.7
	R% at LOQ	70	67	71
	MRMs	196.9->169 (CE 15) 198.9->171(15)	314->258(15) 198.9->171(CE 15)	313.8->257.8 (CE 15) 196.9->107.0 (CE 45)
	ME (%)	-4	-74	41
Tebuconazole (GC-MS/MS)	**RT**	15.2	12.9	14.7
	R%	78	84.8	66.6
	MRMs	125->99(CE 20) 250->125(CE20)	250->125 (CE 30) 226->186 (15)	125->89 (CE 15) 250->125 (CE 20)
	ME (%)	10	334	89
Malathion (GC-MS/MS)	**RT**	11.8	9.4	11.47
	R%	94	82	72
	MRMs	126.9->99 (CE 5) 172.9->99 (CE 15)	173->117(CE15) 143->111(CE 10)	172.9->99.0(CE 15) 157.8->125.0 (CE 20)
	ME (%)	14	121	102
Acetamiprid (LC-MS/MS)	**R%**	na	99	105
	MRMs	na	223->126(CE25) 223->99(CE47)	223.1->126.1 (CE 21) 223.1->73.0 (CE 55)
	ME (%)	na	-35	-45
Thiamethoxam (LC-MS/MS)	**R%**	na	109	86
	MRMs	na	292->211(CE 10) 292->246(CE 10)	292->181.1 (CE 22) 292->211.1 (CE 5)
	ME (%)	na	-73	-31

Como se muestra en la **Tabla 1,** para algunos plaguicidas analizados en todas las matrices y tomados como ejemplo, la matriz tuvo una influencia directa en la optimización de las condiciones instrumentales, principalmente debido a los coextractivos de la matriz específica estudiada que eluyen isobáricamente con los analitos objetivo. En este caso, la forma del pico de los analitos de la matriz en estudio se vió afectada y comprometió la

determinación analítica y los criterios de confirmación, como, por ejemplo, las proporciones de iones. Además, se observó que el Efecto Matriz (EM) también influyó en el tiempo de retención de los analitos. Se observaron cambios en los tiempos de retención (t_R) para la elución de analitos preparados en disolvente frente a los preparados en matriz. Ésta es una consecuencia directa del coeficiente de distribución de los analitos, que tiende a aumentar cuando está presente una "matriz" adicional en la columna cromatográfica. El uso del bloqueo del tiempo de retención como tecnología cromatográfica para GC-MS / MS resultó esencial, para cada combinación analito-matriz, en las determinaciones cualitativas y cuantitativas. En todos los métodos validados en esta tesis; hojas de *Vitis*, boldo, cúrcuma y papa se demostró que la validación del método debe ser específica para cada combinación analito / matriz en cada laboratorio y para cada instrumentación, ya que la optimización de las condiciones instrumentales es un factor específico y no una condición genérica. Por lo tanto, se concluyó que la información bibliográfica y la base de datos de MRM y CE de los fabricantes pueden ser un buen comienzo para los analistas, pero la validación interna es imprescindible para obtener resultados confiables y debe ser específica para cada combinación de matriz y analito y cumplir así los criterios de control de calidad analíticos.
Una conclusión de esta tesis es que el concepto de matrices representativas no es aplicable a cultivos menores.

3.1.2 Compensación de efectos de matriz

Se ha demostrado que las hojas de *Vitis*, las hojas de boldo y el polvo de cúrcuma son matrices ricas en metabolitos secundarios, como ácidos orgánicos, vitaminas, terpenos, alcaloides, fenólicos, taninos, carotenoides, polifenoles, etc. Debido a que los analitos target están presentes en niveles de traza, y los metabolitos secundarios en concentraciones aproximadamente 100-1000 veces más altas, la preparación de la muestra deberá eliminar, tanto como sea posible, los metabolitos secundarios de la planta. Sin embargo, esto no siempre se logra en un 100% a pesar de utilizar los métodos modernos, baratos, rápidos y eficaces como QuEChERS, y una cantidad inevitable de coextractivos estará presente en los extractos para la determinación cromatográfica. Se podría volver a los métodos tradicionales de preparación de muestras, pero eso representaría un incremento en la dispersión de errores. Tomando ventaja de las tecnologías analíticas modernas se prefiere adoptar todos los beneficios vinculados al tipo de enfoques QuEChERS. Los coextractivos de la matriz son impredecibles y pueden causar varios problemas como: supresión de iones

(o en algunos casos su aumento), cromatografía deficiente, falsos positivos debido a la presencia de compuestos isobáricos, recuperaciones bajas de los analitos debido a procesos de partición o adsorción e interferencias con la relación de iones de los iones del analito en el análisis dirigido. Los coextractivos generalmente causan efecto matriz de moderados a altos, por lo general suprimiendo la señal en el análisis por LC y suprimiendo o mejorando la señal en el análisis por GC.

Se observó que el uso de detectores selectivos de masas que emplean analizadores de trampa de iones y / o cuadrupolos simples puede, cuando se producen efectos de matriz elevados, no proporcionar una confirmación objetiva de la identidad del analito. Esta falta de selectividad se aplica especialmente a compuestos que producen iones no específicos de valores bajos de m / z mediante el proceso de ionización por impacto de electrones, por ejemplo, diclorvos. En estas circunstancias, el logro de una identificación sin sesgo y una cuantificación exacta y precisa puede volverse inviable debido a la interferencia de los iones de la matriz que tienen iones con las mismas características que en el analito objetivo. La conclusión es que en un laboratorio analítico se debe ser consciente de este desafío e identificar herramientas alternativas para superar este fenómeno.

La falta de selectividad asociada con la preparación y limpieza de muestras genéricas modernas puede compensarse con la mayor selectividad proporcionada por los espectrómetros de masas de triple cuadrupolo (QqQ) que generalmente implican también una alta sensibilidad cuando se operan en el modo objetivo SRM / MRM. En otras palabras, depende de las capacidades instrumentales del laboratorio poder "desviarse y contabilizar" los efectos de matriz significativos para cumplir con los objetivos analitocs que se le plantean. Esta conclusión está respaldada por los criterios exigidos para el desempeño del método establecidos por las pautas y estándares internacionales, apoyadas en el hecho de que la tecnología analítica se está desarrollando rápidamente, poniendo a disposición técnicas nuevas y más poderosas continuamente [1].

Para permitir que los laboratorios analíticos cumplan su función de garantizar un suministro mundial de alimentos seguros, las autoridades de los países en desarrollo y las organizaciones que fomenten el avance de las capacidades analíticas en los laboaratorios deben invertir esfuerzos en optimizar la aplicación de las capacidades disponibles.También es importantísmo que puedanidentificar y seleccionar las tecnologías emergentes más apropiadas en términos de rendimiento, rentabilidad, longevidad y sostenibilidad. Es importante que el desarrollo o la revisión de los criterios de desempeño analítico sea dictado por un riesgo real más que en nuevas inversiones analíticas y instrumentales. En ese marco

la idoneidad de las técnicas más antiguas y probadas debe mostrar su ajuste a las necesidades de inocuidad alimentaria actuales.

Es de destacar que se demostró que el EM no se puede eliminar y, por lo tanto, se tuvieron que implementar estrategias compensatorias. Usando el sistema instrumental LC-MS / MS fue posible diluir los extractos de cúrcuma y minimizar estos efectos con las estrategias de "diluir y inyectar". Los extractos se diluyeron 1: 5 a 1:10 con acetato de etilo antes de inyectarlos en el sistema cromatográfico. En general, en este trabajo la coinyeccion de los estándares con la matriz (matrix matching calibration) fue la estrategia preferida adoptada para compensar los EM en todas las matrices en estudio. En el caso de GC-MS / MS, los coextractivos de la matriz actuaron como protectores de analitos en los calibradores y permitieron una cuantificación y confirmación adecuadas, lo que garantiza LOQ suficientemente bajos.

En la **Figura 1** se muestra una comparación de los efectos de la matriz observados en GC-MS / MS para cipermetrina, fenarimol, kresoxym metyl, pendimetalin, pirimetanil como analitos que fueron incluidos en el alcance de las hojas de *Vitis*, boldo y cúrcuma en polvo. Estos analitos habían mostrado una falta de robustez en los extractos de papa cuando se usaba QuEChERS como método de preparación de muestras.

Solo para el caso de hojas de *Vitis*, se muestran efectos de matriz opuestos para pirimetanil, con mejora de señal. Para el caso de Boldose observó una supresión de señal en matriz de boldo por GC-MS / MS.

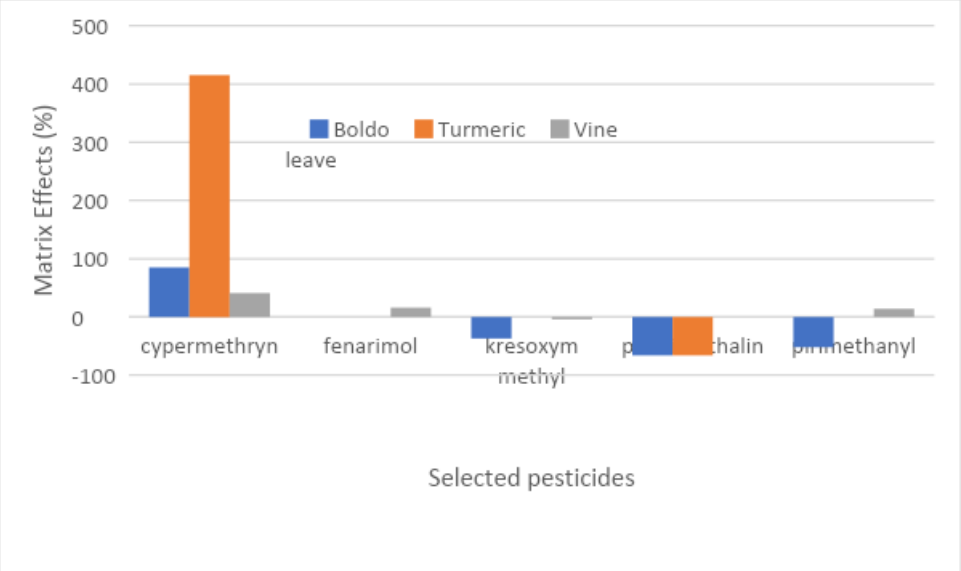

Figura 1: Comparación de los efectos de la matriz en hojas de vid, boldo y polvo de cúrcuma para cypermethyn, fenarimol, kresoxym methyl, pendimethalin y pirimetanil obtenido por GC-MS / MS.

Esta es una confirmación más de que los parámetros de validación requieren un ajuste fino para cada matriz estudiada y confirma la conclusión genérica de que los efectos de la matriz no se pueden predecir y deben ser evaluados en cada combinación matriz / plaguicida Es posible concluir que la principal limitación de los enfoques “multiplex” basados en LC-MS / MS y GC-MS /MS es la incapacidad de superar los efectos de la matriz; sin embargo, hay métodos de compensación disponibles, aunque consumen mucho tiempo con una eficiencia inferior al 100%.

Se demostró en el caso de las hojas de *Vitis*, el boldo y el polvo de cúrcuma que, con el aumento de la complejidad de la matriz de la muestra, las altas variaciones intramatriz no se pueden compensar mediante el uso de un extracto de muestra en blanco "predeterminado" para la calibración ajustada por matriz. Por tanto, es fundamental identificar una muestra compuesta y representativa con el fin de realizar estudios de validación de métodos. Muy a menudo, encontrar un blanco adecuado es una tarea muy desafiante para el químico analítico.

3.1.3 Optimización de estrategias de calibración

Se implementó un trabajo adicional para comparar diferentes estrategias de calibración para la detección de más de 50 pesticidas en hojas de *Vitis* mediante cromatografía de gases acoplada a espectrometría de masas tándem. Se trabajó realizando comparación de matrices, calibración de solventes y calibración de procedimientos. Las curvas de calibración se construyeron usando regresión lineal ponderada, y la pendiente de la curva de calibración del solvente fue la más alta, mientras que la curva de calibración de procedimiento fue la más baja. La **Figura 2** muestra, a modo de ejemplo, el analito zoxamida (**Figura 2**).

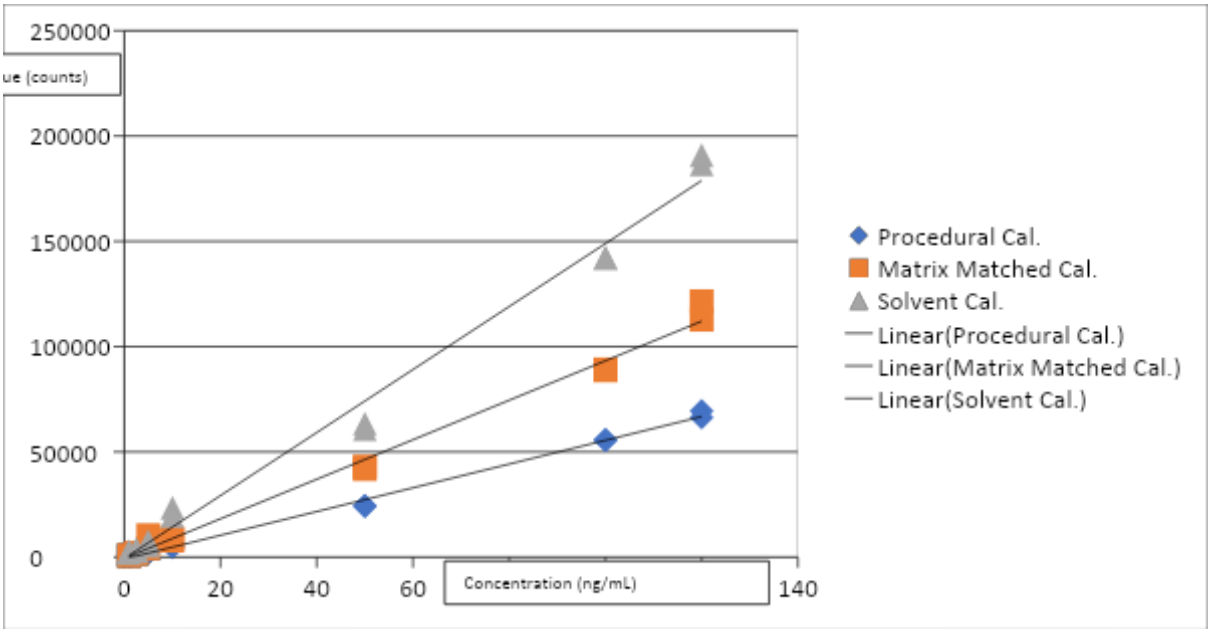

Figura 2: Curvas de calibración para Zoxamide por GC-MS / MS

Los EM de los extractos inyectados en GC- MS / MS se pudieron compensar bien mediante el enfoque de la curva ajustada por la matriz. Por otro lado, la calibración de procedimientos ayudó a compensar los efectos de la matriz y las pérdidas de recuperación, así como la eficiencia de la extracción. Esto es útil cuando es un problema previamente identificado durante los estudios de validación de métodos para la combinación particular de matriz y analito. Se concluyó que los requisitos de calibración individuales se deben evaluar caso por caso y los enfoques de calibración se modificarán en consecuencia y de acuerdo con las directrices de referencia.

En esta tesis no se implementaron investigaciones sobre el uso de estándares internos marcados isotópicamente (ILSTD) para compensar los efectos de la matriz. El trabajo inicial indicó que ILSTD es muy desafiante para las aplicaciones de GC-MS / MS, y LC-MS / MS que son las técnicas cromatográficas preferidas para el formato de ensayo de dilución de isótopos estables (SIDA). El ILSTD no se implementó en esta tesis ya que el el numero de analitos marcados necesarios para el alcance analticos multirresiduo era grande y los costos asociados con el trabajo experimental estaban fuera del presupuesto permitido.

3.1.4 Optimización del submuestreo

Un aspecto que a menudo se pasa por alto es la optimización del paso de submuestreo. Esto asegura la homogeneidad de los analitos en el producto procesado utilizando una cantidad de porción analítica definida. El tratamiento de la muestra se define como el procedimiento (por ejemplo, cortar, triturar, mezclar) utilizado para hacer que la muestra analítica sea aceptablemente homogénea con respecto a la distribución de los analitos, antes de retirar la porción analítica. Como parte de los estudios de validación del método para hojas de *Vitis*, esta tesis también incluyó la verificación de la homogeneidad de las porciones analíticas utilizando de 2 g de muestra. Esta fue la porción analítica sobre la que se realizó la preparación de muestra; extracción con solvente y limpieza del extracto que fue posteriormente analizada utilizando el procedimiento analítico validado para cuantificar / calificar los pesticidas incluidos en el alcance del método para hojas de *Vitis*. Se adoptaron dos enfoques diferentes para verificar que la parte analítica fuera lo suficientemente homogénea y representativa para garantizar que la incertidumbre del submuestreo fuera aceptable.

El primer enfoque consistió en analizar en un solo lote todas las porciones analíticas derivadas de una muestra contaminada naturalmente. En este caso, la variabilidad derivada del análisis de residuos de plaguicidas en la muestra puede aproximarse a la variabilidad del submuestreo, ya que todos los demás factores se mantienen constantes.

El segundo enfoque consistió en evaluar las diferencias que surgen de los experimentos de fortificación utilizando blancos de matriz. La adición se realizó en las hojas de *Vitis* en blanco *antes del procesamiento de la muestra y se comparó con la adición en porciones analíticas individuales después de la homogeneización, ver **Figura 3**. La diferencia entre los dos procedimientos se puede aproximar a la variabilidad que surge de la homogeneización de la muestra y el submuestreo, ya que todos los factores se mantuvieron constantes durante el procedimiento analítico. El procedimiento de adición consistió en colocar todas las hojas de *Vitis* en papel de aluminio limpio y con cuidado usando una jeringa Hamilton con una mezcla de pesticidas a 0.01 mg / kg (ver **Figura 3**). Después de esperar 30 minutos para permitir la absorción de los residuos en la superficie, las hojas de *Vitis* se transfirieron cuidadosamente a un mortero y, utilizando un pistilo y nitrógeno líquido, las hojas se procesaron criogénicamente hasta obtener un polvo fino (Ver **Figura 4**). Las muestras se prepararon según el método validado para hojas de *Vitis*.

Figura 3: Se colocan hojas de *Vitis* sobre papel de aluminio y se llevan a cabo experimentos de adición con una jeringa Hamilton. El disolvente aplicado a la bandeja para lavar el aluminio también se analiza y se resta de los cálculos aplicados para el balance de masa.

Figura 4: B. Maestroni procesando muestras en blanco de hojas de *Vitis* utilizando nitrógeno líquido y un mortero.

Se utilizó una muestra contaminada naturalmente, que resultó positiva para residuos de clorpirifos, para estudiar la homogeneidad del procesamiento de la muestra de acuerdo con

el primer enfoque. Por lo tanto, se seleccionó el clorpirifos como plaguicida de estudio para los experimentos de adición y se incluyó en la mezcla de adición de acuerdo con el segundo enfoque.

La directriz del Codex CAC / GL 59-2006 [2] sobre la estimación de la incertidumbre de los resultados proporciona información para los antecedentes estadísticos y el principio de estimación del procesamiento de muestras a partir de experimentos de adición, de acuerdo con el enfoque 2. Según CAC / GL 59-2006, CV_L es la incertidumbre relativa de la fase de laboratorio de la determinación que puede derivarse del submuestreo, preparación de la muestra, procesamiento de la muestra y pasos analíticos. CV_{SP} es la incertidumbre relativa de la preparación de la porción de prueba, incluido el submuestreo, la preparación de la muestra y el procesamiento de la muestra, y la incertidumbre relativa del CV_A del análisis, incluida la extracción, limpieza, evaporación, derivatización y determinación instrumental.

Los resultados de los experimentos de procesamiento de muestras se resumen en la **Tabla 2** para el pesticida clorpirifos de acuerdo con los dos enfoques.

Tabla 2: Resumen de resultados para clorpirifos obtenidos del experimento con el objetivo de verificar la homogeneidad del submuestreo (o procesamiento de la muestra) de acuerdo con los dos enfoques descritos en el texto.

Approach 1			Approach 2	
nr samples	25		nr samples	9
average residue (µg/kg)	93		average residue (µg/kg)	95.7
CV_{SP}%	6		CV_A (%)	2.2
			CV_L (%)	3.4
			CV_{SP} (%)	2.7

De acuerdo con la **Tabla 2**, ambos enfoques representan menos del 10% de la variabilidad derivada del paso de submuestreo y está en consonancia con los valores estimados para el submuestreo en otros productos básicos [3], [4]. Este resultado permitió concluir que el procesamiento de hojas de *Vitis* utilizando nitrógeno líquido en un mortero estaba dando una muestra aceptablemente homogénea, y que la variabilidad del submuestreo estaba dentro de niveles aceptables. Por el contrario, si este no hubiera sido el caso, se debería haber considerado el uso de porciones analíticas más grandes (es decir,> 2 g) para obtener una mejor estimación del valor real.

También se implementó una verificación rápida del paso de submuestreo para el boldo y la cúrcuma, que se homogeneizaron en polvos finos utilizando enfoques criogénicos. Aunque aparentemente son fáciles de mezclar, los polvos de cúrcuma sorprendentemente mostraron falta de homogeneidad en un primer paso de verificación después de la preparación usando dilución geométrica.

El uso de IMS permitió rápidamente la confirmación de la homogeneidad del submuestreo utilizando la información proporcionada por el perfil de la fracción volátil. El paso de submuestreo tuvo que ser rediseñado para tener en cuenta el mezclado durante 5 minutos en un molino de bolas, seguido de un mezclado usando un mortero y mano durante 15 minutos con la adición de nitrógeno líquido.

3.1.5 Desarrollo y optimización de la preparación de muestras

Antes de que se pueda demostrar la validación, es imperativo desarrollar y / o adaptar un método de preparación de muestras. Desde el enfoque más simplista de “diluir y inyectar”, se puede aplicar una amplia variedad de enfoques de preparación de muestras. En esta tesis, se aplicaron protocolos genéricos de preparación de muestras como la preparación de muestras QuECheRS a los cultivos menores en estudio, y se implementaron combinaciones específicas de limpieza en hojas de *Vitis*, boldo y matriz de cúrcuma. Se utilizaron técnicas de TLC e IMS para identificar las condiciones optimizadas para las combinaciones de analito / matriz estudiadas (**Figura 5**).

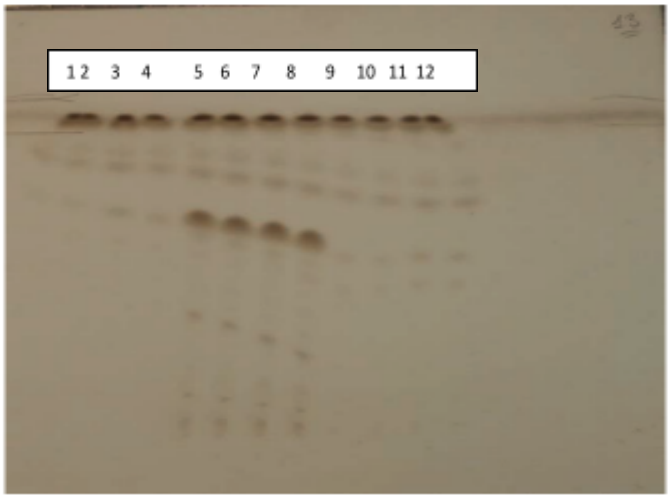

Figura 5: Ejemplo de un conjunto de combinaciones de sal de limpieza (desde 1 a 12, concentraciones diferentes de PSA y $MgSO_4$) aplicadas a la matriz de hojas de *Vitis* en blanco y manchadas en placas de cromatografía de capa fina (TLC).

Una variable importante en todos los estudios fue la adición de agua a las matrices alimentarias, para ayudar al solvente orgánico a extraer mejor los analitos. El material vegetal se hincha con el agua añadida, lo que facilita la extracción de algunos de los analitos más polares. En el caso de disolventes miscibles en agua (acetonitrilo, metanol), los analitos se reparten más fácilmente que cuando se utilizan disolventes inmiscibles en agua (acetato de etilo, diclorometano).

En general, según lo reportado en literatura, la amina primaria secundaria (PSA) en combinación con silica de fase reversa (RP-C18), negro de carbón grafitizado (GCB) y otros sorbentes como florisil, alúmina y cloruro de calcio ($CaCl_2$) demostraron ser efectivos en cierta medida. para eliminar de la matriz compuestos específicos. [5], [6]. En todos los estudios, el método final de preparación de muestras seleccionado fue un compromiso entre la determinación de residuos y un cierto grado de efectos de la matriz. En esta tesis también se logró un esfuerzo por volver más" verde" el proceso analítico evitando el uso de disolventes clorados muy tóxicos como el diclorometano y miniaturizando las condiciones del método. La optimización incluyó una verificación de los parámetros de rendimiento del método, especialmente la recuperación, y la desviación estándar (exactitud y precisión) utilizando el peso de la porción analítica más baja posible, es decir, 2 g de muestra, que resultó ser suficientemente homogénea con respecto a la distribución del analito, y suficiente con respecto al LOQ alcanzado. En consecuencia, esto implicó menores volúmenes de disolventes utilizados para la extracción y menores cantidades de sorbentes para la preparación de muestras, yendo así en la dirección de los principios de la "química verde" [7].

3.1.5.1 Proceso de verificación de homogeneidad

Se implementó un nuevo enfoque para verificar la homogeneidad de las muestras de polvo de cúrcuma utilizando IMS. En la práctica, los componentes volátiles de las muestras de cúrcuma se midieron utilizando un espectrómetro de movilidad iónica por cromatografía de gases de espacio de cabeza (HS-GC-IMS). En un primer intento, se obtuvieron mediante GC-IMS cinco muestras comerciales de cúrcuma en blanco diferentes, una muestra combinada y una muestra auténtica (rizoma de cúrcuma secada en laboratorio / finamente pulverizada) y se compararon en términos de su composición química utilizando el análisis de componentes principales (PCA) como se muestra en la literatura [8]. Cada muestra

produjo una huella química específica. La muestra combinada, preparada mediante dilución geométrica, se compensó en comparación con las muestras elementales originales y, por lo tanto, se consideró insuficientemente homogénea. Por lo tanto, se implementó un nuevo enfoque de preparación de matriz blanco (combinado)El polvo de cúrcuma combinado final obtenido por mezclado en un molino de boles y posoterior homogeinización en mortero fue suficientemente homogéneo y representativo para los estudios de validación del método. La novedad de este enfoque se basa en el uso de IMS como una técnica rápida y no destructiva para la cúrcuma en polvo, para verificar la homogeneidad del paso de submuestreo.

3.2 Validación de métodos analíticos para cultivos menores

El presente trabajo se centra en diferentes aspectos de la validación de métodos, que es el proceso de caracterizar el desempeño que se espera de un método en términos de su alcance, especificidad, precisión, sensibilidad, repetibilidad, reproducibilidad dentro del laboratorio, entre otros, o, en otros términos, definido como el proceso para evaluar la idoneidad del método analítico para su propósito.

Esta tesis tuvo como objetivo investigar todos los aspectos inherentes a la validación del método analítico de cultivos menores. La optimización de la homogeneidad del submuestreo, la eficiencia de extracción para cultivos comercializados como matrices secas, la representatividad de las matrices blanco utilizadas para los estudios de validación, los efectos de la matriz, el LOQ alcanzable y el cumplimiento de los LMR existentes, la optimización cromatográfica, la estabilidad de los tiempos de retención, fueron algunos de los desafíos estudiados. Los ajustes precisos de todas las condiciones y los detalles complejos de preparación de muestras y la optimización demostraron que cada matriz se comporta de manera muy diferente a todas las demás, respaldando así el concepto de que la validación del método debe ser un "mecanismo de movimiento de ajuste fino" para permitir la prueba de la idoneidad. Las pautas de validación de métodos existentes son útiles para proporcionar el punto de referencia deseable para los criterios de desempeño, pero en última instancia, es el ajuste fino específico que el analista puede aplicar, basado en el juicio y la experiencia profesionales, lo que proporciona la mejor compensación para todas las condiciones. Esto se demostró con las hojas de *Vitis*, el boldo y la cúrcuma. En resumen, la mecanización de ciertos pasos analíticos puede representar una ayuda útil para el químico analítico, pero no puede reemplazar el juicio de un analista bien capacitado para

brindar las mejores condiciones para las pruebas. Todos los métodos se aplicaron con éxito al análisis de muestras de mercado reales y se comprobó que eran adecuados para su propósito.

3.3 Verificación de robustez (y ruggeddness)

Se estudió la robustez en dos productos diferentes, la papa y la cúrcuma, un cultivo principal y uno secundario, respectivamente. Se aplicaron diferentes enfoques experimentales: diseño de experimentos (DoE) y comparaciones entre laboratorios. Aunque esos enfoques se aplican a las condiciones intralaboratorio e interlaboratorio, ambos proporcionaron una medida de idoneidad para el propósito de los métodos desarrollados y validados.

Esta tesis consideró que las necesidades de los laboratorios pueden variar según la disponibilidad de esquemas comerciales de ensayos de aptitud y la disponibilidad de colaboradores válidos que estén trabajando en matrices y estudios similares. No siempre es posible identificar tales esquemas o colaboraciones, y el laboratorio analítico necesita tener herramientas a mano para demostrar la idoneidad para el propósito, como una prueba objetiva que a menudo es requerida por los organismos de acreditación durante los procesos de acreditación. Se logró el objetivo de proporcionar a los laboratorios una metodología para validar la robustez o solidez de un método. El estudio en papa mostró que se pueden utilizar varios DoE para generar dicha información, de una manera relativamente rápida y barata. Se implementaron y compararon un diseño de Plackett Burman (PBD) y un diseño de screening definitivo aumentado (DSD) para su uso en laboratorios analíticos de todo el mundo. Si bien el PBD es más barato en términos del número de réplicas necesarias, el DSD proporciona más información al químico analítico, principalmente en la identificación de los parámetros analíticos que deben estar bajo un estricto control mediante el cumplimiento de prescripciones como los procedimientos operativos estándar.

El trabajo interlaboratorio se aplicó a dos matrices: boldo y cúrcuma. En boldo las determinaciones analíticas se realizaron mediante GC-MS / MS, y estos datos contribuyeron a establecer los parámetros de rendimiento del método para la metodología multirresiduos ajustada en hojas de boldo. En el caso de la cúrcuma, se implementó un estudio completo entre laboratorios para estudiar los parámetros clave de rendimiento del método. Una comparación de los resultados confirmó la reproducibilidad de la metodología desarrollada entre laboratorios para 32 compuestos analizables porGC y 34 por LC (ver Anexo 2). Los

dos laboratorios involucrados fueron FEPL en Austria y GACT en Uruguay, Como conclusión general de esta herramienta interlaboratorio, se pudo concluir que el laboratorio analítico requiere un mínimo de inversión financiera para poder implementar el DoE y las pruebas de aptitud y / o estudios colaborativos. Los recursos necesarios deben estar disponibles y ser incluidos en la planificación de los procesos analíticos del laboratorio.

3.4 Suministro de insumos para marcos regulatorios

Para contribuir al suministro de información para la inocuidad alimentaria como insumo del proceso de control de alimentos (marco regulatorio), se implementó un estudio sobre disipación de residuos en hojas de *Vitis* en Siria y Austria con contribuciones de Uruguay. El objetivo era destacar la dependencia del cumplimiento de los residuos de plaguicidas con los posibles LMR en función del marco reglamentario elegido. Para ser más específicos, este estudio ayudó a generar información para el posible establecimiento de los LMR, ya que son prácticamente inexistentes para las hojas de *Vitis*. Sin ser un LMR para un plaguicida neonicotinoide que está definido sólo en la legislación de la UE. La información sobre los valores máximos de residuos, el tiempo de retención y las tasas de disipación, entre otros, es una información importante necesaria para evaluar el impacto de la exposición dietética a plaguicidas por el consumo de productos alimenticios contaminados. Estos parámetros se obtienen de forma experimental o mediante modelos y varían según el tipo de plaguicida, el tipo de cultivo y las condiciones ambientales predominantes. En este trabajo colaborativo se demostró que el WHP o tiempo de carencia varía para los diferentes pesticidas y combinaciones de cultivos y corrobora hallazgos previos [9], [10], [11], [12]. El WHP es información importante que debe generarse para demostrar que los residuos en el cultivo tratado no excederán el límite máximo de residuos. El estudio sobre el comportamiento de disipación de plaguicidas en hojas de *Vitis* ayudó a abordar el papel del laboratorio analítico en la interpretación de residuos químicos de contaminantes, y su papel consultivo para la mejora de buenas prácticas agrícolas (BPA). A partir de este trabajo se pudo demostrar que, debido a las diferencias de condiciones, es decir, condiciones climáticas, aplicaciones, tipo de plagas, etc., cada país debe realizar esfuerzos locales para proteger su mercado y consumidores. Por lo tanto, los LMR establecidos en el CODEX deben considerarse como una armonización de los LMR con los esfuerzos y contribuciones de todos los países del mundo, para tener en cuenta las diferencias regionales y específicas de cada país. Si bien los LMR son un medio para proteger el mercado, los puntos finales

toxicológicos (IDA, etc.) se consideran directamente para la protección de la salud del consumidor y se monitorean indirectamente a través de programas nacionales de monitoreo.

3.5 Contribuciones a la comprensión del análisis de trazas orgánicas de cultivos menores

Otra conclusión de este trabajo es que el concepto de matrices representativas, como se discute en las directrices del Codex [13] y SANCO [14], no es aplicable a cultivos menores. De acuerdo con las directrices antes mencionadas, en la categoría de "productos básicos difíciles o únicos", las especias se enumeran como un ejemplo de productos típicos representativos dentro de la categoría. Esta no es exactamente una indicación específica de lo que debería ser una matriz representativa. La categoría de cultivos menores es muy amplia e incluye productos muy diferentes desde el punto de vista botánico. El atributo cultivo menor se refiere a un aspecto cuantitativo (comercial) como se discutió anteriormente. Se demostró que las optimizaciones específicas del procesamiento de muestras y la determinación de muestras analíticas son estrictamente necesarias en el caso de la validación analítica de plaguicidas y otros contaminantes orgánicos en cultivos menores. Existe una necesidad demostrada de validación específica de cada producto de cultivo secundario, especialmente debido a los efectos de matriz inherentes que desafían la determinación analítica. Además, el documento SANCO de la UE incluye la declaración de que "los productos básicos difíciles solo deben validarse por completo si se analizan y analizan con frecuencia. Si solo se analizan y analizan ocasionalmente, la validación puede reducirse a simplemente verificar los límites de notificación utilizando extractos en blanco enriquecidos". Fue posible demostrar que la validación completa es imprescindible para cada cultivo menor o producto difícil. Se encontraron altos efectos de matriz para las matrices de hojas de *Vitis*, boldo y cúrcuma, requiriendo una validación completa y un conocimiento extendido de las matrices estudiadas para identificar los puntos críticos del procedimiento analítico. Puede que no sea suficiente comprobar los límites de detección utilizando extractos en blanco enriquecidos. Es necesario verificar el submuestreo y la homogeneidad, la preparación de la muestra debe optimizarse junto con la optimización instrumental (transiciones MRM) como se discutió anteriormente. Eventualmente, los cultivos menores, tales como hierbas y especias, son productos básicos que, dentro de

cada tipo, pueden tener tal variedad que es necesario implementar métodos específicos y holísticos para garantizar la inocuidad de los alimentos en cada caso.

3.6 Primeros conocimientos sobre los métodos multiclase

En lugar de los métodos clásicos que están más orientados a una sola clase, se aplicaron métodos modernos de residuos múltiples para la determinación de residuos y contaminantes en muestras de hojas de *Vitis*, boldo y cúrcuma mediante LC-MS / MS y GC-MS / MS. En cada estudio se demostró la importancia de desarrollar, adaptar y validar multirresiduo, multianalito, multiclase o, en otras palabras, “métodos analíticos multiplex” para poder estudiar una amplia gama de analitos o contaminantes orgánicos traza dentro del mismo procedimiento de preparación de muestrasy determinación instrumental. Hasta la fecha, no se dispone de directrices o normas que regulen los niveles de todas las clases de contaminantes y residuos simultáneamente. Sin embargo, en caso de detectar un contaminante o residuo, el analista está obligado a cumplir con los requisitos de la legislación específica para la clase de interés. La tecnología “multiplex” se está aplicando cada vez más debido a sus ventajas de cribado analítico en comparación con el análisis clásico simple. La comunidad analítica reconoce la importancia de poder seleccionar una gran cantidad de productos básicos, sin embargo, los métodos de confirmación deben ser al menos tan selectivos, específicos, precisos, exactos, resistentes, adecuados para el propósito como sea posible y, finalmente, también rápidos, baratos, respetuoso con el medio ambiente y basado en riesgos.

3.7 Estimaciones de incertidumbre

Se realizó una estimación de la incertidumbre en el estudio de la cúrcuma como indicador cuantitativo de la confianza en los datos analíticos generados, y se demostró que la incertidumbre de todos los analitos no excedía el valor por defecto del 50% como se indica en el Codex [2] y la directriz SANTE [14]. Se demostró que un análisis exhaustivo del método no solo identificó el posible cálculo de incertidumbre, sino que también ayudó a identificar los puntos críticos y, cuando fue necesario, ayudó a mejorar el método analítico. Al igual que en el caso de las pruebas de robustez, la estimación de la incertidumbre es una herramienta útil para identificar errores en el procedimiento analítico y corregir pequeñas posibles fuentes de los mismos, que de otro modo pasarían desapercibidas. La **Figura 6**

proporciona un resumen rápido de las posibles fuentes de incertidumbre del procedimiento analítico tal como se identificó en el estudio de la cúrcuma.

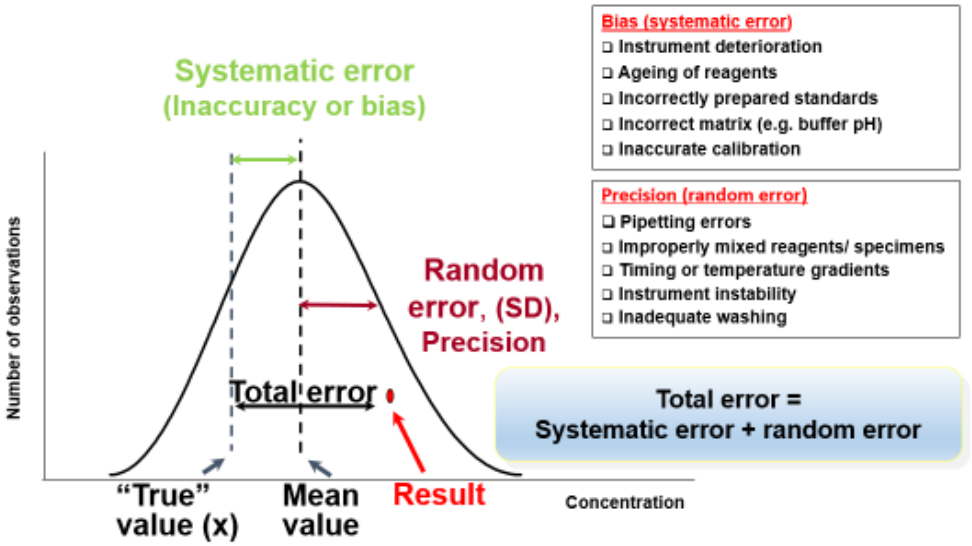

Figura 6: posibles fuentes de errores en el análisis cuantitativo

La evaluación de la incertidumbre se ha convertido en una necesidad en el análisis de contaminantes orgánicos trazas. La evaluación precisa de la medición de la incertidumbre da un valor extra a los datos analíticos y proporciona fiabilidad y confianza en los resultados obtenidos.

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Chapter 4: Final considerations, conclusions and perspectives

4.1. Final considerations and conclusions

This thesis aimed at contributing towards the establishment and validation of processes and tools that combined in an innovative way help to contribute to food safety and environmental sustainability. The choice of matrices included herbs, spices, edible leaves and tubers such as potato. The choice of analytes included specific commodity target substances that help to protect the crop from pests and/or are found as contaminants during growing, processing or storing conditions. Nevertheless, the lack of international crop regulations as well as the nonexistence of established good agricultural practices for some of these minor crops, forced the selection of analytes towards the broadest scope possible. Many of these herbs are produced worldwide and, as a consequence, the agricultural practices applied in the field can be very different. This results in the fact that each testing laboratory needs to validate the analytes under its own instrumental setup and conditions, and as much as possible using "multiplex" methods. Chromatographic instrumentation coupled to tandem mass spectrometry (GG-MSMS and LC-MS/MS) provided the necessary tools to monitor a broad scope of polarities and analytes of pesticides, dyes, persistent organic pollutants and mycotoxins. TLC and IMS were also used to optimize sample preparation methodologies with excellent performance. Optimization of methods resulted in a compromise between possible and measurable parameters against benchmark criteria for performance, such as those from generic guidelines such as from Codex, and the EU.

In addition, it was shown that analytical validation is a must for each minor crops. This provided evidence that the concept of validating representative matrices within commodity groups cannot be applied in the case of minor crops. Individual full method validation needs to be implemented. It is the only way for laboratories to be able to provide performance criteria that cover the variability within each analyte / commodity combination. Matrix effects were the biggest analytical challenge. The exhaustive clean-up was not possible and the coextractives were not only a source of analyte interference but also the reason for a very intensive maintenance of the equipment. Uncertainty estimations, collaborative studies and specific ruggedness / robustness tests were shown to be necessary to provide objective evidence of the goodness and fitness for the purpose of analytical methods. Without a proper MRL vine leaves couldn't be shown to comply with trade requirements; but without a proper indication of pesticide dissipation in vine leaves it would be hard to set adequate MRLs. Herbs and spices need special attention in a case-by-case scenario. The possibility of developing "multiplex" (multiclass) methods including contaminants such as mycotoxins and dyes for such complex matrices was demonstrated. The main goal of a new analytical

method is to be applied indistinctly by any specialized laboratory. The suitability of the method for the multiclass analysis of contaminants in turmeric was proven through interlaboratory trials (see Annex 2).

All of the above are a bunch of aspects that converge to assure food safety.

This thesis emphasised and showed possible ways to overcome the different problems analytical laboratories face in the task. Summarizing, it was shown that only the combination of tools and methodologies which help to enforce an active regulatory framework may enable a fully functional food safety system. Partial applications of tools and methodologies are inadequate to ensure food safety for all and a sustainable environment.

The outcome from this work is a clear demonstration of the new active role of the analytical laboratory in terms of optimization of analytical methods that are fit for the intended purpose. The data they provide are the basis that supports the whole food control system. Routine laboratories must put major attention in the optimization of instrumental conditions, implementation of specific full method validation studies for each minor crop, with a verification of ruggedness and robustness testing using specific DoE, together with uncertainty estimations of the whole analytical process, while setting up the scope of methods based on real evidence and risk based and, where possible, develop and apply screening approaches to increase the number of testing.

The ISO 17025 fosters the participation in interlaboratory testing of analytical methods, in order to provide reliable information to decision makers that ultimately could be included in trustable databases. But food testing for residues, contamination and fraud, which are milestones to ensure food safety, are only aspects of a much broader problem to be solved. Good water supply when food is produced, microbiological, heavy metals and natural / anthropogenic radioactivity contamination, are other aspects, among others, to be considered to grasp a holistic vision of the problem.

4.2. Future work

This thesis highlighted the need for further studies into food safety tools and harmonized methodologies to be able to assess it from a broader perspective. Fragmented approaches to food safety represent the current status of food safety control systems. Limitations exist for food legislation in terms of regulating all residues / contaminants in all food matrices. Much work is still needed to ensure ethics in food production, and testing regimes that are able to ensure the safety of our foods. Food fraud can occur at any point in the farm to fork

chain, and future work for future safety appears to be in the direction of combining food forensics with authenticity and food safety information applying risk concepts to identify evidence of flaws of food safety control systems. Sustainability of food safety in a broader sense can only be achieved within a healthy environment, therefore food safety should also include environment safety. The “uncontrollable and unforeseeable” environment needs to be circumscribed as much as possible. The number of residues and contaminants is continuously growing, which implies an increasing demand for their analysis in many potential ingredients, raw materials (animal or plant origin) and food commodities. To assess human daily intake of these emerging food contaminants / residues, quantification of background levels of both recognized and newly identified contaminants / residues shall be implemented while characterizing emerging food contaminants that have shown to have negative effects on human health.

Related to this, assessing the risk associated with mixtures of contaminants, which are changing continuously, represents a tricky challenge for food safety, and new approaches should be implemented in order to analyse a wider range of compounds in a shorter time. The possibility of performing retrospective data analysis and the capability of performing structural elucidation of unknown or suspected compounds will be desirable objectives to be reached.

Monitoring approaches should shift from specific monitoring of one specific class of contaminants to multiclass / integrated monitoring, through risk-based sampling designs and information gathering from intelligent sources and/or block chain approaches. Analytical innovations should be sought using HRMS untargeted approaches specifically for minor crops, and incorporating in situ testing, also using new analytical portable instrumentation, with a focus on rapid screening approaches. More analytes, new emerging challenges shall be identified, including large studies on the fate and quantitation of active metabolites in food matrices. Method development and validation of more than 1000 target compounds in complex matrices is feasible from a technical perspective but is associated with an overwhelming data management. Therefore, new approaches to macro data analysis in combination to new performance guidelines shall be implemented to reduce the overall workload for full method validation studies. Essential is to strike a compromise between practicality and matrix / analyte combinations. To help guide questions on how to better predict hazard exposure and how to prevent it, risk assessments and evaluations shall be carried out to identify method scope for targeted testing and integrated/ more holistic monitoring programs. Parallel to this, all data generated shall be shared for better understanding of the real food safety situation and to benefit intelligence gathering. Finally,

networking and improved communication shall become the key for enhanced food safety at the global level. Secure, sustainable and safe food are parts of the same problem for the global consumer that need to be dynamically integrated to reach real solutions.

Capítulo 4: Consideraciones finales, conclusiones y perspectivas

4.1. Consideraciones y conclusiones finales

Esta tesis tuvo como objetivo contribuir al establecimiento y validación de procesos y herramientas que combinados de manera innovadora ayuden a contribuir a la inocuidad alimentaria y la sostenibilidad ambiental. La elección de matrices incluyó hierbas, especias, hojas comestibles y tubérculos como la papa. La elección de analitos incluyó sustancias objetivo de productos específicos, que ayudan a proteger el cultivo de plagas y / o se encuentran como contaminantes durante las condiciones de cultivo, procesamiento o almacenamiento. Sin embargo, la falta de regulaciones internacionales de cultivos, así como la inexistencia de buenas prácticas agrícolas establecidas para algunos de estos cultivos menores, obligó a la selección de analitos hacia el alcance más amplio posible. Muchas de estas hierbas se producen en todo el mundo y, como consecuencia, las prácticas agrícolas aplicadas en el campo pueden ser muy diferentes. Esto da como resultado el hecho de que cada laboratorio de pruebas necesita validar los analitos en su propia configuración y condiciones instrumentales, y en la medida de lo posible utilizando métodos "multiplex". La instrumentación cromatográfica acoplada a la espectrometría de masas en tándem (GC-MS / MS y LC-MS / MS) proporcionó las herramientas necesarias para monitorear una amplia gama de polaridades y analitos de plaguicidas, tintes, contaminantes orgánicos persistentes y micotoxinas. También se utilizaron TLC e IMS para optimizar las metodologías de preparación de muestras con un rendimiento excelente. La optimización de los métodos resultó en un compromiso entre los parámetros posibles y mensurables frente a los criterios de referencia para el desempeño, como los de las directrices genéricas, como las del Codex y la UE.

Además, se demostró que la validación analítica es imprescindible para cada cultivo menor. Esto proporcionó evidencia de que el concepto de validar matrices representativas dentro de los grupos de productos no se puede aplicar en el caso de cultivos secundarios. Es necesario implementar la validación del método completo individual. Es la única forma en que los laboratorios pueden proporcionar criterios de desempeño que cubran la variabilidad dentro de cada combinación de analito / producto. Los efectos matriciales fueron el mayor desafío analítico. La limpieza exhaustiva no fue posible y los coextractivos no solo fueron una fuente de interferencias de analitos sino también el motivo de un mantenimiento muy intensivo del equipo. Se demostró que las estimaciones de incertidumbre, los estudios colaborativos y las pruebas específicas de robustez / robustez son necesarias para proporcionar evidencia objetiva de la bondad y la idoneidad para el propósito de los métodos analíticos. Sin un LMR adecuado, no se podría demostrar que las hojas de *Vitis*

cumplen con los requisitos comerciales; pero sin una indicación adecuada de la disipación de plaguicidas en las hojas de *Vitis*, sería difícil establecer LMR adecuados. Las hierbas y especias necesitan una atención especial en un escenario de caso por caso. Se demostró la posibilidad de desarrollar métodos “multiplex” (multiclase) que incluyan contaminantes como micotoxinas y colorantes para matrices tan complejas. El objetivo principal de un nuevo método analítico es ser aplicado indistintamente por cualquier laboratorio especializado. La idoneidad del método para el análisis multiclase de contaminantes en la cúrcuma se demostró mediante ensayos entre laboratorios (ver **Anexo 2**).

Todos los aspectos anteriores convergen para garantizar la inocuidad alimentaria.

Esta tesis enfatizó y mostró posibles formas de superar los diferentes problemas que enfrentan los laboratorios analíticos en la tarea. En resumen, se demostró que solo la combinación de herramientas y metodologías que ayuden a hacer cumplir un marco regulatorio activo puede permitir un sistema de inocuidad alimentaria completamente funcional. Las aplicaciones parciales de herramientas y metodologías son inadecuadas para garantizar la inocuidad alimentaria para todos y un medio ambiente sostenible.

El resultado de este trabajo es una clara demostración del nuevo papel activo del laboratorio analítico en términos de optimización de los métodos analíticos que se adecuan al propósito previsto. Los datos que proporcionan son la base que sustenta todo el sistema de control de alimentos. Los laboratorios de rutina deben poner mayor atención en la optimización de las condiciones instrumentales, la implementación de estudios de validación de métodos completos específicos para cada cultivo menor, con una verificación de la robustez y pruebas de robustez utilizando DoE específico, junto con estimaciones de incertidumbre de todo el proceso analítico, mientras se configura el alcance de los métodos basados en pruebas reales y basados en riesgos y, cuando sea posible, desarrollar y aplicar enfoques de detección para aumentar el número de pruebas.

La ISO 17025 fomenta la participación en pruebas interlaboratorio de métodos analíticos, con el fin de brindar información confiable a los tomadores de decisiones que finalmente podría ser incluida en bases de datos de uso general. Pero las pruebas alimentarias para detectar residuos, contaminación y fraude, que son hitos para garantizar la inocuidad alimentaria, son solo aspectos de un problema mucho más amplio que debe resolverse. El buen abastecimiento de agua en la producción de alimentos, la contaminación microbiológica y por metales pesados y radiactividad natural / antropogénica, son otros aspectos, entre muchos mas, a considerar para captar una visión holística del problema.

4.2. Trabajo futuro

Esta tesis destacó la necesidad de realizar más estudios sobre herramientas de inocuidad alimentaria y metodologías armonizadas para poder evaluarla desde una perspectiva más amplia. Los enfoques fragmentados de la inocuidad alimentaria representan el estado actual de los sistemas de control de la inocuidad alimentaria. Existen limitaciones para la legislación alimentaria en términos de regular todos los residuos / contaminantes en todas las matrices alimentarias. Todavía se necesita mucho trabajo para garantizar la ética en la producción de alimentos y regímenes de prueba que puedan garantizar la seguridad de nuestros alimentos. El fraude alimentario puede ocurrir en cualquier punto de la cadena de la granja a la mesa, y el trabajo futuro para los sistemas de inocuidad alimentaria parece estar en la dirección de combinar la ciencia forense de alimentos con la autenticidad y la información de inocuidad alimentaria. aplicando conceptos de riesgo para identificar con evidencia firme posibles fallas de los sistemas de control de inocuidad alimentaria. La sostenibilidad de la inocuidad alimentaria en un sentido más amplio solo se puede lograr dentro de un medio ambiente saludable, por lo tanto, la inocuidad alimentaria también debe incluir la seguridad ambiental.

El entorno "incontrolable e imprevisible" debe circunscribirse en la medida de lo posible. El número de residuos y contaminantes crece continuamente, lo que implica una demanda creciente para su análisis en muchos ingredientes potenciales, materias primas (de origen animal o vegetal) y productos alimenticios. Para evaluar la ingesta diaria humana de estos contaminantes / residuos alimentarios emergentes, se debe implementar la cuantificación de los niveles de fondo de contaminantes / residuos tanto reconocidos como recientemente identificados, al tiempo que se caracterizan los contaminantes alimentarios emergentes que han demostrado tener efectos negativos en la salud humana.

En relación con esto, evaluar el riesgo asociado con las mezclas de contaminantes, que cambian continuamente, representa un desafío delicado para la inocuidad alimentaria, y se deben implementar nuevos enfoques para analizar una gama más amplia de compuestos en un tiempo más corto. La posibilidad de realizar análisis de datos retrospectivos y la capacidad de realizar una elucidación estructural de compuestos desconocidos o sospechosos serán objetivos deseables a alcanzar.

Los enfoques de monitoreo deben pasar de un monitoreo específico de una clase específica de contaminantes a un monitoreo multiclase / integrado, a través de diseños de muestreo basados en riesgos y recopilación de información de fuentes inteligentes y / o enfoques de cadena de bloques. Se deben buscar innovaciones analíticas utilizando enfoques no dirigidos de HRMS específicamente para cultivos menores, e incorporando pruebas *in situ*, también utilizando nueva instrumentación portátil analítica, con un enfoque en enfoques de detección rápida. Se identificarán más analitos, nuevos desafíos emergentes, incluidos grandes estudios sobre el destino y la cuantificación de los metabolitos activos en las matrices alimentarias. El desarrollo de métodos y la validación de más de 1000 compuestos target en matrices complejas es factible desde una perspectiva técnica, pero está asociado con una gestión de datos abrumadora. Por lo tanto, se implementarán nuevos enfoques para el análisis de macrodatos en combinación con nuevas directrices de rendimiento para reducir la carga de trabajo general para los estudios completos de validación de métodos. Es esencial lograr un compromiso entre la practicidad y las combinaciones de matriz / analito. Para ayudar a orientar las preguntas sobre cómo predecir mejor la exposición a peligros y cómo prevenirla, se deben realizar evaluaciones de riesgo y evaluaciones para identificar el alcance del método para pruebas específicas y programas de monitoreo integrados / más holísticos. Paralelamente, todos los datos generados se compartirán para comprender mejor la situación real de la inocuidad alimentaria y para beneficiar la recopilación de información. Por último, la creación de redes y la mejora de la comunicación se convertirán en la clave para mejorar la inocuidad alimentaria a nivel mundial. Los alimentos seguros, sostenibles y seguros son partes del mismo problema para el consumidor global que deben integrarse dinámicamente para alcanzar soluciones reales.

Annex 1: Acronyms

APCI	Atmospheric pressure chemical ionization
CAC	Codex Alimentarius Commission
EC	European Commission
EFSA	European Food Safety Authorithy
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration (US Federal government)
GACT	Grupo Analysis Contaminantes en Traza
GAPs	Good Agricultural Practices
GCB	Graphitized carbon black
GC−HRMS	Gas chromatography-high resolution mass spectrometry
GC−MS	Gas chromatography-mass spectrometry
GC−MS/MS	Gas chromatography-tandem mass spectrometry
GMPs	Good Manufacturing Practices
HACCP	Hazard Analysis and Critical Control Point
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry
IAEA	International Atomic Energy Agency
ILSTD	Isotopically labelled internal standard
LC−MS	Liquid chromatography-mass spectrometry
LC−MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification/determination
MDLs	Method detection limits
MQL	Method quantification limit
MRM	Multiple reaction monitoring
OCs	Organochlorines
OPs	Organophosphorus (OPs)
PAHs	Polycyclic Aromatic Hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PCBs	Polychlorobyphenils (PCBs)
PLE	Pressurized liquid extraction
POPs	Persistent organic pollutants,
PSA	primary secondary amine
QqQ	Triple quadrupole mass spectrometric detector
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
RSD	Relative standard deviation
SIDA	Stable isotope dilution assay
SIM	Single ion monitoring
SPS	Sanitary and Phytosanitary
SRM	Selected reaction monitoring
TBT	Technical Barriers to Trade

UDELAR	Universidad de la Republica, Uruguay
UHPLC	Ultra-high-performance liquid chromatography
USDA	United States Department of Agriculture
WTO	World Trade Organization

Annex 2: Interlaboratory trial

Supplementary materials to complement the information on the paper Method Optimization and validation for multi class residue analysis in turmeric, and with a special focus on the equipment used for the interlaboratory trial.

Collaborative trial with GACT in Uruguay

1.1. Chemicals, materials, and standards

Ethyl acetate (EtOAc), HPLC grade, was supplied by J.T. Baker (USA). Ammonium formate, anhydrous magnesium sulphate extra pure ($MgSO_4$), sodium citrate dibasic sesquihydrate, primary secondary amine (PSA), and octadecyl reverse phase silica gel (RP-C_{18}) were obtained from Sharlau (Barcelona, Spain). Methanol (MeOH) was purchased from Carlo Erba (France), and trisodium citrate dihydrate was from Merck (Germany). In the GACT laboratory in Uruguay the raw absorbents were individually weighted prior to being used in the clean-up step.

A stock solution of analytes was obtained by FEPL laboratory in Seibersdorf, Austria, and transported under refrigerated conditions via DHL to Uruguay. Working solutions were prepared at 1 mg L^{-1}, by taking appropriate aliquots of the stock solutions.

1.2 Chromatographic equipment

1.2.1. GC-MS/MS

GC–MS/MS analysis was performed by a Shimadzu GCMS-TQ8040 system. The instrument is equipped with a 2010 plus gas chromatograph coupled to a triple quadrupole mass spectrometer. Aliquots of 1 µL of sample extract were injected into the gas chromatograph in splitless mode. The injector temperature was 280 °C, and Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. GC separation was carried out with a Rxi®-5Sil MS

capillary column (5 % diphenyl / 95 % dimethylpolysiloxane; 30 m; 0.25 mm id; 0.25 µm df) by Restek (Bellefonte, PA, USA). The oven conditions were 60 °C initial (1 min), increased to 160 °C at 25 °C/min, increased to 240 °C at 4 °C/min, and finally increased to 290 °C at 10 °C/min (11 min hold). The total run time was 41 min. Interface temperature was 290 °C and the ion source was at 230 °C operated in electron ionization mode (70 eV). Detection was performed with a detector voltage of 1.6 kV. Argon was the collision gas at a constant pressure of 200 kPa. MSMS detection was performed in the multiple reaction monitoring (MRM) mode using transitions and collision energies previously selected for each compound. Lab solution GC-MS/MS solution Version 4.4 from Shimadzu was used for instrument control and data processing.

1.2.2. LC-MS/MS

The analysis was performed with an Agilent 1200 LC system coupled to a 4000 QTRAP LC-MS/MS System from AB SCIEX in the scheduled MS-MS mode. The chromatographic separation was performed on a ZORBAX Eclipse XDB-RPC$_{18}$ (150 mm × 4.6 mm. 5 µm) column and the column oven temperature were set at 20 °C. The mobile phase was a gradient between (A) 0.1 % formic acid in water and (B) 5 mM ammonium formiate in MeOH. The elution program used started with holding B (10 %) for 3 min and increasing to 100 % B over 17 min and then holding for 5 min. Finally, the system returned to the original conditions over 3 min and held for equilibration for 5 min (33 min total run). The injection volume was 5 µL and the flow rate 0.6 mL/min. The MRM mode was performed for tandem MS detection. The optimal MRM conditions for each analyte were optimized using direct infusion in the ESI+ mode. Source temperature was 500 °C, the ionization voltage was 5000 V, curtain gas was nitrogen at 20 psi and the nebulizer gas was air at 50 psi. Scheduled multiple reaction monitoring was used with a setting of a 90 s detection window covering the expected

retention time of each analyte and the target scan time was 2 s for all pesticides. To acquire and process the data Analyst software version 1.6 from AB SCIEX was used.