MULTICOMPONENT PESTICIDE RESIDUE ANALYSIS ON SELECTED SPICES

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By

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Certificate

This is to certify that this thesis entitled "**Multicomponent Pesticide Residue Analysis on Selected Spices**" is an authentic record of research work carried out by **Mr RAMESH BABU N** under my supervision in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry of University of Calicut and further that no part thereof has been presented before for any other degree.

Dr. Joby Thomas. K
(Supervising Teacher)

DECLARATION

I hereby declare that the thesis entitled "**Multicomponent Pesticide Residue Analysis on Selected Spices**", submitted to the University of Calicut in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy in Chemistry is a bonafide research work done by me under the supervision and guidance of Dr. Joby Thomas K., Associate Professor (Retd.), Department of Chemistry, St. Thomas' College, Thrissur.

I further declare that this thesis has not previously formed the basis of any degree, diploma or any other similar title.

27-07-2022 **RAMESH BABU N**

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To

My Family

PREFACE

From historical times when human beings started relying on agriculture for food, protection of crops against pests have been a persistent concern. Controlling pest population and mitigating their adverse effects on crops have been a constant challenge. The use of chemical pesticides as crop protection agents have evolved over time following a path of increasing sophistication, culminating in modern synthetic pesticides which are highly effective against pests and less persistent in nature. These pesticides have played a significant role in ensuring global food security in the modern era.

Since synthetic pesticides function by inhibiting or interfering with biochemical processes in the body of the pests, these are potentially harmful to other living beings as well, including humans. Extended and indiscriminate use of these pesticides results in the accumulation of traces of these chemicals in the agricultural produce, termed as pesticide residues, which in turn cause harmful effects upon consumption of such produce. Health issues like cancer and disorders of the immune, reproductive and nervous systems have been attributed to the presence of pesticide residues in food. This makes pesticide residues a major food safety concern. Many countries across the world have issued increasingly stringent regulations of maximum residue limits (MRLs) for pesticide residues in various food commodities to ensure consumer protection. In this context, testing of pesticide residues in food is important to ensure compliance of food commodities with such regulations.

Analysis of pesticide residues have also evolved over time. For many years, chromatographical techniques with conventional detectors have been the preferred method for trace analysis. With the advent of highly sensitive and selective mass spectrometric techniques, hyphenated instrumentation where gas and liquid chromatography were

coupled with tandem mass spectrometry became the tool of choice for the analytical chemist in testing pesticide residues in food. The sample preparation techniques for pesticide residue analysis have also undergone considerable changes. The classical techniques which relied on solvent extraction and partitioning were time intensive and tedious, and have given way to the modern 'quick, easy, cheap, effective, rugged and safe' (QuEChERS) sample preparation technique which offers simplicity without sacrificing analytical performance.

Spices are considered difficult matrices to analyse because of their complex chemical composition. All spices have some active chemical compounds present in significant concentrations which contribute to their special properties like colour, flavour and aroma. These compounds can potentially interfere with analysis of pesticide residues. Spices are also very diverse, and belong to different classes like dried fruits (e.g., chillies, black pepper), dried seeds (e.g., cumin, fennel), dried floral parts (e.g., saffron), dried roots (e.g., ginger, turmeric) etc. Each class of spices have distinct properties and it is practically difficult to use a single analytical method to cover all major classes of spices. Thus, modern analytical methods for spices need to be sufficiently general to aid easy implementation but also have to be fine-tuned with respect to different classes of spices to ensure analytical performance. This is a gap area which is addressed in this thesis. For convenience, the work presented in this thesis is divided into two parts.

Part I of the thesis deals with developing, optimizing and validating pesticide residue analysis for different classes of spices. The pesticides most commonly used for cultivation of spices in India are covered. Two main instrumentation techniques are used, viz. ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) and gas chromatography coupled to tandem mass spectrometry (GC-MS/MS). One of the most important problems faced in using mass spectrometric

techniques for quantitative analysis is the matrix effect (ME), which makes response of a target analyte different in solvent and matrix extracts. This issue poses significant challenges in high sensitivity trace analysis for pesticide residues, especially in complex matrices like spices. The causes of ME are different in UPLC-MS/MS and GC-MS/MS and have to be addressed differently in developing analytical methods. This is also addressed in Part I of the thesis.

In Part I, the first chapter presents an overview of classical and modern pesticide residue analysis methodology and instrumentation, the origins of ME in LC-MS/MS and GC-MS/MS with different approaches to mitigating these effects, and the processes used for method validation. The analytical protocols and instrumentation methods used for pesticide residue analysis in spices is described in Chapter 2. In Chapter 3, the development, optimization and validation of a multiresidue method for 53 pesticides in six representative spices using UPLC-MS/MS is documented, along with studies on matrix effect and measurement uncertainty calculations. In Chapter 4, the development, optimization and validation of a multiresidue method for 25 pesticides in six representative spices using GC-MS/MS is covered, along with evaluation of matrix effect measurement uncertainty calculations. In Chapter 5, two novel methods for mitigating ME in pesticide residue analysis in spices is explored, viz. use of analyte protectants in GC-MS/MS, and use of surrogate matrix compounds in solvent-based reference standards in LC-MS/MS. In Chapter 6, analysis of a class of broad-spectrum fungicides called dithiocarbamates, which are extensively used in cultivation of spices, using GC-MS is documented. This is followed by select bibliography.

Part II of the thesis deals with application of the methods developed in Part I to real samples for the purpose of evaluation of compliance with national MRLs as well as characterization of food safety hazards due to presence of pesticide residues in commonly

consumed spices. Chapter 1 presents a review of the regulations in India with respect to pesticide residues, the extant MRL regulations, evaluating compliance with MRLs and performing food safety hazard characterizations based on results of analysis. The methodology and instrumentation used in the study is depicted in Chapter 2. In Chapter 3, the results of application of the methods developed in Part I to real samples of six representative spices collected from local markets is covered. A total of 60 samples were analysed for 78 pesticides using UPLC-MS/MS and GC-MS/MS. Based on the results obtained, compliance with the national MRLs and food safety hazard characterization calculations were performed. This is followed by select bibliography.

ABBREVIATIONS

ADI	Acceptable daily intake
AP	Analyte protectants
AQC	Analytical quality control
ASTA	American Spice Trade Association
CRM	Certified reference material
d-SPE	Dispersive solid phase extraction
EI	Electronic ionization
ESI	Elecrospray ionization
FSSAI	Food Safety and Standards Authority of India
GAP	Good agricultural practices
GC	Gas chromatography
GCB	Graphitized carbon black
LOD	Limit of Detection
LOQ	Limit of Quantification
MMC	Matrix matched calibration
MPI	Maximum permissible intake
MrM	Multiresidue method
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NEDI	National estimated dietary intake
NVNA	N-vanillyl nonanamide
PSA	Primary secondary amine
QuEChERS	Quick, easy, cheap, effective, rugged, safe sample preparation method
RSD_r	Relative standard deviation - repeatability precision
RSD_R	Relative standard deviation - reproducibility precision
TMDA	Theoretical maximum daily intake
UPLC	Ultra-high performance liquid chromatograph

ABSTRACT

The use of chemical pesticides as crop protection agents is an indispensable feature of modern agriculture, which helps to ensure global food security. However, extended and indiscriminate use of these pesticides results in the accumulation of traces of these chemicals in the agricultural produce, termed as pesticide residues, which in turn cause harmful effects upon consumption of such produce. This makes pesticide residues a major food safety concern. Many countries across the world have issued increasingly stringent regulations of maximum residue limits (MRLs) for pesticide residues in various food commodities to ensure consumer protection. In this context, testing of pesticide residues in food is important to ensure compliance of food commodities with such regulations.

Spices are considered difficult matrices to analyse because of their complex chemical composition, with significant concentrations of active compounds that contribute to their special properties like colour, flavour and aroma. These compounds can potentially interfere with analysis of pesticide residues. Spices are also very diverse, and belong to different classes like dried fruits, seeds, floral parts, roots etc. which are distinct from one another. It is practically difficult to use a single analytical method to cover all major classes of spices. Thus, modern analytical methods for spices need to be sufficiently general to aid easy implementation but also have to be fine-tuned with respect to different classes of spices to ensure analytical performance. Development of such methods using UPLC-MS/MS and GC-MS/MS, covering different classes of spices, is documented in this thesis.

Part I of the thesis deals with developing, optimizing and validating pesticide residue analysis for different classes of spices. The pesticides most commonly used for cultivation of spices in India are covered. Two instrumentation techniques are used, viz. UPLC-MS/MS GC-MS/MS, with QuEChERS sample preparation method which was

optimized for different classes of spices. One of the most important problems faced in using mass spectrometric techniques for quantitative analysis is the matrix effect (ME), which makes response of a target analyte different in solvent and matrix extracts. This issue poses significant challenges in high sensitivity trace analysis for pesticide residues, especially in complex matrices like spices. Novel ways of addressing ME in pesticide residue analysis in spices is also documented in Part I.

Part II of the thesis deals with application of the methods developed in Part I to real samples for the purpose of evaluation of compliance with national MRLs as well as characterization of food safety hazards due to presence of pesticide residues in commonly consumed spices. A total of 60 market samples were analysed for 78 pesticides using UPLC-MS/MS and GC-MS/MS. Based on the results obtained, compliance with the national MRLs and food safety hazard characterization calculations were performed.

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PART I

PESTICIDE RESIDUE ANALYSIS IN SPICES: METHOD DEVELOPMENT, VALIDATION AND APPLICATION

CHAPTER 1

INTRODUCTION AND REVIEW

Since the advent of organized agriculture in history, crop protection products have been used by farmers to ensure highest possible harvest. Attack by pests have always been a potential hazard to agriculture. Controlling pest population and mitigating their adverse effects on crops have been a challenge to farmers since ancient times. The first recorded use of a chemical pesticide in agriculture is attributed to the work of the Roman scholar Marcus Terentius Varro[1], who lived during 116 BC – 27 BC. He had found that a paste prepared from crushed olives was toxic to ants, moles and weeds. Application of salts to control weeds, and burning sulphur for control of insects, have also been reported in historical documents. The Chinese have historically used arsenic and water mixture to control insects that infested orchards.

Such remedies have existed throughout history, but extensive use of chemical pest control agents began in relatively recent times. The agricultural revolution, which is considered to have begun in Europe in the 19th century[2], ushered in an era of great technological improvement and productivity in agriculture. Since then, the rapidly increasing world population has placed great demands on agriculture for the sustenance of billions of people around the world. Pesticides have been playing an extremely important role in maximising agricultural productivity.

Modern pesticides: an overview

The evolution of pesticides during the course of history followed a path of increasing sophistication[3]. In ancient times, minerals and metals were used for the control of pests. The use of these materials for pest control is thought to have originated in China. Sulphur was one of the earliest substances used for this purpose, and its use still continues

today. Arsenic, along with its compounds like lead arsenate, chromated copper arsenate etc, also has a long history as weedicides and insecticides.

The next stage in the evolution of pesticides saw the use of plant-based bio-control agents. Nicotine, derived from tobacco leaves, was recognized as a useful insecticide in the sixteenth century. A class of plants collectively called in olden days as pyrethrums, now classified as chrysanthemum, were also found to have insecticidal properties, and their dried flower heads were used as natural insecticides. Extracts of the strychnine tree (*Strychnos nux-vomica L.*), were used to control rodents. Extract taken from tuba root (*Derris elliptica L.*) which contains the compound rotenone was widely used for control of fish and insects in south-east Asian countries. The main problems with plant-based pesticides were that they were difficult to obtain in purified form and so could not be produced in quantities needed for large scale agriculture. As a result, attempts were made to develop synthetic pesticides.

The first synthetic pesticides to be prepared were organochlorine insecticides like dichloro-diphenyl-trichloroethne (DDT)[4,5]. During the second world war organophosphate compounds were secretly developed as chemical warfare agents in Germany, After the war many of these were converted to use as pesticides, and some of these chemicals are still used in agriculture. A new class of pesticides, *viz*. carbamates, was also developed and by mid-twentieth century, organochlorine pesticides were largely replaced in agriculture by organophosphates and carbamates.

In 1949, the pesticide allethrin was synthesised in the laboratory, and the old class of pyrethrin pesticides was revived as popular plant protection agents. Since then, a wide variety of synthetic pyrethroids were prepared and they gained popularity due to their low toxicity towards nontarget organisms and reduced persistence in nature. Though their rapid

loss of activity in the environment constraints their application, synthetic pyrethroids are still employed widely in modern agriculture.

Currently, the neonicotinoids[6] class of pesticides is gaining high popularity. They protect the plant by moving into the tissues and thus protect all parts of the plant. They function as neurotoxins to arthropod insects by binding irreversibly to the nicotinic acetylcholine receptors, thus resulting in over-stimulation of nerves and paralyzing the insect[5]. Imidacloprid, the first neonicotinoid pesticide that was synthesised in the laboratory, is considered to be the most extensively used pesticide in the world. In addition to this, many new and emerging classes of pesticides are gaining acceptance in agriculture. Figure 1.1 shows the chemical structures of the most commonly used pesticides in agriculture.

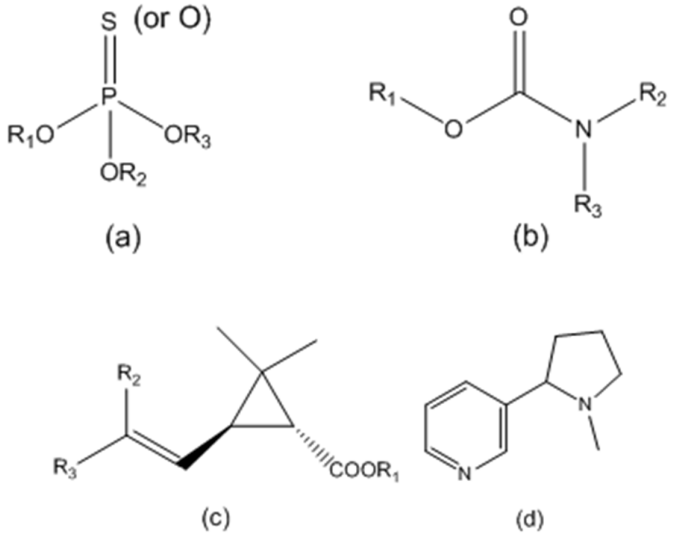


Figure 1.1 General chemical structures of commonly used modern pesticides: (a) organophosphates, (b) carbamates, (c) pyrethroids and (d) nicotine, which is structurally similar to neonicotinoid compounds.

In the Indian context, synthetic pesticides can be broadly grouped into three classes: (a) classical (b) modern and (c) emerging. Table 1.1 lists the major classes of synthetic pesticides that have found extensive use. Most of these pesticides are

neurotoxins, their mode of action being inhibition of essential biochemical pathways in cells and nerve centres. The modern and emerging pesticides have increasing levels of sophistication, including swift action against targeted pests, comparatively low toxicity to non-target organisms and low persistence in nature.

Table 1.1: Classes of synthetic pesticides and mode of functioning

	Class of pesticide	**Examples**	**Mode of functioning**	
1	Organochlorines	BHC, DDT	Affects chloride ion transport at nerve centres	Classical
2	Organophosphates	Ethion, chlorpyrifos	Acetylcholinesterase inhibition	
3	Carbamates	Carbaryl, aldicarb	Acetylcholinesterase inhibition	
4	Pyrethroids	Allethrin, cypermethrin	Affect sodium channels in the axonal membranes	Modern
5	Neonicotinoids	Imidacloprid, acetamiprid	Binds to nicotinic acetylcholine receptors in cells	
6	Macrocyclic lactones	avermectins and milbemycins	Inhibition of chloride ion flow in cells	Emerging
7	Phenyl pyrazoles	Fipronil	Affects chloride ion transport at nerve centres	
8	Nereistoxin analogues	Cartap, thiosultap	Binds to nicotinic acetylcholine receptors in cells	
9	Diamides	Flubendiamide, chlorantraniprole	Releasing stored calcium from the sarcoendoplasmic reticulum	
10	Benzoylureas	Ufenuron, triflumuron	Inhibition of biosynthesis of chitin	
11	Cyclic ketoenols	Spirodiclofen, spiromesifen	Acetyl-CoA-carboxylase inhibition	

From classical to emerging pesticides there is an increasing trend in toxicity and a decreasing trend in environmental persistence. This has important implications on the consequences of injudicious use of these pesticides. The classical pesticides have high bioaccumulation and residues of these pesticides cause chronic diseases in the long term. The modern and emerging pesticides have low persistence but much higher toxicity, so their residues can be more potent and can cause severe health implications in the short

term. In developing countries like India, due to economic constraints, large scale transition to the emerging pesticides is still in the future, and the most common pesticides used in agriculture are still organophosphates, carbamates, pyrethroids and neonicotinoids.

Pesticide residues: environmental and health hazards

Since all the synthetic pesticides function by inhibiting or interfering with biochemical processes in the body of the pests, these are potentially harmful to other living beings as well, including humans. Extended and indiscriminate use of these pesticides results in the accumulation of traces of these chemicals in the agricultural produce, termed as pesticide residues, which in turn cause harmful effects upon consumption of such produce[7–10]. Health issues like cancer and disorders of the immune, reproductive and nervous systems have been attributed to the presence of pesticide residues in food. These chemicals can also affect non-target organisms[11,12] and contaminate natural habitat due to their persistence in nature[13,14].

The issue of pesticide residues came into stark focus in 1962 when Rachel Carson published the classic and popular book *Silent Spring*[15], which clearly documented for the first time the environmental hazards posed by synthetic pesticides. The book had tremendous impact on the worldwide agricultural scenario and also caused the scientific community to consider the environmental and health effects of the use of synthetic pesticides. This eventually led to the creation of the Environmental Protection Agency (EPA), the first of its kind, in the United States of America. Since then, pesticides have been extensively studied not just for their efficiency in crop protection but also for their adverse health effects to non-target organisms and environmental persistence.

The steadily increasing global health consciousness and awareness of matters related to public health have led to the formulation of strict regulatory measures for safety parameters in food, and this has reflected on pesticide residues also. Now, many countries

have fixed maximum residue limits (MRLs) for pesticide residues in food, which are enforced with increasing stringency, both domestically and in food imports[16,17]. The Codex Alimentarius Commission, a multinational standards-setting body under FAO/WHO, has also set up a database for MRLs for pesticide residues in food[18]. In India, the regulations for pesticide residues have been issued by the Food Safety Standards Authority of India (FSSAI)[19].

Analysis of pesticide residues: classical and modern methods

The first example of multi-residue methods (MrMs) for pesticide residue analysis was the Mills method[20], which used acetonitrile extraction followed by diluting with water and partitioning into petroleum ether. This method was effective for nonpolar compounds like organochlorines, but the partially polar pesticides like organophosphorus compounds were seen to be lost during the partitioning into nonpolar solvents. Subsequently, the Luke method[21,22] was developed which used extraction by acetone followed by dilution with water and partitioning into petroleum and dichloromethane. Other methods were derived from these two methods by introducing various combinations of solvents[23,24] in the partitioning step to cover wider ranges of polarity in the target compounds. This class of methods can be called the classical methods of pesticide residue analysis.

It was realized by researchers that the role of water in these methods during partitioning step was critical. Water was added either as part of the initial extraction solvent or added subsequently as diluent. Adding salts[25] to facilitate phase separation by saturating the water layer was seen to directly affect the polarity range covered by these methods. Addition of salts in the Luke method, to separate acetone and water layers and thus avoiding a subsequent partitioning into a nonpolar solvent[26] was also tried out. Other variations tried 'freezing out' water from Luke method extracts by lowering the temperature. Solid phase extraction (SPE) was also used to avoid the partitioning step[27,28]

All these methods were in use extensively for a long time across the world. However, they still had limitations in terms of applicability and the range of pesticides and matrices that could be covered. They were also time consuming and expensive. Moreover, in most of these the methods, partitioning into non-polar solvents in the second step to remove water resulted in partial loss of polar compounds.

In order to overcome the limitations of the classical methods, many alternate approaches were considered by researchers towards the end of the last decade. Most of these new approaches used special ways of sample preparation and extraction. Accelerated solvent extraction, which performed the extraction at high temperatures and pressures, were able to reduce extraction times considerably without affecting thermally labile residues[29]. Matrix solid-phase dispersion (MSPD) was considered a successful process for extraction of residues from semi-solid and solid matrices[30]. This process involved the blending the sample with a support material containing a bound organic phase like octadecylsilyl (C-18). Solid phase microextraction (SPME) was another technique which was used successfully in food matrices[31]. This technique used a fused silica capillary fibre which had a stationary phase coated on the outer surface, which adsorbed the analyte directly. The technique was used in conjunction with GC MS and LC MS analyses[32]. Microwave assisted extraction of residues in agricultural products was also an important sample preparation technique adopted *in lieu* of classical techniques[33]. Another important procedure, mostly adopted in the European Union, involved the use of gel permeation chromatography (GPC) for effecting cleanup of extracts. This method, first included in the German Federal Food Act, subsequently became the DFG-multiresidue S19 method which was adopted across the European Union (European Standard CSN EN 12393-2, 2008). This method was also adapted to include mass spectrometric detection, which enabled

highly sensitive analysis of organochlorine, organophosphorus and carbamate residues in food[34].

All these new techniques, which were developed to overcome the limitations of the classic methods of residue analysis, still had the disadvantage that they required specialized instrumentation and expertise. Also, there was still some level of limitation in applicability of these techniques across various matrices. With the availability of highly sensitive mass spectrometric techniques in recent times, a simple, widely applicable and inexpensive sample preparation technique became the need of the hour.

The QuEChERS sample preparation method

In 2003, Anastassiades and Lehotay published the first research paper involving the novel use of dispersive solid phase extraction (d-SPE) for cleanup of extracts in pesticide residue analysis. They named this the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method, and ushered in a new era in routine regulatory analysis of pesticide residues in food in the laboratories all over the world[35]. The method they developed was shown to perform better than the classical methods used for analysis of pesticides in fruits and vegetables at the time. Also, the new method was able to cover a larger range of pesticide classes than any of the classical methods.

This method addressed the main problem which was faced by classical methods, *viz*. the final extract solution containing water. Partitioning into non-polar solvents left behind polar residues, and the use of salts was also not fully effective. By miniaturizing the entire extraction process and by optimizing the solvent and salt content to minimize the presence of water in the final extract, the developers of QuEChERS found a way around this issue. In the original QuEChERS methodology, the steps followed were as follows:

1. *Homogenization of the sample*. A low sample weight (2-10 g), after thorough homogenization, was used for extraction.

2. *Extraction with a low volume (10-15 ml) of acetonitrile (MeCN)*. The ratio of sample to solvent was typically maintained at 1g ml^{-1} (e.g., 10 g sample + 10 ml MeCN).
3. *Novel use of NaCl + anhydrous magnesium sulphate (Anh. $MgSO_4$) which fully removes water from the organic (MeCN) phase*. In this step, the residues were transferred to the organic phase while a substantial portion of the coextracted material remained in the aqueous phase.
4. *Fast cleanup of the extract* using a d-SPE step with primary-secondary amine (PSA) and anh. MgSO4.

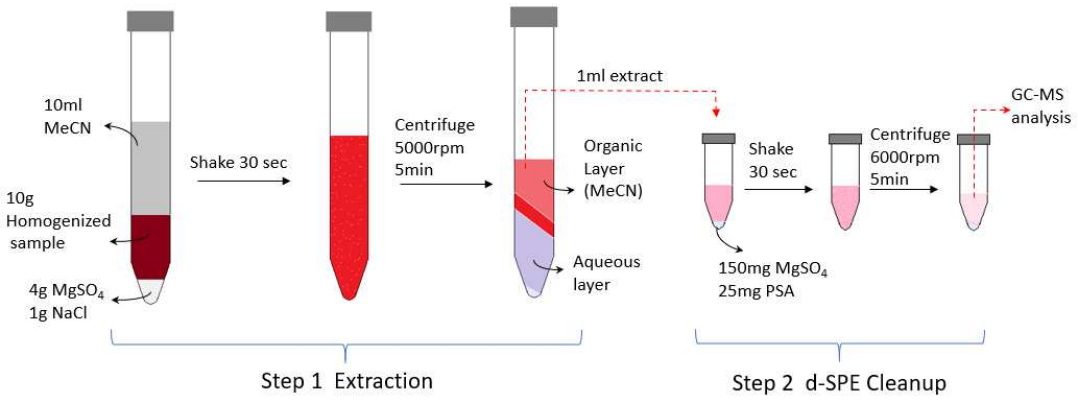


Figure 1.2: Steps of the original QuEChERS method

The importance of use of MeCN in the QuEChERS process is because of the following two properties of this solvent: (a) compared to ethyl acetate (EtOAc) and acetone, MeCN extracts a larger range of pesticide residues, and also limits the coextracted lipophilic compounds from the matrix; (b) MeCN is miscible with water and can penetrate effectively into aqueous samples thereby maximising the extraction process. The addition of anh. $MgSO_4$ produced separation between organic and aqueous phases by saturation of the aqueous phase. Hydration of $MgSO_4$ is an exothermic reaction, and this raises the

temperature of the extract to about 40°C. These features helped in the transfer of the nonpolar residues into the organic layer without the use of nonpolar solvents.

The sodium chloride was added to reduce the extraction of interfering polar compounds. Out of the several combinations of salts studied, the use of 4g Anh. $MgSO_4$ + 1g NaCl was found to give optimal recoveries of pesticide residues, better separation of aqueous and organic phases, less quantity of interfering compounds in the extract, and better chromatographic performance. The d-SPE step was the most novel aspect of the QuEChERS procedure, as compared to the classical methods. In contrast with the traditional SPE techniques, in d-SPE the sorbents were added into the extracted solution directly to bring about the cleanup. Thus, there was no need here for the accessories commonly used in conventional SPE, like columns, cartridges, vacuum manifolds etc. After the first step of extraction with acetonitrile, a 1 ml aliquot was taken for d-SPE cleanup. The cleanup was brought about by $MgSO_4$ and PSA, which was successful in removing polar coextractives like sugars, organic acids and traces of water remaining in the extract after the first step. Even with the d-SPE technique, the extract obtained was not as pure as is desirable. So, the QuEChERS technique requires the use of chromatography coupled with mass spectrometric techniques for obtaining good results.

Modern adaptations of the QuEChERS method

Many modifications were introduced into QuEChERS to adjust the method to address specific challenges[36]. The QuEChERS methodology could be considered as a conceptual framework for sample preparation in pesticide residue analysis, as it is easily modified to suit analytical needs. This amenability for modification is also a drawback, as a broad spectrum of changes and recombinations of the QuEChERS reagents have been published, which is in need of systematic harmonization. Some of the important modifications are now summarized.

It was observed that in the original QuEChERS procedure, certain pH sensitive pesticides showed recovery losses. In the original method, the pH of the extract was adjusted to 4. This was a compromise to accommodate pesticides lost at low pH and those which were unstable at high pH. In fact, it was demonstrated that the use of d-SPE step with PSA in medium and high pH matrices raised the pH value even higher, i.e. to basic range, resulting in degradation of certain pesticides in the extract[37]. To address these issues, buffers were introduced into the extraction process. The two main approaches were (a) strong acetate buffer, pH 4.8 (AOAC Method No. 2007.1)[37] and (b) weak citrate buffer, pH 5.5 (European Union standard method EN 15662)[38]. Figure 1.3 shows the comparison of these two methods with the original QuEChERS[35] method.

The coextractives were found to vary from matrix to matrix in both the above methods. It was also observed that the strong buffer in AOAC method could reduce efficiency of the cleanup using PSA[39]. Due to these issues, other sorbents were also included in the steps with an aim to improve method performance.

Two additives, C-18 sorbent[40] and Graphitized Carbon Black (GCB)[41], when used in conjunction with the original cleanup steps, were seen to improve method performance in a variety of matrices. The role of C-18 was to remove nonpolar lipid interferences from fatty matrices, while GCB was used to remove pigments (e.g., carotenoids and chlorophyll) from certain plant matrices. But the GCB was also found to affect the recoveries of certain planar pesticides like hexachlorobenzene, polyaromatic hydrocarbons etc. The use of zirconium dioxide sorbent for cleanup in high fat matrices[42] was also an important modification. Combining this sorbent with C-18, or entirely replacing C18 and GCB with this sorbent, has been found useful in many matrices. With all these new developments in adapting the QuEChERS methods to suit different matrices and classes

of pesticides, it is important that optimization and validation have to be performed for specific matrix / pesticide combination to obtain acceptable and reliable results.

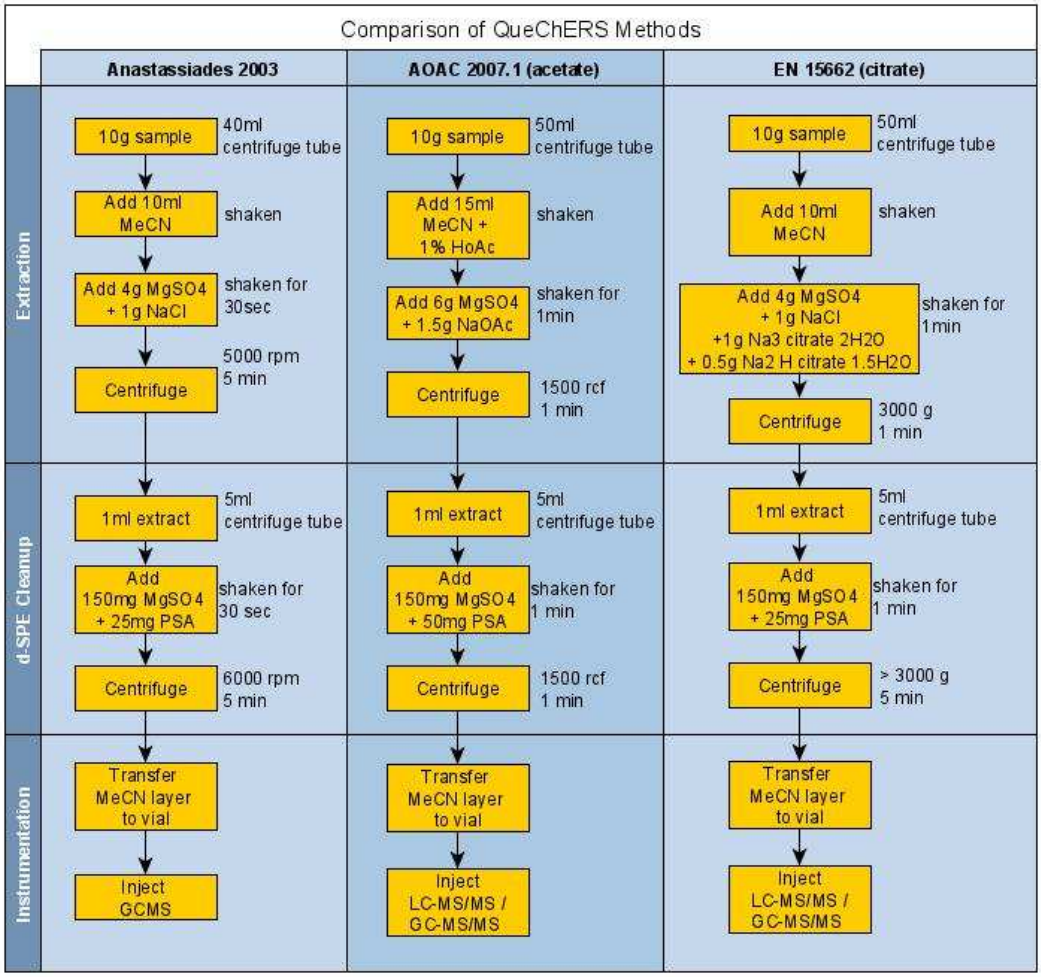


Figure 1.3. Comparison of the original QuEChERS method with the buffered methods

Since the QuEChERS steps involve the use of salts, traces of these can find its way to the final injection volume and potentially affect the ion sources in GC-MS/MS and LC-MS/MS. The use of ammonium salts to address this issue was also an important attempt in QuEChERS modifications. It was observed that ammonium formate could induce phase separation between MeCN and water in the first step of QuEChERS, and as ammonium salts are volatile and avoid deposition in GC liner, its use can improve method

performance[43,44]. Use of ammonium formate can also help in improving ionization in the LC-MS ion source. Additionally, when the MeCN used for extraction contains formic acid, ammonium formate produces buffering action which can improve recoveries in certain pesticides, and coextractives were also found to be less.

Instrumentation in pesticide residue analysis

Mass spectrometry involves separation and detection of ionized fragments arising from a set of target analytes, which have been earlier separated in an HPLC or a GC. The process of generating these ionized fragments is important and is fundamentally different for LC and GC. Mass spectrometer functions under high vacuum, and so the nature of the interfacing between GC/LC and the MS system is also specific to each technique. The ionized fragments, after entering the mass spectrometric system, are analysed using a system of four electrodes named as quadrupoles. These quadrupoles, depending on the potentials applied on it, can perform scanning of the mass fragments, or can specifically isolate and transmit fragments with specific mass / charge (m/z) values.

In modern routine pesticide residue analysis, detection and quantification of a target compound is usually made in the presence of many other compounds that are eluted from the analyte matrix. Since mass spectrometer is a universal detector, the coextracted compounds will produce signals that will interfere with the detection of the target analyte. In order to overcome this issue, mass spectrometry is carried out twice in sequence, which gives the technique the name 'tandem' mass spectrometry and is depicted as MS/MS. In this case, three quadrupoles are placed sequentially in the mass spectrometer. The first quadrupole selects an ion with a specific m/z (typically called the 'parent ion') corresponding to the target analyte from among the ions produced in the ionization source. This ion then undergoes collision induced dissociation in an enclosed quadruple system containing a collision gas (e.g., N_2), producing secondary ion fragments (typically called

'daughter ions'). A third quadrupole then selects ions with a specific m/z value, which are then detected by a channel electron multiplier or an equivalent detection system. This sequence of ion selection by the quadruples is typically termed as multiple reaction monitoring (MRM) transition, and is the method adopted for residue analysis using hyphenated, tandem mass spectrometric techniques. This is schematically shown in Figure 1.4.

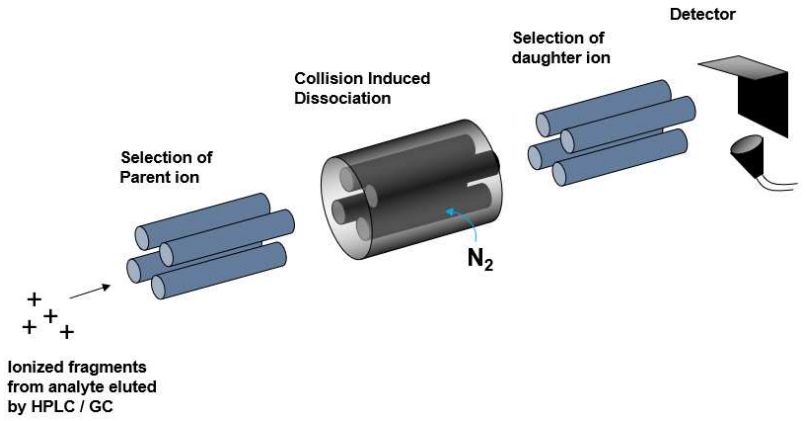


Figure 1.4. Instrumentation in tandem mass spectrometry (MS/MS)

The most important ionization techniques used are Electrospray ionization (ESI) in LC-MS/MS and Electronic Ionization (EI) in GC-MS/MS. Although the process in both cases involves transformation of the analyte molecules into ions, the technique involved in both cases are different and constitute a key distinction between these two mass spectrometric techniques. ESI in LC-MS/MS occurs at atmospheric pressure, and EI in GC-MS/MS takes place in vacuum. Figures 1.5 and 1.6 schematically show both the ionization techniques. Due to the difference in the mechanisms involved in both techniques, their effects on residue analysis are also different. The impact of co-eluting matrix components on method performance, typically termed as matrix effects (ME) in residue analysis, originate in the sample introduction and ionization steps in mass spectrometry.

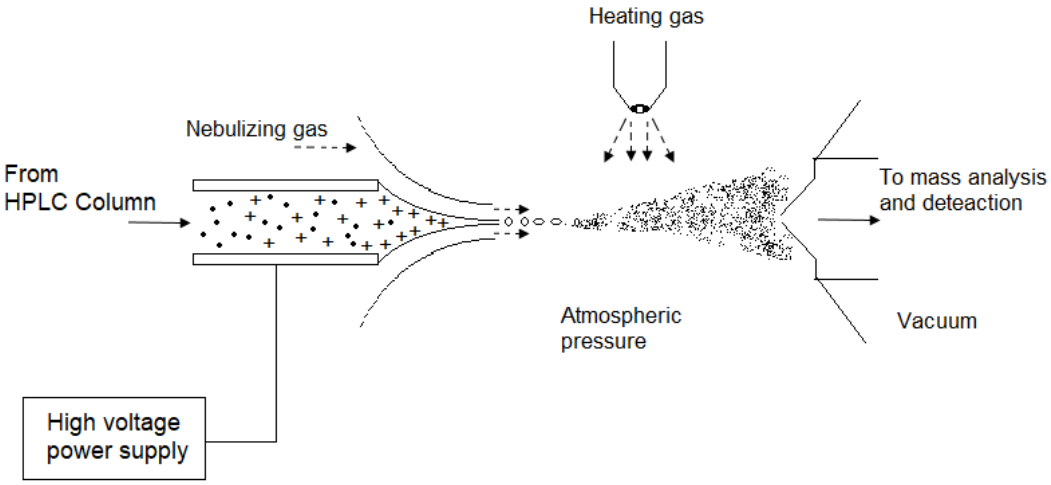


Figure 1.5. Electrospray ionization in LC-MS/MS

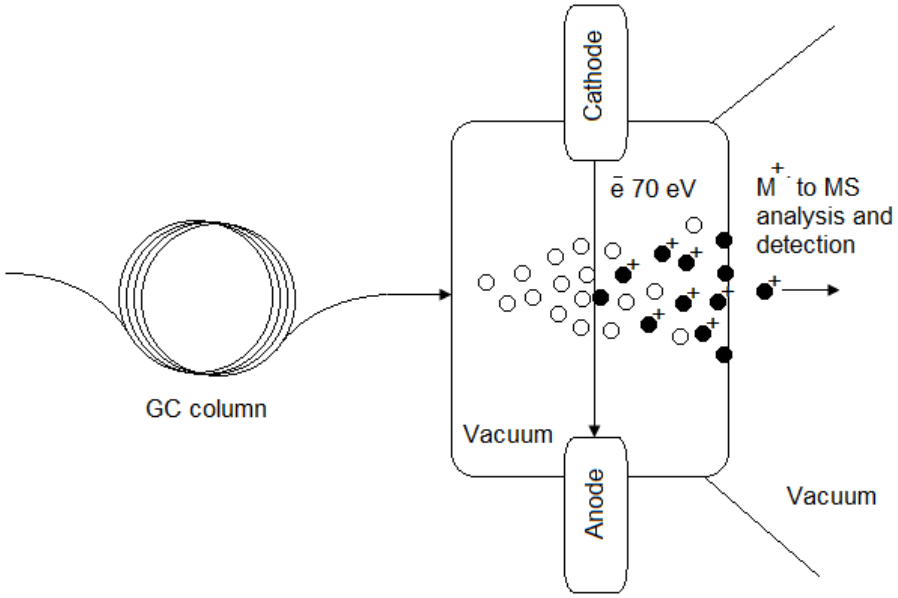


Figure 1.6. Electron ionization in GC-MS/MS

Figure 1.5 shows ESI operating in positive mode, where the applied electrical field in the capillary tube of the probe enables the analyte M to take up a proton from the solvent, leading to the formation of MH^+, which has a mass of 1 atomic mass unit (amu) greater than the molecular mass of the analyte. The droplets loaded with these positive ions

moving out of the tip of the capillary are then broken up by a nebulizer gas and dried by a heating gas, leading to the decrease in the drop size. Further evaporation shrinks these drops and makes the positive charges in the drops come closer together. At a point when the charge density of the drop reaches a critical value called the Rayleigh stability limit, the repulsive forces exceed the surface tension of the drop. This breaks the drop apart leading to a second generation of progeny droplets, in a process known as coulomb explosion. The process continues until free gas phase ions are produced and pulled into the mass spectrometer by appropriate potentials, for mass analysis by the quadrupole system followed by detection of ions[45,46]. This is a soft ionization and does not usually involve degradation of the molecular ion. As is evident from the above description, in ESI the ionization process happens at atmospheric pressure and involves several variables; and requires optimization. A reproducible ionization process from machine to machine without separate optimization is not possible.

On the other hand, in EI shown in Figure 1.6, the conditions are more standardized. The electron impact process where the analyte molecule M passes through an electron beam of 70 eV producing the molecular ion radical $M^{+\cdot}$ happens in vacuum, and the conditions are standardized and repeatable. EI is hard ionization and the molecular ion usually does not survive intact but breaks down into more stable ions and neutral molecules. Since the process is repeatable, libraries of mass spectra are possible in GC-MS analysis[47,48]. Although the mechanisms of ionization are different in GC and LC modes, for quantitative analysis both modes utilize tandem mass spectrometry, or the multiple reaction monitoring (MRM) transitions, as shown in Figure 1.4.

Matrix effects in pesticide residue analysis

Although GC-MS/MS and LC-MS/MS are powerful and highly sensitive techniques for pesticide residue analysis, they are limited by the efficacy of available sample

processing methods. All such methods result in extracts that can pose residual matrix effects, which will affect qualitative identification and quantitative estimation of analytes[49–51]. The reasons for such matrix effects (ME) are mainly the following:

1. Coelution of the analyte with matrix components (interfering molecules of the same mass) which can affect the analyte transitions. This can lead to variation in quantifier: qualifier ion ratio in the sample as compared to that in the standard, creating ambiguity in identification.
2. Suppression or enhancement of MS/MS transition for the analyte, which can pose problems with quantification.

In combination, the effects described above can result in increasing the uncertainty of the analytical method.

In practice, the ME can be understood as the change in response signal in GC-MS/MS or LC-MS/MS for an analyte when it is present in the matrix, as compared to its response when it is present in the solvent. This effect can be enhancing or suppressive. Thus, ME must be evaluated and accounted for to achieve reliability in quantitative trace level analysis. As a result of ME, quantifying pesticide residues in a real-life sample by using a reference standard prepared in solvent will possibly lead to error in the results. In order to account for ME, two techniques are commonly used; (a) preparation and use of reference standards in blank matrices termed as matrix-matched calibration (MMC) standards, and (b) use of internal standards or isotopically labelled standards. Use of isotopically labelled standards might be cost-prohibitive. In practice, the technique adopted in routine analysis is to prepare and use MMC standards where possible, and to optimize extraction techniques to ensure that the coextracted interferences from the matrix are minimized. Matrix effects are usually estimated as the ratio of slopes of the calibration curves (a minimum of five points), in one of the following ways[52,53]:

$$ME\ (\%) = \frac{MMC\ Slope - Solvent\ Calibration\ Curve\ Slope}{Solvent\ Calibration\ Curve\ Slope} \times 100$$

or

$$ME(\%) = \frac{Slope_{matrix-matched}}{Slope_{Solvent}} \times 100$$

In the first case, a negative ME will indicate signal suppression and a positive ME will mean signal enhancement. In the second case, ME values above 100 indicates signal enhancement and below 100 indicates signal suppression. Matrix effects can also be estimated at a particular concentration of an analyte, in which case the following equation is used:

$$ME(\%) = \left(\frac{Response_{Matrix}}{Response_{Solvent}} - 1\right) \times 100$$

Matrix effects can be enhancing or suppressive. Either way, the results are affected and strategies should be adopted to minimize, mitigate or correct for the matrix effects. Matrix effects can also be rotational or translational. Rotational matrix effect involves rotation of the matrix matched calibration curve from the solvent curve either clockwise (suppression) or anticlockwise (enhancement), as shown in Figure 1.7.

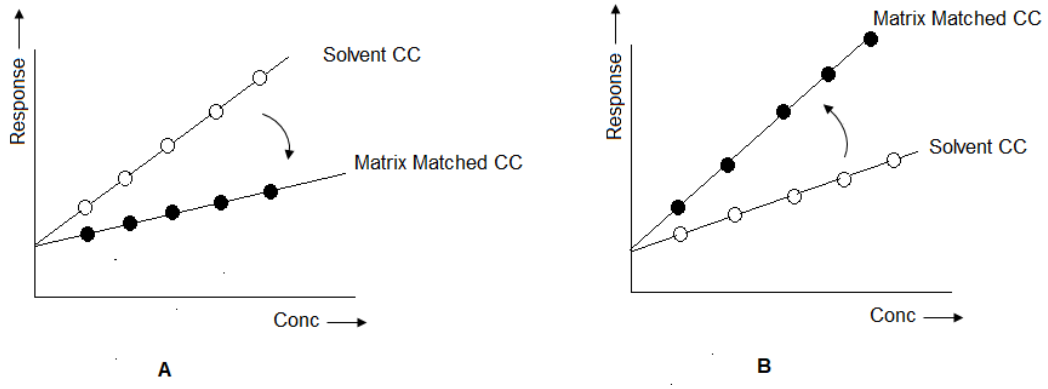


Figure 1.7. Rotational matrix effects: A – suppression, B – enhancement

These effects can be controlled or corrected for by using various methods that will be described below. The translational matrix effects involve the shifting of matrix matched calibration curve without changing its relative angle with the solvent calibration curve. These effects are much more difficult to correct.

Causes of matrix effects in LC-MS/MS

The most commonly used ionization mode used for pesticide residue analysis in LC-MS/MS is ESI[49], and the ME is usually suppressive, although enhancement is also rarely observed. It is very necessary to identify, estimate and correct this suppressive ME in LC-MS/MS as this will otherwise seriously affect the sensitivity of the method, even if highly sensitive instrumentation is used[54].

In LC-MS/MS, the compounds causing ME can have come from the matrix itself (endogenous), e.g. pigments, carbohydrates, lipids, peptides, metabolites of target analytes etc., or introduced into the extract during the sample preparation steps (exogenous), e.g. plastic and polymer residues, phthalates, organic acids, buffers etc[55]. This means that the ME will vary with the matrix and the sample preparation methods adopted. Owing to their higher concentration in the extract, the major contribution to ME will be from endogenous compounds. The ion suppression due to ME can have multiple causes[56,57], such as:

1. Competition between the matrix components and the analyte compounds for available protons in the ESI capillary (positive mode);
2. Competition between matrix components and analyte ions to reach the surface of the droplets in the ESI process[58];
3. Chemical reactions or other interactions between the analytes and matrix compounds[59,60].
4. Presence of mobile phase additives and buffers: The use of mobile phase additives like formic acid is known to improve the ionization process and thus the analyte

response. However, the presence of larger concentration of buffer in the mobile phase can result in suppressive ME[61].

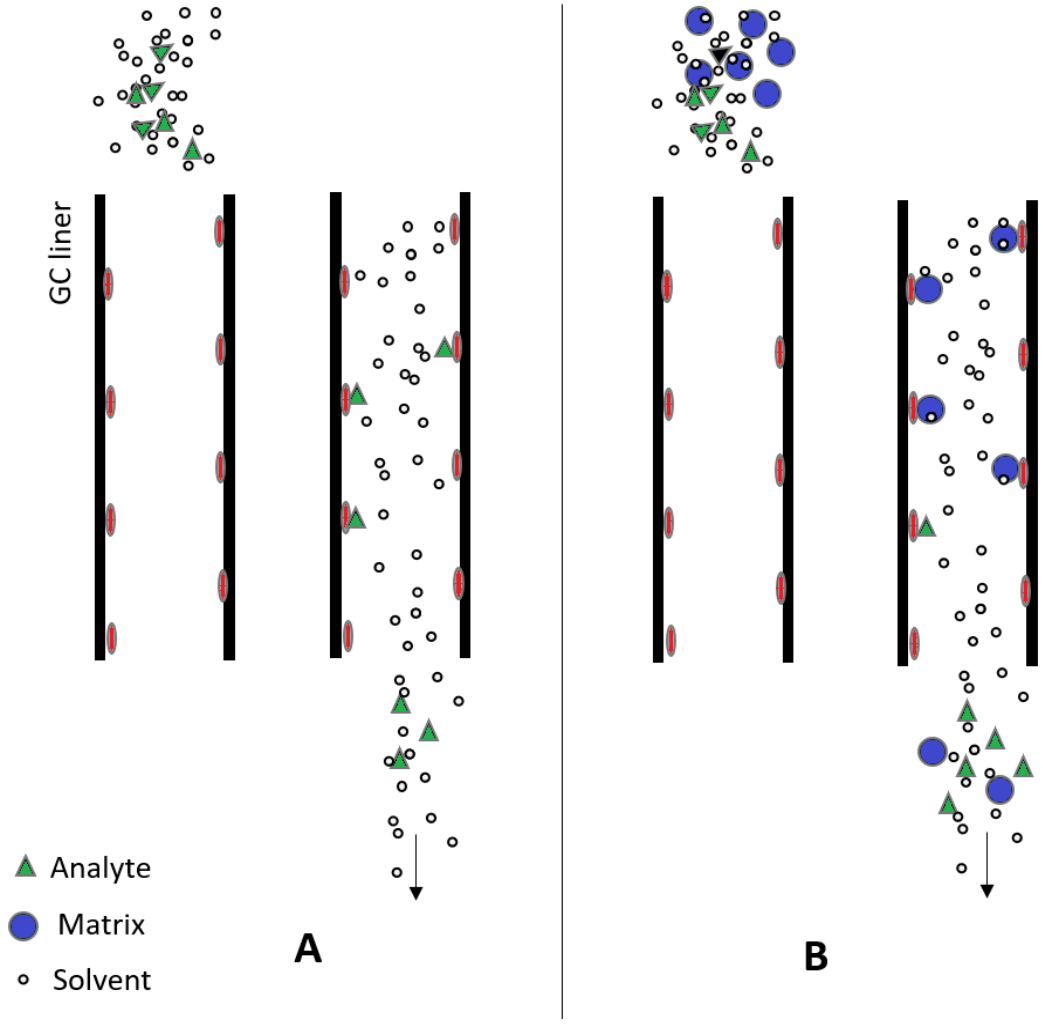
5. Design of the ESI probe: In ESI process, the analyte is ionized in the liquid phase as the effect of a potential applied, and results in the formation of charged droplets. It has been observed that the maximum number of ions formed in ESI is related to the surface area of all the droplets formed, and that this happens usually at an analyte ion concentration of 10^{-5}M. Thereafter the ionization is seen to level off and eventually decrease[55]. In the matrix extracts, as the coeluting compounds would be present in high concentrations and might have ionization properties comparable to the target analyte, this limit of analyte ion concentration is rapidly exceeded, thus suppressing the ionization of the target analyte.

Atmospheric Pressure Chemical Ionization (APCI), another ionization technique used in LC-MS/MS, has been demonstrated to have lower ion suppression because the ions are converted into gas phase before ionization occurs[54]. However, APCI is not extensively used in pesticide residue analysis.

Causes of matrix effects in GC-MS/MS

The presence of matrix effect in gas chromatography analysis was first pointed out, with a probable explanation, in 1993[51]. According to this theory, the free silanol groups in the GC injector, column and detector functioned as active sites to the analyte molecules, and adsorb them. When an analyte is injected in a solvent, the solvent molecules are not absorbed by these sites but the analyte molecules are. Thus, the number of analyte molecules reaching the detector is low. When an injection in GC is made for analyte molecules contained in a real, extracted matrix solution which has coextracted molecules from the matrix, a competition for the available active sites is set up between the analyte molecules and the molecules extracted from the matrix. As the matrix components will be

typically at a much higher concentration than the analyte molecules, most of the active sites are taken up by the molecules from the matrix. Consequently, a larger number of analyte molecules reach the detector resulting in a higher response. This is schematically shown in Figure 1.8.



A – Injection of the analyte in solvent, **B** – injection of analyte in a matrix containing interfering coextractives

Figure 1.8. Schematic representation of matrix effect arising in GC

In pesticide residue analysis, the matrix effect in GC is principally seen to be a composite of four factors[62,63]:

1. Chemical properties of the pesticides being analysed, like polarity, sensitivity to pH conditions and thermal stability;
2. Interference from coextracted matrix compounds, which depends on the nature and composition of the matrix being studied;
3. The number of active sites in the GC injection liner and column;
4. The concentration of the compounds in the solution being studied.

Correcting matrix effects: LC-MS/MS

The common strategies available in method development to minimize ion suppression are (a) fine tuning the extraction to minimize co-extractives; (b) optimizing cleanup to refine the extracts, and (c) optimizing chromatographic conditions to ensure full separation of analyte under study. However, in LC-MS/MS residue analysis the above three strategies are not pursued beyond a point, as the method might result in loss of analytes and / or become unwieldy in terms of time and effort. Hence in practice mostly two approaches are used to account for / mitigate ion suppression effects, viz. alternate calibration techniques and sample extract dilution.

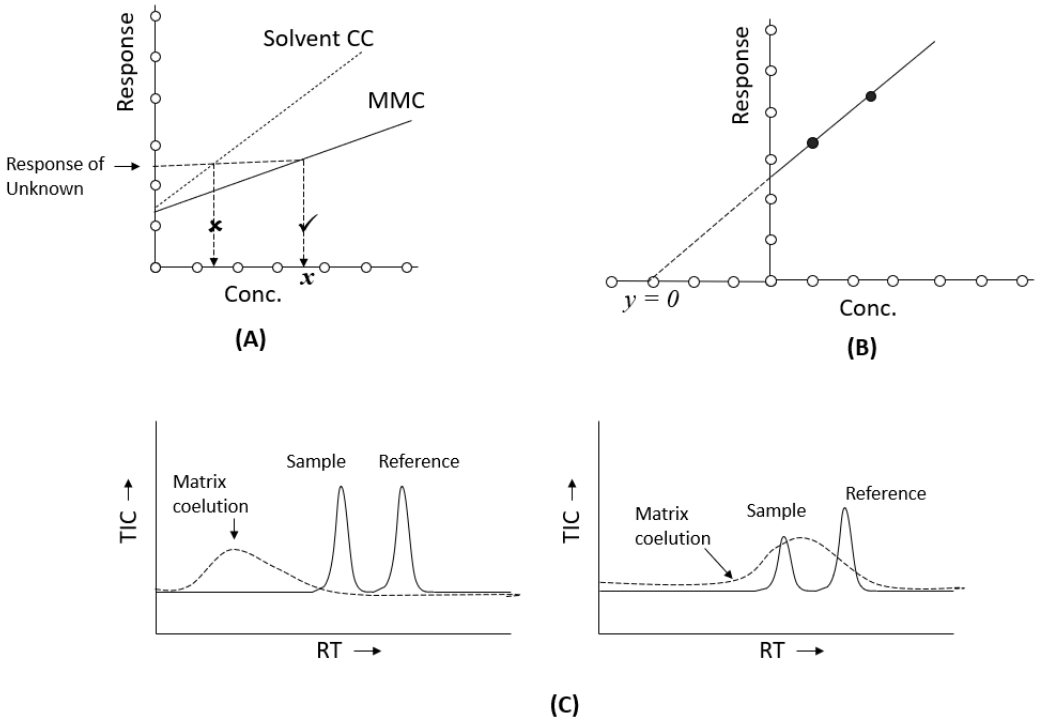
1. Matrix-matched calibration (MMC) is the most commonly used calibration method to account for matrix effects and is now a part of basic validation of the method. In this case, different concentrations of the analyte are prepared in extracts of blank matrix and used for quantification of samples (Figure 1.9A). The chief difficulty in this case is the availability of blank matrices. The blank matrix has to be sufficiently similar to the samples or they will not be able to compensate adequately for the effects posed by the matrix. In case of spices this becomes

especially difficult, as the composition of the matrix within the same spice can vary widely[54,58,64].

2. Standard addition (SA) is another calibration technique that can be used effectively for correcting ion suppression effects even in variable matrices. The technique involves spiking the unknown sample with at least two known concentrations, and plotting a graph using the areas from these spiked samples. The graph is then extrapolated to the negative side of X-axis to get the actual concertation of the analyte in the sample (Figure 1.9B). The chief drawback of this method is that it is time and effort intensive[65–67].
3. Internal standards (IS) work according to the concept that any ion suppression from the matrix will affect both the target analyte and the internal standard identically. Thus, the internal standard chosen should be as close as possible in structure and analytical behaviour to the target analyte(s). In the most commonly used IS procedure, a known volume of internal standard is added to each of the standard and sample (this can be pre- or post- extraction). The ratio of the responses of the analyte and the IS in the sample should be theoretically independent of the instrument sensitivity and other extraneous parameters, and thus can give accurate quantitation of the analyte. The best IS would be the isotopically labelled analyte(s); however, these are difficult to obtain and costly. When isotopically labelled IS are not available, the analyte itself can be used as an IS in what is known as the echo-peak technique. Here, two injections are made in the same HPLC run. First the unknown matrix sample is injected, and after a short time-lag of ~ 30 seconds, the standard solution is also injected. The result will be two well-resolved peaks of the analyte (in the sample and in the standard) eluting close to each other,

so that they undergo similar ME[68]. Thus, matrix effects are compensated (Fig. 1.9C).

4. Dilutions of the extract is a simple and straightforward method to decrease the coeluting matrix components which compete with the target analyte in the ionization process. The signal suppression of the analyte is also consequently reduced. The drawback of the method is that the analyte is also diluted and thus the limits of detection and quantification will increase. Thus, dilution will be most effective when used with high sensitivity instrumentation[69–71].



(A) Matrix-matched calibration; **(B)** Standard addition and extrapolation method; **(C)** Echo peak method – left: no matrix suppression, right: matrix suppression

Figure 1.9. Strategies for addressing matrix effects

Using mobile phase additives such as trifluroacetic acid (TFA) formic acid, acetic acid, ammonium formate, ammonium acetate etc have been known to decrease the consequences of ion suppression in LC-MS/MS by increasing the ionization efficiency[61]. It is typically recommended to use the least possible concentration of these additives to obtain optimum ionization.

Correcting matrix effects: GC-MS/MS

In GC-MS/MS analysis also, the most common method aimed at minimizing the matrix effect on analytical result is matrix matched calibrations[72,73]. Another important approach is the use of analyte protectants[74,75]. Other approaches include use of coated inlet liners in GC[76], the calculation of a 'matrix factor' to apply on the analytical results, use of diverse injection systems in GC[77], use of internal /isotopically labelled standards[78], more efficient cleanup strategies[79], priming of GC system[80], and applying specialized matrix matching using plant extracts[81].

In terms of time and effort, by far the simplest procedure used for minimizing the matrix effects in GC-MS/MS is matrix matched calibration, and hence this is the approach most widely used by analytical laboratories. The matrix enhancement produces larger and well-formed peaks; this means that this phenomenon can be made use of to increase the sensitivity of the method. In the case of matrix matched standards, the matrix components will shield the analytes from the active sites and preserve the response of the analyte. However, this protection capability varies among type and nature of the matrix. E.g., the matrix components should have similar polarity as the analytes so that they can be effectively extracted by the analytical method adopted. The key drawback in this method is that it requires an exactly matching matrix blank, i.e., a sample matrix which is uncontaminated by the target analytes, in order to prepare the calibration standards.

Use of analyte protectants

An alternative to matrix matched calibration in GC-MS/MS, when suitable blank matrices are not available, is the use of analyte protectants (APs). These are compounds added to all final analytical solutions (sample extracts, reference standards, quality control solutions etc) alike, which then would compete for, and block, the active sites in the GC, thus enhancing response for target analytes. It has been noted that in many cases where the appropriate method conditions are met, the use of AP can not only provide an efficient practical solution to ME in GC analysis, but can also increase the sensitivity by improving chromatographic response.

GC liners have been known to cause issues in analysis, like peak broadening and tailing, due to the presence of surface silanols, chemical impurities, ionic charges etc in the active sites on the liner surface which can interact with the analytes. Modern developments in deactivation processes have introduced improvements in the GC system which has greatly increased the inertness of the surfaces, resulting in very few active sites. However, with prolonged use, non-volatile material condenses on these surfaces, and introduces active sites. Prolonged use and exposure to analytical conditions in the GC also lead to loss of effectiveness of the deactivation process. The newly exposed active sites on the surfaces in GC can then take part in various interactions with the analyte like hydrogen bonding, charge interactions, catalysis, degradation of analyte and even partial covalent bonding. This results in increase of ME in GC.

ME in GC is principally observed in the case of analytes that can interact well with the active sites. Stable analytes like hydrocarbons, organohalogen compounds etc interact less with the active sites. However, compounds containing O, N, P, S etc. tend to interact strongly with the active sites, thus causing ME[82,83]. The use of an additive to 'shield' the active sites from the analyte, thereby reducing ME, was first reported in 1993[84]. This

additive was termed as a 'masking agent'. However, at this time the approach was not considered effective for routine use and the main method for addressing ME in GC was still matrix matched calibrations[85]. Later, the developers of the QuEChERS method also successfully demonstrated the use of masking agents in GC analysis[35,86], which they termed Analyte Protectants. They reported that the peak shapes were remarkably improved in GC-MS analysis of QuEChERS extracts of sugary aromatic fruits like apple. for organophosphorus pesticides. So, by adding a similarly volatile sugar derivative to all calibration standards and samples alike, they could obtain excellent recoveries, without using matrix matched standards. In a study of 93 such additives, they found that sugar like compounds containing multiple hydroxyl groups lead to the highest signal enhancements for relatively polar pesticides. It was also found that APs could be used even when matrix matched calibration solutions were used, which gave better sensitivity, narrower and better-shaped peaks and lower detection limits.

Residue analytical methods in spices

The Codex Committee on Spices and Culinary Herbs (CCSCH) classifies spices into 6 classes, viz. dried fruits and berries (e.g., chillies, black pepper), dried roots and rhizomes (e.g., turmeric, ginger), dried seeds (e.g., cumin, fennel), dried floral parts (e.g., mace, saffron), dried bark (e.g., cinnamon, cassia) and dried leaves (e.g., basil, oregano)[87]. Owing to the difficulty posed by most spice matrices due to their diverse chemical nature and possibility of high amount of matrix coextractives, classical methods for residue analysis did not work well in spices[88].

In classical methods, the extent of matrix coextractives nearly always necessitated an extra cleanup step before instrumental analysis, which was usually carried out by solid phase extraction (SPE) or gel permeation chromatography (GPC). Analysis of 170 pesticides in the Chinese spice ginseng has been reported with GPC cleanup and GC-HR-

TOFMS[89]. Using SPE cleanup, analysis of 16 pesticides in black and white pepper using GC-ECD has been reported[90].

Some recent alternative methods which have been reported as successful in spices involve Matrix solid-phase dispersion (MSPD) and dispersive liquid-liquid microextraction (DLLME). MSPD is a sample preparation method in which the sorbents used in SPE are used as grinding aids for sample homogenization. In this technique, the sample with grinding aid is crushed with a mortar and pestle and the mixture is transferred to an SPE column for further analysis. MSPD has the advantages of less chemical consumption and ease of use, but being a manual technique, issues with precision using this technique has been reported. The method has been reported for 163 pesticides in 6 herbs using GC with conventional detectors[91]. Analysis of dried herbs for 10 organophosphorus pesticides has been successfully carried out using DLLME sample preparation and GC-MS[92].

The use of QuEChERS sample preparation in spices has also seen some successes. In capsicum, analysis of 38 pesticides using LC-MS/MS with a limit of quantification (LOQ) of 0.05 mg/kg has been achieved[53]. In cardamom, high sensitivity analysis of residues using both LC-MS/MS[93] and GC-MS/MS[94] have been reported. Good recoveries for pesticides in cumin matrix, with LOQ of 0.01 mg/kg, were achieved using LC-MS/MS[95] and GC-MS/MS[96]. In the herbs chamomile, thyme and marjoram, analysis of 160 pesticides using GC-MS/MS with LOQ ranging from 10-250 μg/kg has been reported[97]. Similar results have been obtained in cinnamon bark using LC-MS/MS[98].

Analysis of dithiocarbamate residues

Dithiocarbamates (DTC) are broad-spectrum antifungal agents, with comparatively low toxicity profiles and low production costs which have led to their widespread application in the control of fungal diseases in plants, especially in

combination with new systemic antifungal agents[99,100]. These compounds are non-systemic, and due to their insolubility in water, are likely to remain at the site of application without much dissipation into the environment. As a consequence of this, the risk of indiscriminate use of this class of fungicides leading to residues in agriculture products above regulatory limits is an important concern.

For regulatory purposes, DTC fungicides are considered together as a group, for which a combined maximum residue limit is assigned. One of the major techniques employed for analysis of DTC residues is converting the DTCs present in the sample quantitatively into carbon disulphide (CS_2), and then analysing the CS_2 evolved using techniques like spectrophotometry[101,102], gas chromatography[100,103–106] and liquid chromatography[107,108]. Alternative approaches have been developed which involve a methylation step, followed by modified QuEChERS extraction and detection of the methylated compounds using LC-MS/MS[109,110]. These methods are able to distinguish between different groups of DTC compounds, but are still limited by the solubility constraints of individual DTC compounds in general. Validation of an analytical method for DTC residues in spices has not been reported until now, and this is addressed in the present work.

Validation of pesticide residue analysis methods

Method validation is typically defined as a set of assessments carried out using the analytical method being validated, in order to ensure that the method is fit for its intended purpose and is capable of providing reliable analytical data[111]. In this work, the various analytical methods developed were validated as per the EU SANTE/12682/2019 document[112]. This ensured that the methods would meet international requirements as well as would be able to produce results that can be reliably used for verifying regulatory compliance of samples.

Scope and objectives of the present investigation

The use of spices to lend flavour, aroma and colour to food is an important and indispensable part of world-wide cuisine. This ubiquitous use of spices makes the presence of pesticide residues in spices a major food safety risk. Spices are generally considered as difficult matrices to analyse for pesticide residues present in trace levels. Some pesticides (e.g., thermally labile, less volatile) are more efficiently analysed using liquid chromatography, whereas other pesticides (thermally stable and volatile) are analysed better by gas chromatography.

Part I of this thesis documents the development and validation of an optimized workflow for high efficiency trace level determination of different groups of pesticides in various categories of spices using UPLC-MS/MS and GC-MS/MS, capable of assessing compliance with modern international maximum residue regulations. The analytical methods developed are based on the QuEChERS sample preparation technique, optimized individually for different classes of spices. The methods are validated as per internationally accepted protocols and acceptance criteria.

Dithiocarbamate fungicides are a separate class of plant protection agents which cannot be analysed as per protocols described above, due to their insolubility in solvents used in QuEChERS methods. A method for assessing total dithiocarbamate residues by converting the compounds to CS_2 is validated for the first time in spices. The developed method is employed for compliance evaluation of real samples against regulatory requirements.

Matrix effects (ME) is an important issue arising in chromatographic and mass spectrometric analyses at trace level concentrations, which adversely affect performance of analytical methods. The ME posed by different categories of spices for LC-MS/MS and GC-MS/MS is assessed. Usually, to address these ME in routine analysis, matrix matched

calibrations (MMC) are used, which is hampered by the difficulty in obtaining suitable blank matrices for preparation of the calibration standards. Two novel strategies are evaluated as alternative to MMC in analysis of spices, *viz.* use of analyte protectants in GC-MS/MS, and use of surrogate matrix compounds in LC-MS/MS.

CHAPTER 2

MATERIALS AND METHODS

This chapter describes the chemicals, reagents, certified reference materials (CRMs), sample preparation techniques, instrumentation methods, optimization schemes and acceptance criteria employed in developing and validating high sensitivity pesticide residue analysis methods in spices. The method validation protocols employed are also explained in this chapter.

Materials

The mass spectrometry grade solvents used for mobile phase preparation in ultra-high performance liquid chromatography (UPLC), *viz.* methanol and acetonitrile, were obtained from Biosolv, USA. The QuEChERS chemicals, principally primary secondary amine (PSA), graphitized carbon black (GCB), and C-18 bulk sorbent were procured from Agilent, India. All other analytical grade chemicals like isooctane, acetic acid, formic acid, sodium chloride, anhydrous magnesium sulphate, ammonium formate, formic acid, sodium citrate dibasic trihydrate, sodium citrate dibasic sesquihydrate etc. were procured from Merck, India. Analyte protectants for GC-MS/MS, *viz.* ethyl glycerol, shikimic acid, sorbitol and d-gluconolactone, and N-vanillyl nonanamide (NVNA) for surrogate matrix experiments in chillies, were purchased from Sigma Aldrich India. All pesticide residue certified reference materials (CRMs) were procured from Dr. Erhenstorfer, Germany. Carrier gas for GC was 99.9995% pure helium obtained from Bhuruka gases, India.

Instrumentation

A 3-digit precision balance (Sartorius BSA223S) was used for weighing all samples for analysis. For reference standard preparations a 5-digit precision balance (Shimadzu AUW220D) was used. Homogenization was carried out in all spices using a

kitchen blender. Certified reference material and stock standards were stored in a -20°C freezer (Remi RQV-300 plus), and intermediate standards were stored at 4°C in a low temperature cabinet (Remi CC-19 plus). Centrifuges for sample preparation with two speeds were used, *viz.* 5000 rpm (Remi CM-8 plus) and 10,000 rpm (Remi C-24 plus). Vortex shaker used was Remi CM-101. For concentration of extracts, a nitrogen-based evaporator from PCI Analytics (N_2 Fastvap) with a Peak nitrogen generator was used. For detection and quantification of analytes, Agilent GC-MS/MS (7890 GC / 7000 C MS) and Waters UPLC-MS/MS (Xevo TQS Micro) were used. For determination of pungency and extractable colour of chillies in surrogate matrix studies, a Shimadzu Prominence HPLC with diode array detector, and a Hitachi UV-VIS spectrophotometer were used.

Preparation of reference standard solutions

All the CRMs procured had certified purity > 95%. The CRMs were first divided into two sets, i.e., those for UPLC-MS/MS analysis and those for GC-MS/MS analysis. The individual pesticide standard stock solutions of 1000 mg L^{-1} of all the CRMs in each set were prepared in acetonitrile or methanol, based on the solubility of the respective compounds. For each set, the intermediate mixed standard at 10 mg L^{-1} was then prepared in acetonitrile and stored at -20°C until analysis. Working solutions and calibration standards of the mixed standard were prepared daily by appropriate serial dilutions.

Sample Selection and homogenization

All the spice samples used in the method development studies were obtained from local markets in dried, whole form, except curry leaves which were obtained fresh and sun-dried to constant weight. All spices had moisture content in the range 7 – 10%. Homogenization of different spices were carried out to simulate their forms in typical culinary usage. The details are given in Table 2.1 below. In all cases, the homogenization was performed using a kitchen blender immediately before commencing experiments. For

spices requiring crushing, the process was continued until the spice matrix was thoroughly broken up to facilitate efficient extraction. For spices requiring grinding, the samples were ground to fine powder and sieved through ASTM 20 (850 μm) mesh before analysis.

Table 1.2 Representative spice matrices with modes of homogenization

Category of spice	Representative matrix	Homogenization
Dried fruits with low pigment content	Cardamom	Crushing
Dried fruits with high pigment content	Chillies	Grinding
Dried roots / rhizomes	Ginger	Crushing
Dried seeds	Cumin	Grinding
Dried leaves	Curry leaves	Crushing
Dried bark	Cinnamon	Grinding

During the method development phase, the spice samples were homogenized and screened using unoptimized sample preparation protocols and instrumental methods. In each spice, the samples which showed absence of the target pesticides were isolated and treated as blanks to be used for the matrix interference evaluation studies and matrix-matched calibrations.

General scheme for method development and optimization

As the diverse groups of spice matrices studied had different nature and properties, analytical methods developed for different spices often needed to be specifically optimized. There were two aspects of method development, *viz.* (a) the sample preparation, which involves extraction of the matrix with suitable solvents followed by cleaning or refining the extracts, and (b) instrumental analysis involving multiple reaction monitoring (MRM) transitions using LC-MS/MS and GC-MS/MS techniques. As pesticides show varying sensitivity in GC and LC analyses, both techniques had to be used for analysis, with different sets of pesticides standardized for each technique. The analytical methods

for pesticides were developed, optimized and validated as per the general scheme given in Figure 1.10.

The sample preparation part of method development included homogenization, optimization of sample weight, moisture content, extraction step, cleanup step and concentration / reconstitution step. The cleanup step had to be optimized separately for both GC and LC analyses for each spice, as the chemistry and mode of action of matrix interference in either technique differ. The concentration and reconstitution of cleaned up extract was required to increase sensitivity of many of the analytes.

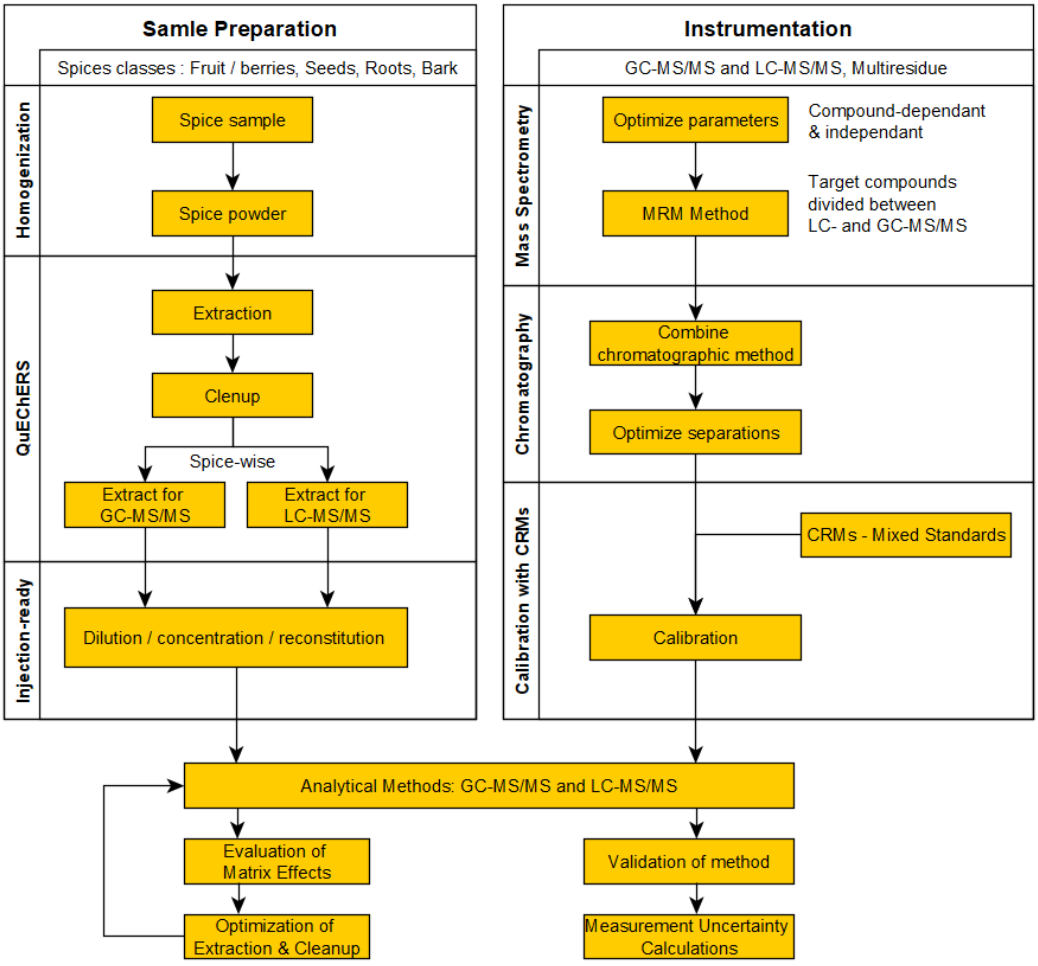


Figure 1.10 General Scheme for method development and optimization

The instrumentation method development involved two parts, (a) chromatographic method and (b) mass spectrometric method. Chromatography was optimized to obtain

good separation and peak shapes for all the analytes. Mass spectrometric method development involved optimization of general parameters in the mass spectrometer and the multiple reaction monitoring (MRM) transitions specific to the analytes under consideration. In both GC-MS/MS and LC-MS/MS, two MRM transitions per analyte were used, the transition with highest response and specificity was taken as the quantifying transition and another transition as the qualifying transition. In all cases, linearity of instrument was assessed using solvent and matrix matched calibration standards, and matrix effects were ascertained. Wherever matrix effects were found significant, matrix matched calibration was used for quantitative analysis.

QuEChERS Sample preparation

Sample preparation in general consisted of extracting the homogenized spice sample in acetonitrile, in the presence of 4 g $MgSO_4$ and 1 g NaCl using a vortex mixer. This was then centrifuged at 5000 rpm, and an aliquot of the supernatant solution was taken for the cleanup step. The QuEChERS cleanup reagents were then added, mixed thoroughly on a vortex shaker, and centrifuged at 10,000 rpm. The supernatant solution was then concentrated and reconstituted as necessary, filtered through a nylon-66 membrane and analysed on a GC-MS/MS or LC-MS/MS with instrumental conditions optimized for residue analysis.

The sample preparation procedures were optimized for different classes of spices separately for UPLC-MS/MS and GC-MS/MS and will be described in Chapters 3 and 4. The initial parameters optimized were sample weight and matrix hydration. Spiked samples were analysed with varying sample weights, moisture content and soaking times. Acetonitrile was used as the extraction solvent, along with 4 g $MgSO_4$ and 1 g NaCl, and sample-solvent ratios were also optimized. Extraction was performed with and without buffering. For buffering, 1 g of sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7.2H_2O$) and

0.5 g of sodium citrate dibasic sesquihydrate ($C_6H_5Na_2O_7.1.5H_2O$) were added along with $MgSO_4$ and NaCl. For the cleanup step, different combinations of four QuEChERS chemicals were used, *viz.* $MgSO_4$, C-18 bulk sorbent, PSA and GCB. Combinations that gave best recoveries for different classes of spices were taken as the optimal cleanup combinations for the respective class of spices.

Method parameters: GC-MS/MS and LC-MS/MS

For general residue analysis in GC-MS/MS, split-less injection was used for the analysis. The GC temperature program was adjusted to obtain optimal separation of analytes in the chromatogram. Electron impact (EI) at 70 eV was used for ionization, and dynamic multiple reaction monitoring (D-MRM) was employed for quantification, where MRM segments for the analytes were set based on retention time (RT) of the analytes. Multiple transitions from the Agilent Methods Library were chosen for the same compound in the initial screening runs, and two MRM transitions with good response, peak shape and low matrix interference were finally selected for each analyte to function as the quantifier (higher response transition) and qualifier.

In LC-MS/MS, electrospray Ionization (ESI) was used in conjunction with segmented MRM for quantification. The compound independent parameters like capillary voltage, desolvation temperature, source gas and cone gas flows were first adjusted to get good spray and optimum ionization. Multiple MRM transitions from the Waters QuantPedia® library were selected for the screening runs, and two MRM transitions with good response, peak shape and low matrix interference were finally selected for each analyte to function as the quantifier (higher response transition) and qualifier.

Five-point calibrations were performed for each analyte for routine quantification runs. A typical routine analysis batch began with a solvent blank and a matrix blank, followed by the test samples. In every analysis batch, a recovery sample spiked with the

analytes in the range 0.01 to 0.1 mg kg^{-1} was included prior to test samples as a quality control (QC) check, and a reference standard in the concentration range of 0.01 to 0.1 µg mL^{-1} was included after every ten test samples to verify stability of response.

Preparation of analyte protectant solution for GC-MS/MS

In GC-MS/MS analysis, the use of analyte protectants as an alternative to matrix matched calibrations was investigated. Stock solutions with concentration of 50 mg ml^{-1} of sorbitol, gluconolactone and shikimic acid were first prepared in 60:40 acetonitrile water mixture. The analyte protectant mixture was then prepared by mixing 2 g of ethylene glycerol, 2 ml of gluconolactone stock solution and 1 ml each of sorbitol and shikimic acid stock solutions in a 10 ml volumetric flask, then making up the solution with 60:40 acetonitrile water mixture.

Analysis of pungency and colour in chillies for surrogate matrix studies

In LC-MS/MS, the use of synthetic compounds as matrix surrogates were evaluated as an alternative to matrix matched calibration using chilli as a representative spice matrix, and for this chilli samples with a wide range of pungency and colour were required. Blank samples of chilli-pepper with varying pungency and colour were obtained from local supermarkets in Kochi, India and tested for pungency and colour using American Spice Trade Association (ASTA) methods 21.3 and 20.1 respectively[113(p3),114].

For pungency analysis, 25 g of powdered chilli sample was refluxed with 200 ml rectified spirit for five hours, allowed to cool, filtered and injected in an HPLC with a C-18 reverse phase column. Detection of capsaisinoids was performed at 280 nm in a diode array detector. Identification of the capsaicinoid compounds based on relative retention time and quantification were performed against an injection of 100 mg kg^{-1} NVNA, and total capsaisinoids were calculated in scoville heat units (SHU)[113]. For extractable colour, about 0.1 g of ground chilli sample was extracted with 100 ml acetone for 16 hours at room

temperature in the dark, and filtered. The absorbance of the extract at 465 nm was determined on a UV-VIS spectrophotometer, and the extractable colour, in ASTA colour units, was calculated[114].

Sample preparation for dithiocarbamate (DTC) analysis

DTC analysis method was developed and optimized for two spices, *viz.* cardamom and black pepper. Analysis of DTC was done by converting all the dithiocarbamates present in the sample to carbon disulphide (CS_2). The hydrolysis reagent used for this purpose was prepared by dissolving 75 g of $SnCl_2$ in 5 L of 4N HCl.

About 25 g sample of the spices was accurately weighed into a 250 ml stoppered glass bottle. The sample (for both whole and crushed forms) was soaked in 50 ml water for 30 minutes. Then, 50 ml isooctane was added, followed by 75 ml of the hydrolysis reagent. The bottle was stoppered and transferred into a covered water bath maintained at 80°C, with shaking at intervals of 1 minute, for 1 hour. The bottle was then immediately transferred to an ice bath, and 2 ml of the supernatant isooctane layer was pipetted out and centrifuged at 5000 rpm for 5 minutes. From the centrifugate, 1 ml of the upper layer was pipetted into a GC autosampler vial, from which 2 µl was injected in the GC-MS system. In order to avoid interference from plastic surfaces, powder free nitrile gloves were used by the analysts, and for volume transfers during analysis, only glass apparatus were used.

Instrumentation for DTC analysis

GC-MS operating in electron ionization (EI) and selected ion monitoring (SIM) modes was used for DTC analysis. Ultrapure helium was used as carrier gas. Injection in split mode was optimized for best response of CS_2 in GC-MS. A temperature gradient program giving good response and peak shape for CS_2, followed by a post-run program which contained a mid-column back flush, were also optimized so as to obtain acceptable accuracy and precision.

A typical routine analysis batch in DTC analysis began with a solvent (isooctane) blank, a reagent blank and a matrix blank, followed by the test samples. In every analysis batch, a recovery sample spiked in the range 0.05 to 0.1 mg kg^{-1} was included prior to test samples as a QC check, and a reference standard in the concentration range of 0.025 to 0.1 µg mL^{-1} was included after every ten test samples to verify stability of response.

Method validation

Within-laboratory method validation was undertaken to ensure that the analytical method developed and optimized was fit for its intended purpose (e.g., assessing compliance of a sample against regulatory limits).

Table 1.3 Acceptable performance criteria for analytical methods

Parameter	**Measured as**	**Performance criterion**
Linearity	From a calibration curve of 5 levels, deviation of calculated concentration from true concentration	≤ ± 20 %
Recovery	Average recovery of each spike level analysed, with $n \geq 5$	70-120 %
Repeatability Precision (RSD_r)	Relative standard deviation of each spike level analysed (same analyst, same day, $n \geq 5$)	≤ 20 %
Within-laboratory reproducibility precision (RSD_R)	Relative standard deviation of 3 replicates of each spike level performed on 3 non-consecutive days (different analysts, $n = 9$).	≤ 20 %
Specificity	Response in reagent blank and blank control samples in the same MRM and at the same retention time as the analyte.	≤ 30 % of LOQ
Ruggedness	Relative standard deviation for results obtained from five combinations of three parameters chosen as variables in the optimized method	≤ 20 %
Ion ratio	Quantifier: qualifier ratio in the sample matrix as compared to average of the ion ratios of calibration standards in the same batch	±30%
Retention time (min)	For the quantifying MRM transition, the retention time of the peak in the sample chromatogram as compared to the peak in the standard chromatogram	± 0.1

Method validation data generated was supported by performance verification and analytical quality control (AQC) checks during experimental runs. This section outlines the procedures followed for (a) validation of analytical methods used for pesticide residue

analysis of spices and spice products by GC-MS/MS and LC-MS/MS techniques, and (b) performing AQC checks during experimental runs subsequent to validation of the methods. The acceptable performance criteria for the different validation parameters are summarized in Table 1.3 above. The methods for calculating the validation parameters are summarized below.

Linearity

Linearity of instrument response was assessed by preparing the calibration curve. The lowest calibration level was chosen to be equal to or lower than the default regulatory limit (typically 0.01 mg kg^{-1}) for which the method was intended to be used. A five-level calibration was used in all linearity studies. A linear calibration function without forcing inclusion of the origin was chosen, with the stipulation that the regression coefficient R^2 obtained was at least 0.9 or higher. The linearity calculations were accepted when the deviation of the back-calculated concentrations of the calibration standards from the true concentrations was not be more than ± 20% for each analyte.

Matrix effect

Spices pose significant matrix effects (MEs) in both GC-MS/MS and LC-MS/MS analysis. These effects arise from the difference in behaviour of the target analytes in the matrix extract as compared to that in the solvent. ME is usually suppressive in LC-MS/MS and enhancing in GC-MS/MS. For assessing ME, one of the following two approaches were followed:

1. In studies undertaken to mitigate the extent of ME at a particular analyte concentration, the same concentration of the analyte was prepared in the solvent as well as the extract from a blank sample, and injected in the GC-MS/MS or LC-MS/MS. ME was then calculated for each analyte as per the following equation:

$$ME(\%) = \left(\frac{R_M}{R_S} - 1\right) \times 100,$$

where R_M and R_S are the responses for a particular concentration of pesticide in the matrix extract and solvent respectively. ME was considered significant if the value was ± 20% or more.

2. In studies to ascertain overall ME posed by a spice towards an analyte, solvent-only and matrix-matched calibration curves were set up and the slopes of the curves were compared. In this case, the ME could be calculated using one of the following equations:

$$ME\ (\%) = \left(\frac{S_m - S_s}{S_s}\right) \times 100$$

or

$$ME(\%) = \frac{S_m}{S_s} \times 100$$

where S_m is the slope of the matrix matched calibration curve, and S_s is the slope of the solvent-only calibration curve.

In the first way of expressing ME, a negative value of ME indicated signal suppression, and a positive value indicated signal enhancement. In second way of expressing ME, a value of less than 100 indicated signal suppression and greater than 100 indicated signal enhancement.

Accuracy and precision

Accuracy was assessed in terms of the recovery from spiked blank samples. Recovery (%) was assessed by spiking each analyte at two levels into blank a matrix. The levels were typically (a) at the limit of quantification (LOQ) and (b) 2-10 times LOQ of the method. A minimum of five replicates of each of the two spike levels were analysed.

The recovery (%) for each experiment was then calculated as $\frac{C_x}{C_s} \times 100$, where C_x is the calculated concentration from the analysis and C_s is the spiked concentration.

Subsequently, the average recovery was calculated at each spike level. Precision was calculated in terms of relative standard deviation (RSD), in two stages, *viz*. repeatability and reproducibility. Repeatability (RSD_r) or intra-day precision was calculated as the RSD from results of five replicates of each spike level, performed on the same day. Reproducibility (RSD_R) or inter-day precision, was determined by RSD of 3 replicates of each spike level performed on 3 non-consecutive days ($n = 9$).

Limit of Quantification

Although there are various methods for determining the limit of quantification (LOQ) based on slope of calibration curves, signal-to-noise ratios etc, in the present study, DG SANTE guidelines were followed[112]. Thus, LOQ was taken as the lowest spiked level which satisfied all the acceptable performance criteria of validation parameters. For each analyte and matrix, the lowest spiked level giving average recovery ($n = 5$) in the range 70-120% with an associated intra-laboratory repeatability of $RSD_r < 20\%$ was taken as the LOQ.

Specificity

To assess the specificity of the method to an analyte the response in the reagent blank and control sample for the quantifying MRM transition at the retention time of the compound were compared with the response of the analyte at the LOQ level in the blank matrix. Specificity is calculated as $\frac{R_b}{R_{LOQ}} \times 100$, where R_b is the response of the analyte in the reagent blank or the control sample, and R_{LOQ} is the response of the analyte spiked at the LOQ level in the matrix.

Ruggedness

The ruggedness (also called robustness) of an analytical method is defined as the resistance to change in the results produced by the analytical method when minor deviations are made from the experimental conditions described in the validated procedure. To assess ruggedness, three different variables were chosen covering extraction and instrumentation process, and five combinations of these variables fixed for analysing blank samples spiked at the same analyte concentration, in the range LOQ to 5 times LOQ. The RSD of the results obtained were calculated to assess the ruggedness of the method.

Evaluation of measurement uncertainty

Evaluation of measurement uncertainty is one of the most important requirements in method development of trace analyses, and is typically defined as the dispersion of values that can be reasonably attributed to the measurand. Measurement uncertainty was individually calculated for all analytes in GC-MS/MS and LC-MS/MS analyses. In each case, contributing factors to overall measurement uncertainty were assessed and a cause-effect diagram was constructed, including aspects from standard purity, weighing of standards, volume measurements, accuracy (recovery) and precision (repeatability). Each significant contributing factor was then labelled as Type-A (data form a series of observations) or Type-B (all other data) uncertainties. For Type-B uncertainties, rectangular distribution was assumed in all cases. The standard and relative uncertainties were then calculated, and then combined together to obtain the combined uncertainty. From this value the expanded uncertainty was then calculated by multiplying with a coverage factor k (typically taken as $k = 2$, for 95% confidence limit).

CHAPTER 3

RESIDUE ANALYSIS IN SPICES BY UPLC-MS/MS

Development and validation of high sensitivity, multiresidue analysis in representative matrices chosen from different categories of spices, using ultra-high performance liquid chromatography and tandem mass spectrometry (UPLC-MS/MS) is documented in this chapter. Sample homogenization, extraction, cleanup and instrumental analysis of residues of 53 LC-amenable pesticides that are commonly applied in spice cultivation, were optimized and validated for six spices, *viz.* cardamom, chillies, ginger, cumin, curry leaves and cinnamon.

Liquid chromatographic and mass spectrometric conditions were tuned to obtain desired high sensitivity responses for the target analytes with multiple reaction monitoring (MRM) detection. MRM transitions for each analyte which showed good response, peak shapes and low matrix interference were identified and used for quantification. Starting from a general QuEChERS sample preparation profile as explained in Figure 1.10, specific schemes were devised to suit the different classes of spices by using different combinations of QuEChERS cleanup reagents and identifying the combination that gave best recoveries in each selected matrix.

The matrix effects (MEs) posed by different classes of spices in UPLC-MS/MS were evaluated and addressed. An integrated methodology for high sensitivity multiresidue analysis of the LC-amenable pesticides for the six spices, using specifically optimized sample preparation scheme followed by UPLC-MS/MS analysis, was developed. Validation of this analytical scheme was conducted as per SANTE Guidelines[112]. Measurement uncertainty was calculated for all target analytes at the limit of quantification level (LOQ) level.

General analytical scheme and establishment of blanks

As there is considerable difference in nature and composition of spices from different groups, it is clear that the analytical methods had to be tailored and optimized to suit the different groups of spices. The general procedure followed was as follows:

(a) The liquid chromatographic and mass spectrometric parameters were optimized for the 53 analytes under consideration to obtain good separation and response for all compounds.

(b) Spice samples belonging to each category were screened using a basic unoptimized QuEChERS sample preparation method and the optimized UPLC-MS/MS method above. Samples which were free from incidence of pesticides under consideration were selected as blanks for extraction / cleanup optimization and later ME studies.

(c) The extraction and cleanup steps of the QuEChERS were then optimized for each spice matrix. For this, various combinations of extraction and cleanup reagents were studied. The combination of reagents that gave best recovery and precision results were taken as the optimized sample preparation method for each respective matrix.

(d) Using the optimized sample preparation method, extracts were prepared from blank samples of each spice matrix. These extracts were gravimetrically analysed to understand matrix load which indicated the extent of matrix interferences. ME was then assessed by comparing slopes of solvent-only and matrix-matched calibration curves.

(e) Using the optimized sample preparation and chromatographic methods, method validation was conducted for all spice matrices and fitness for intended purpose was assessed as per the acceptance criteria summarized in Table 1.3.

(f) Measurement uncertainty at the established limit of quantification (LOQ) was calculated from the validation data in a representative spice matrix, cumin, for all 53 analytes.

UPLC-MS/MS method optimization

For the UPLC mobile phase, two solvent systems were considered, viz. an acetonitrile-water system and a methanol-water system. In either case, an elution profile with gradient curve no. 6 starting with high aqueous concentration (98:2), passing through high organic concentration (1:99) and returning to the starting composition was found to give good separation of analytes on the C-18 column. This profile was then combined with a buffer system, viz. 5 mM ammonium formate / 0.1% formic acid. In all, four combinations of UPLC mobile phases were assessed: acetonitrile - water system with and without buffer, and methanol – water system with and without buffer.

Table 1.4 Optimized UPLC-MS/MS method parameters

Instrumentation	**Parameters**
UPLC	
Column	Waters XBridge® BEH C-18 2.5mm, 2.1x100mm
Mobile Phase	A: Water with 5mM ammonium formate and 0.1% formic acid B: methanol with 5mM ammonium formate and 0.1% formic acid Flow 0.5 ml/min Gradient: Initial A:B 98:2, 5 min A:B 50:50 curve 6, 7 min A:B 40:60 curve 6, 11 min A:b 25:75 curve 6, 14 min A:b 1:99 curve 6, 17 min A:B 98:2 curve 6. Total runtime 21 min.
MS/MS	
Capillary voltage	0.6 kV
Desolvation temp.	600°C
Source gas	1100 L/hr
Cone gas	50 L/hr

Of the four combinations of mobile phase studied, methanol-water composition was in general seen to be better than acetonitrile-water composition in obtaining good peak shape and resolution. It was also observed that the use of buffers improved the response

and peak shapes in general. Thus, methanol-water mobile phase containing ammonium formate / formic acid (5 mM / 0.1%) buffer was finalized as the mobile phase. The detailed mobile phase gradient profile is given in Table 1.4 above. The optimized chromatogram for 53 pesticides at 0.01 mg L^{-1} is shown in Figure 1.11.

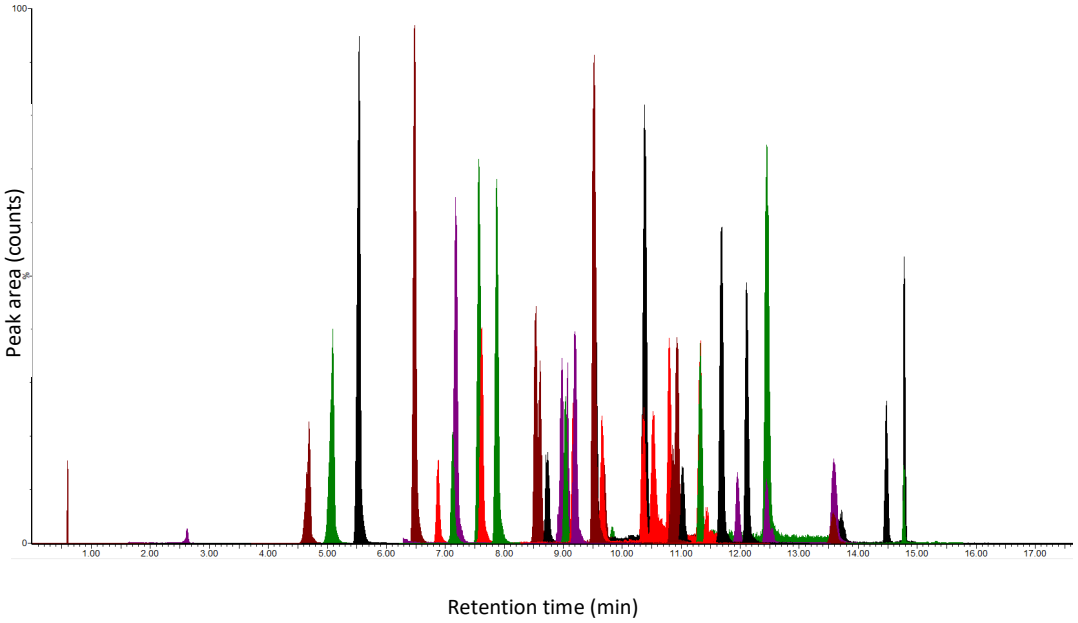


Figure 1.11 Optimized UPLC-EI-MS/MS chromatogram

As electrospray ionisation (ESI) was used for analysis, optimization of mass spectrometric conditions centred around two sets of parameters, *viz.* the compound-independent parameters which included capillary voltage, desolvation temperature, source gas flow and cone gas flow, and the compound-dependent parameters which included collision energy and cone voltage. Optimizing the compound-independent parameters was required to obtain consistent ionization of the analyte molecules and a stable spray. The optimized values of these parameters are given in Table 1.4. Two MRM transitions were used to monitor each analyte, with the transition having the higher response used for quantification, and the other transition being used as the qualifier. The compound dependant parameters were optimized individually for each MRM transition. Figure 1.12

shows the points of application of these parameters along the ion-path of the mass spectrometer.

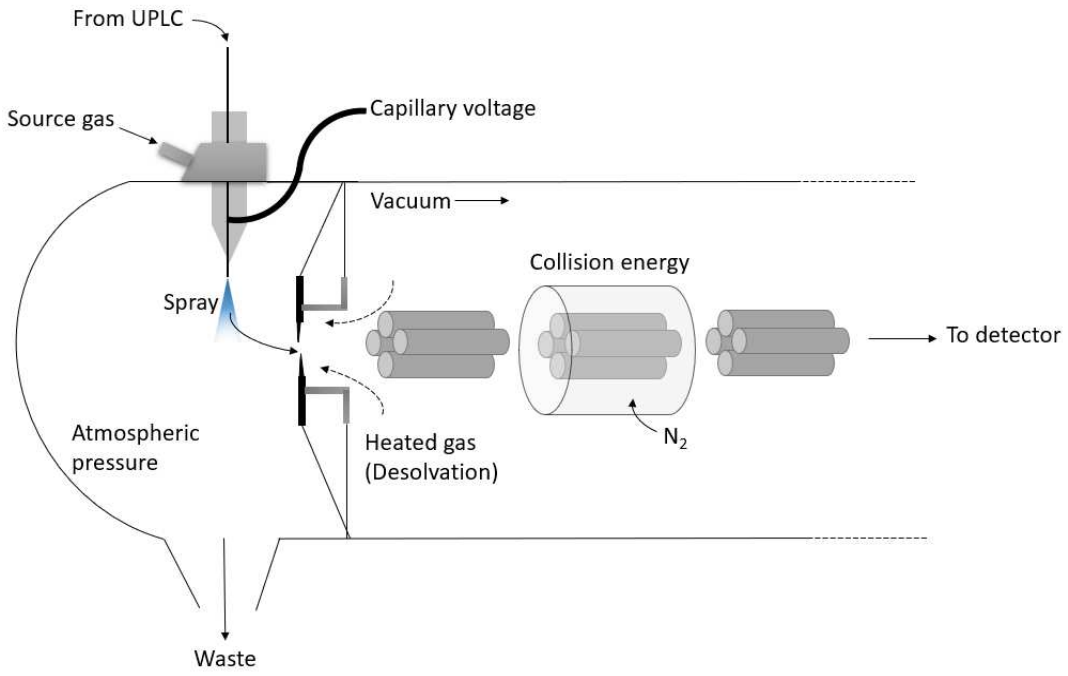


Figure 1.12 Schematic diagram showing mass spectrometric parameters: LC-MS/MS

. The retention times and the optimized compound dependant parameters for each MRM transitions of the 53 analytes are shown in Table 1.5.

Table 1.5. Optimized compound-dependent parameters in UPC-MS/MS

Pesticide	**T_R (min)**	**Quantifying transition (m/z)**	**Qualifying transition (m/z)**	**Collision Energy (V)**	**Cone Voltage (V)**
Acephate	12.62	183.9/142.95	183.9/49	20/18	10
Acetamiprid	5.09	223/126	223/56.1	15/20	30
Amectoctardin	8.53	276.16/244.07	276.16/168.06	24/14	16
Azoxystrobin	8.6	404/329	404/372	30/25	25
Bifenazate	9.55	301.1/198	301.1/170	20/10	25
Boscalid	8.92	342.9/139.9	342.9/307	20/45	25
Buprofezin	12.45	306.1/201	306.1/57.4	25/10	10

Carbaryl	6.88	202.1/145.1	202.1/127.1	25/10	25
Carbofuran	6.48	222.11/165.1	222.11/123	20/10	5
Chlorpyrifos	13.72	349.9/97	349.9/198	16/16	20
Cyantraniliprole	7.13	475.2/286	475.2/444	16/16	20
Cycloxydim	11.95	326/180	326/280	22/16	34
Cyprodinil	9.58	226/93	226/108	35/25	5
Diazinon	10.8	305.1/169	305.1/96.9	35/22	20
Dimethenamid	8.54	276/244	276/168	26/14	17
Emamectin benzoate	14.48	886.6/158	886.6/126	30/35	20
Ethion	13.59	385/199	385/142.9	25/10	30
Fenarimol	9.84	331/81	331/268	30/25	20
Fenbuconazole	10.35	337/70.1	337/125	30/20	15
Fenhexamid	9.68	301.96/55.18	301.96/97.11	35/25	35
Fenpyroximat	14.78	422.2/366.1	422.2/138.1	30/20	5
Flupicolide	8.98	383/172.999	383/109.06	66/20	40
Flutriafol	7.57	302.1/70.2	302.1/123.1	20/25	15
Fluxapyroxad	9.2	382.2/362	382.2/342	20/10	20
Hexaconazole	11.33	314/70.1	314/159	20/25	15
Imidacloprid	4.69	256.1/209.1	256.1/175.1	20/15	25
Iprobenfos	10.37	289/91	289/205	20/10	9
Malathion	9.08	331/127	331/99	20/15	10
Mandipropamid	9.04	411.8/328.1	411.8/125	35/15	35
Mehtiocarb	8.71	226/169	226/121	20/10	25
Metalaxyl	7.61	280.1/220.1	280.1/192.1	20/15	10
Methamidophos	0.6	142/93.9	142/124.9	13/13	15
Methoxyfenozide	9.2	369.2/149.1	369.2/313.23	15/10	15/5
Penthiopyrad	10.93	360.1/177.1	360.1/276	47/21	30
Phenthoate	10.52	321/79.1	321/135	40/20	9
Phosalone	11.42	367.9/181.9	367.9/110.9	42/14	12
Pirimiphos methyl	10.92	306.1/108.1	306.1/164.1	32/22	25
Procloraz	11.02	375.84/307.92	375.84/70.12	24/16	10
Profenofos	12.54	372.9/302.6	372.9/127.9	40/20	25
Pyraclostrobin	11.33	388.1/193.9	388.1/163	25/12	5
Quinalphos	10.37	299/96.9	299/162.9	30/24	15
Quinoxyfen	13.57	308/197	308/161.9	35/30	15
Spinosad A	11.68	732.6/142	732.6/98.1	35/30	35
Spinosad D	12.44	746.52/142	746.52/98.1	35/31	40
Spirodiclofen	14.76	411.14/71.16	411.14/313.1	15/10	35
Spirotetramat	9.65	374/330	374/302	30/15	20
Tebuconazole	10.85	308/70.1	308/125	20/35	10
Thiacloprid	5.54	253/126	253/90.1	35/20	40
Thiodicarb	7.17	355.08/88.1	355.08/108.1	16	17
Thiophanate	7.88	371/151	371/93.1	50/22	28
Triadimefon	9.17	294.1/69.3	294.1/197.2	20/15	25
Triazophos	9.53	314.1/161.9	314.1/118.9	35/18	22
Trifloxystrobin	12.11	409/186	409/145	40/16	10

QuEChERS sample preparation

The spices considered under the study were representative matrices from different categories of spices, *viz*. cardamom (dried fruits with low pigment content), chillies (dried fruits with high pigment content), ginger (dried roots / rhizomes), cumin (dried seeds), curry leaves (dried leaves) and cinnamon (dried bark). Homogenization of the spices were performed to simulate normal culinary usage, as explained in Table 1.2. Using the homogenized matrices, extraction and cleanup steps were optimized.

In the optimization experiments for general parameters like sample: water ratio and sample weight, an extraction step with sample: solvent ratio of 1:5 with 4g anh. $MgSO_4$ and 2 g NaCl, followed by vortexing for 1 minute and centrifuging at 5000 rpm for 5 minutes was followed. A basic, unoptimized cleanup profile as reported in the original QuEChERS method[35] was used, with 1 ml extract cleaned up using 150 mg anh. $MgSO_4$ and 25mg PSA, with vortexing for 30 seconds and centrifuging at 10,000 rpm for 5 minutes and injected in UPLC-MS/MS.

Five representative pesticides, viz. imidacloprid, ethion, chlorpyrifos, quinalphos and spirodeclofen were chosen to perform these initial optimizations, because of the uniformly good response obtained for these pesticides in all matrices under consideration. Subsequently the cleanup parameters were also optimized matrix-wise to obtain best recovery and precision. In all the optimization steps detailed below, matrix matched calibration (MMC) was employed in UPLC-MS/MS quantitative analysis. The calibration standards were set up using blank extracts prepared using the method steps being optimized.

Optimization of sample: water ratio

All the spices studied were low-moisture products and contained only about 8-12% average moisture content. Direct extraction of the matrices showed low accuracy and

precision, showing that rehydration of the matrices was essential to achieve efficient extractability of analytes. For this, water was added to 2 g of homogenized spice sample fortified at 50 μg kg^{-1} with the five representative pesticides, and allowed to soak in order to ensure rehydration. It was observed that the minimum soaking time required to ensure consistent results was 30 minutes. For lower soaking times, results obtained were not repeatable, and for higher soaking times, there was no significant improvement in precision. Thus 30 minutes was chosen as the optimal soaking time.

For optimizing the moisture content, the sample (g): water (ml) ratios 1:2, 1:4, 1:6 and 1:8 were used with a soaking time of 30 minutes ($n = 5$ in each case), and the accuracy and precision data for a fortification level of 50 μg kg^{-1} were compared to arrive at the optimum sample: water ratio for 5 the representative analytes chosen. It was observed when the spice samples were extracted directly without addition of moisture and using basic QuEChERS cleanup, the recovery and precision were poor, but with rehydration of the matrices, the recovery of all pesticides increased significantly.

The precision of analysis was also seen to be significantly affected by hydration. Recovery values were low when extracted without hydration for the five pesticides in all spice matrices and were in the ranges 28.2-51.8% for cardamon, 35.8-52% in cumin, 37-56% in ginger, 23.4-55.4% in chillies and 38.6-54% in curry leaves, with high standard deviations. This showed that even with proper homogenization, hydrating the matrix was important to ensure optimum extraction by the solvent. Hydration was seen to increase the recoveries by 20% or above in all cases.

These effects are shown in Figure 1.13, where the recovery values for the 5 pesticides in six spices are plotted against various sample: water ratios.

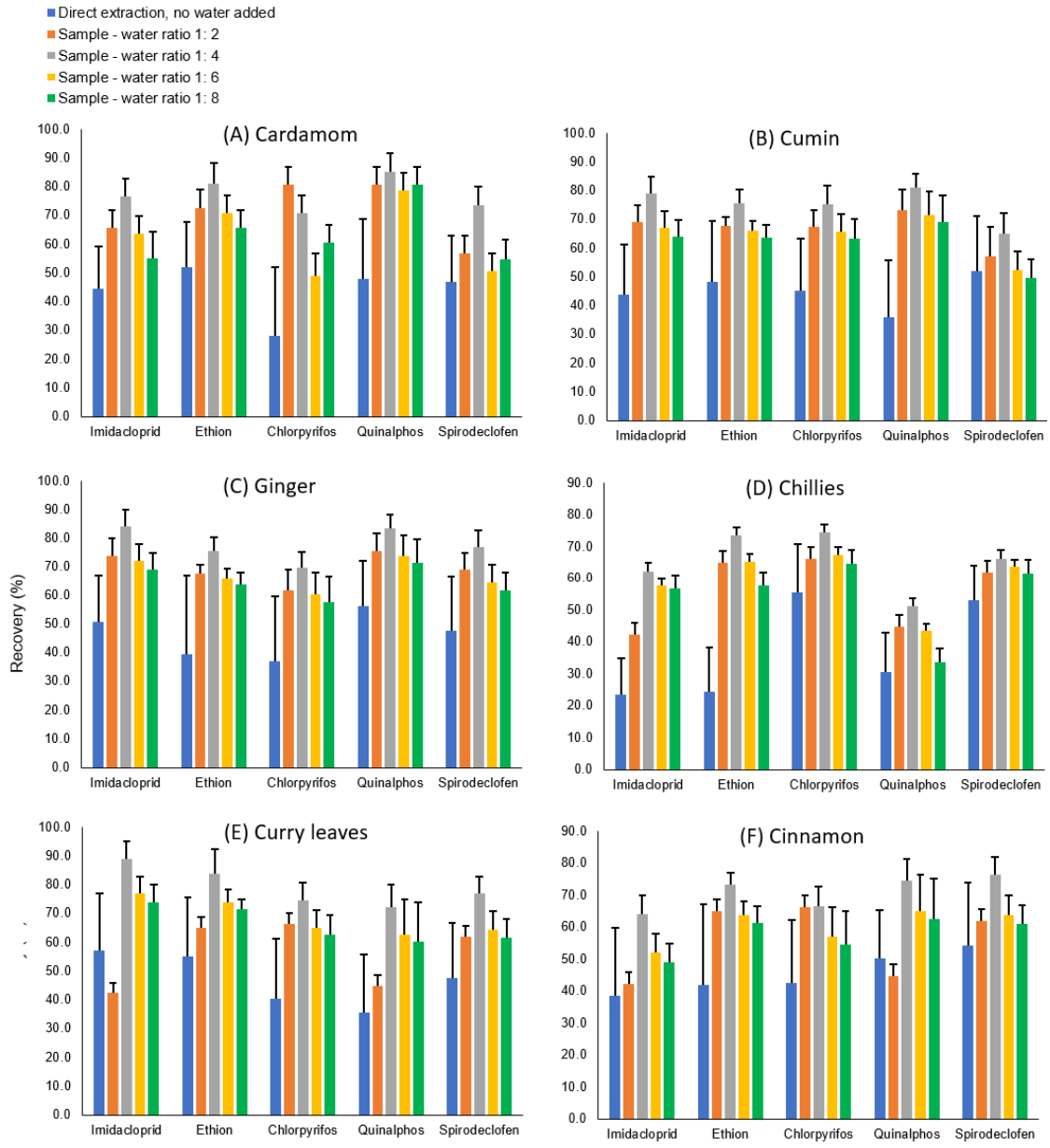


Figure 1.13 Effect of moisture content on extraction efficiency in spices

Precision (RSD_r) values were seen to be significantly improved by hydrating of the matrix, changing from 21- 84% without hydration, < 20% after hydration. It was observed that the even with unoptimized cleanup step, hydration with sample: water ratio of 1:4 could achieve recoveries in the range of 70-80%, except in the case of chillies, where recoveries of two pesticides, imidacloprid (62.2%) and spirodiclofen (66.2%) were seen

to be lower. The recoveries obtained at this sample: water ratio was consistently the highest except in one case, *viz*. chorpyrifos in cardamom, where 1:2 ratio showed a slightly higher recovery than 1:4 (+10%). However, following the major trend, the sample: water ratio of 1:4 was taken as optimal for the spices under consideration.

Optimization of sample weight

For optimizing the amount of sample taken for analysis, four sample weights were chosen, *viz.* 1 g, 2 g, 4 g and 6 g (n = 5 in each case). The homogenized samples of each of the spice matrices were first spiked with the five representative analytes at 50 μg kg^{-1}. Water was then added at the sample-water ratio of 1:4 and soaked for 30 minutes, as optimized earlier. The samples were then extracted, cleaned up and analysed in LC-MS/MS and the average recovery values were calculated.

It was observed that there were no large changes in average recovery with sample weight, but precision was seen to be significantly affected. Typical results for a representative spice, cardamom, for the five analytes are shown in Figure 1.14 where the average recoveries for the five analytes are plotted against sample weight. The same pattern was seen to recur in other matrices also.

Recovery values ranged between 69.6-88.8%, and there no significant difference in average recoveries for each compound with increase in sample weight. However, precision values showed discernible changes. The sample weight of 1 g showed high RSD_r values (14-20 %), but higher sample weights, i.e., 2 g and 4 g, showed better precision (RSD_r 3-11%). For the highest sample weight of 6g, precision was seen to decrease (RSD_r 11-17%). This is probably because spices contain significant amounts of crude fibre which makes perfect homogenization difficult, and increasing sample weight consequently would decrease the precision. As 2 g was the lowest sample weight which showed good

recovery and precision, this was chosen to be the optimal sample weight for all spices under consideration.

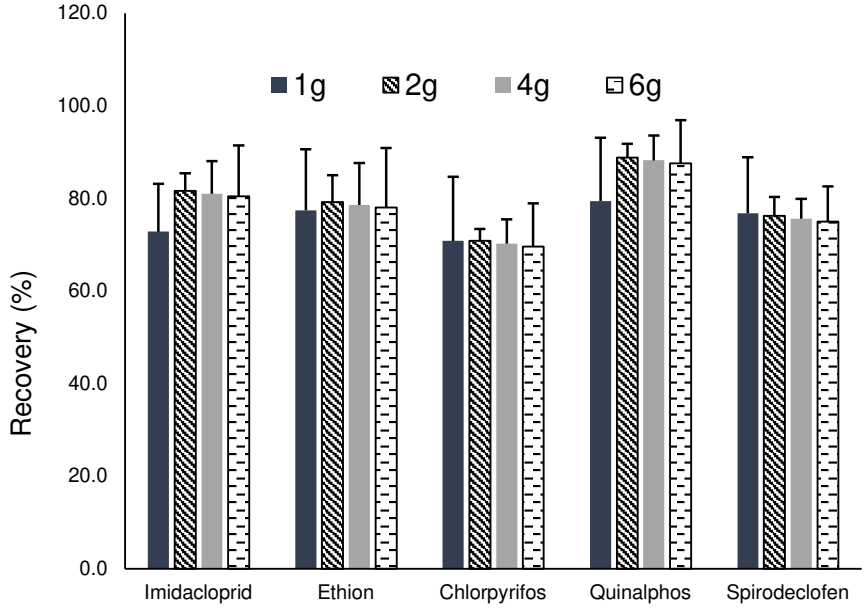


Figure 1.14 Effect of sample weight on recovery and precision in cardamom

Buffering during extraction step

It was noted during the initial optimization steps that for certain pH dependant pesticides, especially diazinon, carbaryl, chlorpyrifos, fenhexamid and malathion, there was a level of inconsistency in the repeatability of recovery values. Thus, before optimizing the cleanup step, the effect of buffer salts in the extraction efficiency in the six spices was studied. Using the optimized extraction parameters, recovery studies with and without citrate salts showed that for these pesticides, method performance improved considerably in the presence of citrate salts. For diazinon, carbaryl, chlorpyrifos and malathion, recovery values with addition of citrate salts increased by 13, 19, 17 and 24% in cardamom, 17, 18, 14 and 20% in cumin, 18, 25, 13 and 13% in ginger and 15, 12, 10 and 13% in chillies. For fenhexamid, recovery value increased by 19% in chillies. In all other cases, the variation in recovery values was minor, within ±8% for all compounds in

all spice matrices. However, it was deemed beneficial to include sodium citrate salts in the extraction step to improve overall method performance, and this was adopted to complete the optimization of the extraction step.

Optimization of cleanup step

To optimize the cleanup step, four QuEChERS reagents were considered, *viz*. anh. $MgSO_4$, PSA, C-18 endcapped sorbent and GCB. The use of $MgSO_4$ was to remove excess water from the extract and thus facilitate recovery of nonpolar residues. PSA contains primary and secondary amino groups that removed acidic interferences from the extracts. GCB acted by reducing pigments from the extracts, but it is known to affect recoveries of planar pesticides and this factor was also taken into consideration during the optimization step. The C-18 sorbent was used to remove non-polar interferences.

Spices typically have relatively high amounts of non-polar volatile oil content, of varying chemical compositions, in addition to other active chemical compounds. In cardamom the volatile oil content is around 8 - 9%, in ginger 0.7 - 4% and in cumin 2.7 - 4.3%. Chillies have capsaicinoid content, responsible for their pungency, ranging from 2000 - 5000 mg kg^{-1}. The colour in chillies, arising carotenoid content, range from 0.1 – 0.3%, or 1000 - 3000 mg kg^{-1}. All these factors contribute to matrix co-extractives which can potentially interfere with analytical performance. Also, as soaking spice samples in water was seen to be very important to obtain good recovery and precision, a natural consequence is the increased water content in the extract which has to be addressed to manage the recovery of non-polar pesticides.

In view of these factors, different combinations of cleanup chemicals were studied. After several initial trials, it was concluded that anh. $MgSO_4$ and PSA were required in the cleanup step in all spice extracts, and fine-tuning of accuracy and precision could be done based on the amounts of C18 and GCB. Thus, the following four combinations were

finalized for optimization studies: **(A)** 300 mg MgSO4 + 75 mg PSA + 50 mg C18, **(B)** 300 mg MgSO4 + 75 mg PSA + 50 mg C18 + 20 mg GCB, **(C)** 300 mg MgSO4 + 75 mg PSA + 75 mg C18 and **(D)** 300 mg MgSO4 + 75 mg PSA + 75 mg C18 + 20 mg GCB. The spice samples were first extracted with the already optimized extraction parameters like sample weight, sample-water ratio and soaking time. About 2g of the homogenized samples were extracted with 10 ml acetonitrile with 4g anh. $MgSO_4$ and 2 g NaCl, followed by vortexing for 1 minute and centrifuging at 5000 rpm for 5 minutes. From the centrifugate, 2 ml extract was taken to optimize the cleanup step. Each combination from (A) to (D) were applied to 5 samples of each of the four spices spiked at 0.01 mg kg^{-1}, then average recoveries and repeatability precision (RSD_r) were assessed. Figure 1.15 shows the overall average recoveries for the five representative compounds, viz. imidacloprid, ethion, chlorpyrifos, quinalphos and spirodiclofen, obtained for the four cleanup combinations in the six spice matrices studied.

It was seen that with no cleanup, i.e., by analysis of the crude extract as such, the average recoveries ranged from ~ 20 - 65% in all the matrices studied, which is considerably below the minimum limits of acceptable method performance. It was also noted that the repeatability precision in most spices were low, with the RSD_r values clustering relatively closer to the higher limit of the acceptable criteria of 20%. This proved that cleanup was an essential step in achieving acceptable method performance in spices. In cardamom, without cleanup the average recoveries of the selected pesticides ranged from 51.4-75.0%, with RSD_r values ranging from 5-10%. Out of the four cleanup combinations studied, the best recoveries were obtained for (C), i.e., with 300 mg $MgSO_4$ + 75 mg PSA + 75 mg C18. The average recoveries (*n=5*) using this combination ranged from 83.7 - 97.8%, with RSD_r in the range 4-8%. Thus, combination **(C)** was taken as the optimized cleanup combination in cardamom. It was observed that the effect of cleanup

was in increasing the accuracy of the method, and precision values did not improve much with cleanup.

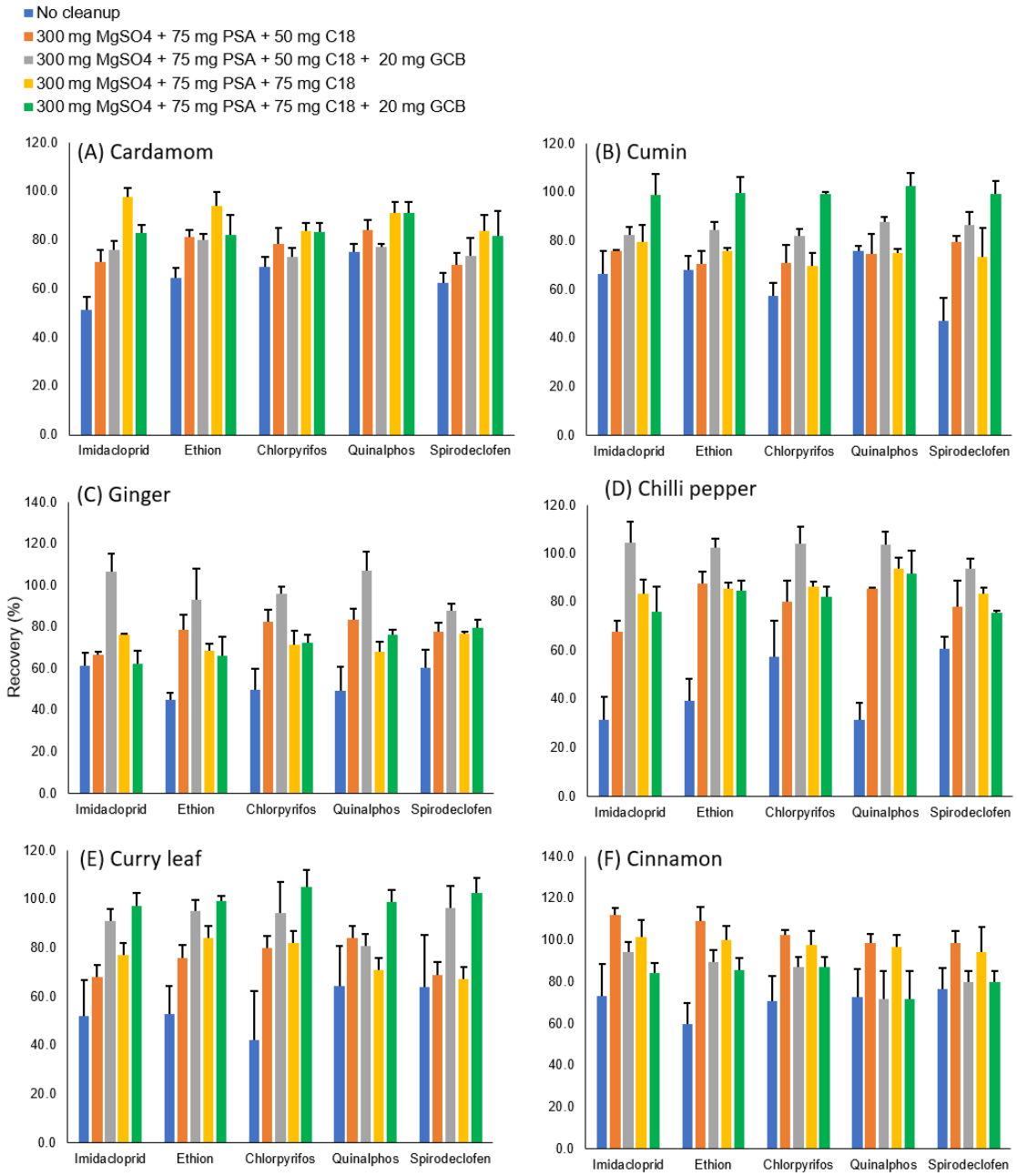


Figure 1.15 Optimization of cleanup procedures in spices: UPLC-MS/MS

In cumin, without cleanup the average recoveries of the selected pesticides ranged from 47.1-75.8%, with RSD_r values ranging from 3-20%. Out of the four cleanup

combinations studied, the best recoveries for ethion, chlorpyrifos, quinalphos and spirodiclofen were obtained for **(B),** i.e., with 300 mg $MgSO_4$ + 75 mg PSA + 50 mg C18 + 20 mg GCB, while in imidacloprid, the best recovery was obtained with combination (A), i.e. 300 mg $MgSO_4$ + 75 mg PSA + 75 mg C18 + 20 mg GCB. For the pesticides giving best performance with combination **(B)**, the recoveries ranged from 82.0-86.4% with RSD_r values from 7-11%. for imidacloprid, the average recovery with combination (B) was 82.3% with RSD_r of 1% while with combination **(D)** it was 98.7% with RSD_r of 7%. Considering that for imidacloprid the average recovery with combination (B) was within the acceptable limits of 70-120%, and had better precision than what was obtained with combination **(D)**, it was concluded that for cumin the optimal cleanup combination could be taken as combination **(B)**. It was observed that the effect of cleanup in cumin was in increasing both the accuracy and precision of the method considerably.

In ginger, without cleanup the average recoveries of the selected pesticides ranged from 45.1 - 61.4%, with RSD_r values ranging from 7-23%. Out of the four cleanup combinations studied, the best recoveries for all the five selected pesticides were obtained for **(B)**, i.e., with 300 mg $MgSO_4$ + 75 mg PSA + 50 mg C18 + 20 mg GCB. With this combination, the average recoveries obtained were in the range 87.7-107.2%, with RSD_r values ranging from 3 - 17%. It was thus concluded that for cumin the optimal cleanup could be taken as combination **(B)**. It was observed that the effect of cleanup in cumin was in increasing the accuracy, and precision was not seen to be improved significantly.

In chilli pepper also, the best recoveries were obtained with combination **(B)**, which was taken as the optimal cleanup combination for this spice. Here, the recoveries improved from 31.6-60% (RSDr 8-30%) without cleanup, to 93.8-104.6% (RSD_r 5-8%) with cleanup combination **(B)**. In curry leaves, the optimal cleanup combination turned out to be combination **(D)**, i.e., 300 mg $MgSO_4$ + 75 mg PSA + 75 mg C18 + 20 mg GCB.

Here, the recoveries improved from 42-64.5 (RSD_r 25 - 48%) without cleanup to 97.3-104.9% (RSDr 2-7%) with cleanup combination **(D).** Finally, for cinnamon, the optimal cleanup combination was identified as combination **(A)**, i.e., 300 mg $MgSO_4$ + 75 mg PSA + 50 mg C18. Here, recovery improved from 59.8-76.6% (RSD_r 13-21%) without cleanup to 98.6-112% (RSD_r 2-7%) with cleanup combination **(A)**. In all the spice matrices, accuracy (% recovery) and precision (RSD_r) values obtained using the optimized cleanup combination were well within the acceptable criteria of 70-120% and ≤ 20% respectively.

Table 1.6 Optimized extraction and QuEChERS cleanup scheme for LC-MS/MS

Process	Cardamom	Cumin	Ginger	Chillies	Curry leaves	Cinnamon
Extraction						
Sample weight (g)	2	2	2	2	2	2
Add water (ml) / soak time (min)	8/30	8/30	8/30	8/30	8/30	8/30
Add acetonitrile (ml)	10	10	10	10	10	10
Add $MgSO_4$ anh. (g)	4	4	4	4	4	4
Add NaCl (g)	1	1	1	1	1	1
Add Sodium citrate tribasic dihydrate (g)	1	1	1	1	1	1
Add sodium citrate dibasic sesquihydrate (g)	1	1	1	1	1	1
	Vortexed 30 sec, centrifuged 5000 rpm 5 min.					
Cleanup						
Volume taken for cleanup (ml)	2	2	2	2	2	2
Add PSA (mg)	75	75	75	75	75	75
Add C18 sorbent (mg)	75	50	50	50	75	50
Add GCB (mg)	0	20	20	20	20	0
Add $MgSO_4$ anh (mg)	300	300	300	300	300	300
	Vortexed 30 sec, centrifuged 10000 rpm 5 min.					
Concentration and reconstitution						
Cleaned extract evaporated to dryness (ml)	2	2	2	2	2	2
Reconstituted in 1:1 MeOH:H_2O (ml)	1	2	2	2	2	2

Concentration and reconstitution

The solution obtained after extraction and cleanup is in acetonitrile, whereas the mobile phase used in LC-MS/MS analysis is methanol-water. It was observed that changing the final extract from acetonitrile to methanol enhanced method performance and

also improved peak shapes. Thus, at the end of the optimized cleanup step, 2 ml of the extract was evaporated under nitrogen to near dryness and reconstituted with 1 ml, 1:1 methanol water solution. This introduced a concentration of the residues thus considerably enhancing the sensitivity of the method. The presence of water in the final injection solution was also seen to improve the peak shapes in some of the pesticides like acetamiprid. Table 1.7 above summarizes the optimized extraction, cleanup and concentration methodologies for all the spices studied, for analysis of the 53 pesticides using LC-M/MS.

Matrix load with optimized cleanup

The effect of the optimized cleanup step on the matrix load in the final solution is evident from the results of the gravimetric studies shown in Figure 1.16. The load of potentially interfering matrix co-extractives (mg ml^{-1}) in the extract was reduced after cleanup by 53% in cardamom, 51% in cumin, 50% in ginger, 57% in chillies, 39% in curry leaves and 57% in cinnamon.

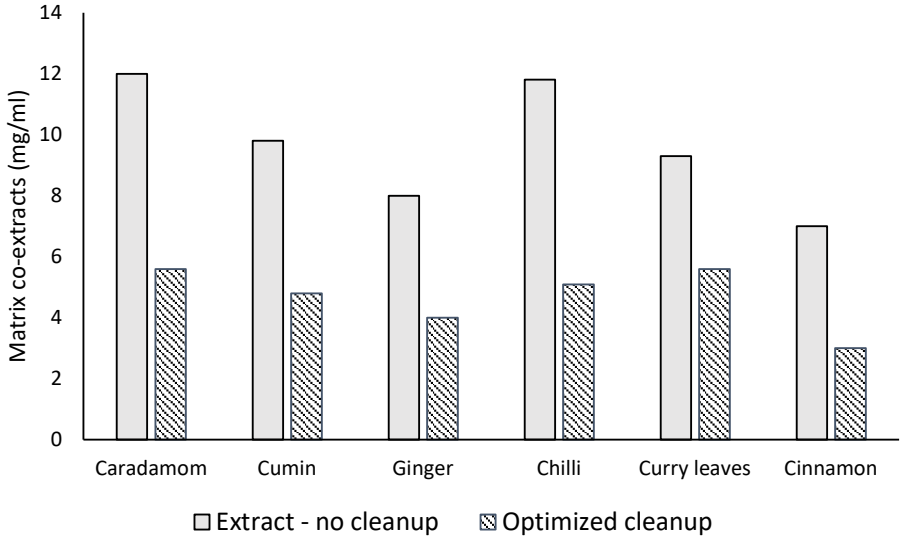


Figure 1.16 Matrix load in cleaned extracts: UPLC-MS/MS

Evaluation of matrix effects

In spite of the efficient cleanup steps which were optimized of all spices, it is evident from Figure 1.16 above that there is still considerable amount of matrix components remaining in the extract to cause interference to quantification. The assessment of matrix effects (MEs) was thus considered to be of importance in optimizing overall method performance.

The MEs were calculated using the following equation:

$$ME(\%) = \frac{Slope_{matrix-matched}}{Slope_{Solvent}} \times 100$$

ME between 80-120% are considered negligible, or soft ME, and does not require matrix matched calibration for reliable quantitative results. ME between 50-80% (suppression) and 120-150% (enhancement) are considered medium. ME lower than 50% (suppression) and higher than 150% (enhancement) are considered strong[52,115].

The ME posed by the spice matrices were uniformly suppressive and ranged from medium to strong. In cardamom, the ME ranged from 25-80%, in cumin between 10-46%, in ginger between 35-89% in chillies between 11-67%, in curry leaves from 40-83% and in cinnamon 45-79%. Thus, the highest suppression was observed in cumin and chillies.

Only 4 pesticides showed matrix suppression in the low ranges (ME > 80%), viz. fenhexamid (88%), fenpyroximat (89%) ad flutriafol (87%) in ginger matrix and pyroaclostrobin (80%) in cardamom matrix. When matrix suppression is low, i.e., ME is between 80 - 100%, results estimated using solvent-only calibration curves will not have large errors. However, with ME < 80%, using solvent-only calibration curves will lead to considerable underestimation of results.

In spices, the ME values were > 80% only in 1.8% cases in all the spice - pesticide combinations studied. This meant that for 98.2% of the analytes studied, ME manifested as response suppression in the medium and high ranges. Thus, it was concluded that matrix

matched calibration could not be avoided in all four spices so as to obtain reliable results. Table 1.7 shows the comparison of calibration equations ($y = mx + c$, where y represents the response, x the concentration of analyte, m the slope and c the y-intercept) and regression coefficients (R^2) for the analytes studied, in solvent and spice matrices. The matrix effects observed in the analytes in four representative spices are shown in Figure 1.17.

From the above data, it is evident that matrix effect is a significant aspect of pesticide residue analysis in spices using LC-MS/MS, and without addressing this issue, reliable method performance is not possible. Thus, matrix-matched calibration was fixed as a necessary requirement in the optimized methods. This posed the additional difficulty of ensuring the availability of blank matrices for the preparation of matrix matched calibration solutions. An attempt to address this issue to some extent is made in the studies outlined in Chapter 5.

Method performance

The method performance evaluation was performed based on the criteria given in Table 1.3. For all pesticides and spice matrices, good linearity could be established with R^2 values between 0.98-0.99, as shown in Table 1.7. All the optimized methods achieved the criteria of ≤ 20 % deviation in back-calculated concentrations from the true concentrations using five-point calibration curves. Average recoveries obtained were well within the acceptability criteria of 70-120%. Repeatability Precision (RSD_r, same analyst, same day, $n \geq 5$), and within-laboratory reproducibility precision (RSD_R, of 3 replicates of each spike level performed on 3 non-consecutive days, different analysts, n = 9) met the acceptability criteria of ≤ 20 % in all spike levels for all pesticides and spice matrices.

Table 1.7 Linearity equations and correlation coefficient values for pesticides analyzed by LC-MS/MS

Pesticide	Regression equation, R^2 value						
	Solvent	**Cardamom**	**Cumin**	**Ginger**	**Chillies**	**Curry leaves**	**Cinnamon**
Acephate	874x - 233, 0.9952	454x - 205, 0.9932	192x - 184, 0.9922	507x - 182, 0.9902	297x - 238, 0.9862	103x - 529, 0.9864	166x + 1803, 0.9871
Acetamiprid	19728x + 24531, 0.9952	13218x + 21588, 0.9912	1973x + 19380, 0.9872	13218x + 19134, 0.9862	6116x + 25022, 0.9912	12733x - 285, 0.9939	1358x + 7249, 0.9868
Amectoctardin	22375x - 353, 0.9981	9845x - 311, 0.9921	5146x - 279, 0.9931	14320x - 275, 0.9911	9397x - 360, 0.9891	8388x - 601, 0.9873	1280x + 29450, 0.9913
Azoxystroin	12353x + 1181, 0.9941	7165x + 1040, 0.9881	4200x + 933, 0.9881	7659x + 922, 0.9921	3459x + 1205, 0.9871	5561x + 14936, 0.9882	3229x + 5198, 0.9903
Bifenazate	23099x - 593, 0.9896	15476x - 522, 0.9866	7392x - 468, 0.9806	12704x - 463, 0.9876	15476x - 605, 0.9826	531x + 4225, 0.9815	3803x - 137, 0.9882
Boscalid	3380x - 35, 0.9933	2602x - 31, 0.9843	1048x - 28, 0.9923	1521x - 27, 0.9873	777x - 36, 0.9893	11534x + 10545, 0.9831	13868x - 524, 0.9901
Buprofezin	49527x - 663, 0.9951	33183x - 583, 0.9901	13868x - 524, 0.9901	17335x - 517, 0.9931	17830x - 676, 0.9881	7471x + 5544, 0.9878	432x + 27305, 0.9924
Carbaryl	1728x + 34564, 0.9914	933x + 30416, 0.9924	432x + 27305, 0.9924	1158x + 26960, 0.9924	639x + 35255, 0.9894	9496x + 22521, 0.9963	13009x + 1396, 0.9941
Carbofuran	37168x + 1767, 0.9951	21558x + 1555, 0.9891	13009x + 1396, 0.9941	27876x + 1378, 0.9941	12266x + 1803, 0.9871	10530x + 3975, 0.9913	787x + 8594, 0.9829
Chlorpyrifos	1789x + 10878, 0.9819	876x + 9573, 0.9669	787x + 8594, 0.9629	1180x + 8485, 0.9699	751x + 11096, 0.9659	4839x - 692, 0.9924	913x + 19532, 0.9882
Cyantraniliprole	9938x - 569, 0.9988	3677x - 501, 0.9918	2783x - 450, 0.9908	6361x - 444, 0.9898	1590x - 580, 0.9958	3844x - 372, 0.9939	385x + 5525, 0.9835
Cycloxydim	8267x - 156, 0.9952	3803x - 137, 0.9882	1653x - 123, 0.9872	4960x - 122, 0.9872	1819x - 159, 0.9912	1980x - 785, 0.9923	5592x + 13790, 0.9841
Cyprodinil	236x + 11621, 0.9877	130x + 10226, 0.9847	52x + 9181, 0.9847	139x + 9064, 0.9887	57x + 11853, 0.9997	913x + 19532, 0.9882	1358x + 7249, 0.9868
Diazinon	21039x - 678, 0.9954	11151x - 597, 0.9884	5049x - 536, 0.9874	10309x - 529, 0.9864	4839x - 692, 0.9924	385x + 5525, 0.9835	1280x + 29450, 0.9913
Dimethenamid	24025x - 365, 0.9979	14895x - 321, 0.9909	6006x - 288, 0.9909	12733x - 285, 0.9939	3844x - 372, 0.9939	5592x + 13790, 0.9841	5146x - 279, 0.9931
Emamectin benzoate	11650x + 770, 0.9953	6291x - 678, 0.9873	3961x - 608, 0.9893	8388x - 601, 0.9873	1980x - 785, 0.9923	1358x + 7249, 0.9868	4200x + 933, 0.9881
Ethion	8300x + 19149, 0.9962	5312x + 16851, 0.9932	3652x + 15127, 0.9912	5561x + 14936, 0.9882	913x + 19532, 0.9882	5592x + 10680, 0.9831	7392x - 468, 0.9806
Fenarimol	856x + 5417, 0.9905	368x + 4767, 0.9865	300x + 4279, 0.9875	531x + 4225, 0.9815	385x + 5525, 0.9835	2207x + 5615, 0.9858	1048x - 28, 0.9923
Fenbuconazole	17476x + 13519, 0.9911	7864x + 11897, 0.9821	5592x + 10680, 0.9831	11534x + 10545, 0.9831	5592x + 13790, 0.9841	2667x + 22809, 0.9923	13868x - 524, 0.9901
Fenhexamid	8489x + 7107, 0.9918	4584x + 6254, 0.9848	2207x + 5615, 0.9858	7471x + 5544, 0.9878	1358x + 7249, 0.9868	3370x + 4026, 0.9923	432x + 27305, 0.9924
Fenpyroximat	10669x + 28873, 0.9993	6722x + 25408, 0.9953	2667x + 22809, 0.9923	9496x + 22521, 0.9963	1280x + 29450, 0.9913	8040x + 18755, 0.9904	1358x + 7249, 0.9868
Flupicolide	14041x + 5096, 0.9953	10952x + 4485, 0.9903	3370x + 4026, 0.9923	10530x + 3975, 0.9913	3229x + 5198, 0.9903	4153x + 5977, 0.9919	1280x + 29450, 0.9913
Flutriafol	30923x + 23741, 0.9974	19791x + 20892, 0.9904	8040x + 18755, 0.9904	26903x + 18518, 0.9934	7422x + 24215, 0.9944	8300x + 19149, 0.9962	3229x + 5198, 0.9903
Fluxapyroxad	18056x + 7566, 0.9939	10111x + 6658, 0.9919	4153x + 5977, 0.9919	13361x + 5901, 0.9919	3070x + 7717, 0.9919	856x + 5417, 0.9905	1848x + 5374, 0.9828
Hexaconazole	23678x - 789, 0.9934	13023x - 694, 0.9924	4262x - 623, 0.9884	17048x - 615, 0.9904	8051x - 805, 0.9884	17476x + 13519, 0.9911	282x + 970, 0.9947
Imidacloprid	15187x - 266, 0.9964	10175x - 234, 0.9944	3341x - 210, 0.9884	9416x - 207, 0.9884	5467x - 271, 0.9874	8489x + 7107, 0.9918	5684x + 36169, 0.9968

Iprobenfos	44698x + 194, 0.9966	24584x + 171, 0.9896	14303x + 153, 0.9896	28607x + 151, 0.9906	13856x + 198, 0.9896	10669x + 28873, 0.9993	6369x - 572, 0.9961
Malathion	14856x + 1308, 0.9839	10102x + 1151, 0.9869	6091x + 1033, 0.9819	9656x + 1020, 0.9879	5348x + 1334, 0.9889	14041x + 5096, 0.9953	1592x - 515, 0.9924
Mandipropamid	9253x + 11353, 0.9902	5644x + 9990, 0.9862	3516x + 8969, 0.9852	5089x + 8855, 0.9842	1481x + 11580, 0.9872	1748x + 3310, 0.9872	7981x - 370, 0.9935
Mehtiocarb	4483x + 33510, 0.9867	2331x + 29489, 0.9887	1435x + 26473, 0.9147	2869x + 26138, 0.9887	1524x + 34180, 0.9847	10827x + 40289, 0.9938	1593x - 5298, 0.9899
Metalaxyl	18905x - 810, 0.9960	8318x - 713, 0.9930	8318x - 640, 0.991	10209x - 632, 0.9870	6239x - 826, 0.9870	8861x - 637, 0.9951	1356x + 5668, 0.9885
Methamidophos	198x + 14601, 0.9639	133x + 12849, 0.9829	46x + 11535, 0.9869	109x + 11389, 0.9809	73x + 14893, 0.9869	1137x - 574, 0.9954	4928x + 5976, 0.9963
Methoxyfenozide	2731x + 4244, 0.9882	1803x + 3734, 0.9832	546x + 3353, 0.9802	1748x + 3310, 0.9872	1038x + 4329, 0.9822	12144x - 412, 0.9915	1632x + 24504, 0.9962
Penthiopyrad	8586x + 3839, 0.9966	5409x + 3379, 0.9936	1631x + 3033, 0.9886	5238x + 2995, 0.9956	2662x + 3916, 0.9926	4249x - 5901, 0.9929	2003x + 128, 0.9893
Phenthoate	9306x + 76635, 0.9886	5956x + 67439, 0.9876	2419x + 60542, 0.9956	4839x + 59776, 0.9896	2140x + 78168, 0.9866	2035x + 6314, 0.9905	13883x - 170, 0.9925
Phosalone	3230x + 146, 0.9913	2003x + 128, 0.9893	1421x + 115, 0.9833	2519x + 114, 0.9853	807x + 149, 0.9843	7008x + 6657, 0.9943	4467x + 5987, 0.9878
Pirimiphos methyl	23530x - 193, 0.9955	13883x - 170, 0.9925	2588x - 152, 0.9905	17412x - 150, 0.9875	6118x - 197, 0.9865	5238x + 2995, 0.9956	883x + 1081, 0.9907
Procloraz	7702x + 6803, 0.9918	4467x + 5987, 0.9878	1848x + 5374, 0.9828	5314x + 5306, 0.9898	1617x + 6939, 0.9888	4839x + 59776, 0.9896	10827x + 40289, 0.9938
Profenofos	1226x + 1228, 0.9977	883x + 1081, 0.9907	282x + 970, 0.9947	748x + 958, 0.9887	417x + 1253, 0.9917	2519x + 114, 0.9853	8861x - 637, 0.9951
Pyraclostrobin	13534x + 45783, 0.9978	10827x + 40289, 0.9938	5684x + 36169, 0.9968	8662x + 35711, 0.9938	2707x + 46699, 0.9908	2707x + 46699, 0.9908	1137x - 574, 0.9954
Quinalphos	13845x - 724, 0.9981	8861x - 637, 0.9951	6369x - 572, 0.9961	9138x - 565, 0.9971	2354x - 738, 0.9921	2354x - 738, 0.9921	12144x - 412, 0.9915
Quinoxyfen	4550x - 652, 0.9964	1137x - 574, 0.9954	1592x - 515, 0.9924	3367x - 509, 0.9934	2002x - 665, 0.9884	1356x + 5668, 0.9885	4249x - 5901, 0.9929
Spinosad A	34698x - 469, 0.9985	12144x - 412, 0.9915	7981x - 370, 0.9935	22207x - 366, 0.9905	14920x - 478, 0.9935	4928x + 5976, 0.9963	2035x + 6314, 0.9905
Spinosad D	6640x - 6706, 0.9979	4249x - 5901, 0.9929	1593x - 5298, 0.9899	4382x - 5231, 0.9959	2722x - 6840, 0.9949	1632x + 24504, 0.9962	7008x + 6657, 0.9943
Spirodiclofen	3083x + 7175, 0.9915	2035x + 6314, 0.9905	1356x + 5668, 0.9885	2065x + 5597, 0.9855	1079x + 7319, 0.9835	12393x - 126, 0.9962	2888x + 27296, 0.9902
Spirotetramat	10950x + 7564, 0.9973	7008x + 6657, 0.9943	4928x + 5976, 0.9963	7008x + 5900, 0.9953	3723x + 7716, 0.9893	9538x - 3155, 0.9898	29836x - 141, 0.9942
Tebuconazole	6278x + 31018, 0.9982	2888x + 27296, 0.9902	1632x + 24504, 0.9962	4144x + 24194, 0.9932	3390x + 31638, 0.9902	10897x + 12613, 0.988	14471x - 3514, 0.9918
Thiacloprid	45901x - 160, 0.9972	29836x - 141, 0.9942	12393x - 126, 0.9962	36262x - 125, 0.9892	18820x - 163, 0.9952	4138x + 5065, 0.9873	11822x + 6412, 0.9933
Thiodicarb	32888x - 3993, 0.9988	14471x - 3514, 0.9918	9538x - 3155, 0.9898	23351x - 3115, 0.9978	7564x - 4073, 0.9928	12366x - 299, 0.9951	51525x - 378, 0.9961
Thiophanate	26577x + 15966, 0.997	17807x + 14050, 0.993	10897x + 12613, 0.988	11960x + 12454, 0.991	5050x + 16286, 0.992	18905x - 810, 0.9960	17890x + 36481, 0.9988
Triadimefon	11822x + 6412, 0.9933	6502x + 5642, 0.9913	4138x + 5065, 0.9873	7566x + 5001, 0.9873	3783x + 6540, 0.9843	198x + 14601, 0.9639	23530x - 193, 0.9955
Triazophos	51525x - 378, 0.9961	22671x - 333, 0.9891	12366x - 299, 0.9951	32461x - 295, 0.9981	13396x - 386, 0.9931	2731x + 4244, 0.9282	7702x + 6803, 0.9918
Trifloxystrobin	17890x + 36481, 0.9988	13239x + 32103, 0.9918	4830x + 28820, 0.9908	12702x + 28455, 0.9938	5367x + 37211, 0.9978	8586x + 3839, 0.9966	1226x + 1228, 0.9977

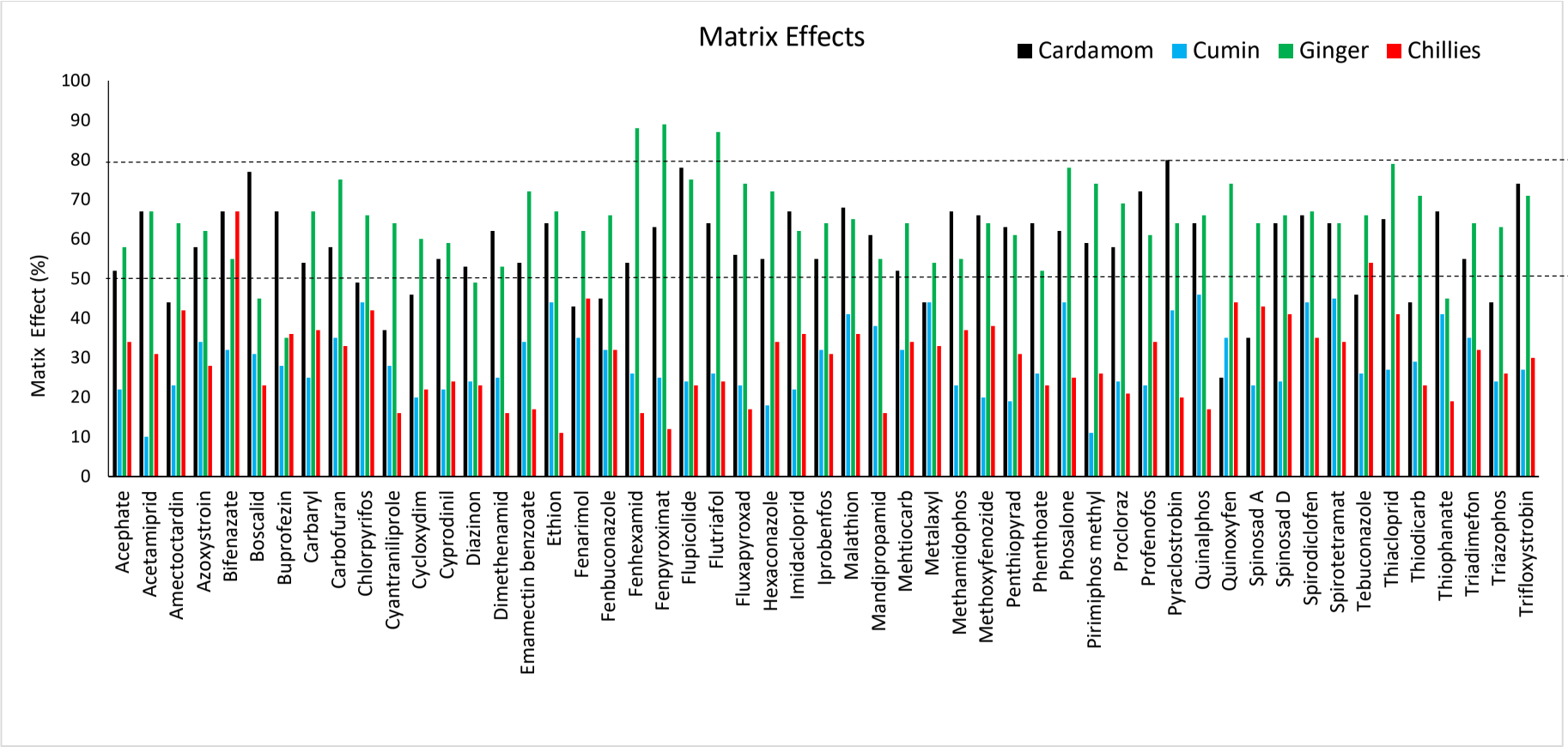


Figure 1.17 Matrix effects (%) of pesticides in four representative spices

The stated limit of quantification (LOQ) of the method, taken as the lowest spike level which could achieve the performance criteria for accuracy and precision, was fixed uniformly at 0.01 mg kg^{-1}, although in some cases limits of 0.005 mg/kg could be demonstrated. Specificity, assessed as the response in reagent blank and blank control samples in the same MRM and at the same retention time as the analyte, could meet the requirement of ≤ 30 % of LOQ in all the optimized methods. For the study of method ruggedness, three variables in the sample preparation method, *viz*. sample weight, sample: water ratio and extraction solvent volume were chosen. By varying these three variables by 20%, five different combinations were created, and each combination was applied in duplicate to a blank cardamom sample spiked with all analytes at 0.03 mg kg^{-1} (n = 10). The RSD value obtained was 14.36%, which was within the acceptability criteria, indicating that the method was sufficiently rugged to withstand small changes in the optimized method conditions.

Measurement uncertainty calculation

Uncertainty of measurement is defined as a value associated with a result that characterises the dispersion of the values that can be reasonably attributed to the measurand[116]. It is typically measured by first identifying the various components that can contribute to the uncertainty of the method using a cause-and-effect diagram, and then quantifying the uncertainties associated with each step.

Type A uncertainties are those arising from repeated measurements and Type B comprise of all other measurements. For the study, cumin was taken as a reference matrix for spices. Figure 1.18 shows the factors considered in this study for assessing method uncertainty.

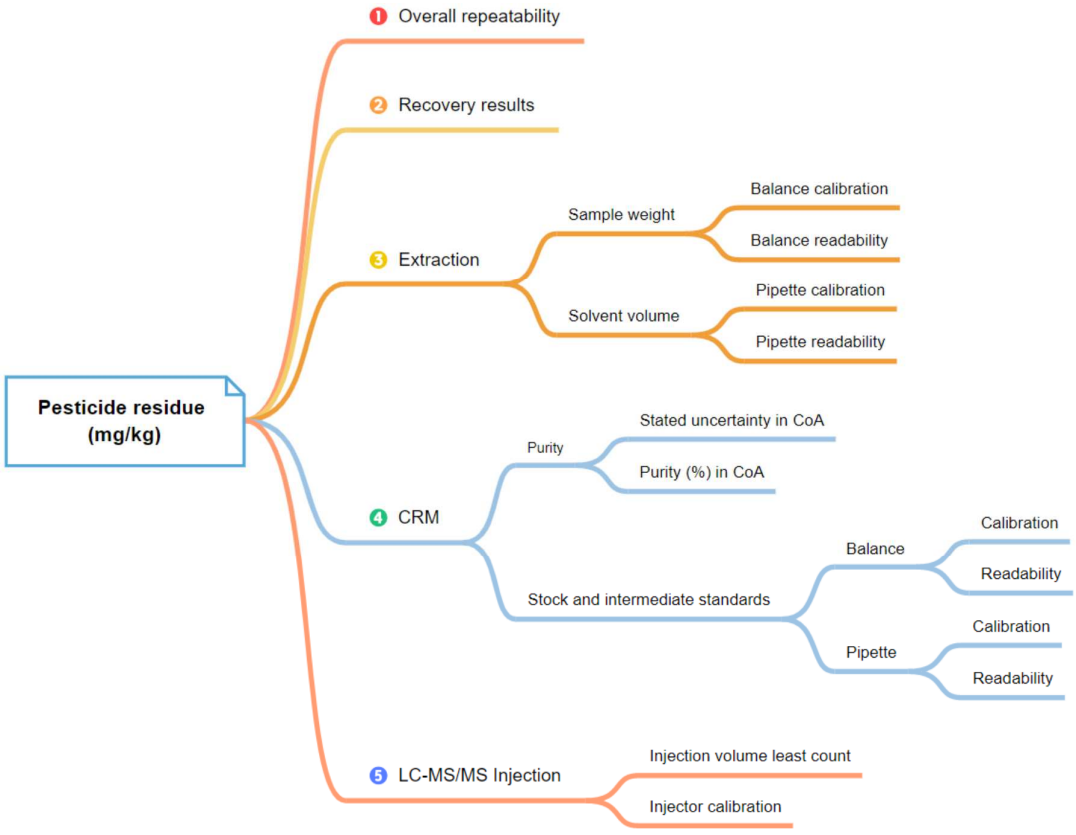


Figure 1.18 Uncertainty components for residue analysis

Uncertainty was evaluated at the LOQ level of 10 µg kg^{-1} (0.01 mg kg^{-1}) which was achieved using the optimized methods developed. Table 1.9 shows the relative standard uncertainties specific to each analyte. Uncertainty component related to precision was assessed from the repeatability results of spike level 10 µg kg^{-1}, n = 5 as (standard deviation of measurements)/$\sqrt{n}$. The uncertainty component related to accuracy was calculated from the average recovery value R as (100-R)/ $\sqrt{3}$, considering recovery error as Type B uncertainty with rectangular distribution. The uncertainty component with respect to standard purity is calculated from the percentage of purity P and uncertainty value U_{CRM} stated on the certificate, as $\frac{U_{CRM}}{P \times \sqrt{3}}$.

Tale 1.8 Relative standard uncertainty components at reference value 10 µg kg^{-1}

Compound	U (precision)	U (trueness)	U (CRM purity)
Acephate	0.1538	0.1910	0.0029
Acetamiprid	0.0806	0.3321	0.0029
Amectoctardin	0.1397	0.0907	0.0029
Azoxystrobin	0.1669	0.4393	0.0029
Bifenazate	0.1397	0.0907	0.0029
Boscalid	0.1704	0.2629	0.0029
Buprofezin	0.1426	0.1608	0.0029
Carbaryl	0.1704	0.2629	0.0029
Carbofuran	0.0700	0.3335	0.0029
Chlorpyrifos	0.0597	0.2436	0.0010
Cyantraniliprole	0.0769	0.0593	0.0029
Cycloxydim	0.1669	0.4393	0.0029
Cyprodinil	0.1397	0.0907	0.0029
Diazinon	0.0841	0.2216	0.0029
Dimethenamid	0.1048	0.0371	0.0029
Emamectin benzoate	0.1114	0.1824	0.0030
Ethion	0.0455	0.4223	0.0030
Fenarimol	0.1704	0.2629	0.0030
Fenbuconazole	0.0894	0.2084	0.0029
Fenhexamid	0.1274	0.1495	0.0030
Fenpyroximate	0.0769	0.0593	0.0029
Flupicolide	0.1877	0.1202	0.0029
Flutirafol	0.0675	0.2443	0.0030
Fluxapyroxad	0.1361	0.0018	0.0014
Hexaconazole	0.1114	0.1824	0.0029
Imidacloprid	0.0860	0.1998	0.0029
Iprobenfos	0.1274	0.1495	0.0029
Malathion	0.1717	0.3345	0.0030
Mandipropamid	0.0561	0.2230	0.0029
Mehtiocarb	0.0860	0.1998	0.0030
Metalaxyl	0.1704	0.2629	0.0029
Methamidophos	0.1336	0.2302	0.0030
Methoxyfenozide	0.1710	0.3029	0.0030
Penthiopyrad	0.0660	0.3966	0.0029
Phenthoate	0.1336	0.2302	0.0011
Phosalone	0.2239	0.1119	0.0011
Pirimiphos methyl	0.1710	0.3029	0.0030
Procloraz	0.0845	0.0906	0.0029
Profenofos	0.4064	0.0389	0.0010
Pyraclostrobin	0.1107	0.1462	0.0029
Quinalphos	0.1336	0.2302	0.0029
Quinoxyfen	0.1361	0.0018	0.0030
Spinosad A	0.0661	0.0149	0.0030

Spinosad D	0.2239	0.1119	0.0030
Spirodiclofen	0.1286	0.3805	0.0011
Spirotetramat	0.0860	0.1998	0.0030
Tebuconazole	0.1313	0.1665	0.0059
Thiacloprid	0.0740	0.0347	0.0029
Thiodicarb	0.0612	0.0801	0.0029
Thiophanate	0.1710	0.3029	0.0030
Triadimefon	0.2370	0.0369	0.0029
Triazophos	0.1278	0.1410	0.0029
Trifloxystrobin	0.1806	0.1838	0.0010

For the standard preparation and extraction steps, the uncertainty components (U_x) were taken as common for all analytes. These were all Type B components, so rectangular distribution was assumed and the standard uncertainty was calculated as $U_s = U_x / \sqrt{3}$, and relative uncertainty was then calculated as $RU_s = U_s/R$ where R is the reference value.

Tale 1.9 Common relative standard uncertainty components – UPLC-MS/MS analysis

Activity	**Step**	**Ref. value**	**Parameter**	**U_X**	**Type**	**U_S**	**RU_S**
Stock standard Preparation	Weighing	0.01 g	Balance readability	0.0001 g	B	0.00006	0.00577
Stock standard Preparation	Weighing	0.01 g	Balance calibration	0.0002 g	B	0.00009	0.00866
Stock standard Preparation	Measuring volume	10 ml	Pipette readability	0.1 ml	B	0.05774	0.00577
Stock standard preparation	Measuring volume	10 ml	Pipette calibration	0.013 ml	B	0.00751	0.00075
Intermediate standard Preparation	Measuring volume	1 ml	Pipette readability	0.1 ml	B	0.05774	0.05774
Intermediate Std Prep	Measuring volume	10 ml	Pipette calibration	0.013 ml	B	0.00751	0.00075
Sample Weight	Weighing	2 g	Balance readability	0.001 g	B	0.00058	0.00029
Extraction volume	Measuring volume	10 ml	Balance calibration	0.013 ml	B	0.00751	0.00075
Sample injection	Measuring volume	2 ml	Injector readability	0.5 ml	B	0.28868	0.14434

Table 1.9 shows above these relative standard uncertainty components. From the uncertainty components, the combined uncertainty was then calculated as

$$U_C = \sqrt{U_1^2 + U_2^2 + \cdots + U_n^2}$$

The expanded uncertainty was then calculated as $U_E = k \times U_C$. For 95% confidence limit (CL), the value of *k* was taken as 2. In reporting results, the format used was $X \pm U_E$ *@ 95% CL.*

Figure 1.19 below shows the expanded uncertainty values in percentage for the reference value of 10 mg kg^{-1}, for various pesticides studied. The calculated expanded uncertainty values ranged from 3.40 - 9.90%. For the purpose of reporting results, a uniform expanded uncertainty of ± 10% at the reference value of 10 mg kg^{-1} was adopted.

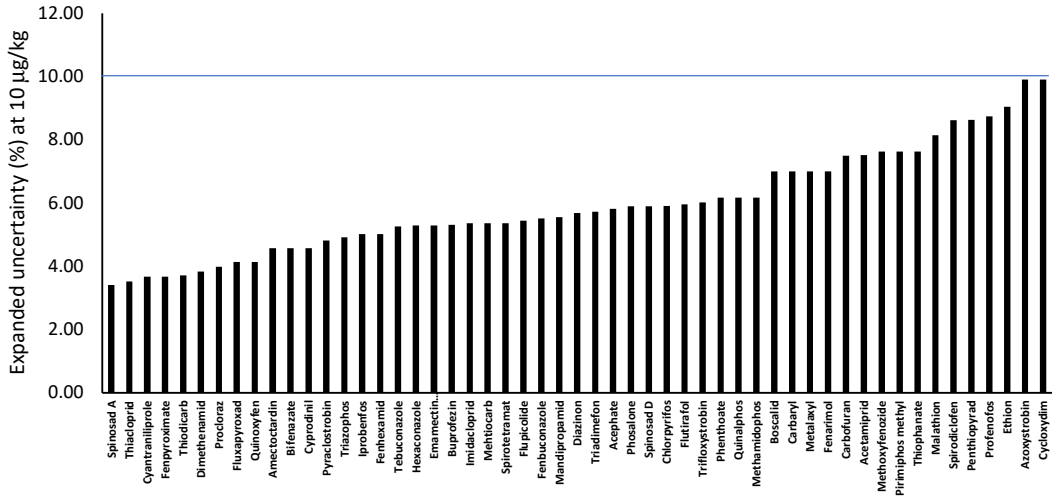


Figure 1.19 Expanded uncertainty for 95% confidence limit for UPLC-MS/MS analysis

Conclusions

A versatile, efficient and sensitive analytical method for pesticide residues using UPLC-MS/MS in six selected spices was developed, optimized specifically for different matrices, and validated. The matrices selected were representatives from different categories of spices, viz. cardamom (dried fruits with low pigment content), chillies (dried fruits with high pigment content), ginger (dried roots / rhizomes), cumin (dried seeds), curry leaves

(dried leaves) and cinnamon (dried bark). Extraction parameters were optimized to obtain efficient transfer of analytes from the spice matrices to solvent, and spice-specific cleanup steps were optimized to obtain accuracy and precision levels meeting internationally accepted method performance requirements. Matrix effects were assessed in various spices, and it was concluded that with medium to high matrix suppression noted in all spices, matrix-matched calibration was an essential requirement to obtain trouble-free quantitation at low concentration levels. Limit of quantification of 10 mg kg^{-1} or better were obtained in all analytes and matrices. Measurement uncertainty at limit of quantification was calculated as ±10% with 95% confidence limit for all analytes. The developed method can be used for regulatory compliance evaluation of spices as per national and international maximum residue limit requirements.

CHAPTER 4

RESIDUE ANALYSIS IN SPICES BY GC-MS/MS

Validation of high sensitivity, multiresidue analysis in representative matrices chosen from different categories of spices using gas chromatography and mass spectrometry is documented in this chapter. Sample homogenization, extraction, cleanup and instrumental analysis using GC-MS/MS, of residues of 25 GC-amenable pesticides that are commonly applied in spice cultivation, were optimized and validated for six spices, *viz*. cardamom, chillies, ginger, cumin, curry leaves and cinnamon.

Gas chromatographic and mass spectrometric conditions were tuned to obtain the desired high sensitivity responses for the target analytes in multiple reaction monitoring (MRM) detection. Starting from a general QuEChERS sample preparation profile as explained in Figure 1.10, specific schemes were devised to suit the different classes of spices by using various combinations of QuEChERS cleanup reagents and identifying the combination that gave best recoveries in each selected matrix. The matrix effects posed by different spices in GC-MS/MS were evaluated and addressed. An integrated methodology for high sensitivity multiresidue analysis of the GC-amenable target analytes in different spices, using an optimized sample preparation scheme, followed by GC-MS/MS analysis was developed. Validation of this analytical scheme was conducted as per SANTE guidelines[117] and measurement uncertainty was evaluated.

General analytical scheme and establishment of blanks

The analytical scheme followed in this chapter was similar to that followed in the case of LC-amenable compounds as described in Chapter 3 and followed the following sequence:

(a) The gas chromatographic and mass spectrometric parameters were optimized for 25 analytes to obtain good separation and response for all compounds.

(b) Spice samples belonging to each category were screened using a basic unoptimized QuEChERS sample preparation method and the optimized GC-MS/MS instrumentation method. Samples which were free from incidence of pesticides under consideration were selected as blanks for matrix effect and method optimization studies.

(c) The extraction and cleanup steps of the QuEChERS were then optimized for each spice matrix. For this, various combinations of extraction and cleanup reagents were studied. The combination of reagents that gave best accuracy and precision were taken as the optimized sample preparation method for each spice matrix.

(d) Using the optimized sample preparation method, extracts were prepared from blank samples of each spice matrix. These extracts were gravimetrically analysed to understand matrix load which indicated the extent of matrix interferences.

(e) Matrix effect was then assessed by comparing slopes of solvent-only and matrix-matched calibration curves. In GC-MS/MS, matrix effect observed is generally enhancement in response, and in some pesticides, solvent-based reference standards failed to give acceptable responses. Thus, in all the optimization studies, matrix matched calibration standards were used, prepared from blank extracts using the same extraction/cleanup steps used in the studies.

(f) Using the optimized sample preparation and instrumental methods, method validation was conducted for all spice matrices and fitness for intended purpose was assessed as per the criteria outlined in SANTE 12682 guidelines[112]. Measurement uncertainty at the established limit of quantification (LOQ) was

calculated using the validation data in a representative spice matrix, cumin, for all the analytes.

GC-MS/MS method optimization

After screening multiple MRM transitions for the target analytes, the transitions which showed lowest matrix interference for the spices under consideration were identified and used for analysis.

Table 1.10 Gas chromatographic and mass spectrometric conditions

Parameter	**Set Values**
Chromatographic parameters	
Injection volume	2 ml
Injector program	70°C (0.1 min), ramp at 450°C/min to 3250°C (2 min), ramp at 10°C/min to 250°C
Column	DB-5MS (15m, 250 mm, 0.25 mm) x 2, with mid-column backflush
Column flow	0.9 ml/min
Oven program	60°C (1 min), ramp at 40°C/min to 170°C (0 min), ramp at 10°C to 310°C (3 min). Total run time 20.75 min.
Mass spectrometric parameters	
Ion source	EI
Filament current	35 mA
Electron energy	70 eV
Source Temperature	300°C
Collision cell quench flow (He)	2.25 ml/min
Collision gas flow (N2)	1.5 ml/min

Once the MRMs were identified, retention time based dynamic MRM (D-MRM) was applied for each analyte, which improved response and peak shapes as shown in Figure 1.20 below. Two MRM transitions per analyte were used, with the transition giving higher response used as the quantifying transition. The second transition was used as the qualifying transition for confirming identity of the residues in samples. A mid-column backflush technique was used in which two 15 m columns were connected by a central backflush valve, which was seen to improve the method precision considerably, especially when large number of samples were analysed in a single batch run. Temperature programmes for the injector and column oven were tuned to obtain good separation and

response for the compounds under consideration. The optimized instrumental method is summarized in Table 1.10 above and the final MRMs for the 25 analytes under consideration are summarized in Table 1.11.

Table 1.11 Optimized MRM transitions in GC-MS/MS

Compound	Quantifier Transition	Qualifier Transition	RT (min)	Dwell time (ms)	CE (V)
Azinphos-methyl	104.9 / 51	104.9 / 77.1	14.78	6.5	15
Bifenthrin	181.2 / 165	181.2 / 166.2	14.45	7.2	15
Chlorothalonil	263.8 / 229	265.8 / 231	8.538	5.9	20
Chlorpyrifos-methyl	285.9 / 93	287.9 / 92.9	9.525	5.2	20
Cyfluthrin isomers (sum)	162.9 / 91	162.9 / 127	16.96	6.9	15
Cyhalothrin (Gamma)	197 / 161	141 / 91.1	14.65	6.7	15
Cyhalothrin (lambda)	208 / 181	181.1 / 152	14.45	6.5	15
Cypermethrin isomers (sum)	181.1 / 152	164.9 / 91	17.06	6.3	15
Deltamethrin	181 / 152	250.7 / 172	18.82	14.5	15
Dichlorvos	109 / 79	184.9 / 93	4.92	20.79	15
Disulfoton	88 / 60	142 / 81	8.895	5.9	5
Endosulfan a	194.9 / 159	194.9 / 160	11.87	6.7	15
Endosulfan b	206.9 / 172	194.9 / 158.9	12.92	7.4	15
Esfenvalerate	167 / 125	167 / 89	18.01	11.3	15
Ethoprophos	157.9 / 97	157.9 / 114	7.5	6.3	10
Fenitrothion	277 / 260	277 / 109	10.05	4.7	5
Fenpropathrin	207.9 / 181	264.9 / 89	14.95	7.6	15
Fenvalerate	167 / 125	167 / 89	18.01	11.3	15
Fipronil	366.8 / 213	368.8 / 214.8	10.93	5.9	15
Iprodione	313.8 / 56	313.8 / 244.9	13.87	7.4	15
Parathion	290.9 / 109	138.9 / 109	10.03	4.8	10
Parathion-methyl	262.9 / 109	125 / 47	9.218	6.9	10
Phorate	121 / 65	230.9 / 128.9	7.894	7.5	10
Piperonyl butoxide	176.1 / 131	176.1 / 117.1	13.98	6.7	15
Vinclozolin	187 / 124	197.9 / 145	9.561	4.9	20

RT – retention time, CE – collision energy

Sample preparation method optimization

The spices considered under the study were representative matrices from different categories of spices, viz. cardamom (dried fruits with low pigment content), chillies (dried fruits with high pigment content), ginger (dried roots / rhizomes), cumin (dried seeds), curry leaves (dried leaves) and cinnamon (dried bark). Homogenization of the spices were performed to simulate normal culinary usage, as explained in Table 1.2.

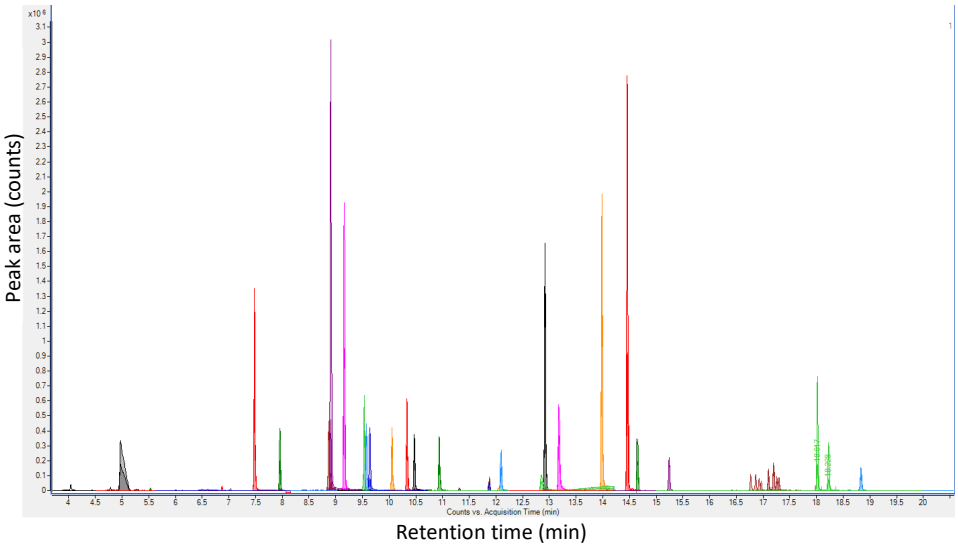


Figure 1.20 Optimized chromatogram in GC-MS/MS

The extraction steps, including sample weight, sample-water ratio, soaking time, sample-solvent ratio and use of buffering salts were adopted as optimized in Chapter 3 for UPLC-MS/MS analysis. Thus, 2 g homogenized samples were taken from each spice, soaked in 8 ml water (sample-water ratio 1:4) for 30 minutes, and extracted with 10 ml acetonitrile, with the addition of 4 g anh. $MgSO_4$, 1 g NaCl, 1g of sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7$. 2 H_2O) and 0.5 g of sodium citrate dibasic sesquihydrate ($C_6H_5Na_2O_7$. 1.5 H_2O). This mixture was then vortexed thoroughly, and centrifuged at 5000 rpm for 5 minutes. From the supernatant extract, 2 ml was pipetted out and used for optimization of the cleanup steps specific for each spice.

Optimization of cleanup conditions

Like in the case of LC-MS/MS, cleanup of spice extracts was required in GC-MS/MS also because matrix effects are more pronounced and critical in the latter case. Because of the nature of gas chromatography, presence of large amount of pigments in the final extracts is likely to cause charring in the GC injection liner and thus result in

inconsistent responses. Also, as a result of matrix hydration in the extraction step, chances of traces of water being present in the final extract is high and this has to be removed to preserve chromatographic performance and ensure safety of the GC capillary column[118–120]. The cleanup step was designed to address these critical issues. As explained in Chapter 1, in the d-SPE step, anh. $MgSO_4$ and PSA are used for removing polar coextractives like sugars, organic acids and traces of water remaining in the extract after the extraction step. The role of C-18 is to remove nonpolar lipid interferences, while GCB is used to remove pigments. Thus, all four of the d-SPE reagents were used for optimization of the cleanup step.

Initial screening studies using various combinations of the four cleanup reagents, *viz.* PSA, GCB, C18 and $MgSO_4$, it was established that cleanup of all spice extracts needed $MgSO_4$ and C18, and fine tuning could be done based on the amounts of PSA and GCB. Accordingly, four final combinations of cleanup reagents were used for optimization, *viz*. **(A)** 100 mg $MgSO_4$ + 100 mg C18 + 25 mg PSA, **(B)** 100 mg $MgSO_4$ + 100 mg C18 + 75 mg PSA, **(C)** 100 mg MgSO4 + 100 mg C18 + 25 mg PSA + 10 mg GCB and **(D)** 100 mg MgSO4 + 100 mg C18 + 25 mg PSA + 30 mg GCB. Five representative compounds, viz. bifenthrin, disulfoton, fenitrothion, fenpropathrin and vinclozolin, with good response and peak shape in the optimized GC-MS/MS DMRM conditions, were chosen to be used for optimizing the cleanup conditions based on recovery and precision data. The spice samples were first extracted with the already optimized extraction parameters like sample weight, sample-water ratio and soaking time as detailed in Chapter 3. About 2 g of the homogenized samples were extracted with 10 ml acetonitrile with 4 g $MgSO_4$ and 2 g NaCl, followed by vortexing for 1 minute and centrifuging at 5000 rpm for 5 minutes. From the centrifugate, 2 ml extract was taken to optimize the cleanup step. Each combination from **(A)** to **(D)** were applied to 5 samples of

each of the four spices spiked at 0.01 mg kg^{-1} with the representative pesticides, then average recoveries and repeatability precision (RSD_r) were assessed. The results of optimization are summarized in Figure 1.21 below.

In all cases, extracts without cleanup showed poor recovery and precision. In cardamom, without cleanup, recoveries for the representative pesticides were in the range 48.8-71.5% with RSDr (n = 5) in the range 9-37%. In the other spices the values of recovery and precision were as follows: cumin - recoveries 51.9-69.4% (RSDr 19-25%), ginger - recoveries 50.9-65.9% (RSDr 11-28%), chillies - recoveries 37.0-54.4% (RSDr 23-44%), curry leaves - recoveries 42.0-64.0 % (RSDr 22-48%) and cinnamon - recoveries 59.8-76.6% (RSDr 13-21%).

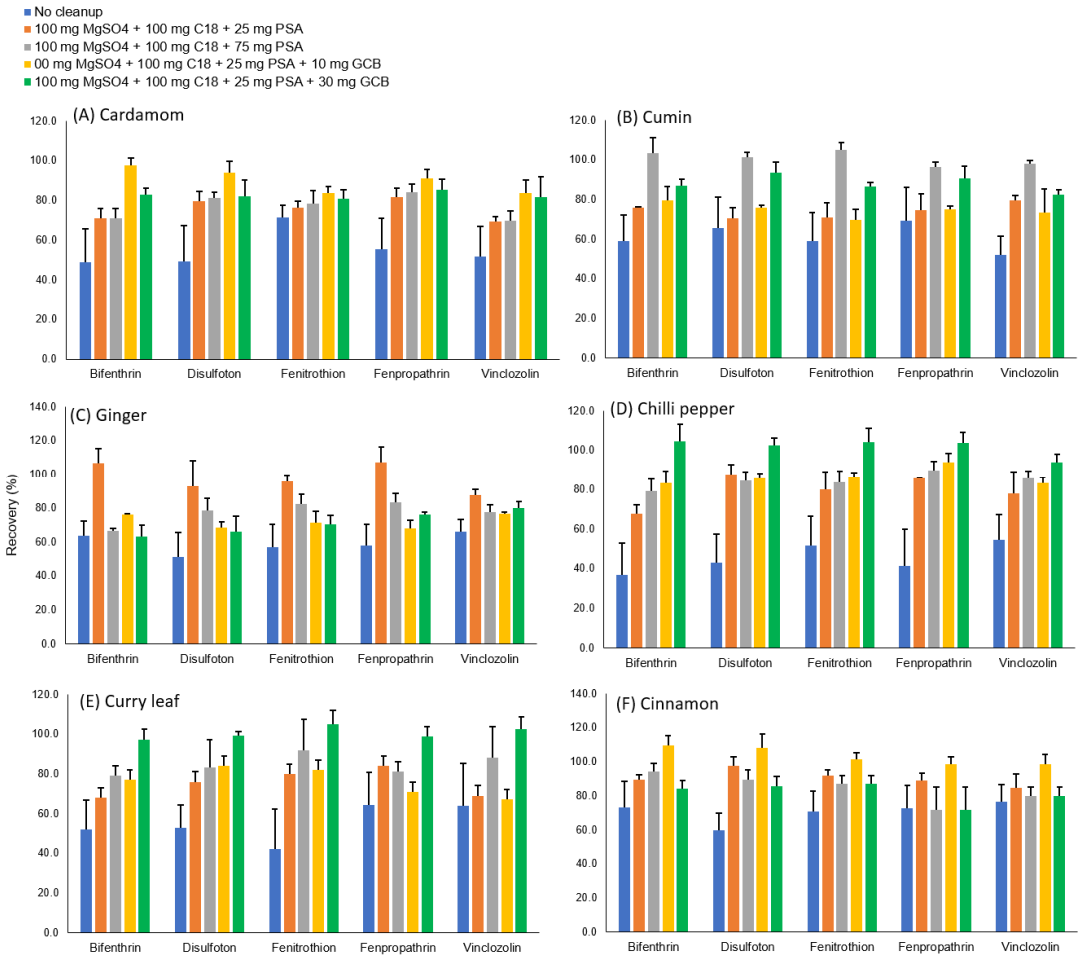


Figure 1.21 Optimization of cleanup procedure in spices: GC-MS/MS

The uniformly low recovery results in all cases without cleanup indicates that cleanup was an essential step for good method performance in analysing spices with GC-MS/MS. The precision values without cleanup were especially poor in the case of the two spices with most pigments, *viz.* chillies and curry leaves. This is possibly due to the deposition of pigments in the GC injector liner which gets charred on heating and cause response variations. Both the accuracy (recovery %) and the precision values were seen to considerably improve with the introduction of the cleanup step.

In cardamom and cinnamon, out of the four cleanup combinations studied, the best recoveries were obtained for **(C),** i.e., with 100 mg $MgSO_4$ + 100 mg C18 + 25 mg PSA + 10 mg GCB. In cardamom, the average recoveries for the five representative pesticides, *viz.* bifenthrin, disulfoton, fenitrothion, fenpropathrin and vinclozolin, using this combination ranged from 86.2-99.7%, with RSD_r in the range 4-11%. In cinnamon, the average recoveries were in the range 98.5-108.1% with RSDr in the range 4-7%. Thus, combination **(C)** was taken as the optimized cleanup combination in cardamom and cinnamon.

In cumin, out of the four cleanup combinations studied, the best recoveries for the five representative pesticides were obtained for **(B)**, i.e., with 100 mg $MgSO_4$ + 100 mg C18 + 75 mg PSA. The recovery values for this combination ranged from 96.2-104.8% with RSD_r values in the range 2-8%, so this combination was considered as the optimum cleanup step for cumin. In ginger, the optimized cleanup combination was **(A)**, i.e., 100 mg $MgSO_4$ + 100 mg C18 + 25 mg PSA, with average recoveries in the range 87.7-107.2% and RSD_r in the range 3-17%. In chillies and curry leaves, the optimized cleanup combination turned out to be **(D)**, i.e., 100 mg MgSO4 + 100 mg C18 + 25 mg PSA + 30 mg GCB. The average recoveries for chillies were in the range 93.8-104.6% with RSDr in

the range 5-8%, while in curry leaves the average recoveries in the range 99.2-104.9% with RSDr in the range 2-7%.

In all the spice matrices, accuracy (recovery %) and precision (RSD_r)values obtained using the optimized cleanup combination were well within the acceptable criteria of 70-120% and ≤ 20% respectively. In cumin and ginger, use of GCB, which reduces pigmentation in the extract, was not seen to be required. The requirement for PSA, which limits acidic cooextractives in the extract, turned out to be higher in cumin (75 mg), owing to the nature of this matrix. Cardamom and cinnamon required 10mg of GCB in the cleanup step. As expected, the requirement of GCB was seen to be highest in chillies and curry leaf (30 mg). Using higher amounts GCB for removing pigmentation is not generally advisable as it can adsorb planar pesticides and reduce recovery of such compounds, but this effect was not observed in the case of the target analytes used in the present study.

The effect of the optimized cleanup procedure in each of the spices, in terms of the matrix load (mg/ml) measured gravimetrically in the spice extracts is shown in Figure 1.22.

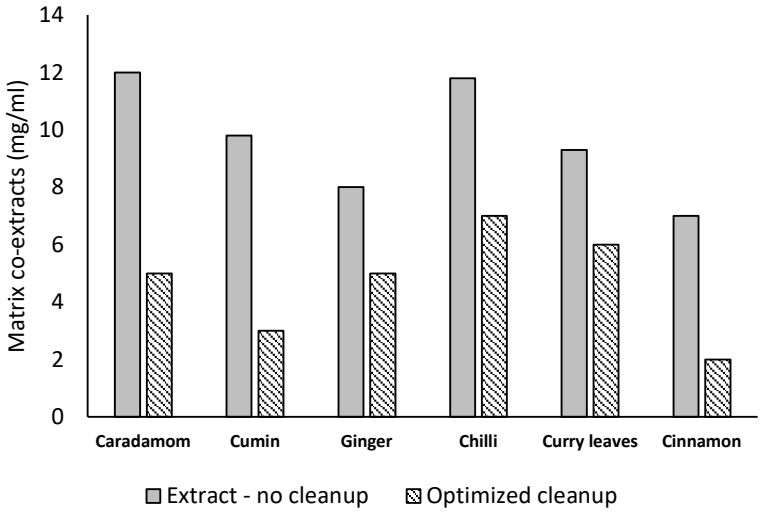


Figure 1.22 Matrix load in cleaned extracts: GC-MS/MS

The highest matrix load was in the extract was observed in the case of cardamom (12 mg/ml) and the least was for cinnamon (7 mg/ml). After cleanup, the highest reduction in matrix load was observed in cinnamon (71.4%), followed by cumin (69.3%), cardamom (58.3%), chillies (40.7%), ginger (37.5%) and curry leaves (35.4%). The reduction in matrix load is seen to translate directly into the considerable increase in accuracy and precision in the results in the cleaned-up extracts. The summary of the optimized sample preparation method for the 25 target analytes in six spices for analysis by GC-MS/MS is given in Table 1.12 below.

Table 1.12 Optimized extraction and QuEChERS cleanup scheme for GC-MS/MS

Process	**Cardamom**	**Cumin**	**Ginger**	**Chillies**	**Curry leaves**	**Cinnamon**
Extraction						
Sample weight (g)	2	2	2	2	2	2
Add water (ml) / soak time (min)	8/30	8/30	8/30	8/30	8/30	8/30
Add acetonitrile (ml)	10	10	10	10	10	10
Add $MgSO_4$ anh. (g)	4	4	4	4	4	4
Add NaCl (g)	1	1	1	1	1	1
Add Sodium citrate tribasic dihydrate (g)	1	1	1	1	1	1
Add sodium citrate dibasic sesquihydrate (g)	1	1	1	1	1	1
	Vortexed 30 sec, centrifuged 5000 rpm 5 min.					
Cleanup						
Volume taken for cleanup (ml)	1	1	1	1	1	1
Add PSA (mg)	25	75	25	25	25	25
Add C18 sorbent (mg)	100	100	100	100	100	100
Add GCB (mg)	10	0	0	30	30	10
Add $MgSO_4$ anh. (mg)	100	100	100	100	100	100
	Vortexed 30 sec, centrifuged 10000 rpm 5 min.					

Matrix effects in GC-MS/MS

In GC-MS/MS, the matrix effect manifests as response enhancement of analytes in the matrix extract as compared to pure solvent, and is considered to originate because of competition for active sites in the injection system of the GC between analytes and the compounds coextracted from the matrix[83]. Although modern developments in GC

technology have enhanced the inertness of the various components of the injection system, some active sites remain in these components. When an analyte is injected in solvent, the analytes get adsorbed on these active sites, and as a consequence the amount of analyte molecules that goes into the column gets reduced, resulting in diminished response. However, in matrix, the coextractives from the matrix will compete with the analyte for these active sites. Being in much higher concentration than the trace level analytes, the matrix compounds will saturate the active sites, thereby resulting in a much higher fraction of the analyte molecules entering the column culminating in enhanced response. On the whole, this means that matrix matched calibration is mostly unavoidable in GC-MS/MS, as solvent based calibration standards might offer poor response below acceptability criteria. However, the downside of this approach is that injecting large number of matrix matched calibration standards in the GC will result in deposition of the matrix in the injection liner and cause charring, and this will result in unexpected drop or inconsistency in response. Thus, optimized cleanup is a critical step in GC-MS/MS analysis of pesticide residues in spices, as it removes the matrix coextractives to a substantive extent, thereby extending life of the injection liner and still provides considerable extent of response enhancement due to the remaining matrix coextractives.

Figure 1.23 shows the effect of cleanup in removing matrix components in two representative spices, cardamom and chillies. From the MS full-scan total ions chromatogram (TIC) of spice extracts with and without cleanup, it is seen that the high boiling, early eluting compounds are not much affected by cleanup, but there is reduction in amount of matrix coextractives at later retention times. From the recovery and precision studies using the optimized cleanup methods specific to each spice, it is evident that the reduction in matrix coextractives thus achieved by cleanup is enough to bring the method

performance within acceptable criteria. Further studies on ME in GC-MS/MS and alternate methods for mitigating these effects are further addressed in detail in Chapter 5.

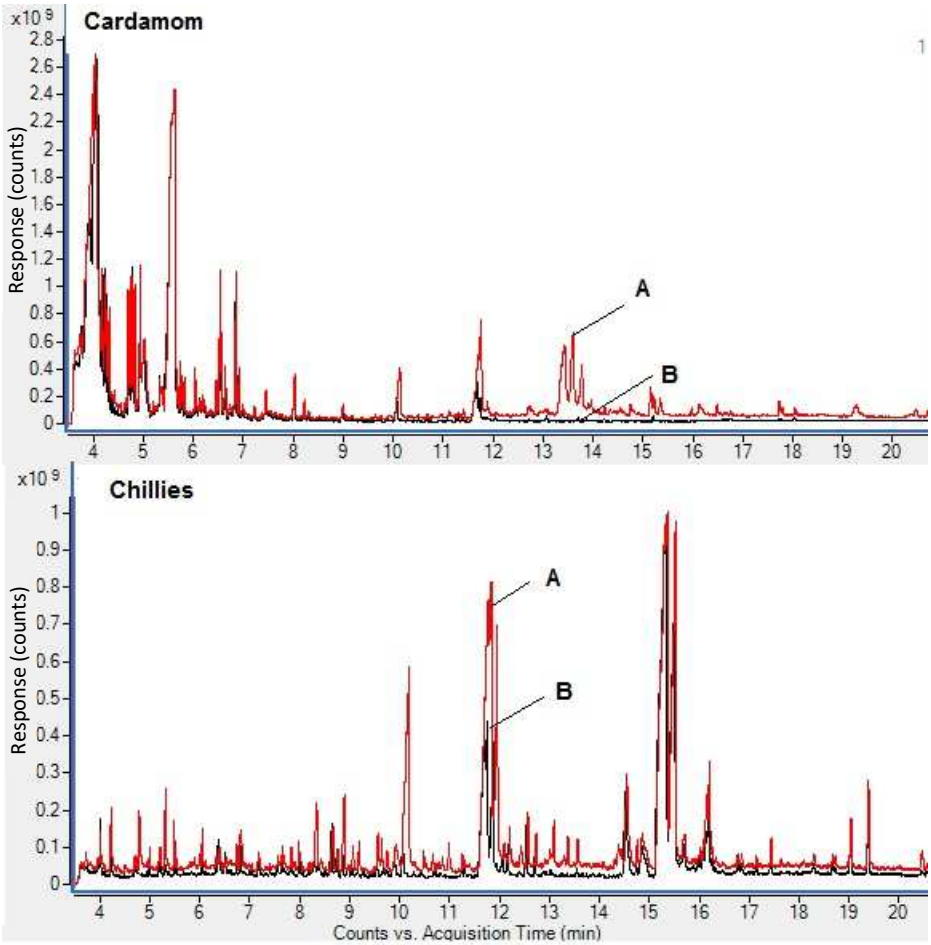


Figure 1.23 Full-scan TIC for extracts of chillies and cardamom, without cleanup (A) and with cleanup (B)

Method performance

The method performance evaluation was performed based on the criteria given in Table 1.3. For all pesticides and spice matrices, good linearity could be established with R^2 values 0.98 or better. All the optimized methods achieved the criteria of ≤ 20 % deviation in back-calculated concentrations from the true concentrations using five-point

calibration curves. Average recoveries obtained were well within the acceptability criteria of 70-120%. Repeatability Precision (RSD_r, same analyst, same day, n = 5), and within-laboratory reproducibility precision (RSD_R, of 3 replicates of each spike level performed on 3 non-consecutive days, different analysts, n = 9) met the acceptability criteria of ≤ 20 % in all spike levels for all pesticides and spice matrices. Table 1.13 summarizes the key validation parameters using the optimized sample preparation and instrumentation methods in a representative spice matrix, cumin.

Table 1.13 Validation parameters for target analytes in cumin as a representative matrix

Compound	R^2 (matrix matched)	Repeatability[a]		Reproducibility[b]	
		Av. Rec (%)	RSD_r	Av. Rec (%)	RSD_R
Azinphos methyl	0.9904	92.3	12	86.3	16
Bifenthrin	0.9921	88.9	4	83.4	7
Chlorothalonil	0.9836	92.3	3	90.6	14
Chlorpyrifos-methyl	0.9811	93.6	6	93.2	12
Cyfluthrin isomers (sum)	0.9901	104.1	7	96.0	12
Cyhalothrin (Gamma)	0.9812	103.1	4	92.9	8
Cyhalothrin (lambda)	0.9813	102.9	3	92.5	15
Cypermethrin isomers (sum)	0.9932	102.1	8	97.5	16
Deltamethrin	0.9866	89.6	7	77.9	13
Dichlorvos	0.9803	92.1	3	110.1	7
Disulfoton	0.9932	96.1	4	94.5	6
Endosulfan a	0.9904	100.8	5	96.3	5
Endosulfan b	0.9812	110.3	7	98.3	8
Esfenvalerate	0.9865	113.3	2	114.0	5
Ethoprophos	0.9932	93.8	4	94.9	4
Fenitrothion	0.9963	90.0	9	85.6	12
Fenpropathrin	0.9839	98.1	5	79.0	6
Fenvalerate	0.9932	115.2	2	114.3	6
Fipronil	0.9869	113.0	6	106.0	10
Iprodione	0.9811	112.3	4	98.3	6
Parathion	0.9899	86.6	6	75.6	7
Parathion-methyl	0.9951	91.7	6	99.2	3
Phorate	0.9887	107.6	3	94.1	5
Piperonyl butoxide	0.9937	97.6	5	85.3	7
Vinclozolin	0.9961	108.8	8	102.2	9

[a]Spike level 10 μg kg^{-1}, same analyst, same day, n = 5.

[b]Spike level 10 μg kg^{-1}, 3 replicates performed on 3 non-consecutive days, different analysts, n = 9.

The stated limit of quantification (LOQ) of the method, taken as the lowest spike level which could achieve the performance criteria for accuracy and precision, was fixed uniformly at 0.01 mg kg^{-1}. Specificity, assessed as the response in reagent blank and blank control samples in the same MRM and at the same retention time as the analyte, could meet the requirement of ≤ 30 % of LOQ.

Assessment of Measurement Uncertainty

For measurement uncertainty calculations in GC-MS/MS analysis, the same sequence of steps outlined in Chapter 3 was followed.

Table 1.14 Relative standard uncertainties specific to each analyte compound, at a reference value of 10 μg kg^{-1}

Compound	U (precision)	U (trueness)	U (CRM purity)
Azinphos methyl	0.1607	0.3707	0.0029
Bifenthrin	0.1063	0.3770	0.0010
Chlorothalonil	0.4487	0.0890	0.0029
Chlorpyrifos-methyl	0.2689	0.2491	0.0029
Cyfluthrin isomers (sum)	0.1308	0.1597	0.0002
Cyhalothrin (Gamma)	0.0878	0.1686	0.0002
Cyhalothrin (lambda)	0.4582	0.0555	0.0030
Cypermethrin isomers (sum)	0.1628	0.4942	0.0029
Deltamethrin	0.0928	0.1230	0.0030
Dichlorvos	0.0952	0.2129	0.0030
Disulfoton	0.1041	0.1149	0.0031
Endosulfan a	0.1882	0.3211	0.0013
Endosulfan b	0.0892	0.4687	0.0029
Esfenvalerate	0.0492	0.3207	0.0029
Ethoprophos	0.0626	0.3130	0.0029
Fenitrothion	0.2314	0.1333	0.0030
Fenpropathrin	0.1276	0.0783	0.0029
Fenvalerate	0.0898	0.5465	0.0029
Fipronil	0.1171	0.0174	0.0029
Iprodione	0.0494	0.1324	0.0015
Parathion	0.0876	0.3296	0.0031
Parathion-methyl	0.0779	0.2138	0.0029
Phorate	0.1645	0.0501	0.0029
Piperonyl butoxide	0.2314	0.3207	0.0029
Vinclozolin	0.1276	0.3130	0.0029

Cumin was chosen as the representative matrix for the study. The uncertainty components detailed in Figure 1.18 in Chapter 3 holds good in the present case also. Uncertainty was evaluated at the limit of quantification level of 10 µg kg^{-1} (0.01 mg/kg) which was achieved using the optimized methods developed. Table 1.14 shows the relative standard uncertainties specific to each analyte. Uncertainty component related to precision was assessed from the repeatability results of spike level 10 µg/kg, n = 5 as (standard deviation of measurements)/$\sqrt{n}$.

The uncertainty component related to accuracy was calculated from the average recovery value R as (100-R)/ $\sqrt{3}$, considering recovery error as Type B uncertainty with rectangular distribution. The uncertainty component with respect to standard purity is calculated from the percentage of purity P and uncertainty value U_{CRM} stated on the certificate, as $\frac{U_{CRM}}{P \times \sqrt{3}}$. For the standard preparation and extraction steps, the uncertainty components were taken as common for all analytes. These were all Type B components, so rectangular distribution was assumed and the standard uncertainty was calculated as U_s = U / $\sqrt{3}$, and relative uncertainty was then calculated as U_s/R where R is the reference value. Table 1.15 below shows these relative standard uncertainty components.

From the uncertainty components, the combined uncertainty was then calculated as

$$U_C = \sqrt{U_1^2 + U_2^2 + \cdots + U_n^2}$$

The expanded uncertainty was then calculated as $U_E = k \times U_C$. For 95% confidence limit (CL), the value of k was taken as 2. Figure 1.24 below shows the expanded uncertainty values in percentage for the reference value of 10 mg kg^{-1}, for various pesticides studied. In reporting results, the format used was $X \pm U_E$ *@ 95% CL.*

Tale 1.15 Common relative standard uncertainty components: GC-MS/MS analysis

Activity	Step	Ref. value	Parameter	Ux	Type	SUx	RSUx
Stock standard Preparation	Weighing	0.01 g	Balance readability	0.0001 g	B	0.00006	0.00577
Stock standard Preparation	Weighing	0.01 g	Balance calibration	0.0002 g	B	0.00009	0.00866
Stock standard Preparation	Measuring volume	10 ml	Pipette readability	0.1 ml	B	0.05774	0.00577
Stock standard preparation	Measuring volume	10 ml	Pipette calibration	0.013 ml	B	0.00751	0.00075
Intermediate standard Preparation	Measuring volume	1 ml	Pipette readability	0.1 ml	B	0.05774	0.05774
Intermediate Std Prep	Measuring volume	10 ml	Pipette calibration	0.013 ml	B	0.00751	0.00075
Sample Weight	Weighing	2 g	Balance readability	0.001 g	B	0.00058	0.00029
Extraction volume	Measuring volume	10 ml	Balance calibration	0.013 ml	B	0.00751	0.00075
Sample injection	Measuring volume	2 ml	Injector readability	0.5 ml	B	0.28868	0.14434

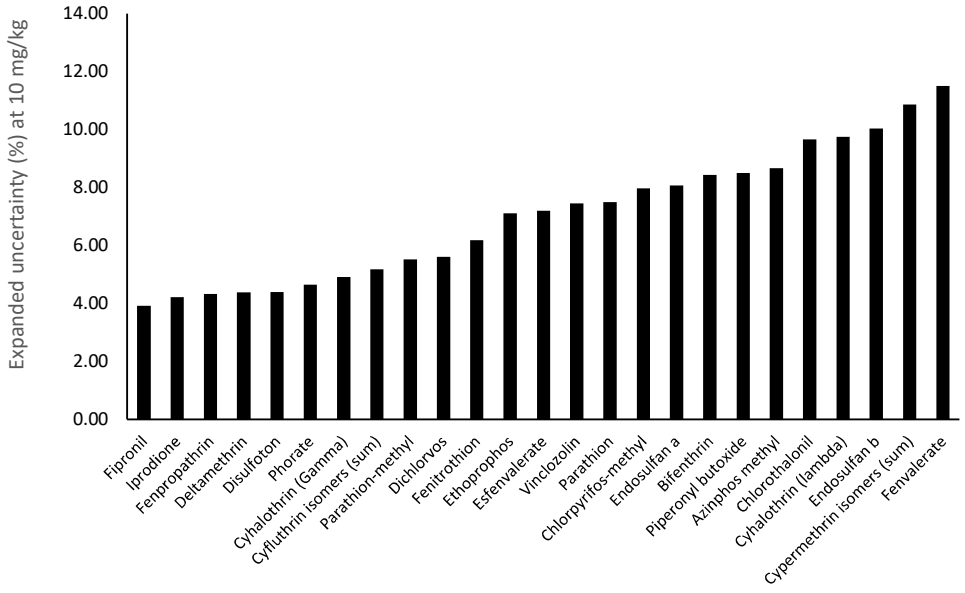


Figure 1.24 Expanded uncertainty at 95% confidence limit for GC-MS/MS analysis

For all the analytes studied with GC-MS/MS, the expanded measurement uncertainty values at the LOQ of 10 μg kg^{-1} was below 10%, except in the cases of cypermethrin isomers (10.9%) and fenvalerate (11.5%). The lowest measurement uncertainty obtained was for Fipronil (4.9%).

Conclusions

An efficient and sensitive analytical method for analysis of residues of 25 pesticides using GC-MS/MS in six selected spices was developed, optimized for different spice matrices, and validated. The matrices selected were representatives from different categories of spices, viz. cardamom (dried fruits with low pigment content), chillies (dried fruits with high pigment content), ginger (dried roots / rhizomes), cumin (dried seeds), curry leaves (dried leaves) and cinnamon (dried bark). Extraction parameters were optimized to obtain efficient transfer of analytes from the spice matrices to solvent, and spice-specific cleanup steps were optimized to obtain accuracy and precision levels meeting internationally accepted method performance requirements. Matrix effects were assessed in various spices, and it was noted that high matrix effects, mostly manifesting as response enhancement, was present in all cases. Thus matrix-matched calibration was an essential requirement to obtain trouble-free quantitation at low concentration levels. Limit of quantification of 10 μg kg^{-1} was obtained in all analytes and matrices. Expanded measurement uncertainty at limit of quantification was calculated in the range of 4.9-11.5% with 95% confidence limit for all analytes at LOQ. A common measurement uncertainty value of 12% at LOQ was adopted, covering all the compounds studied. The developed method can be used for regulatory compliance evaluation of spices as per international maximum residue limit requirements.

CHAPTER 5

MITIGATION OF MATRIX EFFECTS IN SPICES

Spices are typically considered as difficult matrices in trace analysis using chromatography and mass spectrometry because of the high level of matrix effects. Matrix effects (ME) in mass spectrometry manifest as a difference in response between the same concentration of an analyte when present in a solvent and in an extract containing matrix compounds. The nature of ME differs considerably in GC-MS/MS and LC-MS/MS, chiefly owing to the mechanism through which they occur. In gas chromatography, matrix effect arises due to competition for active sites in the injection system between analyte molecules and other molecules present in the injected solution. The concentration of compounds other than the analyte will be much higher in a matrix extract containing the analyte than in a solvent-based reference standard of the analyte. As a consequence, in gas chromatography the ME manifests as enhanced response for the analyte in the matrix extract than in the solvent[83,85]. In contrast, in liquid chromatography, matrix effect arises in the electrospray ionisation source (ESI) due to competition for protons for ionization, and usually manifests in the form of signal suppression[46,54,55]. The origin and nature of these effects were described in detail in Chapter 1. This chapter documents two different approaches undertaken to mitigate matrix effects posed by spices in GC-MS/MS and UPLC-MS/MS respectively.

Quantitation problems due to matrix effects

Measurement of pesticide residues always take place in the extract from a matrix. Since ME causes the response for an analyte to vary in a solvent and an extract solution, using a solvent-based reference standard for calibrating the analysis instrument will always result in substantial quantification errors. In GC-MS/MS there will be matrix enhancement

of the analyte signal, so if a solvent-based calibration curve is used for quantification this will result in gross overestimation of the result. The case will be reversed in the case of LC-MS/MS, as there is matrix suppression of the analyte signal. So, using a solvent-based calibration curve here will result in gross underestimation of the result. This problem can be addressed in different ways, as described in Chapter 2. By far the most common way to accomplish this is by using matrix-matched calibration standards, prepared from samples known to be free from the analyte under consideration, as shown schematically in Figure 1.25. This was the approach followed in Chapters 3 and 4, for optimizing the sample preparation methods for spices. Another way to mitigate ME is to use additives in the solvent-based calibration standard to mimic the matrix, so as to equalize the response of an analyte in solvent and matrix extract. The use of this approach in GC-MS/MS and LC-MS/MS is considered in the following sections.

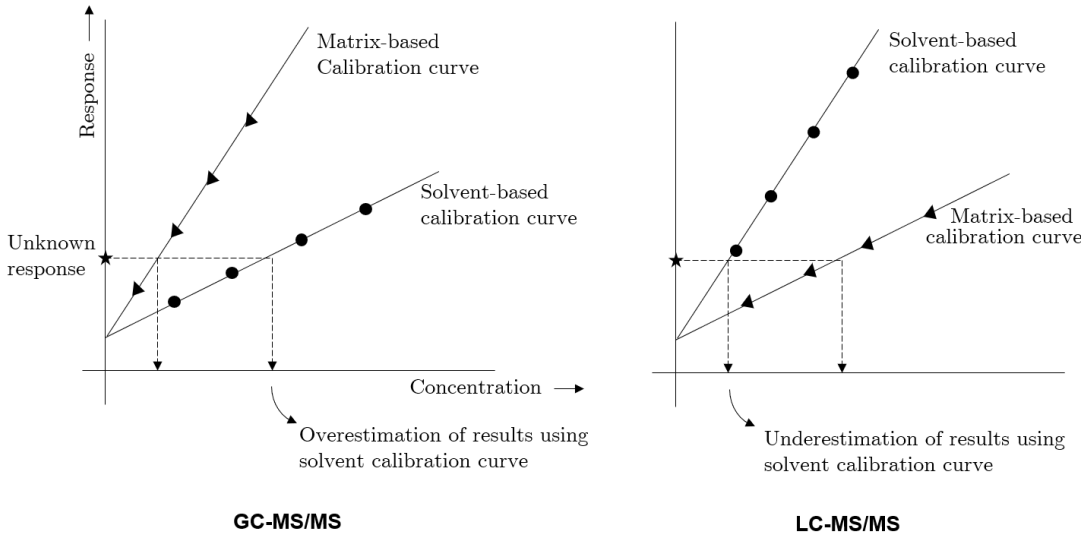


Figure 1.25 Schematic representation of quantitation issues due to matrix effects

Matrix Effects in GC-MS/MS

As explained in Chapter 4, in GC-MS/MS, solvent-based standards give relatively low response as compared to matrix-based standards and thus matrix-matched calibration is in general an unavoidable procedure in trace level quantitation. Availability of blank spice matrices free from large number of pesticide compounds is a difficult task, and preparing blank extracts and matrix-based standards for each analysis is also time-, labour- and resource-intensive. In view of this, an alternate approach for mitigating matrix effects is to use additives in the solvent standard which would behave in a similar manner as the matrix and thus reduce the difference in response of analytes in solvent and matrix extracts. These additives, in the context of GC-MS/MS, are typically called analyte protectants, because they 'protect' the analytes from getting absorbed in the actives sites in the GC injection system.

Table 1.16 List of analytes and GC-MS/MS retention times (t_R)

Compound	**t_R (Min)**
Ethoprophos	7.426
Phorate	7.899
Disulfoton	8.859
Chlorpyrifos-methyl	9.491
Vinclozolin	9.524
Parathion-methyl	9.596
Fenitrothion	10.008
Parathion	10.431
Fipronil	10.904
Piperonyl butoxide	13.936
Bifenthrin	14.406
Fenpropathrin	14.599
l Cyhalothrin	15.189
g Cyhalothrin	15.369
Cyfluthrin Isomers	16.669
Cypermethrin Isomers	17.048
Fenvalerate I	17.965
Fenvalerate II	18.176
Deltamethrin	18.777

The most effective analyte protectants (APs) are compounds with multiple hydroxyl groups, which can bind to the active sites in the GC injection system through hydrogen bonds and thus block these active sites from interacting with the target analytes. In order to accomplish this, the APs are added to both the solvent-based calibration standards as well as test solutions, at concentrations far exceeding the expected concentration of the target compounds. In this section, the efficacy of using APs as an alternative to matrix matched calibration in spices is explored. The list of pesticides used in this study, with their corresponding retention times in GC-MS/MS, are given in Table 1.16 above.

Matrix effects in GC-MS/MS analysis of pesticides

In evaluating the matrix effects in spices using GC-MS/MS, three representative spices, *viz.* cardamom, cumin and chillies were studied. Blank samples of these three spices were extracted using the optimized sample preparation and cleanup procedures developed in Chapter 4. These cleaned extracts were used for preparation of calibration standards. A comparison of the calibration curves in the solvent and the three matrix extracts in a representative analyte, bifenthrin, is shown in Figure 1.26 below. It was observed that all matrices showed considerable response enhancement as compared to the solvent standard. The highest enhancement was seen in cumin, followed by chillies and cardamom. For the solvent-based calibration curve, both the response, expressed in peak area, and the linearity, expressed as the regression coefficient R^2 where low. Matrix matched calibration standards in all three spices showed marked increase in response as well as linearity.

Matrix effects were calculated using the following equation:

$$ME\ (\%) = \left(\frac{S_m - S_s}{S_s}\right) \times 100$$

where S_m is the slope of the matrix matched calibration curve, and S_s is the slope of the solvent-only calibration curve.

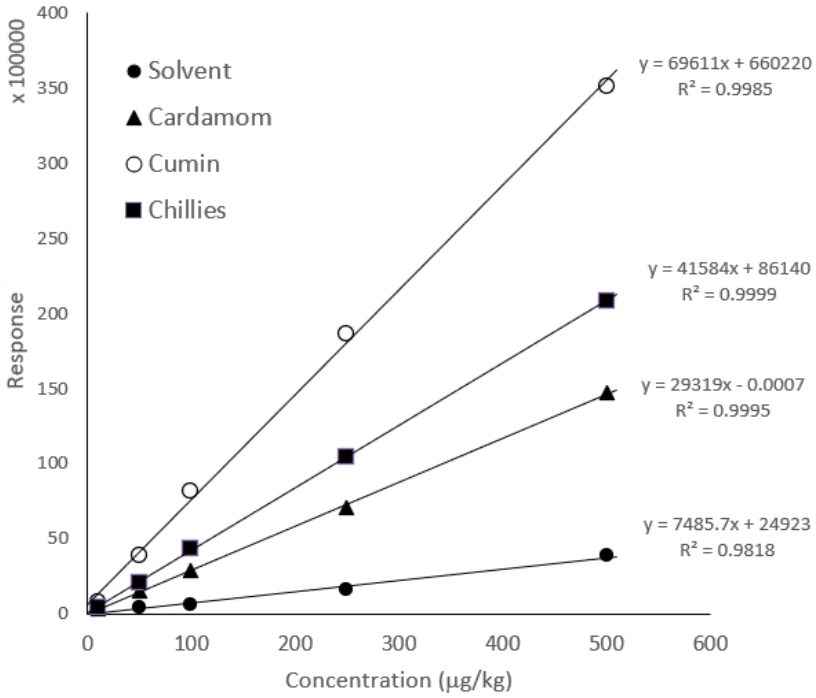


Figure 1.26 Comparison of calibration curves in solvent and spice extracts in bifenthrin

The matrix effects exhibited by the three representative spices for various pesticides is shown in Figure 1.27. It was seen that for most of the pesticides, there was considerable matrix enhancement, except in the case of vinclozolin, where a small amount of signal suppression was observed. The highest matrix effects were observed for Fenitrothion and parathion. In nearly all cases cumin showed the highest matrix effect, except in the case of fenitrothion and methyl parathion, where chilli showed the highest matrix effects. The matrix effect observed in cardamom was the lowest in call cases. Overall, the matrix effects ranged from -27% in the case of vinclozolin (cumin) to 64,107% in the case of fenitrothion (chillies). These high values made it necessary that without

addressing these matrix effects, reliable quantitation will not be achieved in all three spices. By default, matrix matched calibration is used for this purpose, which is time consuming and tedious. In the following sections, use of APs as viable alternatives to matrix matched calibration in GC-MS/MS is investigated.

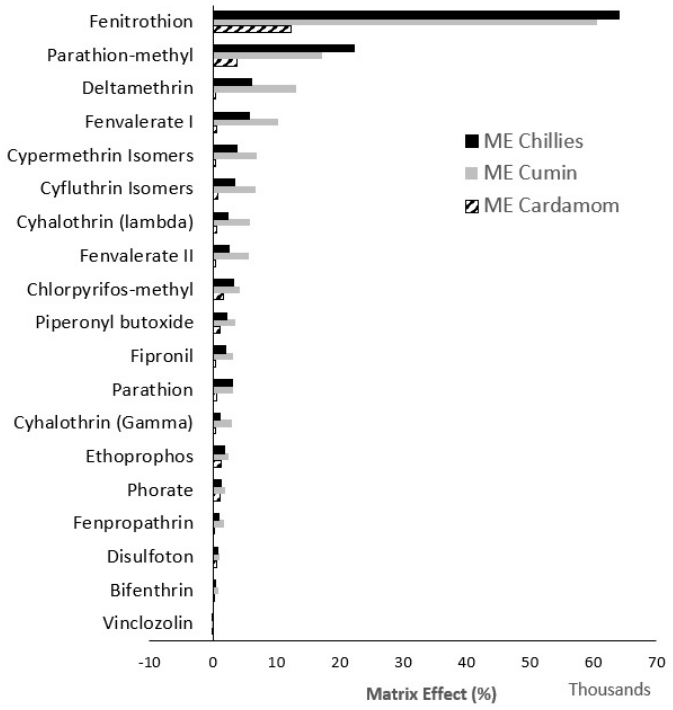


Figure 1.27 GC-MS/MS Matrix effects for pesticides observed in three spices

Chemicals as analyte protectants

Out of the several compounds reported in literature as having good analyte protectant effects, the four chemicals shown in Figure 1.28, covering different volatility ranges are known to give best results [74,75,80,86]. Thus, these four compounds, *viz.* ethylene glycerol, shikimic acid, sorbitol and delta-gluconolactone were selected as APs for studies on mitigation of matrix effect in analysis of spices by GC-MS/MS. All these compounds have multiple hydroxyl groups. Using a mixture of these compounds in a solvent-based

calibration standard can protect the analyte molecules from getting adsorbed in the active sites of the GC injection system and thus result in response enhancement and better peak shapes.

The compounds were used to prepare an AP mixture ensuring that the concentrations of the APs were much higher than the expected concentration of the analyte, and the effects of addition of this mixture in solvent-based standards were studied.

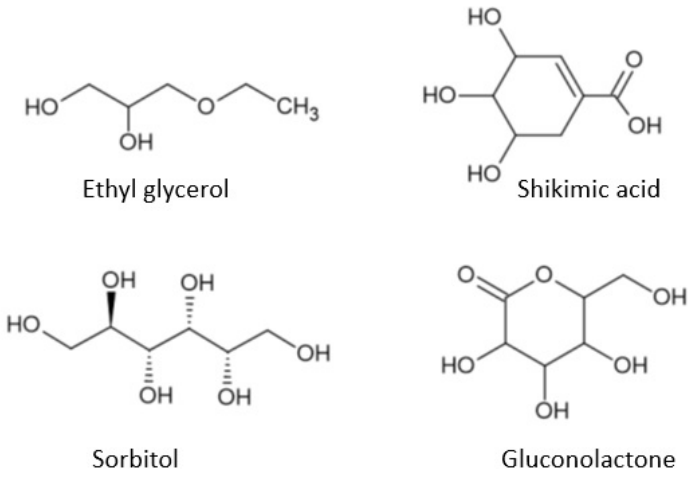


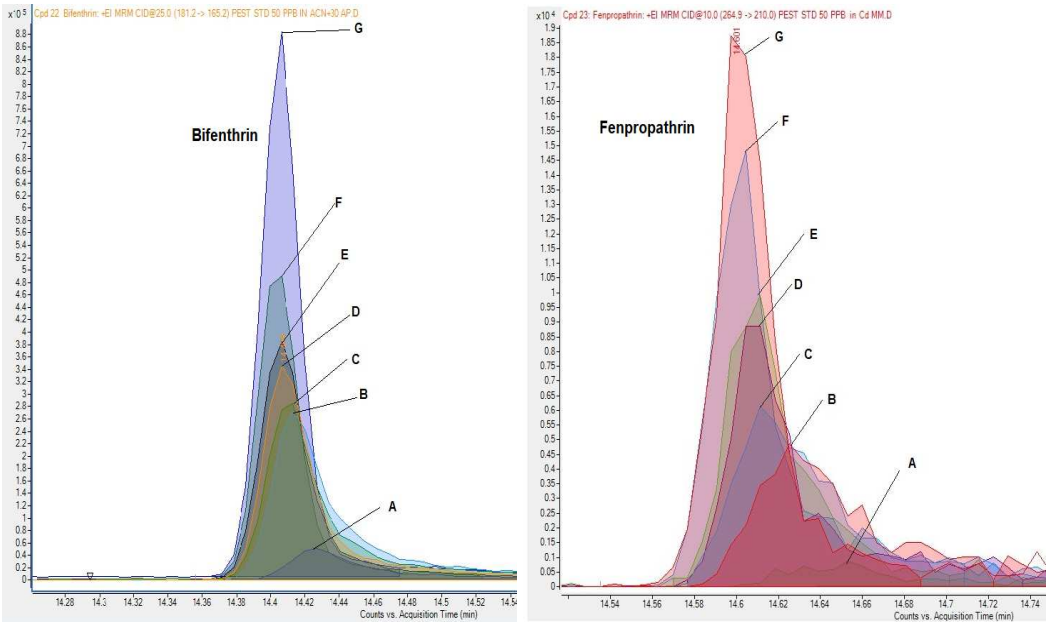
Figure 1.28 Chemical structures of analyte protectants used in the study

The AP mixture for the study was prepared as described in Chapter 2. For assessment of mitigation of matrix effects, varying quantities of the AP mix solution (10, 20, 30, 50 and 100 µl) per ml of sample extract were added to solvent-based calibration standards at 50 mg kg^{-1} concentration, and the increase in responses of the analytes thus achieved was compared with the responses in spiked solutions of blank matrix extracts at the same concentration.

It was observed that the addition of AP mixture had its positive effect on peak shape of the analytes. Figure 1.29 shows the comparison in response of two representative compounds bifenthrin and fenpropathrin at 50 µg kg^{-1} concentration, in extract from

cardamom matrix and in acetonitrile with varying amounts of AP mixture added. Bifenthrin shows high sensitivity in GC-MS/MS, whereas fenpropathrin shows comparatively lower sensitivity. It was seen that the effect of AP is more marked in fenpropathrin when compared to bifenthrin.

As expected, matrix matched standards gave the best peak shapes and highest responses, and in solvent-based standards the peak shapes and responses were poor. The peak shapes progressively improved with addition of increasing quantities of AP mixture in solvent-based standards (10, 20, 30, 50 and 100 µl). Beyond 100 µl addition, the AP mixtures were not seen to produce significant improvement in peak shapes.



A: no AP added, **B**: 10 µl, **C**: 20 µl, **D**: 30 µl, **E**: 50 µl, **F**: 100 µl, **G:** 50 mg kg^{-1} matrix-matched standards in cumin extract.

Figure 1.29 Effect of volume of AP mix added on peak shapes in 50 mg kg^{-1} solvent-based standard in two representative analytes.

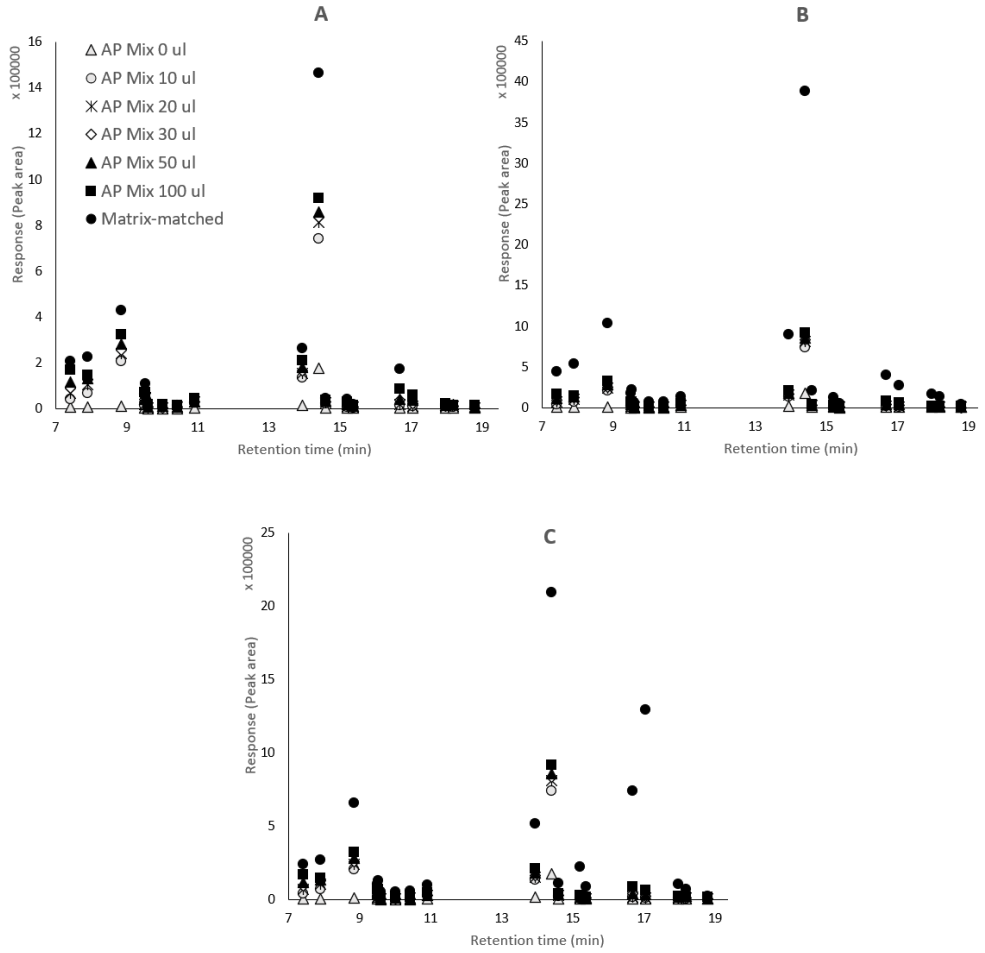
The comparison of responses in solvent-based standards at 50 mg kg^{-1} concentration for various analytes, with and without AP mix addition, as compared to the matrix matched standards at the same concentration, is given in Figure 1.30 for the spices cardamom, cumin and chilli. Here, the responses of standards (peak areas) are plotted against the retention times of the analytes (see Table 1.16).

As in the case of peak shapes, it was seen that response of solvent-based standards in all analytes increased markedly with increase in AP mix volume added, the highest response enhancement observed in the case of 100 µl. Addition of higher volumes of AP mix did not produce marked increase in response. The enhancement effects could be discerned most clearly in analytes that exhibited high sensitivity in GC-MS/MS, e.g., disulfoton (8.859 minutes), bifenthrin (14.406 minutes).

As cumin showed the most matrix effect, the influence of AP mix in solvent standards was least effective in this spice. For cardamom and chilli, the response in solvent standards was increased to a level closer to the matrix matched standards. For analytes exhibiting low sensitivity in GC-MS/MS, the enhancement due to addition of AP brought the peak areas close to that of the matrix matched standards.

From the indications from peak shapes and response enhancement, addition of 100 µl mixture of AP solution to solvent standards promised the most effective mitigation of matrix effects. To verify this, matrix effects were assessed for each analyte in solvent-based standards at 50 mg kg^{-1} with 100 ml of AP mix added, and compared with matrix effects observed at the same concentration in extracts of three spices.

The matrix effects were calculated as per the equation $ME(\%) = \left(\frac{R_M}{R_S} - 1\right) \times 100$, where R_M and R_S are the responses for a particular concentration of pesticide in the matrix extract and solvent respectively.

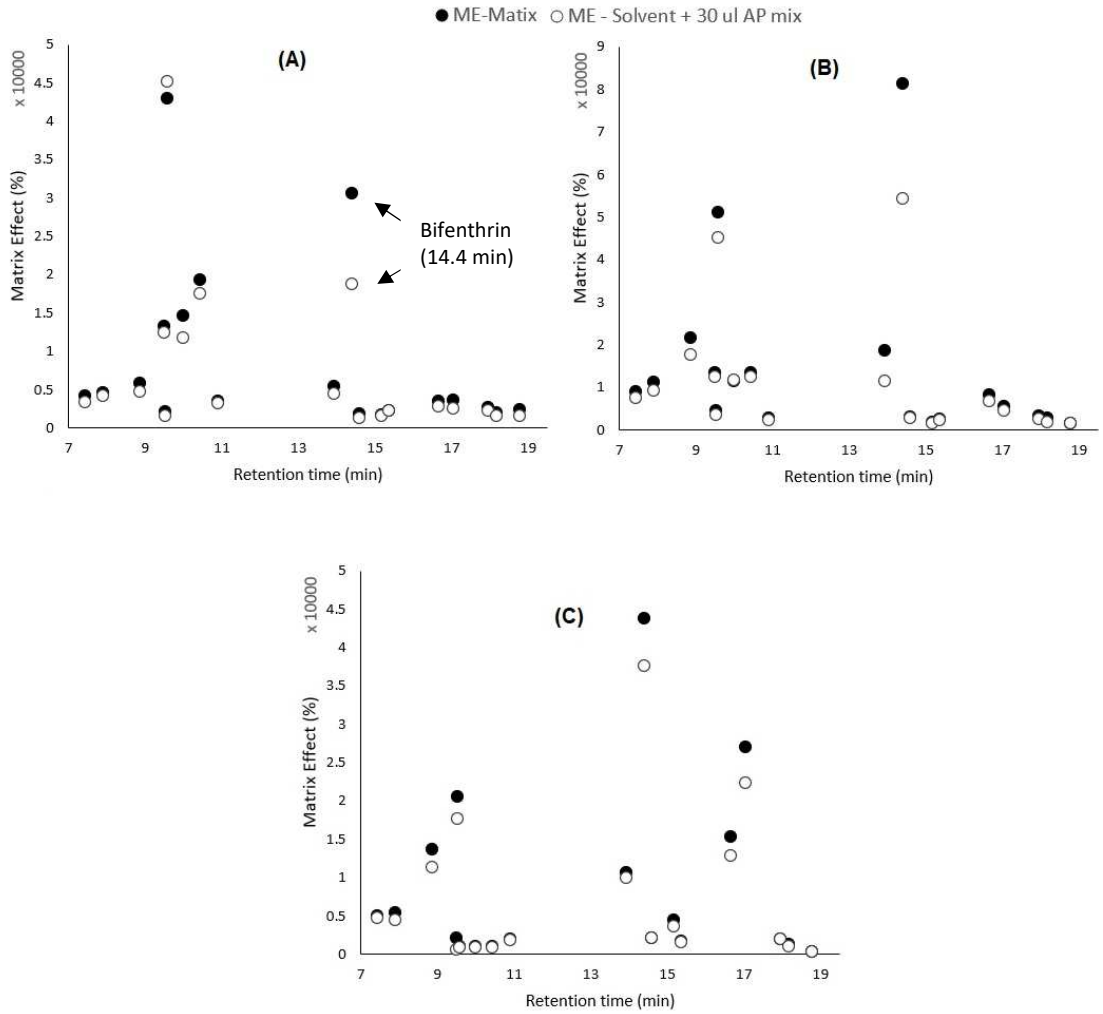


A: cardamom, **B**: cumin **C**: chillies

Figure 1.30 Effect of volume of AP mix added on response in 50 mg kg^{-1} solvent-based standard as compared to response in matrix matched standards.

The efficiency of action of AP were evaluated in terms of the closeness between the MEs observed in the AP-added solvent standard and the matrix matched (MM) standard, at a fixed concentration of 50 µg kg^{-1}. For complete compensation of errors due to matrix effects, the MEs in both AP-added solvent standard and the MM standard should be equal. Larger the deviation of the ME in AP-added solvent standard as compared to that in the MM standard, the lower the efficiency of the action of AP for a particular analyte. So, the efficiency of action of AP was calculated as $E_{AP} = \frac{ME_{AP}}{MM_{AP}} \times 100$, where ME_{AP} is

the matrix effect observed for an analyte in solvent standard containing AP mix, and ME_{MM} is the matrix effect observed in the matrix matched standard of the same concentration.



(A) cardamom, **(B)** cumin, **(C)** chillies

Figure 1.31 Comparison of matrix effects for 50 µg kg^{-1} standards in solvent containing 100 µl AP mix /ml of extract and in extracts of three spices

Considering the fact that ME ≤ ±20% is taken as low (or "soft") ME which does not affect quantification drastically[52,112], the same benchmark was used for E_{AP} also. Thus, $E_{AP} \geq 80\%$ in an analyte was taken as acceptable performance of the AP in mitigating matrix effect in that analyte. Figure 1.31 above shows the comparison of matrix effects in

solvent standard containing AP mix and standard in blank extract for three spices, cardamom, cumin and chillies.

In cardamom, satisfactory mitigation of matrix effects was obtained for 73.6% of the analytes, with EAP values 80.3% and above in these cases. For the remaining analytes, the EAP values ranged from 61.5-73.9%. The lowest effect of AP was observed in the case of bifenthrin. In cumin, the number of analytes exhibiting satisfactory mitigation of matrix effect was slightly lower at 68.4%, having EAP 81.3% and above.

For the remaining analytes the EAP values were between 61.1 and 79.3%. Here, the analyte with lowest EAP was piperonyl butoxide, followed by bifenthrin (66.6%). The best results with regard to analyte protection was observed in the case of chillies, with 84.2% of the analytes showing satisfactory EAP values, 82.4% and above. The remaining compounds had EAP values between 73.9 and 82.4%. Thus, it was seen that addition of 100 µl of AP mix solution per ml of spice extract in the calibration standards could mitigate matrix effects for a large number of analytes, and this was concluded as the optimal amount of analyte protectants for GC-MS/MS analysis of residues in spices. The most efficient analyte protection was obtained in the case of chilli matrix, with coverage of 84.2% of analytes tested, followed by cardamom, with 73.6 % of analytes and cumin, 68.4 % of analytes. The effect of AP was seen to be lower in compounds like bifenthrin which experienced relatively high response enhancement in GC-MS/MS. On the whole, the use of APs was found to be an efficient and convenient way for mitigating matrix effects in GC-MS/MS analysis of residues in spices.

Matrix Effects in LC-MS/MS

In LC-MS/MS also, ME pose hindrance to reliable identification and quantification of analytes at the sensitivity levels demanded by present regulatory requirements for pesticide residues. Accordingly, minimizing matrix effects is an integral part of method

development in high sensitivity pesticide residue analysis. Spices in general possess the special property of having a few prominent chemical compounds, in relatively higher concentrations, that contribute to special properties of colour, aroma and flavour. Because of the prominence of such compounds, it is likely that these compounds also contribute to the matrix effects posed by a particular spice, and thus, using synthetic analogues of these prominent compounds as matrix surrogates in LC-MS/MS calibration standard solutions offers the possibility of mitigating matrix effects in a manner analogous to the use of analyte protectants in GC-MS/MS. Such a study using chillies as a representative spice is covered in this section.

Matrix surrogates to mitigate matrix effects in chillies

In chilies, the chemical compounds that contribute to the pungency are capsaicinoids[121], and those that contribute to the red colour are carotenoids[122]. Pungency in chillies is typically measured in Scoville Heat Units (SHU)[123]. Normally, the capsaicinoid contents in various varieties of chilli range from 100 (very mild) to over 1,500,000 SHU (extremely hot). For normal culinary applications all over the world, chilli-peppers of medium to high pungency, i.e., 30,000 - 80,000 SHU (2000 - 5000 mg kg^{-1}), are used. Among the capsaicinoids, the three most important compounds are capsaicin (CAP), nordihydrocapsaicin (NHC) and dihydrocapsaicin (DHC).

For analysis of capsaicinoid compounds in chillies using HPLC, the synthetic analogue of capsaicinoids, N-vanillyl nonanamide (NVNA) is used as a reference standard in HPLC. This is because pure capsaicin, owing to its pungency, is difficult to handle in laboratory conditions. Relative retention times are then used for identification of the capsaicinoids[124]. Colour in chilli-peppers is usually measured in the American Spice Trade Association (ASTA) colour units, which represents the extractable colour from chilli-peppers in acetone based on absorbance at 460 nm[114]. Normally, the colour in chilli-

peppers range from 40 – 160 ASTA units. The combination of colour and pungency vary widely in different varieties of chillies.

Analysis of pesticide residues in chillies using LC-MS/MS is prone to matrix effects, mainly due to these two classes of compounds in this spice matrix, which produce pungency and colour in this spice. Owing to the fact that chilli-peppers are commercially cultivated most extensively in developing countries where systematic adherence to good agricultural practices is not the norm, it is difficult to obtain pesticide-free matrices for preparation of matrix-matched calibration (MMC) standards. Thus, use of MMC standards for chilli-peppers for routine use in the laboratory is not always feasible. The standard addition technique can effectively account for matrix effects without the need for blank matrix, but this method requires at least two injections per sample and is not practical in routine testing where large numbers of samples are to be analysed. Use of internal standards also has limitations with respect to cost and applicability. So, the possibility of adding the prominent matrix compound present in chillies to solvent-based calibration standards to try and equalize the response of analytes in solvent and matrix, was explored.

Study of composition of chilli extracts after cleanup

The effect of the optimized QuEChERS sample preparation method developed in Chapter 3 on the two main classes of compounds in chilli-pepper matrix, viz. capsaicinoids and carotenoids, was assessed by comparing the extent of reduction of these compounds at the end of the cleanup step.

Blank samples of chillies with varying pungency and colour were screened for pungency and colour using the methods described in Chapter 2. Based on the results of the screening, samples of varying pungency and colour combinations were selected for further evaluations. In the matrix effect study, to represent the range of pungency in chilli-pepper used in typical culinary applications, two chilli-pepper matrices representing low and high

ends of the pungency range commonly used for culinary applications, labelled as **MC1** (pungency 38,100 SHU and colour 106 ASTA units) and **MC2** (pungency 84,600 SHU and colour 81 ASTA units) were selected.

To compare the effects of cleanup on the capsaicinoid content, the extracts before and after cleanup step from the sample MC2 (higher pungency) was injected in HPLC with UV detection at 280 nm under the same conditions used for capsaicinoid estimation. It was observed that the peaks corresponding to the capsaicinoids showed negligible change in peak areas in the extracts before and after cleanup, indicating that the capsaicinoids were left largely unaffected.

To identify the effect of cleanup step on the carotenoid content, after making 100 times dilution of the extracts from **MC1** and **MC2** samples, absorbance at 460 nm was measured on a UV-VIS spectrophotometer, before and after cleanup. It was observed that there was significant reduction in absorbance after the cleanup step, indicating the reduction in the carotenoid content. For the extract from **MC1** (colour value of 106 ASTA units), the decrease in absorbance was 74%, and for the extract from **MC2** (colour value 81 ASTA units), the decrease was 87%. Thus, it was concluded that the optimized cleanup step in the LC-MS/MS sample preparation method principally affected the carotenoids and not capsaicinoids. The effects of cleanup step on the capsaicinoid and carotenoid content are shown in Figure 1.32.

As capsaicinoids from the chilli matrix are largely unaffected by the sample preparation steps, it is evident that these compounds would be the major contributors to matrix effects in this spice. Thus, by using a compound analogous to capsaicinoids in the solvent-based standards, similar to the way analyte protectants are used in GC, the possibility of mitigating matrix effects in chillies could be explored.

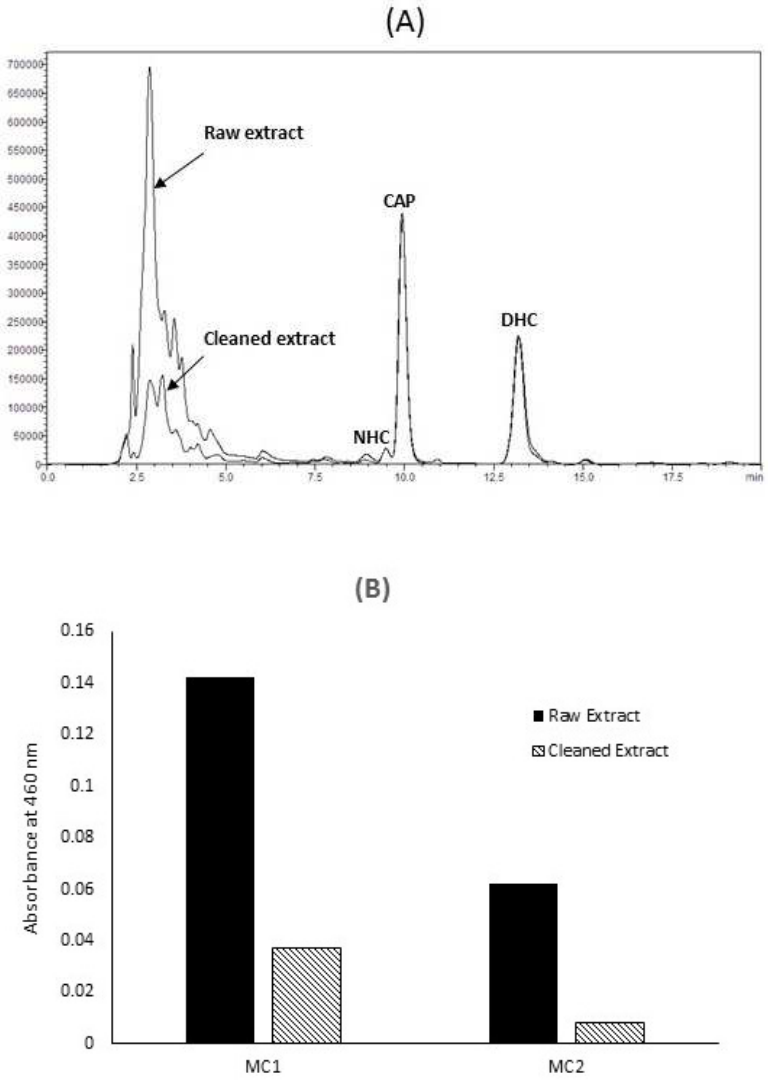


Figure 1.32 Effect of optimized cleanup step during sample preparation, on **A** - capsaicinoid content (NHC: nordihydrocapsaicin, CAP: capsaicin, DHC: dihydrocapsaicin) and **B** - carotenoid content of the chilli extract.

The naturally occurring range of capsaicinoids in chilli-peppers used in normal culinary applications is 2000 - 5000 mg kg^{-1}. This is very much higher than expected concentrations of the target compounds in pesticide residue analysis, and as such high endogenous concentrations will always be present in chilli-pepper extracts. It offered the possibility of using a matrix surrogate compound in calibration solutions prepared in acetonitrile to account for matrix effect in chillies. Synthetic capsaicin or NVNA, which

is a close analogue to the capsaicinoids, was deemed to be a good candidate for use as a matrix surrogate. Figure 1.33 shows the structures of the main capsaicinoids and NVNA.

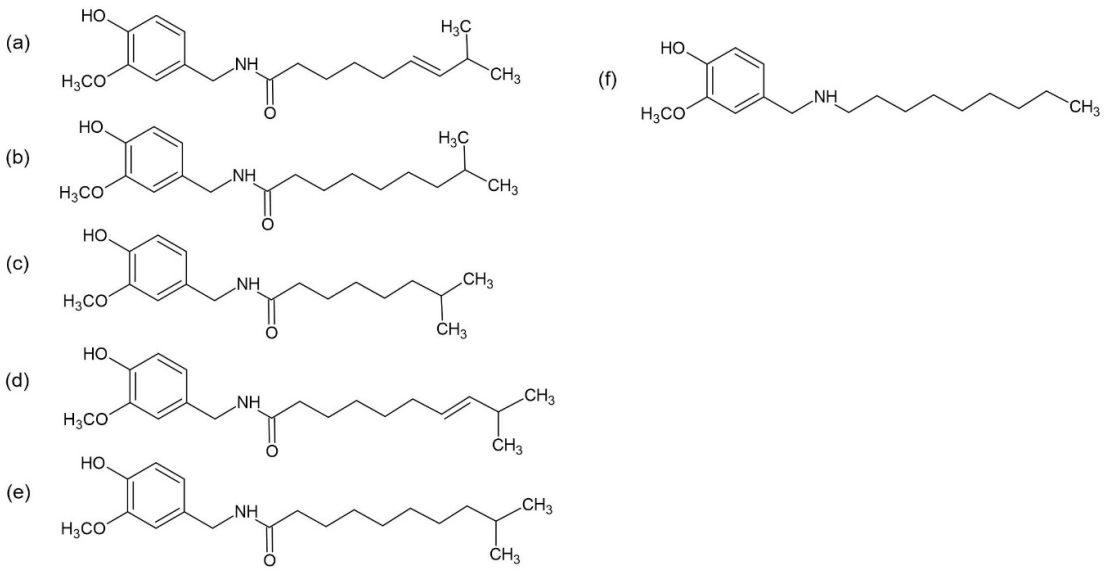


Figure 1.33 Chemical structures of capsaicinoids in chilli-peppers and synthetic capsaicin: (a) capsaicin, (b) dihydrocapsaicin, (c) nordihydrocapsaicin, (d) homocapsaicin, (e) homodihydrocapsaicin, (f) N-vanillylnonanamide (NVNA, synthetic capsaicin).

Use of NVNA as a matrix surrogate for analysis of chilli samples

While selecting chilli matrices for this study, there were two important constraints. The first was that the two matrices chosen, *viz.* **MC1** and **MC2**, should not have traces of any of the pesticides used for evaluation of the matrix effects. Secondly, matrices themselves had to meet requirements of capsaicin content (high and low pungency respectively). Because of these constraints, the number of analytes fixed for the study were limited to the following 29 compounds: acephate, ametoctradin, buprofezin, carbaryl, carbofuran, cyantraniliprole, dimethenamid, emamectin benzoate, ethion, fenarimol, fenhexamid, fenpyroximat, fluopicolide, hexaconazole, imidacloprid, iprobenphos, metalaxyl, methiocarb, methoxyfenozide, pirimiphos-methyl, pyraclostrobin, quinalphos, quinoxyfen, spinosad-A, spinosad-D, spirodiclofen, thiacloprid, triadimefon and

trifloxystrobin. The optimized chromatographic and mass spectrometric conditions for these pesticides were covered in tables 1.4 and 1.5 respectively in Chapter 3.

Post extraction spiked solutions of pesticide standards at 0.01 mg kg^{-1} were prepared in acentonitrile, containing concentrations of NVNA ranging from 10 to 50 mg kg^{-1}. that the Matrix effects were then calculated using the following equation for each analyte:

$$ME\ (\%) = \left(\frac{R_{matrix}}{R_{solvent}} - 1\right) \times 100,$$

where R_{matrix} and $R_{solvent}$ are the responses for 0.01 mg kg^{-1} analyte concentration in the matrix extract and solvent respectively. The matrix effects posed by these solutions were compared with those for the same concentration of pesticides in extracts from the samples **MC1** and **MC2**. From the results it became evident that increase in NVNA concentration reduced the difference between matrix effects of the extracts and the surrogate solution, but even at NVNA concentration of 50 mg kg^{-1}, the matrix effect in surrogate solution remained considerably lower.

In order to avoid using higher concentrations of NVNA in the surrogate matrix, this approach was coupled with dilution of extracts. Thus, post extraction spikes of 0.01 mg kg^{-1} were prepared in extracts of **MC1** and **MC2** diluted to 10%, 25%, 50% and 75% and the matrix effects in these solutions were compared to those in the surrogate matrix solution containing 50 mg/kg NVNA. It was observed that good agreement between matrix effects could be obtained by combining 50% extract dilution with calibration using surrogate matrix solution containing 50 mg/kg NVNA. The matrix effect values for the undiluted extracts and 50% diluted extracts are shown in Table 1.17.

For an analyte concentration of 0.01 mg kg^{-1}, matrix effects seen in 50% diluted extracts were found to be closely matching with the matrix effect seen in an acetonitrile solution containing 50 mg kg^{-1} NVNA matrix surrogate. This is shown in Figure 1.34.

Table 1.17 Comparison of matrix effect between extracts of samples MC1 and MC2, with and without dilution

Compound	Sample MC1		Sample MC2	
	ME (%), 0% dilution	**ME (%), 50% dilution**	**ME (%), 0% dilution**	**ME (%), 50% dilution**
Acephate	-35.54	-28.56	-38.33	-32.66
Imidacloprid	-26.3	-20.3	-30.6	-28.2
Ametoctradin	-22.77	-15.25	-28.7	-21.6
Thiacloprid	-44.41	-32.9	-52.3	-39.94
Carbofuran	-37.51	-33.47	-41.69	-38.2
Carbaryl	-44.26	-30.93	-57.27	-36.77
Cyantraniliprole	-18.98	-15.51	29.3	-19.86
Metalaxyl	-29.11	-24.02	-23.13	-29.58
Dimethenamid-P	-34.22	-26.14	-45.97	-32.73
Methiocarb	-32.18	-26.48	-39.53	-31.81
Fluopicolide	-9.18	-1.38	-15.74	-6.73
Triadimefon	-2.29	1.6	-8.35	-2.3
Methoxyfenozide	-27.38	-10	-40.11	-28.01
Fenhexamid	-99.42	-85.51	-99.76	-95.91
Fenarimol	-22.64	-8.24	-42.35	-20.1
Quinalphos	-3.08	5.32	-9.32	1.4
Iprobenphos	-14.3	-8.43	-19.94	-10.47
Pirimiphos methyl	-22.72	-9.79	-29.04	-17.97
Hexaconazole	-11.21	-1.42	-18.15	-6.52
Pyraclostrobin	-25.17	-7.39	-3.54	-4.13
Spinosad A	-14.41	-2.49	-20.32	-8.6
Trifloxystrobin	-16.05	-1.7	-22.3	-8.16
Buprofezin	-15.9	-9.84	-29.53	-15.15
Spinosad D	-18.02	-4.71	-25.63	-8.08
Quinoxyfen	-13.94	-7.16	-6.09	-10.76
Ethion	-20.31	-8.96	-25.37	-10.12
Emamectin benzoate	-12.38	-2.6	-18.6	3.45
Spirodiclofen	-10.35	0.04	-17.32	0.56
Fenpyroximate	-27.04	-8.13	-32.72	-12.06

For 0.01 mg kg^{-1} concentration of pesticides, the difference in matrix effect (%) between 50% diluted extract and in 50 mg/kg NVNA solution varied from -8.9 to 12.5 in **MC1** extract and from -19.6 to 20.9 in **MC2** extract. Moreover, this difference was within ±10 for 93% of the pesticide studied in the case of **MC1**, and for 70% in the case of **MC2**.

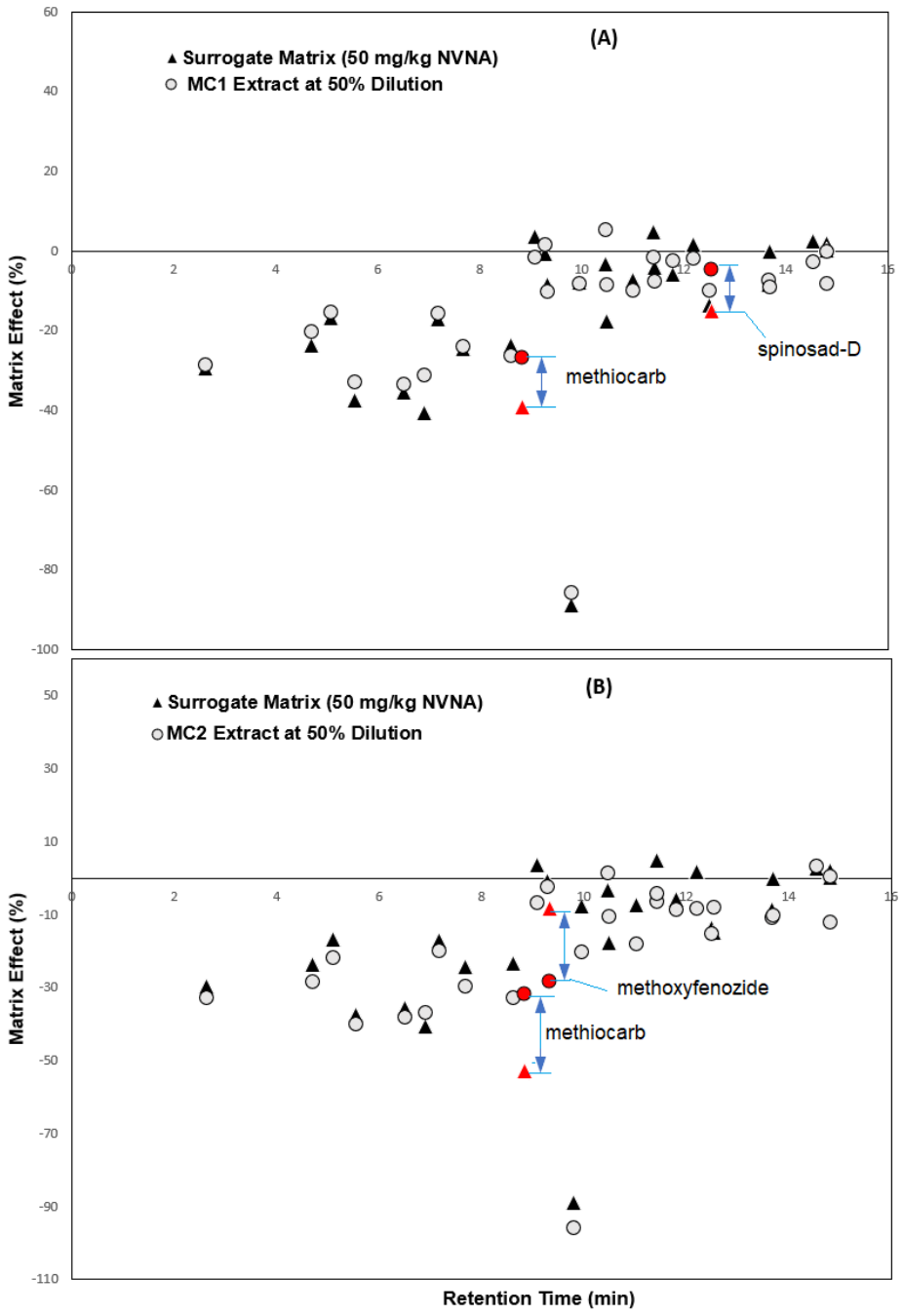


Figure 1.34 Matrix effects of pesticides at concentration of 0.01 mgkg^{-1} in (A) surrogate matrix with 50 mg kg^{-1} NVNA & MC1 (38,100 SHU) matrix extract diluted to 50%, and (B) surrogate matrix with 50 mg kg^{-1} NVNA & MC2 (84,600 SHU) matrix extract diluted to 50%.

The increase in variation in matrix effect for the sample with higher pungency shows that the ability of NVNA to function as a matrix surrogate is more effective in chilli-

pepper with medium pungency. In **MC1** matrix, the variation in matrix effect (%) between extract with 50% dilution and in solvent containing 50 mg kg^{-1} NVNA surrogate ranged from -8.9 in ethion to +12.5 in methiocarb. In addition to methiocarb and ethion, high variations were observed in spinosad-D (+10.2), fenpyroximat (-8.5), quinalphos (+8.4), iprobenfos (+9.2) and carbaryl (+9.8). For all other analytes, the variation was < ±10. Also, variation was < ± 5 in the case of 65% of the analytes, and in two cases were nearly equal to zero, *viz*. metalaxyl (+0.4) and acephate (+0.9). For the matrix MC2 with higher pungency, the picture was more complex. Here, the variation in matrix effect (%) between analytes in extract with 50% dilution and in solvent containing 50 mg kg^{-1} NVNA surrogate ranged from -19.6 in methoxyfenozide to +20.87 in methiocarb. In addition to methoxyfenozide and methiocarb, highest variations were observed in fenpyroximat (-12.4), fenarimol (-12.3), hexaconazole (-11.34), pirimiphos methyl (-10.6), flupicolide (-10.5) and ethion (-10.6). Except in the case of methiocarb, none of the compounds showed variation > +10. Here, only 51% of the analytes showed variation of < ±5.

Overall, it was clear that use of 50 mg/kg NVNA solution as a matrix surrogate, coupled with 50% extract dilution, was viable in the case of chilli-peppers with a wide range of pungency for commonly used pesticides in the cultivation of this spice. The process could be seen to be most effective in the case of medium pungency chillies, and the surrogate performance decreased when the pungency of the matrix increased.

Application to real samples

The effectiveness of the surrogate matrix method in compensating for the matrix effect in chillies was studied by analysing real samples with incurred residues using this new method. The methodology chosen was to analyse same set of samples first with an established method and then by the newly developed method, so that comparison of the results indicated the accuracy of the new method.

In the present case the established method chosen was standard addition[65–67]. This method is frequently used to analyse samples with incurred residues where a blank matrix for preparation of matrix matched calibration standards is not available.

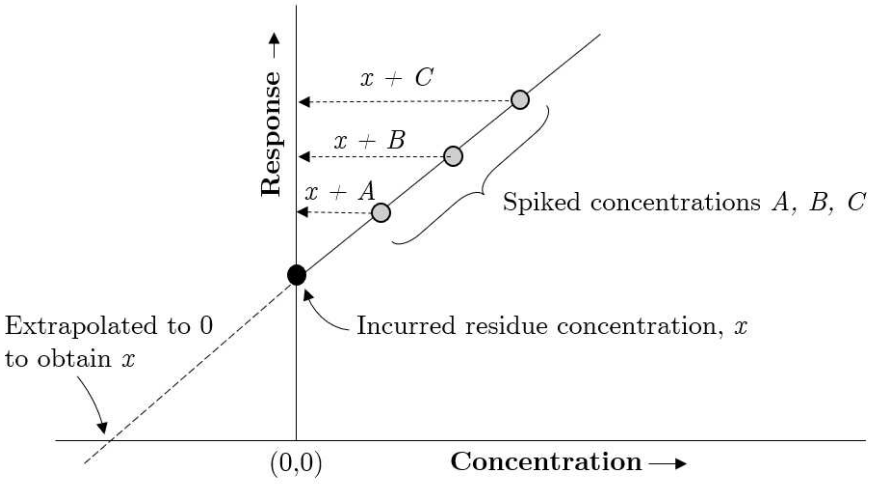


Figure 1.35 Schematic illustration of standard addition technique

The technique involves spiking different aliquots from the extract of the sample with incurred residues with 2 – 3 concentration levels of the analyte being tested for, and injecting in the LC-MS/MS. The resulting calibration line is then extrapolated to the X-axis resultant calibration line was extrapolated to the X-axis to obtain the incurred residue concentration[112], as shown in Figure 1.35. This post-extraction standard addition effectively accounts for matrix effect without the requirement of a blank matrix.

For the present study, three chilli samples with pungency values 36,200, 44,200 and 58,100 SHU and with incurred residues ranging from 0.01 to 0.1 mg kg^{-1} were first analysed in triplicate using the standard addition method. Three aliquots from extracts of these samples were spiked with 0.05, 0.10 and 0.15 mg kg^{-1} of the detected analytes and injected in UPLC-MS/MS, and the resultant calibration line was extrapolated to the X-axis

to obtain the incurred residue concentration. The same samples were further analysed in triplicate using the approach developed in this study, i.e., 50% diluted QuEChERS extracts of test samples quantified against solvent-based calibration standards containing 50 mg kg^{-1} NVNA solution as matrix surrogate. The average results obtained from the use of standard addition technique and the surrogate-matrix based calibration were compared to assess the efficacy of the latter approach for quantification of residues in chilli-peppers. `The compounds detected in the three samples were imidacloprid, buprofesin, quinalphos, ethion, metalaxyl, carbofuran, carbaryl and iprobenfos, and the residue concentrations ranged from 0.070 to 0.102 mg kg^{-1} (70 – 102 μg kg^{-1}). The comparison of the average results obtained in both the experiments is shown in Figure 1.36.

The errors, taken as deviation of the average result obtained by surrogate matrix method from the average results obtained by standard addition method, ranged from -0.08 to +0.09 mg/kg. Overall precision was seen to better in the case of surrogate matrix calibration approach, with %RSD in the range 1.1 - 13.3, as compared to the standard addition approach, 3.4 - 15.6. It was seen that there was close agreement between the results obtained by the two approaches. As expected, the largest variations in results between the two methods was observed in the sample which showed the maximum pungency.

The practical application of this approach becomes evident when a routine analysis batch in the laboratory contains a large number of chilli-pepper samples and a suitable blank matrix is not available for preparation of matrix matched calibration standards. Standard addition method, which is the most common course of action in such cases necessitates at least one screening run of all samples and then two further injections with standard addition for all samples which show incidence of residues. Using 50 mg kg^{-1}

NVNA based surrogate matrix for preparation of calibration standards, coupled with 50% dilution of the QuEChERS extract, can thus significantly save effort and instrument time.

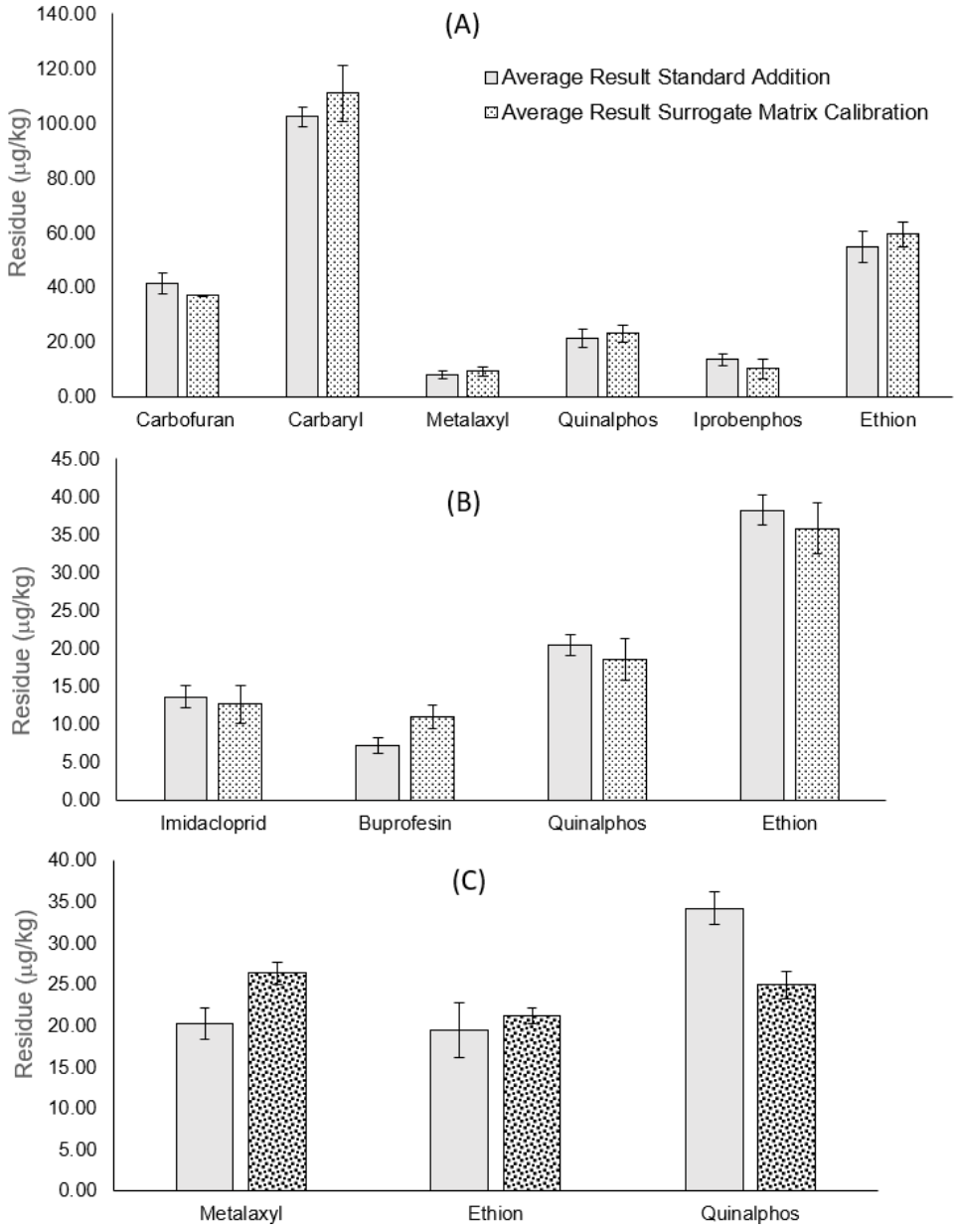


Figure 1.36 Comparison of average values of residue results (µg/kg) obtained using 3-point standard addition and surrogate matrix-based calibration (n=3) for three chilli-pepper samples with incurred residues: (A) sample S1 (36,200 SHU), (B) Sample S2 (44,200 SHU) and (C) Sample S3 (58,100 SHU).

This approach could be extensible to other spices also, because spices typically contain a small number of active compounds in concentrations high enough to make major contribution to matrix effects, e.g., curcuminoids in turmeric, piperine in black pepper etc.

Conclusions

Spices pose considerable matrix effects in pesticide residue analysis using both GC-MS/MS and LC-MS/MS. In GC-MS/MS, this effect involves enhancement in responses of analytes, whereas in LC-MS/MS, suppression in response of analytes is normally observed. In either case, matrix effect seriously undermines analytical accuracy when using solvent-based reference standards, and matrix matched calibration is the most used technique to address the issue of matrix effects. However, this requires availability of blank matrices and additional work to prepare matrix matched calibration standards. In this Chapter, two alternate ways of addressing matrix effects, in GC-MS/MS and LC-MS/MS respectively, were explored.

The use of a mixture of analyte protectants containing ethylene glycerol, shikimic acid, sorbitol and δ-gluconolactone, was found to be an efficient and convenient way of mitigating matrix effects in spices without the use of matrix matched calibration standards. In the study using 3 representative spices, *viz.* cardamom, cumin and chillies, and 19 representative analytes, it was found that adding 100 µl of AP mix / ml of the solvent-based calibration standards showed ME in close approximation to MMC standards. The best results for the use of AP in mitigating ME was found in chillies, followed by cardamom and cumin.

Spices are characterized by certain chemical compounds that contribute to the principal properties like aroma, colour, pungency, flavour etc, and are also present in relatively large quantities in the matrix. This offers the possibility of mitigating matrix effects by adding synthetic analogues of such compounds to solvent calibration standards

as matrix surrogates. This was successfully demonstrated using chillies as a representative spice. The capsaicinoids present in chillies were identified as the main chemical component that causes matrix effects in this spice, and by adding 50 mg kg^{-1} of N-vanillyl nonanamide (synthetic capsaicin) in solvent based calibration standards and introducing 50% dilution in QuEChERS extract of the spice was seen to reduce the matrix effect in chillies to <10% in 93% of the pesticide compounds studied in the case of medium pungency chillies, and for 70% of the compounds in the case of higher pungency chillies. These approaches were tried out successfully in real samples with incurred residues.

CHAPTER 6

ANALYSIS OF DITHIOCARBAMATES IN SPICES

Dithiocarbamate (DTC) fungicides are used extensively for the control of fungal diseases in plants due to their comparatively low toxicity profiles and their low cost of manufacture. Their use is prevalent in two important spices, *viz*. small cardamom or Malabar cardamom (*Elettaria cardamom*) and black pepper (*Piper nigrum*), both of which are traded globally and used extensively across the world.

DTC fungicides are generally non-systemic in nature and, due to their low solubility in water, are likely to remain at the site of application without much dissipation into the environment. Thus, monitoring residues of DTC for compliance with international regulations and for assessing food safety risks is an important consideration. Regulatory agencies have stipulated maximum residue limits (MRLs) for DTC residues in these two spices. For example, the Codex Alimentarius Commission has fixed an MRL of 0.1 mg kg^{-1} for DTC residues in cardamom and black pepper[125,126]. The European Union (EU) also has set a maximum residue limit (MRL) of 0.1 mg kg^{-1} for DTC residues in cardamom and seed spices[127]. This chapter documents the development and validation of a GC-MS method for analysis of DTC residues in cardamom and black pepper, which is sensitive enough to meet the requirements of the international regulatory MRLs mentioned above.

Structure of DTC compounds

The dithiocarbamate class has a number of compounds. Based on their chemical structure, DTC compounds can typically be categorized into three subclasses, viz. dimethyldithiocarbamates (DMDs), ethylenebisdithiocarbamates (EBDs), and propylenebis dithiocarbamates (PBDs). Another subclass of compounds that belong to both DMD and EBD are also defined, called polycarbamates[99,109]. Typically, these

compounds exist complexed with transition metal ions, with the exception of thiram, dazomet and milneb, of which thiram is the simplest compound. Metiram is a mixture of polythiuram disulfides and zinc ammoniate bis(dithiocarbamate). Figure 1.37 shows the chemical structures of important DTC compounds.

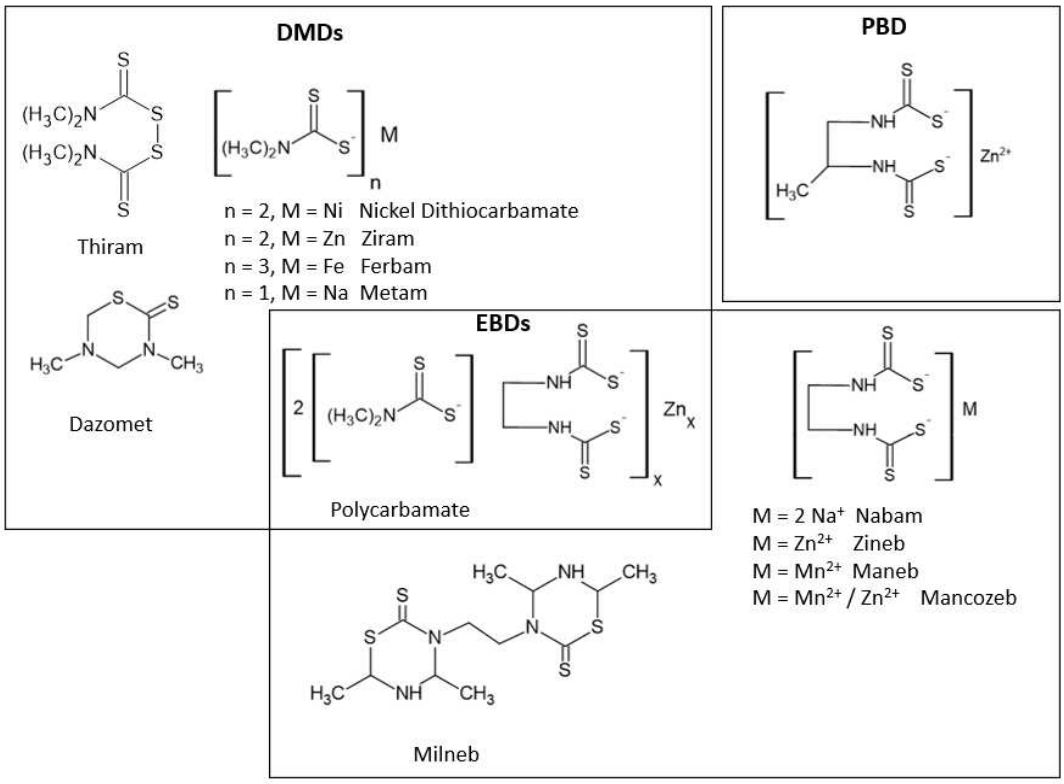


Figure 1.37 Chemical structures of important dithiocarbamate fungicides

There are two important problems associated with the analysis of DTC residues: solubility and stability[99]. Among the DTC compounds, ziram, ferbam and thiram are sparingly soluble in water, and soluble in some organic solvents like chloroform, carbon disulphide, acetone and acetonitrile. The compounds metam and nabam are soluble in water, but less so in organic solvents. Apart from these compounds, the majority of the DTC compounds are practically insoluble in water and organic solvents alike.

The solubility issues among DTC compounds mean that it is practically impossible to devise a single extraction method that can reliably extract all the DTC compounds together. Apart from this solubility issue, DTC compounds become unstable when coming into contact with plant extracts with low pH, and decompose into carbon disulphide (CS_2) and the corresponding amino compound. Thus, extracting a homogenized plant matrix using polar or organic solvents, which is the normal method for residue analysis, is not found to be effective in the case of DTC residues. So, the most effective method to analyse DTC compounds is to convert them quantitatively to CS_2, absorb the CS_2 thus evolved in a nonpolar solvent, and quantify the CS2 as representing total DTC compounds present.

Since DTC compounds are non-systemic and are expected to be present only as a surface contamination, homogenization is not considered to be an important step in DTC analysis. However, spices are usually used in ground / crushed forms in culinary applications and spices like cardamom and black pepper have significant amounts of nonpolar essential (volatile) oils in them. Thus, the possibility of interference of the chemical components in these essential oils, in the formation of CS_2 evolved from DTC compounds during analysis, is an important factor to be considered in optimizing this method for spices.

The sample preparation and extraction methods for DTC analysis were detailed in Chapter 2. The method involves cleavage of DTC compounds using a mixture of $SnCl_2$ and HCl, and CS_2 which gets released is absorbed into isooctane. The total CS_2 thus produced is analysed using GC-MS using selected ion monitoring (SIM) mode. Thiram was used as a representative DTC compound in all recovery studies, considering its simple chemical formula, and taking into account that 1 mole of thiram corresponds to 2 mols of CS_2 as shown in Figure 1.38. The purity of the thiram reference standard (99.5%) was

accounted for in the recovery studies, and the control samples used for spiking were screened to ensure absence of DTC residues before commencing the optimization studies.

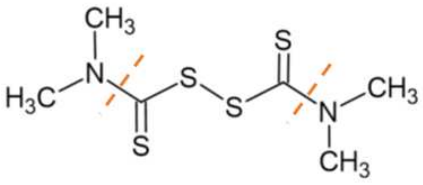
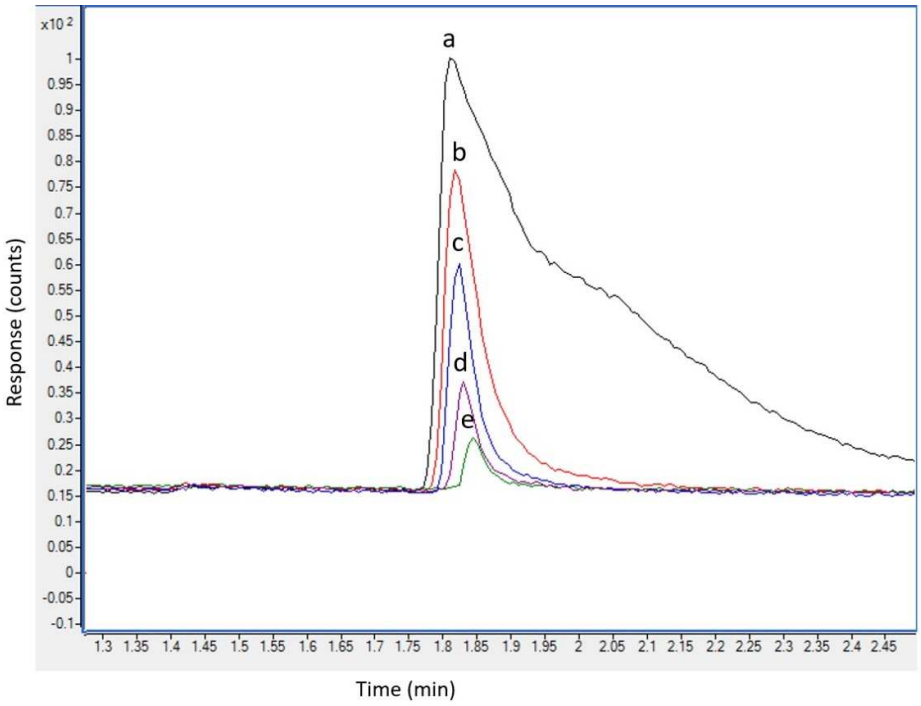


Figure 1.38 Cleavage of thiram to form CS_2

Optimization of instrumental conditions

Splitless injection in GC was observed to be unsuitable for obtaining good chromatographic resolution under the experimental conditions used. Hence optimization of CS_2 on GC-MS, by monitoring the ion with m/z 76, was done in split injection mode. At a low split ratio of 0.1:1, the response was good but the peak shape was not suitable for quantitative analysis.

On progressively increasing split ratio to optimize peak shape and response, it was noted that for lower split ratios of 0.1:1 and 10:1, the peak shapes obtained were not appropriate, and for higher split ratios of 50:1 and 100:1, the peak shapes were better but responses were low. Thus, the medium split ratio of 20:1 with a corresponding split flow of 22.066 mL min^{-1}, which afforded good response and peak shape, was adopted as the optimum setting with a retention time of 1.82 minutes. Optimization of split ratio based on the peak shape and response level obtained for CS_2 concentration of 0.1 mg kg^{-1} is shown in Figure 1.39.



(a) 0.1:1 / 0.11033 mL/min, **(b)** 10:1 / 11.033 mL/min, **(c**) 20:1 / 22.066 mL/min, **(d)** 50:1 / 55.165 mL/min, **(e)** 100:1 / 110.33 mL/min

Figure 1.39 Optimization of the injection mode in GC-MS. split ratio / split flow

The use of post-run, mid-column backflush facility was seen to be important in obtaining good chromatographic performance. This feature is schematically shown in Figure 1.40. This facility in the GC allowed the flow of carrier gas to be reversed after the elution of the target analyte peak is completed, thereby flushing out the remaining volatiles in the injection. This gives additional protection to the column, and helps to keep the column clean and ready for the subsequent injection by the time the run is completed. Although including this extended the total runtime by 10 minutes, it was found to be extremely helpful in maintaining consistency of instrument response, especially after multiple injections in a batch run for complex spice matrices like cardamom and black pepper. The optimized GC and MS parameters are summarized in Table 1.18.

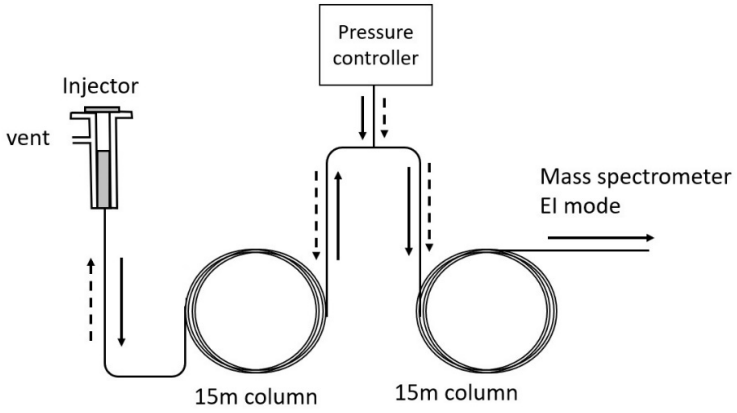
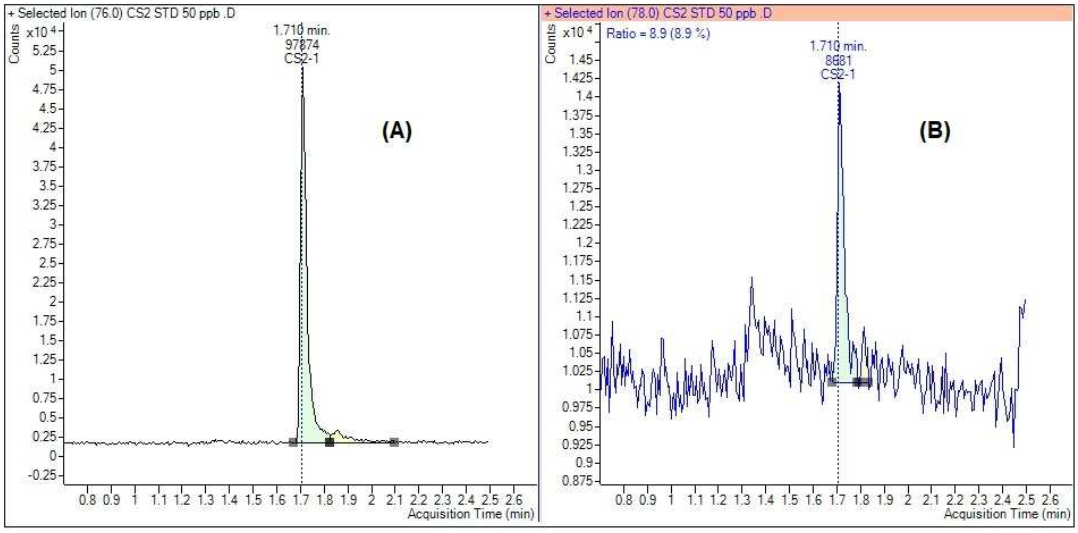


Figure 1.40 Schematic of the post-run, mid-column backflush option in GC. Solid arrows indicate the flow of carrier gas during run, and dotted arrows indicate the flow of carrier gas after the post run, mid column back flush is initiated.

Table 1.18 Optimized GC-MS parameter settings for analysis of CS_2

Parameter	**Settings**
GC parameters	
Injector temperature	150°C
Split injection	Split ratio 20:1 (split flow 22.066 ml/min)
Carrier flow	He, 1.1 ml/min
Injector conditions	Temperature program: 70°C hold 0.1 min, ramp at 450°C/min to 325°C, hold 2 min, cool at 10°C/min to 250°C.
Injection volume / mode	2 µl / split ratio 20:1
Column	Agilent 19091M-431 DB-5MS (30 m x 250 µm x 0.25 µm 5% diphenyl / 95% dimethylpolysiloxane)
Column conditions (run)	Temperature program of 14 min: 40°C hold 5 min, ramp at 40°C / min to 200 and hold for 5 min
Column conditions (post run)	Post-run program of 10 min at 310°C: Mid-column back flush, with inlet pressure at 2 psi, backflush column flow -2.553 ml/min, and onward column flow 2.967 ml/min.
MS parameters	
Source temperature	230°C
Ionization / electron energy	Electron ionization (EI) / 70 eV
Detector voltage	1500V
Ion source temperature	230°C
Damping gas flow	0.6 ml/min
Emission current	250 µA
MS analysis	Selected ion monitoring (SIM), m/z 76 and 78 with unit resolution and dwell time 200 ms

In GC-MS SIM mode, the ion with m/z 76 was used for quantitation, and the ion with m/z 78 with a response of ~ 9% of the quantifier ion was used for confirmation, as shown in Figure 1.41. The ratio between the responses of these ions was observed to be maintained between 8.5 to 10.9 in all the cardamom and black pepper samples analysed, which complied with the compound identification requirement using single quadruple MS techniques as per DG-SANTE guidelines.



(A) m/z = 76, **(B)** m/z = 78, at LOQ concentration of 0.05 mg kg^{-1}

Figure 1.41 Chromatogram of CS_2 in GC-MS, SIM mode

Method validation

Accuracy was assessed in terms of the percentage recovery of thiram as a representative compound for DTC, in cardamom and black pepper in both whole and crushed (cardamom) / ground (black pepper) forms. The fortification levels were 0.1 mg kg^{-1} (which represented the Codex MRL for DTC in the two spices), 0.5 mg kg^{-1} and 1 mg kg^{-1}. Method precision was assessed in terms of relative standard deviation and HorRat values[128]. The results are summarized in Table 1.19.

Table 1.19 Accuracy (% recovery), intra-day precision (RSD_r, n = 5) and inter-day precision (RSD_R, n=9) for dithiocarbamates (as CS_2) in cardamom and black pepper (whole and crushed/ground forms)

Fortification level (mg/kg)	Mean recovery (% ± SD)	RSD_r (%)	Predicated RSD_r (%)[a]	$HorRat_r$[b]	RSD_R (%)
Cardamom					
0.1	78 (±5)	6.7	22.4	0.30	14.8
0.5	82 (±6)	6.7	17.6	0.38	13.2
1.0	90 (±8)	7.8	15.9	0.49	12.5
Cardamom crushed					
0.1	73 (±6)	7.4	22.4	0.33	10.8
0.5	85 (±10)	11.8	17.6	0.67	12.1
1.0	93 (±6)	6.8	15.9	0.43	9.2
Black pepper					
0.1	81 (±6)	7.2	22.4	0.32	13.1
0.5	91 (±5)	5.3	17.6	0.30	14.2
1.0	97 (±8)	8.1	15.9	0.51	12.9
Black pepper ground					
0.1	78 (±5)	7.1	22.4	0.31	11.6
0.5	76 (±7)	8.2	17.6	0.47	9.8
1.0	81 (±9)	10.6	15.9	0.67	12.1

RSDr: repeatability relative standard deviation; ; RSD_R: reproducibility relative standard deviation.

[a] Predicted RSD is calculated as $PRSD_r = 2C^{-0.15}$

[b] $HorRat_r$ is calculated as RSD_r / PRSD

In whole cardamom, the average recoveries (n = 5) were 75, 86, and 98% at spiking levels of 0.1, 0.5 and 1 mg kg^{-1} respectively, while in the ground cardamom, the recoveries were 78, 82 and 90% respectively for the same spiking levels. Between whole and crushed cardamom, the variation of recovery levels was in the range 3 to 5%, indicating that the extent of interference of matrix components (including essential oils, which would have been released in higher quantity to the reaction medium in the crushed form) in cardamom was minimal. The standard deviations in the recovery values were marginally higher in the crushed samples as compared to the whole samples. The intra-day repeatability values (RSD_r) in the whole form were 6.7 to 7.8% in whole and 6.8 to 11.8 in crushed forms. overall, the HorRat values in both whole and crushed forms of cardamom were within the acceptable range of 0.3 - 1.3. The inter-laboratory precision (RSD_R) for whole cardamom

was between 12.5 to 14.8%, while that in crushed cardamom was slightly lower at 9.2 to 10.8%.

In black pepper also similar trends were observed in recoveries and standard deviation. For whole black pepper, recovery values obtained were 81, 91 and 97 % for 0.1, 0.5 and 1.0 mg kg^{-1} fortification levels respectively. In ground samples, these recovery values were lower, at 78, 76 and 81% respectively. The same range standard deviations, ± 5 to ±9, observed for black pepper for both whole and ground samples, is possibly due to the higher homogeneity was higher in the ground black pepper samples. The HorRat values for whole and ground black pepper were also within the acceptable range. As in the case of cardamom, the inter-day precision (RSD_R) values in ground black pepper (9.8 to 12.1%) were slightly lower than those for whole black pepper (12.9 to 14.2%). In all cases, the intra- and inter-day precision values were well below 20%, which is the acceptance limit for this parameter. Limit of detection at 0.025 mg kg^{-1} and limit of quantification at 0.05 mg kg^{-1} were established in both the spice matrices.

Analysis of whole and crushed (cardamom) / ground (black pepper) forms of naturally contaminated samples showed that there was very little effect of matrix components (including essential oils) in the spices on the CS_2 generation process. The comparison of average results from replicate analysis (n=3) for whole and homogenized form of the two spices (crushed cardamom/ground black pepper), in 5 naturally contaminated samples of each spice, showed only minor variations between whole and homogenized forms. In black pepper the variation between whole and ground forms was between -0.6% to 0.9% and cardamom the variation was between 0.3% - 0.6%.

This result, considered along with the fact that DTC are generally non-systemic, indicates that comminution of samples is not required in routine analysis of DTC residues using this method. Although the results for recovery and precision from fortified samples

varied slightly between whole and crushed/ground forms, the values were well within acceptable tolerance limits.

Matrix effects and effects of sample comminution

To assess the matrix effect, matrix-matched calibration curves of CS_2 were plotted using extracts from blank samples of black pepper and cardamom in the range 0.125 to 1 μg mL^{-1} and compared with the calibration curve for CS_2 in isooctane plotted in the same concentration range. The comparison is shown in Figure 4.2

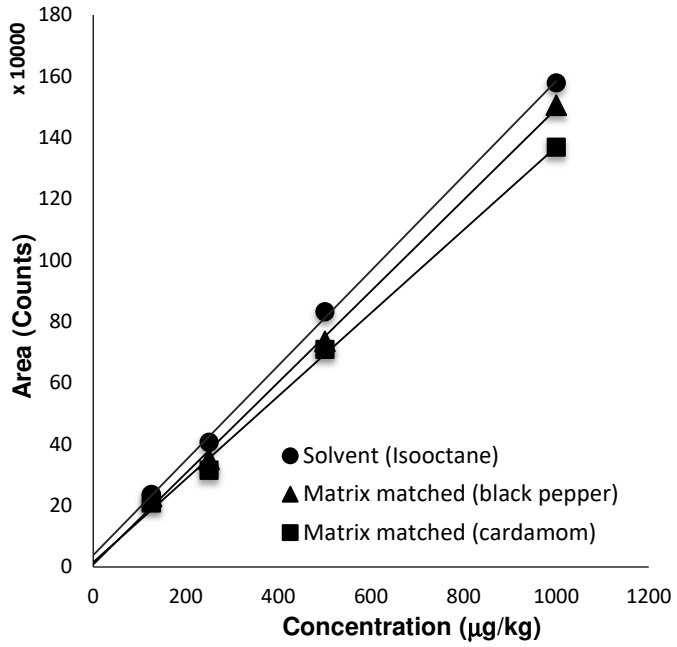


Figure 1.42 Matrix matched calibration curves for CS_2 in isooctane, black pepper extract and cardamom extract

Matrix effect (ME) was calculated using the following equation[50,53]:

$$ME\ (\%) = \frac{(Slope\ of\ matrix\ matched\ curve - slope\ of\ solvent\ curve)}{Slope\ of\ solvent\ curve} \times 100$$

The calibration equations and regression coefficients for the solvent and matrix-matched calibration curves for cardamom and black pepper, and the calculated matrix effects for

the two spices, is given in Table 1.20. In both the spices, the average matrix effect was suppressive: -3.8% for black pepper and -12.4% for cardamom. As the matrix effects observed were low, for routine analysis solvent-based calibration curves were employed for quantitative determination.

Table 1.20 Matrix effect (ME, %) in black pepper and cardamom

Matrix	**Calibration equation**	**Regression coefficient (R^2)**	**ME (%)**
Solvent	y = 1545x + 39036	0.9992	-
Black pepper	y = 1486x + 8373	0.9975	-3.8
Cardamom	y = 1354x + 15566	0.9972	-12.4

A matrix enhancement in low ranges (<10%) has been observed previously in fruits and vegetables while using CS_2 analysis in GC-MS[100]. In the case of spices, however, there is a small amount of matrix suppression in the signal. For GC analysis in general, the type matrix effect expected is signal enhancement due to the interactions of the analyte and matrix molecules with the active sites in the GC injection system and column[79,83,85]. This is because the molecules from the matrix, being in higher concentration than the analyte molecules, will occupy and block the available active sites and thus increase the number of analyte molecules entering the mass spectrometer. In the present case, this mechanism does not seem to be operating, as it is likely that the isooctane extract injected does not contain sufficient concentration of matrix components to cause the expected matrix enhancement effect. It is more likely that coeluting peaks might play a role in affecting the ionization of the analyte in EI, thus resulting in a small amount of signal suppression. This seems to be consistent with the approximate essential oil content in the two spice matrices, black pepper (oil content ~ 4%, observed signal suppression -3.8%) and cardamom (oil content ~ 8%, observed signal suppression -12.4%). However, as the extent of matrix effect was observed to be low the use of matrix-matched calibration was not needed in quantitative analysis. This offers the possibility that the method could be adapted for

testing DTC residues in other classes of spices, like fruits (e.g., chillies), roots and rhizomes (e.g., turmeric, ginger), bulbs (e.g., garlic) etc where the use of DTC compounds for fungal disease control is prevalent.

Safety evaluation of DTC in cardamom and black pepper

Twenty-six cardamom samples and twelve black pepper samples were collected in whole form from local markets in Kochi, Kerala, and analysed using the optimized method for DTC. The results were evaluated against the Codex MRL of 0.1 mg kg^{-1} in cardamom and black pepper.

The results were further assessed from the point of view of consumer safety, in terms of the theoretical maximum daily intake (TMDI, mg $person^{-1}$ day^{-1}) as compared against the maximum permissible intake (MPI, mg $person^{-1}$ day^{-1}). The MPI was calculated as the acceptable daily intake (ADI, mg $kg^{-1}day^{-1}$) of DTC multiplied by the average body weight of a child, taken as 16 kg[129]. The ADI values assigned by the Codex Joint Meeting of Pesticide Residues (JMPR) was used for the calculations of MPI. The TMDI was calculated as the average incidence level of DTC (mg kg^{-1}) in cardamom and black pepper multiplied by the average consumption of cardamom and black pepper taken as 0.0038 kg and 0.014 kg respectively[130].

Out of the 26 market samples studied for cardamom, 73.1% were found to be in compliance with the Codex limit of 0.1 mg kg^{-1}. In the case of the 11 black pepper samples studied, the compliance level was 72.7%. As per the risk evaluation of DTC by the Codex joint meeting on pesticide residues (JMPR), the ADI for the DTC compounds[131] were fixed as, thiram: 0 - 0.01 mg kg^{-1}, ferbam & ziram: 0 - 0.02 mg kg^{-1}, and mancozeb, maneb, zineb & metiram: 0 - 0.03 mg kg^{-1}[74,75,80,86]. Although mancozeb is the most prominent DTC compound used for spice cultivation in India, the more stringent ADI assigned to thiram, i.e., 0.01 mg kg^{-1}, was used for the calculations of MPI. The comparison of the

calculated MPI values based on the ADI for DTC residues and the TMDI values based on the average incidence level of DTC residues in real-life samples studied for the two spices, are given in Table 1.21.

Table 1.21 Safety evaluation of dithiocarbamate residues in cardamom and black pepper

Spice	Average incidence (mg/kg)	Consumption (kg/person/day)[a]	ADI (mg/kg/ day)	MPI (mg/person / day)[b]	TMDI (mg/person / day)
Cardamom	0.09 (n =26)	0.0038	0.01	0.16	0.00034
Black pepper	0.13 (n =11)	0.0140	0.01	0.16	0.00178

[a] Median quantity of spice intake per day[130]
[b] ADI multiplied by average body weight of a child, taken as 16 kg

During the safety evaluation, it was seen that for both the spices, the TMDI values (0.00034 and 0.00178 mg person^{-1} day^{-1} for cardamom and black pepper respectively) were much below the MPI values of 0.16 mg person^{-1} day^{-1}, indicating that there was no significant health risk with respect to DTC residues in the samples studied.

Conclusion

The method of analysis of dithiocarbamate residues by acid hydrolysis and reduction to carbon disulphide followed by absorption into isooctane and analysis by GC-MS SIM method, which was earlier reported in vegetables and fruits, has been extended to spices for the first time. The GC chromatographic conditions were optimized with split injection. The novel use of a post-run GC program implementing mid-column backflush, which gave good consistency in instrument response though large batches, was seen to be important in the routine analysis DTC residues in complex matrices like spices. Validation of the method in two spices, viz. cardamom and black pepper, was performed using thiram as a representative compound for dithiocarbamate residues. Method validation parameters like accuracy, precision, linearity, and range were assessed and found acceptable as per

international standards. LOD at 0.025 mg kg-1 and LOQ at 0.05 mg kg^{-1} were established in both spice matrices studied. These levels are adequate for compliance assessment of spice samples against the Codex MRLs. Recovery studies in the whole and crushed / ground forms of the spices, and the assessment of matrix effects in both spices, proved that there is no significant impact of matrix interference in the optimized analytical method. This offers the possibility of extending the method to other classes of spices also.

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PART II

APPLICATION OF ANALYTICAL METHODS TO REAL SAMPLES

CHAPTER 1

INTRODUCTION AND REVIEW

Throughout the world from historical times, developing countries are known to be the major producers of spices. Asian and African countries have geoclimatic conditions conducive to the cultivation of many important spices. India, since time immemorial, has been known as the land of spices. India is also the world's largest producer, consumer as well as exporter of spices[1]. Each state in India is home to one or more commercially prominent spices. However, one of the important issues faced by developing countries like India, especially in plantation crops like spices, is that a major share of cultivation happens in small and marginal farms. In such places, due to various constraints, adoption of good agricultural practices, and optimal and judicial application of pesticides, are not widespread. Chances of indiscriminate use of pesticides are high in such situations, and thus the chances of incidence of residues in the food produced, at levels above the regulatory limits, are comparatively high. Assessment of dietary risks due to pesticide residues in food thus becomes an important concern. This chapter reviews the process of fixing maximum residue limits for pesticides by national regulatory authorities in India and the common techniques used for dietary hazard characterization due to pesticide residues.

Risks due to pesticide residues

Agricultural production is beset by attacks from a lot of pests like insects, mites, fungi, weeds, vertebrate pests etc. Although pesticides are tremendously important from the point of view of ensuring productivity of agricultural commodities by controlling these pests, uncontrolled and indiscriminate use of pesticides produce residues in food which are harmful to non-target organisms also. Thus, pesticide residues pose food safety risks,

and are regulated by legislation in most countries. To manage these issues, a set of authorized good agriculture practices (GAP) are usually adopted for different agricultural commodities. These practices lay down recommended safe dosages for approved pesticides, application conditions etc., which have been demonstrated in the field to have effective control of target pests without resulting in large amounts of pesticide residues. Many countries across the world have fixed maximum residue limits (MRLs) for pesticides in raw agriculture commodities, based on GAP practices and pattern of consumption of agricultural products.

In India, the use of chemicals for pest management in crops and animals is regulated by the Central Insecticide Bureau and Registration Committee (CIB&RC). Only pesticides which are registered by this body can be legally used in India. The MRLs for the registered pesticides in agriculture commodities are fixed, based on requirements, by the Food Safety and Standards Authority of India (FSSAI). These MRLs are derived using data from supervised field trials, taking into account toxicological information of the pesticides and dietary exposure data.

Fixing MRLs for raw commodities

As mentioned above, the usual practice of setting MRLs is derived based on statistical analysis of data from GAP based supervisory field trials for a commodity using the pesticides under consideration. However, supervisory field trials are cost- and labour-intensive. So, whenever there is a felt need, there is also the provision to fix MRLs based on monitoring data in commodities which do not have GAP trial data[2]. The MRL calculations are usually done using the MRL Calculator developed by the Organisation for Economic Cooperation and Development (OECD), United States Environmental Protection Agency[3]. This calculator is an Excel sheet which takes residue monitoring data from field trials as input and calculates the following three values as output: supervised

trial median residues (STMR), highest residue, mean residue and the maximum residue limit.

In a typical multi-location supervised field trial, residue data is generated at different interval days after application of the pesticide as per GAP and a set pre-harvest interval (PHI), typically taken as 3 days. This data is loaded as input in the OECD MRL calculator, which will calculate the MRL value based on appropriate statistical approaches. A dataset of 10-15 results is considered to generate MRL values of acceptable uncertainty levels.

Dietary exposure and hazard estimation of residues

When pesticide residues are present in food commodities (raw or processed), a consumer of such food is exposed to these residues. The extent of such exposure from a food commodity is based on the dietary consumption pattern that commodity, which is expressed as g person^{-1} day^{-1}. Consumption pattern for long term effects is used for MRL calculations, and consumption pattern for short term effects, based on 95th percentile, is used for short term risk assessment. For spices[2], the consumption pattern for long term effects used for MRL calculations ranges from 2 - 3 g person^{-1} day^{-1}, which is much lower than that for staple foods. A comparison is shown in Table 2.1 below.

Estimation of dietary hazard (or risk) due to pesticide residues require two steps, *viz.* hazard identification and hazard characterization. For these steps, data from toxicological studies as well as human epidemiological studies are used. The two important values for hazard identification are acceptable daily intake (ADI) and acute reference dose (ARfD). To calculate these values, first the no-observed-adverse-effect-level (NOAEL) is estimated toxicologically, which is defined as the largest concentration of a substance that causes no detectable adverse effect on a target organism. Typically, NOAEL is divided by a safety factor of 100 to arrive at ADI or ARfD in units of mg kilogram-body-weight-^{1} day^{-1}. ADI is usually expressed as a range while ARfD is

expressed as a value. The safety factor may vary depending on the test organism and the compound being studied.

Table 2.1: Comparison of dietary consumption of cereals and spices[2]

Commodity	**Dietary consumption (g person^{-1} day^{-1})***
Cereals	
Bajra	113
Barley	59
Maize	100
Ragi	56
Rice (Milled and parboiled)	257
Sorghum (Jowar)	163
Wheat (whole flour)	192
Other cereals / millets	45
Spices	
Cardamom	2
Black pepper	2
Coriander	3
Cumin	2
Fenugreek	2
Ginger	3
Chillies (dried	3
Garlic	2
Other spices	2

*FSSAI recommended dietary consumption for long-term effects (for MRL calculation).

To perform hazard characterization due to residues, the national estimated daily intake (NEDI) values are used. NEDI is calculated as follows:

$$NEDI = \sum STMR \times \frac{F_i}{bw}$$

Where STMR is the supervisory trial median residue (mg kg^{-1}), F_i the per capita food consumption (kg person^{-1} day^{-1}) and bw the average body weight (kg). Next, the ADI is converted to per person per day by multiplying ADI by 60, where 60 kg is taken as the reference body weight for dietary risk assessment in Indian context[2]. Dietary hazard is then

characterized by comparing NEDI with ADI. If this comparison indicates that the residues generated by field trials will not generate dietary intakes exceeding the ADI, then the calculated MRL value based on the field trial is adopted as the actual MRL under the GAP conditions. Ideally, NEDI ≤ 80% of ADI means that there is minimum hazard due to pesticide dietary exposure.

Fixing MRLs for processed commodities

The process described above summarized the fixing of MRLs in raw agriculture commodities. However, such commodities undergo processing before reaching the consumer, thus fixing MRLs of processed foods also requires consideration[2,4]. The steps of processing can affect the pesticides in the raw commodity, and based on the physicochemical properties of the residue, the final product can contain a residue concentration which is different from the raw commodity.

Processing can involve several widely different operations, ranging from simple to complex. Washing, peeling, grinding, cooking, oil extraction etc can all considered as processing steps. Some of these can result in reduction of residues (e.g., washing) while soe others can result in magnification of residues (e.g., oil extraction, preparation of spice oleoresins). Other steps like cooking can convert a residue into a metabolite which can be more toxic than the original residue. All these factors contribute to the complexity of fixing MRLs for processed foods. To arrive at MRL for a processed food, the processing factor is calculated:

$$P_f = \frac{R_p}{R_r}$$

where R_p and R_r are the residue concentrations (mg kg^{-1}) in the processed and raw commodity respectively.

Except in cases where residue gets concentrated on processing, processing factor need not be applied if $R_r \leq LOQ$. A value of $P_f > 1$ indicates residue enrichment, while P_f

< 1 indicates residue reduction. The processing factors are calculated through a rigorous and involved procedure involving multiple independent trials and processing steps. In cases where P_f is available, compliance evaluation is done after multiplying the MRL by this factor.

Dehydration factors in spices

A specifical case of processing factor which is applicable to spices involve the case were processing involves only loss of moisture. Spices are typically dry commodities with moisture content reduced to 8-10%. This results in residue concentration, though the risk associated with the absolute quantity of pesticide present as residue does not change. Thus, a dehydration factor needs to be applied to the MRL before performing compliance evaluation in spices. This is calculated as follows:

$$DF = \frac{1}{(1 - \frac{W}{100})}$$

where W is the water content (%) in the spice.

The European Spice Association (ESA) has published a study in which dehydration factors ranging from 5 to 10 were calculated for various spices and herbs[5]. FSSAI has adopted a dehydration factor of 10 for dried chillies, thus the MRL of dried chillies is ten times the MRL of fresh chillies[6].

Assessing compliance with MRLs

In general, the result of an analysis for a pesticide in a commodity can be directly compared against the corresponding MRL. A result which is at or above MRL indicates non-compliance ('fail'). A result which is below the MRL indicates compliance ('pass'). Statistically, however, the measurement uncertainty associated with the result also has to be considered while evaluating MRL compliance. In this situation, there are four possibilities to be considered, which shown schematically in Figure 2.1. The mode of

reporting of results will thus depend on the testing laboratory selecting the appropriate strategy for assessing compliance, which is stated formally as the 'decision rule'.

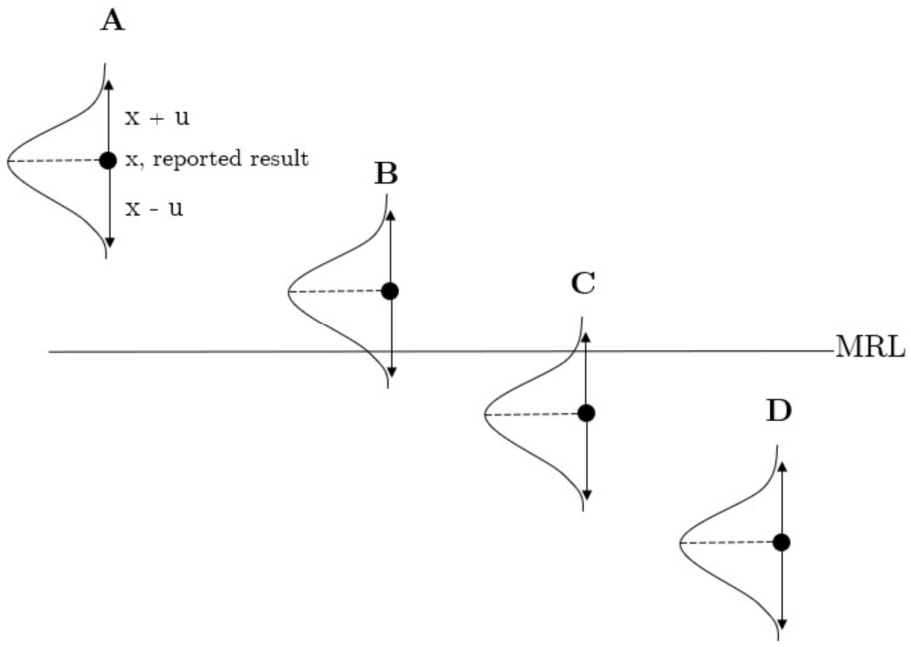

Figure 2.1 Using measurement uncertainty in assessing MRL compliance

There is no ambiguity in the cases A and D in Figure 2.1, as in both these cases the sample passes or fails even when uncertainty values are accounted for. However, in cases B and C, applying uncertainty will affect the decision on compliance. In case B, even though the absolute value of the result is above MRL, there is a possibility of the sample passing if negative uncertainty is considered. In case C, even though the absolute value of the result is below MRL, there is a possibility of sample failing when positive uncertainty is considered. In cases like pesticide residues where there is a potential hazard to the consumer of the food, decision rule C is usually applied.

Scope and objectives of the present study

In the present study, the sample preparation and instrumentation methods developed in Part 1 will be applied to real samples of spices collected from markets, in

order to establish the capability of these methods for regulatory compliance evaluation against FSSAI MRLs. The residues detected in the samples will be characterized to gain insights into the levels and types of residue contaminations that can occur in different spices. These results will be used to perform an estimate of food safety hazard associated with presence of pesticide residues in spices.

CHAPTER 2

MATERIALS AND METHODS

This chapter describes the chemicals, reagents, certified reference materials, sample preparation techniques and instrumentation methods employed for multiresidue analysis in real samples of six spices, *viz.* chillies, cardamom, cumin, ginger, cinnamon and curry leaves. Performing compliance evaluation against FSSAI MRLs for these spices using the results obtained, and characterization of food safety hazards in the tested samples based on these results, are also described.

Materials

The mass spectrometry grade solvents used for mobile phase preparation in UPLC, *viz.* methanol and acetonitrile, were obtained from Biosolv, USA. The QuEChERS chemicals, principally primary secondary amine (PSA), graphatized carbon black (GCB), and C-18 bulk sorbent were procured from Agilent, India. All other analytical grade chemicals like isooctane, acetic acid, formic acid, sodium chloride, anhydrous magnesium sulphate, ammonium formate, formic acid, sodium citrate dibasic trihydrate, sodium citrate dibasic sesquihydrate etc. were procured from Merck, India. All pesticide residue certified reference materials (CRMs) were procured from Dr. Erhenstorfer, Germany. Carrier gas for GC was 99.9995% pure helium obtained from Bhuruka gases, India.

Instrumentation

A 3-digit precision balance (Sartorius BSA223S) was used for weighing all samples for analysis. For reference standard preparations a 5-digit precision balance (Shimadzu AUW220D) was used. Homogenization was carried out in all spices using a kitchen blender. Certified reference material and stock standards were stored at -20°C in a freezer (Remi RQV-300 plus), and intermediate standards were stored at 4°C in a low temperature cabinet (Remi CC-19 plus). Centrifuges for sample preparation with two

speeds were used, *viz.* 5000 rpm (Remi CM-8 plus) and 10,000 rpm (Remi C-24 plus). Vortex shaker used was Remi CM-101. For concentration of extracts, a nitrogen-based evaporator from PCI Analytics (N_2 Fastvap) with a Peak nitrogen generator was used. For detection and quantification of analytes, Agilent GC-MS/MS (7890 GC / 7000 C MS) and Waters UPLC-MS/MS (Xevo TQS Micro) were used.

Sample collection

Samples were collected from local markets in Kochi, Kerala in whole, dried forms. Branded spices in retail packs were not considered in this study. Instead, samples were procured in loose from as detailed in Table 2.2. Ten samples of each spice were collected. The curry leaf samples were obtained in fresh and then sun-dried to constant weight before commencing analysis, as the method development had been performed in the dried form of this spice.

Table 2.2 Details of samples collected for survey

Spice samples	Number of samples	Weight collected (kg)
Cardamom (whole)	10	1
Cumin (whole)	10	1
Ginger dried (whole)	10	0.5
Cinnamon (whole)	10	0.25
Curry leaves (fresh)	10	0.5

Sample preparation and extraction

The samples of the different spices were homogenized as described in Part 1 (Table 1.2) before analysis. Each sample was analysed in duplicate. QuEChERS based sample preparation, as optimized for each of the six spices in Part 1, Chapters 3 (LC-MS/MS) and 4 (GC-MS/MS), were used for analyses.

Instrumental analysis

For all analyses, reference standards were prepared as described in Part I, Chapter 2. A total of 78 residues were analysed in the six spices considered for the study, with GC-MS/MS being used for analysis of 25 residues and LC-MS/MS being used for the analysis of 53 residues. Instrument conditions used were as described in Part I, Chapters 3 and 4. Matrix matched calibrations were used in all analyses. Average of the results from duplicate analysis was used to perform compliance evaluations and hazard characterization calculations.

Assessing compliance with Indian MRLs

In India, the national MRLs are issued by the FSSAI, in the Food safety and standards (contaminants, toxins and residues) regulation[6]. This regulation is frequently updated, and in the latest version available online, there are MRLs issued for 213 pesticides. However, the number of MRLs issued for spices is only 23. Out of the six spices considered in the present study, specific MRLs are available only for 3 spices, *viz.* chillies, cumin and cardamom. MRLs are available for a class named leafy vegetables, in which curry leaves could be included. However, in this study, only dried curry leaf was considered. Thus, dehydration factor would need to be applied in this case while considering compliance with MRLs for leafy vegetables. Out of the 78 pesticides commonly used in spices covered in this study, MRLs are available only for 23, and these are summarized in Table 2.3.

For compliance evaluation, the concentrations of the pesticides observed in the study were compared against the MRL values as described above, considering decision rule C (see Figure 2.1). In cases were FSSAI has not fixed MRLs for a spice-pesticide combination, the default MRL of 0.01 mg kg^{-1} was used for evaluating compliance.

Table 2.3 Available national MRLs for pesticides for various spices

No.	Pesticide	FSSAI MRL (mg kg^{-1})
1	Fipronil	Chillies: 0.1
2	Spinosad A	Chillies: 0.1
3	Spinosad D	Chillies: 0.1
4	Thiodicarb	Chillies: 0.1
5	Thiacloprid	Chillies: 0.2
6	λ Cyhalothrin	Chillies: 0.5
7	Deltamethrin	Chillies: 0.5
8	Pyraclostrobin	Cumin: 0.02, Chillies: 0.5
9	Fenpropathrin	Chillies: 2
10	Quinalphos	Cardamom: 0.01, Chillies: 2
11	Triazophos	Chillies: 2
12	Imidacloprid	Chillies: 3
13	Tebuconazole	Chillies: 4
14	Triadimefon	Chillies: 4
15	Trifloxystrobin	Chillies: 4
16	Cyantraniliprole	Chillies: 5
17	Hexaconazole	Chillies: 5
18	Azoxystrobin	Cumin: 0.03, Chillies: 10
19	Fenpyroximate	Chillies: 10
20	Acetamiprid	Chillies: 20
21	Buprofezin	Chillies: 20
22	Spirotetramat	Chillies: 20
23	Carbaryl	Chillies: 50, Leafy vegetables: 10

Characterization of food safety hazards

For each spice, the pesticide with the highest incurred residue concentration was used to perform the hazard assessment study. The theoretical maximum daily intake (TMDI) was calculated as the average incidence level of the residue (mg kg^{-1}) multiplied by the average consumption of the respective spice (g $person^{-1}$ day^{-1}) taken from FSSAI guidelines for calculating MRLs[2,7], as summarized in Table 2.1. The acceptable daily intake (ADI) values assigned for these pesticides by the Codex Joint Meeting of Pesticide Residues (JMPR) was used as the threshold criteria. The ADI multiplied by the average body weight of a child, taken as 16 kg, gave the maximum permissible intake (MPI, mg $person^{-1}$ day^{-1})[7]. The TMDI values were then compared with the MPI values to arrive at a

characterization of hazards associated with the incidence of the residues in the tested samples of spices. When the TMDI value calculated for a pesticide based on the average incurred residues in a sample was found to be less than the MPI value for that pesticide, the incurred residue was considered as not posing significant food safety hazard to humans. If the TMDI value exceeded the MPI value, the food safety hazard posed by the incurred residue was considered as significant.

CHAPTER 3

APPLICATION OF MULTRIRESIDUE METHODS TO REAL SAMPLES

Spices are essential ingredients in Indian food, and nearly all aspects of the national cuisine incorporate spices in some form. As a result of this, food safety risks in spices becomes an important consideration. In this chapter, applying the multiresidue analysis methods developed in Part I to real samples, for establishing the ability of these methods to be used effectively for routine food safety evaluations, is documented. Characterization of food safety hazards due the presence of pesticide residues in spices were performed taking into account two aspects, *viz.* (a) compliance to the national maximum residue limits (MRLs) in spices, and (b) the consumption pattern of spices.

A survey was conducted by collecting samples (whole form) each for six spices, *viz*. cardamom, cumin, ginger, chillies, cinnamon and curry leaves from local markets in Kochi, Kerala. These samples were analysed in duplicate using the QuEChERS sample preparation and instrumentation methods developed and optimized in Part 1, Chapters 3 (LC-MS/MS) and 4 (GC-MS/MS), for 78 commonly used pesticides in India. The details of sample collection were explained in Part 2, Chapter 2. The average results obtained for each pesticide were then used to perform safety evaluations in the five spices studied.

Residue analysis results

Out of the total 78 residues tested in 60 samples of various spices, incidence of 30 compounds were observed across all the samples tested. Among the tested compounds, the highest percentage of pesticides were detected in cardamom (20.5%), followed by chillies (17.9%), cumin (14.1%), ginger (8.9%), cinnamon (5.1%) and curry leaves (7.6%). The total residue load, calculated as the sum of concentrations of all the incurred residues in all the samples of a particular spice, was taken as a measure of the extent of residue

contamination in that spice. The highest residue load was observed in cumin (13.94 mg kg^{-1}), followed by cardamom (12.58 mg kg^{-1}, chillies (8.9 mg kg^{-1}, curry leaves (3.76 mg kg^{-1}), ginger (2.46 g kg^{-1}) and cinnamon (0.56 mg kg^{-1}). These results are summarized in Figure 2.2 below.

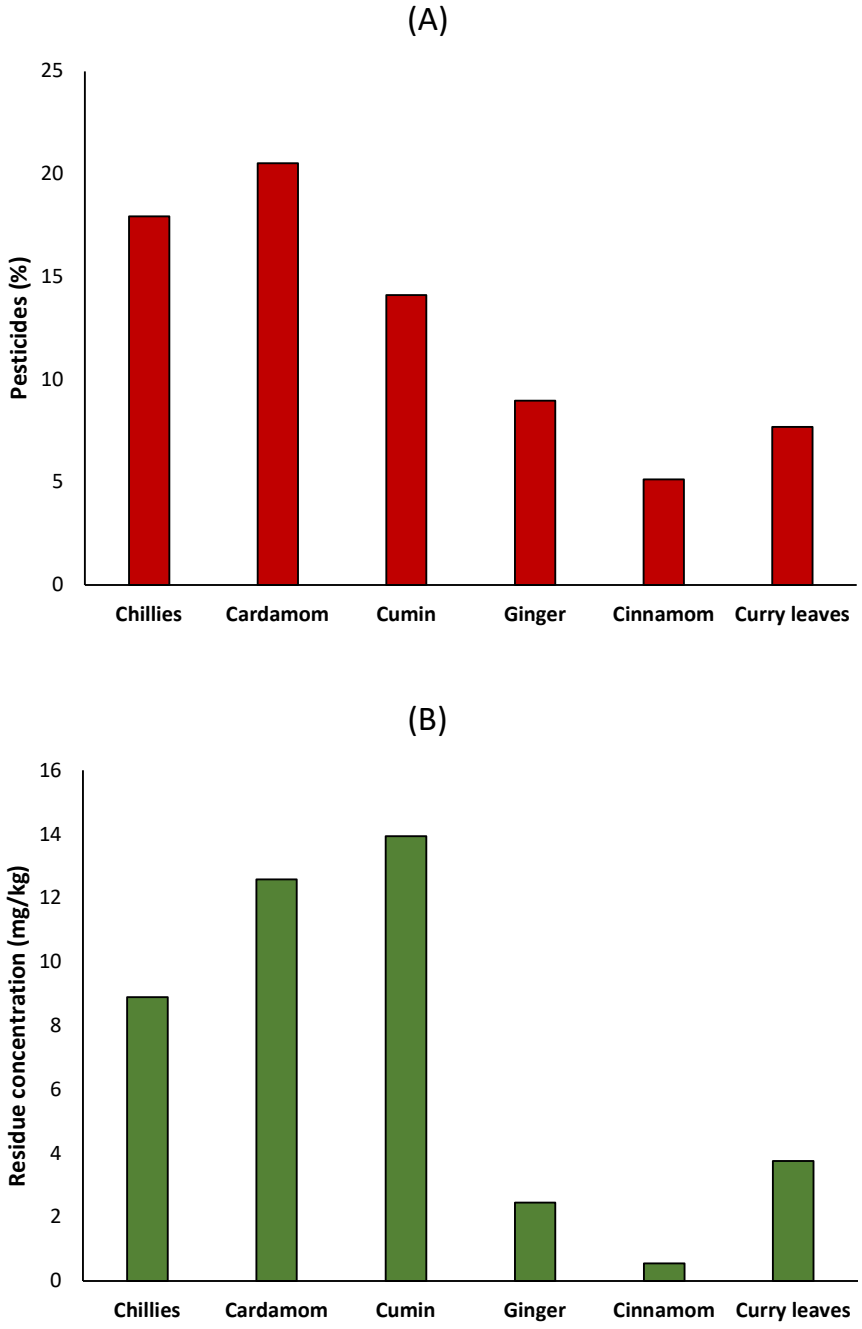

Figure 2.2 Incidence of pesticide residues: (A) residues detected in spices (%) out of the 78 compounds tested; (B) overall residue load in the samples of each spice tested

Incidence of residues in spices

In chillies, out of the 10 samples tested, residues were detected in all except 2 samples. The number of residues detected were 14, and for these the concentrations ranged from 0.02 - 3.00 mg kg^{-1}. The pesticide which showed highest incurred concentration was profenofos, which occurred in 4 samples. Acetamiprid, triazophos, azoxystrobin, fipronil were detected in 3 samples each, and quinalphos, chlorpyrifos, Imidacloprid, ethion, metalaxyl, γ cyhalothrin, cypermethrin, pyraclostrobin and spirotetramat were detected in 2 samples each. The cumulative pesticide load in chillies was the third highest among all samples tested, at 8.9 mg kg^{-1}. The details are summarized in Figure 2.3.

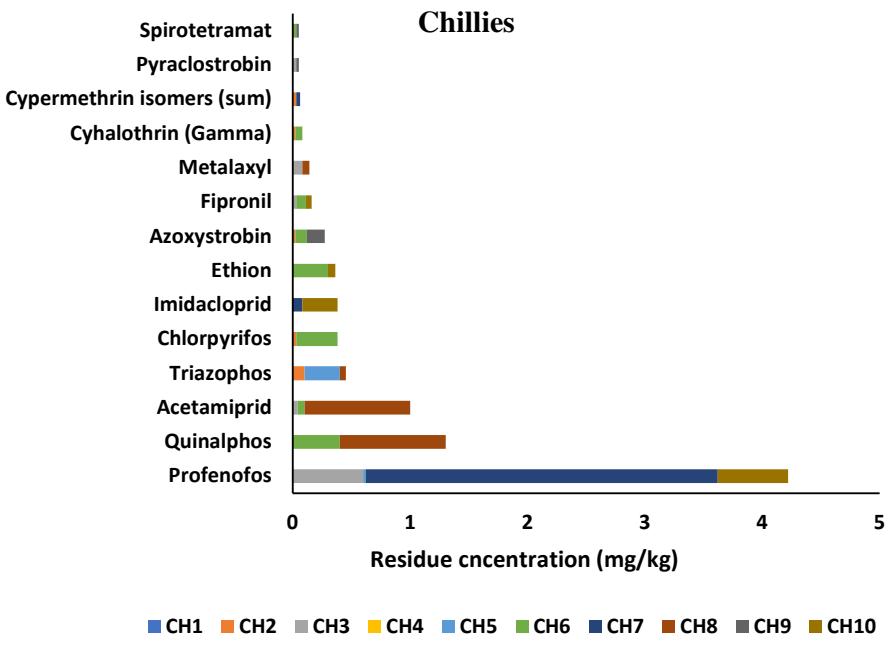

Figure 2.3 Pesticide residues detected in chillies

In cardamom also, residues were detected in all except 2 samples. The number of residues detected were 16, and for these the concentrations ranged from 0.02 - 1 mg kg^{-1}. The highest incurred value was for acetamiprid, at 1 mg kg^{-1}. Incidence of the pesticides were as follows: quinalphos - 6 samples; metalaxyl, γ cyhalothrin - 5 samples; phorate, chlorpyrifos, imidacloprid and triazophos - 4 samples; acetamiprid, hexaconazole and profenofos - 3 samples; methamidophos and carbofuran - 2 samples; buprofezin, parathion-methyl, azinphos-methyl and deltamethrin - 1 sample each. The cumulative pesticide load in all cardamom samples was the second highest among all spices tested, at 12.6 mg kg^{-1}. The details are summarized in Figure 2.4.

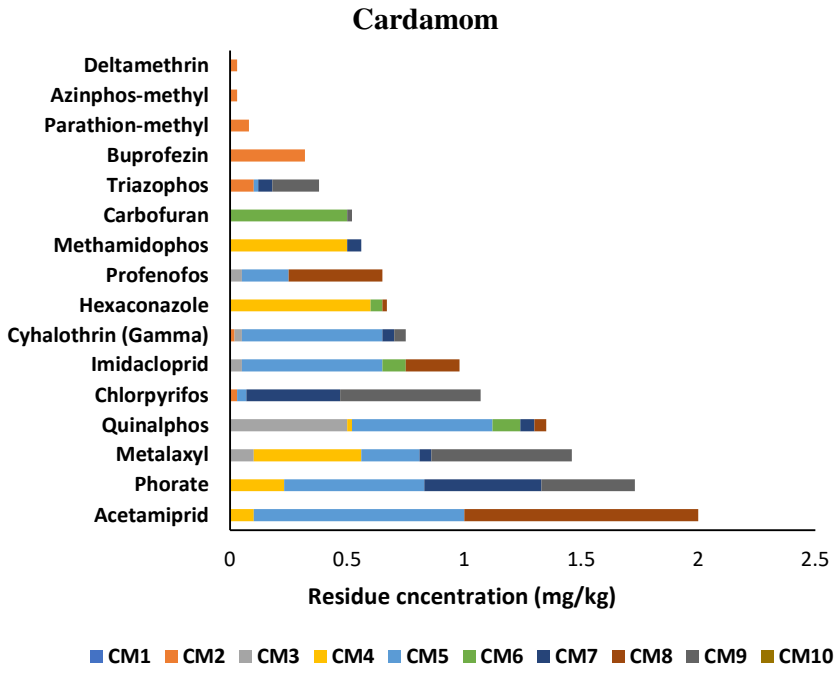

Figure 2.4 Pesticide residues detected in cardamom

In cumin, residues were detected in all except 3 samples. The number of residues detected were 11, and for these the concentrations ranged from 0.03 - 3 mg kg^{-1}. The highest incurred value was for hexaconazole, at 3 mg kg^{-1}. Incidence of the pesticides were as follows: Imidacloprid - 5 samples; profenofos - 4 samples; carbaryl, quinalphos,

chlorpyrifos, spirotetramat and triazophos - 3 samples; hexaconazole and boscalid - 2 samples; iprobenfos and azoxystrobin - 1 sample. The cumulative pesticide load in cumin was the highest among all samples tested, at 13.94 mg kg^{-1}. The details are summarized in Figure 2.5.

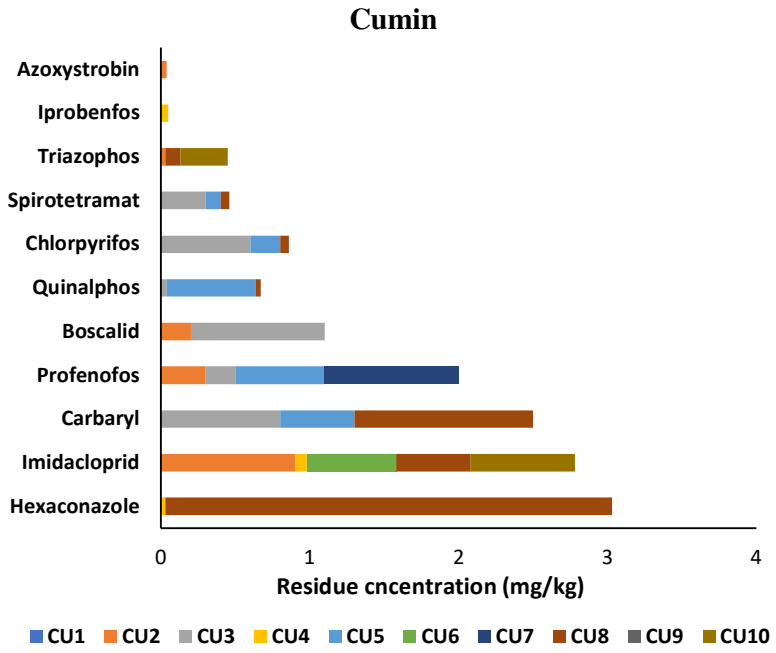

Figure 2.5 Pesticide residues detected in cumin

Ginger, cinnamon and curry leaves in general showed lower pesticide incidence. In ginger, residues were detected in 6 samples. The number of residues detected were 7, and for these the concentrations ranged from 0.02 – 0.6 mg kg^{-1}. The highest incurred value was for triazophos, at 0.6 mg kg^{-1}. Incidence of the pesticides were as follows: triazophos, hexaconazole, imidacloprid and fenbuconazole in 3 samples; chlorpyrifos in 2 samples; g cyhalothrin and phorate in 1 sample each. The cumulative pesticide load in ginger was 2.46 mg kg^{-1}. The details are summarized in Figure 2.6.

In cinnamon, residues were detected only in 4 samples, which made it the cleanest spice in the survey. The number of residues detected were 4, and for these the concentrations ranged from 0.02 – 0.2 mg kg^{-1}, again the lowest among the tested spices.

The highest incurred value was for fipronil, at 0.2 mg kg^{-1}. Incidence of the pesticides were as follows: Imidacloprid in 3 samples, fipronil, acephate and malathion in 2 samples. The cumulative pesticide load in cinnamon was only 0.56 mg kg^{-1}. The details are summarized in Figure 2.7.

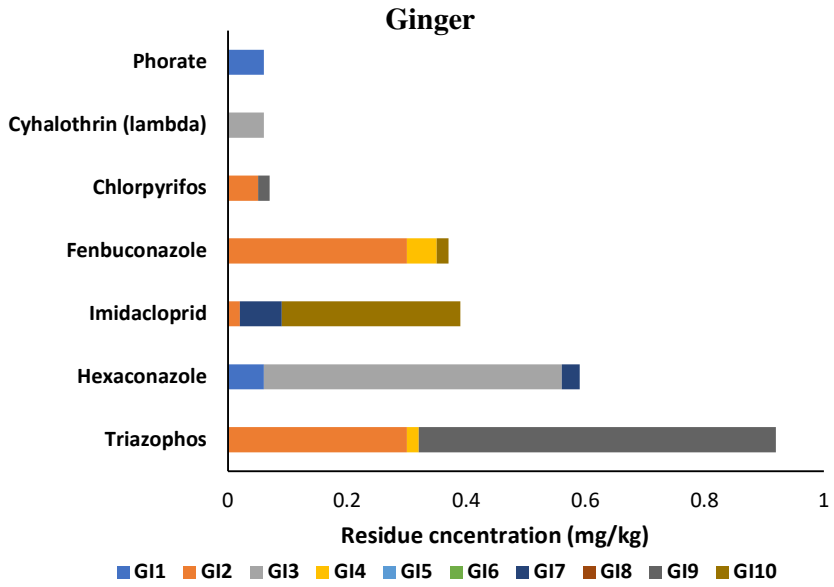

Figure 2.6 Pesticide residues detected in ginger

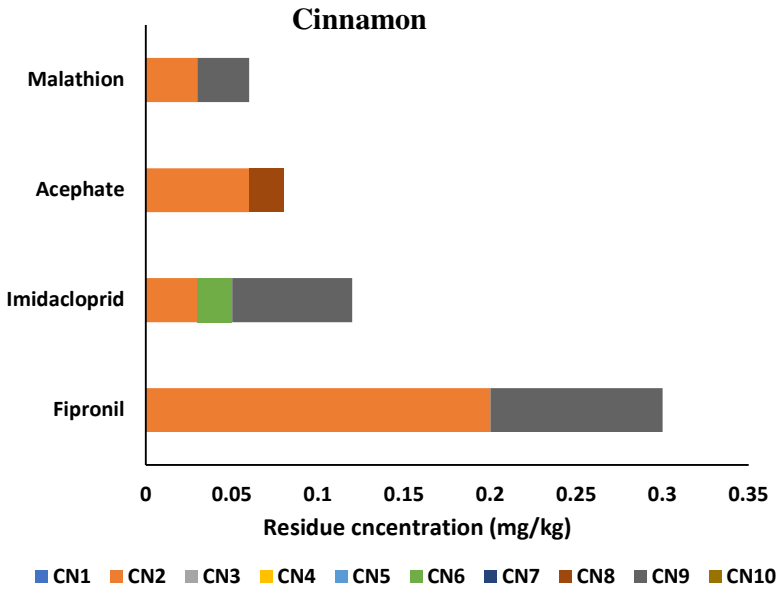

Figure 2.7 Pesticide residues detected in cinnamon

In curry leaves, residues were detected in 6 samples. The number of pesticides detected were 6, and for these the concentrations ranged from 0.03 – 0.5 mg kg^{-1}. The highest incurred value was for chlopryrifos, at 0.5 mg kg^{-1}. Incidence of the pesticides were as follows: chlorpyrifos and profenofos in 4 samples; cypermethrin, triazophos and bifenthrin in 3 samples; and fipronil in 2 samples. The cumulative pesticide load in ginger was 4.03 mg kg^{-1}. The details are summarized in Figure 2.8.

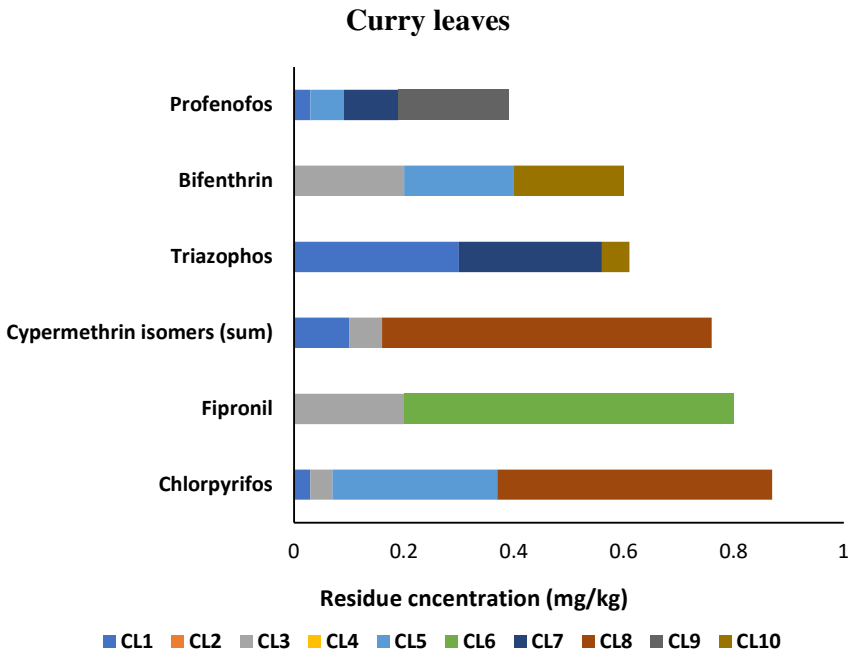

Figure 2.8 Pesticide residues detected in curry leaves

Compliance with national MRLs in spices

In India, the FSSAI sets national MRLs for foods, including spices. The MRLs in spices for the pesticides included in this study were compiled in Table 2.x. For spices, MRLs are available for only 23 out of the 78 pesticides covered in this study. As per the extant regulations, where an MRL is not specified for a commodity / pesticide combination, then the default MRL of 0.01 mg kg^{-1} is considered to apply. Although this scenario occurs mostly because the studies required for fixing MRLs as per FSSAI

guidelines[2] have not been carried out for these combinations, it is adopted in order to ensure consumer safety. Also, since 0.01 mg kg^{-1} is near the quantification limit for current level of instrumentation techniques, this rule meant that for the 55 pesticides without MRLs, any reported value was tantamount to non-compliance as per FSSAI regulations.

Chillies have liberal MRLs under FSSAI, as a distinction is made between chilli and dried chilli, and a dehydration factor of 10 is applied for dried chilli[6]. Thus, in all cases where MRLs have been fixed for chilli, the MRL of dried chilli is fixed 10 times the MRL of chilli. So, even though 14 compounds had been detected in this spice, the number of pesticides above MRLs were only 6, as shown in Figure 2.9 below. Among these, the pesticide detected at concentrations above the MRL highest number of times was profenofos, in 40% of the samples. The other four residues were detected at concentrations above MRL in 20% samples.

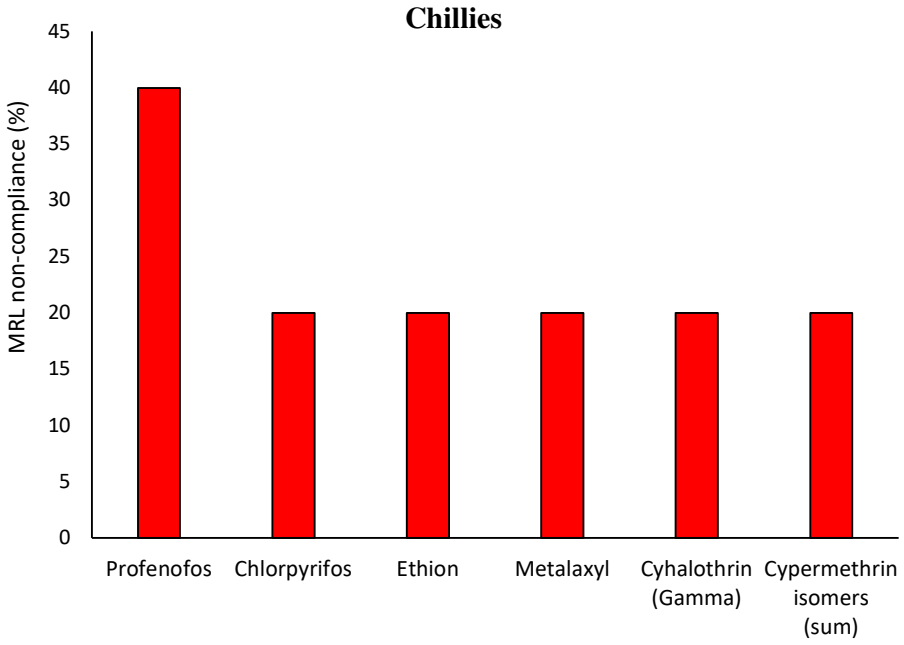

Figure 2.9 Pesticides detected above MRL levels in chillies

In cardamom, the situation was more complex, with all 16 compounds detected were at concentrations above MRL. The reason for this was that FSSAI has established

specific MRL for cardamom in only one compound, *viz*. quinalphos, at 0.01 mg kg^{-1}, and dehydration factor is not seen to be applied in the FSSAI regulations for any spice other that chilli. The MRL for quinalphos in cardamom can be contrasted with the MRL for the same compound in chillies which is 2 mg kg^{-1} (see Table 2.1). As per FSSAI rules, the default MRL to be used in cases where specific MRL is not fixed for a commodity / pesticide combination is also 0.01 mg kg^{-1}, and thus all the pesticide detected in cardamom were found to be at above-MRL concentrations. Among these, the most detected compound was quinalphos, which occurred in 60% of the samples. Metalaxyl and γ cyhalothrin were detected at above-MRL concentrations in 50% of the samples. The pesticides which were found to be above MRLs in the least number of samples were buprofezin, parathion methyl, azinphos methyl and deltamethrin, which were detected in one sample each. Figure 2.10 summarizes the incidence of pesticide residues above MRL levels in cardamom.

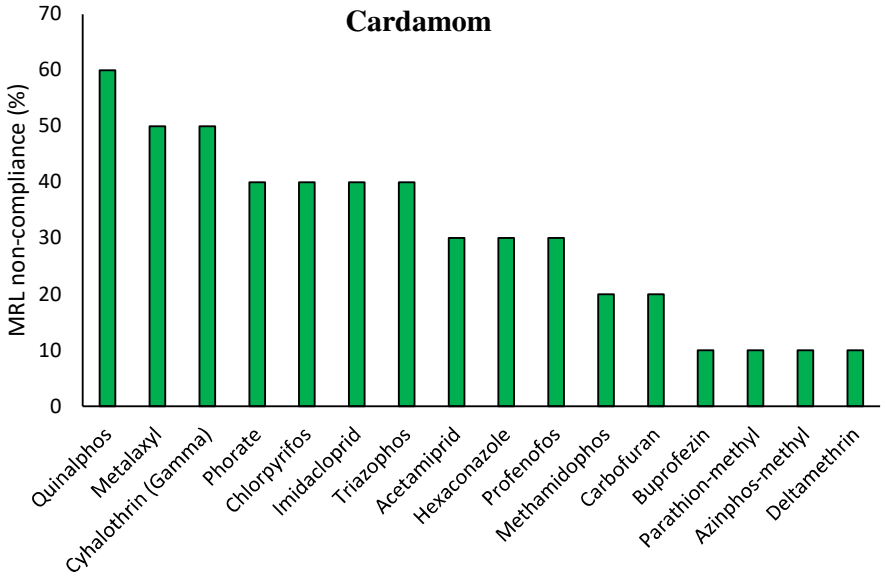

Figure 2.10 Pesticide residues detected above MRL levels in cardamom

In cumin also, all the 11 pesticide residues detected were found to be above MRL levels. As in the case of cardamom, the reason for this is the lack of MRLs in cumin. Only pyraclostrobin has MRL in cumin (Table 2.1), which was not detected in the present study. Thus, the default MRL of 0.01 mg kg^{-1} had to be applied for all the detected pesticides.

Imidacloprid was the pesticide with the greatest number of incidences above MRL, in 50% of the samples studied. Azoxystrobin and iprobenfos were the pesticides with least number of incidences above MRL in cumin. Figure 2.11 summarizes the incidence of pesticide residues above MRL levels in cumin.

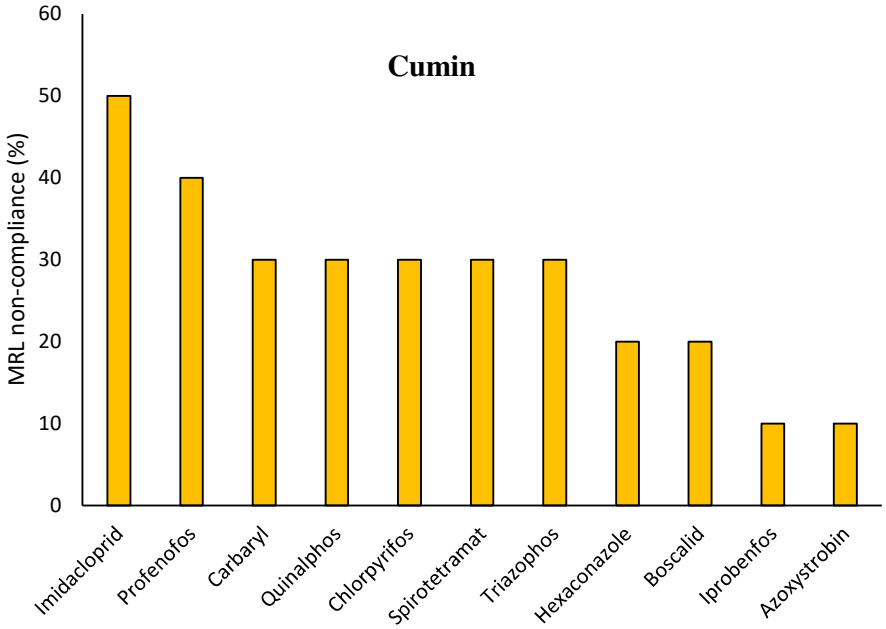

Figure 2.11 Pesticide residues detected above MRL levels in cumin

MRLs for ginger were not available as per FSSAI regulations, and thus the default MRL of 0.01 mg kg^{-1} had to be applied. Thus, all the 7 pesticides detected in ginger had to be considered as above MRL, with triazophos having the highest incidence above MRL and

phorate having the lowest. Figure 2.12 summarizes the incidence of pesticide residues above MRL levels in ginger.

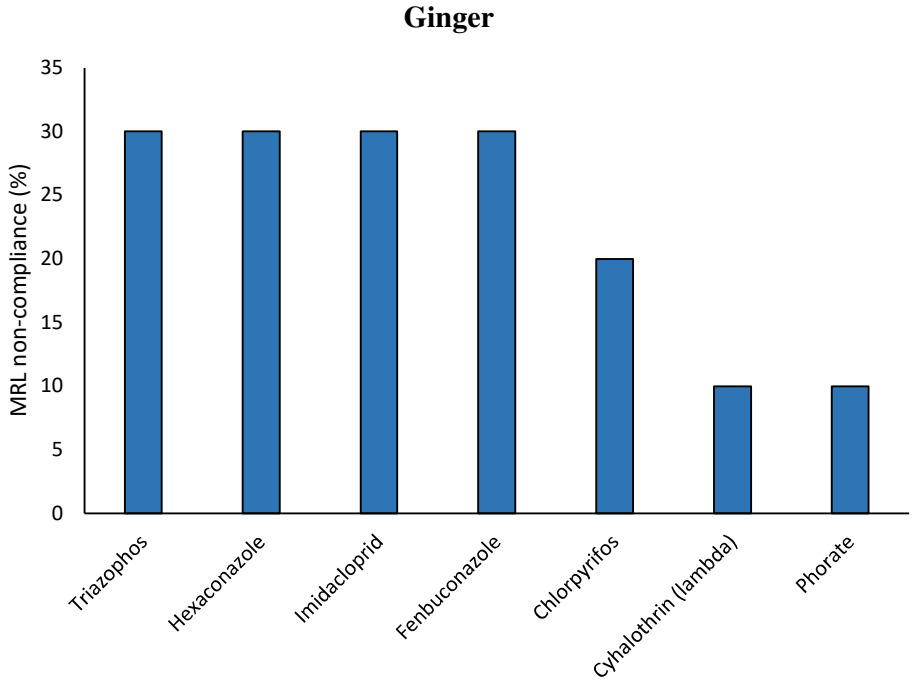

Figure 2.12 Pesticide residues detected above MRL levels in ginger

There were no MRLs available in the case of cinnamon also, and thus all detected pesticides had to be treated as above MRL. Among all the spices studied, cinnamon had the least number of incident pesticides as well as the least cumulative pesticide load. This is probably because cinnamon belong to the dried bark class of spices, and thus the extent of pest infestation in bark is less. The major pest related issues in cinnamon occur on leaves and shoots, thus foliar insecticides are often used in cultivation of cinnamon[8]. This is consistent with the type of pesticides detected in the study. Out of the four detected pesticides imidacloprid showed the highest incidence above MRL and malathion showed

the least. Figure 2.13 summarizes the incidence of pesticide residues above MRL levels in cinnamon.

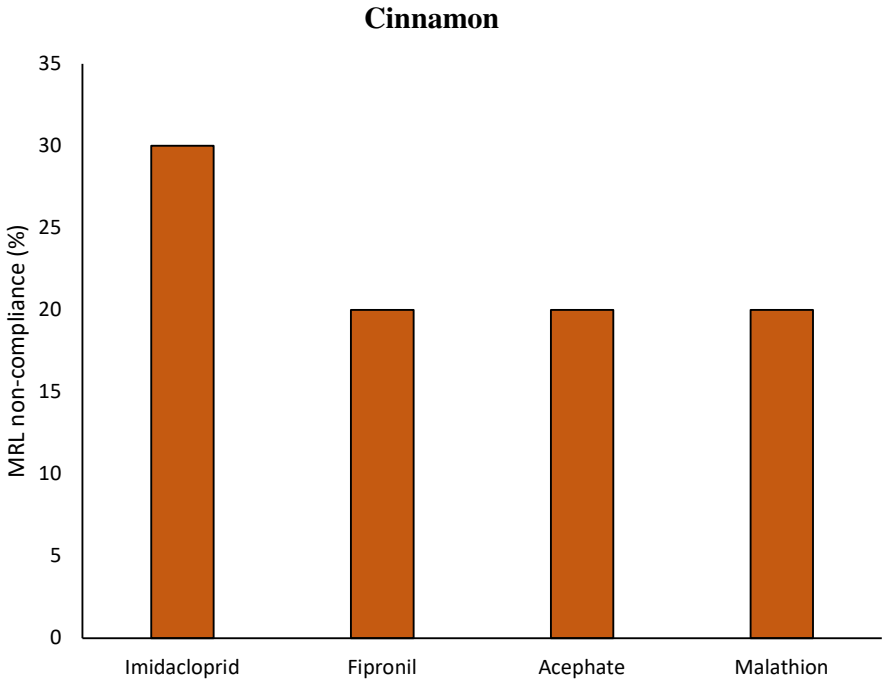

Figure 2.13 Pesticide residues detected above MRL levels in cinnamon

MRLs specific to curry leaves is not available under FSSAI, but the MRLs for leafy vegetables could be considered to apply for this spice. In the present study, dried curry leaves were tested and this affords the possibility of using a dehydration factor while evaluating compliance. However, for pesticides, MRL for leafy vegetables was available only for carbaryl, which was not detected in the curry leaf samples in the present study. Thus, the default MRL of 0.01 mg kg^{-1} had to be considered to apply in curry leaves also, necessitating that all the pesticides detected in curry eaves I the study had to be considered to be above the MRL level. Among the six pesticides detected in curry leaf, chlorpyrifos had the maximum incidence above MRL and fipronil had the least. Figure 2.14 summarizes the incidence of pesticide residues above MRL levels in curry leaves.

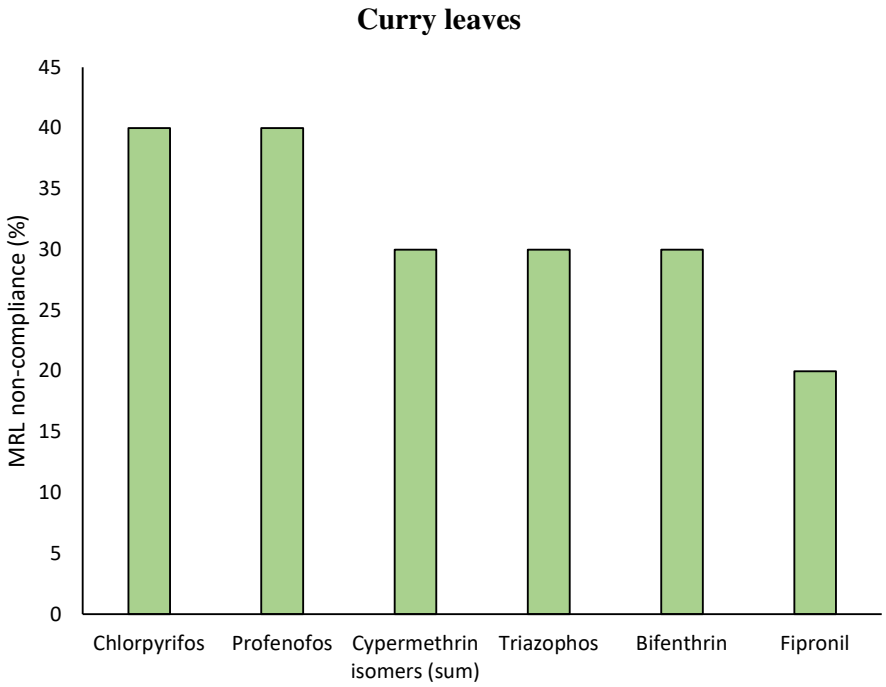

Figure 2.14 Pesticide residues detected above MRL levels in curry leaves

Food safety hazards in spices due to pesticide residues

Overall, 30 pesticides were detected among all the spice samples studied. These belonged to the following classes: organophosphates (12), pyrethroids (5), β-methoxyacrylates (2), carbamates (2), neonicotenoids (2), triazoles (2), acylalanines (1), cyclic ketoenols (1), oxathiines (1), phenyl pyrazoles (1) and thiadiazines (1). It can be noted that majority of compounds detected belonged to the classical and modern classes, while the detection of new generation pesticides was low (see Table 1.1). The pesticides chlorpyrifos, imidacloprid and triazophos were the most commonly detected pesticides occurring in 5 spices each, followed by cypermethrin, fipronil, hexaconazole and quinalphos which were detected in 3 spices each. The detected pesticides are commonly used for control of feed and storage pests, insects and fungal attacks. A summary of the different classes of pesticides detected in the various spices tested is shown in Table 2.4.

Table 2.4 Classes of pesticides detected in the spice samples studied

Pesticide	Class	Detected in	Usage
Acephate	Organophosphates	Cinnamon	Foliar and soil insecticide[9]
Acetamiprid	Neonicotenoids	Cardamom, chillies	Insecticide (aphids)[10]
Azinphos-methyl	Organophosphates	Cardamom	Foliar insecticide[11]
Azoxystrobin	β-methoxyacrylates	Chillies, cumin	Fungicide[12]
Bifenthrin	Pyrethroids	Curry leaves	Insecticide[13]
Boscalid	Oxathiines	Cumin	Fungicide[14]
Buprofezin	Thiadiazines	Cardamom	Insecticide[15]
Carbaryl	Carbamates	Cumin	Insecticide[16]
Carbofuran	Carbamates	Cardamom	Pesticide[17]
Chlorpyrifos	Organophosphates	Cardamom, curry leaves, cumin, Chillies, ginger	Pesticide[18]
Cyhalothrin γ and λ	Pyrethroids	Cardamom, chillies, ginger	Insecticide[19]
Cypermethrin	Pyrethroids	Curry leaves, chillies	Insecticide[20]
Deltamethrin	Pyrethroids	Cardamom	Insecticide[20]
Ethion	Organophosphates	Chillies	Insecticide[21]
Fenbuconazole	Triazoles	Ginger	Fungicide[22]
Fipronil	Phenyl pyrazoles	Curry leaves, cinnamon, Chillies	Insecticide[23]
Hexaconazole	Triazoles	cumin, cardamom, ginger	Fungicide[24]
Imidacloprid	Neonicotinoids	Cumin, cardamom, ginger, chillies, cinnamon	Insecticide[25]
Iprobenfos	Organophosphates	Cumin	Fungicide[26]
Malathion	Organophosphates	Cinnamon	Insecticide[27]
Metalaxyl	Acylalanines	Cardamom, chillies	Fungicide[28]
Methamidophos	Organophosphates	Cardamom	Insecticide[29]
Parathion-methyl	Organophosphates	Cardamom	Insecticide[30]
Phorate	Organophosphates	Cardamom, ginger	Acaricide[31] (mites, ticks)
Profenofos	Organophosphates	Chillies, cumin, cardamom, curry leaves	Insecticide[32]
Pyraclostrobin	β-methoxyacrylates	Chillies	Fungicide[33]
Quinalphos	Organophosphates	Cardamom, Chillies, cumin	Pesticide[34]
Spirotetramat	Cyclic ketoenols	Cumin, chillies	Insecticide[35]
Triazophos	Organophosphates	Ginger, curry leaves, chillies, cumin, cardamom	Acaricide[36] (mites, ticks)

Although there are a number of pesticides detected in the spices studied, in assessing MRL compliance, two factors need to be considered: (a) low number of MRLs in FSSAI regulations in spices, and (b) the comparatively low intake of spices as compared

to other staple foods. The first factor necessitates that for majority of spice – pesticide combinations, the default MRL of 0.01 mg kg^{-1} needs to be considered for compliance assessment. Since this concentration level is very close to the quantification level of mass spectrometric instruments used for analysis of pesticide residues, any quantified value will result in non-compliance with MRL regulations. This is, however, a legal requirement and does not necessarily mean there is high food safety hazard due to pesticide residues in spices. The second factor of extent of consumption of spices as compared to staple foods directly illustrates this point. A comparison of dietary consumption data for staple foods like cereals and millets, as compared to spices was shown in Table 2.1. It can be seen that spices are consumed at very low quantities as compared to staple foods. Thus, a spice in which incidence of a pesticide occurs at a particular concentration will pose much lower food safety risk as compared to a staple food with the same incidence concentration. For example, the incidence of the pesticide propiconazole at 0.06 mg kg^{-1} in the spice cumin (default MRL 0.01 mg kg^{-1}, dietary consumption 2 g $person^{-1}$ day^{-1}) will pose much less food safety risk than the same concentration in rice (MRL 0.05 mg kg^{-1}, dietary consumption 257 g $person^{-1}$ day^{-1}) even though the MRL is exceeded in both cases.

Hazard characterization of residues in spices

For the hazard characterization, the residue which showed highest incidence in each spice was considered. The theoretical maximum daily intake (TMDI, mg $person^{-1}$ day^{-1}) was calculated by multiplying the residue concentration (mg kg^{-1}) by the average consumption of the spice (kg $person^{-1}$ day^{-1}). The maximum permissible intake (MPI, mg $person^{-1}$ day^{-1}) was calculated by multiplying the acceptable daily intake (ADI, mg kg body $weight^{-1}$ day^{-1}) by the average body weight of a child, taken as 16 kg[7]. The results of the evaluation are shown in Table 2.5.

Table 2.5 Hazard characterization in spices

Spice	Highest residue concentration		ADI (mg kg^{-1} day^{-1})[a]	Average Consumption (kg $person^{-1}$ day^{-1})[b]	TMDI (mg $person^{-1}$ day^{-1})	MPI (mg $person^{-1}$ day^{-1})
	Pesticide	Conc. (mg kg^{-1})				
Chillies	Profenofos	3	0.03	0.003	0.009	0.48
Cardamom	Acetamiprid	1	0.07	0.002	0.002	1.12
Cumin	Hexaconazole	3	0.005	0.002	0.006	0.08
Ginger	Triazophos	0.6	0.001	0.003	0.0018	0.016
Cinnamon	Fipronila	0.2	0.0002	0.002	0.0004	0.0032
Curry leaves	Chlorpyrifos	0.5	0.01	0.002	0.001	0.16

[a] ADI values are from the online Codex Pesticide MRL Database[37]
[b] Average consumption values for spices are taken from FSSAI Guidance Manual 2021[2]

It is seen that in all the spices studied, the theoretical maximum daily intake values are considerably less than the maximum permissible intake values, thereby showing that the incident residue levels in spices obtained in the study does not indicate any food safety hazard to consumers.

Conclusions

The residue analysis methods developed in Part I were applied to real samples of the spices chillies, cardamom, cumin, ginger, cinnamon and curry leaves collected from local markets for assessing the compliance against the national pesticide MRL regulations issued by FSSAI. The methods used QuEChERS sample preparation followed by GC-MS/MS and LC-MS/MS instrumentation as optimized in Part I, Chapter 3 and 4. In the study, analysis of 78 pesticides in 60 samples were covered.

The maximum number of pesticides were detected in cardamom, whereas the total residue load was seen to be maximum in cumin. The least number of pesticides and residue load was observed in cinnamon. Among the samples tested, 30 pesticides were seen to occur at levels above MRL levels in the spices tested. Maximum pesticide incidences as levels above MRLs were observed in cardamom, followed by cumin ad chillies. Cinnamon showed the least number of pesticides with concentrations above MRLs. Majority of the

pesticides detected in the spices belonged to organophosphates, carbamates and pyrethroid classes, with only few occurrences of new generation pesticides. Although 30 compounds were found in the study to be above MRL levels, it was noted for majority of these compounds, specific MRLs for spices were not available and so the default MRL of 0.01 mg kg-1 had to be used, which caused any quantified value to be above the MRL level. It was also observed that the consumption levels of spices were very small when compared to those for staple foods, which minimized the risks associated with pesticide residues in spices, and this was confirmed by risk characterization calculations.

The capability of the sample preparation and instrumental methods developed for multicomponent pesticide residue analysis in spices, for assessing compliance with national regulations and for assessing food safety risks associated with presence of pesticide residues, was thus effectively demonstrated.

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SUMMARY AND RECOMMENDATIONS

Summary

Presence of pesticide residues in food is one of the most important food safety hazards, which are known to cause adverse health effect in consumers. As a result, the amount of pesticide residues in food are strictly regulated by many countries across the world by issuing maximum residue limits (MRLs). In India, the MRLs for pesticides in various foods are fixed by the Food Safety and Standards Authority of India (FSSAI), taking into account various factors, including legality of usage of the pesticides based on good agricultural practices (GAP), toxicological evaluation of the pesticides and consumption pattern of the food in which the MRL is fixed. Typically, the MRLs are in the ranges of low parts per millions (mg kg^{-1}). To evaluate compliance of a particular food with respect to pesticide residues, the food has to be analysed with a sensitivity that enables direct comparison with the MRLs.

Spices, which are used extensively in India for culinary preparations, are prone to incidence of pesticide residues and the associated health hazards. Spices are generally considered difficult matrices to analyse owing to their complex chemical nature. Besides being low moisture commodities, spices contain active compounds that impart colour, flavour and aroma to foods, and these compounds can pose interferences in high sensitivity analysis of pesticide residues in these matrices. Since spices belong to different classes like dried roots, fruits and berries, seeds, bark, floral parts etc., applying a single analytical method for pesticide residues to different classes of spices is not practical.

In the present work, an effective and structured analytical framework for analysis of residues of 78 pesticides commonly used for cultivation of spices in India was developed and validated for six spices belonging to different classes, *viz.* chillies,

cardamom, cumin, ginger, cinnamon and curry leaves. The methods employed high sensitivity chromatography and tandem mass spectrometry techniques, *viz.* UPLC-MS/MS and GC-MS/MS. The issue of matrix effects observed in mass spectrometric analysis of pesticide residues in spices, which introduce qualitative and quantitative errors, were addressed in this work. Two novel strategies to mitigate these matrix effects, *viz.* the use of analyte protectants in GC-MS/MS, and the use of active components in spices as surrogate matrix compounds in solvent-based reference standards in UPLC-MS/MS, were successfully implemented.

Dithiocarbamates (DTC) are a class of broad-spectrum fungicides extensively employed in the cultivation of spices. A sensitive analytical method in which DTC residues were quantitatively converted to carbon disulphide, absorbed into isooctane and detected in GC-MS using selected ion monitoring technique was successfully validated in two spices, cardamom and black pepper.

The developed residue analysis methods using UPLC-MS/MS and GC-MS/MS were successfully applied to real spice samples collected from retail markets for performing compliance evaluation of these samples with the national MRL regulations in India. Characterization of food safety hazards associated with presence of pesticide residues in spices, based on the results of these analyses, were also performed.

Recommendations

Spices typically contain active chemical compounds which contribute to their special properties and which are present in relatively high concentrations. Many of these active compounds, or chemical analogues of such compounds, have been synthesised are readily available as reference standards. The novel strategy developed in the present work for using synthetic capsaicin as surrogate matrix compound in solvent-based standards to mitigate matrix effects in chillies can be extended to many other spices (e.g., curcumin for

analysis of residues in turmeric, piperine for analysis of residues in black pepper, etc.). The analytical method for DTC compounds validated in cardamom and black pepper also affords possibility for extending to other classes of spices. In analysis of samples from retail markets, the residues detected were mostly organophosphates, pyrethroids and carbamates. This showed that the adoption of new generation pesticides in India for cultivation of spices is still not widespread. This is an avenue of improvement which can potentially result in considerably lowering food safety hazards in Indian spices.

PUBLICATIONS

i. **Ramesh Babu Natarajan,** Joby Thomas Kakkassery, Ranjith Arimboor, Joby Jacob, Binumol Thankan, Development and validation of a GC-MS method for analysis of dithiocarbamate fungicide residues in the spices cardamom (*Elettaria cardamomom*) and black pepper (*Piper nigrum*). *Journal of Food Science and Technology,* **2022,** 1-11 https://doi.org/10.1007/s13197-022-05462-9

ii. **Ramesh Babu Natarajan**, Joby Thomas Kakkassery, Anaswara Raveendran, Amrutha Ravi, Mohit Mohan. Determination of pesticide residues in four major spices using UPLC-MS/MS and optimized QuEChERS sample preparation workflow. *Oriental Journal of Chemistry*, **2022** (38)3 727-737. http://dx.doi.org/10.13005/ojc/380325

CONFERENCE PRESENTATION

i. **N. Ramesh Babu,** K. Joby Thomas. Impact of colour and pungency principles in chilli on the detection of organophosphorus residues by GC-MS/MS and LC-MS/MS techniques, in the *International Conference on Chemistry and Physics of Materials*, organized by St. Thomas College Autonomous, Thrissur during 19-21 December 2018**.**

TO BE COMMUNICATED

i. **Ramesh Babu Natarajan,** Joby Thomas Kakkassery, Anaswara Raveendran, Mohit Mohana and Amrutha Ravi, High sensitivity pesticide residue analysis in chilli-peppers by UPLC-MS/MS: mitigation of matrix effects using combined matrix-surrogate and dilution approaches.