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.

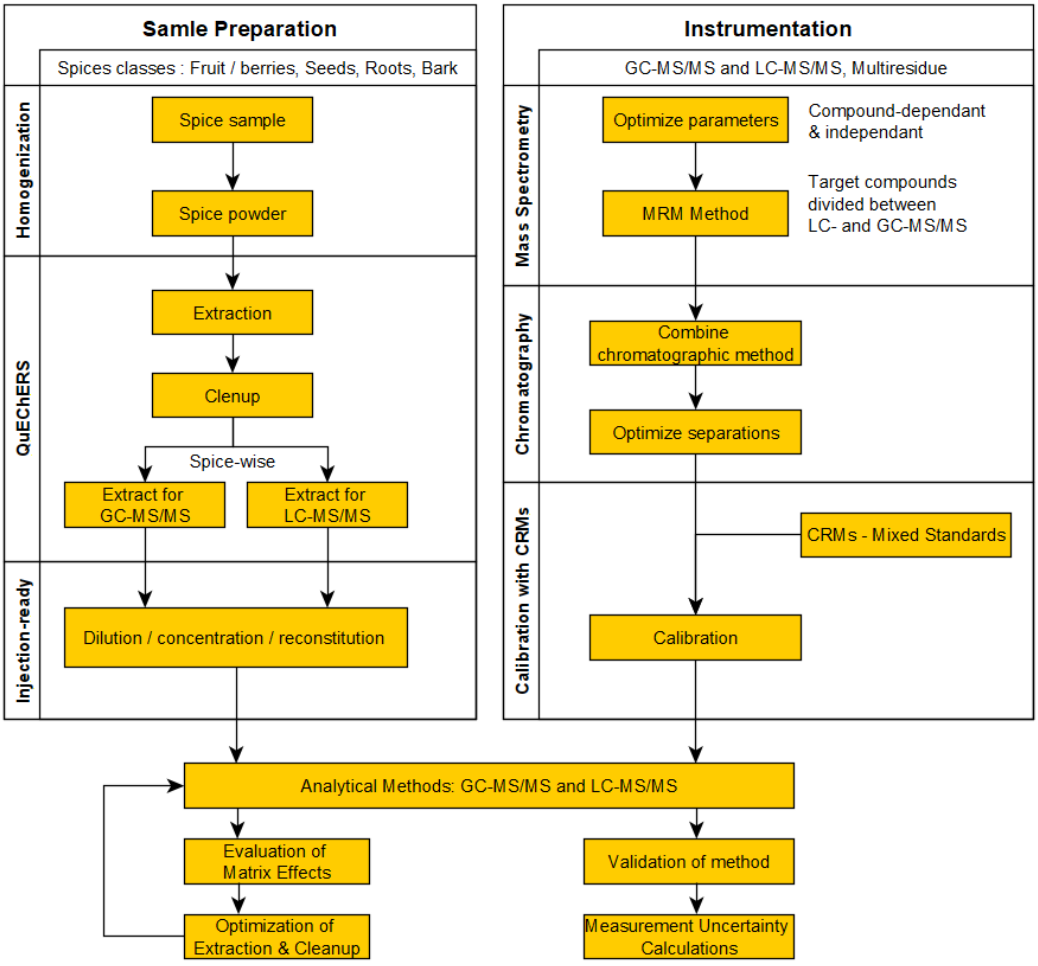

Figure 1.10 General Scheme for method development and optimization

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).