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.

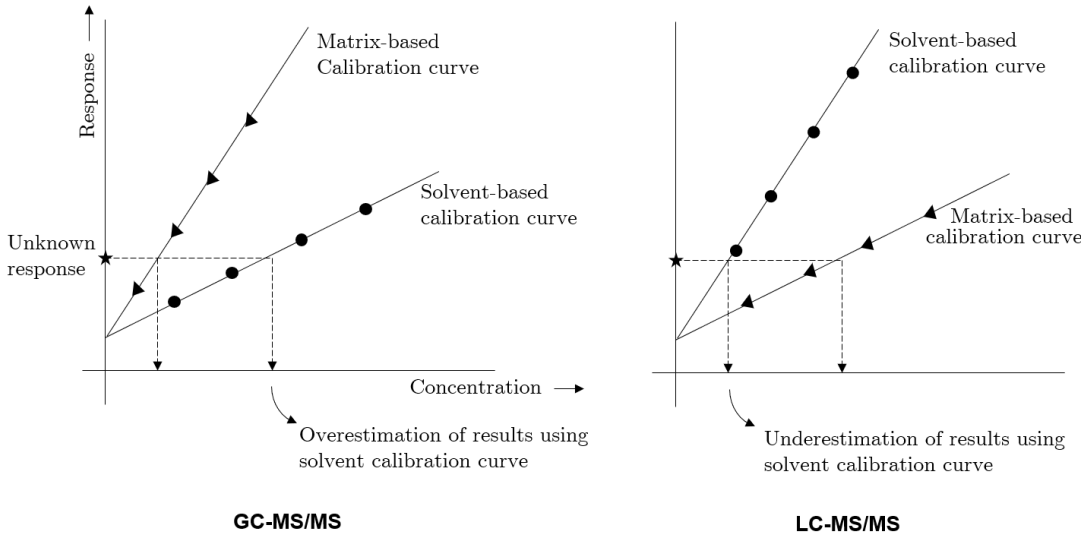


Figure 1.25 Schematic representation of quantitation issues due to matrix effects

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.

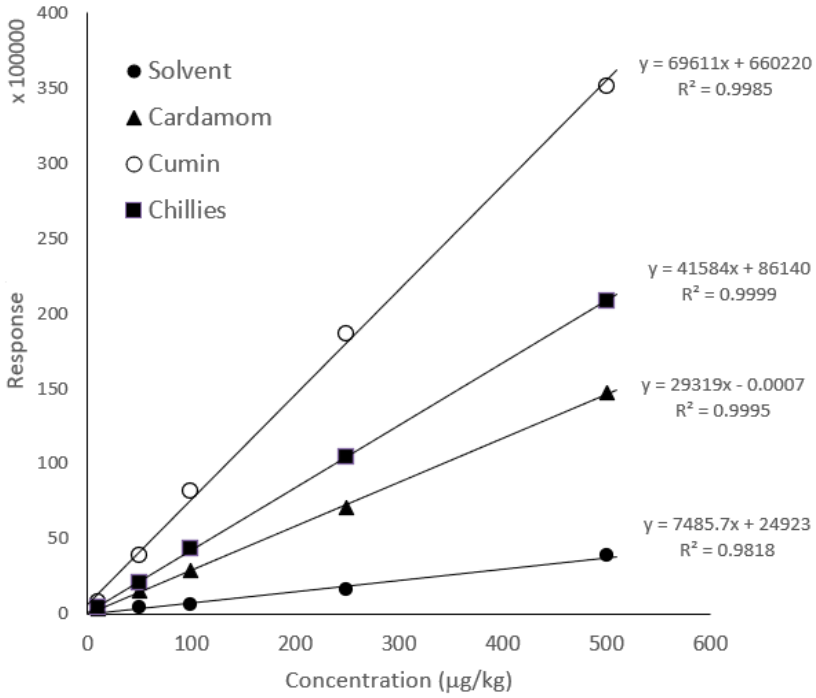


Figure 1.26 Comparison of calibration curves in solvent and spice extracts in bifenthrin

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.

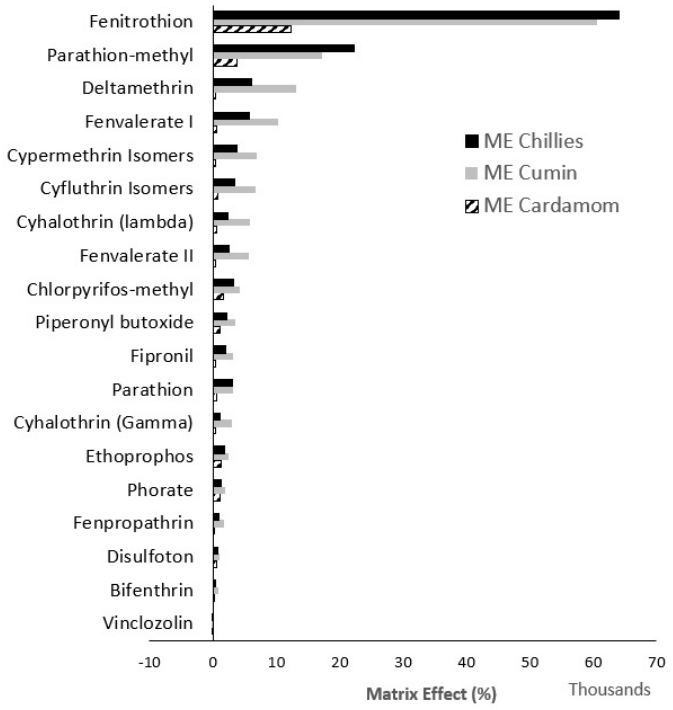


Figure 1.27 GC-MS/MS Matrix effects for pesticides observed in three spices

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.

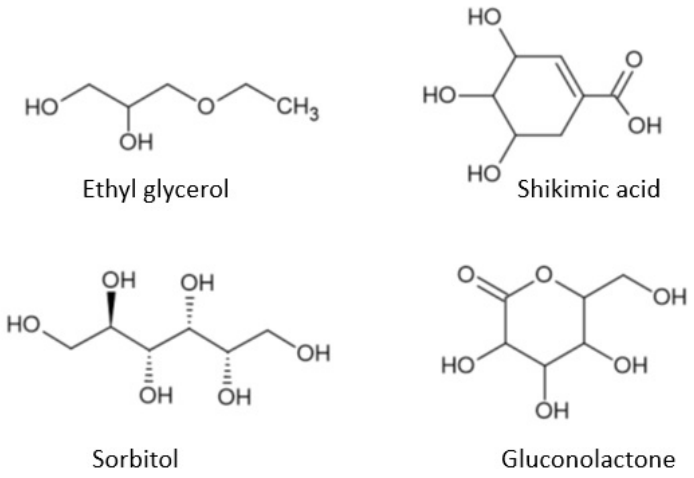


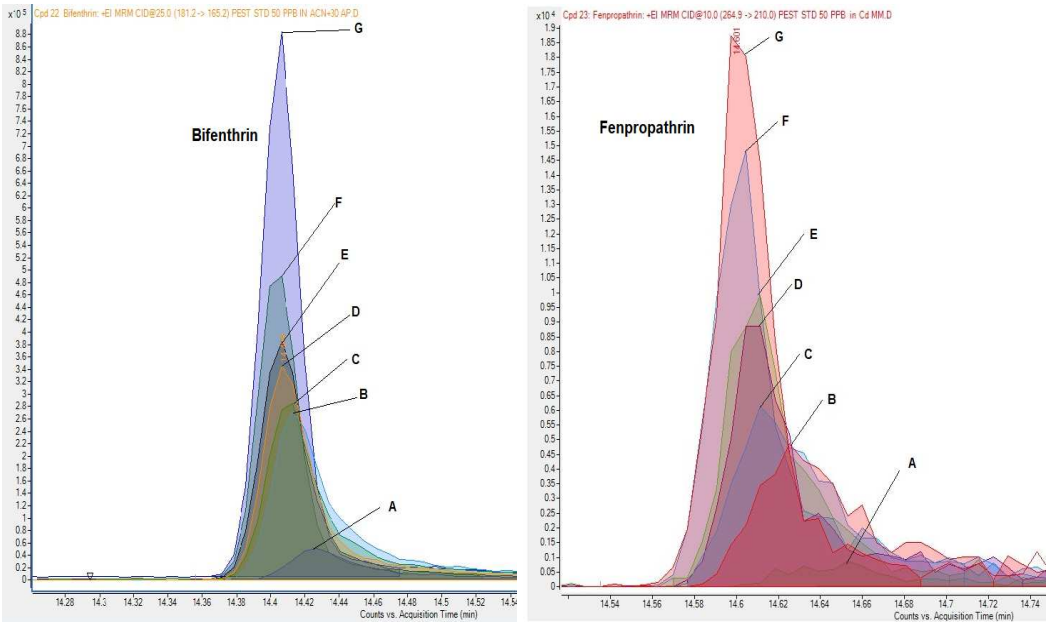
Figure 1.28 Chemical structures of analyte protectants used in the study

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.

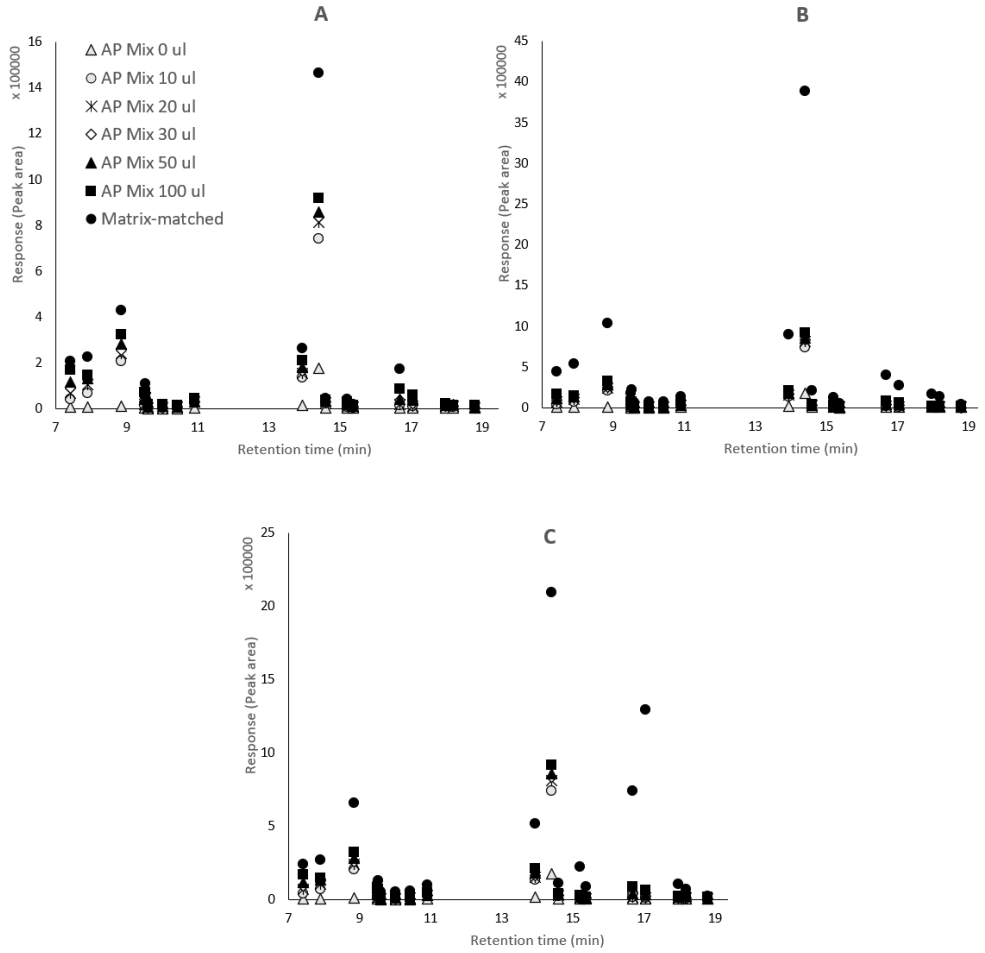
The comparison of responses in solvent-based standards at 50 mg kg^{-1} concentration for various analytes, with and without AP mix addition, as compared to the matrix matched standards at the same concentration, is given in Figure 1.30 for the spices cardamom, cumin and chilli. Here, the responses of 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.

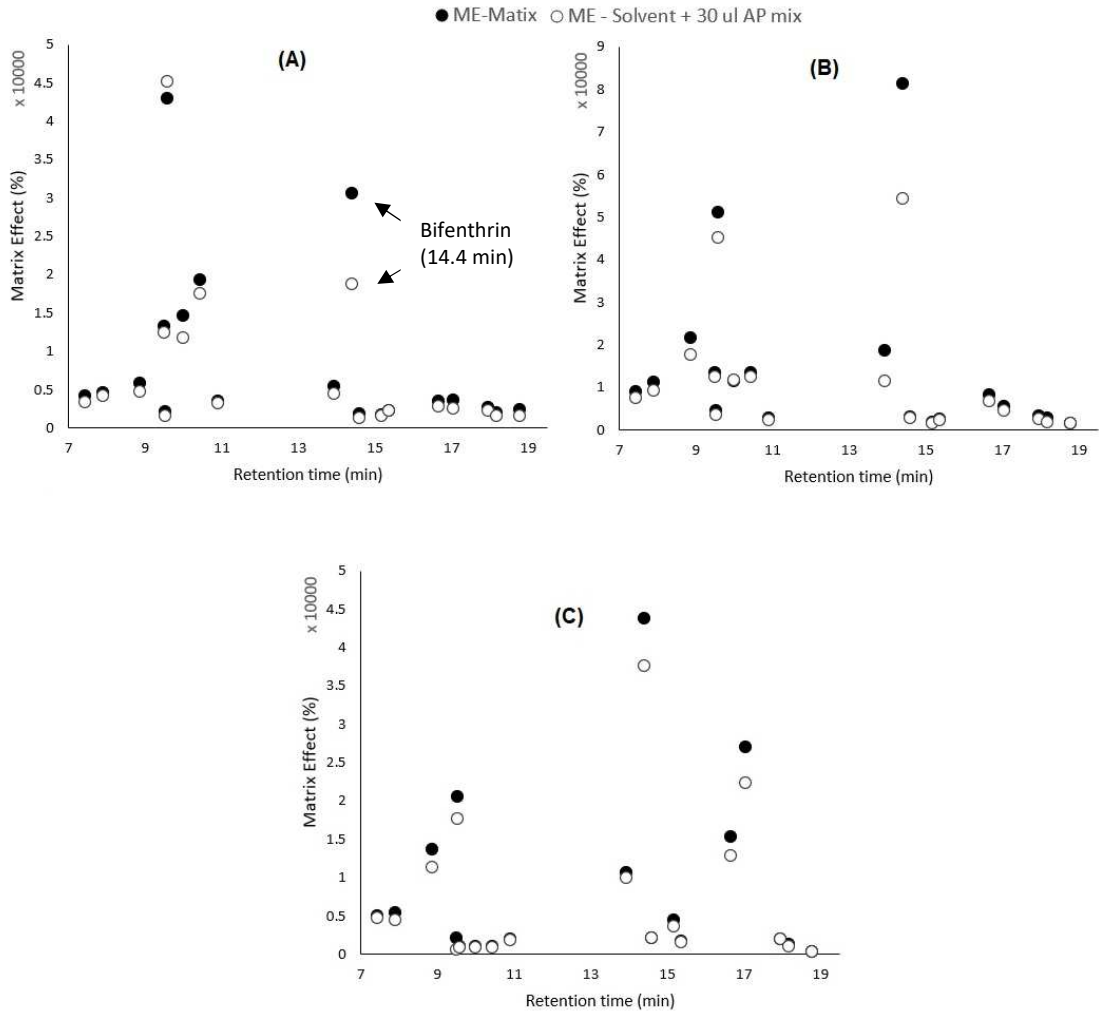


A: cardamom, **B**: cumin **C**: chillies

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.

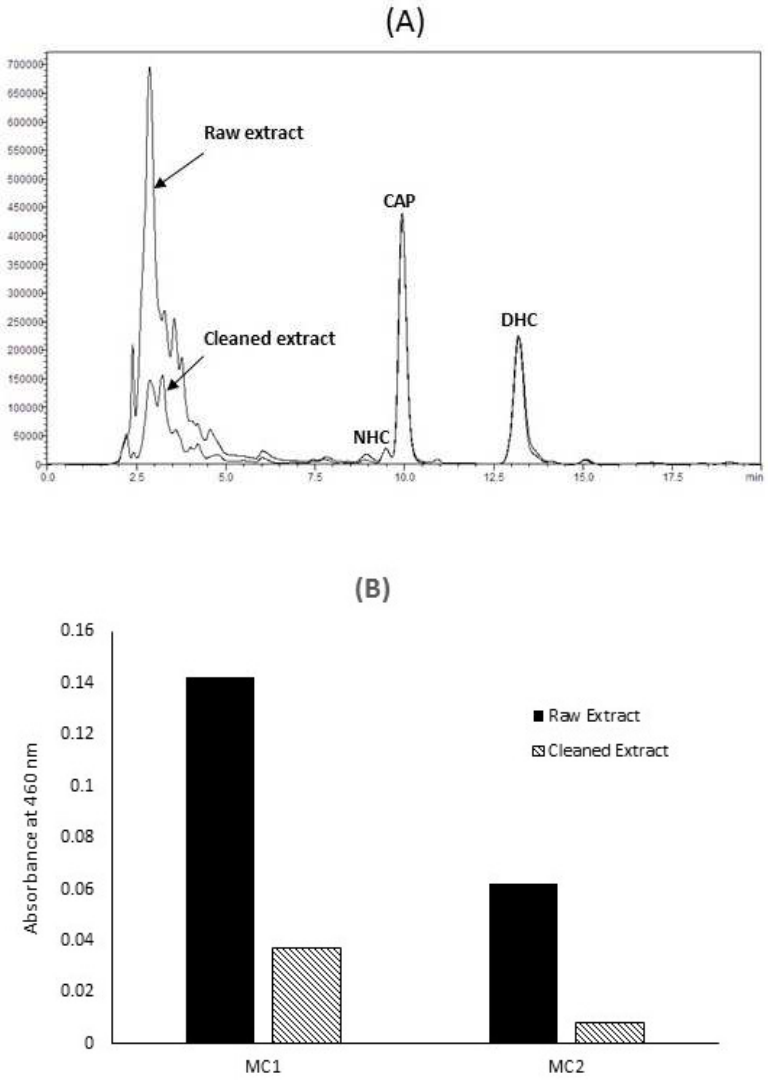


Figure 1.32 Effect of optimized cleanup step during sample preparation, on **A** - capsaicinoid content (NHC: nordihydrocapsaicin, CAP: capsaicin, DHC: dihydrocapsaicin) and **B** - carotenoid content of the chilli extract.

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.

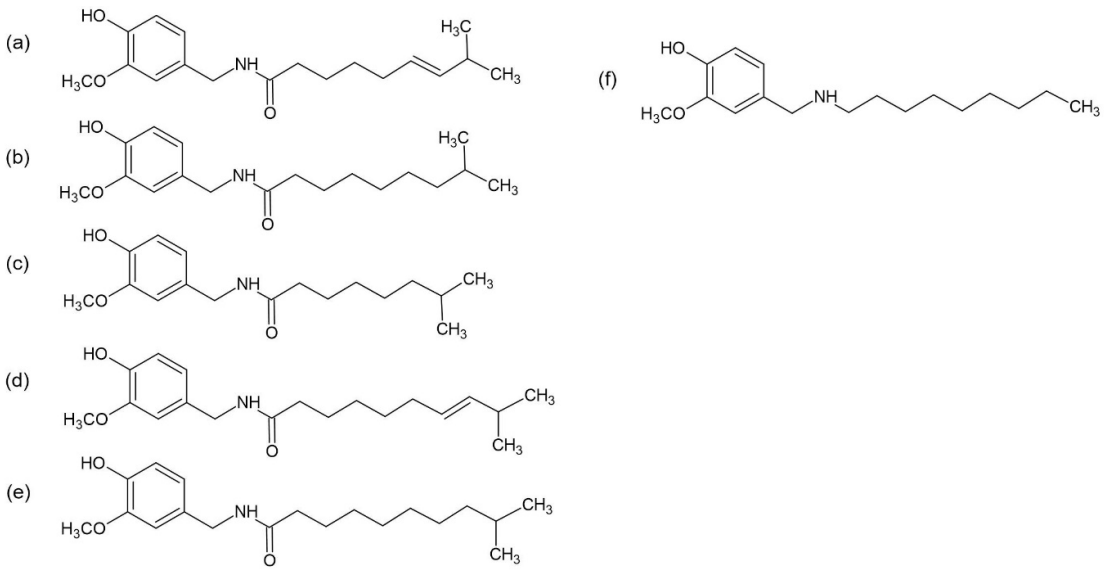


Figure 1.33 Chemical structures of capsaicinoids in chilli-peppers and synthetic capsaicin: (a) capsaicin, (b) dihydrocapsaicin, (c) nordihydrocapsaicin, (d) homocapsaicin, (e) homodihydrocapsaicin, (f) N-vanillylnonanamide (NVNA, synthetic capsaicin).

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**.

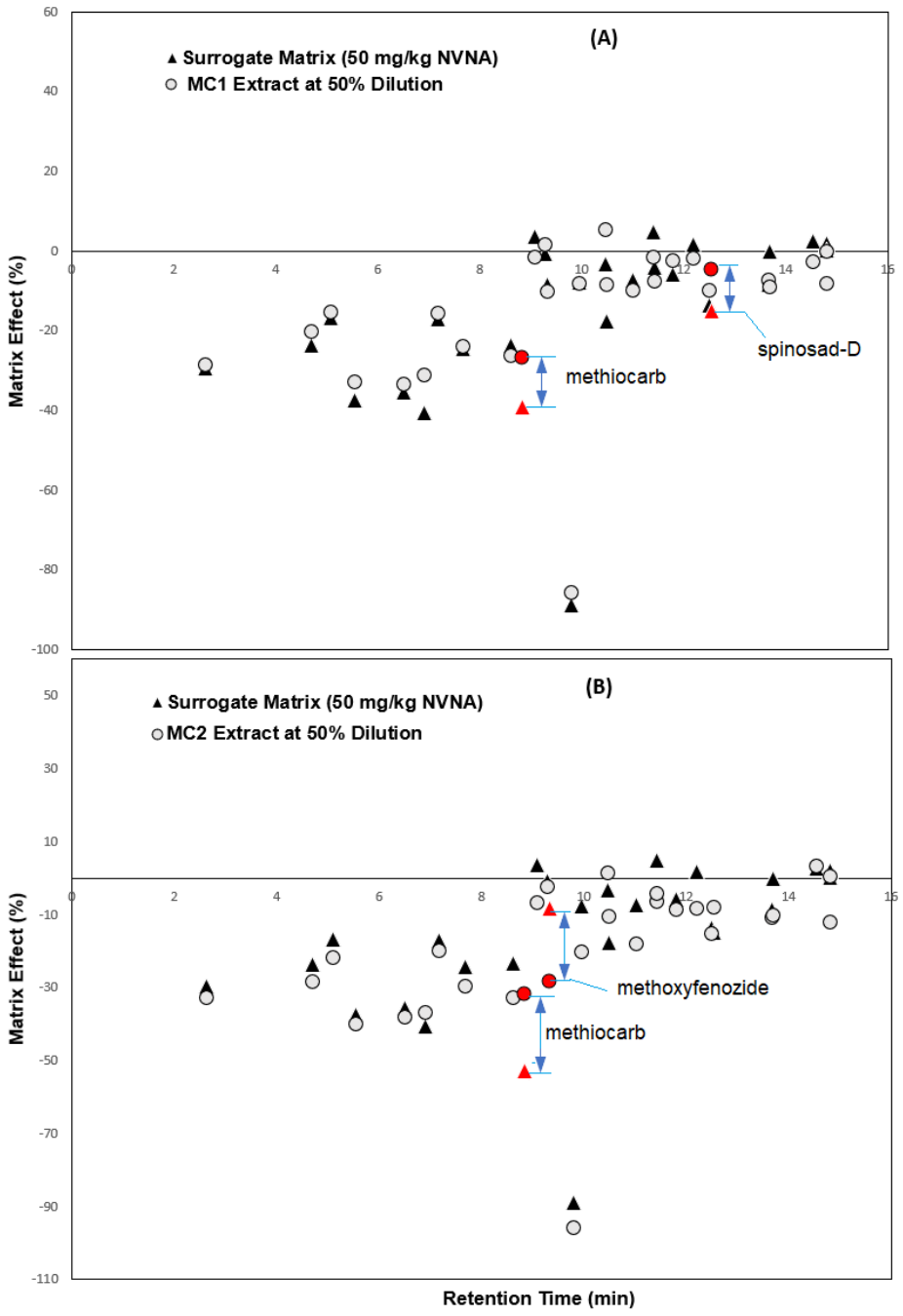


Figure 1.34 Matrix effects of pesticides at concentration of 0.01 mgkg^{-1} in (A) surrogate matrix with 50 mg kg^{-1} NVNA & MC1 (38,100 SHU) matrix extract diluted to 50%, and (B) surrogate matrix with 50 mg kg^{-1} NVNA & MC2 (84,600 SHU) matrix extract diluted to 50%.

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.

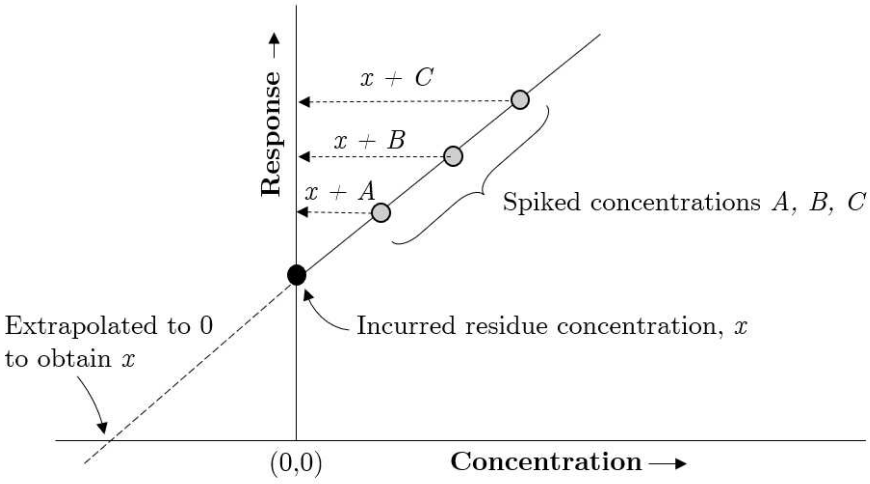


Figure 1.35 Schematic illustration of standard addition technique

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.

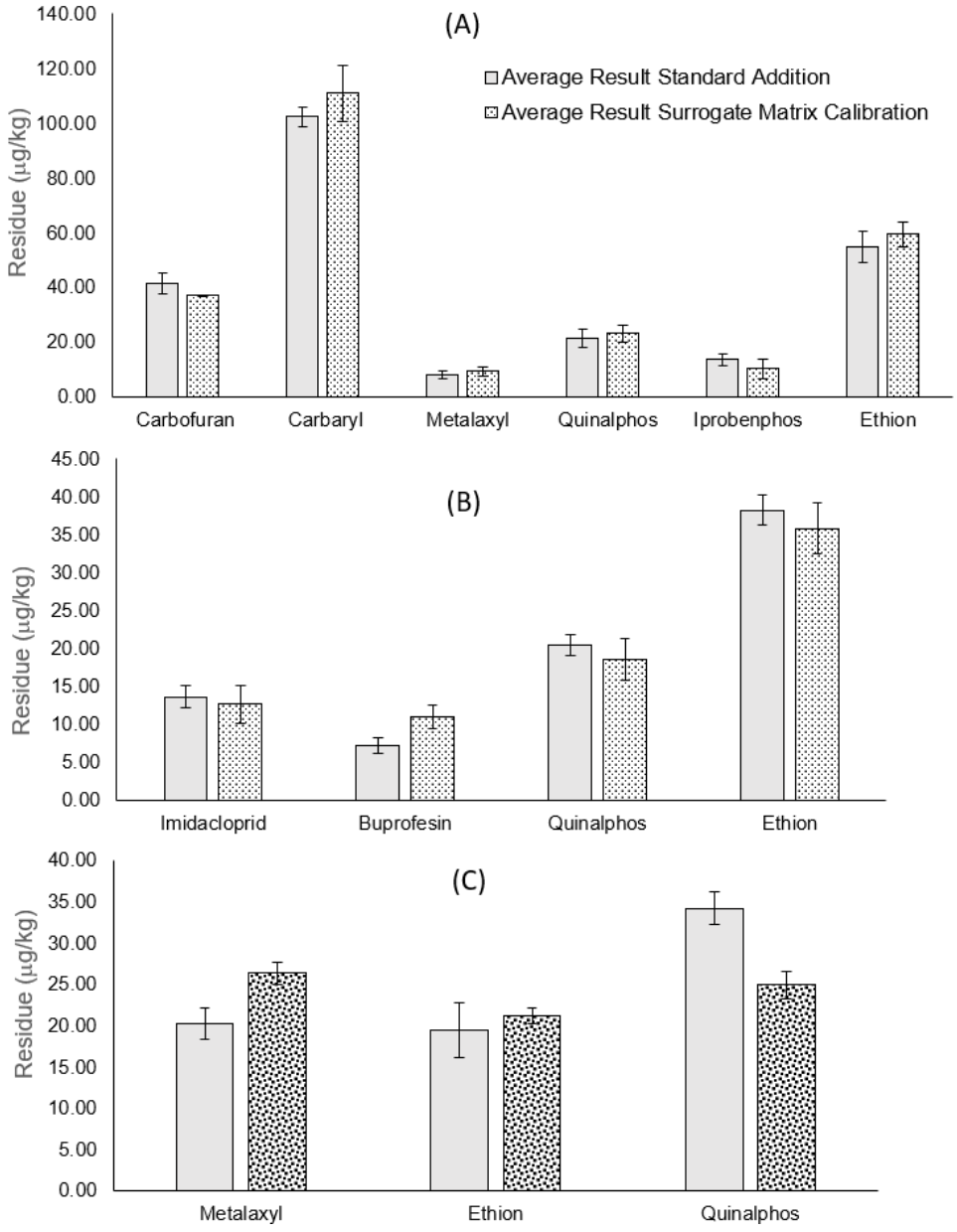


Figure 1.36 Comparison of average values of residue results (µg/kg) obtained using 3-point standard addition and surrogate matrix-based calibration (n=3) for three chilli-pepper samples with incurred residues: (A) sample S1 (36,200 SHU), (B) Sample S2 (44,200 SHU) and (C) Sample S3 (58,100 SHU).

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.