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.
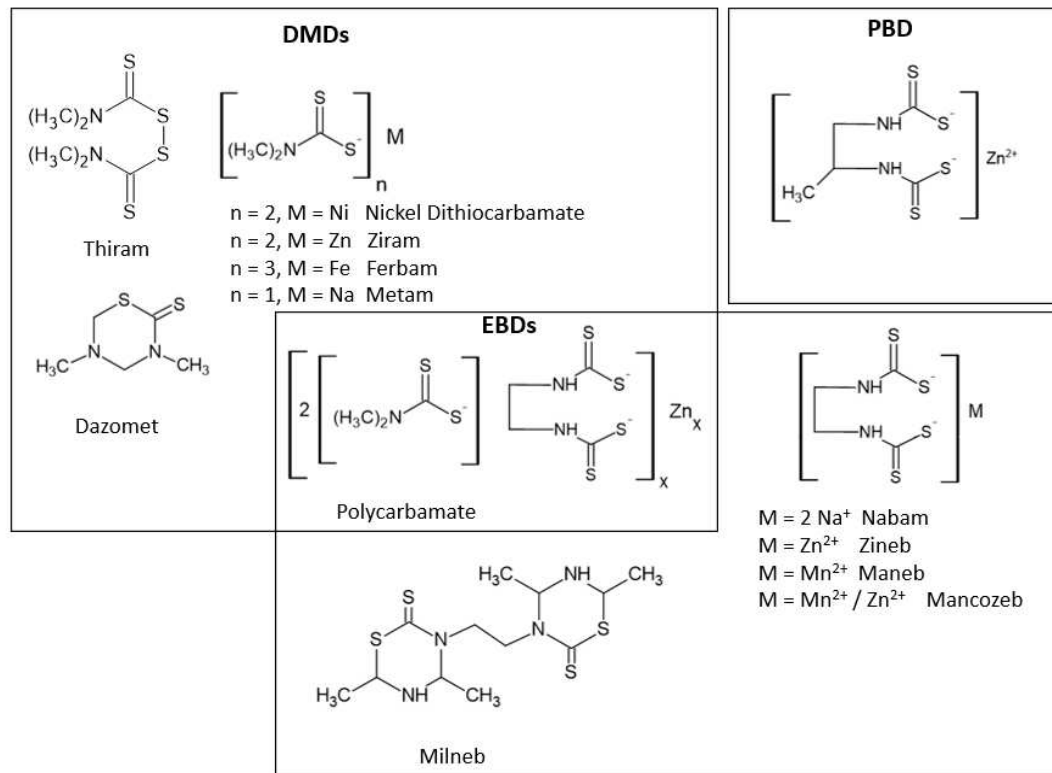

Figure 1.37 Chemical structures of important dithiocarbamate fungicides

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.
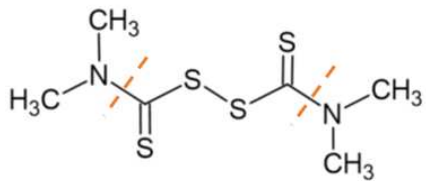

Figure 1.38 Cleavage of thiram to form CS_2

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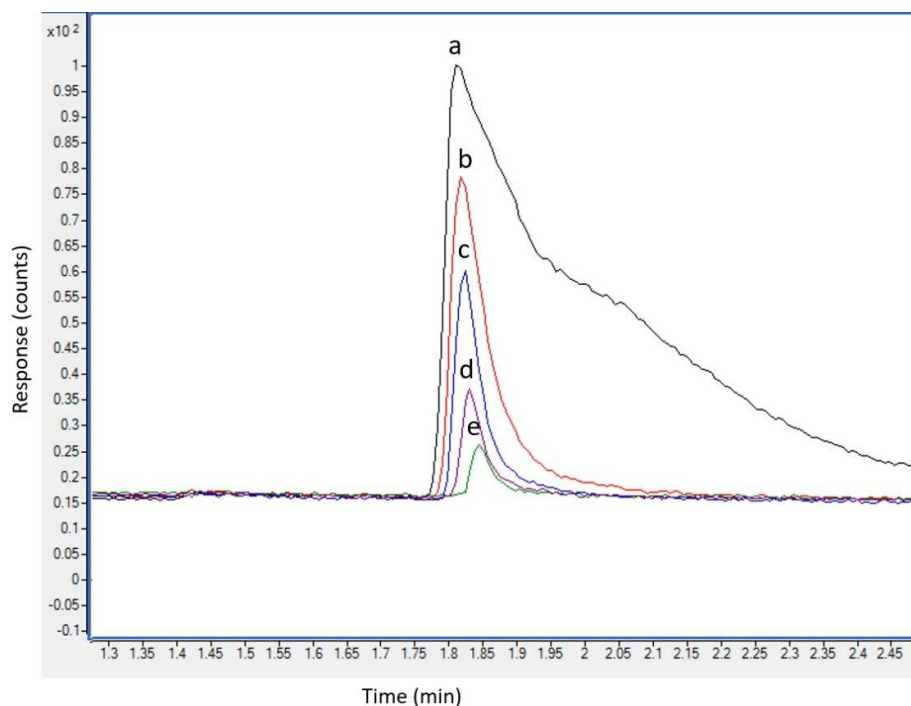
(**a**) 0.1:1 / 0.11033 mL/min, (**b**) 10:1 / 11.033 mL/min, (**c**) 20:1 / 22.066 mL/min, (**d**) 50:1 / 55.165 mL/min, (**e**) 100:1 / 110.33 mL/min

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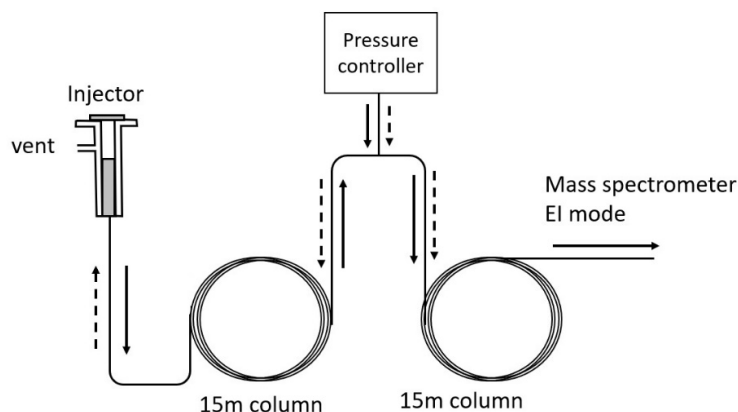
Figure 1.40 Schematic of the post-run, mid-column backflush option in GC. Solid arrows indicate the flow of carrier gas during run, and dotted arrows indicate the flow of carrier gas after the post run, mid column back flush is initiated.

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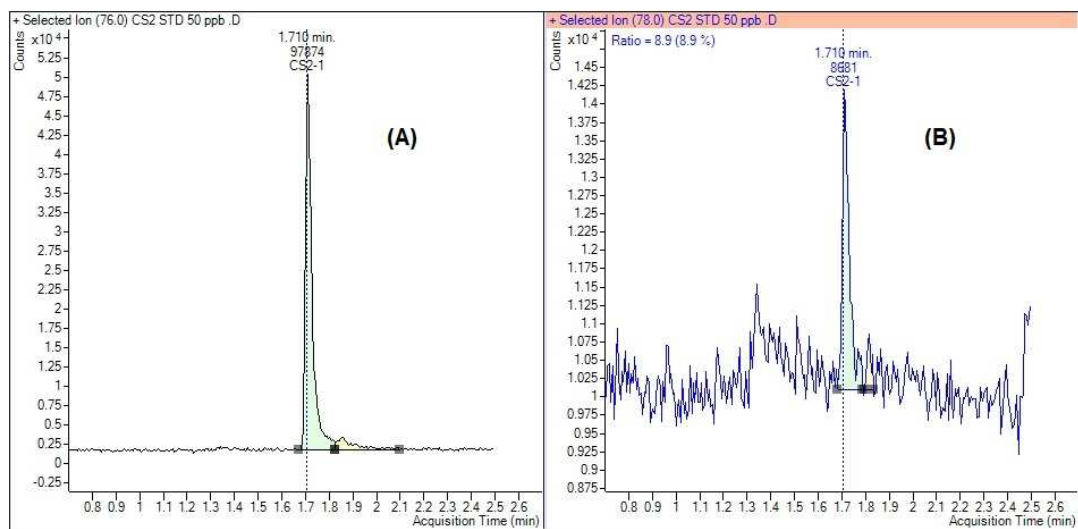
(**A**) m/z = 76, (**B**) m/z = 78, at LOQ concentration of 0.05 mg kg^{-1}

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.

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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
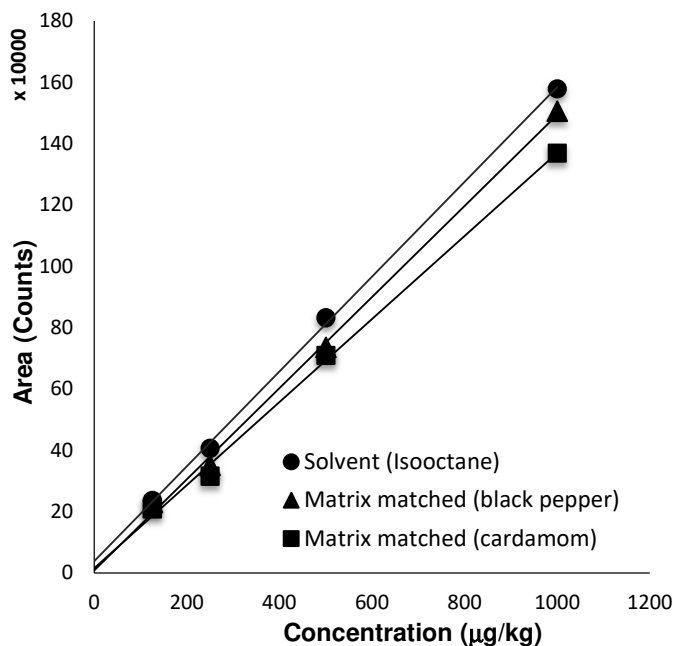

Figure 1.42 Matrix matched calibration curves for CS_2 in isooctane, black pepper extract and cardamom extract

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.