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Assessment of organochlorine pesticide residues in Indian flue-cured tobacco with gas chromatography-single quadrupole mass spectrometer

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Abstract Presence of pesticide residues in tobacco increases health risk of both active and passive smokers, apart from the imminent potential health problems associated with it. Thus, monitoring of pesticide residue is an important issue in terms of formulating stringent policies, enabling global trade and safeguarding the consumer's safety. In this study, a gas chromatography-single quadrupole mass spectrometry (GC-MS) method based upon quantifier-qualifier ions (m/z) ratio was employed for detecting and assessing ten organochlorine pesticide residues (α-HCH, β-HCH, γ-HCH, δ-HCH, 2,4-DDT, 4,4-DDT, endrin, α-endosulfan, β-endosulfan and endosulfan sulphate) in 152 flue-cured (FC) tobacco leave samples from two major tobacco growing states, Karnataka and Andhra Pradesh, of India. In the majority of samples, pesticide residue levels were below the limit of quantification (LOQ). In few samples, pesticide residues were detected and they found to comply with the guidance residue levels (GRL) specifications of the Cooperation Center for Scientific Research Relative to Tobacco (CORESTA). Detection of the phase out pesticides like DDT/HCH might be due to transfer of persistent residues from the environmental components to the plant. This is the first report on these ten organochlorine pesticide residues in Indian FC tobacco.

Keywords Organochlorine pesticides · Indian tobacco · Residue assessment · GC-MS

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Introduction

Use of plant protection chemicals, mainly pesticides, has become integral part of modern agricultural production systems around the world, not only to protect the crop but also to secure the optimum crop yield. However, indiscriminate and non-judicious use of pesticides along with absence of good agricultural practices often results in presence of pesticide residues in the produce. Exposure of consumers to these commodities containing pesticide residues is undesirable because of the health hazards associated with these pesticides. Thus, to monitor the pesticide residues in food commodities, several national and international bodies follow stringent rules to minimize the exposure of consumers to the pesticides. Publics' exposure to pesticides, up to a significant level, can also be through mainstream and passive smoking of tobacco (Clapp and Shelar 1972; Clark et al. 1998). Tobacco (*Nicotiana tabacum* L.), one of the world's leading commercial crops, is grown extensively

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in countries such as India, China, Brazil, USA, etc. for the production of tobacco leaf (economic part). The pesticides applied to tobacco during its cultivation may remain in the leaf till harvesting and even after curing/post harvest processing in the final manufactured product (Morris 1972; Chapman 2003). While smoking, the pyrolytic products from tobacco plant matrix may interact with the pesticides or with the pyrolytic products of pesticides and results in the formation of more toxic smoke than that from the sole pesticide residues (Lorenz et al. 1987) which is being inhaled by mainstream smoker and several other passive smokers. Clapp and Shelar (1972) reported that rate of transfer of pesticides from tobacco into smoke averaged about 12 % of that in the tobacco before combustion. Transfer of organochlorine pesticides like DDT, its derivatives, and pyrolytic products into mainstream smoke may reach up to 20 % of the residues in the unsmoked tobacco (Bowery et al. 1965; Atallah and Dorough 1975). The per cent transfer of some other organochlorine pesticides like endosulfan (15.3–16.3 %), endrin (18.18–31.58 %), γ-HCH (3.1–40 %) etc. from unburned tobacco to mainstream smoke have also been noticed (Rodgman and Perfetti 2013). Thus, presence of pesticide residues in tobacco is likely to increase the harmful effect of smoke for active and passive smokers.

In India, tobacco is cultivated in about 0.4 M ha and yielding 0.76 Mt of dry leaf produce (FAO 2012). India is the world's second largest producer (after China) and exporter (after Brazil) of tobacco and nearly $ 901.95 million revenue is generated from its export (Tobacco Board 2013). Apprehensions related to the accumulation of pesticide residues at toxic levels in the final produce drives the food safety regulations to become more and more stringent in most countries. Tobacco being non-food crop has attracted less attention regarding regulations of pesticide residues. But reports on pesticide residues in smoke have drawn attention of national and international bodies (CEC 1976; GAO 2003) across the world, and stringent policies have been formulated to regulate the pesticide residue in tobacco. Recently, in 2013, the Agro-Chemical Advisory Committee (ACAC) of the Cooperation Center for Scientific Research Relative to Tobacco (CORESTA) has issued pesticide guidance residue levels (GRLs) in tobacco. In spite of several awareness campaigns about the imminent potential health problems associated with tobacco, millions of people, particularly in lower and middle-income countries, still indulge in cigarette smoking (WHO 2011), and presence of pesticide residues, further, increases health risk of smokers directly and non-smokers exposed to tobacco smokes. Thus, monitoring of pesticide residue is an important issue in terms of formulating stringent policies, enabling global trade and safeguarding the consumer's safety.

To our knowledge, there exists no information on the pesticide residue levels in FC tobacco grown in India. This study aimed to assess residue levels of ten organochlorine pesticides (α-HCH, β-HCH, γ-HCH (lindane), δ-HCH, 2,4-DDT, 4,4-DDT, endrin, α-endosulfan, β-endosulfan and endosulfan sulphate) in tobacco leaves from two major tobacco growing states of India. Since accurate estimation of residue is prerequisite for effective monitoring and formulating strategies, efforts were made to standardize and validate a multiresidue analysis method. A gas chromatography-single quadrupole mass spectrometry (GC-MS) method based upon quantifier-qualifier ions was employed for detection of these pesticides.

Materials and methods

Collection of tobacco samples and chemicals

The cured leaf samples of FC tobacco collected from Karnataka and Andhra Pradesh, the two principal tobacco-producing states of India, were used in the present study. In all, 152 representative leaf samples were drawn from seven districts including Mysore (12.08° N 76.32° E) and Hassan (13° N 76° E) districts in Karnataka and Prakasam (15.50° N 80.05° E), Krishna (16.17° N 81.13° E), Nellore (14.43° N 79.97° E), East Godavari (16.57° N 82.15° E) and West Godavari (16.43° N 81.09° E) districts in Andhra Pradesh. The leaf samples of 0.5 kg each were collected and stored at 4 °C and processed for pesticide residue analysis within 10 days (Rahman et al. 2012).

Certified reference standards of all the test pesticides were of >98 % purity and purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile, the extraction solvent was of specially dried residue analysis grade and purchased from Thomas Baker (Mumbai, India). The adsorbent, florisil, was received from Sisco Research Laboratories (Mumbai, India). The other reagents viz., sodium chloride and anhydrous sodium sulphate were of analytical reagent grade and purchased from Merck (Mumbai, India).

Selection of pesticides and preparation of standard solutions

A total of ten organochlorine pesticides (Table 1) was considered in this study, which are typically GC-amenable and belonged to the list of chemicals issued by CORESTA (2013) for tobacco. Most of these organochlorine pesticides are banned and are not recommended for agricultural uses in tobacco cultivation in the Indian subcontinent. The prolonged persistence of these organochlorine pesticides coupled with their low GRL values in tobacco was the main criteria for selecting these pesticides.

The stock solutions of the individual pesticide standards were prepared by accurately weighing 10 (±0.01) mg of each analyte in volumetric flasks (certified "A" class) and dissolving the same in 10 mL hexane. These were stored in dark vials in a refrigerator at −20 (±2) °C. An intermediate stock standard mixture of 10 μg mL^{-1} was prepared by mixing the appropriate quantities of individual stock solutions followed by requisite volume makeup with hexane and stored at −20 (±2) °C. Stability of the working solvent standards was checked against freshly prepared working standards (1 μg mL^{-1}) from the intermediate stocks as per SANCO guidelines (SANCO/12495 2011). A working standard mixture of 1 μg mL^{-1} was prepared by diluting the intermediate stock solution, from which the calibration standards were prepared by serial dilution with hexane. A set of six calibration standards at 0.005, 0.01, 0.025, 0.050, 0.10 and 0.25 μg mL^{-1} was freshly prepared from the working standard mixture of 1 μg mL^{-1} concentration by appropriate dilutions. Matrix-matched standards at the same concentration levels were prepared by extracting control tobacco and spiking the extract with appropriate volumes of the working standard solutions.

Processing of tobacco samples

The leaf samples were oven dried at 60 °C for 2 h. The dried leaves (after removing mid rib) were powdered, homogenized, sieved (through 1 mm) and used for extraction. Then 1 g powder was taken in a 150-mL Erlenmeyer conical flask, and 20 mL of acetonitrile/water (1:1) mixture was added to the flask. Our laboratory observation showed that the resulting coloured solvent extract from the tobacco matrix extracted with acetonitrile/water (1:1) mixture became clear upon florisil clean-up as compared to extraction with pure acetonitrile. This indicated chances of matrix interference from tobacco samples were less for acetonitrile/water (1:1) mixture. Further, use of acetonitrile/water (1:1) mixture ensured low use of solvent and reduced the cost of solvent. The samples were agitated for

Table 1 Instrument parameters with Rt, SIM ions, LOQ, matrix effect, recovery and GRL of ten organochlorine pesticides

Pesticide name	Rt (min)	SIM ions (m/z)			LOQ (mg kg^{-1})	Matrix effect (%) at 0.05 mg kg^{-1}	Recovery (% RSD) at 0.05 mg kg^{-1}	GRL (mg kg^{-1})
		Quantifier	Qualifier 1[a]	Qualifier 2[a]				
α-HCH	5.821	181	183 (99)	217 (70)	0.006	14.4	105 (±9)	0.05[b]
β-HCH	6.255	181	183 (97)	217 (78)	0.006	16.8	99 (±12)	
δ-HCH	6.765	181	217 (52)	183 (99)	0.012	21.8	108 (±10)	
Lindane	6.373	181	183 (99)	217 (59)	0.006	14.4	102 (±14)	0.05
Endrin	10.642	263	81 (86)	243 (65)	0.012	38.9	85 (±15)	0.05
α-Endosulfan	9.715	195	170 (91)	206 (95)	0.012	15.8	82 (±10)	1.00[c]
β-Endosulfan	10.835	195	197 (87)	170 (60)	0.012	18.4	81 (±9)	
Endosulfan sulphate	11.851	272	274 (95)	237 (48)	0.012	39.6	72 (±11)	
2,4-DDT	11.085	235	237 (65)	165 (51)	0.006	32.2	96 (±8)	0.20[d]
4,4-DDT	11.915	235	165 (46)	237 (66)	0.006	26.1	85 (±8)	

GRL guidance residue level

[a] Relative abundance of qualifier 1 and 2 was calculated relative to the quantifier m/z considering its intensity as 100 %

[b] Total HCH=(α-HCH+β-HCH+δ-HCH)

[c] Total endosulfan=(α-endosulfasn+β-endosulfan+endosulfan sulphate)

[d] Total DDT=(sum of 2,4- and 4,4-DDT, 2,4- and 4,4-DDD (TDE), 2,4- and 4,4-DDE) as per the official residue definition of CORESTA (2013)

45 min over an orbital shaker at 150 rpm and filtered. The filtrate was partitioned with 40 mL of hexane, and the coloured hexane fraction was collected for clean-up. A column was prepared by fabrication of a column bed which was made of 2 g florisil (60/100 mesh) (activated at 200 °C for 6 h, followed by deactivation with 2 % distilled water) sandwiched between two layers of anhydrous sodium sulphate (2 g each layer). Florisil worked as an adsorbent for removal of large organic molecules from tobacco matrix extract like pigments, lipids, long-chain organic acids and other matrix compounds. Anhydrous sodium sulphate played dual purposes. First, it acted as a protective layer for underneath adsorbent layer of florisil while loading the sample for column clean-up, and secondly, it removed the traces of water from the solvent extract before injecting to the instrument. Before clean-up, the column was eluted with pure hexane and then coloured hexane extract was passed through the column. Clear and colourless hexane fraction was collected from the column and evaporated to dryness under reduced pressure. The residuum was re-dissolved in a volume of 2.5 mL hexane and analyzed by a QP-2010 Plus GC-MS (single quadrupole, Shimadzu Corporation, Kyoto, Japan).

Instrument condition

The GC system (GC 2010 Plus) was equipped with ZB-5 (5 % diphenyl, 95 % dimethylpolysiloxane, 30 m (l)× 0.25 mm (id), 0.1 μm film thickness) capillary column and autosampler. Ultra pure grade helium (INOX Limited, Hyderabad) was used as the carrier gas. The GC separation of pesticides was achieved by formulating an optimized oven temperature program that started from an initial temperature of 100 °C (hold for 0.5 min), ramped at the rate of (@) 30 °C min^{-1} up to 180 °C (hold 1 min), @ 10 °C min^{-1} up to 240 °C (hold for 2 min), @ 10 °C min^{-1} up to 250 °C min (hold for 1 min), @ 10 °C min^{-1} to 260 °C (hold 2 min) and finally @ 40 °C min^{-1} up to 320 °C (hold for 10 min). This program resulted in a run-time of 18.67 min. A typical GC-MS batch consisted of five matrix-matched multiresidue calibration standards, samples, one matrix blank and one recovery sample for performance check after a set of every six samples. The detector voltage was set at 1 kV, and the data acquisition was carried out in the selected ion monitoring (SIM) mode with compound-specific m/z ions for selective identification of each pesticide (Table 1). The injector temperature was maintained at 250 °C in a splitless mode and 2 μl of the sample volume was injected. The carrier gas (helium) flow was maintained at 3 mL min^{-1} at the linear velocity of 64.4 cm s^{-1}.

Method performance

The calibration curves for all the compounds in pure solvent and matrix were obtained by plotting the peak area against the concentration of the corresponding calibration standards at six calibration levels ranging between 0.005–0.25 mg mL^{-1}. The sensitivity of the method was evaluated in terms of limit of quantification (LOQ). The LOQ is the concentration at which the S/N is ≥10 in matrix extract (Table 1). Recovery of the studied pesticide mixture from tobacco matrix was studied at 0.05 mg kg^{-1} level of fortification with six replications (Table 1). The matrix effect was evaluated by spiking untreated tobacco samples with the pesticide mixture at 0.05 mg kg^{-1} level. The peak area response of individual pesticides in hexane was compared with that of the corresponding response in the matrix-matched standard at the same concentration level (Table 1). A negative value of ME (%) indicates matrix-induced signal suppressions, whereas a positive value indicates enhancement in the signal.

Results and discussion

Calibration curves with $R^2 \geq 0.997$ for all the test compounds indicated good linearity of the method. The LOQ values for all pesticides ranged from 0.006-0.012 mg kg^{-1} which were much below the GRL values of CORESTA (Table 1). The present method showed 72–108 % recovery range with relative standard deviation (RSD) less than 15 % which met the internationally accepted recovery criteria (EURACHEM/CITAC Guide CG4 2000; CORESTA 2013). The matrix-induced enhancements were observed for all studied pesticides and, therefore, matrix-matched standards were used for quantification (Table 1).

Recently, Rahman et al. (2012) used a high-performance liquid chromatography (HPLC) system with a photodiode array detector for detection of some pesticides on the basis of retention time (Rt) using. But tobacco is a known complex matrix, and therefore, the detection of residues on the basis of any universal detector makes the trace level detection often challenging

(Chen et al. 2013). Hence, true detection of pesticide residue is of great significance matter for matrices like tobacco.

The method employed in the present study enabled the separation and detection of the studied pesticides in a tobacco matrix fortified at 0.05 mg kg^{-1} level as evident from Fig. 1. This method, where detection of pesticide was based on GC-MS with confirmation on the basis of the quantifier-qualifier ions (m/z) ratio, minimized the chances of false detection of pesticide residues. Further, it effectively and efficiently detects the pesticide isomers with similar quantifier-qualifier ions. For example, the m/z=181 ion is the quantifier ion and m/z=183 and 217 are the qualifier ions for both alpha and delta isomer of HCH (Fig. 1, inset). Though all quantifier-qualifier ions are similar, but the ratios of quantifier to qualifier ions (m/z) are different and they are very specific for a particular isomer, and this feature increases the selectivity of the method. It is also evident from the relative abundance data shown in Table 1. The present method also enables easy detection of pesticide isomers with different quantifier-qualifier ions. For example, the m/z=195 ion is the quantifier ion for both alpha and beta isomers of endosulfan, while the qualifier ions are m/z=170 and 206 for α-endosulfan and m/z=197 and 170 for β-endosulfan (Fig. 1, inset).

Table 2 shows the status of studied organochlorine pesticide residues in Indian FC tobacco samples. The total DDT residue was detected in four samples (detection range 0.06–0.12 mg kg^{-1}) out of 70 samples from Mysore district of Karnataka and only in one sample (detection of 0.05 mg kg^{-1}) from West Godavari district of Andhra Pradesh. These detection levels were 1.7–3.3 times lower than that of the GRL of total DDT (0.2 mg kg^{-1}). DDT is a phased out chemical for agricultural use in India, with a limited and legally restricted application for public health purposes. The reason for detection of DDT in those limited samples could be because of the transfer of persisting residues of DDT from environmental components (Mukherjee and Gopal 2002; Bakore et al. 2004; Rahman et al. 2012). Traces of total endosulfan residues (ranging from 0.08–0.16 mg kg^{-1}) were detected in two samples from Mysore district (out of 70 samples) of Karnataka and in two samples from Prakasam district (out of 28 samples), one sample from Nellore district (out of 6 samples) and one from West Godavari district (out of 32 samples) of Andhra Pradesh. These residues were 6.25–12.5 times lower than that of the GRL of total endosulfan (1.00 mg kg^{-1}). Lindane was detected only in three samples (detection range 0.02–0.03 mg kg^{-1}) from Prakasam district of Andhra Pradesh which were 1.67–2.5 times below the CORESTA issued GRL (0.05 mg kg^{-1}). The total HCH was detected only in one sample from East Godavari district of Andhra Pradesh. The important point to note that none of the samples from East Godavari were found to have lindane, but one sample had total HCH which is a

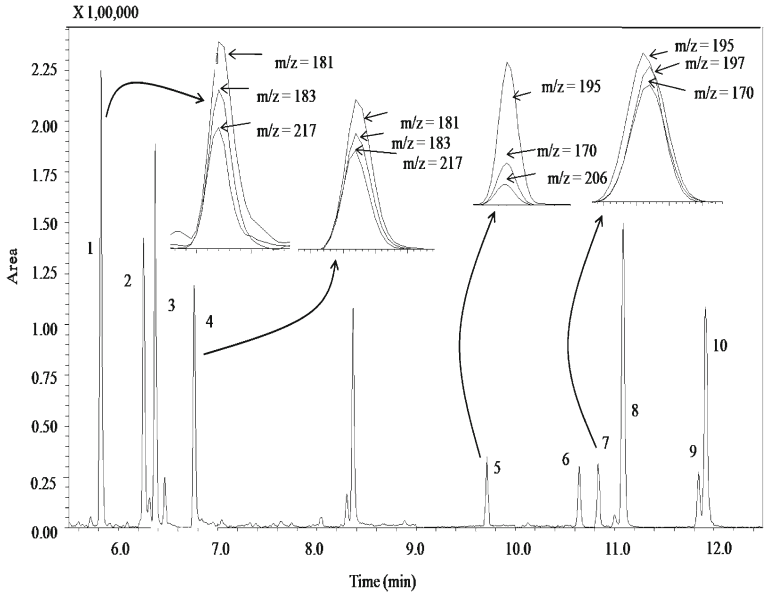

Fig. 1 Multiresidue chromatogram of pesticide mixture at 0.05 mg kg^{-1} fortification level in tobacco matrix (*1* α-HCH, *2* β-HCH, *3* lindane, *4* δ-HCH, *5* α-endosulfan, *6* endrin, *7* β-endosulfan, *8* endosulfan sulphate, *9* 2,4-DDT, *10* 4,4-DDT); *Inset*: ion fragmentation of *1* α-HCH, *4* δ-HCH, *5* α-Endosulfan and *7* β-endosulfan

Table 2 Status of organochlorine pesticide residues in the collected FC tobacco samples

State	District	No. of samples analyzed	No. of samples with positive detection	Pesticide detected (frequency of detection)	Concentration range (mg kg^{-1})	Remarks as per GRL values of CORESTA
Karnataka (12.97° N 77.56° E)	Mysore (12.08° N 76.32° E)	70	5	Total endosulfan (2)	0.12–0.13	Residues below GRL
				Total DDT (4)	0.06–0.12	Residues below GRL
	Hassan (13° N 76° E)	9	0	No detection	–	–
Andhra Pradesh (17.36° N 78.47° E)	Prakasam (15.50° N 80.05° E)	28	3	Lindane (3)	0.02–0.03	Residues below GRL
				Total Endosulfan (2)	0.06–0.13	Residues below GRL
	Krishna (16.17° N 81.13° E)	3	0	No detection	–	–
	Nellore (14.43° N 79.97° E)	6	1	Total endosulfan (1)	0.16	Residues below GRL
	East Godavari (16.57° N 82.15° E)	4	1	Total HCH (1)	0.01	Residues below GRL
	West Godavari (16.43° N 81.09° E)	32	2	Total endosulfan (1)	0.08	Residues below GRL
				Total DDT (1)	0.05	Residues below GRL

GRL guidance residue level

combination of HCH isomers (α-HCH, β-HCH and δ-HCH). This indicated that though there was no application of lindane (γ-HCH), but the encountered residues of total HCH might be a resulting from the transfer of persisting HCH residues from environmental components to the plant (Kumari et al. 2004; Prakash et al. 2004; Mahdavian and Somashekar 2013). The tobacco samples collected from the Hassan district of Karnataka and Krishna district of Andhra Pradesh were also devoid of all ten organochlorine pesticide residues under study. Table 3 shows comparative status of organochlorine pesticide residues in tobacco leaf/products reported earlier. As compared to these reports, present study found trace levels of some pesticide residues in few collected tobacco samples. The residue levels in these positively detected samples were much below their GRLs issued by the CORESTA (2013).

Table 3 Comparative status of organochlorine pesticide residue levels in tobacco leaf/product

Pesticide	Tobacco leaf/product	Country	Residue level (mg kg^{-1})	Remarks (no. of times more than the HCD in present study)	Reference
DDT	Cigarette	Australia	18.8–53.2	157–433	Morris 1972
	Cigarette	Finland	3.6–7.5	30–62.5	Lowman 1972
	Tobacco leaf	Bangladesh	4.0	33.3	Rahman et al. 2012
	Tobacco leaf	Australia	0.2–1.3	1.67–10.8	Speck 1988
	FC tobacco leaf	India	0.05–0.12		Present study
HCH	Tobacco leaf	Australia	1.29–9.7	43–323	Moser 1984
	FC tobacco leaf	India	0.02–0.03		Present study
Endosulfan	Tobacco leaf	Australia	0.4–1.8	2.5–11.2	Speck 1988
	FC tobacco leaf	India	0.06–0.16		Present study
Endrin	Tobacco leaf	Australia	0.3		Speck 1988
	FC tobacco leaf	India	BLQ		Present study

HCD highest concentration detected, *FC* flue-cured, *BLQ* below limit of quantification

Conclusions

The present method with internationally acceptable recovery level and matrix effect ensures true detection of pesticide residues at trace levels. This is the first report on ten chlorinated pesticide residues (α-HCH, β-HCH, γ-HCH (lindane), δ-HCH, 2,4-DDT, 4,4-DDT, endrin, α-endosulfan, β-endosulfan and endosulfan sulphate) in Indian FC tobacco. Results showed that in the majority of samples, pesticide residue levels were below the limit of quantification (LOQ). The exact reason for the presence in traces of the residues of DDT/α-HCH/β-HCH/δ-HCH that are phased out and out of use is not clearly known. One possible source for these pesticide residues could be previously contaminated environment. Based on the results obtained, we conclude that the levels of organochlorine pesticide residues in FC tobacco grown in India are very low and negligible. However, monitoring the pesticide residues in tobacco at regular intervals is of immense importance in terms of formulating stringent policies, enabling global trade and safeguarding the consumer's safety.

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