Bull. Environ. Contam. Toxicol. (1995) 54:444–450

Environmental Contamination and Toxicology

Determination of Chlorothalonil in Produce by Enzyme Immunoassay

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Received: 3 February 1994/Accepted: 1 August 1994

Chlorothalonil, 2,4,5,6-tetrachloroisophthalonitrile, is a broad-spectrum non-systemic fungicide extensively used on fruits, vegetables and other agricultural products worldwide. The maximum residue limit (MRL) for chlorothalonil in agricultural commodities ranges from 0.3 to 15 ppm. Its residues in crops have been analyzed commonly by GC with electron-capture detection (Valverde-Garcia, et al. 1993, Di Muccio A et al. 1993, Newsome and Collins 1989, El-Nabarawy and Carey 1988, Lesser and Massil 1987, Bicchi et al. 1985) or HPLC with either UV (Jongen et al. 1991) or photoconductivity and UV in tandem detection methods (Gilvydis and Walters, 1988). More recently, immunoassays for chlorothalonil have been reported (Lawruk et al. 1993, Fitzpatrick et al. 1993a) and are applied primarily to water samples. Indepth reviews for the applications of immunochemical methods have been presented (Giese J 1993, Van Emon and Lopez-Avila 1992, Gazzazz SS, et al. 1992).

The surge of interest in immunochemical methods for environmental analysis is partly due to its sensitivity, selectivity and cost-effectiveness. The recent availability of a variety of antibodies and commercial immunoassay kits also reflects the high profile of this powerful analytical tool. Since many applications of commercial kits have been focused on relatively clean water analysis, the question as to their broader utility in the determination of residues in substrate such as food by food industries or regulatory agencies arose.

The present study represents the development of a sample preparation procedure and the application of a commercial immunoassay kit to the rapid screening of foods for chlorothalonil. The produce examined includes celery, broccoli, tomato, cucumber, cauliflower, carrot, cantaloupe, apple, grape and snow peas.

MATERIALS AND METHODS

The RaPID Assays® kits for chlorothalonil, the magnetic separation rack and the RPA-I RaPID Photometric Analyzer™ with pre-programmed quantification methods, were supplied by Ohmicron, Newtown, Pennsylvania. The kit contained anti-chlorothalonil antibody coupled to magnetic particles, chlorothalonil coupled to peroxidase, enzyme substrate solution, peroxidase inhibitor, buffered saline diluent, and standards of chlorothalonil in water. A chlorothalonil analytical standard, obtained from the pesticide repository of the Food Research Division, was used for spiking, and was dissolved in acetonitrile for the recovery study at concentrations of 1 mg/mL and 0.1 mg/mL.

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To monitor the recovery of chlorothalonil in various commodities, produce were obtained from local supermarkets and about 1 kg each were blended. Ten g of previously homogenized produce was spiked with 10-100 μL of the spiking solutions to give 4-5 concentrations, including the MRL. These fortified samples were incubated at room temperature for 30 min prior to extraction.

The following method was developed for sample preparation of food commodities. One hundred mL of 70% aqueous methanol was added to a 10 g sample of the blended produce in a 250 mL bottle and the mixture was vortex mixed for 10 min. After the solids were removed by filtration (Whatman no. 1 filter paper), the filtrate was diluted serially with the supplied buffered saline diluent to the appropriate range. The kit had been optimized for water samples by the manufacturer to produce a standard curve with a linear range of 0.07-5 ng/mL. A 10^2-fold dilution of extract was used to quantify chlorothalonil in samples at concentrations of less than 0.1 ppm, a 10^3 dilution for concentrations between 0.1 ppm and 5 ppm, and a 10^4 dilution for those greater than 5 ppm. All determinations were done in duplicate, although the kit only requires the standards be run in duplicate. Since the c.v. for essentially all duplicate samples were less than 10%, it is more cost effective to run samples in duplicate rather than triplicate by the commercial kit.

For the recovery of surface wash chlorothalonil residues, the surface of 10 g pieces from various commodities were streaked with 0.5-20 ppm of chlorothalonil. Samples were air dried over-night and then extracted by vortexing with 70% aqueous methanol for 10 min. The mixtures were centrifuged (Beckman TJ-6) at 4°C at 2000 rpm for 10 min. The clear supernatants were analyzed without filtration.

The principle of RaPID Assays®, a magnetic particle-based ELISA, has been previously described (Rubio et al., 1991). The assay, as applied to foods was carried out according to the procedure for water samples specified in the kit, except all determinations were done in duplicate. Briefly, the standard, control or samples, chlorothalonil-enzyme conjugate and anti-chlorothalonil antibody coupled to magnetic particles were incubated in the polystyrene tube at room temperature for 30 min. The magnetic particles were separated, washed, and the enzyme substrate added. After another 20 min incubation, colour development was stopped by addition of the enzyme inhibitor. The absorbance of the colour in the sample and standard tubes was read at 450 nm and the amount of chlorothalonil determined by reference to the standard curve. The standard curve consisted of a linear regression of the ln concentration of chlorothalonil against the percentage decrease in absorbance (B/B_0). Results were printed out in ppb in 1 mL of diluted extract automatically from the analyzer. Final results were obtained by multiplying by the appropriate dilution factor and dividing by the sample weight.

RESULTS AND DISCUSSION

Low minimum detection limits in pesticide immunoassays are often not attainable for food samples due to the various matrix effects. Most of the immunochemical procedures only provide data on water samples, especially the commercial immuno-

assay kits. We present a simple and rapid analytical procedure and its recovery data for chlorothalonil in various produce using a commercial RaPID Assays chlorothalonil kit (Ohmicron). Produce were simply homogenized and extracted in 70% methanol. The filtrates were diluted and analyzed without any clean-up procedure. Sample clean-up steps are routinely performed prior to GC and HPLC analysis (Valverde-Garcia, et al. 1993, Di Muccio A et al. 1993, El-Nabarawy and Carey 1988, Lesser and Massil 1987, Bicchi et al. 1985, Jongen et al. 1991, Gilvydis and Walters, 1988). Most immunoassays do not require sample clean-up, especially in water samples. The other reported chlorothalonil immunoassay required C_{18} solid phase extraction clean-up for food crops (Fitzpatrick et al. 1993b).

Good recoveries were obtained on all the commodities studied. Some experiments were done in 1-3 replicates and averaged values were quoted. Mean recoveries ranged from 92-111% for the 10 commodities at 4-5 levels of fortifications (Table 1). No blank samples contained any quantifiable chlorothalonil at the detection limit of 0.07 ppb, indicating the matrix effect was essentially non-existent. This is probably due to the specificity and sensitivity of the antibodies. It was not the case if the extracts were centrifuged at 2000 rpm at 4°C for 10 min instead of filtering, indicating low speed centrifugation is not an efficient way of removing all interfering matrices. Recoveries were variable or exceeded 100% (Table 2), even though the samples appeared clear. The sample blanks did not show any matrix effect in the form of quantifiable chlorothalonil.

Table 1: Percent recoveries of chlorothalonil from various commodities.

	chlorothalonil added (ppm)							
	0.5	0.75	1	5	10	15	20	MEAN ± SD
Carrot	96	100	100[1]	93	[3]	_	_	97 ± 2.9
Celery	_	_	90	90	102	102[1]	91	95 ± 6.0
Broccoli	80	_	80	90[1]	92	_	_	86 ± 5.5
Cauliflower	114	_	110	88[1]	107	_	_	105 ± 10.0
Tomato	96	_	100	98[1]	108	_	_	101 ± 4.6
Cucumber	102	_	95	101[1]	112	_	_	102 ± 6.1
Cantaloupe	98	_	100	114[1]	108	_	_	105 ± 6.4
Snow peas[2]	100	_	90	84	94	_	_	92 ± 5.8
Apple[2]	92	_	110	114	116	_	_	108 ± 9.5
Grape[2]	108	_	110	110	117	_	_	111 ± 3.4

[1] Maximum residue limit. [2] No tolerance set. [3] Analysis not available. All data are the means of duplicate determinations. Control samples do not contain any quantifiable chlorothalonil.

The snow peas, apple and grape were included in the recovery exercise although chlorothalonil is not registered for use in these commodities. With the prevailing global economy, it is foreseeable that imported commodities may contain illegal pesticides. In fact, chlorothalonil has been found in imported snow peas and apples. Grape was included since residue data were lacking (FAO/WHO, 1990) and its MRL

may vary in other countries. For example, the MRL for chlorothalonil on grapes in New Zealand is 25 ppm.

Table 2: Effect of centrifugation compared to filtration on recovery of chlorothalonil in extracts from various commodities

	chlorothalonil added (ppm)				
	0.5	1	5	10	MEAN ± SD
Cauliflower	40	90	118	137	96 ± 36.5
	(114)	(110)	(90)	(107)	(105 ± 9.1)
Tomato	126	130	128	139	131 ± 5.0[1]
	(96)	(100)	(98)	(108)	(101 ± 4.6)
Cucumber	176	190	186	209	190 ± 12.0[1]
	(102)	(95)	(101)	(112)	(102 ± 6.1)

[1] Significant at $p < 0.01$, two-way ANOVA, when compared with corresponding filtration method. All data are expressed in % recoveries. Values in parenthesis are recoveries from extracts after filtration for comparison.

Table 3: Intra-assay and inter-assay coefficients of variation of recoveries in various commodities fortified at 5 ppm.

		Intra-assay		Inter-assay	
	N	MEAN ± SD	CV (%)	MEAN ± SD	CV (%)
Cucumber	5	104 ± 8.6	8.3	88 ± 15.2	17.3
Tomato	5	105 ± 10.6	10.1	95 ± 13.1	13.8
Carrot	5	102 ± 7.7	7.5	96 ± 5.2	5.4
Cauliflower	5	93 ± 3.5	3.8	90 ± 9.1	10.2

All mean data are expressed in % recoveries.

Good intra-assay and inter-assay coefficient of variations (cv) of recoveries were obtained, ranging from 4-10% and 5-17% respectively on 4 commodities fortified at 5 ppm (Table 3). Separate spiked samples, extractions and assays were performed for the interassay trials. Chlorothalonil appeared to be relatively unstable in produce extracts (Table 4), despite the fact that it is stable in normal storage temperature, pH and UV radiation (FDA Surveillance Index, 1981). When the same extracts were stored at room temperature on the laboratory bench for 3 days, chlorothalonil levels dropped more than 50%. We, therefore, recommend all sample extracts be analyzed on the same day.

The effect of solvents on the extraction efficiency of chlorothalonil was examined (Table 5) since it is soluble in all organic solvents (The Pesticides Manual, 1983). Extraction of various commodities with 100% methanol, hexane or methylene chloride gave unacceptable results. Methanol gave high and low results, while hexane and

methylene chloride gave low recoveries in various commodities. When hexane or methylene chloride was used, 5 mL of the extracts were carefully dried in a rotary evaporator at 30°C and the residues reconstituted in 5 mL of 70% methanol before assay. Since acetonitrile was shown to give irreproducible results in fruit and vegetables in a multiresidue method (Lee et al. 1991), it was not examined. Acetone was not attempted. Seventy percent aqueous methanol consistently gave reproducible and good recoveries on all the commodities studied.

Table 4: Stability of chlorothalonil in three day old extracts stored at room temperature

	chlorothalonil added (ppm)				
	0.5	1	5	10	MEAN ± SD
Cauliflower	42	52	50	50	49 ± 3.8[1]
	(114)	(110)	(90)	(107)	(105 ± 9.1)
Cantaloupe	20	10	42	62	34 ± 20.1[1]
	(98)	(100)	(114)	(108)	(105 ± 6.4)
Snow peas	30	8	54	79	43 ± 26.5[1]
	(100)	(90)	(84)	(94)	(92 ± 5.8)

[1] Significant at $p < 0.01$, two-way ANOVA, when compared with corresponding fresh sample. All data are expressed in % recoveries. Values in parenthesis are recoveries from fresh extracts for comparison.

Table 5: Effect of extraction solvent on recoveries in various commodities fortified at 5 ppm.

	Methanol	70% Methanol	Hexane	Methylene chloride
Broccoli	68	_[1]	_	_
celery	113	_	_	_
Tomato	176	98	48	62
Cucumber	_	101	46	44
Carrot	_	93	30	20
Cauliflower	_	88	74	44

[1] Analysis not available. All data are expressed in % recoveries.

Since chlorothalonil is a non-systemic fungicide, with no evidence of ^{14}C-chlorothalonil translocation in plants (FAO surveillance Index, 1981), a 70% methanol surface wash chlorothalonil residue method was examined. The recoveries were high and variable (Table 6). Filtering the extracts did not appear to improve the assay, since essentially identical results were obtained in selected samples with or without filtering.

Table 6: Percent recovery of surface wash residue of chlorothalonil from various commodities.

	chlorothalonil added (ppm)							
	0.5	0.75	1	5	10	15	20	MEAN ± SD
Carrot	256	253	260	210	[1]	_	_	245 ± 20
Celery	_	_	114	131	122	138	130	130 ± 6
Broccoli	148	_	170	186	88	_	_	148 ± 37
Cauliflower	202	_	240	254	239	_	_	234 ± 19
Tomato	266	_	280	258	87	_	_	223 ± 79
Cucumber	318	_	270	260	110	_	_	239 ± 80

[1] Analysis not available. All data are the means of duplicate determinations. Control samples did not contain any quantifiable chlorothalonil.

Our results showed that in the laboratory with a careful selection of sample preparation, a commercially available immunoassay kit for water samples can be suitable for the determination of chlorothalonil in a complex food matrix. Although real-world samples with incurred chlorothalonil residues were not done in this study, we do not foresee any major problem since the antibodies do not cross react with its 4-hydroxy metabolite at the detection range. Immunoassay has the advantage of being sensitive, yet simple and rapid and does not require sample clean-up. Many samples can be screened for chlorothalonil in a short time by the commercial kit. Typically, twenty five commodity extracts can be analyzed in duplicate within three hours.

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