Simultaneous Determination of Aldicarb, Ethiofencarb, Methiocarb and Their Oxidized Metabolites in Grains, Fruits and Vegetables by High Performance Liquid Chromatography

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ABSTRACT

Three carbamate pesticides, aldicarb (A0), ethiofencarb (E0), methiocarb (M0) and six of their oxidized metabolites, sulphoxides (A1, E1, M1) and sulphones (A2, E2, M2) were simultaneously determined. Five grams of a sample were homogenized with acetone, and then treated with dichloromethane-hexane mixture (1:1) and sodium chloride (NaCl), homogenized and centrifuged. The organic layer was removed and the aqueous residue was re-extracted a second time with dichloromethane-hexane mixture. The combined organic extracts were evaporated in vacuo. The residue was dissolved in dichloromethane and charged on a Sep-Pak® aminopropyl cartridge. Carbamates were eluted from the cartridge with 1% methanol in dichloromethane. The eluate was evaporated to dryness and 0.5 ml of methanol and 1.5 ml of 0.001 N-hydrochloric acid (HCl) solution were added. Individual carbamates were separated by gradient elution high performance liquid chromatography (HPLC) using octyldecylsaline (ODS) column. Derivatization of separated carbamates to fluorescent derivatives was achieved in-line. Recovery of pesticides and their oxidized metabolites from rice, apple, cabbage and other foods ranged from 60 to 103% following fortification at 20 ppb. Detection limits were 1 ppb for A1, A2, E1, E2, 2 ppb for A0, E0, M1 and 4 ppb for M0 and M2 (S/N>3).

Key Words: Aldicarb, ethiofencarb, methiocarb, metabolites, high performance liquid chromatography

Aldicarb, ethiofencarb and methiocarb are N-methyl functional in their carbamate insecticides all possessing a thioether group. They are oxidized at the thioether resulting conversion to the corresponding sulphoxides or sulphones (Fig. 1) after application to crops (2-4). There are several methods for quantifying these compounds, including gas chromatography followed by flame photometric detection (2,10,12) or atomic emission detection (16) and HPLC followed by ultraviolet (UV) absorption detection (3-5), mass spectrometric detection (13) or fluorescence detection (1,6-9,11,14,15).

Quantification of carbamates by HPLC using post-column derivatization and fluorescence detection was first described by Moye et al. (14). This technique was modified for use on food samples by Krause (17). This technique is the official method of analysis in the United States (2). In recent years de Kok et al. (6-9) reported an improved procedure using solid phase extraction clean-up and automated injection. Page and French (15) used this procedure in conjunction with the Luke’s method of extraction.

In Japan the Ministry of Public Welfare has specified tolerance levels for aldicarb and ethiofencarb, which have been in effect since May 1, 1993. The tolerance levels for aldicarb are very low for some crops, such as 20 ppb for rice or soybeans. For satisfactory quantitative accuracy, a detection limit of 2 ppb would be required. However, the detection limit of de Kok is 5 ppb (9) and that of Page and French, 10 ppb (15). In addition, de Kok reported rather low recovery (below 30%) for aldicarb sulphone (9).

In the present study, improvement in detection limits and simultaneous determinations of aldicarb, ethiofencarb, methiocarb and their metabolites have been conducted.

In the following, the names of carbamates are abbreviated by their initials. The number of sulfur-binding oxygens is given as A0 for aldicarb, E1 for ethiofencarb sulphoxide and so on (Fig. 1).

Figure 1. Chemical structure of aldicarb, ethiofencarb, methiocarb and their oxidized metabolites.
MATERIALS AND METHODS

Samples
Grains, vegetables and fruits were purchased from markets in Osaka.

Reagents
a) Analytical standards: 50 mg of A0 (Riedel-de-Haën) was dissolved in 50 ml of acetone (1 mg/ml, standard solution). The standard solution was diluted by acetone (0.2 μg/ml, working solution) and was added to the sample to test recovery. The standard solution was diluted by 25% methanol in 0.001 N-HCl for liquid chromatography. A1 and A2 (Riedel-de-Haën), E0, E2, E3, M0, M1, M2 (Nihon Bayer Agrochem K.K.) were treated similarly as A0. E1 was synthesized and purified following the report of Cabras (3) and also treated similarly.
b) Distilled water (Wako Pure Chemicals Co., Wako, TX), methanol and acetonitrile (E. Merck AG, Darmstadt, Germany) were HPLC grade. Dichloromethane, n-hexane, diethyl ether and ethyl acetate were pesticide analysis grade (Ishizu Seiyaku Ltd.). Ethanol was special grade (Katayama Chemical).
c) Hydrochloric acid (Katayama Chemical), sodium sulphate (NaSO4) and NaCl (Ishizu Seiyaku Ltd.) were special grade.
d) Sep-Pak® aminopropyl, C18, silica and florisoril solid phase extraction columns were product of Millipore Corporation. Membrane filter cartridges of 0.45 μm pore size were product of Japan Millipore Ltd.
e) Mobile phase A: 5% methanol in distilled water, mobile phase B: 80% methanol in distilled water.
f) Sodium hydroxide (NaOH) solution: 2 g of NaOH (Ishizu Seiyaku Ltd.) was dissolved in 1,000 ml of distilled water.
g) o-Phthalaldehyde (OPA) solution: OPA was special grade for biochemical analysis (Wako). Three hundred milligrams of OPA was dissolved in 20 ml of ethanol, and 18.54 g of boric acid (Katayama Chemical), 4.0 g of NaOH and 100 ml of 3-mercaptothiopionic acid (Tokyo Chemical Industry Co., Ltd.) were added. The entire mixture was diluted to 1,000 ml with distilled water.
h) 25% methanol in 0.001 N-HCl: 250 ml of methanol was added to 750 ml of 0.001 N-HCl solution.
i) Filter paper: paper for filtration of organic layers was product of Toyo Advantec Co. Its diameter was 9 cm.

Apparatus
An electric homogenizer (Nihonseiki Co. Ltd.), rotary evaporator (Shibata Scientific Technology Ltd.) and centrifuge (Kokusan Co.) were used.

High performance liquid chromatography
a) Apparatus: the HPLC system consisted of an automatic controller (Shimadzu SCL-10A), an automatic injector (Shimadzu SIL-10A), two pumps for mobile phase (Shimadzu LC-10AD), and two pumps for reaction solvents (Lab-Quatec Co., Ltd. MP-302). Reaction solvents were introduced to the line after column separation. Degasser apparatus was used to remove the air from mobile phase and reaction solutions (Lab-Quatec Co., Ltd. GT-104). An oven was used for column and OPA addition reaction (Shimadzu CTO-10A). A second oven was used for hydrolysis (Shimadzu CRB-6A). Quantification was achieved with a fluorescence detector (Shimadzu RF-10A) and integrator (Shimadzu C-R7A). The system was equipped with a stainless steel column (4.6 mm i.d. × 250 mm) packed with Shimadzu STR-ODS II.
b) Operating parameters: mobile phase flow rate was adjusted to 1.0 ml/min during analysis. The system was equilibrated at 15% mobile phase B in mobile phase A, then 3 min after injection, a 32 min gradient to 100% mobile phase B was begun. When the gradient was completed, the mobile phase was returned to 85% A, 15% B and held for 15 min to re-equilibrate the column. The other conditions were as follows: flow rate of reaction solvents, 0.35 ml/min; temperature for column separation and OPA addition, 50°C; temperature for hydrolysis, 90°C; excitation and emission wavelengths of fluorescence detector, 340 and 455 nm, respectively.

Extraction
a) Modified de Kok’s procedure: 10 g of sample was put in a stainless steel cup to which 20 ml of acetone was then added followed by homogenization for 2 min. To the mixture, 40 ml of CH2Cl2/n-hexane mixture (1:1) was added and homogenized again for 2 min. The mixture was transferred to a centrifuge tube and centrifuged by 3,000 rpm at room temperature for 3 min. The organic layer was dehydrated by the addition of anhydrous sodium sulphite (Na2SO4). The extract was filtered through filter paper, evaporated to ca. 1 ml in vacuo at 40°C, and dried up under gentle stream of nitrogen. The residue was dissolved in 1 ml CH2Cl2.
b) Improved procedure 1: almost same as a) except that extraction with CH2Cl2/n-hexane was done twice.
c) Improved procedure 2: almost same as b) except that 2 g of NaCl was added to the sample mixture before extraction.
d) Final procedure: 5 g of chopped sample was put in a stainless steel cup to which 20 ml of acetone was added followed by homogenization for 2 min. To the mixture 1 g of NaCl and 30 ml of CH2Cl2/n-hexane mixture (1:1) were added and homogenized again for 2 min. The mixture was transferred into a centrifuge tube and centrifuged by 3,000 rpm for 3 min. The organic layer was carefully taken and 40 ml of CH2Cl2/n-hexane was added to the aqueous residue, shaken vigorously and centrifuged again. The organic layers were combined and dehydrated by the addition of anhydrous Na2SO4. The extract was filtered through filter paper, evaporated to ca. 1 ml in vacuo at 40°C and dried up under gentle stream of nitrogen. The residue was dissolved in 1 ml of appropriate solvent for solid phase extraction.

Clean-up
a) Test of various cartridges: Sep-Pak® aminopropyl, silica, florisoril and C18 cartridges were tested for their ability to purify carbamates from onion. Water and methanol were used for charge and elution of extract for C18 cartridge. Dichloromethane, ethyl acetate, diethyl ether, acetone and methanol were used for aminopropyl, silica and florisoril cartridges. The cartridges were conditioned by 10 ml of solvent, which is the same one for application of sample extract. The extract was dissolved in 1 ml of appropriate solvent and charged on each cartridge followed by elution with appropriate solvents mixture. Eluant was concentrated by evaporation in vacuo and dried under nitrogen stream. Then 0.5 ml of methanol and 1.5 ml of 0.001 N-HCl were added to the extract.
b) Final procedure: the extract was dissolved in 1 ml of CH2Cl2, and charged on Sep-Pak® aminopropyl cartridge, followed by elution with 10 ml of 1% methanol in CH2Cl2. The eluate was removed as previously described, dissolved in 0.5 ml of methanol followed by addition of 1.5 ml 0.001 N-HCl solution. Then it was filtered through a membrane filter cartridge. Sep-Pak® aminopropyl cartridge was conditioned by 10 ml of CH2Cl2 prior to use.

Clean-up of fatty samples
Extracts from unpolished rice, corn and soybeans were additionally cleaned up by acetonitrile/n-hexane partition prior to solid phase extraction. Extracts from these samples were dried and redissolved in 20 ml of acetonitrile and 20 ml of n-hexane
(saturated with acetonitrile) was added. The mixture was shaken vigorously for 5 min and the lower layer was taken. The acetonitrile layer was dried, redissolved in 1 ml CH₂Cl₂ and subjected to solid phase extraction.

**Quantification**

Forty microliters of the sample solution was automatically injected into the HPLC for residue analysis. The concentration of individual carbamates was calculated based on peak area calibration curves. The calibration curves were drawn for injected amounts of 0.1, 0.2, 1, 10 and 100 ng for individual carbamates. Each injection was done three times to test reproducibility. For the routine work 2 ng of standard carbamates was injected to HPLC three times per day.

**Recovery test**

Chopped samples were fortified with each 20 ppb of carbamates 2 h before extraction. Recovery data represents five replications for rice and onion, and two replications for the other crops.

**RESULTS AND DISCUSSION**

**High-performance liquid chromatography conditions**

Figure 2 shows a typical chromatogram of nine carbamates separated by HPLC with post-column reaction and fluorescence detection. A fluorescence detector is highly sensitive, but great care should be given to methanol and distilled water used for the mobile phase. In this study, even ultra-pure water prepared by Milli-Q water purification system (Millipore) failed to give a flat baseline. Commercial water for HPLC analysis gave reproducible results.

Most previous studies have used 2-mercaptoethanol to produce fluorescence derivatives (1,6-9,11,14,15). However, 3-mercaptopropionic acid can be used more conveniently, since its odor is weaker than that of 2-mercaptoethanol. This replacement did not change the response of the derivatives to the detector.

**Linearity and limit of detection**

The linear dynamic range of the detector response for A0, A1, A2, E0, E1, E2 and M1 was checked and appeared to be from 0.2 to 100 ng injected on-column. That of M0 and M2 was 1 to 100 ng. The detection limits at the sample to noise ratio 3 are indicated in Table 1.

**Extraction**

To obtain minimum detection limits, the sample should be concentrated. De Kok's procedure (6) was modified purpose. Ten grams of onion were fortified with 20 ppb of carbamates and extracted, followed by clean-up using Sep-Pak® aminopropyl. Individual carbamates were quantified by HPLC. Table 2 shows the results. The recovery of A1 was poor, as also reported by de Kok. A1 is the most polar compound of the nine and, thus, may remain in the aqueous residue of the onion. Double extraction with CH₂Cl₂/n-hexane gave better results (improved procedure 1), and the addition of NaCl before extraction process was also effective (improved procedure 2). However, the recovery of A1 was still below 60%. The sample weight was reduced to 5 g with satisfactory results (final procedure).

An attempt to use ethyl acetate instead of CH₂Cl₂ was not successful due to the poor recovery at A1.

**Clean-up**

Solid phase extraction (SPE) using Sep-Pak® aminopropyl, silica, florisil and C18 cartridges were carried out for sample purification. Sep-Pak® C18 gave the best recovery, though not adequate purification ability; particularly for A1. When Sep-Pak® silica or florisil was used, 30% of E0 was converted to E1. Sep-Pak® aminopropyl was best for clean-up. When diethyl ether or ethyl acetate were used instead of CH₂Cl₂, 99 or 10% of E0 was converted to E1 with diethyl ether or ethyl acetate, respectively.

**R.T.(min)**

![Figure 2. Typical chromatogram of pure carbamates and carbamate metabolites. Concentrations: 0.05 μg/ml for each compound.](image)

![Figure 3. Chromatograms of fortified rice extract with or without partition by acetonitrile and n-hexane. Rice was fortified with 20 ppb of carbamates. (a) without partition. (b) with partition.](image)
TABLE 1. Detection limits of N-methylcarbamates by HPLC with post-column reaction and fluorescence detection (S/N=3).

<table>
<thead>
<tr>
<th></th>
<th>A0</th>
<th>A1</th>
<th>A2</th>
<th>E0</th>
<th>E1</th>
<th>E2</th>
<th>M0</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>

Injection volume was 40 µl. Injection was repeated three times. The concentration of sample to noise ratio 3 was regarded as detection limit.

TABLE 2. Recoveries of added carbamates from onion by various way of extraction.

<table>
<thead>
<tr>
<th>Extraction</th>
<th>A1</th>
<th>A2</th>
<th>E2</th>
<th>M1</th>
<th>M2</th>
<th>A0</th>
<th>E0</th>
<th>M0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified de Kok’s procedure</td>
<td>20</td>
<td>56</td>
<td>79</td>
<td>63</td>
<td>64</td>
<td>74</td>
<td>55</td>
<td>71</td>
</tr>
<tr>
<td>Improved procedure 1</td>
<td>30</td>
<td>71</td>
<td>82</td>
<td>79</td>
<td>68</td>
<td>78</td>
<td>82</td>
<td>84</td>
</tr>
<tr>
<td>Improved procedure 2</td>
<td>59</td>
<td>91</td>
<td>88</td>
<td>87</td>
<td>74</td>
<td>68</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Final procedure</td>
<td>73</td>
<td>82</td>
<td>86</td>
<td>75</td>
<td>45</td>
<td>94</td>
<td>78</td>
<td>82</td>
</tr>
</tbody>
</table>

Fortification level was 20 ppb. All extracts were subjected to solid phase extraction clean-up using Sep-Pak® aminopropyl. Recoveries are average of duplicates.

- Second extraction was done with CH₂Cl₂ and n-hexane, twice.
- Two grams of NaCl was added before second extraction.
- Sample weight was 5 g.

TABLE 3. Recoveries of carbamates from fortified rice.

<table>
<thead>
<tr>
<th>Clean-up method</th>
<th>A1</th>
<th>A2</th>
<th>E2</th>
<th>E1</th>
<th>M1</th>
<th>M2</th>
<th>A0</th>
<th>E0</th>
<th>M0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep-Pak® NH₄⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partition + Sep-Pak NH₂</td>
<td>87</td>
<td>85</td>
<td>88</td>
<td>91</td>
<td>89</td>
<td>91</td>
<td>70</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

Average of duplicates.

- Five grams of unpolished rice were fortified with 20 ppb of carbamates followed by addition of acetone and 1 g of NaCl, and extracted with CH₂Cl₂/n-hexane (1:1), cleaned up by Sep-Pak® NH₄⁺ cartridge.
- Sample extract was cleaned up by CH₃CN/n-hexane partition before solid phase extraction.
- Recovery could not be calculated because of interfering peak.

The addition of HCl solution to the final sample solution not only stabilizes carbamates but also eliminates impurities. For some samples, precipitation occurred following the addition of HCl solution to the extract, and precipitates were removed by a membrane filter. This did not affect recovery.

Clean-up of fatty samples

Fat-containing samples, such as unpolished rice or soybeans, gave fatty final extracts difficult to filter through a membrane. A1, A2, E1 and M1 could not be determined because interfering peaks appeared on the chromatograms (Fig. 3). Acetonitrile/n-hexane partition prior to solid-phase clean-up was effective for removing fatty materials. A single partition was sufficient for achieving satisfactory recovery, as shown in Table 3.

Recovery test

Rice and onion were fortified with 20 ppb of the nine carbamates followed by a residue analysis. Average of recovery and the standard deviation for five trials are and

![Typical chromatograms of fruit or vegetable extract.](Figure 4) The broken lines indicate the peaks of carbamates corresponding to levels of 20 ppb. (a) Apple extract. (b) Potato extract.
TABLE 4. Recoveries (%) and standard deviation (SD) of the carbamate pesticides and their metabolites in fortified rice and onion.

<table>
<thead>
<tr>
<th>Crops</th>
<th>A1</th>
<th>A2</th>
<th>E2</th>
<th>E1</th>
<th>M1</th>
<th>M2</th>
<th>A0</th>
<th>E0</th>
<th>M0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice* recovery</td>
<td>85</td>
<td>86</td>
<td>88</td>
<td>87</td>
<td>86</td>
<td>77</td>
<td>88</td>
<td>74</td>
<td>61</td>
</tr>
<tr>
<td>SD</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Onion recovery</td>
<td>75</td>
<td>88</td>
<td>87</td>
<td>89</td>
<td>87</td>
<td>79</td>
<td>83</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>SD</td>
<td>5</td>
<td>8</td>
<td>5</td>
<td>4</td>
<td>8</td>
<td>11</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Fortification level was 20 ppb. Each value is calculated from the result of 5 trials.

* Partitioning purification with acetonitrile and n-hexane was necessary.

TABLE 5. Recoveries (%) of the carbamate pesticides and their metabolites in fortified grains, vegetables and fruits.

<table>
<thead>
<tr>
<th>Crops</th>
<th>A1</th>
<th>A2</th>
<th>E2</th>
<th>E1</th>
<th>M1</th>
<th>M2</th>
<th>A0</th>
<th>E0</th>
<th>M0</th>
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<tbody>
<tr>
<td>Cornb</td>
<td>90</td>
<td>90</td>
<td>91</td>
<td>95</td>
<td>85</td>
<td>80</td>
<td>102</td>
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<td>70</td>
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<tr>
<td>Soybeanb</td>
<td>102</td>
<td>91</td>
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<td>93</td>
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<td>77</td>
<td>92</td>
<td>84</td>
<td>62</td>
</tr>
<tr>
<td>Peach</td>
<td>81</td>
<td>92</td>
<td>87</td>
<td>95</td>
<td>90</td>
<td>90</td>
<td>87</td>
<td>89</td>
<td>84</td>
</tr>
<tr>
<td>Apple</td>
<td>77</td>
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<td>87</td>
<td>88</td>
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<td>87</td>
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<td>Banana</td>
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<td>93</td>
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<td>86</td>
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<td>Sweet potato</td>
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<td>94</td>
<td>95</td>
<td>89</td>
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<td>92</td>
<td>93</td>
<td>75</td>
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<td>Potato</td>
<td>74</td>
<td>88</td>
<td>91</td>
<td>84</td>
<td>86</td>
<td>83</td>
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<td>Cucumber</td>
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<td>92</td>
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<td>Egg plant</td>
<td>77</td>
<td>88</td>
<td>93</td>
<td>91</td>
<td>83</td>
<td>83</td>
<td>77</td>
<td>89</td>
<td>79</td>
</tr>
</tbody>
</table>

Fortification level was 20 ppb.

* Average of duplicates.

b Acetonitrile/n-hexane partition was necessary.

75 to 89% for onion. Standard deviation was 1 to 11%.

Fourteen grains, vegetables and fruits were also tested for recovery. Table 5 shows the results. Recovery was from 60 to 103%. The detection limits of the present method were 1 ppb for A1, A2, E1, E2, 2 ppb for A0, E0, M1, and 4 ppb for M0 and M2. These are calculated from data in Table 1. Typical chromatograms are shown in Fig. 4. No interfering peaks appeared on the chromatograms except for a peak of from cabbage extract that had a retention time essentially the same as that of E1.

Individual fortification tests for sulphides, sulphoxides and sulphones were conducted, and interconversion between them during extraction was not detected.

CONCLUSION

In the present study, detection limits of aldicarb and its oxidized metabolites were improved by devising concentration and purification of sample extracts. The proposed method can be adopted for analysis of aldicarb in crops under the new tolerance levels in Japan. Simultaneous determination of ethiofencarb, methiocarb and their metabolites have been also achieved.

ACKNOWLEDGMENTS

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