*Food Chemistry* **35** (1990) 51–58

# Determination of Methyl 2-Benzimidazolecarbamate in Fruit Juices by Immunoassay

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(Received 7 February 1989; accepted 21 February 1989)

*ABSTRACT*

*A modified enzyme immunoassay method has been developed to determine methyl 2-benzimidazolecarbamate (MBC), the degradation product of benomyl, in fruit juices and concentrates. The analysis is complete within 30 min and up to eight samples can be run simultaneously. Samples are quantified using a hand-held battery-powered photometer which makes possible analysis in processing and quality-control laboratories. MBC concentration is linear from 1 to 26 ng/g (100 to 2600 pg/tube). For juices greater than 26 ng/g, dilutions must be made. The lower limit of detection was 10 ng/g ppb for juices and 30 ng/g for concentrates. Percent coefficients of variation (%CV) ranged from 16·6 to 4·5 for standards and 25·0 to 12·0 for samples. Agreement between this method and high-performance liquid chromatography (HPLC) was good.*

## INTRODUCTION

Benomyl, a systemic benzimidazole fungicide, is used on 43 different food crops in the US as either a preharvest or postharvest treatment. Benomyl



*Food Chemistry* 0308-8146/89/$03·50 

breaks down quite rapidly to its metabolite methyl-2-benzimidazole-carbamate (MBC) (Peterson & Edgington, 1969; Sims *et al.*, 1969). Tolerances, which include MBC, range from 0·2 to 10 μg/g. Recently, there has been some concern as to the safety of benomyl and MBC (Anonymous, 1987). Because of possible health effects (Anon., 1987), widespread use (Bardalaye & Wheeler, 1985), and insufficient residue data (Bardalaye & Wheeler, 1985), there is a need to monitor benomyl and MBC in food commodities.

Methodology available to quantify benomyl and MBC in food usually employs chromatography—gas (Pyysalo, 1977) or high-performance liquid chromatography (HPLC) (Spittler *et al.*, 1984; Bardalaye & Wheeler, 1985; Monico-Pifarre & Xirau-Vayreda, 1987)—or immunoassay (Newsome & Shields, 1981; Newsome & Collins, 1987). Since benomyl decomposes rapidly to MBC in many organic solvents (Chiba & Doornbos, 1974), most procedures analyze for total MBC by converting benomyl to MBC (Chiba & Singh, 1986), but there are a few methods that can quantify each (Chiba & Veres, 1980; Chiba & Singh, 1986). However, chromatographic techniques are expensive, require clean-up steps, may lack sensitivity, and are time consuming. Thus the newer less expensive technology of immunoassay should be very useful in screening food items for chemical residues.

This paper describes modifications to Newsome's (Newsome & Collins, 1987) MBC enzyme linked immunoassay method that enables a quicker, more sensitive and field adaptable assay. MBC results are reported for fruit juices and concentrates purchased from local supermarkets.

## MATERIALS AND METHODS

### Reagents and materials

Benomyl (99·9%) was obtained from the EPA, Triangle Park, North Carolina, while MBC (98%) was a gift from DuPont, Wilmington, Delaware. The MBC standard was prepared in methanol (6·8 μg/ml) and appropriate dilutions were made with phosphate buffer to yield standards containing 1·6, 3·2, 6·4, 12·8 and 25·6 ng/g for the immunoassay. The benomyl standard was used for HPLC analysis to check for the presence of benomyl in fruit juices. It was dissolved in methanol (5·0 μg/ml).

Phosphate buffer, 0·67M and pH 7·2, was prepared by weighing 8·9 g of potassium phosphate monobasic (anhydrous) and 11·7 g of potassium phosphate dibasic (anhydrous) into separate 1 liter volumetric flasks. These solutions were brought to volume with HPLC grade water. Three parts dibasic to two parts monobasic were mixed to obtain pH 7·2 buffer.

Solvents were HPLC grade. All reagents pertaining to the preparation of

immunogens for producing antisera to MBC were previously described (Newsome & Collins, 1987).

Fruit juices and concentrates were purchased from supermarkets in Bangor, Maine.

## Apparatus

A liquid chromatograph equipped with a Waters Associates Model 510 pump (Milford, Massachusetts), a Vici Instruments Model EC6 Valco pneumatic injector (Houston, Texas), and a Hewlett-Packard Model 1040A photodiode array detector (Avondale, Pennsylvania) was used to analyze MBC and benomyl in fruit juices and concentrates. Kits from Immuno-Systems (Scarborough, Maine) were used to determine MBC in fruit juices and concentrates by immunoassay.

## Immunoassay method for MBC

Standards and samples (juices and concentrates were first diluted in buffer, 100 $\mu$l of the juice or concentrate with 900 $\mu$l phosphate buffer) were analyzed by adding 100 $\mu$l to an antibody-coated polystyrene tube (ImmunoSystems) followed by 160 $\mu$l of enzyme conjugate (horseradish peroxidase covalently bound to MBC, ImmunoSystems). The tubes were incubated for 15 min at room temperature and then rinsed four times with distilled water to remove unreacted sample and enzyme conjugate. To the rinsed tubes were added a 160 $\mu$l each of substrate (hydrogen peroxide) and chromogen (tetramethylbenzidine, ImmunoSystems). The tubes were incubated at room temperature for 10 min before adding one drop of 2·5M sulfuric acid to stop the reaction. The sulfuric acid causes formation of a yellow color. Color intensity was measured by reading the difference in optical density ($\Delta$OD) between the control (no MBC added) and each sample and/or standard at 450 nm with a hand-held battery-powered differential photometer from Artel Inc. (Windham, Maine).

As many as eight samples and a control can be analyzed simultaneously. A control was run with each set of eight tubes since its OD value was used to measure the $\Delta$OD/OD values (where $\Delta$OD is the difference in optical density of the samples or standards from the control divided by the optical density of the control read against water) of the standard and samples. A standard curve was run by analyzing a set of standards (0·0, 1·6, 3·2, 6·4, 12·8 and 25·6 ng/g in buffer) at the beginning and end of each day with the average values used to quantify MBC in the samples. A plot was made of $\Delta$OD/OD values versus log of MBC concentration (pg/tube) which was used to calculate MBC concentration in the samples.

**HPLC method for MBC**

The HPLC method is a modification of the reversed-phase method developed by Chiba and Singh, 1986. A 100 ml aliquot of juice or concentrate mixture (dissolve 25 ml of juice concentrate in 75 ml water) was added to a 500 ml separatory funnel containing 100 ml water followed by the addition of 2 g of sodium carbonate. This mixture was shaken for 30 s to dissolve the carbonate. The juice was extracted twice for 2 min each with two 50-ml aliquots of methylene chloride. The combined organic layers were passed through anhydrous sodium sulfate into a 250 ml round-bottom flask. The extract was rotary evaporated at 40°C to near dryness (approximately 2 ml). It was then transferred to a 7 ml vial and dried under nitrogen. The dried sample was dissolved in 0·5 ml methanol, sonicated for 1 min and filtered through a 0·45 $\mu$m nylon filter (Gelman Science Inc., Ann Arbor, Michigan). The operating conditions for the HPLC were as follows: (1) column—Vydac 218TP54, stainless steel, 25 cm × 4·6 mm i.d. (Separations Group, Hesperia, California); (2) mobile phase—acetonitrile–methanol–water–acetic acid–monoethanolamine–tetrahydrofuran (125 + 700 + 2 + 0·75 + 15 ml); (3) flow-rate—1·0 ml/min; (4) wavelength—286 nm; (5) absorbance range—0·04 absorbance units full scale; (6) injection volume—25 $\mu$l. All benomyl peaks were checked for purity using the peak purity mode on the photodiode array detector.

## RESULTS AND DISCUSSION

The immunoassay showed a linear relationship (Fig. 1) from 1·0 to 26·0 ng/ml (100 to 2600 pg/tube) which was observed between the logarithm of the MBC concentration and the $\Delta$OD/OD at 450 nm. For samples having greater than 26·0 ng/ml a dilution must be made. A $\Delta$OD of 0·80 or larger indicates a sample concentration of more than 26·0 ng/ml which means the sample should be diluted and the analysis rerun.

As with any analytical procedure, consistency and accuracy from day to day is important. Reproducibility results of the MBC immunoassay for standards and fruit juices can be seen in Tables 1 and 2. Table 1 shows the consistency of the data obtained from analyzing standards, on different days (two sets per day every other day) for a duration of 1 month. Percent coefficients of variation (%CV) for the standards ranged from 16·6 to 4·5, which are excellent for a residue screening technique.

Table 2 represents the reproducibility of MBC determination in fruit juices and concentrates. Samples were analyzed on different days (one set every other day) over a period of 2 weeks. The %CV are higher than the

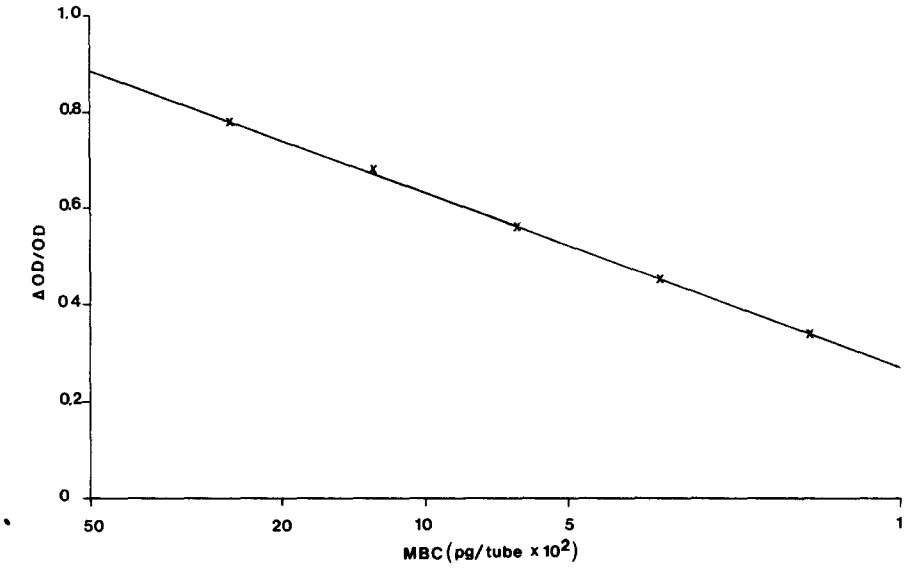


**Fig. 1.** Typical standard curve for MBC immunoassay.

standards but they are excellent for a screening technique: they ranged from 25·0 to 12·0. Unlike the standards, the %CV for the juices were slightly larger and do not decrease with higher concentrations of MBC. This tends to indicate that there may be a matrix effect with juices on the reaction between the antibody and enzyme conjugate, but causes no significant problems in quantitation since the amount reported by the immunoassay was in good agreement with the HPLC results. All benomyl peaks were ascertained as being pure according to the results given by the photodiode array detector. If one did observe a significant matrix effect then one could add 100 $\mu$l of juice free of MBC to the standards which would nullify such a matrix effect. We have observed significant matrix effects with an atrazine immunoassay method for corn and were able to alleviate the problem using corn extract

**TABLE 1**
Reproducibility of the MBC Immunoassay Standards

| | *Amount MBC added in ng/g* | | | | |
|---|---|---|---|---|---|
| | *1·6* | *3·2* | *6·4* | *12·8* | *25·6* |
| Number of standards analyzed | 16 | 17 | 17 | 17 | 17 |
| %CV | 16·6 | 12·0 | 9·2 | 7·5 | 4·5 |

%CV, Percent coefficients of variation.

**TABLE 2**
Reproducibility of the MBC Immunoassay on Fruit Juices and Concentrates Containing MBC

| | *Amount MBC in ng/g* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *15* | *35* | *42* | *56* | *58* | *96* | *367* |
| Number of samples analyzed | 11 | 9 | 10 | 8 | 10 | 9 | 11 |
| %CV | 25·0 | 21·0 | 25·0 | 13·0 | 18·0 | 12·0 | 15·0 |

%CV, Percent coefficients of variation.

that was free of atrazine residues to dissolve the standards instead of the buffer (Bushway *et al.*, 1989).

Comparisons were made between the HPLC and immunoassay methods for MBC determination in fruit juices and concentrates (Table 3). Twenty-six commercial juices and concentrates were analyzed once by both techniques. For the majority of the samples the methods agreed well at both low and high MBC concentrations making the immunoassay an excellent screening test since a screening test has to be rapid, inexpensive and semi-quantitative with no false negatives. If a sample is shown to be positive at the level that is unacceptable, then it can be analyzed by one of the more quantitative classical methods. The concentrates vary somewhat at the lower levels of MBC which is most likely caused from a matrix effect. Thus, the lower limit of detection of MBC by immunoassay appears to be 30 ng/g for juice concentrates and 10 ng/g for juices.

Because of the acidic conditions of fruit juice and the processing conditions, one would not expect to find benomyl. An experiment was performed whereby we added benomyl to juices and after an hour all of the benomyl was converted to MBC. It appears that juices would only contain MBC making this screening test even more applicable.

The cross-reactivity of the MBC antibody has been described earlier by Newsome and Collins (1987), who observed that the antibody does not cross-react with any important MBC metabolites found in food. Thus it is quite specific to MBC.

Since the antibody is quite specific to MBC, it can be used as a confirmation test for MBC that has been isolated by HPLC. The peak that corresponds to MBC can be collected from the HPLC, evaporated to dryness, and then analyzed by immunoassay for confirmation. This has been tried in our laboratory and the immunoassay works well as a confirmatory test.

Although tube-type immunoassays are limited by the number of samples

**TABLE 3**
Comparison of the Immunoassay and HPLC Methods for Determination of MBC residues in Fruit Juices and Concentrates

| *Sample* | *MBC found (ng/g)* | |
|---|---|---|
| | *Immunoassay* | *HPLC* |
| Apple concentration | 365 | 367 |
| White grape concentration | <10 | <10 |
| Apple concentration | 81 | 96 |
| Apple concentration | 194 | 200 |
| Apple concentration | <10 | <10 |
| Apple concentration | 42 | 35 |
| Grape concentration | <10 | <10 |
| Grape concentration | 31 | <10 |
| Orange concentration | 25 | <10 |
| Pineapple concentration | <10 | <10 |
| Berry concentration | 33 | <10 |
| Cran-Raspberry concentration | <10 | <10 |
| Citrus concentration | 36 | <10 |
| Apple juice | 52 | 56 |
| Apple raspberry juice | 33 | 42 |
| Apple cherry juice | 63 | 58 |
| Apple juice | 26 | 35 |
| Apple cherry juice | 18 | 15 |
| Apple juice | <10 | <10 |
| Apple apricot juice | <10 | <10 |
| Apple cherry juice | <10 | <10 |
| Apple cherry juice | <10 | <10 |
| Apple juice | <10 | <10 |
| Apple juice | <10 | <10 |
| Apple cherry juice | 260 | 236 |
| Apple raspberry juice | 130 | 100 |

that can be analyzed simultaneously (eight samples and a control with this test) compared to the plastic well configuration, it does have the advantages of less incubation time and is inexpensively field adaptable. With this tube system, actual samples can be screened in a processing or quality control facility by using a hand-held battery-powered photometer.

## ACKNOWLEDGEMENT

This manuscript is number 1350 of the Maine Agricultural Experiment Station.

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