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Determination of benzomate in plant tissues by high-performance liquid chromatography

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Benzomate (3-chloro-α-ethoxyimino-2,6-dimethoxybenzyl benzoate) is a non-systemic acaricide which is effective against *Panonychus ulmi* and *P. citri* on apple and citrus. It was introduced in 1972 by the Nippon Soda Co. under the trade mark Citrazon[1].

Analytical procedures for benzomate involving decomposition by acid to the phenol analogue followed by a colorimetric determination method have been described[2]. However, these methods have the disadvantages of being both complicated and time-consuming. Recently, Roseboom and Berkhoff[3] and Ono and Toyama[4] reported the high-performance liquid chromatography (HPLC) of benzomate residues in apple and citrus. These methods are superior to the former colorimetric ones. However, the extracts still had to be cleaned up by column chromatography or thin-layer chromatography in order to eliminate interfering compounds.

In this paper, a simple method for determining benzomate residues in plant tissues by HPLC using microparticulate packings is described.

EXPERIMENTAL

Apparatus

The HPLC system was a TRI-ROTAR chromatograph with a UVIDEC-100 UV detector (Japan Spectroscopic Co.). The spectrophotometer was a Hitachi Model 323 and the blender was a Polytron (Kinematica, Switzerland).

Materials

Benzomate was obtained from the Nippon Soda Co. (Japan). The organic solvents were of pesticide-residue-analysis grade. A standard solution of benzomate was prepared in methanol. Packing materials HP-01, SN-01 and SC-02 were purchased from the Japan Spectroscopic Co. and Nucleosil $7C_{18}$, Nucleosil $5C_{18}$ and Nucleosil 5CN from Macherey, Nagel & Co. Acetonitrile and *n*-hexane used in liquid–liquid partition were prepared as follows: approximately equal volumes of acetonitrile and *n*-hexane were shaken vigorously in a separating funnel for 3 min at room temperature. After standing to allow complete separation of the layers, each layer was collected.

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Extraction of benzomate from plant tissues

The orange pulp and apple were homogenized in a mixer. A 50-g portion of the homogenate was transferred to a 500-ml flask, 100 ml of methanol was added and the flask was shaken for 30 min at room temperature.

The orange peel was finely chopped. A 10-g portion of the chopped sample was transferred to a 500-ml flask, 100 ml of methanol was added, and the mixture was blended at high speed for 1–2 min and then shaken for 15 min at room temperature.

Isolation of benzomate from crude extract

The mixture was filtered through a filter paper under suction. The extraction was then repeated using an additional 50-ml portion of methanol. The combined methanol extract was transferred to a 500-ml separating funnel, 150 ml of water (200 ml, orange peel) and 5 g of sodium chloride were added and the benzomate was extracted with two 100-ml portions of dichloromethane. After phase separation and filtration of the dichloromethane layer through Whatman 1 PS filter paper, the filtrate was transferred to a 500-ml round-bottomed flask and evaporated to dryness under reduced pressure and below 40°C. The dried residue was dissolved in 50 ml of acetonitrile and the solution was transferred to a 200-ml separating funnel and washed with two 50-ml portions of *n*-hexane, discarding the *n*-hexane layer after phase separation. After addition of 10 ml of water to the acetonitrile layer, the solution was washed with 25 ml of *n*-hexane by shaking gently for 10–20 sec. Care was taken not to shake too vigorously and/or for long periods to prevent losses resulting. The *n*-hexane layer was discarded after phase separation and 50 ml of dichloromethane was then added to the acetonitrile layer which was then shaken vigorously for 5 min. After phase separation, the dichloromethane–acetonitrile layer was filtered through a bed of anhydrous sodium sulphate, and then evaporated to dryness under reduced pressure.

The dried residue was dissolved in 2.0 ml of methanol, and the benzomate was assayed by the liquid chromatographic method described below.

Liquid chromatographic analysis of benzomate

A column (250 × 4.6 mm I.D., stainless steel) packed with Nucleosil $7C_{18}$ and with acetonitrile–water (70:30) as the mobile phase was used for the analysis of orange pulp and apple. The column pressure was 80 kg/cm^2. A column of the same size packed with Nucleosil $5C_{18}$ and with acetonitrile–water (65:35) as the mobile phase was used for the analysis of orange peel. The column pressure was 135 kg/cm^2. Other conditions were as follows: flow-rate, 1.0 ml/min; UV detector, 235 nm and 0.03 absorbance units full scale; chart speed, 5.0 mm/min. Chromatography was carried out at ambient temperature.

In practice, a 50- or 10-g sample was taken for the assay, the final volume of extracted sample was made up to 2.0 ml, and then 3 μl (orange pulp and apple) or 10 μl (orange peel) of the solution were chromatographed under the conditions described above.

The amount of benzomate was determined by comparing the peak height with the working curve for benzomate. The working curve for the relationship between the amount of benzomate and peak height was linear over the range 3–100 ng.

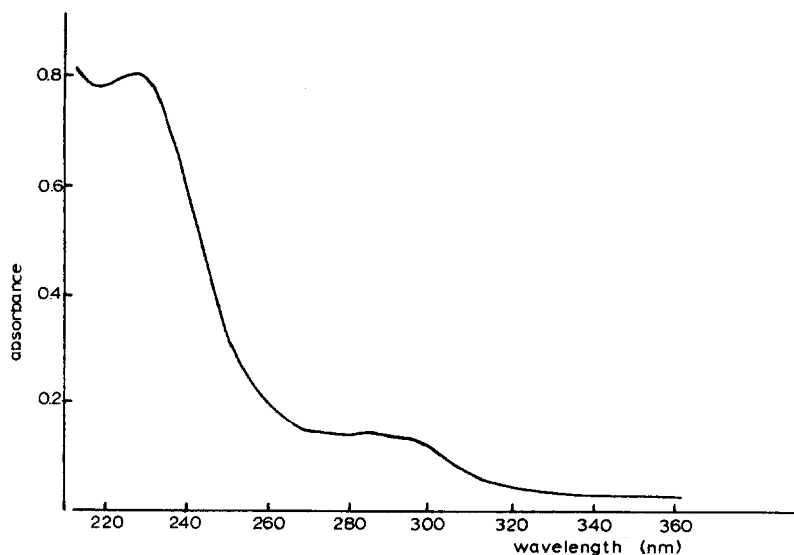

Fig. 1. UV absorption spectrum of 10 μg/ml solution of benzomate in methanol.

TABLE I

RELATIVE SENSITIVITY AT VARIOUS WAVELENGTHS

Wavelength (nm)	*Relative sensitivity (100 at 235 nm)*
235	100
254	34
285	9
295	9

TABLE II

PROPERTIES OF HPLC COLUMN PACKINGS

Column packing	*Functional group*	*Mean particle size (μm)*	*Number of theoretical plates per 25 cm**
HP-01	Polystyrene–divinylbenzene co-polymer	10	400
SN-01	Aminopropyl	10	3600
Nucleosil 5CN	Cyanopropyl	5	7300
SC-02	Octadecyl	10	3500
Nucleosil 7C_{18}	Octadecyl	7	5900
Nucleosil 5C_{18}	Octadecyl	5	9100

* Determined by injection of 60 ng of benzomate into the chromatograph.

TABLE III

CHARACTERIZATION OF BENZOMATE

Column packing	*Mobile phase*	*Column pressure (kg/cm²)*	*Retention time (min)*	*Capacity factor*	*M.D.Q.**	*Resolution***		
						Orange peel	*Orange pulp*	*Apple*
HP-01	Methanol	40	15.4	2.8	50	0	1.0	—***
SN-01	Acetonitrile–water (70:30)	30	3.0	0.5	10	0	0	0
Nucleosil 5CN	Acetonitrile–water (70:30)	150	4.6	0.8	3	0	0	0
SC-02	Acetonitrile–water (70:30)	30	10.0	3.2	8	—***	0.3	0.7
SC-02	Acetonitrile–water (65:35)	35	13.4	5.1	10	—***	1.1	0.7
Nucleosil $7C_{18}$	Acetonitrile–water (70:30)	80	10.4	3.6	3	0	2.0	1.9
Nucleosil $7C_{18}$	Acetonitrile–water (65:35)	120	14.0	5.3	4	0	1.7	0.9
Nucleosil $5C_{18}$	Acetonitrile–water (70:30)	120	12.9	4.8	3	0.7	2.4	1.4
Nucleosil $5C_{18}$	Acetonitrile–water (65:35)	135	17.5	7.2	4	1.5	2.5	3.7

* Minimum detectable quantity (ng per injection).

** Resolution of benzomate and nearest interfering peak from sample.

*** Benzomate was shoulder peak of large interfering peak from sample.

TABLE IV

LOWER LIMITS OF DETECTION

Sample	*Sample weight (g)*	*Minimum detectable quantity (ng)*	*Final volume (ml)*	*Injection volume (µl)*	*Lower limit of detection (ppm)*
Orange pulp	50	3	2	3	0.04
Orange peel	10	4	2	10	0.08
Apple	50	3	2	3	0.04

RESULTS AND DISCUSSION

HPLC operational conditions

Benzomate in methanol has three maximum absorptions (λ_{max}) at 228, 285 and 295 nm with molar extinction coefficients (ε) of $2.91 \cdot 10^4$, $3.64 \cdot 10^3$ and $3.27 \cdot 10^3$ l mol^{-1} cm^{-1}, respectively (Fig. 1). Table I shows the relative sensitivity of benzomate at various wavelengths: the highest sensitivity was at 235 nm and this wavelength was selected for detection. Any wavelength shorter than 235 nm could not be used because the acetonitrile–water mixture used the HPLC mobile phase has a high UV background.

Several reversed-phase columns were investigated as to their ability to separate benzomate and extraneous sample peaks. Table II listed the types of columns tested. Among these columns, the octadecyl-bonded (ODS) type gave good results (Table III). The aminopropyl and cyanopropyl columns held benzomate weakly and the polystyrene–divinylbenzene column was even less sensitive. Separation efficiency was increased by using an ODS column with a smaller particle size and an increased capacity factor. Complete baseline separation of benzomate and the interfering peaks (resolution > 1.5)[5] was obtained by the following combinations: for orange pulp and apple, Nucleosil $7C_{18}$ and acetonitrile–water (70:30); for orange peel, Nucleosil $5C_{18}$ and acetonitrile–water (65:35). Acetonitrile rather than methanol in the mobile phase was used because the pressure was lower in acetonitrile than in methanol: this is due to the lower viscosity of acetonitrile, a factor which is necessary for a small-particle column.

Recovery and detection limit

Table IV shows the lower limits of detection, which are almost the same as

TABLE V

RECOVERIES FROM PLANT TISSUES

Plant tissue	*Fortification (ppm)*	*Recovery (%)**	*Coefficient of variation (%)*
Orange pulp	1.0	74.7 ± 1.9	2.5
Orange peel	2.5	76.8 ± 3.7	4.8
Apple	1.0	78.1 ± 2.1	2.7

* Mean of four determinations ± standard deviation.

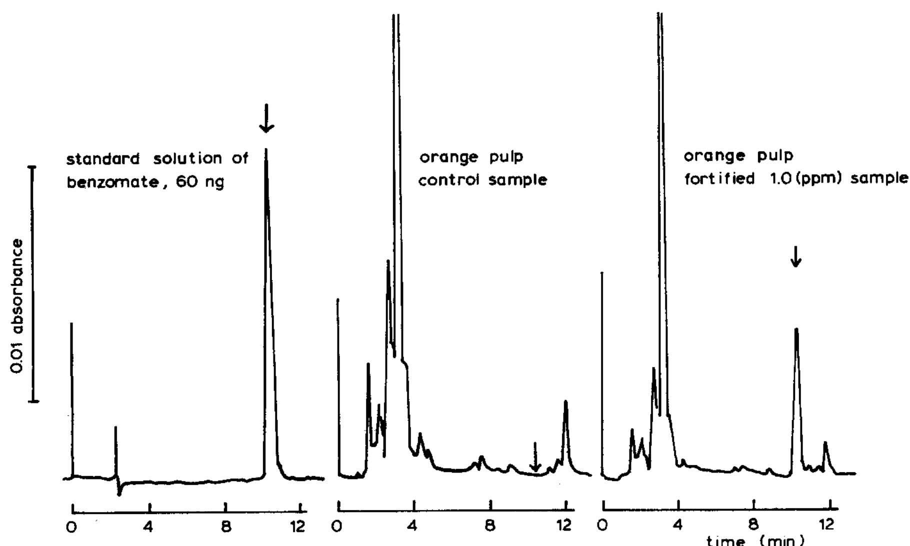

Fig. 2. Chromatograms of orange pulp extracts. For operating conditions see text. Sample: 50 g. Final volume: 2 ml for control, 4 ml for fortified sample. Injection volume: 3 μl. The arrow indicates the retention time of benzomate.

those determined by the methods of Ono and Toyama[4] and Roseboom and Berkhoff[3]. The recoveries for benzomate are given in Table V. Known amounts of benzomate were added to various plant-tissue homogenates and determined by the proposed procedure. The recoveries were acceptable and the reproducibility of the method was good.

Fig. 2 shows liquid chromatograms of a standard solution of benzomate, a control sample and a fortified orange pulp extract. Benzomate is completely separated from other extraneous peaks.

The detection wavelength was set at near as possible to the maximum sensitivity of benzomate and the injection volume was minimized in order to keep the column clean, whereas the sensitivity of detector was set as high as possible.

The columns (Nucleosil $7C_{18}$ and Nucleosil $5C_{18}$) used for the residue analysis of benzomate functioned well throughout the analyses without deterioration for about three months. By this method five or six samples can be analysed in less than one day by one analyst.

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