Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1022 (2004) 145–150
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Rapid simultaneous determination of *o*-phenylphenol, diphenyl, thiabendazole, imazalil and its major metabolite in citrus fruits by liquid chromatography-mass spectrometry using atmospheric pressure photoionization

Naoki Yoshioka*, Yumi Akiyama, Kiyoshi Teranishi

Hyogo Prefectural Institute of Public Health and Environmental Sciences, 2-1-29, Arata-cho, Hyogo-ku, Kobe 652-0032, Japan

Received 20 May 2003; received in revised form 9 September 2003; accepted 9 September 2003

Abstract

A simple and rapid simultaneous analytical method of four post-harvest fungicides, *o*-phenylphenol (OPP), diphenyl (DP), thiabendazole (TBZ), imazalil (IMZ) and its major metabolite R14821 (IMZ-M) in citrus fruits was developed. These compounds were extracted under basic conditions with diethyl ether. The organic layer was washed with water and evaporated at moderate pressure adding methanol. These compounds were determined by liquid chromatography–mass spectrometry (LC–MS) using atmospheric pressure photoionization (APPI). The recoveries of the five compounds added to citrus fruits at 1 μg/g ranged from 67 to 100%, with relative standard deviations (R.S.D.) ranging from 2 to 8%. The detection limits (S/N = 3) were 0.01 μg/g and 0.05 μg/g (DP).

Keywords: Food analysis; Atmospheric pressure photoionization; Citrus fruits; *o*-Phenylphenol; Diphenyl; Thiabendazole; Imazalil; Pesticides

1. Introduction

o-Phenylphenol (OPP), diphenyl (DP), thiabendazole (TBZ) and imazalil (IMZ) are widely used for post-harvest treatments of citrus fruits (Fig. 1). The maximum residue limits (MRLs) for these fungicides in Japan are 10, 70, 10, 5 μg/g, respectively, and MRLs of Codex are 10 (OPP), 10 (TBZ), 5 (IMZ) μg/g. Since they are frequently detected in imported citrus fruits, many commercial samples must be inspected to ensure the food safety. Additionally, IMZ is easily metabolized to R14821 [1-(2,4-dichlorophenyl)-2-(1*H*-imidazole-1-yl)-1-ethanol], which is often detected in citrus fruits [1]. In the United States, sum of IMZ and R14821 (IMZ-M) is regulated [2], so the survey of residual IMZ-M is also required.

A large number of analytical procedures for these fungicides have been reported in the literature. Simultaneous analytical methods for OPP, DP and TBZ [3,4], for TBZ and IMZ [5–7] in citrus fruits were reported. While systematic analytical methods for OPP, DP, TBZ and IMZ were reported [8–10], the sample extract must be divided into two fractions, and analyzed separately. No reports have described a simultaneous method of these 4 fungicides. Moreover, there are few reports about analytical methods of IMZ-M [1,11–13], which are described only for IMZ and IMZ-M.

Recently, liquid chromatography–mass spectrometry (LC–MS) has been introduced for the analysis of these post-harvest fungicides [7,14]. High selectivity of MS detector can simplify the clean-up steps of the sample extract. OPP, TBZ and IMZ can be determined by LC–MS using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), however, nonpolar compounds such as DP cannot be ionized by these two ionization techniques.

Atmospheric pressure photoionization (APPI), which is suitable for analyzing low or nonpolar compounds such as polyaromatichydrocarbons (PAHs) [15] or mycotoxins [16], has been available as a new ionization method for LC–MS. This APPI source is based on a high-emission gas discharge

* Corresponding author. Tel.: +81-78-511-6640; fax: +81-78-531-7080.
E-mail address: naoki_yoshioka@pref.hyogo.jp (N. Yoshioka).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.09.021

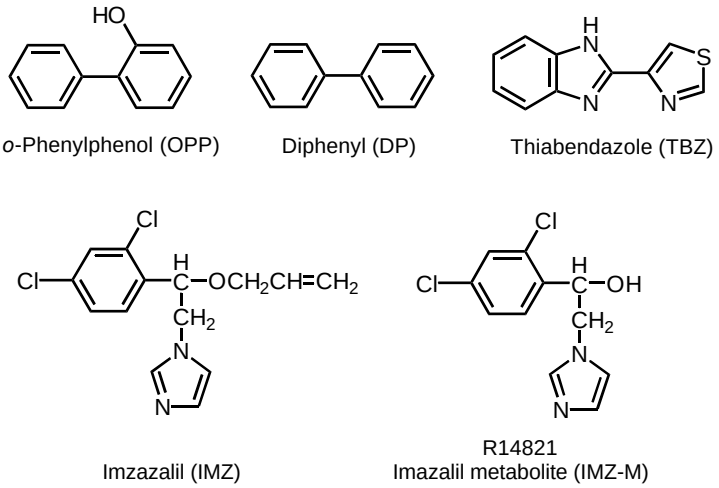

Fig. 1. Chemical structures of four fungicides and its metabolite.

lamp that generates vacuum-ultraviolet photons of 10.0 eV energy. So the analyte of which ionization potential is lower than 10.0 eV can be ionized [17,18]. As the ionization potential of DP is 8.2 eV [19], it can be readily ionized by APPI. Then, we tried to determine DP simultaneously with other fungicides by APPI.

This paper describes a rapid and simultaneous analytical method of OPP, DP, TBZ, IMZ and IMZ-M in citrus fruits by APPI-LC–MS.

2. Experimental

2.1. Chemicals and reagents

OPP, DP, TBZ were obtained from Wako Pure Chemical (Osaka, Japan), IMZ and IMZ-M, 1-(2,4-dichlorophenyl)-2-(1*H*-imidazole-1-yl)-1-ethanol were purchased from Kanto Chemical (Tokyo, Japan). Diethyl ether and methanol (HPLC grade), sodium chloride and sodium hydroxide were supplied by Wako Pure Chemical. Silicone treated filter paper 1PS (Whatman, Maidstone, UK) was used for dehydration. Stock solutions (250 μg/ml) and working solutions were prepared in methanol and stored at 4 °C.

2.2. Sample preparation

A 50 g of chopped citrus fruits were homogenized with 50 g of water using a blender for 3 min. A 10 g of homogenate was placed in a 50 ml centrifuge tube. Ten ml of water and 10 g of sodium chloride were added to the tube. The content was adjusted to pH 12 with 1 mol/l sodium hydroxide solution. Diethyl ether (25 ml) was added and the tube was shaken for 10 min on a reciprocal shaker, and then centrifuged at 2500 rpm for 5 min. The upper organic phase was removed and added to a separatory funnel. A further 25 ml of diethyl ether was added to the tube, shaken for 5 min and centrifuged for 5 min, and the upper organic phase was removed and combined with the first extract in the separatory funnel. The combined extract was washed with 15 ml of water and the extract was dehydrated by filtrating with a silicone treated filter paper. After addition of methanol (3 ml) as a keeper, the extract was evaporated on a rotary evaporator at 30 °C and 200 hPa until all of diethyl ether was evaporated. The volume of the residue was adjusted to 5 ml with methanol, and 5 μl of the sample solution was injected into the LC–MS system.

The 0.1 ml of 50 μg/ml standard solution (OPP, DP, TBZ, IMZ and IMZ-M) was fortified to 10 g of the homogenate which contained 5 g of citrus fruit. Each fortification level was 1 μg/g to the sample.

2.3. Instrument and conditions

An Agilent (Palo Alto, CA, USA) 1100 LC–MS system consisted of an autosampler, a binary pump and a mass detector was used. The mass detector system equipped with an APPI ion source. Inertsil ODS-3 (150 mm × 3 mm i.d., 5 μm, GL Sciences, Tokyo, Japan) was used as an analytical column. Water was purified using Milli-Q water purification system (Millipore, Bedford, MA, USA).

In the mobile phase consisted of methanol and water at a flow rate of 0.5 ml/min. In gradient-elution analysis, the first mobile phase was 60% methanol, increased linearly to 100% in 10 min, and held at 100% for 2 min. Separations were carried out at 40 °C. A return to the initial conditions was carried out in 8 min.

The mass detector operated under the conditions of 450 °C vaporizer temperature, 350 °C drying gas (N_2) temperature, 7 l/min drying gas flow, 60 psi nebulizer gas (N_2) pressure and 1400 V of capillary voltage. The fragmentor voltage was set to 100 and 200 V simultaneously. The selected ions and parameters used for time-scheduled SIM are listed in Table 1.

Table 1
The ions used for time-scheduled SIM

Time (min)	Compound	Ion mode		Fragmentor voltage (V)	*m/z*	Relative abundance[a] (%)
3.0–4.5	TBZ	Negative	Target	100	200	100
			Qualifier	200	173	64
4.5–6.0	IMZ-M	Positive	Target	100	257	100
			Qualifier	200	189	22
6.0–7.5	OPP	Negative	Target	100	169	100
			Qualifier	200	141	48
8.5–10.0	IMZ	Positive	Target	100	297	100
			Qualifier	200	159	25
10.0–12.0	DP	Positive	Target	100	154	100
			Qualifier	200	153	48

[a] Relative abundances indicate 100 times the ratio of intensity of the qualifier ion at 200 V to the target ion at 100 V.

3. Results and discussion

3.1. Extraction and purification

Diethyl ether, ethyl acetate, acetone and methanol are often used for extraction of fungicides from citrus fruits. As acetone and methanol are hydrophilic solvents and elimination of water-soluble interferences by using these solvents is difficult, they were excluded for the extraction solvent. With ethyl acetate in extraction, much emulsion layer occurred during extraction. In addition, diethyl ether, which has lower boiling point than ethyl acetate, is readily evaporated on a rotary evaporator at lower temperature without volatilization of DP. Therefore, diethyl ether was chosen as an extraction solvent.

Since OPP is acidic, TBZ, IMZ and IMZ-M are basic and DP is a neutral compound, it is difficult to extract the five compounds simultaneously from citrus fruits. The pH range 4–12 was studied and essentially the same recoveries were obtained for all test compounds for pH > 8, with losses occurring for IMZ and IMZ-M at pH < 7. The optimum pH was found around pH 10. In order not to lower the pH of the water phase under 10 during extraction with diethyl ether by the strong acidic buffer action of citrus fruit extracts, the content was adjusted to pH 12 before shaking.

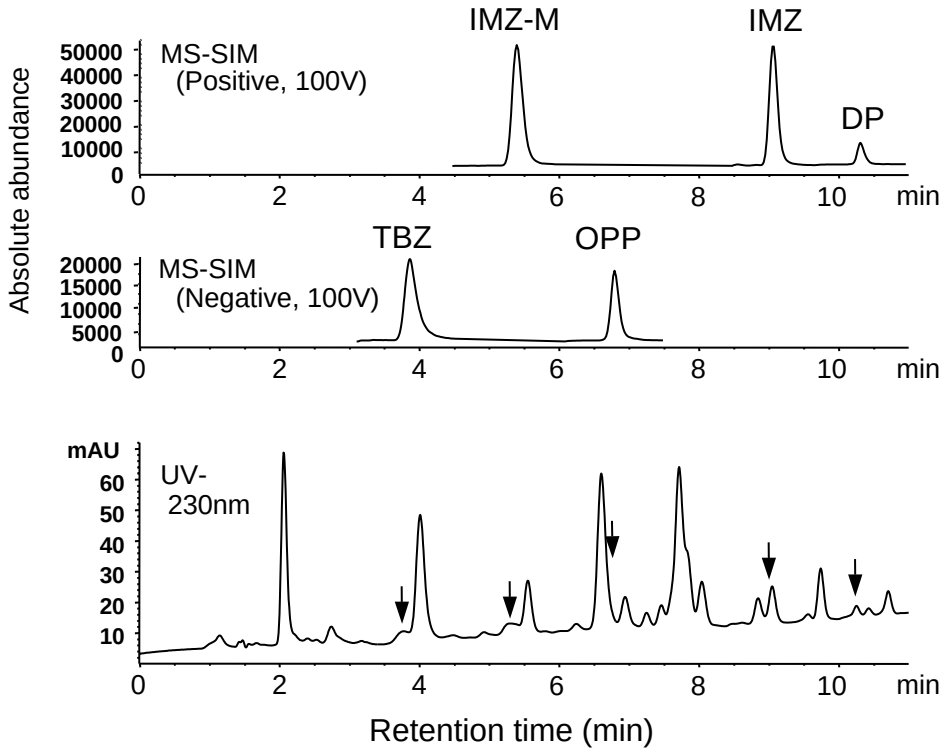

Fig. 2. Comparison of chromatograms of orange sample fortified with the fungicides at 1 μg/g (detection: MS-SIM and UV 230 nm).

As the polarities of these five compounds are different, clean-up by liquid–liquid partition is also difficult. Then minimum purification of the extract was examined. After the ether extract from the fortified orange sample was just washed with water, the extract was evaporated and re-dissolved in methanol, then analyzed by HPLC with both MS and UV detectors. The chromatograms are shown in Fig. 2. The target compounds were not distinguished from the neighboring peaks by UV detection and appear as minor peaks. When using a MS detector, the analytes were detected free from interferences, as the most abundant peaks in the chromatogram. From these results, high selectivity by LC–MS enabled minimum clean-up of the extract, and just washing with water was found to be sufficient for LC–MS analysis.

3.2. Optimization of LC–MS conditions

Sensitivities of the four compounds except for DP by APPI were as good as by APCI. Addition of ammonium acetate, which is often used for a buffer of mobile phase at positive ion mode, suppressed the ionization of OPP ($[M-H]^-$). Therefore, only methanol and water were chosen as a mobile phase.

Ionization by APPI is influenced by the capillary voltage and the vaporizer temperature. To optimize the ionization conditions, these effects were investigated by changing the parameters.

Sensitivities of these five compounds were examined by changing the capillary voltage from 1000 to 1800 V. Other conditions were described in experimental section. For OPP, TBZ, IMZ and IMZ-M, setting capillary voltage to 1300 V gave maximum peak areas, and 1450 V gave maximum for DP.

Next, by changing vaporizer temperature from 300 to 500 °C, as the vaporizer temperature went up, the peak areas became smaller for OPP, TBZ, IMZ and IMZ-M. In contrast, the peak area of DP increased. To get the high sensitivity especially for DP, the capillary voltage was set to 1400 V and the vaporizer temperature was 450 °C for the determination of the five compounds simultaneously.

A standard solution of each compound was analyzed by SCAN mode at both positive and negative ion modes, with fragmentor voltage of 100 and 200 V. TBZ, IMZ and IMZ-M were ionized at both ion modes. DP was ionized at only positive ion mode and OPP was at only negative ion mode. Although the peak height of DP was lower than the other compounds, it was sensitive enough to detect by SIM mode.

The mass spectra of each compound were obtained. M^+ ion was detected in DP (*m*/*z* 154) and $[M+H]^+$ ions were detected in TBZ (*m*/*z* 202), IMZ (*m*/*z* 297) and IMZ-M (*m*/*z* 257) at positive ion mode. $[M-H]^-$ ions were detected in OPP (*m*/*z* 169) and TBZ (*m*/*z* 200) at negative ion mode. At fragmentor voltage of 200 V, fragmentor ions (TBZ: *m*/*z* 173, IMZ-M: *m*/*z* 189, OPP: *m*/*z* 141, IMZ: *m*/*z* 159, DP: *m*/*z* 153) were also detected. These ions were used as qualifier ions to confirm the presence of fungicides by comparing the ratio of peak intensities to the parent ion peaks at fragmentor voltage of 100 V. These relative abundances are listed in Table 1. TBZ were ionized at both positive and negative ion modes, however, negative ion mode was chosen for time-scheduled SIM because the relative abundance of the fragmentor ion at negative was higher than that at positive.

As TBZ is eluted early, because of its high polarity, it is difficult to separate it from the interference peaks. Ion-pair reagents such as sodium 1-pentanesulfonate [3] or sodium dodecyl sulfate [9] are often used in order to increase the retention time. However, stabilization of the mobile phase takes longer when using these ion-pair reagents, and these reagents shorten the column lifetime. Even though TBZ elutes early without ion pair reagents, this caused no problems if APPI-SIM mode detection was used.

3.3. Analytical performance and application

Table 2 shows the recoveries of the five compounds from lemon, orange and grapefruit. The fortification level was set to 1 μg/g because MRLs of these compounds are relatively high (5–70 μg/g) and half of the positive citrus samples contained more than 1 μg/g of the fungicides from our previous investigation [20]. The inter-day recoveries from orange sample were tested over 5 working days. The recoveries

Table 2
Recoveries of the five compounds fortified to citrus fruits at 1 μg/g

Compound	Intra-day						Inter-day[a]	
	Lemon (recovery (%))		Orange (recovery (%))		Grapefruit (recovery (%))		Orange (recovery (%))	
	Mean	R.S.D.%	Mean	R.S.D.%	Mean	R.S.D.%	Mean	R.S.D.%
OPP	85	3	94	2	76	2	92	3
DP	71	3	67	7	73	3	75	7
TBZ	87	8	100	2	82	2	98	12
IMZ	71	6	75	5	78	6	77	9
IMZ-M	87	7	82	4	84	3	87	5

($n = 5$).
[a] Inter-day recovery study was carried out over 5 working days.

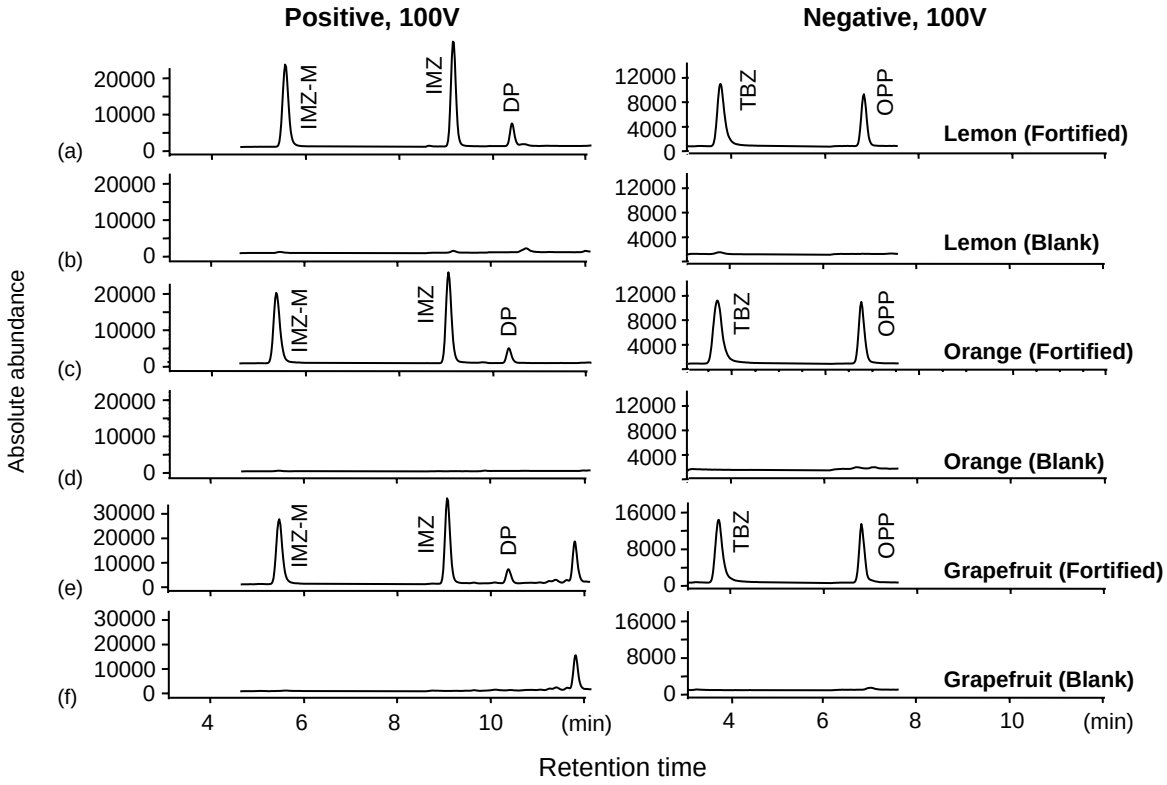

Fig. 3. SIM chromatograms of lemon, orange and grapefruit samples fortified at 1 μg/g: (a) lemon (fortified); (b) lemon (blank); (c) orange (fortified); (d) orange (blank); (e) grapefruit (fortified); (f) grapefruit (blank).

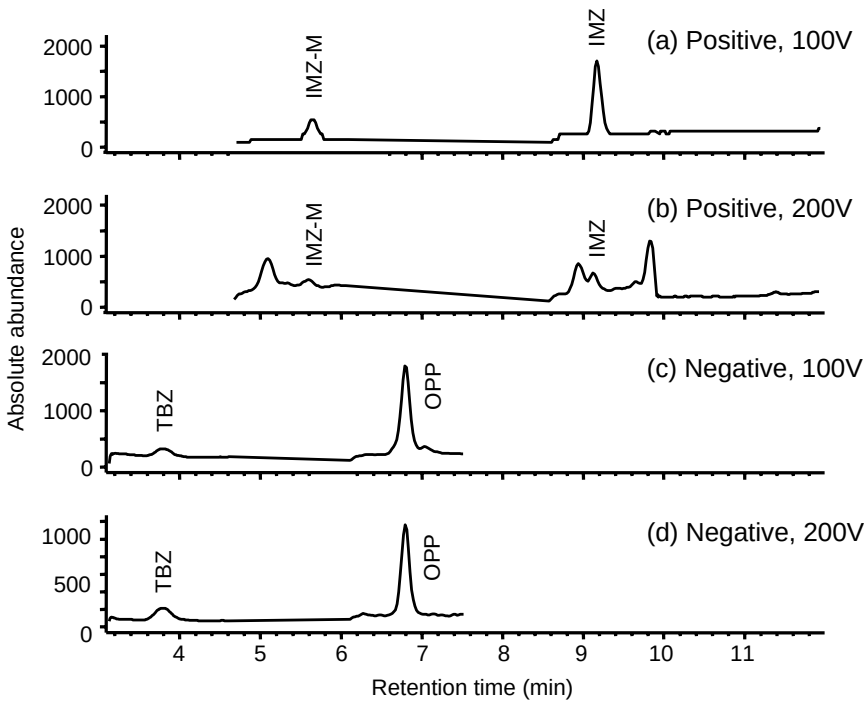

Fig. 4. SIM chromatograms of an orange sample containing OPP (0.15), TBZ (0.01), IMZ (0.05) and IMZ-M (0.02 μg/g). (a) Ion mode: positive, fragmentor voltage (100 V); (b) positive (200 V); (c) negative (100 V); (d) negative (200 V).

ranged from 67 to 100%, and the relative standard deviations (R.S.D.) at intra- and inter-day were within 8 and 12%, respectively. The results are satisfactory for residue analysis of the post-harvest fungicides.

Typical SIM chromatograms of lemon, orange and grapefruit samples are shown in Fig. 3. They were fortified at the level of 1 μg/g. There were few peaks in the blank chromatograms, and both of the retention times and abundance ratios (qualifier ions/target ions) were not matched with those of fungicide peaks. So, they did not interfere with the determination of the five compounds.

Fig. 4 presents the SIM chromatograms of an orange sample containing OPP, TBZ, IMZ and IMZ-M in both the positive and negative ion mode. The abundance ratios, qualifier ions (200 V)/target ions (100 V) were 0.41 (OPP), 0.69 (TBZ), 0.24 (IMZ) and 0.29 (IMZ-M). That is, the four compounds were all identified with less than 10% uncertainty on the basis of the relative abundances given in Table 1.

The detection limits (S/N = 3) were 0.01 μg/g for OPP, TBZ, IMZ and IMZ-M and were 0.05 μg/g for DP. The detection limits of DP was higher than the other compounds, however, it was enough for the routine analysis.

Twenty samples of citrus fruits obtained from local markets at Hyogo prefecture in Japan were analyzed by using this method. OPP in 8 samples (0.02–2.46 μg/g), TBZ in 11 samples (0.01–2.88 μg/g), IMZ in 17 samples (0.01–1.43 μg/g), and IMZ-M in 14 samples (0.01–0.22 μg/g) were detected, respectively. DP was neither detected in 20 samples (<0.05 μg/g). IMZ-M was detected in citrus samples of which IMZ concentrations were over 0.05 μg/g, and the ratio of IMZ-M to IMZ was 2–40%. None of the samples exceeded the MRLs.

4. Conclusions

We developed a simple and rapid analytical method for the determination of OPP, DP, TBZ, IMZ and IMZ-M in citrus fruits. In this method, these compounds can be determined simultaneously and simple extraction steps can reduce the time of sample preparation. This method is suitable for the routine analysis. Ten citrus samples require 2 h for sample preparation and 3 h for LC–MS analysis, so 20 samples can be inspected in a day.

References

[1] H. Matsumoto, Jpn. J. Food Chem. 7 (2000) 22.
[2] http://www.epa.gov/pesticides/reregistration/imazalil/ImazProductChem.pdf.
[3] S. Hori, M. Ueguchi, T. Nishimune, H. Yokoyama, J. Antibact. Antifung. Agents 18 (1990) 57.
[4] Y. Yamazaki, T. Ninomiya, J. AOAC Int. 82 (1999) 1474.
[5] Y. Ito, Y. Ikai, H. Oka, J. Hayakawa, T. Kagami, J. Chromatogr. A 810 (1998) 81.
[6] S. Navickiene, M.L. Ribeiro, J. High Resol. Chromatogr. 22 (1999) 303.
[7] M. Fernandez, R. Rodriguez, Y. Pico, J. Manes, J. Chromatogr. A 912 (2001) 301.
[8] Y. Tonogai, Y. Tsumura, Y. Nakamura, Y. Ito, J. Food Hyg. Soc. Jpn. 33 (1992) 23.
[9] M. Nakazato, K. Tadano, H. Ogawa, H. Ushiyama, Y. Kawai, T. Kobayashi, Y. Tateishi, Y. Tamura, Jpn. J. Toxicol. Environ. Health 41 (1995) 392.
[10] Y. Chatani, T. Chikamoto, M. Munehisa, T. Adachi, M. Komatsu, J Food Hyg. Soc. Jpn. 37 (1996) 187.
[11] Pesticide Analytical Manual, vol. II, US Food and Drug Administration, Rockville, MD, 1995, Sec. No. 180.413.
[12] H. Matsumoto, J. AOAC Int. 84 (2001) 546.
[13] N. Kimura, T. Nagayama, I. Takano, M. Kobayashi, Y. Tamura, Y. Tateishi, K. Kitayama, K. Saito, J. Food Hyg. Soc. Jpn. 44 (2003) 63.
[14] X. Pous, M.J. Ruiz, Y. Pico, G. Font, Fresenius J. Anal. Chem. 371 (2001) 182.
[15] T.J. Kauppila, T. Kuuranne, E.C. Meurer, M.N. Eberlin, T. Kotiaho, R. Kostiainen, Anal. Chem. 74 (2002) 5470.
[16] M. Takino, S. Daishima, T. Nakahara, Rapid Commun. Mass Spectrom. 17 (2003) 1965.
[17] D.B. Robb, T.R. Covey, A.P. Bruins, Anal. Chem. 72 (2000) 3653.
[18] M. Takino, S. Daishima, T. Nakahara, Rapid Commun. Mass Spectrom. 17 (2003) 383.
[19] http://webbook.nist.gov/chemistry/.
[20] Y. Akiyama, N. Yoshioka, M. Tsuji, J. AOAC Int. 85 (2002) 692.