Sunil Kumar Singh[1]
P. Padmaja[2]
S. Y. Pandey[1]

[1]Department of Chemistry, Jai Research Foundation, Vapi, India
[2]Department of Chemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, India

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Research Article

Fast ultrasound-assisted extraction followed by capillary gas chromatography combined with nitrogen–phosphorous selective detector for the trace determination of tebuconazole in garlic, soil and water samples

A fast and an efficient ultrasound-assisted extraction technique using a lower density extraction solvent than water was developed for the trace-level determination of tebuconazole in garlic, soil and water samples followed by capillary gas chromatography combined with nitrogen–phosphorous selective detector (GC–NPD). In this approach, ultrasound radiation was applied to accelerate the emulsification of the ethyl acetate in aqueous samples to enhance the extraction efficiency of tebuconazole without requiring extra partitioning or cleaning, and the use of capillary GC–NPD was a more sensitive detection technique for organonitrogen pesticides. The experimental results indicate an excellent linear relationship between peak area and concentration obtained in the range 1–50 μg/kg or μg/L. The limit of detection (S/N, 3 ± 0.5) and limit of quantification (S/N, 7.5 ± 2.5) were obtained in the range 0.2–3 and 1–10 μg/kg or μg/L. Good spiked recoveries were achieved from ranges 95.55–101.26%, 96.28–99.33% and 95.04–105.15% in garlic, Nanivaliyal soil and Par River water, respectively, at levels 5 and 20 μg/kg or μg/L, and the method precision (% RSD) was ≤5%. Our results demonstrate that the proposed technique is a viable alternative for the determination of tebuconazole in complex samples.

Keywords: Capillary gas chromatography / Extraction efficiency / Low-density extraction solvent / Organonitrogen pesticides / Ultrasound-assisted extraction
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1 Introduction

Tebuconazole, ((*RS*)-1-*p*-chlorophenyl)-4, 4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol, is a new generation azole group fungicide [1]. It is widely used for controlling soil-borne and foliar disease such as powdery mildew diseases in leaf spot, rust and root rot of crops acting as steroid demethylation (Sterol biosynthesis inhibitors) [2]. Garlic (*Allium sativum* L) is an important vegetable, consumed by people all over the world. This crop is prone to various fungal diseases, mainly orange rust (*Puccinia allii*). Many studies indicate tebuconazole (Folicur) provides good protection against garlic rust disease [3]. Pesticides in fruits and vegetables can be a significant source for human exposure [4, 5], and soil is also an important component of the environment, acts as a sink for the pesticides used in agriculture and their residue from soil can reach in water bodies by leaching and runoff [6]. Pesticides are inherently toxic molecules, and the presence of pesticides residue in food products and ground water is extremely hazardous to human beings, as ground water is a major source of drinking water. Therefore, a rapid, simple and an efficient analytical method is needed for the determination the residues of tebuconazole in food products and environment in order to minimize the risk.

A variety of extraction techniques have been employed over the years to determine pesticides and their degradation products, including liquid–liquid extraction (LLE) [7], SPE [8, 9], solid-phase microextraction (SPME) [10, 11], semipermeable membrane device (SPMD) [12, 13] and supercritical fluid extraction [14], followed by various chromatographic techniques such as GC and LC coupled with nitrogen–phosphorus (NPD) [15], electron-capture (ECD) [16],

Correspondence: Dr. P. Padmaja, Department of Chemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, India
E-mail: p_padmaja2001@yahoo.com

Abbreviations: DAP, Days after planting; **ECD**, electron-capture detection; **IT-MS**, ion trap mass spectrometry; **LLE**, liquid–liquid extraction; **NPD**, nitrogen–phosphorous selective detector; **QuEChERS**, quick, easy, cheap, effective, rugged and safe; **RSD**, relative standard deviation; **SPMD**, semipermeable membrane device; **SPME**, solid-phase microextraction; **UAE**, ultrasound-assisted extraction

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diode-array [17], fluorescence [18] and MS detection systems [19]. Most of the pre-concentration techniques are expensive, complicated, time-consuming and have low extraction efficiency. Ultrasound-assisted extraction (UAE) has a high extraction efficiency, low solvent consumption, low equipment cost, is easy to operate, has no hassle related to sample preparation (e.g. partitioning and clean-up procedure), is easy to perform and requires a low extraction temperature. This extraction process is fast in comparison with traditional methods because of the contact surface area between the solid or liquid sample, and the liquid phase of extraction solvent is much greater due to the occurrence of particle disruption [20]. These advantages make it a quick and efficient method for the pre-concentration of analytes from complex matrices.

The aim of this work is to determine the harvest time trace amount of tebuconazole in garlic, Nanivaliyal soil and expected contamination in Par River water samples by applying ultrasound radiation in aqueous samples contained in round-bottomed glass tubes along with ethyl acetate. The UAE accelerates the emulsification of ethyl acetate in order to enhance the extraction efficiency of tebuconazole followed by centrifugation for layer separation prior to capillary GC combined with NPD using 35% phenylmethylpolysiloxane bonded phase capillary column for separation. The capillary GC–NPD is a more sensitive detection technique for tebuconazole. The UAE is an alternative extraction technique for common Soxhlet, QuEChERS (quick, easy, cheap, effective, rugged and safe), SPE and shaking flask extraction [22–23], and [24] for pesticides from complex matrices.

2 Materials and methods

2.1 Reagents and standards

A reference standard of tebuconazole (purity 99.24%) was supplied by United Phosphorus (Mumbai, India), and a formulation of tebuconazole, Folicur 25.9% EW (Bayer Crop Science, India) was purchased from a local market. Ethyl acetate and anhydrous sodium sulfate (AR grade, Merck, India) were also purchased from the local market. The distilled water used was taken from Milli-Q water system. The stock solutions of tebuconazole (500 mg/L) were prepared in ethyl acetate.

2.2 Instrumentation and operating conditions

The capillary gas chromatograph was equipped with a NPD and spit mode injector (Perkin Elmer, USA, and Model, Clarus 500). The Dura Bond mid-polar fused-silica capillary column with low bleed (DB-35 column: packing with 35% phenylmethylpolysiloxane, dimension: 30 m × 0.25 mm I.D., 0.25 micron film thickness) was connected with the GC, and the instrument was controlled by Total Chrom software for data integration. The optimum temperature parameters were 200 to 275°C at a rate of 25°C/min (hold for 8.0 min), 260 and 290°C for column oven, injector port and detector, respectively. The 2 μL volume of aliquot test solutions were injected into the split mode injector (split ratio, vent/column, 2:1). At optimum conditions as carrier gas, helium and flow rate, 1.0 mL/min, hydrogen gas rate 1.5 mL/min and air 95 mL/min, attenuation 32, range 1, bead background 0.5 mV and time constant 200. At this optimum condition, the excellent linear relationship was observed between detector response and concentration, the method was found with good sensitivity and precision. An ultrasonic water bath (fixed frequency 35 kHz, Spectra Lab, India) was used for extraction; the round-bottomed glass tube of 15 mL capacity with interchangeable polypropylene cap (Borosil, India) was used as a sample container for extraction, and refrigerated centrifuge (Rota 4RV/FM, Plasto Crafts, India) was used for the centrifugation of samples.

2.3 Soil sample collection and characterization

Well-aerated moist surface soil was collected from different spots of the selected experimental plot in Nanivaliyal, Gujarat, India, from the depth of 10 to 15 cm using a hand auger, and the collected soil was passed through 2.0 mm sieve. The field was free from any chemical contamination. The soil samples were characterized for different physicochemical properties, viz., the percentage of organic carbon, cation exchange capacity, moisture content, oven dry weight, total nitrogen content and water holding capacity were 0.60%, 10.57 meq/100 g soil, 5.66%, 18.93 g, 0.006% and 52.77%, respectively. The mean pH (0.01 M $CaCl_2$ suspension) and corresponding mean pH (distilled water suspension) of the soil were 6.97 and 7.02. The (particle size distribution) contents of coarse sand, fine sand, silt and clay were 36.00, 36.21, 10.56 and 10.57%, respectively. The soil characterization methods were prescribed by Walkley and Baruah [25,26]. The moisture content was maintained approximately at 60% of the maximum water holding capacity, using distilled water.

2.4 Samples preparation for recovery

The white variety of garlic was purchased from the local market. Whole garlic bulbs were blended into paste, and 5 g homogenized garlic was weighed accurately and characterized. Nanivaliyal soil samples were put into 15 mL screw-capped round-bottomed glass tubes separately, and 10 mL Par River water sample was taken out into amber glass tubes. Each sample was separately spiked at a level of 5 and 20 μg/kg or μg/L in five replicates using tebuconazole standard stock solution. A volume of 2 mL distilled water and 5 mL ethyl acetate was added into garlic and Nanivaliyal soil samples tubes separately, and 2 mL ethyl acetate was added into Par River water sample tubes, and samples-contained tubes placed on ultrasonic bath (Frequency of 35 kHz, Spectra Lab, India) at 30°C for 15 min then centrifuged for 10 min at 4000 rpm. The organic layer was separated; moisture from the extracts was removed by passing through sufficient amount of sodium sulfate, and extracts were filtered through a 0.2 μm PTFE syringe filter, the filtrate was dried under gentle stream of

nitrogen gas. The residues were reconstituted with 1 mL ethyl acetate and then 2 μL of aliquot was injected in the optimum conditions into the capillary GC–NPD for recovery analysis.

2.5 Pesticide application and sampling

Field experiments were conducted to determine the harvest time residue of tebuconazole in garlic vegetable, Nanivaliyal field soil and expected contamination in Par River water because the Par River flowed approximately 30–50 m from the selected plot (5 × 5 m). The garlic was planted 4 by 4 inches apart in triple rows on 25 October 2008. Clove tops were covered with 1 to 1½ inches of soil. After plantation, nine irrigations were given, i.e. first irrigation just after sowing, second at 15 days after sowing and remaining seven irrigations at an interval of 10–15 days and applied 50% RDN as urea + 50% N through bio compost for achieving higher bulb yield. The formulation of tebuconazole, Folicur 25.9% EW was applied 65 days after planting (DAP) at a rate of 1 L/ha as a post-emergent spray on garlic vegetable, and the second spray was applied after 15 days of first application. The fall-planted garlic was harvested in July 2009. Fifteen garlic bulbs were selected randomly and ploughed soil samples from four to five spots of the plot were collected for residue analysis. The soil samples were mixed thoroughly, air dried and passed through a 2 mm sieve. The sample was spread on a glass plate and divided into four parts. Soil from two opposite quarters was retained, rejecting the remaining two. The process was used to obtain 500 g of representative soil sample, and the moisture content was maintained approximately at 60% of the maximum water holding capacity, using distilled water. Water samples were collected in triplicate into amber-coloured glass bottles from the Par River, and the representative water sample was processed for analysis.

3 Results and discussion

3.1 Effect of the solvents of an extraction

For the ultrasound extraction method, the selection of extraction solvents should obey the following principles: the target analytes must be dissolved in the extraction solvents, the solvent must have good chromatography behaviour and the solvents must be sufficiently immiscible in the aqueous phase, hence after extraction no extra partition step would be required, the presence of water is simply removed by the excess of sodium sulfate. Therefore, ethyl acetate was tested, and the effect of this solvent on the performance of UAE was investigated. It was observed that the good recovery of tebuconazole was found in ethyl acetate (see Table 1) because it allowed good penetration of bonding analytes, and its polarity was sufficient to extract the target analyte from garlic, soil and water samples; therefore, in this work ethyl acetate was a suitable solvent for extraction. The clean-up steps were omitted to avert the loss of the tebuconazole, so it was detected sensitively.

3.2 Influence of samples matrix on recovery

The fungicide recovery in soil may be affected by the physiochemical properties of soils [27, 28], particularly, the soil pH, cation exchange capacity and water contents, and characteristics of fungicides. The good recovery in soil is attributed to soil buffering capacity, which will have led to aqueous extraction, having taken place at the pH of the soil in water. In this study, distilled water was used as aqueous extractant instead of buffer because of the pH values and cation exchange capacity of soil, and pH values of garlic pest and water samples were 7.02, 10.57 meq/100 g soil, 5.66% and 5.97, 7.19, respectively; it is very close to the characteristics of tebuconazole, having a pK_a value of 5.76. The pH value of soil, garlic pest and water samples determined the existing form of the tebuconazole in sample used for good extraction efficiency, which was controlled by addition of distilled water in aqueous extraction of tebuconazole, so it showed good recoveries (see Table 1).

3.3 Effect of sonication and centrifugation time

In UAE, the sonication time was investigated to obtain the quantitative recoveries of tebuconazole. Different extractions

Table 1. Recoveries, precision (% RSD, $n = 5$), linear range, correlation coefficient, limits of quantitation (S/N) = 7.5 ± 2.5 and limits of detection (S/N) = 3 ± 0.5 of the UAE technique

Samples	Blanks	Fortification levels (μg/kg or μg/L)	Recoveries (%)	RSD[a] (%)	Linear range (μg/kg or μg/L)	Correlation coefficient (R^2)	LOQ[b] (μg/kg or μg/L)	LOD[c] (μg/kg or μg/L)
Nanivaliyal soil		5	99.33	4.85				
	–	20	96.28	3.40	1–50	0.9977	10	3
Par River water		5	105.15	3.78				
	–	20	95.04	1.33	1–50	0.9981	1	0.2
Garlic bulb		5	101.26	4.58				
	–	20	95.55	2.71	1–50	0.9978	5	2

a) The RSD (%) = five replicate experiment of each sample at spiked levels 5 and 20 μg/kg or μg/L separately.
b) The limit of quantitation (S/N) = 7.5 ± 2.5.
c) The limit of detection (S/N) = 3 ± 0.5.

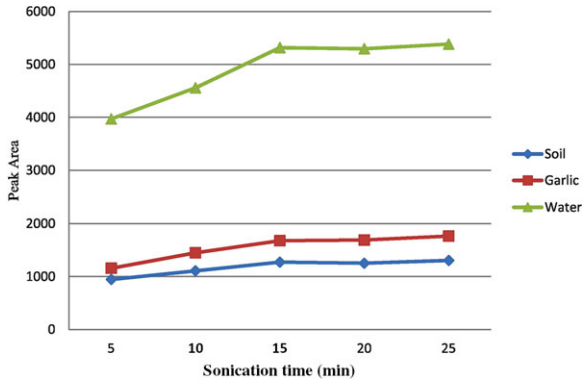

Figure 1. Effect of sonication time on extraction efficiency: Organic solvent (ethyl acetate) volume added 1 mL; constant centrifugation time 5 min; spiked sample concentration 20 μg/kg or μg/L for garlic, soil and water samples separately.

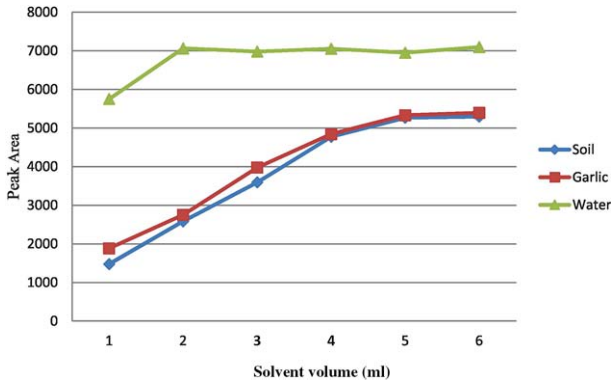

Figure 3. Effect of organic solvent (ethyl acetate) volume on extraction recoveries: Sonication time 15 min; Centrifugation time 10 min; spiked sample concentration 20 μg/kg or μg/L for garlic, soil and water separately.

were done using increasing sonication times to establish the kinetic of the extraction. The effect of the sonication times of 5, 10, 15, 20 and 25 min were studied. Figure 1 shows that the extraction efficiency versus sonication time is remarkably important. It reveals that the UAE method is time dependent. The transition of the analytes from the aqueous phase to extraction solvents phase took 15 min. The 15 min is the transition time for contact in the surface area between the extraction solvent and the aqueous phase during the formation of cloudy solution. Then to achieve the equilibrium state, the centrifuging time was also studied from 5 to 20 min with the rotation speed of 4000 rpm. Figure 2 shows that the extraction efficiency of tebuconazole reached the maximum value at 10 min centrifugation and remained unchanged with a further increase in the centrifuging time up to 20 min. Therefore, the sonication time and centrifugation time for subsequent analysis were 15 and 10 min, respectively.

3.4 Effect of solvent volume

In order to examine the effect of extraction solvent volume for extraction of tebuconazole from garlic, soil and water samples

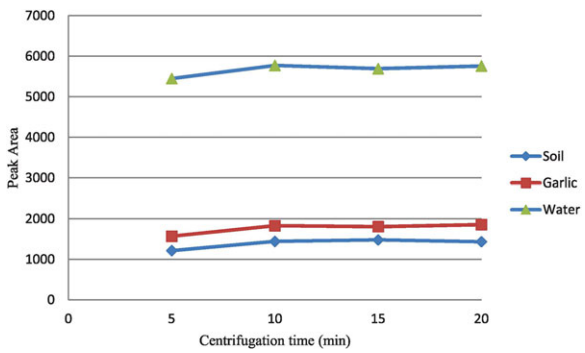

Figure 2. Effect of centrifugation time on extraction efficiency: Organic solvent (ethyl acetate) volume added 1 mL; constant sonication time 15 min; spiked sample concentration 20 μg/kg or μg/L for garlic, soil and water samples separately.

containing different volumes of ethyl acetate ranging from 1 to 6 mL were taken. The effect of extraction solvent volume was investigated according to Fig. 3; the extraction efficiency increased when the volume of ethyl acetate was increased from 1 to 6 mL with 1 mL intervals, and then remained almost constant at 5 mL in garlic and soil, and 2 mL in water samples. Therefore, volumes of 5 and 2 mL ethyl acetate were selected for further studies.

3.5 Feature of the method

The optimum experimental conditions were validated by evaluating the parameters such as the linear range, correlation coefficients, LOD, LOQ and %RSD as listed in Table 1. It was found that this method has excellent linearity between the peak area and concentration of tebuconazole in the range between 1 and 50 μg/kg or μg/L in each of the garlic vegetable, Nanivaliyal soil and Par River water samples separately. The detection limits of the method, calculated on the basis of signal to noise ratio of 3 ± 0.5 ($S/N = 3 \pm 0.5$) of UAE, were in the range of 0.2 to 3 μg/kg or μg/L. The quantification limits, calculated on the basis of signal to noise ratio of 7.5 ± 2.5 ($S/N = 7.5 \pm 2.5$) of UAE, were in the range of 1 to 10 μg/kg or μg/L. The method precision was investigated for tebuconazole using five replicate experiments of each sample separately at fortified level 5 and 20 μg/kg or μg/L, and the RSD was obtained $\leq 5\%$.

3.6 Analysis of real garlic, soil and water samples

To evaluate the applicability of the proposed method, the extraction and determination of the tebuconazole residue at harvest time in garlic vegetable and Nanivaliyal soil samples and expected contamination in Par River water samples were performed according to the procedures described herein. As a result, there were no harvest time residues of the tebuconazole found in the garlic vegetable and Nanivaliyal soil and no contamination in Par River water. Thus, to assess matrix

effects, the garlic pest, Nanivaliyal soil and Par River water samples were spiked with the standard solution of the tebuconazole at the concentrations level of 5 and 20 μg/kg or μg/L each, respectively. For each concentration level, five replicate experiments for a whole analysis process were made. The recoveries of the method were expressed as the mean percentage between the amounts found and the ones added. The extraction performance was evaluated and the results are given in Table 1. The mean recoveries of the tebuconazole in garlic vegetable, Nanivaliyal soil and Par River water samples

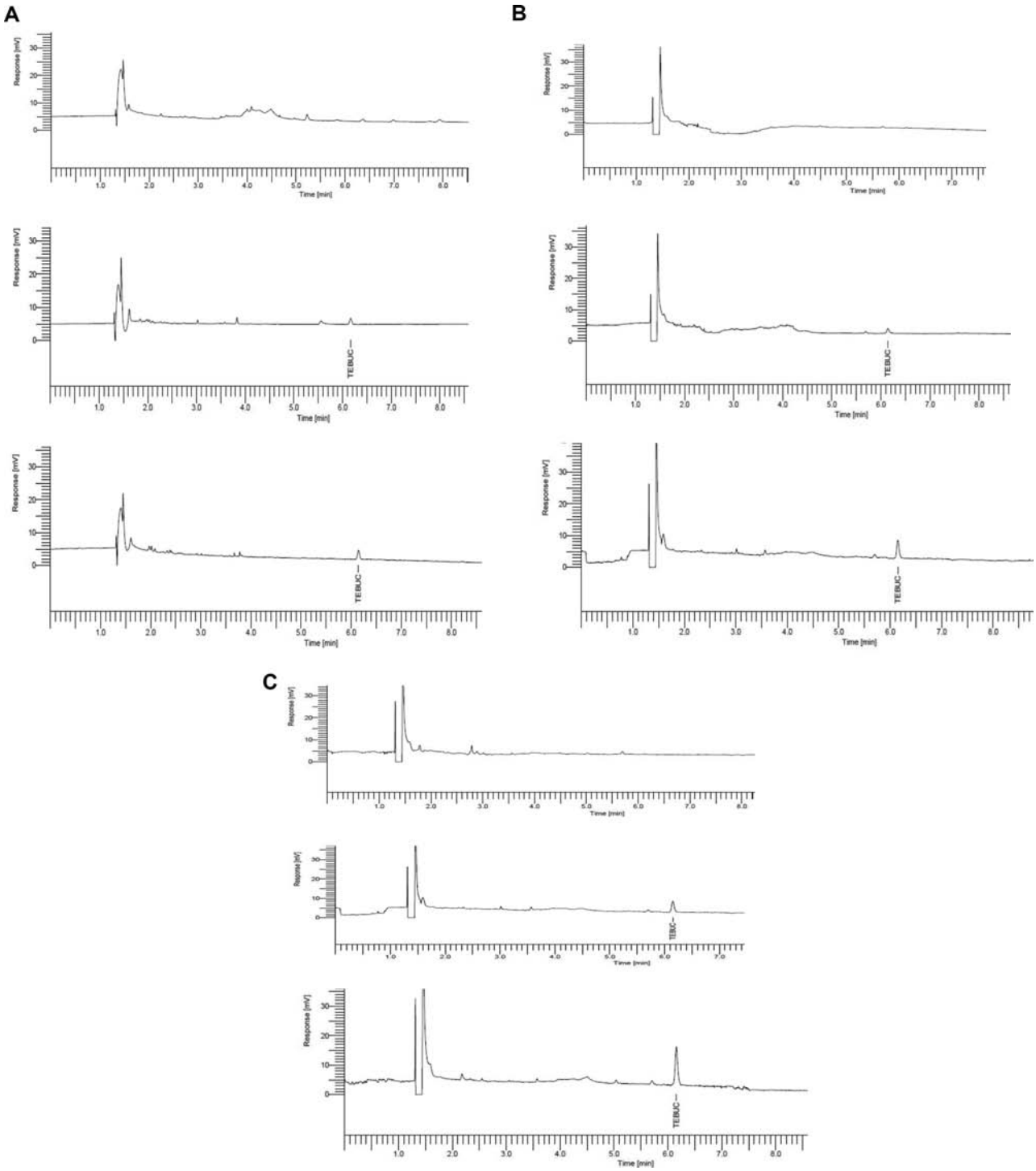

Figure 4. (A) Typical chromatograms of the real garlic vegetable (B) Nanivaliyal soil and (C) Par River water samples before and after being spiked of the tebuconazole standard solution at concentration level 5 and 20 μg/kg or μg/L for each, respectively; the retention time (RT) of tebuconazole in spiked samples is 6.16 min.

at spiked concentration levels 5 and 20 μg/kg or μg/L were in the range of 95.55–101.26, 96.28–99.33, and 95.04–105.15%. Figure 4 shows the typical chromatograms of the real garlic vegetable, Nanivaliyal soil and Par River water samples before and after being spiked of the tebuconazole standard solution at level 5 and 20 μg/kg or μg/L for each separately. The retention time (RT) of tebuconazole in spiked samples is at 6.16 min.

Li [29], Wang [30] and Huang [31] reported methods using enantiomeric enrichment, QuEChERS (quick, easy, cheap, effective, rugged and safe) and titanate nanotube array micro-solid-phase extraction (μSPE), sample preparation techniques for trace level determination of tebuconazole and its metabolite in vegetables, fruits, soil and water samples coupled with different analytical tools like reversed-phase LC–MS/MS, GC–NPD, GC with ion trap (IT) MS and HPLC–UV. The LODs reported by Li were less than 0.6 μg/kg, whereas the limit of LOQ did not exceed 2.0 μg/kg. Wang reported LOD and LOQ of 0.4–7 and 1.2–20 μg/kg for GC–IT-MS/MS and GC–NPD, and Huang reported the LODs of the method in the range of 0.016–0.086 μg/L in water samples. The LOD of the method for tebuconazole in this study is better than that reported by Wang and Li, in fruit, vegetable, soil and water samples, while the LODs of the method for tebuconazole in water samples presented by Huang are somewhat lower than that obtained in the present method.

4 Concluding remarks

It is concluded that the proposed method would have good selectivity in comparison with some of the most recent and efficient extraction techniques, such as an enantioselective enrichment, QuEChERS and μSPE. The results demonstrate that the proposed method can consume low amount of organic solvent and is fast in comparison with the traditional methods, as the contact surface area between solid or liquid sample and the liquid phase of the extraction solvent is much greater due to the occurrence of particle disruption, ease to operate and more efficient because ultrasound radiation was applied to accelerate the emulsification of the ethyl acetate in aqueous samples to enhance the extraction efficiency of tebuconazole without requiring extra partitioning or cleaning, and the use of capillary GC–NPD was a more sensitive detection technique for organonitrogen pesticides despite the application of selective detection principles for organonitrogen pesticides. The NPD was discovered with low detection limits and good selectivity by the observation that an alkali salt in the flame of the system enhanced the ionization of N and P containing compounds [32, 33]. Tebuconazole is a triazole group organonitrogen pesticides containing three nitrogen atoms in ring; it is polar in nature hence the separation of tebuconazole with a low-bleed mid-polar narrow-bore column, DB-35 (35% phenylmethylpolysiloxane stationary phase), and the use of capillary GC–NPD for the low-level detection of tebuconazole was selected. It has been proved that the proposed method provides a good sensitivity, reproducibility and recovery (see Table 1). The results demonstrate that the proposed technique is a viable alternative for determination of tebuconazole in complex samples.

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The authors have declared no conflict of interest.

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