

**RESIDUE LEVELS OF THIABENDAZOLE IN ORANGE
FRUITS AFTER ITS
APPLICATION AS A POST HARVEST FUNGICIDE**

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ABSTRACT

This study was conducted to estimate the exposure of the Kuwaitis population to pollution with thiabendazole [TBZ] residues from consuming orange fruits treated with TBZ as a post harvest fungicide. Residue levels of TBZ in some orange fruits that are popularly consumed in Kuwait was evaluated using an analytical procedures which involves ethyl acetate extraction for TBZ, followed by clean up using a solid phase extraction [SPE] cartridges consisting of anion exchange and primary / secondary amine bonded silica in tandem. TBZ residues were determined by a reversed phase high performance liquid chromatography with fluorescence detection [RP –HPLC – FL]. Recoveries through the method were satisfactory [above 90% with % RSD < 11%), Method detection limit [LOD] and quantification limit [LOQ] were 0.015 and 0.5 ug / g respectively. The developed method was applied to determine TBZ residues levels in orange fruits samples obtained from local market. Results showed that residues of TBZ were found in all of the orange fruit samples, and 20-30 % of these samples contain residues more than the maximum residue limits for TBZ [10 ug / g] set by Food and Agriculture Organization / World Health Organization [FAO / WHO] .

INTRODUCTION

Thiabendazole [2-(4-Thiazolyl) benzimidazole] is a post harvest systemic fungicide widely used to control diseases occurring during the storage, transportation and trading of fruits particularly citrus fruits and vegetables, (PMC ,1994). It is usually applied to orange fruits as a dip treatment combined with natural wax. It has been reported that a home made and commercial orange juice, highly consumed by children, may contain certain levels of TBZ residues (Dejonckheere et. al.,1996; Perret et. al.,2002 and Albero et al 2004). In this regard to protect the consumers from the harmful impact of TBZ residues in orange fruits, the maximum residue levels (MRL's) for it in Europe was (5mg TBZ/kg fruit), as reported by EC (1993) .In Japan tolerance level of 10mg/kg for TBZ in hole orange fruits has been established (Yammazaki and Ninimiyia, 1999 as) is the same of FAO/WHO (1993) . It is true that residues of TBZ in treated orange fruits and orange juice over the previously mentioned MRL levels constitute a hazard source for consumers . Therefore concerns about potential environmental contamination and the health hazardous to man in Kuwait from consumption of TBZ treated orange fruits has prompted periodical investigation in its residual levels (identification and qualification). High performance liquid chromatographic (HPLC) methods have been developed for determination the residue levels of TBZ in orange fruits and orange juices. A simple, sensitive and rapid methods for determination of TBZ residue levels in whole orange fruits based on extraction of TBZ from samples with ethyl acetate. and cleaned up by passing through tandam solid phase extraction (SPE) columns consisting of anion exchange and primary/secondary amine bonded silica (limit of detection, LOD = 0.1ug/g) and / or HPLC - FL (limit of detection LOD = 0.01ug/g) as reported by Gilvydis and Walters (1990), Muccio et.al. (1995), Iko et al (1998) and Yamazaki and Ninimiya (1999). The aim of this work was conducted to investigate the residue levels of thiabendazole in imported orange fruits.

MATERIALS AND METHODS

1. Apparatus:

- a. Solid phase extraction cartridges (SPE-cartridges): Bond Elut SAX, 500 mg and Bond Elut PSA 500 mg (Varian sample preparation products, Harbor City, CA, USA).
- b. SPE vacuum manifold: Vac Elut 24 (Varian sample preparation products, Harbor City, CA, USA).
- c. Filtration device for sample solution: A 10 ml syringe with luer – lock tip, fitted with nylon 13 mm diameter disposable filter unit, 0.45 µm por size. (Millipore, Bedford, MA, USA).
- d. Speed cutter: Model MK-K74 (Matushita, Osaka, Japan).
- e. Omni – Mixer Homogenizer : Sorvall (Norwalk, CT,USA).
- f. Liquid chromatograph : HPLC analysis were carried out on a Hewlett – Packard (HP) Model 1050 instrument consisting of an auto-sampler (set to inject 20 µl) a pumping unit, and a Hewlett Packard Model 1046 a spectra fluorometric detector. Chromatograms were recorded on Hewlett Packard model 3396 integrator.
- g. Filtration system under vacuum for filtration of mobile phase: Re-usable filtration system under vacuum consisting a glass filter holder (300ml), vacuum filter flask (1 liter) and vacuum pressure pump (Millipore, Bedford, USA)
- h. Filter for filtration the mobile Phase: Durapore membrane filter (Hydrophilic), Pore size 0.45 µm, 47 mm, HVLP 04700, (Millipore, Bedford, USA).
- i. Centrifuge – GP, Bechman (USA).

2. Chemicals and reagents:

a. Chemicals:

Water – HPLC grade was prepared from water purification system, Mili Q 2 plus (Millipore, Bedford, MA, USA). Resistivity of water must be >12 meg ohms-cm. Methanol HPLC grade (BDH, Poole, UK): Phosphoric acid 85% (BDH, Poole, UK), triethylamine 99% (Sigma – Aldrich, Germany) and sodium acetate anhydrous (BDH, Poole, UK) were of analytical reagent grade. Ethyl acetate and sodium anhydrous sulfate (Merck, Darm stadt, Germany) were of pesticide residue analysis grade. 1- decane sulfonate sodium salt

98% (Aldrich, USA). TBZ analytical reference standard (99%) was purchased from Sigma Chem. Co. (St Louis, USA).

b. Reagents:

- Ion pairing solution (4.1 mM): was prepared according to PAM (1994) as following: Pipet 7.0 ml of phosphoric acid into 200 ml. HPLC grade water and dissolve 1.0 g, 1-decane sulphonate sodium salt in this mixture. For this mixture pipet 10.0 ml of triethylamine and dilute to 1 liter with HPLC grade water and adjust the pH of solution to 2.5.
- TBZ standard stock solution. TBZ standard stock solution was prepared at 0.1mg/ml in methanol and stored at -18°C . Working standard solutions was made up with methanol. All working standard solution of TBZ were stored at 4°C .

3. Analytical procedures

a- Sample collection, preparation and extraction:

Orange fruit samples (Abo Sura and Valencia) were collected from the central market Shuwaikh, Kuwait. Ten samples, each sample consist 2 boxes (30 – 36 fruits / box) were collected from each type of the orange fruits Abosura and Valancia. From each box 10 orange fruits were randomly taken. Each whole orange fruit was sliced using a knife into 8 pieces from top to bottom. One slice from each fruit was collected and chopped well in speed cutter. Chopped orange samples must be store at -18°C in polyethylene bags if not used for extraction and analysis immediately. A 25g of chopped orange fruits was homogenized in homogenizer [Omni Mixer] for 10 min. with the presence of 50ml of ethyl acetate, 1g anhydrous sodium acetate and 20g anhydrous sodium sulfate. After centrifugation at 3000 rpm for 10 min, the supernatant was transferred to an 100 ml conical flask. The residual plug was extracted with another 20 ml of ethyl acetate added to the supernatant in the previous conical flask The ethyl acetate extract was evaporated to 1 ml in 25 ml round bottom flask in rotary vacuum evaporator at 40°C .

b- Purification of the extracts (Cleanup):

The resulting extracts from the extraction step were purified according to Yamazaki and Ninomyia (1999) with a little modification using a primary / secondary amine (PSA) Bond Elut PSA and Bond Elut SAX cartridges in tandem preconditioned with 20 ml of methanol followed by 20 ml ethyl acetate. The elute was evaporated carefully to

1 ml in a rotary evaporator at 40°C and adjust the volume to 2 ml or appropriate volume with methanol. Filter this solution through a filtration device for sample solution into 2 ml vials before HPLC – analysis. Purified extracts must be store at – 18°C if not used for analysis immediately.

c- HPLC – analysis:

Analysis of TBZ in purified extracts was done by HPLC – equipped with fluorescence detector (HPLC –FL) HP – Model 1050 according to Gilvydis and walter (1990) and Yamazaki and Ninomiya (1999). The separation of TBZ from plant matrix was performed on a Inertsil ODS2 (CI8) column (5µm, 250x4.6mm I.D.) (Chrompack, USA) with suitable guard column. The mobile phase was prepared by mixing manually 65 parts from ion pairing solution (see reagents) with 35 parts of methanol. Mix thoroughly and filter this solution through the filtration system under vacuum for simultaneously remove particulate matter and degas the mobile phase. More degas the mobile phase by ultra-sonification for 5 min. The flow rate of the mobile phase was 1 ml. / min. The fluorescence detector (FL) was operated at excitation wave length of 280nm and emission wave length of 310 nm.

d- Recovery experiments:

Recovery experiments were carried out. For that purpose a 25 g finally chopped untreated orange fruits samples (Blank) were fortified at rates of 0.01, 0.1, 0.5, 1, 5 and 10mg/g (5 replicates). TBZ was extracted from these samples purified and analyzed as described above.

e- Quantification:

For quantification of TBZ residues in orange fruits, a 20 µl from purified extract of sample was injected into HPLC – FL operated at conditions as described before. An external standard method was used for quantifying the TBZ residues using a peak areas of tested samples and the average response factor of the multi-level calibration curve within the linear range of the FL detector of HPLC. Calibration curve was obtained by plotting the absolute peak areas versus the concentration of standard calibration solution of TBZ in the orange, 0.01ug – 50 ug/ml.

f- Method sensitivity (limit of detection) (LOD) and limit of quantification (LOQ)According to PAM (1994)

The method sensitivity (limit of detection) (LOD) was determined by the conventional method (using the fortified orange fruit samples with TBZ and the above mentioned analytical procedures) in which the detection limit was calculated as the lowest concentration of TBZ which provides a peak height three times the average base line noise obtained from non – fortified sample of orange fruits (Blank). The limit of quantification [LOQ] was determined corresponding a value 10 times the back ground noise.

RESULTS AND DISCUSSION

1. Evaluation for the analytical procedures.

In this study the analytical procedures for extraction, clean up and analysis of TBZ residues in orange fruits were done according to the methods of PAM (1994), Ito et al. (1998) and Yamazaki and Ninomiya (1999). So, each analytical procedure was evaluated for its suitability. According to PAM (1994), the HPLC – FL system was tested for its suitability (system suitability test, SST). For that purpose the repeatability of HPLC – FL injection, (calculated as the relative standard deviation, %RSD, of 5 repetitive of HPLC – FL injection), the efficiency of the used column (N), and the asymmetry factor (As) for TBZ chromatographic peak were determined. These parameters were determined by analyzing a standard solution of TBZ at 1 ug/ml. The solution was injected 5 times into HPLC – FL operated under prescribed conditions and %RSD for the resulting peak areas and retention times were determined. Under the operated condition, %RSD values obtained for TBZ retention time and peak area were 1.5% and 1.8% respectively. In addition, N was found 1500 and As = 1.1, Therefore the repeatability achieved with the used HPLC – FL apparatus was satisfactory according to PAM (1994).

Quantification of TBZ was carried out via the FL detector with reference to an external standard of comparable concentrations in the range 0.01ug/ml – 50ug/ml (the calibration curve). The obtained calibration curve of TBZ was found linear over the concentration range studied with a correlation coefficient $r = 0.9997$. These results indicate that this HPLC – FL method is suitable for quantification of TBZ in orange fruit samples. The limits of detection (LOD) of the used method were determined by considering a value 3 times the background noise obtained for blank samples, whereas the limits of quantification LOQ were determined considering a value 10 times the background noise. The LOD for TBZ is 0.015 and the limit of quantification is 0.5mg/kg. To establish the performance of the used analytical procedures (extraction and clean up), untreated orange fruits (free from TBZ) were fortified at levels of 0.01, 0.1, 1, 5 and 10ug/g with TBZ analytical reference standard and processed according to the analytical procedures. Recoveries of TBZ and the corresponding relative standard deviation (%RSD) are listed in table 1. The average recoveries of TBZ were ranged 90 – 98% with %RSD < 10.8% in Abo Sura orange fruits and were ranged 93 – 99.2% with %RSD < 11% in Valencia orange. From all these previously data it can be concluded that the developed analytical procedures demonstrate satisfactory recoveries, low detection and quantification limits, good reproducibility and accuracy. Furthermore, the main feature of the method include a clean up for orange fruit extracts based on simple operation and use of disposal cartridges in tandem with substantial savings of glass wares, reagents and time compared with other methods (Gilvydis and walter, 1990; PAM ,1994; Muccio et. al., 1995 and Nakazato et. al., 1995). The clean up procedure is selective towards TBZ and virtually no interfering peaks were obtained at the retention time (17.5 min) of the analyte in the chromatograms of fortified orange fruits (fig.1). Also, as shown in fig.2, it is clearly that TBZ well separated from interfering sample co-extractives. All these favourable analytical features of the developed analytical procedures allowed its application for the determination of TBZ residue levels in orange fruit samples. In addition the developed analytical procedures can be used as a routine technique in monitoring programmes in Kuwait to determine low residue levels of TBZ in imported orange fruits.

Table- 1 Recovery (%) and %RSD of TBZ added to orange fruit samples at 5 different fortified levels.

Fortification Level Ug / g	<u>Abosura</u> % Recovery % RSD	<u>Valancia</u> % Recovery % RSD
0.01	90.0 7.5	93.0 9.0
0.1	92.3 8.5	95.2 11.0
1.0	95.1 5.5	96.3 8.5
5.0	98.0 10.5	98.3 7.5
10.0	97.0 10.8	99.2 6.0

- a. Each Value is the mean of 5 replicates
- b. Relative standard deviation.

2 – Residue levels of TBZ in Orange fruits :

The analytical procedures which have been developed were applied for the determination of TBZ residue levels in the imported orange fruits that are most popularly consumed in Kuwait, Abosura and Valancia. Ten samples from each type were collected from the central market of vegetables and fruits, Shuwaikh. As shown in table 2, residues of TBZ were found in all analyzed samples in the range 3,4 – 13.4 ug / g. Also results revealed that 30 % of the Abosura and 20 % of Valancia orange fruit samples contain TBZ residue levels exceeding the maximum residue limit [10ug /g] as established by FAO / WHO (1993). Results of some national monitoring programs which have been conducted on screening of pesticide residues in total diets showed that: In Kuwait, TBZ residues were found only in imported pears at maximum concentration of 0.63ug/g(Sawaya et. al., 1999). In USA, 60% of the analyzed samples of fruit juices were positive for pesticides, TBZ is included,(FDA, 1999). In Belgium, 49.1% of analyzed orange fruits samples were positive for TBZ residues with a maximum concentration of 3.45 µg/g,(Dejoncheere et.al.,1999).

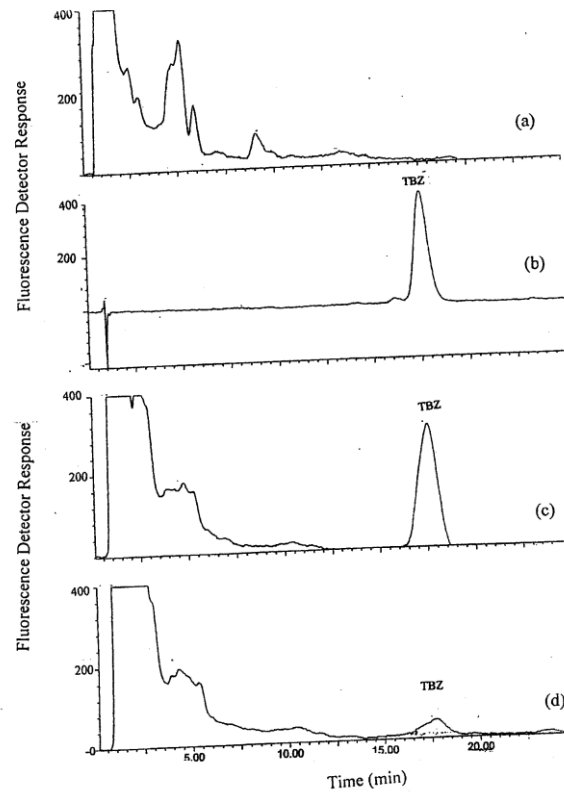


Figure : 1 - Typical HPLC- FL chromatograms of :
(a) – Extract blank orange fruits (free of TBZ) (25 ug / 2 ml).
(b) TBZ standard solution for LC at 10ug / ml
(c) Extract of orange fruits fortified with TBZ at 10 ug / g (25g / 2 ml)
(d) Extract of orange fruits fortified with TBZ at 0.1 ug / g (25g / 2 ml).

In the light of these data and our data, it can be concluded that, as a consequence of TBZ use as a post harvest fungicide on orange fruits, the presence of its residues in oranges is unavoidable. To keep residue levels of TBZ as low as possible a series of measures of Good Agricultural Practice (GAP) which includes: optimum dosage; safe interval between application and consumption of treated fruits, and number of applications must be applied. So, importing of orange fruits from countries applied these measures are acceptable. The presence of TBZ in some samples at levels over the FAO/WHO MRL (10ug/g), indicate that periodically monitoring programs for screening of pesticide residues (include TBZ residues) must be conducted in total diets of people in Kuwait in order to follow up the changes in the levels of these pesticide residues. In addition strengthening the capabilities of laboratories that are responsible for the day – to – day analyses of pesticide residues in food has become mandatory to protect the man and his environment in Kuwait from hazardous chemicals.

Table. 2 – Levels of TBZ [$\mu\text{g} / \text{g}$]* residues found in orange fruits using the proposed analytical procedures.

Sample No	$\mu\text{g} / \text{g} \pm \% \text{RSD}$	
	Abosura	Valancia
1	5.5. \pm 8.0	12.3 \pm 7.5
2	5.6 \pm 9.0	5.4 \pm 8.2
3	11.2 \pm 6.0	11.3 \pm 11.1
4	8.2 \pm 7.1	8.3 \pm 10.5
5	13.4 \pm 11.0	7.5 \pm 11.8
6	3.4 \pm 10.1	3.4 \pm 6.5
7	3.5 \pm 8.2	6.5 \pm 9.2
8	13.1 \pm 8.5	8.1 \pm 8.3
9	9.4 \pm 6.7	5.6 \pm 9.2
10	7.2 \pm 9.2	7.1 \pm 10.3

RSD = Relative standard deviation

* Each value is the mean of 3 replicates

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الملخص العربي

مستويات متبقيات مبيد الثيابندازول في ثمار البرتقال المعاملة بعد الحصاد بالمبيد الفطري

فيصل علي الشريقي ، علي محمد سبتي ، نايف المطيري ، مصطفى عبد العليم خليفة

تهدف هذه الدراسة إلى إلقاء الضوء على مستوى متبقيات مبيد ثيابندازول (TBZ) مبيد فطري يستخدم في معاملة ثمار البرتقال لحمايتها من التلف أثناء التخزين (في ثمار البرتقال المستوردة من الخارج والتي تستهلك في دولة الكويت لذا تم تطوير طريقة تحليل تشتمل على استخلاص المتبقيات بواسطة خلاص الإيثيل ethyl acetate متبوعا بالتنقية بواسطة استعمال SPE cartridges ، وتم تقدير كمية المتبقيات باستخدام جهاز (RP-HPLC-FL) ، وأوضحت النتائج أن كفاءة الطريقة مرضية حيث كانت نسبة الاسترجاع أكثر من 90 % وكانت قيم LOD ، LOQ المقدره هي 0.015 ، 0.5 ميكروجرام/جرام على التتابع . كما أوضحت نتائج تقدير المبيد (TBZ) على ثمار البرتقال المجموعة من السوق المحلي أن كل هذه الثمار تحتوي على متبقيات هذا المبيد ، وأن 20-30% من هذه العينات تحتوي على متبقيات المبيد بنسبة عالية عن الحد المسموح به دوليا (10 ميكروجرام/جم) والمقدرة بواسطة WHO/FAO .