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Araştırma Makalesi/Reserach Article

Verification of An Effective Method for Some Pesticide Residues Determination in Nectarine and Peach Samples

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Abstract

Method verification is an internal quality control procedure verifying the suitability of the method for specific purposes. All methods should be verified prior to analyses of real samples. LOQ, range of linearity, precision, accuracy and recovery criteria have to be taken into consideration while performing the method verification. This study was conducted to verify QuEChERS procedure for the analysis of boscalid, chlorpyrifos and tebuconazole in nectarine and peach. Samples spiked at 3 levels of 3 different pesticides. LC-MS/MS was used for chromatographic separation. Matrix calibration (MC) was used to compensate the matrix effect. QuEChERS method revealed that all pesticide LOQs were less than MRL. Recovered boscalid, chlorpyrifos and tebuconazole from matrices were recorded as 123.73% (RSD= 8.89%; n=52), 108.37% (RSD= 14.95%; n=52) and 110.34% (RSD= 15.22%; n=52), respectively. Overall recovery of the QuEChERS-AOAC 2007.01 method was 113.96 %, with RSD value of 14.52 % (n=156; SD=16.55). All recovery ranges were noted as 66.42-139.68%. These values were fit the SANTE recovery ranges (60–140%). MC curves 3 pesticides were linear (R^2 ≤0.999). Some other verification criteria, such as accuracy, linearity, precision and recovery were within the specified limits. According to the results of this study, the QuEChERS method is suitable for the analyses of chlorpyrifos, tebuconazole and boscalid in case of nectarine and peachs.

Keywords: Peach, Nectarine, Pesticide Residues, Method Verification, QuEChERS

Nektarin ve Şeftali Örneklerinde Bazı Pestisit Kalıntılarının Belirlenmesi İçin Etkin Bir Yöntemin Doğrulanması

Öz

Metot doğrulama, yöntemin belirli bir amaca uygunluğunu doğrulayan bir iç kalite kontrol prosedürüdür. Gerçek örneklerin analizinden önce tüm analiz metotları doğrulanmalıdır. Metot doğrulamasını gerçekleştirmek için LOQ, doğrusal aralık, kesinlik, doğruluk ve geri kazanım gibi kriterler dikkate alınmalıdır. Bu çalışma nektarin ve şeftalide boscalid, chlorpyrifos ve tebuconazole analizi için QuEChERS analiz prosedürünü doğrulamak amacıyla yapılmıştır. Örnekler 3 farklı seviyede 3 pestisitle spike edilmiştir. Kromatografik ayrıştırma LC-MS/MS ile yapılmıştır. Matris etkisini elimine etmek için matrisli kalibrasyon (MC) kullanılmıştır. QuEChERS metodu-LC-MS/MS analiz sistemi, pestisitlerin LOQ'larının MRL'nin altında olduğunu göstermiştir. Örnek matrislerinden boscalid, chlorpyrifos ve tebuconazole geri alımları, sırasıyla, 123.73% (RSD= %8.89, n=52), %108.37 (RSD= %14.95; n=52 ve %110.34 (RSD= %15.22%; n=52) olarak bulunmuştur. QuEChERS-AOAC 2007.01 methodunun tüm geri alımı, %14.52 RSD değeri ile, %113.96 (n=156; SD=16.55) olmuştur. Geri alım sınırları %66.42-139.68 arasında olmuştur. Bu değerler SANTE geri kazanım limitleri (%60–140) ile uyumludur. Mtrisli kalibrasyon (MC) eğrileri 3 pestisit için de doğrusal (R^2 ≤0.999) olarak bulunmuştur. Geri alım, kesinlik, doğruluk ve doğrusallık gibi bazı diğer yöntem doğrulama kriterleri gerekli aralıklarda bulunmuştur. Nektarin ve şeftali örneklerinde, chlorpyrifos, tebuconazole and boscalid kalıntılarının analizleri için QuEChERS yöntemi uygun bulunmuştur.

Anahtar Kelimeler: Şeftali, Nektarin, Pestisit Kalıntısı, Metot Doğrulama, QuEChERS

Introduction

Analytical procedure must be ready to detect residues of pesticide in food for the purpose of export/import certification, monitoring, field-application trials, risk assessment, verification of organic food and marketing to consumers. For whatever purpose, the methods must be robust, yield accurate results, meet LOQs, reliable and cover the scope of analytes and matrices (Krynitsky and Lehotay, 2002).

Method validation (also the method verification) is one of the quality requirement in pesticide residue analysis. The terms of validation and verification are offenly confused each other. The EUROCHEM Guide and SANTE Document define method validation as the process of defining an analytical requirement, and confirming that the method under consideration has capabilities consistent with what the application requires. Method verification is basically the process of confirmation, through provision of objective evidence, that specified requirements have been fulfilled. If a laboratory applies a standarized method or prevalidated method into its condition without any change in the procedure, the laboratory just needs to verify that it can perform the method by meeting the method performance criteria. The extend of verification study is less than what is needed for validation study. If the original method was not changed throughout the study, the term of "*method verification*" is more appropriate than "*method validation*" (Omeroğlu et al., 2012; EUROCHEM, 2014; Magnusson and Örnemark, 2014; SANTE, 2019).

The method verification process has a direct efffect on quality of analytical results. Though a verification cannot rule out all possible problems, they should address the most common ones. All methods must be verified prior to analyses of real sample matrix. Verification is carried out to show the method fitness for the specified goal, which means that the analytes and food samples were evaluated to reach LOQs, acceptable recovery and reproducibility. Verification is also necessity of OECD-GLP and ISO 17025 quality systems. Verification studies are carried out with the following parameters: accuracy, bias, detection limit, linearity, precision (repeatability and reproducibility), robustness, ruggedness, scope, selectivity, sensitivity and specifity. These criteria cannot be copied from any literature and laboratory. The method is acceptable when the criteria were fit withe specified limit. Method validation/verification is necessary; (a) when there is a significant change (new equipment and relocation of instrument, (b) when new updated software is uploaded to the instrument, (c) when warning is received from the quality control unit that the performance of the method changes over time, (d) when it is necessary to show that the same results are obtained with the existing method and the newly developed method (Green, 1996; Tiryaki, 2016; EURACHEM/CITAC, 2000).

The most common methods developed in 1960s and 1970s (Mills et al., 1963; Luke et al., 1975; Specht and Tilkes, 1995), use large amount of solvent and thus they are costly and produce a lot of waste in each sample. Anastassiades et al. (2003) developed a more cost-effective method named QuEChERS (quick, easy, cheap, effective, rugged and safe), a simple procedure for the pesticide residue analyses of in agrcultural commodity. This method was very successful in the analyses and suitable for multi-residue methods. Then the QuEChERS procedure was modified using citrate buffers or acetate to tackle some trouble pesticides (Lehotay et al., 2005; Pay et al., 2007). With joint work, these two changes became AOAC International Official Method 2007.01 (Lehotay, 2007) and standard method EN (European Norm) 15662 (Mastovska et al., 2010), respectively.

Recovery limits and their RSDs are described in the EU SANCO 2004 and 2006 document depending on the amount of analyte in the samples or the fortification levels (Table 1). Lower recovery range is acceptable as the analyte concentration decreases. However, this is shown as a single limit for recovery and RSD values as 60-140% and ≤ 20%, respectively in SANCO/SANTE documents published in 2007 and after.

Table 1. Recovery limits and RSD values based on analyte concentrations and/or spiking levels

Concentration range (µg/kg)	RSD (%)	Mean recovery range (%)
≤ 1	35	50-120
> 1 ≤ 10	30	60-120
> 10 ≤ 100	20	70-120
> 100 ≤1000	15	70-110
> 1000	10	70-110

Source: Anonymous 1999; SANCO, 2004.

Anastassiades et al. (2003) applied the QuEChERS method on different pesticides and found the recovery between 85-101% and the RSD value less than 5%. Dashtbozorgi et al. (2013) optimized the QuEChERS procedure for the residue analysis of 19 pesticides in cucumber and tomato. Overall method recovery was found to be 86-104% with an RSD of 12%. As a result, the method developed for the cucumber and tomato product was found to be effective.

A number of studies have been performed on method verification with several pesticides and food commodities. The QuEChERS method verified; for the analyses of chlorpyrifos, dimethoate, indoxacarb and imidacloprid residues in apple (Tiryaki, 2016), for the analyses of chlorpyrifos, acetamiprid, pirimiphos methyl and formetanate HCl residues in cucumber (Çatak and Tiryaki, 2020), for the analyses of chlorpyrifos, acetamiprid and formetanate HCl residues in Capia peper (Polat and Tiryaki, 2020) and for the analyses of emamectin-benzoate, penconazole and imidacloprid in tomato (Polat and Tiryaki, 2019). The overall recoveries of methods in these 4 studies were 90.2%, 97.7%, 104.9% and 107.1%; respectively.

Nectarine and peach and are significant fruits for Turkey for both export and consumption of our own people. In 2020, nectarine and peach were produced 162,244 and 729,804 tones, respectively. 40,370 and 138,102 tonnes of these productions for nectarine and peach, respectively, belongs to Çanakkale Province (TSI, 2021). A lot of pesticides is used by farmers against the pest. Therefore, the importance of pesticide residues emerges. Thus, it is necessary to work with verified residue analtical methods by using local peach and nectarine matrices. The reliability of the data has a significant role in pesticide residue analyses. This study findings will provide important results for the next studies

This work focused to verify a reliable and cheap method for the analysis of some pesticides in nectarine and peach. Spiked fruit samples were subjected to QuEChERS AOAC 2007.01 analytical method, followed by determination using LC-MS/MS. The analytical data reliability and method verification were checked by SANTE parameters (SANTE, 2019).

Materials and Methods

Chemicals and reagents

Tebuconazole (99.9% purity), boscalid (99.0% purity) and chlorpyrifos (99.0% purity) were purchased from Dr. Ehrenstorfer Laboratories. Acetonitrile (ACN), magnesium sulfate hepta hydrate ($MgSO_4*7H_2O$) and sodium acetate (NaAC were purchased from Merck, with the purities of 99.0%, 99.5% and 99.0% , respectively.Primary Secondary Amine (PSA, particle size of 40 µm) was purchased from Agilent.

Standards and solutions

Boscalid, chlorpyrifos and tebuconazole working solutions (1.0 mg/ml were prepared with ACN from stock solution (400 mg/ml) of 3 pesticides. Matrix-matched solution of pesticide were used to eliminate matrix effect (Kanrar et al., 2010). Matrix-matched calibration (MC) for tebuconazole, chlorpyrifos and boscalid were prepared in ACN with the ranges of 1-200 ng/ml, 2-400 ng/ml and 1-200 ng/ml, respectively. According to CODEX_040e (CAC, 2003) and SANTE Guidelines (SANTE, 2019), represenative sample MC was used for quantifications and for elimination of matrix effect. Therefore, representing Class II (low or no chlorophyll content and high water content), apple was used instead of nectarine and peach matrices. Fortification solutions for 3 pesticides representing to 0.1, 1.0 and 10.0 x MRL were prepared in ACN.

Instrumentation

LC-MS/MS (Waters I Class Plus UPLC + Xevo TQ-S micro MS Detector; ESI + mode) device was used for chromatographic analyses. An acquity UPLC BEH C_{18} column (1.7 µm, 2.1 x 100 mm) was connected to LC-MS/MS. Flow rate, total run time and injection volume were 0.35 ml/ min 15 min, and 1 µl, respectively. A gradient program consisting of 10 mM $NH_4CH_3CO_2$ in methanol (B) and 10 mM $NH_4CH_3CO_2$ in water pH 5 (A) was used. The other glasswares and equipments used in the study were balance, centrifuge, vortex Waring blender, GC vials and centrifuge tubes (50- ml).

Fortification experiments (recovery test)

Recovery is the most important parameter of method verification. In general recovery studies are carried out 5 spiked samples at LOQ (lowest spike level) and 5 spiked samples at 2-10 x LOQ to meet the identification and method performance criteria for precision and recovery (SANTE 2019).

Another critical point is spiking levels must cover wide residue ranges including MRL level. So, we spiked at 3 MRL level in triplicate (3 anaytical porsion). Considering 3 GC vial for each analytical porsion, it means 27 recovery data. About 1 kg (EC, 2002) pesticide free (blank) peach and nectarine samples were homogenized in a Waring blender. 15 g (analytical portion) well homogenized sample fortified with 100 µl fortification solution at desired concentration, equal to 0.1, 1.0 and 10.0 x MRL levels (Table 2). Spiked sample tube was vortexed for 30 s and left for 15 min to interact with the matrix. Extraction and clean-up procedures were followed below explained. Analytical procedure was verified with the SANTE requirements (SANTE, 2019). Linearity of the method was also tested with the range of 2-400 ng/ml (for chlorpyrifos) and 1-200 ng/ml (for boscalid and teboconazole). Recoveries were calculated with Equation 1 (Çatak and Tiryaki, 2020).

$$Recovery = \frac{Measured\ concentration, \mu g/kg}{Spiked\ concentration, \mu g/kg} x100 \qquad (1)$$

Table 2. Spiking parameters for three pesticides

Spike	Code	Spiking level (µg/kg)		
		Boscalid	Chlorpyrifos	Tebuconazole
0.1 x MRL	F1/1-3	500	8	60
1.0 x MRL	F2/1-3	5000	80	600
10.0 x MRL	F3/1-3	50000	800	6000
Control	F0/1-3	-	-	-

Extraction and clean-up

The QuEChERS AOAC 2007.01 method described by Lehotay (2007) was used in this study. 15 ml of ACN containing 1% acetic acid was added to the spiked samples, and sample was vortexed vigorously for 1 min. 12.3 g magnesium sulfate hepta hydrate (equal to 6 g $MgSO_4$) and 1.5 g NaAC were added to sample. Then, the mixture was immediately mixed for 1 min by Vortex to prevent conglomerate of $MgSO_4$. The mix centrifuged at 5000 rpm for 5 min. The clean-up of the procedure was performed by transferring 8 ml aliquot of upper ACN layer into 50-ml tube containing clean-up reagents [(50 mg PSA + 307 mg magnesium sulfate hepta hydrate) / per ml extract]. Tube was capped tightly and mixed by Vortex for 30 s. The extracted material was centrifuged at 6000 rpm for 3 min. Later on 1000 µl of cleaned extract was transferred to vial in triplicate for LC/MS detection. All analytical steps of the method summarized in Figure 1.

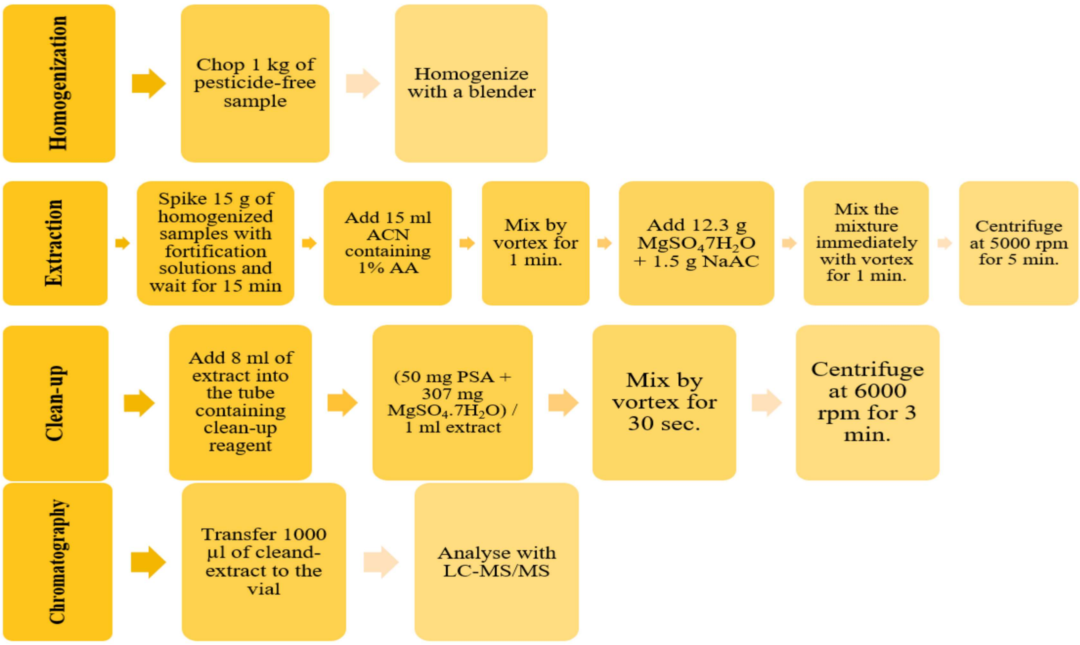

Figure 1. Analytical procedure of QuEChERS AOAC 2007.01 method

Results and Discussion

Method reliabilty studies

Linearity

The pesticide retention times (tR, min) in the extract must comply with the calibration standard (±0.1 min) (Brankovi et al., 2019). The tR of pesticides were 8.75 min, 9.77 min and 10.94 min for boscalid, teboconazole and chlorpyrifos, respectively. Linearity was evaluated by using represantative apple MC. Calibration range, analytical function and correlation coefficient of 5-point calibration curve were presented in Table 3. The matrix-matched calibration curve of boscalid, chlorpyrifos and tebuconazole were linear with the indicated ranges. Correlation co-efficient (R^2) for 3 analyte were $\geq$ 0.999. Tiryaki et al. (2008) indicated that regression equation in MCs is known analytical functions and they were used for analytes quantification.

Table 3. Calibration details of three analyte with 5-level MC.

Pesticide	Calibration range (ng/ml)	Analytic function (Linear equation)	Correlation coefficient (R^2)
Boscalid	1-200	$y = 5.5914\ x^2 + 1295x - 910.30$	0.999955
Chlorpyrifos	2-400	$y = 0.0394814x^2 + 1866x + 125.58$	0.999831
Tebuconazole	1-200	$y = 227797x^2 + 49156.5x + 1405.04$	0.999985

Limit of quantification (LOQ)

Limit of detection is the minimum quantity of the analyte that detectable with acceptable certainty (SANTE, 2019). MRL values and identified LOQs are shown in Table 4 for three pesticide and peach and nectarine. All LOQs were smaller than MRL values.

Table 4. Identified LOQ values from LC-MS/MS system and EU-MRL values for peach and nectarines

Pesticide	LOQ (µg/kg)	EU-MRL (µg/kg)
Boscalid	1	5000
Chlorpyrifos	2	80
Tebuconazole	1	600

Precision and trueness

Repeatability (RSD %) was used as a tool to evaluate precision and recovery (accuracyQ %) was used as a tool accuracy of the method (EURACHEM, 2014; SANTE, 2019).

Precision assessment (the closeness of repeated analyses), were performed for the within laboratory repeatability of the analyses. The recovery is the ratio of found residue to the spiking level of pesticide (Eq. 1). Since MRL values and spike levels for peach and nectarine matrices were the same, recovery values were evaluated by combining them. The mean recoveries and their RSD and SD values for each of the 3 spike levels of all 3 pesticides are provided in Table 5. The lowest recovery and highest recovery for matrices (for both nectarine and peach) were determined as 87.69% and 139.68%, 66.42% and 139.25.0% and 80.00% and 136.55% for the boscalid, chlprpyrifos and tebuconazole, respectively. Overall recovered boscalid, chlorpyrifos and tebuconazole from matrices were 123.73% (SD=11; RSD= 8.89%; n=52), 108.37% (SD=16.21; RSD= 14.95; n=52) and 110.34% (SD=16.79 ; RSD= 15.22%; n=52), respectively. Overall recovery of the QuEChERS-AOAC 2007.01 method (accuracy) was 113.96 %, with the RSD value of 14.52 % (n=156; SD=16.55). All recovery ranges were 66.42-139.68%.

In a previous study, mean recovery of acetamiprid, azoxystrobin and thiacloprid in peach were 98.6%, 95.3% and 80.6%, respectively (Galietta et al., 2011). Findings of present study were fit with the indicated mean recovery ranges (60–140%) and repeatabilities (RSD $\leq$ 20%) (SANTE, 2019). These results also showed that QuEChERS AOAC 2007.01 method may present an accurate and rapid procedure to analyse residue of pesticides in nectarine and peaches.

Table 5. The results of method verification study

	Boscalid			Chlorpyrifos			Tebuconazole		
Fortification, µg/kg	500	5000	50000	8	80	800	60	600	6000
Measured, µg/kg	631.56	6495.87	57824.68	10.03	82.67	763.78	76.70	672.88	5402.32
Mean recovery	126.31	129.92	115.65	125.35	103.33	95.47	127.83	112.15	90.04
SD	6.40	4.58	13.84	8.34	3.92	15.12	5.97	4.53	7.64
RSD (%)	5.07	3.53	11.96	6.65	3.79	15.83	4.67	4.04	8.48
n	18	16	18	18	16	18	18	16	18
Recovery range %	87.69-139.68			66.42-139.25			80.00-136.55		
Overall recovery	123.73			108.37			110.34		
SD	11.00			16.21			16.79		
RSD (%)	8.89			14.95			15.22		
n	52			52			52		
Overall recovery of the QuEChERS-AOAC 2007.01 method (accuracy): 113.96 % (n=156; SD=16.55; RSD%=14.52)									

Conclusion

All pesticide residue analytical methods should be verified prior to analyse of real sample matrix. Even if any method used in modern laboratory, verification is a requirement for internal quality control. In present study, performed with boscalid, chlorpyrifos, and tebuconazole pesticides and nectarine and peach matrices, verification parameters viz. accuracy, linearity, precision and recovery were found to be within the specified limits. The method verification showed high performance in terms of accuracy precision and recovery. Consequently, it is concluded that the aims of present study were met through QuEChERS AOAC 2007.01.

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Authors' Contributions

Both authors have participated sufficiently in the work and take responsibility for the content.

Conflicts of Interest Statement

The authors declare no competing interests.

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