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Development of a gas chromatographic method for the determination of Chlorpyrifos and its metabolite Chlorpyrifos-oxon in wine matrix

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ABSTRACT

A reliable method has been developed for the determination of Chlorpyrifos (CP) and its metabolite Chlorpyrifos-oxon (CPO) in wine sample using pulsed splitless technique coupled with gas chromatography by using electron capture detector. In this study, a quick, easy and cheap sample preparation method (QuEChERS) based on liquid extraction with acetonitrile, followed by dispersive solid phase extraction using primary secondary amine was tested for the separation and quantification of CP and CPO in wine samples. The accuracy of the developed method was tested upon recovery studies and it was calculated as $(92.3 \pm 18.2)\%$ for CP and $(96.6 \pm 16.1)\%$ for CPO. LOD and LOQ values of CP were found as 0.04 and 0.15 ng/mL and 0.49 and 1.62 ng/mL for CPO respectively. By using the pulsed splitless injection mode, the sensitivity of the determination of CP and its metabolite CPO in wine samples was improved compared to splitless technique. CP content of analyzed wine sample was found as 2.05 ± 0.15 ng/mL with a RSD of 7.6% and CPO content was found as 4.99 ± 0.15 ng/mL with a RSD of 3.0% ($n = 3$). The expanded measurement uncertainties were calculated as 17% and 6% for CP and CPO, respectively.

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1. Introduction

Chlorpyrifos (CP) is an organophosphorus pesticide defined as an endocrine disruptor [1,2] that has been used extensively on grape to control various pests especially *Lobesia botrana* [3]. A wide range of noxious effects of the CP on humans have been described. It causes tens of thousands of deaths per year world wide and is a pro-poison that requires metabolic activation to become a potent phosphorylating agent, namely Chlorpyrifos-oxon (CPO) [4].

Many organophosphates readily undergo conversion from thion (P=S) to oxon (P=O) species. This reaction occurs in the environment under the influence of oxygen and light and in the body primarily by the action of liver microsomes [5,6]. This reaction also occurs for CP and transformation product CPO and this compound is about 3000 times more potent than CP in its inhibition of acetylcholinesterase, which leads to neurotoxicity [7]. CP is metabolized by oxidative desulfuration to oxon analog and the parental and oxon form is hydrolyzed to trichloropyridinol (TCP). Conversion scheme of CP to CPO is given in Fig. 1.

In many routine food laboratories, the analytical studies focus on to actual pesticide compounds rather than their metabolites. However, the toxicological properties of some pesticides could remain in their metabolites and therefore, it is necessary to design efficient strategies for the investigation of pesticide metabolite [8]. These metabolites can also be produced as a result of pre-treatment during sample preparation step [9,10].

Wine can be considered one of the most consumed drinks in the world. Recently, monitoring of the pesticides and their metabolites is an important parameter in the quality control of wines [11–13]. Considering the wine making process that includes a desulfurization step, the oxon derivative of CP, which is more toxic than the actual compound, could be formed and then, transferred into the wine [6].

A few numbers of analytical methods for the determination of residues of CP and its main metabolite CPO simultaneously in various matrices have been developed previously. These methods involved extraction of the compounds with appropriate solvents, cleanup of the extract and subsequent determination by chromatographic and immunoassay methods. Simultaneous determination of those analytes were done by using gas chromatography (GC) in milk and cream samples by flame photometric (FPD) [14], in tomatoes and green beans by pulse flame photometric (PFPD) [15], in apple juice by mass spectrometric (MS) [16], in air and fog samples by nitrogen phosphorus (NP) detection [17], in elm bark and soil by electron capture (ECD) detection [18,19]. CP and CPO have been determined especially in clinical samples using liquid chromatographic techniques. These compounds were analyzed in mussels by using diode array detector (DAD) [20], in rat plasma and urine by UV detector [21,22], in urine [23] and rat brain tissue samples [7] by MS detector. An alternative to chromatographic methods enzyme

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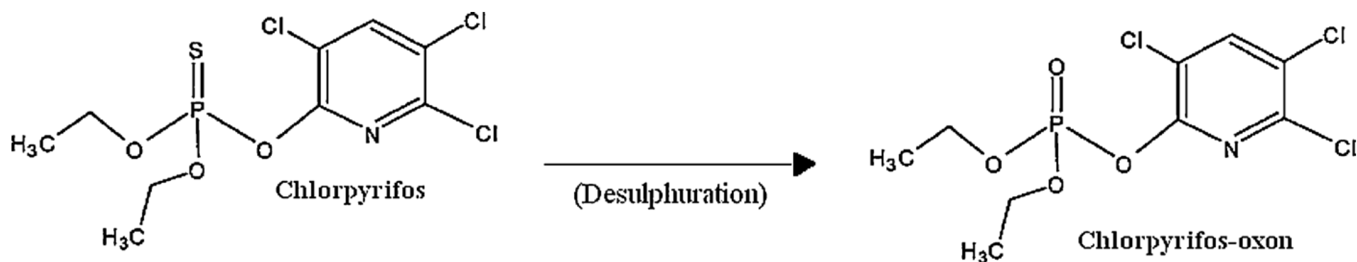

Fig. 1. Conversion scheme of CP to CPO.

based sensors [24,25] has also been applied to the detection of CP and its oxon metabolite.

Classical liquid or gas chromatographic analyses are very sensitive and standardized techniques. Nevertheless, they often are excessively laborious and time-consuming [26]. Literature survey revealed that the methods mostly differ in the sample preparation step prior to the analysis. In recent years simplification and increasing automation of sample preparation steps are one of the modern trends in analytical chemistry. Quick, easy, cheap, rugged and safe (QuEChERS) method is a sample preparation technique for pesticide multiresidue analysis that has already been widely accepted by the international community of pesticide residue analysts [27,28].For pesticides in wine no uniform limits have been established yet, except for procymidone for which the European Union has established maximum residue limit (MRL) of 0.5 mg/kg [29]. MRL value for CP in wine grape has been given as 0.5 mg/kg for CP [30]. The MRL requires very sensitive methods for pesticide determination in wine. Recent developments in Turkish wine sector demand reliable and accurate analysis methods for pesticide residue to meet the basic requirements to compete in world market. Therefore, the determination of pesticides residue levels with their metabolites is of special concern to ensure the safety consumption of wine products.

This study is the first attempt that deals with QuEChERS sample preparation and gas chromatography with ECD technique by pulsed splitless injection to determine CP and its CPO metabolite in wine samples. The validation of the methods described have been carried out according to European norms EN-45000 [31,32], keeping in mind in the following criteria: specificity, limit of detection (LOD), limit of quantification (LOQ), accuracy (recovery from 70 to 100%), precision (repeatability, RSD < 20%), sensitivity (linearity, $r > 0.99$) and practicality (cost, complexity, etc.).

In the method validation procedures, the estimation of the uncertainty is one of the main focuses of interest especially for the ISO standard 17025 [33] due to its importance in showing the data quality. Therefore an estimation of the measurement uncertainty (MU) of the analytical results was also performed in this study in order to reduce the effect of uncertainty sources of critical stages of the analytical method on overall MU budget.

2. Experimental

2.1. Chemicals and reagents

Pesticide certified standards of Chlorpyrifos (O,O′-diethyl O-[3,5,6-trichloro-2-pyridyl] phosphorothioate) and Chlorpyrifos-oxon (O,O′-diethyl O-[3,5,6-trichloro-2-pyridyl] phosphate) were purchased by Labor Dr Ehrenstrofer-Schafers (Augsburg, Germany). A standard mix (10 mg/L) was prepared by dissolving the selected compounds in acetone obtained from Lab Scan (Sowinskiego, Poland), and stored in the dark at 4 °C. More diluted solutions were prepared just before use.

Reagent grade anhydrous sodium chloride, magnesium sulfate, sodium hydroxide were purchased from Fluka (Steinheim, Germany). Sodium citrate and sodium citrate sesquihydrate were also obtained from J.T Beaker Primary secondary amine (PSA) bonded silica (40 μm Bulk) was obtained from Supelco (Bellefonte, PA, USA). The sorbent was used as 25 mg/mL of sample extract.

The red wine sample was taken from a local supermarket. It has an alcohol percentage of 11–12% and it is from grapes of a local village (September 2004). The measurements were made at ambient laboratory temperature and all the solutions were allowed to attain this temperature prior to measurement.

2.2. Instrumentation

GC analyses were performed with a gas chromatograph from Hewlett-Packard (HP) Model 6890 Series, equipped with HP 7673A auto sampler and HP ECD detector systems. A DB-1 fused silica column (30 m × 0.32 mm I.D. and film thickness 0.25 μm) was used. The split–splitless injector and ECD detector were operated at 250 and 300 °C, respectively. The sample (3 μL) was injected in the pulsed-splitless mode with a pressure of 100 psi and the oven temperature program was started as follows: 70 °C for 2 min, rising to 150 °C by a ramp of 25 °C/min then rising to 200 °C by a ramp of 3 °C/min finally 8 °C/min ramp was applied until 280 °C, held for 10 min. The same program was used in the splitless injection mode. Flow rate was 1 mL/min.

Centrifugation was performed by using Nüve NF 800 centrifuge and stirring of the samples was carried out using an IKA basic vibratory stirrer (IKA Labortechnik, HS501 digital).

2.3. Sample preparation procedure

The sample (10.0 g) was weighed into a 50.0 mL Teflon centrifuge tube. For the standard addition and matrix-matched procedures, the sample was spiked with certain volume of mixed standard solution. Acetonitrile (10.0 mL) was then added and the sample was vortexed for 1 min. In the next step, anhydrous $MgSO_4$ (4.0 g), sodium citrate (1.0 g), anhydrous NaCl (1.0 g) and sodium citrate sesquihydrate (0.5 g) were added and the sample was homogenized immediately for 2 min with a vortex mixer. The pH was made 7.0 by dropwise addition of NaOH (20%) solution. The content of the tube is then centrifuged at 3000 rpm for 5 min and 6 mL of organic phase was transferred to a tube containing 0.15 g PSA + 0.9 g anhydrous $MgSO_4$. The extract was centrifuged and finally 1 mL of it was placed into the GC vial to carry out the chromatographic analysis.

2.4. Estimation of uncertainty calculations

The uncertainty of the measurement obtained by the proposed method was estimated using the bottom-up approach on the basis of in-house validation data [34].

The amount of CP and CPO residues in wine W_R is calculated by using Eq. (1):

$$W_R = \frac{C_A \cdot V_{End}}{M_{Sample} Rec} \quad (1)$$

where C_A is the concentration of the identified analyte in the sample solution; V_{End} is the volume of the final sample solution, in mL; M_{Sample} is the sample mass, in g; Rec is the recovery; the relevant uncertainty sources are shown in the cause and effect diagram in Fig. 2. The concentration results of pesticides were affected by these sources.

The overall combined uncertainty of the developed analytical method can be estimated from the general expression can be calculated using Eq. (2).

$$\frac{U(W_R)}{W_R} = \sqrt{\left[\frac{U(V_{End})}{V_{End}}\right]^2 + \left[\frac{U(M_{Sample})}{M_{Sample}}\right]^2 + \left[\frac{U(C_A)}{C_A}\right]^2 + \left[\frac{U(Rec)}{Rec}\right]^2 + U(repeat)^2} \quad (2)$$

3. Results and discussion

3.1. Calibration studies

Since the CP molecule can be determined sensitively by using ECD, this detector was the choice for the analysis of this pesticide in grape and wine samples [35–38]. The calibration studies were conducted by injecting the standard solutions containing both CP and CPO into the column to determine dynamic ranges, limit of detection and limit of quantification of the GC-ECD method. Table 1 summarizes calibration data obtained by injection of the standard solutions in splitless and pulsed splitless injection modes.

In splitless injection mode, the calibration curves display a good linearity for both compounds. It was revealed that, LOD value for CP (0.05 ng/mL) is much lower than that of CPO (35.0 ng/mL). According to these results, CP can be sensitively determined but CPO cannot be detected since the concentration levels expected will be far below the sensitivity levels. A well formed and distinctive peak for CPO can only be observed for concentrations higher than 50 ng/mL.

This lack of sensitivity for the oxon metabolite compared to the parent molecule can be attributed to the structural alteration, mainly the electronegative groups within the CPO molecule. For improving the sensitivity, injection volume was increased to 3 μL and by this means 20 ng/mL of CP sample was injected to the column. In comparison to normal splitless technique, large volume injection gave more sensitive results as expected.

Another technique used in such cases is pulsed splitless technique where the sample was transferred to the column very fast. In this manner, decomposition of the analyte was prevented and large volume injection can be done. After injection, the column flow rate is automatically reduced to normal values for chromatographic analysis.

The same concentration of CP (20 ng/mL) was injected to GC system by using pulsed splitless mode. Fig. 3 shows the comparison of the two CP signals by using splitless mode alone and pulsed splitless mode.

As can be seen from the chromatogram, the response of the CP signal was improved by using pulsed splitless technique. In this technique, a pressure was applied to the sample in the injection port. For optimizing the main parameters, the effect of pressure was also studied. The dependence of response factor of CPO (200 ng/mL) onto the injection pressure was studied for 5, 10, 20, 50 and 100 psi pressures and best results were obtained with 100 psi. Therefore, further calibration studies were carried out by using this pressure and injection volume of 3 μL.

Working standard concentrations ranging from 100 to 300 ng/mL of pure CPO metabolite were prepared by using consecutive dilution in acetone. Fig. 4 shows the comparison of overlaid chromatograms of CPO obtained by using splitless and pulsed splitless technique.

As can be seen clearly from the chromatogram, sufficiently well resolved single peaks were obtained and the analytical signal of CPO was improved by using pulsed splitless technique. Analytical characteristics data obtained with pulsed splitless injection method were given in Table 1.

The sensitivity of the method was assessed by calculating LOD and LOQ values which are given in Table 1. LOD, for each compound was defined as the lowest concentration to exceed the mean baseline value of blank wine sample prepared by QuEChERS method by three times as signal/noise ratio. LOQ, was evaluated as signal-to-noise ratio (S/N) equal to 10. By using this pulsed-splitless injection mode, the sensitivity of the determination of CP and its metabolite CPO in wine samples was improved compared to splitless technique. According to results given in Table 1, the most sensitive measurements were made in this work compared to the literature data's. RSD values of the analysis were obtained between 1–12% ($n = 3$) for CP and 0.3–19% ($n = 3$) for CPO.

3.2. Recovery study

The use of matrix-matched calibration solutions is a common approach that is used to circumvent errors associated with matrix-induced enhancement or suppression effects in GC. The method was tested upon spiking of wine sample (as a blank) with a mix standard solution to be 5 ng/mL of CP and 50 ng/mL of CPO. The accuracy of each sample preparation was, then, assessed comparing the concentration level found from standard added sample with that of blank sample. The recovery assays were replicated three times.

The extraction procedure described above was used to determine the average recoveries of CP and CPO from wine samples. The values found show that the extraction method is efficient in extracting the residues of both compounds from wine matrix, since the average recoveries of CP and CPO was 92.3 ± 18.2% (at 5 ng/mL level) and 96.6 ± 16.1% (at 50 ng/mL level) respectively.

3.3. Quantitative analysis of wine sample prepared by QuEChERS method

When high metabolite concentrations are found in the sample, the analysis method accompanying by a number of different separation techniques can be conducted to provide more accurate results for analyte of interest.

Lehotay et al. demonstrated the effectiveness of QuEChERS method for more than 200 pesticides in food matrixes [28]. This method is quick, effective, cheap and robust. Jiang et al. have also analyzed pesticide residues of wine sample by using this method with MS detector [39]. Since, carrying out the detection of selected analyte (CP and CPO) in our study within red wine sample by means of ECD may serve more selective and sensitive way of determination option particularly in cases where LOD level of pesticides set by restrictive regulation and any doubt created by Type II error. As a result, the modified method offers a reliable, an accurate and reproducible way of determination which can be exploited for the rapid requirement of quality control process for wine samples.

Sample preparation procedure of wine sample by QuEChERS method was given in Section 2. Standard addition method was used in the analysis. QuEChERS sample preparation technique was

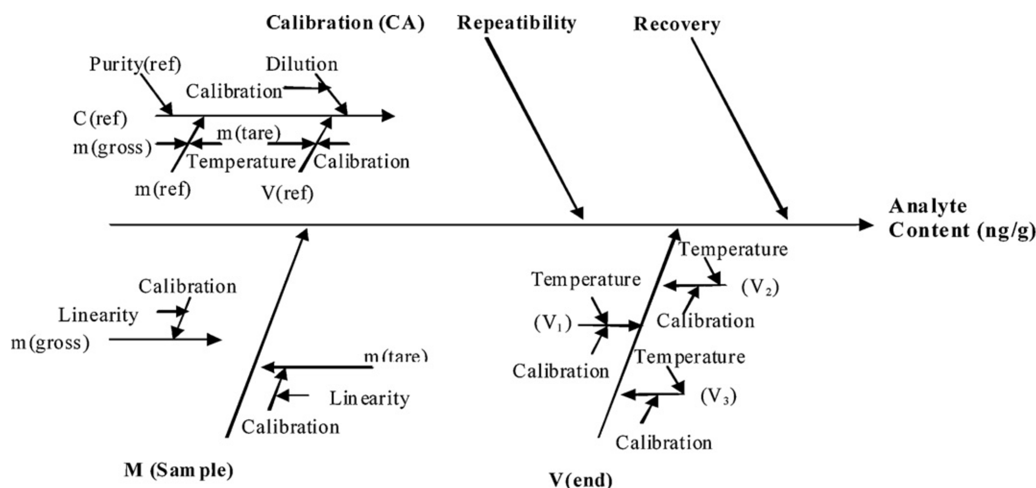

Fig. 2. Cause and effect diagram for the determination of CP and CPO in wine samples.

Table 1
Analytical characteristics data obtained with splitless and pulsed splitless injection methods.

Parameter	Splitless		Pulsed splitless	
	CP	CPO	CP	CPO
t_R (min)	21.4	20.9	21.4	20.9
Linear working range (ng/mL)	0.20–250.0	150.0–300.0	0.2–300.0	5.0–300.0
Linear equation	26.712 × +26.4	0.390 × −12.6	65.245 × +431.4	28.896 × −1143.6
R^2	0.9996	0.9996	0.9990	0.9990
LOD (ng/mL)	0.05	35.00	0.04	0.49
LOQ (ng/mL)	0.17	115.50	0.15	1.62

employed for the blank samples and for the standard spiked samples. 10, 100 and 200 μL of 10 ng/mL of CP standard solution and 2, 4 and 8 μL of 724 ng/mL CPO was added to 10.0 g of wine sample. CP content of the analyzed wine sample was found as 2.05 ± 0.15 ng/mL with a RSD of 7.6% (n = 3) and CPO content was found as 4.99 ± 0.15 ng/mL with a RSD of 3.0% (n = 3). Standard addition chromatograms of CPO and CP and the standard addition calibration curves can be seen in Fig. 5.

As can be seen from results above, pesticide residues in wine samples can be characterized and determined sensitively via this manner. The concentration levels found for CP pesticide residue was below the levels (0.50 mg/kg) established by the Turkish Food Codex and the Codex Alimentarius Maximum Residue Levels (MRLs) for grapes, thus, causes no problems in terms of food safety for CP in wine. But no uniform MRL have been established for CPO metabolite in wine.

3.4. Estimation of the uncertainty

3.4.1. Estimation of the uncertainty derived from the volume of the final sample $u(V_{End})$

The volumetric calibration standard uncertainty of 10 mL of the pipette u(cal), is calculated from the manufacture's specification within the range of ±0.0014 mL. The standard uncertainty is calculated assuming a triangular distribution; $u_{cal} = 0.0014/\sqrt{6}$. In the calculations of uncertainty derived from the reading and filling effects were omitted. The temperature range leads to an uncertainty in the determined volume, due to a considerable larger

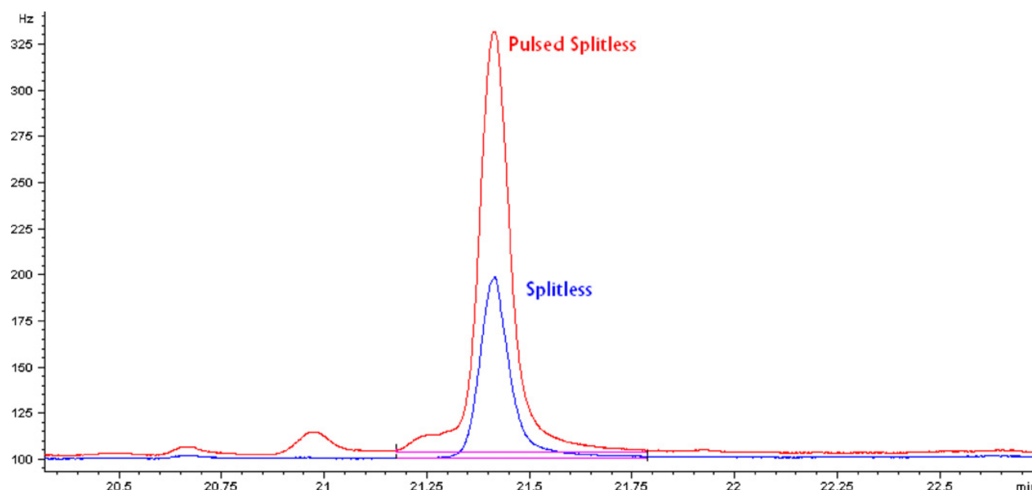

Fig. 3. Comparison of the 20 ng/mL CP signal by using splitless mode alone and pulsed splitless mode (injection volume: 3 μL).

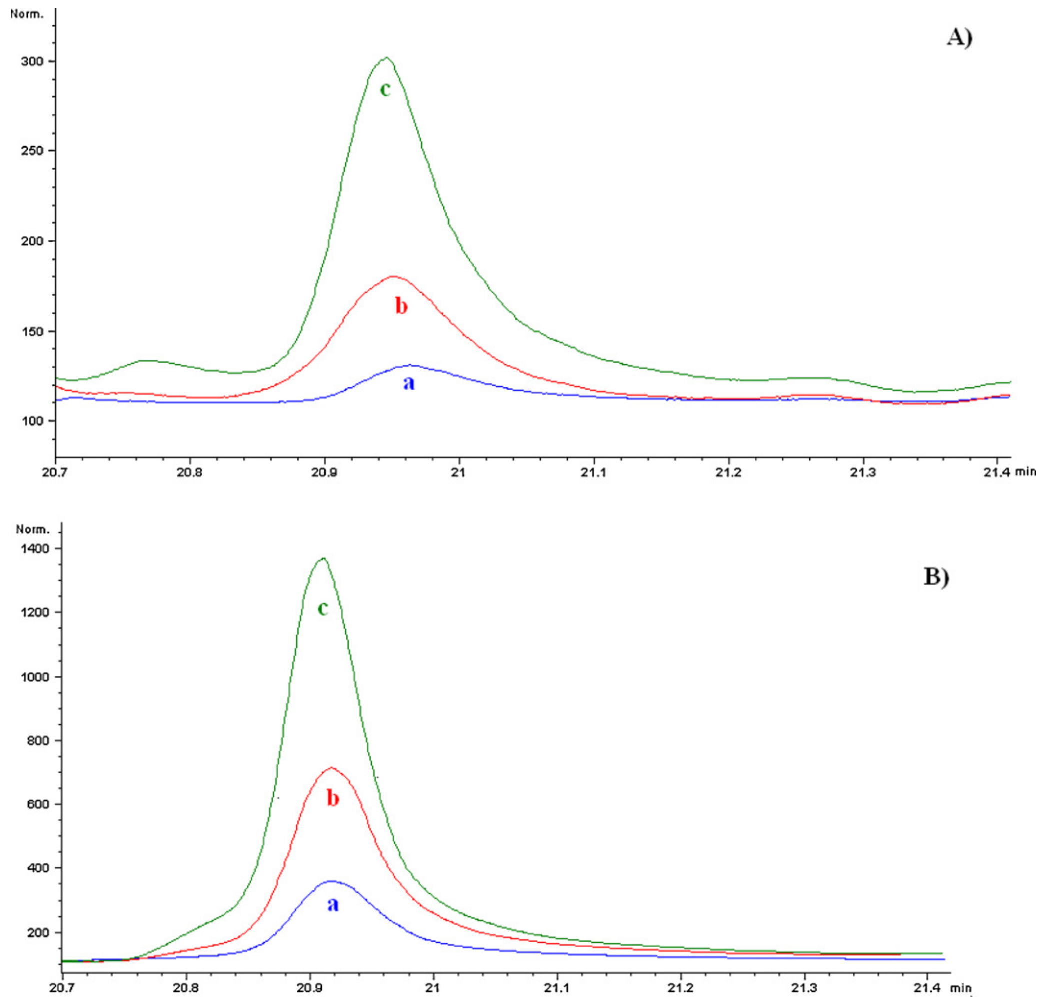

Fig. 4. Comparison of splitless and pulsed splitless injections of CPO overlaid chromatograms for (a) 100, (b) 200 and (c) 300 ng/mL CPO by using splitless (A) and pulsed splitless technique (B) with the injection volume of 3 μL and a pressure of 100 psi.

volume expansion of the liquid in the pipette. For further calculations the volume expansion of acetonitrile was also assumed. The respective relative volume expansion coefficient of acetonitrile is $1370 \times 10^{-6}/^{\circ}C$ [40]. The possible temperature variation in our laboratory is within the limits of ±4 °C. The standard uncertainty of the temperature effect was given as $u_{vtemp} = 4VQ/\sqrt{3}$. In this equation V is the measured volume and Q is the average coefficient of volume expansion of the liquids. Because of the rectangular distribution, the volume expansion term was divided by 3. Thus, volume expansion uncertainties of $u(V_1) = 0.0316$ for 10 mL [41]. Two uncertainty components are combined according to Eq. (3).

$$u(V_1) = \sqrt{(u_{vcal})^2 + (u_{vtemp})^2} \quad (3)$$

Same calculations were also done for 6 mL and 1 mL volumes and the combined uncertainty of V_{End} were calculated.

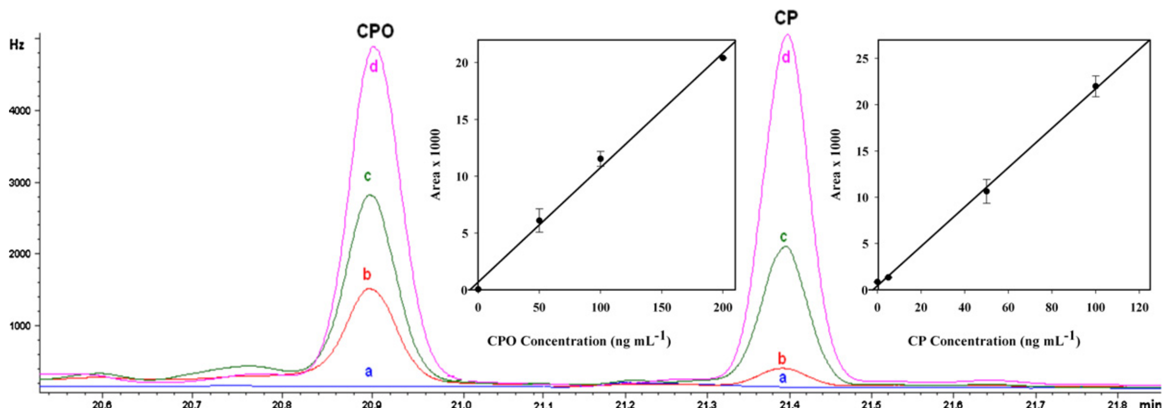

Fig. 5. Standard addition chromatograms and standard addition calibration curves of CPO and CP in wine prepared by QuEChERS method (a) sample and sample spiked with the standard solution to be, (b) 50 ng/mL CPO and 5 ng/mL CP, (c) 100 ng/mL CPO and 10 ng/mL CP and (d) 200 ng/mL CPO and 20 ng/mL CP.

3.4.2. Estimation of the uncertainty derived from the sample mass $u(M_{Sample})$

The balance manufacturer quotes ±0.00017 g fort the linearity contribution which is assumed to show a rectangular distribution and is converted to standard uncertainty of $0.00017/\sqrt{3}$. The contribution for the linearity has to be accounted for twice, once for the tare and once for the gross mass, leading to an uncertainty of $u(M_{\text{sample}}) = \sqrt{2 \times (9.81 \times 10^{-5})^2}$.

3.4.3. Estimation of the uncertainty derived from the concentration of the identified analyte in the sample solution $u(C_A)$

The concentration of the identified analyte in the sample solution can be calculated from the calibration curve. The uncertainty coming from the preparation of the calibration standard solutions was included in the calibration curve. The uncertainty of the calibration curve [42] for each analyte was calculated according to Eq. (4):

$$u(C_{\text{A}}) = \left(\frac{S}{b}\right)\left[\left(\frac{1}{p}\right) + \left(\frac{1}{n}\right) + \frac{(c_{\text{o}} - \bar{c})^2}{S_{xx}}\right] \tag{4}$$

where b is the slope; p is the number of measurements to determine c_{o}; n is the number of measurements for the calibration; c_{o} is the determined concentration of the analyte; $\bar{c}$ is the mean value of the different calibration standards set to make the calibration curve; S is the standard deviation of the residuals of the calibration curve and S_{xx} is calculated as Eq. (5);

$$S_{xx} = \sum \left(c_j - \bar{c}\right)^2 \tag{5}$$

where c_j is the concentration of the each calibration standard used to build up the calibration curve. The calculation of the uncertainty associated with the linear least square fitting procedure was done for each analyte and resulted as 1.9454 and 16.3926 for CP (n = 4) and, CPO (n = 4), respectively. Number of measurements to determine c_{o} is 2.

3.4.4. Estimation of the uncertainty derived from the recovery u(Rec)

The bias study of the developed analytical method was investigated during the in house validation study using spiked samples which is a common approach that is used to circumvent errors associated with matrix-induced enhancement or suppression effects in GC [43]. The method was tested upon spiking 10.00 g of wine sample (as a blank) with a mix standard solution to be 5 ng/mL of CP and 50 ng/mL of CPO.

The accuracy of the method was, then, assessed comparing the concentration level found from standard added sample with that of blank sample. The recovery assays were replicated three times. The standard uncertainty was calculated as the standard deviation of the mean $u(\overline{\text{Rec}} = S_{\text{Rec}}/\sqrt{n})$. According to this equation, standard uncertainty associated with recovery was done for each analyte and resulted as 0.1051 and 0.0929 for CP and CPO respectively.

3.4.5. Estimation of the uncertainty derived from the repeatability u(repeat)

The standard deviation of the CP measurements were given as 0.076 is divided by the square root of 3 to obtain the standard uncertainty of the triple determination. Uncertainty derived from the repeatability can be calculated as $u_{\text{repeat}} = 0.076/\sqrt{3} = 0.0439$. Same calculations were also done for CPO.

Diagram of the standard uncertainty of the analyzed pesticides can be seen in Fig. 6.

The overall combined uncertainty of the method was calculated by using the general expression in Eq. (2) and the calculated

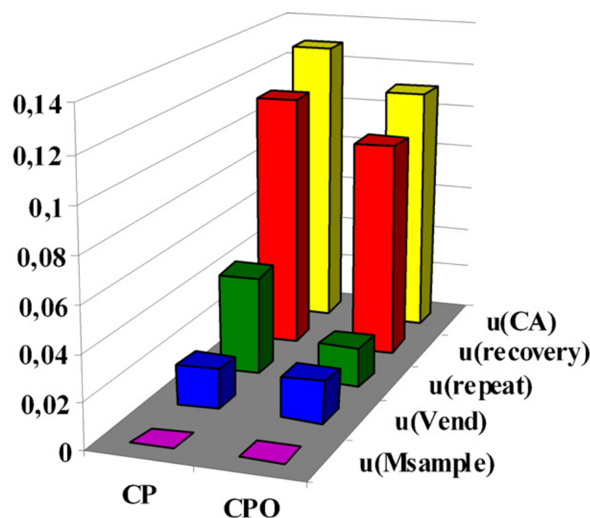

Fig. 6. Diagram of the standard uncertainty of the analyzed CP and CPO.

expanded relative uncertainties $u(k = 2)$ for these analyte were found as 0.35 and 0.29 for CP and CPO respectively.

It can be concluded from Fig. 6 that two sources of measurement uncertainty exist among the all parameters considered were recovery and calibration for estimation of measurement uncertainty budget. When this fact closely examined, it is interesting to note that the number of concentration levels for plotting the calibration curve was lesser (n = 4). This Figure suggested that estimation of MU for pesticides mostly relied on the contribution from the repeatability, calibration, dilution and recovery of the method employed. In order to decrease the uncertainty of the analytical method, it would be convenient to decrease the effects of previously mentioned stages in the sample preparation and clean-up process and increase the number of measurements.

4. Conclusion

This method is the first validated method to quantify CP and its main metabolite CPO in wine samples sensitively. In this study, the signal of CP and CPO was improved by applying pulsed splitless technique and therefore metabolite can be detected in ECD detector.

The proposed method is an economic simplified extraction and clean-up procedure that maintains adequate sensitivity for the detection of CP and its CPO metabolite in wine samples and can easily be adopted by laboratories that do not have expensive equipment such as MS detector. The sample clean-up method used in this work will save time and money over other routine extraction techniques.

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