

Determination of Simazine and Terbutylazine in Olive Oil by High Performance Liquid Chromatography with Ultraviolet Detector

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An effective matrix solid-phase dispersion extraction for the determination of simazine and terbutylazine in olive oil is described. The proposed methodology is based on partitioning liquid-liquid extraction and low temperature precipitation followed by matrix solid-phase dispersion (MSPD), using aminopropyl as dispersant material with a clean-up performed in the elution step with florisil and graphitized carbon (60:40, w/w). Related important factors influencing the extraction efficiency, such as, type of eluent and its volume, type of adsorbent for clean-up step, amount of dispersant material and the ratio of mobile phase were studied and optimized. The method was validated in terms of accuracy and precision (intra-day and inter-day), using spiked samples at different concentration levels. The recoveries were satisfactory (> 91 %) and limits of detection and quantification as μg of herbicides/g of olive oil sample were: 0.0127 and 0.0540 $\mu\text{g g}^{-1}$ for simazine, 0.027 and 0.14 $\mu\text{g g}^{-1}$ for terbutylazine, respectively.

Key Words: Herbicide, Adsorption, HPLC-UV, Matrix solid-phase dispersion, Low temperature, Simazine, Terbutylazine.

INTRODUCTION

"Virgin olive oil" is obtained from the fruit of the olive tree (*Olea europaea*), exclusively by mechanical and/or physical means without any subsequent treatment. Over the past few decades, knowledge gained about the nutritional health benefits of this oil has increased the demand for this product worldwide¹. In agricultural practice for olive groves, the use of insecticides and herbicides provides an unquestionable benefit for crop protection. However, these pesticide residues can persist up to the harvest stage, making the contamination of the olives used to produce olive oil possible². Consequently, both the European Union and the Codex Alimentarius Commission of the Food, Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) have established maximum residue limits (MRLs) in olives and olive oil that cover a large number of pesticides³.

s-Triazine, one kind of herbicides with high power for weed control, have been used as the selected herbicides for crop protection in modern agriculture. However, herbicides containing an *s*-triazine ring are relatively persistent in the environment. Simazine and terbutylazine are synthetic chemicals that are widely used as a

selective triazine herbicides to control the growth of broadleaved weeds and annual grasses in field, berry fruit, nuts, vegetable and ornamental crops, turfgrass, orchards and vineyards. The most widely used methods for the determination of triazine herbicides are chromatographic techniques including gas chromatography^{4,5} and high-performance liquid chromatography⁶. Pesticide residue determination in olive oil is high demanding task considering the inherent complexity of the matrix and its hydrophobic nature. Many multiresidue procedures employing different cleanup techniques and a variety of detection methods have been reported for the determination of pesticide residues in olive oil. The most commonly used methodology is based on gas chromatography (GC)^{7,8} after a comprehensive clean up step. In most cases the clean-up steps are based on liquid-liquid partitioning extraction with solvents of different polarity^{9,10}, gel permeation chromatography (GPC)^{11,12}, solid phase extraction¹³, size exclusion chromatography or adsorption column chromatography¹⁴. In order to separate the low molecular mass herbicides from the higher molecular mass fat constituents of the oil, such as triglycerides. Clean-up is the most laborious, but is the key to the whole process, since small amounts of co-extracted lipids can harm the chromatographic system and cause signal suppression. Additionally, clean-up with an alumina column or the use of solid-matrix partition steps do not allow pesticides to be recovered well from olive oil¹⁵. Furthermore, the elution of the target analyte from the solid phase makes it necessary to use organic solvents¹⁶. Recovery depends on the nature of the analyte and extracting solvent¹⁷. Also, interferences caused by lipids co-eluting from clean-up step adversely affect the analytical performance. In this sense, olive and other edible oils need a rigorous clean-up because of their peculiar matrices⁵. Recently, a multiresidue method for the determination of triazines and organophosphorous pesticides using matrix solid-phase dispersion (MSPD) followed by GC/MS and ion trap MS techniques was reported¹⁸. Hernando and co-workers used acetonitrile as extracting solvent and clean up by primary and secondary amine (PSA), anhydrous MgSO₄, C₁₈ for analysis of multi-class pesticides in olive oil with liquid chromatography ion trap mass spectrometry¹⁹. Analysis of herbicides in olive oil samples by use of LC-TOF-MS was reported by Garcia-Reyes and co workers²⁰. The method was based on MSPD using aminopropyl as a sorbent material and florisil as a clean-up reagent. The proposed methodology reported here is a modification of the method for multiresidue analysis as outlined by Garcia-Reyes and assistants, consists of a preliminary liquid-liquid extraction steps and low temperature precipitation of the oil matrices from the acetonitrile layer followed by matrix solid-phase dispersion using aminopropyl, [adsorbents with weak anion exchange and polar capabilities (NH₂)] as a sorbent material to remove fatty acids and combination of florisil and graphitized carbon black as clean-up reagent. Graphitized carbon black (GCB) has a strong affinity for planar molecules and thus effectively removes pigments such as chlorophyll and carotenoids, as well as sterols present in foods²¹. This work is focused on the development and evaluation of a simple, cheap and efficient sample preparation strategy based on matrix solid-phase dispersion (MSPD) coupled with HPLC-UV determination.

EXPERIMENTAL

Simazine and terbutylazine analytical standards were purchased from Fluka (HPLC grade 99.9 %). Individual pesticide stock solution ($1000 \mu\text{g mL}^{-1}$) was prepared in pure methanol and maintained at $-19 \text{ }^\circ\text{C}$. Working standard solutions were prepared by appropriate dilutions in methanol and then stored in a refrigerator ($4 \text{ }^\circ\text{C}$). Matrix matched standards were prepared by adding working standard solutions to blank olive oil. HPLC grade acetonitrile, *n*-hexane and methanol were obtained from Merck (Darmstadt, Germany). Analytical grade acetone and dichloromethane were purchased from fisher scientific. 3-(Fmoc-amino) propyl bromide was purchased from Fluka (HPLC grade, purity; $\geq 97 \%$). Florisil cartridges with a configuration of 500 mg/6 mL from Agilent Technologies, GCB cartridges with a configuration of 500 mg/6 mL from Alltech Inc, ultra pure silica gel (230 mesh) from Silicycle (Quebec, Canada) and neutral alumina from merck. A Milli-Q-Plus ultrapure water system from Millipore (Milford, MA) was used throughout the study to obtain the HPLC-grade water used during the analyses.

The chemical structures of the herbicides studied in this work are shown in Fig. 1.

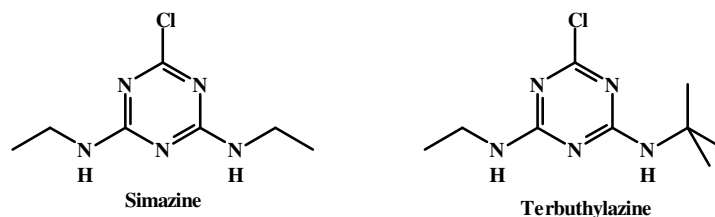


Fig. 1. Structures of the selected herbicides

HPLC analysis was performed by using an Agilent Technologies HPLC 1100 system including ultraviolet detection, a binary pump and a sample injector with 20 μL loop. The chromatographic separation of simazine and terbutylazine was carried out at $25 \text{ }^\circ\text{C}$ on a reversed phase symmetry C_{18} column ($4.6 \text{ mm} \times 150 \text{ mm}$ i.d., particle size $5 \mu\text{m}$). The mobile phase consisted of acetonitrile-water (with 10 % methanol) at the ratio of 85:15 v/v. The flow rate was kept at 1 mL min^{-1} and the absorbance was measured at a wavelength of 223 nm.

Extraction and clean-up: An aliquot of $5.00 \pm 0.01 \text{ g}$ homogenous virgin olive oil samples were weighed in 50 mL screw-capped centrifuge tubes and dissolved in 4 mL of *n*-hexane. Each sample was fortified with an appropriate volume of working standard solution for the recovery experiments. After agitation in a vortex mixer for 10 min, the samples were allowed to stand for 20 min for equilibration. Two-steps liquid-liquid partitioning was undertaken with 20 mL acetonitrile/*n*-hexane (80:20, v/v) (two extractions with 10 mL each), the mixtures were vigorously shaken for 4 min using a vortex mixer and then centrifuged on a kubota-2420 apparatus at 3000 rpm. In order to remove the oil, precipitation was carried out at $-20 \text{ }^\circ\text{C}$ for 2 h

(low temperature method), so triglycerides were precipitated to the bottom of the test tubes in the *n*-hexane layer and the acetonitrile extract rose to the top. Finally, an aliquot of the acetonitrile extracts were transferred to a 10 mL glass test tubes. The extracts were then carefully evaporated to make the final volume approximate 2 mL using rotary vacuum evaporator below 40 °C. This remaining extracts were gently blended with 1.5 g aminopropyl as dispersing phase into a glass mortar using a glass pestle until a homogenous mixture was obtained. The mixtures were transferred into a minicolumn containing florisil and graphitized carbon (60:40, w/w). These minicolumns were connected to a vacuum system for solid-phase extraction. Elution step was carried out by gravity flow with 16 mL of acetonitrile. The final extracts were collected into the graduated conical tubes and evaporated until near dryness, then dissolved with 1:1 acetonitrile/water and filtered through a 0.2 µm PTFE membrane filter paper (Millex FG, Millipore, Milford, MA) prior to HPLC analysis.

RESULTS AND DISCUSSION

Optimization of MSPD procedure: A series of preliminary experiments were conducted to select the optimum operation conditions of the matrix solid-phase dispersion step to achieve the highest recoveries for the herbicides.

Some parameters influencing the extraction efficiency, such as type and volume of eluting solvent, kind of clean-up sorbents, amount of dispersant material (aminopropyl) and the ratio of mobile phase were investigated and optimized.

The nature of the eluting solvent is an important factor since the target analytes should be efficiently desorbed while the remaining matrix components should be retained in the column. Several organic solvents, such as methanol, chloroform, acetone and *n*-hexane (1:1, v/v), dichloromethane and methanol (7:1, v/v) and acetonitrile were studied as the extractants for MSPD of two herbicides from olive oil samples. Olive oil samples were spiked at 0.1 µg g⁻¹ level. Fig. 2 presents the extraction recoveries of pesticides for the tested eluting solvents. When chloroform was used as the extracting solvent emulsification occurred and affected the recoveries of two *s*-triazines. Under this condition the average recovery was 35.6 % the lowest of all extractants used. The recoveries of the two herbicides were the highest (the average recoveries of simazine and terbuthylazine were 97.82 and 100.7 %, respectively) when acetonitrile was used as the eluting solvent. Therefore, further experiments were carried out using acetonitrile as the eluent. The volume of the eluent is another factor that should be considered. A series of experiments were designed and investigated by changing the volume of acetonitrile from 5-25 mL. According to Fig. 3, the average recoveries of the two analytes increased with the increasing volumes of MeCN between 10-16 mL, when this volume was more than 16 mL, the recovery of simazine remained constant. However, recovery of terbuthylazine only reached its maximal value when the volume of MeCN is more than 16 mL. Therefore, in all subsequent experiments, 16 mL of MeCN was used as the eluent.

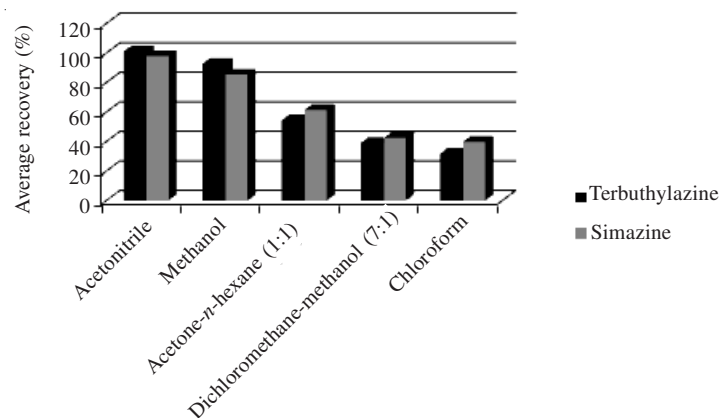


Fig. 2. Effect of solvent type on the recoveries of simazine and terbutylazine

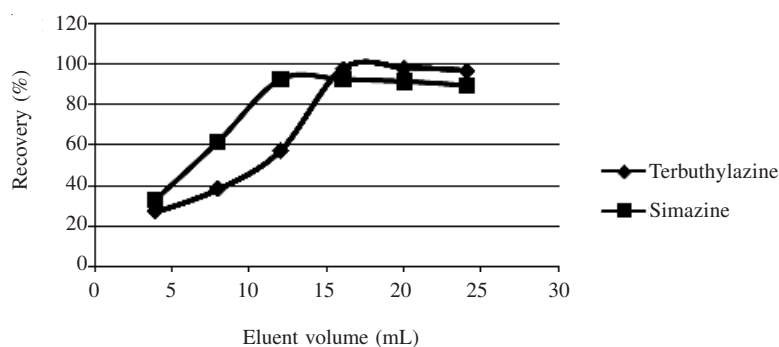


Fig. 3. Influence of the eluent volume on the recoveries of simazine and terbutylazine

In order to reduce cost and analysis time as well as to obtain high clean-up efficiency, different amount of adsorbent material (aminopropyl) and types of clean-up reagents were tested. From Fig. 4, the average recoveries of the two analytes were increased with the increasing the amounts of aminopropyl up to 1.5 g and then reached a plateau. So 1.5 g of aminopropyl was selected to be optimum amount for preconcentration step. Among several clean-up reagents, such as silica gel, alumina, florisil, graphitized carbon and mixed florisil-graphitized carbon (60:40, w/w), the latest was used to remove the co-extractant because it produced a transparent and colourless solution with average recovery of 95 and 93 % for simazine and terbutylazine, respectively. The results obtained are shown in Fig. 5. In this study the extracts had a dark yellow colour when alumina (mean recovery: 49 %) was used as the clean-up reagent and the result were almost similar to silica gel (mean recovery: 36 %). The recoveries were desirable when florisil and graphitized carbon were used individually (mean recovery: 90 %) but the extracts had a yellow colour when florisil was used alone. The excellent recoveries and colourless extracts were obtained when a mixture of florisil and graphitized carbon (60:40, w/w) was used.

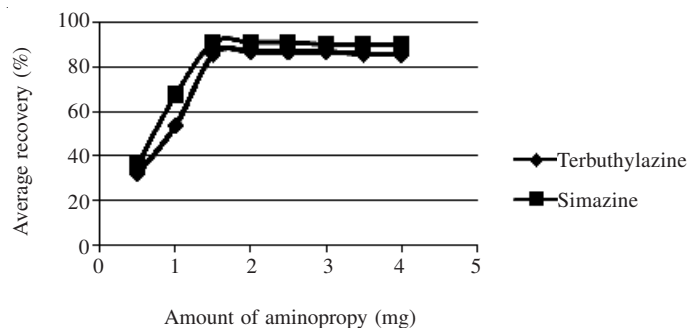


Fig. 4. Effect of the amount of aminopropyl on the recoveries of simazine and terbuthylazine, (ACN as extracting solvent, Florisil/GCB (60:40) as clean-up sorbent, n=3)

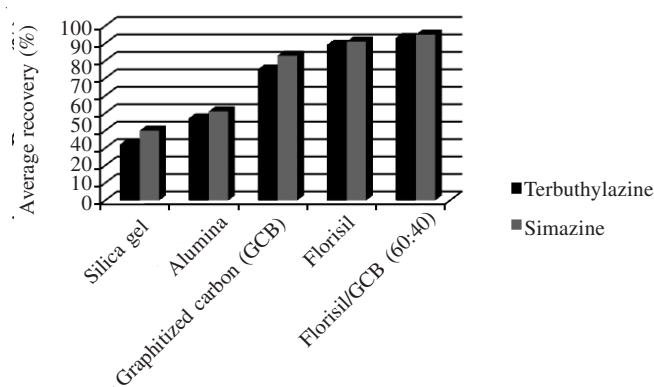


Fig. 5. Effect of different clean-up sorbents on the recoveries of simazine and terbuthylazine, (ACN as elution solvent, NH₂ as dispersing phase, n=3)

Analytical performances

Linearity and repeatability: Under the above optimum conditions, some characteristics of the present method were investigated. Linear range, limit of detection and accuracy were obtained. Seven standard solutions with concentrations in the range of 0.05-1.50 $\mu\text{g mL}^{-1}$ were subjected to HPLC-UV analysis. The relationships between the analyte concentration (X) and peak area of measured signal (Y) are noted as regression equations $Y = 34.65X + 0.232$ with correlation coefficient of 0.9991 for simazine and $Y = 46.52X + 1.209$ with correlation coefficient of 0.9987 for terbuthylazine. The standard solutions were used to determine the intra-day (triplicates at each concentration, one day) and inter-day (triplicates at each concentration, one week) repeatability by assaying the olive oil samples under the selected optimal conditions. The results of intra-day and inter-day repeatability are illustrated in Table-1. Relative standard deviations (RSD) were between 0.46 and 3.40 % for the intra-day and between 3.36 and 5.89 % for inter-day assays.

TABLE-1
INTRA-DAY AND INTER-DAY REPEATABILITY OF THE ASSAYS

Concentration injected (mg/kg)	Intra-day repeatability RSD % (n = 3)	Inter-day repeatability RSD % (n = 7)
Simazine		
0.1	2.68	5.17
1.0	1.49	3.36
10.0	0.46	4.32
Terbutylazine		
0.1	3.40	5.79
1.0	1.45	4.47
10.0	0.76	3.78

Detection and quantification limits: The limit of detection of a method is the lowest analyte concentration that produces a response detectable above the noise level of the system; typically, three times the noise level. The limit of quantification is the minimum injected volume that gives precise measurements. Mathematically, the LOD and LOQ were calculated from the data of the calibration curve as follows: (LOD = $3S_B/m$, LOQ = $10 S_B/m$), where S_B = the standard deviation of the blank; m = the slope of the calibration curve. The measured LOD and LOQ values of the proposed method expressed as μg of herbicides/g of olive oil sample were 0.0127 and 0.054 $\mu\text{g g}^{-1}$ for simazine and 0.027 and 0.14 $\mu\text{g g}^{-1}$ for terbutylazine, respectively. These LODs are below the required maximum residue level (MRL) of 0.1 $\mu\text{g g}^{-1}$ for these two herbicides in olive oil.

Accuracy and precision studies: To evaluate the effectiveness of the proposed method, it was applied to the analysis of a total of seven samples of marketing olive oil. Fortunately, in most cases, pesticide residues were not found. Only in one olive oil sample, simazine was found at concentration levels near the limit of detection (below the authorized maximum residue level). Accuracy and precision were calculated by spiking the samples with suitable amounts of the analytes, over suitable concentration ranges (0.1, 0.5 and 1.0 $\mu\text{g g}^{-1}$). The method validation studies for spiked samples indicated that the present method provides good recoveries and reasonable precision for simazine and terbutylazine at three levels. On the other hands, relative error illustrates the accuracy, so mathematically; the accuracy is the average relative standard deviation of the analysis of a set of data from the mean of population:

$$\text{Relative error (\%)} = \frac{\sum \frac{\bar{X} - \text{True}}{\text{True}}}{N}$$

Table-2 refers, the recoveries were found to be in the range of 91.3-101.8 with the RSD form 1.32-5.82 % and the relative errors were ranged between 0.6 and 1.80 % when $N = 3$.

TABLE-2
PERCENTAGE RECOVERIES AND RELATIVE ERRORS OF SIMAZINE AND
TERBUTHYLAZINE IN OLIVE OIL AT THREE DIFFERENT SPIKED LEVELS

	Spiked level (mg/kg)	Mean value	Recovery (%)	RSD (%)	Relative error (%) (n = 3)
Simazine	0.1	0.0970	97.32	3.25	-1.2
	0.5	0.4730	93.41	4.61	-1.8
	1.0	1.0200	101.80	1.32	0.7
Terbutylazine	0.1	0.0952	91.30	5.82	-1.30
	0.5	0.4850	96.40	2.56	-0.76
	1.0	0.9760	97.81	3.35	-0.60

Clean-up efficiency: The clean-up efficiency was assessed by determining the amount of oil co-extracted from the samples into the extract. This was done gravimetrically after clean-up using florisil/GCB (60:40, w/w) cartridges. From the results obtained, the amount of oil co-extracted for olive oil samples after clean-up procedure was $1.6 \pm 0.4 \text{ mg g}^{-1}$ (n = 7). This value represented 0.2 % of the sample mass. The results showed that the clean-up step was able to remove 99.8 % of the lipid using this method, sufficient for the chromatographic system to maintain its separation efficiency for more than 100 samples injection.

Conditions of HPLC: A HPLC method was used with a reversed phase symmetry C₁₈ column. Sample injection volume was 20 μL and temperature of the column was controlled at 25 °C. The flow rate was kept at 1 mL min⁻¹ and the absorbance was measured at wavelength 223 nm. Two peaks of the two analytes could not be resolved completely when the ratio of the mobile phase (acetonitrile/ water) was 90:10 v/v and the peaks were delayed as well. However, when the mobile phase consists of acetonitrile and water (with 10 % methanol) at the ratio of 85:15 v/v the two peaks could be separated completely and the retention times were reduced (Fig. 6).

Conclusion

An affective, simple and reliable low temperature method followed by matrix solid-phase dispersion (MSPD) extraction procedure was successfully applied to the analysis of simazine and terbuthylazine in olive oil. A multi-factor categorical design was employed for the optimization of extraction/clean-up stage and the type of adsorbent in the co-column was the most important variable. The recommended analytical procedure consists of blending of analytes with 1.5 g of aminopropyl, cleaning-up with a co-column containing florisil and graphitized carbon (60:40, w/w) and elution with 16 mL of MeCN. After validation of method, the analytical results confirmed that the described MSPD-HPLC-UV procedure provides good recoveries with limit of detection well below those set by the international regulations for pesticide residues in olive oil. The developed sample pretreatment procedure offered many obvious advantages such as low cost, simplicity, rapidness, easy to operate, sensitiveness and good repeatability.

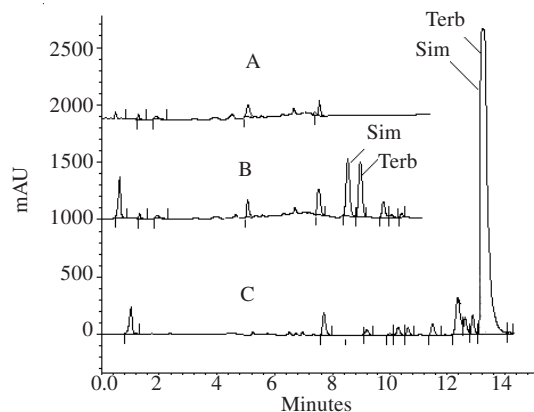


Fig. 6. Chromatograms obtained for (A) blank sample, (B) simazine and terbutylazine in spiked olive oil ($0.1 \mu\text{g g}^{-1}$); separated peaks, when acetonitrile: water (85:15, v/v, with 10 % MeOH) was used as the mobile phase, (C) overlapped peaks, when acetonitrile: water (90:10, v/v) was used as the mobile phase

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