Development of an indirect enzyme immunoassay for the determination of thiabendazole in white and red wines

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A competitive indirect enzyme immunoassay for the determination of fungicide thiabendazole in white and red wine samples was developed. The assay had a detection limit of 0.005 ng mL\textsuperscript{-1}, a dynamic range from 0.01 to 2 ng mL\textsuperscript{-1}, and was precise with intra- and inter-assay coefficient of variation values less than 5\% and 8\%, respectively. Concerning the sample preparation procedure, it was found that filtration of the wine samples through a 0.45 μm pore size polytetrafluoroethylene (PTFE) filter followed by 30 times dilution with the assay buffer eliminated completely matrix interferences for both white and red wines derived from different grape varieties. This was demonstrated by the accurate determination of thiabendazole amounts in spiked wine samples (\% recovery 90.6–108). In addition, the results obtained for certain spiked wine samples were in good agreement with those received by an LC-MS/MS method for determination of thiabendazole in wines. Taking into account the dilution factor, the thiabendazole concentrations that could be determined in wines ranged from 0.3 to 60 ng mL\textsuperscript{-1}. The maximum residue limit (MRL) of thiabendazole in wine grapes is 50 μg kg\textsuperscript{-1}. Thus, the proposed enzyme immunoassay can be applied for the fast and parallel analysis of a high number of white and red wine samples with minimal sample pre-treatment.

Keywords: thiabendazole; indirect enzyme immunoassay; wine samples; LC-MS/MS

1. Introduction

Analysis of food for pesticide residues has revealed the presence of numerous substances that are used to either increase crop yields or preserve raw product quality [1]. The presence of pesticide residues to both unprocessed and processed foodstuff can be the result of non-compliance with the good agricultural practices but also of post-harvest treatment of raw foods such as fruits and vegetables to avoid quality deterioration [2]. It goes without saying that in both cases the presence of pesticide residues in the products offered for consumption may pose a severe threat to human health.

Thiabendazole (Figure S1A; supplementary data) is a systemic fungicide applied either pre-harvest to control diseases or post-harvest to avoid product quality deterioration during storage [3]. Like all pesticides of benzimidazole group, thiabendazole acts by compromising...
cytoskeleton formation through a selective interaction with β-tubulin and thus by disrupting fungal mitotic process [4].

Competent authorities have performed risk assessment for thiabendazole and in order to protect consumers from its toxic effects [5], maximum residue limits (MRLs) are set in many commodities, amongst them table and wine grapes for which the MRL was set at 50 μg kg⁻¹ of product [6]. With regard to stability versus different food processing procedures, studies have showed that thiabendazole is stable during pasteurisation, cooking, brewing and wine-making process [7]. Thus, although MRLs have not been set for wine, it is expected that if thiabendazole has been used to control fungus development in grapes it would be also present in wine.

To ensure food safety and fair practice in international trade, reliable and accurate methods for the determination of pesticide residues are of high importance. Chromatography, liquid or gas, in combination with different types of detectors is the main technique for multi-residue pesticide analysis [8–14] providing high sensitivity, accuracy and reproducibility. Nonetheless, beside the relatively high instrumentation cost, chromatographic methods require time-consuming sample preparation to minimise matrix interferences, large volumes of toxic organic solvents and highly trained personnel. Methods based on the native fluorescence of benzimidazole compounds have been also developed for the determination of this class of pesticides in food and environmental samples which, however, also required cumbersome extraction procedures in order to achieve good accuracy and detection sensitivity [15–18]. Thus, methods for pesticides detection and/or quantification involving simpler sample preparation procedures are still in demand.

To this end, immunoanalytical methods represent attractive alternatives to chromatographic ones for the determination of environmental contaminants since in most cases these methods do not require complex sample preparation procedures [19]. With regard to thiabendazole, a number of methods based on analyte-specific monoclonal or polyclonal antibodies have been developed for its determination in food products including fruit juices [20,21], fruits and vegetables [22–26]. However, to our knowledge, there is not an immunochromatographic method for determination of thiabendazole in wines, despite the fact that thiabendazole is one of the fungicides used for protection of grapes from fungi. Here we present the development of an indirect enzyme immunoassay for the determination of thiabendazole in white and red wines based on a thiabendazole–protein conjugate as solid-phase reagent and a mouse monoclonal anti-thiabendazole-specific antibody (depicted in Figure S2; supplementary data). Emphasis was given to find a simple sample preparation procedure for both white and red wines that alleviated all matrix effects. The developed assay was evaluated using wines, either white or red, prepared from different grape varieties (Greek, Spanish and Czech). Spiked wine samples analysed by the developed immunoassay were also analysed following a LC-MS/MS method after application of a modified QuEChERS sample preparation method.

2. Experimental
2.1 Materials

The mouse monoclonal antibody (Mab clone LIB-TN3C13) against thiabendazole, and the thiabendazole conjugate with bovine serum albumin (BSA-TN3C) were purchased at an initial concentration of 1 mg mL⁻¹ from the Grupo de Inmunotecnologia of Universidad Politécnica de Valencia (Valencia, Spain) [20]. The thiabendazole derivative shown in Figure S1B (supplementary data) was used for the preparation of both the immunogen and BSA conjugate. Goat antimouse IgG (Fc-specific)-peroxidase (anti-mouse IgG-HRP) conjugate, thiabendazole (PESTANAL®, analytical standard) and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
(ABTS) were obtained from Sigma-Aldrich (St. Louis, MO). Absolute ethanol was from Carlo Erba SpA (Milano, Italy) and bovine serum albumin (BSA) from Acros Organics (Geel, Belgium). LC-MS grade acetonitrile (ACN) and water were obtained from Fisher Scientific GmbH (Wien, Austria). 96-Well polystyrene plates were from Greiner Bio-One GmbH (Frickenhausen, Germany). Fluoropore™ polytetrafluorethylene (PTFE) membrane filters, 13 mm with 0.45 μm pore size were purchased from Merck Millipore (Darmstadt, Germany). The water used throughout was doubly distilled.

2.2 Instrumentation

Optical density of the microtitration wells was measured at 405 nm using a Victor3 1420 Multilabel Counter (Perkin Elmer, Milano, Italy). The tandem mass spectrometer Agilent 6490 QqQ-MS/MS system (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 1200 HPLC system with a binary pump system equipped with a reversed-phase C8 analytical column of 4.6 mm × 150 mm and 5 μm particle size (Agilent Zorbax SB) was used for the detection of thiabendazole in wines.

2.3 Pesticide standard solutions

A 1 mg mL⁻¹ stock thiabendazole solution was prepared in absolute ethanol and stored at −20° C. Standard solutions were prepared from this stock by serial dilutions in 10 mM phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 0.2% (w/v) BSA (assay buffer).

2.4 Wine samples

White and red wines were commercial products obtained from local supermarkets. In addition, samples from a small-scale producer of organic wine were also used (Attiki region, Greece). All wines employed in the study were tested by the LC-MS/MS method and no thiabendazole could be detected. Wine samples containing known concentrations of thiabendazole were prepared through analyte spiking with the same thiabendazole solution used for the preparation of standard solutions in assay buffer.

For the enzyme immunoassay, the wine samples were passed through a PTFE filter with 0.45 μm pore size to remove suspended particles and then diluted 30 times with assay buffer.

2.5 ELISA buffers

The coating buffer was 0.05 mol L⁻¹ carbonate buffer, pH 9.3. The washing buffer was 0.01 mol L⁻¹ phosphate buffer, pH 7.4, 0.9% (w/v) NaCl, containing 0.05% (v/v) Tween 20. The blocking solution was 0.01 mol L⁻¹ phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 2% (w/v) BSA. The assay buffer was 0.01 mol L⁻¹ phosphate buffer, pH 7.4, 0.9% (w/v) NaCl, 0.2% (w/v) BSA. The H₂O₂/ABTS peroxidase substrate solution contained 0.03% (v/v) H₂O₂ and 0.0019 mol L⁻¹ ABTS in 0.1 mol L⁻¹ citrate-phosphate buffer, pH 4.5.

2.6 Indirect enzyme immunoassay

Microtitration wells were coated overnight with 100 μL per well of a 0.2 μg mL⁻¹ solution of BSA–thiabendazole conjugate solution in coating buffer. Then, the wells were washed three times with 300 μL of washing buffer and blocked through incubation with 300 μL of blocking solution for 1 h. After washing as previously, 50 μL of standard solutions or 50 μL of wine
sample filtered and diluted 30 times in assay buffer and 50 μL of a 0.1 μg mL\(^{-1}\) anti-thiabendazole antibody solution in the same buffer were added per well and incubated for 1 h under shaking. Wells were then washed three times with 300 μL of washing solution, and 100 μL of a 0.5 μg mL\(^{-1}\) goat anti-mouse IgG–HRP conjugate solution in assay buffer containing 0.05% (v/v) Tween 20 was added in each well and incubated for 30 min while shaking. After washing as previously, 100 μL of chromogenic peroxidase substrate (H\(_2\)O\(_2\)/ABTS) was added in each well and the optical density was measured at 405 nm after 30 min. All steps of the ELISA method were performed at 22°C.

### 2.7 Data analysis

The analytical curve was created by plotting the signal of the different standards expressed as percentage of the zero standard signal (maximum signal, \(B_0\)) against the standard solutions concentration in logit/log scale. The analytical curve was linearised by applying the logit transformation on the y-axis values following the formula \(\text{logit}(B_x/B_0) = \ln[(B_x/B_0)/100 - (B_x/B_0)]\), where \(B_x\) is the signal obtained for each one of the standards and \(B_0\) is the signal obtained for the zero standard solution. Statistical analysis was performed using the OriginPro 8 software (OriginLab Corporation, Northampton, MA).

### 2.8 LC-MS/MS detection of thiabendazole

Thiabendazole was detected in wine samples by using an already validated multi-residue LC-MS/MS method [7]. White and red wine samples were spiked with thiabendazole at concentrations between 1.5 and 150 ng mL\(^{-1}\) and then were subjected to extraction. In detail, 15 g of sample, 3 mL of acetonitrile and 12 mL of water were added to a volumetric tube and shaken for 1 min. Then, the mixture was centrifuged for 5 min at 4000 rpm, the supernatant was transferred to another tube and diluted 10 times with a 1:9 (v/v) acetonitrile/H\(_2\)O mixture. Finally, the extract was filtered through a PTFE filter with 0.45 μm pore size and 10 μL were injected for LC-MS/MS analysis. LC-MS/MS conditions were as follows: the mobile phases were acetonitrile (mobile phase B) and water with 0.1% (v/v) formic acid (mobile phase A) with a flow rate 0.6 mL min\(^{-1}\). The gradient elution started with 20% mobile phase B for 3 min, followed by a linear gradient up to 40% B over 2 min and then up to 100% A in 12 min, after which the mobile phase composition was maintained at 100% A for 3 min. The re-equilibration time was 8 min. The MS/MS was set at positive ion spray ionisation mode (ESI+), with nitrogen as nebulising and collision gas, gas temperature of 120°C, gas flow of 15 L min\(^{-1}\), nebulising gas at 25 psi, sheath gas temperature of 200°C, sheath gas flow of 12 L min\(^{-1}\) and capillary voltage at 4500 V. The acquisition parameters for thiabendazole were 202 > 175 > 131 and the retention time of its chromatographic peak was 3.5 min.

### 3. Results and discussion

#### 3.1 Development of indirect enzyme immunoassay for thiabendazole

Several parameters were optimised with regard to the assay performance such as the pH and composition of the washing, blocking and assay buffer, the concentration of BSA–thiabendazole conjugate used for coating and of anti-thiabendazole antibody, and the temperature at which the immunoreaction is performed. The parameters were selected based on the absorbance measured at 405 nm for \(B_0\) and the half maximal inhibitory concentration (IC\(_{50}\)), that is the concentration of analyte providing a 50% \(B_0\) signal inhibition which is an indication of assay sensitivity. The
parameters that mainly affected the $B_0$ signal and the assay sensitivity were the concentration of BSA–thiabendazole conjugate and of anti-thiabendazole antibody, the pH and composition of the assay buffer as well as the temperature at which the assay was performed. The selection of optimum assay conditions is discussed in detail below.

3.1.1 Selection of BSA–thiabendazole and anti-thiabendazole antibody concentration
The BSA–thiabendazole conjugate concentration used for coating and the anti-thiabendazole antibody concentration were determined through antibody titration experiments using several combinations of different concentrations of the reagents used. As shown in Figure S3 (supplementary data), $B_0$ absorbance values at 405 nm of about 1 were obtained for BSA–thiabendazole/anti-thiabendazole antibody concentration combinations of 100/200, 200/100 and 500/50 ng mL$^{-1}$. The standard curves obtained with these combinations indicated that the more sensitive analytical curve with an IC$_{50}$ value of 0.2 ng mL$^{-1}$ was obtained using 200 ng mL$^{-1}$ of BSA–thiabendazole conjugate for coating along with 100 ng mL$^{-1}$ of anti-thiabendazole antibody.

3.1.2 Selection of assay buffer
The effect of assay buffer pH was tested using buffers with pH values ranging from 4.0 to 9.0. In particular, the buffers used were: sodium acetate buffer, pH 4.0 and 5.0; sodium phosphate buffer, pH 6.0 and 7.4; and sodium carbonate buffer, pH 8.0 and 9.0. All buffers were prepared at 0.01 mol L$^{-1}$ concentration and contained 0.9% (w/v) NaCl and 0.2% (w/v) BSA. In Table 1 the $B_0$ as well as the IC$_{50}$ values of the analytical curves obtained by using the different assay buffers are presented. As it is shown, when the pH was increased from 4.0 to 7.4, the maximum signal value was increased by 50% without affecting, however, the IC$_{50}$ value. Further increase of the pH resulted in slight signal decrease while the assay sensitivity was negatively affected, that is the IC$_{50}$ was increased. Thus, the assay buffer selected for further experimentation was 0.01 mol L$^{-1}$ phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 0.2% (w/v) BSA since it provided low IC$_{50}$ and adequate $B_0$ value.

Since non-ionic detergents, such as Tween 20, are known to suppress non-specific binding signal, the effect of its presence in all buffers was tested. Tween 20 is considered compatible with all assay buffers.

Table 1. Effect of assay buffer pH, Tween 20 concentration in assay buffer and assay temperature on the IC$_{50}$ and $B_0$ values.

| Parameter tested | IC$_{50}$ (ng mL$^{-1}$) | $B_0$ (at 405 nm) |
|------------------|--------------------------|------------------|
| pH               |                          |                  |
| 4.0              | 0.23                     | 0.843            |
| 5.0              | 0.25                     | 0.912            |
| 6.0              | 0.26                     | 1.045            |
| 7.4              | 0.28                     | 1.241            |
| 8.0              | 0.35                     | 1.129            |
| 9.0              | 0.37                     | 1.035            |
| % Tween 20 (v/v) |                          |                  |
| 0                | 0.20                     | 1.267            |
| 0.05             | 0.34                     | 1.283            |
| 0.1              | 0.43                     | 1.256            |
| Assay temperature|                          |                  |
| 37°C             | 0.35                     | 1.360            |
| 22 °C            | 0.19                     | 1.258            |
| 4°C              | 0.12                     | 0.572            |
with immunoassay methods [27], when compared to ionic ones that are characterised as harsh detergents and denature proteins. It was found that the inclusion of Tween 20 in washing, blocking and second antibody dilution buffer at a concentration of 0.05% (v/v) eliminated non-specific binding without affecting assay performance. On the contrary, as it is presented in Table 1, inclusion of Tween 20 in the assay buffer at concentrations 0.05% and 0.1% (v/v) increased the IC_{50} value by 30% and 65%, respectively, indicating that its presence during the immunoreaction deteriorated the assay sensitivity. Therefore, Tween 20 was included in washing, blocking and second antibody dilution buffers but excluded from the assay buffer.

3.1.3 Selection of assay temperature

The effect of assay temperature on the signal and assay sensitivity was tested by performing the immunoreaction at: 4°C, 22°C and 37°C. The \( B_0 \) signals as well as the IC_{50} values obtained by performing the assay at different temperatures are presented in Table 1. As shown by increasing the assay temperature from 4°C to 22°C, a 2.2 times higher \( B_0 \) signal was achieved, while the IC_{50} value was increased by 40%. Further elevation of the assay temperature to 37°C resulted in approximately 10% increase of \( B_0 \) signal, while the assay sensitivity was deteriorated (80% increase of IC_{50} value). Thus, 22°C was selected as the most appropriate temperature to perform the assay as it provided high \( B_0 \) signal and adequate sensitivity.

3.2 Analytical characteristics of the assay and wine samples analysis

A typical linearised analytical curve obtained using the optimum assay conditions with the standard solutions prepared in assay buffer, as well as its 95% confidence limits, are shown in Figure 1. The linear regression equation is:

\[
\text{logit} \left( \frac{B_x}{B_0} \right) = -2.043 \ (\pm 0.028) \ \log C -1.532 \ (\pm 0.032), \ R^2 = 0.9985, \ p < 0.0001 \quad (1)
\]

where \( C \) is the thiabendazole concentration in nanograms per millilitre. The bias for concentrations up to 1 ng mL\(^{-1}\) was ±3%, whereas for 2 ng mL\(^{-1}\) it reached +8%.

The assay detection limit was determined as the concentration corresponding to mean value –3 standard deviations (SD) of \( B_0 \) signal (\( n = 16 \)) and was 0.005 ng mL\(^{-1}\). The quantification limit was determined as the concentration corresponding to mean value –6 SD of \( B_0 \) signal (\( n = 16 \)) and was 0.01 ng mL\(^{-1}\). The linear range of the assay extended up to 2 ng mL\(^{-1}\).

3.3 Sample preparation and wine analysis

Regarding the sample preparation procedure for wine analysis using the developed enzyme immunoassay, at first the extraction protocol used for the LC-MS/MS method was applied. This sample preparation procedure involved extraction with an acetonitrile/water mixture, centrifugation, filtration and further dilution of the samples with an acetonitrile/water mixture. Apart from being relatively laborious, the analytical curve obtained using as matrix wine that has been treated according to this sample preparation procedure was poorly correlated to the thiabendazole curve obtained using standard solutions prepared in assay buffer. In Figure 2 extended standard curves prepared using standard solutions in assay buffer or in wine sample extracted following the LC-MS/MS preparation procedure without further dilution (10% (v/v) acetonitrile) and after five times
dilution with assay buffer (2% (v/v) acetonitrile) are presented. In case of wine sample, appropriate amounts of thiabendazole were spiked in the wine so as to have the same concentrations after the different solution. To define higher and lower asymptotes, final concentrations ranging from 0.002 up to 10 ng mL$^{-1}$ were used. As it is shown, the $B_0$ signal obtained with the wine extract without further dilution was approximately 40% lower than that obtained when the assay buffer was used as zero standard. Furthermore, the detection limit and the IC$_{50}$ value of the curve prepared in the extracted wine sample were 20 and 4 times higher, respectively, when compared with those obtained from the standard curve in assay buffer. Same results were obtained following the LC-MS/MS preparation procedure using assay buffer instead of wine sample, thus indicating that the presence of acetonitrile strongly affects the performance of the anti-thiabendazole antibody. The negative acetonitrile effect on the anti-thiabendazole antibody was eliminated when at least a five times dilution of the wine extract with assay buffer was employed. In other words, the acetonitrile content of the sample should be equal to or lower than 2% (v/v), as it is indicated in Figure 2.

![Figure 1](image1.png)

**Figure 1.** Typical thiabendazole analytical curve. Each point is the mean value of four replicates ± SD. Middle line corresponds to linear regression; side lines represent the 95% confidence limits of the curve.

![Figure 2](image2.png)

**Figure 2.** Thiabendazole analytical curves obtained using standard solutions prepared in assay buffer (■), in wine processed according to the sample preparation protocol for LC-MS/MS analysis without further dilution (○) or in wine processed according to the sample preparation protocol for LC-MS/MS analysis and then diluted five times with assay buffer (●). Each point is the mean value of four replicates ± SD.
However, due to the higher sample dilution needed the detection limit was increased to 0.5 ng mL\(^{-1}\) in the wine.

To avoid the use of organic solvents and to simplify the sample preparation procedure, filtration and dilution of wine with assay buffer was examined. As it is shown in Table 2, dilution of wine equal to or higher than 30 times with assay buffer provided \(B_0\) and IC\(_{50}\) values almost identical to those obtained in assay buffer. Thus, a 30-times dilution was selected for further experimentation.

In order to test if the proposed sample preparation procedure could be applied to both white and red wine, four white and four red wines from different grape varieties, in which thiabendazole was not detected through LC-MS/MS analysis, were spiked with different concentrations of thiabendazole so that after 30 times dilution the expected concentrations would be within the working range of the analytical curve. In Table 3, the recovery values obtained for these wine samples at different spiking levels are presented. For all wines tested, the recovery values ranged from 90.6% to 108% indicating that the method developed could accurately determine thiabendazole in wine samples. The accuracy of the developed method was also evaluated through comparison with the LC-MS/MS. For this reason wine samples spiked with four different levels of thiabendazole were analysed with the validated LC-MS/MS method (EU Reference Laboratory for Pesticide Residues in Fruit & Vegetables, University of Almeria), applying an appropriate sample preparation protocol. The same spiked samples were supplied as unknown to Immunoassay-Immunosensors Lab (Institute of Nuclear and Radiological Sciences and Technology, Energy and Safety of NCSR ‘Demokritos’) to be analysed by the developed ELISA. The results obtained are presented in Table 4. The linear regression equation of the correlation curve (Figure S4; supplementary data) is \(y = 1.033(±0.010)x − 0.71(±1.14)\), indicating good agreement of results obtained with the two methods.

To determine the assay precision, three control samples with low, medium and high concentration were prepared in both white and red wines to cover the concentration range of the analytical curve. The intra-assay coefficient of variation (%CV) was determined by running four replicates of each control in the same day while the inter-assay %CV was determined by measurements performed in 10 consecutive days and their values for the three controls were less than 5% and 8%, respectively.

As it was mentioned, the limit of detection of the ELISA developed was 0.005 `ng mL\(^{-1}\) and corresponds to 0.15 ng mL\(^{-1}\) in non-diluted wines, which is well below the limit set by the EU guidelines in table and wine grapes, for which the MRL is set at 50 μg kg\(^{-1}\) of product [6].

Compared with other methods for thiabendazole determination, the developed immunoassay was much more sensitive than ELISAs reported for thiabendazole detection in potatoes [22–24], and in vegetables and fruits [25] with detection limits of 10, 3 and 9 ng mL\(^{-1}\), respectively, and 3–4 times more sensitive than an ELISA developed for thiabendazole determination in fruit juices [20]. In comparison to a strip-based immunoassay for the detection of thiabendazole in fruits juices [21], the ELISA developed is at least 10 times more sensitive, whereas is 25 times

### Table 2. Effect of wine dilution on analytical characteristics of the thiabendazole assay.

| Wine dilution factor | \(B_0\) signal ± SD (\(n = 4\)) | IC\(_{50}\) (ng mL\(^{-1}\)) |
|----------------------|---------------------------------|-----------------------------|
| 0 (assay buffer)     | 1.311 ± 0.014                   | 0.22                        |
| 10                   | 1.041 ± 0.003                   | 0.71                        |
| 20                   | 1.097 ± 0.008                   | 0.51                        |
| 30                   | 1.288 ± 0.012                   | 0.23                        |
| 50                   | 1.265 ± 0.011                   | 0.23                        |
## Table 3. Thiabendazole recovery in white and red wine samples.

| White wine variety | Amount added (ng mL\(^{-1}\)) | Amount determined (ng mL\(^{-1}\)) | % Recovery |
|--------------------|-------------------------------|-----------------------------------|------------|
| Moschofilero       | 1.50                          | 1.45 ± 0.03                       | 96.7 ± 2.0 |
|                    | 3.00                          | 3.10 ± 0.13                       | 103 ± 4.3  |
|                    | 6.00                          | 5.74 ± 0.28                       | 95.7 ± 4.7 |
|                    | 15.0                          | 15.1 ± 0.61                       | 101 ± 4.1  |
|                    | 30.0                          | 27.3 ± 1.10                       | 91.0 ± 3.7 |
|                    | 60.0                          | 64.2 ± 1.56                       | 107 ± 2.6  |
| Savatiano          | 1.50                          | 1.47 ± 0.04                       | 98.0 ± 2.7 |
|                    | 3.00                          | 2.85 ± 0.12                       | 95.0 ± 4.0 |
|                    | 6.00                          | 6.2 ± 0.17                        | 103 ± 2.8  |
|                    | 15.0                          | 14.6 ± 0.44                       | 97.3 ± 2.9 |
|                    | 30.0                          | 32 ± 0.95                         | 107 ± 3.2  |
|                    | 60.0                          | 63 ± 2.85                        | 105 ± 4.8  |
| Assyrtiko          | 1.50                          | 1.43 ± 0.05                       | 94.6 ± 3.3 |
|                    | 3.00                          | 3.21 ± 0.11                       | 107 ± 3.7  |
|                    | 6.00                          | 5.56 ± 1.60                       | 92.7 ± 2.7 |
|                    | 15.0                          | 14.4 ± 0.22                       | 96.0 ± 1.5 |
|                    | 30.0                          | 32.4 ± 0.73                       | 108 ± 2.4  |
|                    | 60.0                          | 62.1 ± 2.41                       | 104 ± 4.0  |
| Organic wine (Savatiano) | 1.50                        | 1.45 ± 0.06                       | 96.7 ± 4.0 |
|                    | 3.00                          | 2.73 ± 0.10                       | 91.0 ± 3.3 |
|                    | 6.00                          | 6.18 ± 0.17                       | 103 ± 2.8  |
|                    | 15.0                          | 15.8 ± 0.34                       | 105 ± 2.3  |
|                    | 30.0                          | 27.3 ± 1.23                       | 91.3 ± 4.1 |
|                    | 60.0                          | 65.0 ± 2.97                       | 108 ± 4.9  |

| Red wine variety   | Amount added (ng mL\(^{-1}\)) | Amount determined (ng mL\(^{-1}\)) | % Recovery |
|--------------------|-------------------------------|-----------------------------------|------------|
| Agiorghitiko       | 1.50                          | 1.57 ± 0.02                       | 105 ± 1.3  |
|                    | 3.00                          | 2.82 ± 0.13                       | 94.0 ± 4.3 |
|                    | 6.00                          | 6.38 ± 0.13                       | 106 ± 2.2  |
|                    | 15.0                          | 15.6 ± 0.47                       | 104 ± 3.1  |
|                    | 30.0                          | 32.3 ± 1.41                       | 108 ± 4.7  |
|                    | 60.0                          | 62.9 ± 0.84                       | 105 ± 1.4  |
| Syrah              | 1.50                          | 1.47 ± 0.04                       | 98.0 ± 2.7 |
|                    | 3.00                          | 2.72 ± 0.12                       | 90.6 ± 4.0 |
|                    | 6.00                          | 6.08 ± 0.17                       | 101 ± 2.8  |
|                    | 15.0                          | 14.4 ± 0.46                       | 96.0 ± 3.1 |
|                    | 30.0                          | 27.3 ± 1.12                       | 91.0 ± 3.7 |
|                    | 60.0                          | 63.7 ± 2.89                       | 106 ± 4.8  |
| Zweigelt           | 1.50                          | 1.53 ± 0.03                       | 102 ± 2.0  |
|                    | 3.00                          | 2.84 ± 0.10                       | 94.6 ± 3.3 |
|                    | 6.00                          | 5.73 ± 0.25                       | 95.5 ± 4.2 |
|                    | 15.0                          | 16.2 ± 0.41                       | 108 ± 2.7  |
|                    | 30.0                          | 32.5 ± 1.24                       | 108 ± 4.1  |
|                    | 60.0                          | 64.1 ± 1.80                       | 107 ± 3.0  |
| Organic wine (local Greek variety) | 1.50                        | 1.55 ± 0.02                       | 103 ± 1.3  |
|                    | 3.00                          | 2.88 ± 0.08                       | 96.0 ± 2.7 |
|                    | 6.00                          | 6.19 ± 0.19                       | 103 ± 3.2  |
|                    | 15.0                          | 14.1 ± 0.56                       | 94.0 ± 3.7 |
|                    | 30.0                          | 28.2 ± 0.51                       | 94.0 ± 1.7 |
|                    | 60.0                          | 62.2 ± 2.52                       | 104 ± 4.2  |
more sensitive than an SPR immunosensor [26] which, however, does not employ labels. On the other hand, chromatographic techniques for the determination of thiabendazole in wines have limits of detection either similar to [14] or few times higher [13] than those achieved by the developed ELISA. It should be noted that the detection limit of the LC-MS/MS method used for comparison in the present study was 0.02 ng mL\(^{-1}\) for white and 0.04 ng mL\(^{-1}\) for red wines. Although LC-MS/MS can perform multi-residue analysis, which is of great importance for pesticides monitoring, the main advantage of the method developed is the very simple sample preparation procedure that completely avoids the use of organic solvents, the much lower cost of instrumentation and analysis and the capability to analyse a great number of samples in short time.

4. Conclusions

A competitive enzyme immunoassay for the determination of fungicide thiabendazole in white and red wines was developed. The method could be applied for determination of thiabendazole in wine after filtration and dilution of the sample 30 times with assay buffer. The method was sensitive with a limit of detection of 0.005 ng mL\(^{-1}\) which corresponds to 0.15 ng mL\(^{-1}\) in the undiluted wine. This detection limit is far below the MRL of 0.05 mg kg\(^{-1}\) of product set by the EU for thiabendazole in foods and beverages. In addition, the method was accurate with recoveries ranging from 90.6% to 108%, which is precise and fast (assay duration 2 h). Moreover, the results correlated well with those determined by a validated LC-MS/MS method, which however required a more complicated sample treatment prior to the analysis. Thus, the developed immunoassay could be used for the accurate detection of thiabendazole in both white and red wine samples regardless of the grapes variety used for wine production.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/03067319.2015.1100727.
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