A SALTING-OUT ASSISTED LIQUID-LIQUID EXTRACTION FOR THE DETERMINATION OF TEBUCONAZOLE, CARBOFURAN AND IMIDACLOPRID IN WHITE AND ROSÉ WINES

Fernando J. G. Daglia*, Josiele A. M. Conrado*, Luiz André J. Silva* and Alex D. Batista*,
*Instituto de Química, Universidade Federal de Uberlândia, 38400-902 Uberlândia – MG, Brasil

INTRODUCTION

Wine is an alcoholic beverage consumed worldwide, especially in European countries, such as Portugal, Italy and France, produced by the fermentation of different kinds of grapes.1 Grapes production and wine quality are highly influenced by climate, which delimitates the wine production areas and increases the occurrences of plagues. Thus, the use of pesticides is almost mandatory to achieve high production and high-quality grapes. Consequently, pesticides residues have been found in wines in the range of micrograms per liter.2,3 Although pesticides are found in wines at low concentrations levels, they can cause chronic effects on human health.4 Despite the increasing concern about food safety, the European Union has no specific legislation for pesticides levels in wines.5 Grapes are very susceptible to several plagues as they are produced at limited areas which increase the use of pesticides. Part of these pesticides is transferred to the wine during its production.6

A variety of pesticides have been applied to grapes for plagues control.7 Their application depends on the grape type and the region of the production. Tebuconazole is a systemic fungicide with a broad spectrum of action worldwide used in the control of pathogenic fungi in nuts, cereals, vegetables and fruits, especially in grapes.8 Imidacloprid is synthetic insecticide employed for control of insects in a variety of fruits, including grapes. Although it presents low toxicity, it can cause fertility problems in males when subjected to chronic exposure, even at low concentrations.9 In some countries with poor monitoring pesticides programs, forbidden pesticides are still employed which makes necessary the development of analytical methods for these chemicals. Carbofuran is a forbidden pesticide in U.S.A and Europe due its high toxicity and persistence on the environment. It was widely used as insecticide, nematicide and acaricide with a wide spectrum of activity against several agriculture pests.10

Several analytical techniques have been used for the residual determination of pesticides in wines. Due to the complexity of the wine samples, the proposed procedures for pesticides determination usually employ separation techniques, mainly liquid and gas chromatography with different detectors.9 The complexity of the wine samples and the low pesticides concentration levels found on these samples make mandatory the use of sample preparation steps for the removal of potential interferents and preconcentration of the analytes. Several techniques have been employed for this purpose, such as a solid-phase microextraction,11 stir bar sorptive extraction,12 matrix solid-phase dispersion13 and single drop microextraction.14

In order to overcome some limitations of existing multiresidues methods, a new procedure for the extraction of pesticides in agricultural food samples was presented, and it was named as QuEChERS (quick, easy, cheap, effective, rugged and safe).15 The unnecessary use of specific instrumentations, its excellent performance and compatibility with most separation techniques popularized the QuEChERS method.16 It is based on three steps: (i) extraction with acetonitrile; (ii) partition promoted by the addition of salts and (iii) dispersive solid phase extraction. Despite the satisfactory performance of the QuEChERS procedure, it consumes a relatively high amount of organic solvents and salts and involves many manipulation steps compared to microextraction techniques.

The aim of these work was to develop a cleaner alternative sample preparation to QuEChERS protocol based on salting-out assisted liquid-liquid extraction for the extraction of tebuconazole, carbofuran and imidacloprid from white and rosé wines.

EXPERIMENTAL

Chemicals and solutions

Ultrapure water (18 MΩ cm) was employed to prepare solutions and mobile phases. Acetonitrile (≥ 99.9 %) obtained from Merck was used in QuEChERS procedure and as a mobile phase component. Tebuconazole (≥ 98.0%), carbofuran (≥ 98.0%) and imidacloprid (≥ 98.0%) were purchased from Sigma Aldrich and stock solutions were prepared by dilution in methanol (HPLC grade, ≥99.9%, from Sigma Aldrich) and working solutions by dilution in the mobile
phase. Sodium chloride, magnesium sulphate and sodium acetate were obtained from Synth (Diadema, Brazil). Different wine samples (white and rosé wines) were obtained from local markets.

Salting-out assisted liquid-liquid extraction procedure

The SALLE was performed in a 1.5-mL Eppendorf® tube by the adding of 0.5 mL of sample (rosé or white wine), 0.5 mL of acetonitrile followed by manual shaker during 1 min. 50 mg of sodium chloride and 150 mg of sodium acetate were added into the flask and the mixture was shaken for 1 min. For the separation of organic and aqueous phases, the flask was centrifugated at 895 g-force by 5 minutes. 100 µL of the organic upper phase was collected by a microsyringe and injected into an HPLC-UV system for separation and detection.

All the optimization experiments were performed employing wines from producers that do not adopt the use of pesticides during the grape production to guarantee the absence of unknow concentrations of the target analytes during the optimization step. All the optimization experiments employed samples spiked with 500 µg L⁻¹ of each pesticide.

Instrumentation

A centrifuge from Enterprise, model C 1008-P, was used for phase separation during LLME procedure. Chromatographic analyses were performed on a Shimadzu LC-6AD equipped with an SPD-M20A diode array detector, a C18 CLC-ODS column (Phenomenex, 150 × 4.6 mm d.i., 5 µm) kept at 35°C. An SIL-20AC Autosampler was employed for sample injection and a LC-20AT pump for solvents delivery. Gradient elution was performed employing a mobile phase composed by acetonitrile (A) and water (B) as follow: start, 80% B; 10 min, 20% B; 15 - 20 min, 80% B with a flow rate of 1.0 mL/min⁻¹. Analysis time was 20 min including the chromatographic separation and column conditioning for the next analysis. Sample volume injected into the column was 20 µL. Detection was performed at 296 nm for imidacloprid and at 221 nm for tebuconazole and carbofuran. All the chromatographic analyses were performed at LabSolutions Software provided by Shimadzu.

RESULTS AND DISCUSSIONS

Salting-out assisted liquid-liquid extraction procedure optimization

The parameters optimization of the SALLE aimed to achieve a condition with high analytes extraction efficiency with minimum extraction of potential interference species. The salt added to the acetonitrile and wine mixture changes the physical-chemical properties of the system resulting in two phases system and partitioning the analytes preferably to the organic phase (salting-out effect). The efficiency of three different salts was evaluated as presented in Figure 1a. The binary mixture of NaCl and CH₃COONa achieved the best performance. The first salt (NaCl) contributes for the decreasing of the extraction of polar interferents from the matrix and improves solubility of the analytes in the organic phase, improving their extraction.¹⁷ The condition (B) without NaCl presented lower analytes extraction. The original QuEChERS procedure employs high amounts of salts and a high fraction of the added salts remains insoluble.¹⁵ Condition A and B presented a small amount of insoluble salts, while condition C (best composition) achieved complete solubilization of the salts, which indicates that the use of saturated mixtures is not mandatory to attain high analytes extraction efficiency. The proposed procedure employed 25-fold less salts without the need for saturated mixtures, which results in a cheaper and environmentally friendly procedure, as it produces less residues.

The influence of the proportion of NaCl and CH₃COONa was evaluated (Figure 1b) and no significant differences were observed for Carbofuran extraction in the evaluated conditions. Imidacloprid presented the best extraction efficiency at the condition with a higher proportion of CH₃COONa and tebuconazole at the higher proportion of NaCl. The acetate hydrolysis increases the pH of the aqueous phase changing the distribution of charged and uncharged species of the analytes, which could improve or compromise the extraction into the organic phase. Thereby, the proportion of NaCl and CH₃COONa was set at 1:3.

The original QuEChERS method employs a dispersive solid phase extraction (d-SPE) step after separation of organic and aqueous phases. This step involves the use a sorbent material, usually functionalized with a primary secondary amine (PSA) to remove polar interferences (including fatty acids, other organic acids, sugars and pigments, such as anthocyanins).¹⁶ The efficiency of the proposed SALLE procedure was evaluated without the d-SPE step and similar responses were achieved, indicating that this step can be avoided (Figure 2). Thereby, the SALLE is faster and cheaper than the conventional QuEChERS procedure. The high performance of the proposed strategy without d-SPE step can be explained by the fact that white and rosé wines are relatively less complex matrices compared to other food and environmental samples.

The performance of the optimized SALLE procedure was also compared to the conventional QuEChERS methodology by a statistical t-test (t_calc = 1.72, t_table = 2.13, n=5) and no significant differences were observed as presented in Figure 2. The same efficiency was achieved by the proposed procedure employing

![Figure 1](https://example.com/figure1.png)

**Figure 1.** (a) influence of the different salts on the pesticides extraction from white wine by SALLE; (b) influence of the proportion of NaCl and CH₃COONa on the extraction of pesticides from white wine by SALLE. Evaluation performed adding 500 µg L⁻¹ of each pesticide.
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25-fold less salt amount, 20-fold less acetonitrile and sample volumes, additionally to the absence of the d-SPE step. These features make the proposed strategy cheaper, faster and environmentally friendly. Chromatograms obtained by SALLE and QuEChERS are presented at Figure 3.

Analytical features

Calibrations were performed employing nine solutions with different concentrations of the pesticides and linear responses were observed from the 0.5 up to 1000.0 µg L⁻¹ with satisfactory linear coefficients as presented at Table 1. Limits of detection and quantification were experimentally estimated by decreasing concentration until signal noise ratio above three and nine, respectively. The repeatability of the SALLE was evaluated by the analysis of nine replicates of wine sampled added with 100 µg L⁻¹ of each pesticide and it was estimated at 1.4%, 2.4% and 2.2% for imidacloprid, carbofuran and imidacloprid, respectively. Recovery studies at three different concentration levels were performed for accuracy evaluation as presented at Table 2. Best recoveries, in most result, were achieved for higher concentration additions. The lowest evaluated concentration provided lower recoveries values. However, most of the values are accepted according to the European Commission that defines that procedures for pesticides determination on food samples from animal or vegetal origin can present recoveries on the range of 60 to 120% with relative standards deviation up to 30%. This highlights the potential of the presented procedure for the simultaneous analysis of these three pesticides. The procedure was also evaluated for the analysis of red wine samples. However, recoveries were unsatisfactory due to the more complex matrix composition.

The proposed procedure presents advantages compared to previous SALLE published procedures as shown at Table 3. It consumes smaller amount of organic solvent per analysis with one of the smallest RSD, which demonstrate a good precision of the procedure.

Application to real samples

Thirty samples (fifteen rosé wines and fifteen white wines) were analysed by the SALLE and all the pesticides were presented below the limit of detection. These samples were spiked with the pesticides and analysed again as presented at Table 4. The results highlight the applicability of the proposed procedure to the analysis of the analytes in rosé and white wines. Despite the higher matrix complexity of the rosé wines compared to white wines, good analytical features were obtained for these samples.
Table 2. Recoveries from white and rosé wine samples

| Pesticide   | Added (µg L⁻¹) | Recovery (%) White wine | Added (µg L⁻¹) | Recovery (%) Rosé wine |
|-------------|----------------|-------------------------|----------------|-----------------------|
|             |                | White wine              |                | Rosé wine             |
| Imidacloprid| 500            | 106                     | 500            | 106                   |
|             | 100            | 99                      | 100            | 105                   |
|             | 20             | 91                      | 20             | 93                    |
| Carbofuran  | 500            | 94                      | 500            | 99                    |
|             | 100            | 92                      | 100            | 102                   |
|             | 20             | 89                      | 20             | 93                    |
| Tebuconazole| 500            | 103                     | 500            | 108                   |
|             | 100            | 97                      | 100            | 100                   |
|             | 20             | 89                      | 20             | 94                    |

Table 3. Comparison of the proposed SALLE procedure to similar works previously presented

| Analyte / Sample          | Organic solvent / volume | Salt | RSD (%) | Recoveries (%) | Ref. |
|---------------------------|--------------------------|------|---------|----------------|------|
| Biogenic amines / Fish, meat | Acetonitrile/ 2.0 mL   | NaCl | 1.3 – 15 | 80 -115        | 20   |
| Cyanotoxins / water       | Acetonitrile/ 2.0 mL   | (NH₄)₂SO₄ | 3.9 – 14.6 | 61.5 – 99.5 | 21   |
| Insecticid / Honey        | Acetonitrile/ 1.0 mL   | CH₃COONa | 5.3 – 3.4 | 99.2 – 104.3 | 22   |
| Hydroyxymethylfurfural / Honey | Acetonitrile/ 3 mL | Na₂CO₃ | 1.6 -2.9 | 94.1 – 95.0 | 23   |
| Tylosin A, desmycosin / Honey | Acetonitrile/ 5 mL | CH₃COONa | < 7   | 90.2 - 111.2 | 24   |
| Vitamin D3 / Milk         | Acetonitrile/ 3 mL   | (NH₄)₂SO₄ | 1.6 - 11.4 | 94.4 - 113.5 | 25   |
| Pesticides / Wine         | Acetonitrile/ 0.5 mL | NaCl / CH₃COONa | 1.4 – 2.4 | 80 - 108 | This work |

Table 4. Analysis of rosé and white wines spiked with 100 µg L⁻¹ of each pesticide by the SALLE procedure

| Sample     | Imidacloprid | Carbofuran | Tebuconazole |
|------------|--------------|------------|--------------|
| Rosé wine  | 105.5 ± 2.5  | 101.3 ± 0.9 | 102.8 ± 2.4  |
| White wine | 101.3 ± 1.9  | 105.9 ± 1.1 | 105.7 ± 2.9  |
|            | 106.9 ± 2.1  | 102.8 ± 2.0 | 101.9 ± 1.9  |
|            | 105.9 ± 2.4  | 109.5 ± 1.2 | 109.1 ± 3.1  |
|            | 103.1 ± 1.8  | 104.1 ± 1.4 | 108.6 ± 2.6  |
|            | 109.8 ± 1.7  | 110.3 ± 2.3 | 100.2 ± 2.4  |
|            | 100.5 ± 2.0  | 108.5 ± 1.9 | 107.1 ± 2.9  |
| Rosé wine  | 104.9 ± 1.9  | 105.9 ± 0.9 | 99.8 ± 3.2   |
| White wine | 107.4 ± 2.4  | 103.8 ± 1.8 | 107.5 ± 1.7  |
|            | 99.8 ± 1.8   | 107.5 ± 1.3 | 102.7 ± 2.3  |
|            | 101.8 ± 2.8  | 102.8 ± 1.7 | 101.4 ± 2.7  |
|            | 106.5 ± 1.7  | 105.1 ± 2.4 | 108.8 ± 3.2  |
|            | 102.8 ± 0.9  | 104.7 ± 2.2 | 100.2 ± 1.6  |
|            | 100.3 ± 1.9  | 108.4 ± 1.5 | 103.5 ± 2.0  |
| Rosé wine  | 98.6 ± 2.8   | 107.9 ± 2.0 | 100.8 ± 1.1  |
| White wine | 109.5 ± 2.6  | 93.5 ± 1.2  | 99.1 ± 1.2   |
|            | 104.3 ± 3.0  | 97.9 ± 1.9  | 98.7 ± 2.0   |
|            | 108.3 ± 2.1  | 93.8 ± 2.0  | 101.3 ± 1.8  |
|            | 107.8 ± 2.2  | 91.7 ± 2.4  | 97.3 ± 2.5   |
|            | 101.6 ± 2.7  | 92.8 ± 1.7  | 103.7 ± 3.0  |
|            | 109.7 ± 1.9  | 99.3 ± 1.3  | 99.5 ± 2.1   |
|            | 110.1 ± 3.0  | 95.7 ± 2.5  | 104.7 ± 1.8  |
| White wine | 109.5 ± 2.0  | 100.2 ± 2.3 | 102.8 ± 2.7  |
|            | 109.4 ± 2.7  | 94.7 ± 1.4  | 100.3 ± 3.2  |
|            | 106.3 ± 1.9  | 98.8 ± 1.8  | 98.7 ± 2.0   |
|            | 108.7 ± 2.0  | 90.1 ± 2.0  | 103.8 ± 2.1  |
|            | 110.3 ± 2.5  | 99.6 ± 2.3  | 106 ± 1.7    |
|            | 105.7 ± 3.1  | 101.3 ± 1.4 | 97.8 ± 3.3   |
|            | 103.9 ± 1.7  | 95.4 ± 2.1  | 99.4 ± 2.6   |
|            | 107.6 ± 2.6  | 93.1 ± 2.3  | 98.9 ± 1.5   |

CONCLUSIONS

A SALLE was presented for the extraction of pesticides from white and rosé wines. The proposed procedure has remarkable advantages compared to the conventional QuEChERS procedure such as significant reduction on the consumption of sample and reagents and the absence of the d-SPE step, which makes the procedure cheaper, faster and environmentally friendly compared to conventional QuEChERS methodology. These features fulfil the requirements originally proposed by the methodology that should be quick, easy, cheap, effective, robust and safe. The proposed procedure can be applied to routine analysis of carbofuran, tebuconazole and imidacloprid in white and rosé wines.

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