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High-throughput modified QuEChERS method for the determination of the mycotoxin tenuazonic acid in wine grapes

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A reliable, simple, fast, inexpensive and robust sample preparation approach for tenuazonic acid (TA) determination in grapes by liquid chromatography with ultraviolet detection (HPLC-UV) is proposed. The method is based on a modified QuEChERS (quick, easy, cheap, effective, rugged and safe) approach using ethyl acetate as extraction solvent. Its involves extraction of 2.5 g sample (plus 2.5 mL 1% formic acid acidified water) with 5 mL of ethyl acetate; drying/cleaning-up of extract with 0.25 g anhydrous $CaCl_2$ and determination by HPLC-UV. The method was optimized and validated achieving a limit of quantification (LOQ) of 0.05 μg g^{-1}. The overall recoveries were 96%, 82% and 97% for grape samples spiked at 0.05, 0.5 and 5 μg g^{-1}, respectively. The method showed excellent reproducibility with RSDs for the above data ≤8% and Horwitz ratios <0.11. The procedure was applied to evaluate the occurrence of TA in grapes and it was quantified at concentrations ranging between 0.057 and 0.595 μg g^{-1}. The methodological development allows a high throughput routine quality control of grapes, adding a valuable tool for the winemaking industry. The method could be applied in an average laboratory and help to understand possible effects related to *Alternaria* rotting on the final wines elaborated. As well, this is the first report of TA presence in grapes used for wine elaboration, so the results add new knowledge to a growing research area.

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1. Introduction

Alternaria is a fungal genus with a number of species that can infect crops at pre- and post-harvest causing considerable losses due to rotting of fruits and vegetables.[1] Some *Alternaria* species are known to produce secondary metabolites toxic for human health, and among them tenuazonic acid (TA) is one of the most harmful.[2,3] Additionally, TA has been associated to pre-cancerous changes in the esophageal mucosa of mice fed with TA at 25 mg kg^{-1} body weight per day during 10 months and with human hematological disorders such as onylai, a form of thrombocytopenia.[4,5] Presence of TA has already been reported in different food commodities such as tomato products, olives, cereals, carrots and fruits juices, amongst others.[3,6–10] Although there is no current regulation about the presence of *Alternaria* mycotoxins in foods, in 2011, the panel of European Food Safety Authority (EFSA) published its scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. They suggested that *Alternaria* toxins are of high concern for public health and encourage more studies to assess the real extent of food contamination.[11]

Alternaria spp. is part of the main wine grape mycobiota from different winemaking regions worldwide.[12] Due to its opportunistic pathogenic nature, it has been associated to grape berry rot in the field under conducive environmental conditions,[13–15] increasing the chances to found their mycotoxins.[16] There are few reports of natural occurrence of TA in grapes and its derived products such as wines and grape juices.[7] Mikušová *et al.* reported the production of mycotoxins in wine grapes grown in Slovakia, observing that TA had the highest concentrations between the toxic compounds produced by *Alternaria* spp. in dried berries.[17] Recently, Prendes *et al.* reported the prevalence of *Alternaria alternata* as the main component of the wine grape mycobiota of the DOC San Rafael (*Vitis vinifera* L. cv. Malbec), Mendoza, Argentina at harvest time.[16] They also described a high frequency and levels of TA production by *A. alternata* strains isolated from the grapes.

Due to the increase of wine consumers' awareness and attention to health risks related to food safety, monitoring TA occurrence to assess the extent of mycotoxin contamination in wine grapes is of great concern worldwide.[18] In this way, the development of robust and innovative analytical methods to evaluate levels of TA in grapes is a subject of actual interest.

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Both, industry and consumers show awareness, the first to perform quality control of the material used for the production of its wines, the later to establish concentration limits for this toxin and to know the real exposure though the food.

Several methods for assessment of TA in foods have been reported, generally based on high performance liquid chromatography (HPLC) coupled with UV and mass spectrometry (MS) detection.[3,16,19–22] Mass spectrometry has not been widely developed and most of the available methods applied a derivatization step to allow the compatibility with electrospray ionization source (ESI).[21,22] This is due to the relatively high acidity ($pK_a = 3.5$) of TA which requires working at low pH conditions to obtain acceptable peak shapes in HPLC by suppressing the on column ionization.[8] Additionally, TA is a metal chelating compound, which showed irreproducible chromatographic behavior unless modifiers like $Zn(II)SO_4$ were added to the mobile phase.[22] This approach is also incompatible with MS detection, restricting the direct detection without derivatization to UV absorption.[22]

Because of the complexity of grape samples, which includes various compounds of different chemical nature, and the low concentration expected of TA, sample preparation is critical for its highly sensitive and selective determination. Various strategies have been reported in the literature for the extraction of TA in foods. In general, TA is extracted from solid and liquid matrices with organic solvents (acetonitrile, ethyl acetate, methanol, chloroform) or different solvent mixtures by liquid–liquid extraction (LLE), being the acidification of extraction solvent crucial to achieve good recoveries.[3,7,8,10,19,22] For the purification and concentration of extracts, solid phase extraction (SPE) is the most selected technique by using different sorbent materials such as C_{18} and hydrophilic–lipophilic balance (HLB).[6,10,22] In recent years, the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method has been widely used as sample preparation technique. From its introduction by Anastassiades *et al.*,[23] QuEChERS has acquired excellent reputation due to its simplicity and applicability to several analytes and matrices of different chemical nature. In fact, the method allows excellent results in terms of analytical performance and simplification of quantification in complex matrices.[24] Different modifications of salting-out and d-SPE steps were introduced with the aim to reduce extraction solvent volume and co-extracted interferences prior to instrumental analysis.[24–28] QuEChERS with different modifications was recently applied for the determination of different *Alternaria* toxins in a variety of samples.[2,8,21,29–32] Only two of these works determined TA in samples of apple and tomato processed products using HPLC-MS/MS;[30,32] but its optimization and validation for the extraction of TA from grapes and its quantification by HPLC-UV has not been reported yet. Considering the different nature of grape matrix to thus previously reported, the challenge of recovering TA due to its complex chemical behavior and the absence of previous exhaustive optimization of sample preparation conditions, the validation of a specific high throughput analytical approach is needed.

In this work the development, validation and application of a modified QuEChERS-based extraction method followed by HPLC-UV for the reliable quantification of the mycotoxin TA in grapes is reported. Sample preparation conditions were optimized in order to maximize the sensitivity and selectivity of the methodology. The performance of the proposed method was evaluated in terms of limits of detection (LODs), limits of quantification (LOQs), recoveries (accuracy) and linear working range. Additionally, the validated analytical method was applied to determine the presence of TA in wine grapes collected from the DOC San Rafael, Mendoza, Argentina.

2. Experimental

2.1. Standards, solvents and sorbents

Copper salt of TA (≥98%) was purchased from Sigma-Aldrich (Steinheim, Germany). The salt was converted into its free form as described in the literature.[8,20,22] Stock solutions of TA were prepared in methanol (MeOH). Further dilutions were prepared monthly in MeOH and stored in brown bottles at −20 °C to ensure stability.

HPLC-grade MeOH, acetonitrile, hexane and formic acid (FA) were purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Ethyl acetate was from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

Analytical grade sorbents (50 μm particle size) for d-SPE, including primary-secondary amine (PSA), octadecylsilane (C_{18}) and graphitized carbon black (GCB) were both obtained from Waters (Milford, MA, USA). Reagent grade NaCl, $MgSO_4$ and $CaCl_2$ for method development were purchased from Biopack (Buenos Aires, Argentina). Sodium phosphate monobasic (NaH_2PO_4) and *o*-phosphoric acid were obtained from Sigma-Aldrich. The NaH_2PO_4 buffer at 0.1 mol L^{-1} used for chromatographic phase was prepared by dissolving 11.98 g of salt in ultrapure water, adjusting pH to 3 with concentrated phosphoric acid and bringing to 1 L final volume.

2.2. Samples

During the 2015 vintage, grapes of 13 vineyards of *Vitis vinifera* L. cv. Malbec representative of the DOC San Rafael wine grape-growing region (Mendoza, Argentina) were sampled at harvest time. The total geographical area selected for sampling was located between 34.3° and 34.8° S latitude, 67.4° and 68.5° W longitude, with an altitude ranging between 500 and 800 m a.s.l. Briefly, an independent sample was taken from each vineyard containing grape bunches collected at 1.5 m from the ground from 12 plants homogeneously distributed in the vineyard (a bunch per plant) to reach 1 kg approximately, following a procedure previously described.[33] Additionally, a sample was collected from a visually infected bunch found in one of the thirteen vineyards sampled. All samples were kept in plastic bags and placed in ice cooled boxes during transportation to the laboratory. Then, each sample was ground completely in a laboratory mixer, three replicates aliquots of each were collected in 50 mL plastic tubes and finally stored at −20 °C until submitted to the extraction procedure. Extraction conditions were optimized with aliquots of a grape sample took from

the field spiked with TA at 1.88 μg g^{-1} level. This sample was also used to perform matrix-matched calibration and recovery studies at different concentration levels. Non-spiked aliquots were analyzed for taking into account during calculations of recoveries as is described later.

2.3. Sample preparation procedure

Homogenized grape sample (2.5 g) was weighed into 50 mL PTFE centrifuge tubes. Then, 2.5 mL of ultrapure water (acidified with 1% FA) were added. After slurring the sample with water, 5 mL ethyl acetate were added and the tube vigorously hand-shaken for 1 min to ensure adequate homogenization of sample and extraction solvent. For phase separation, 4 g of $MgSO_4$ and 1 g of NaCl were added; the tube was shaken for 1 min and centrifuged for 10 min at 8000 rpm. Thereafter, the top layer was transferred to a tube containing 0.25 g anhydrous $CaCl_2$, vortexed for 30 s and centrifuged at 8000 rpm for 5 min. The supernatant was collected in a glass khan tube and evaporated to dryness (SpeedVac concentrator). Finally, the residue was re-suspended in 0.5 mL mobile phase [MeOH : 0.1 M NaH_2PO_4 (2 : 1 v/v), adjusted to pH 3.2] and 20 μL were injected in the HPLC-UV. Fig. 1 shows a scheme of the modified QuEChERS procedure.

2.4. HPLC-UV analysis

TA was determined using a HPLC-MWD system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany). The HPLC instrument was a Dionex Ultimate 3000 consisting of vacuum degasser unit, autosampler, quaternary pump and chromatographic oven. The detector was a Dionex MWD-3000 (RS) model with an analytical flow cell operated with a data collection rate of 5 Hz, a band width of 4 nm and a response time of 1.000 s. The working wavelength for the analyte was 279 nm. The Chromeleon 7.1 software was used to control all the acquisition parameters of the HPLC-MWD system and also to process the obtained data.

HPLC separations were carried out in a Kinetex XB-C_{18} column (4.6 mm × 150 mm, 5 μm) Phenomenex (Torrance, CA, USA) using a previous reported method as a reference with modifications.[20] The mobile phase consisted in a mixture of MeOH and 0.1 M NaH_2PO_4 pH 3 (2 : 1 v/v), adjusting the blend to pH 3.2 with phosphoric acid. Samples were analyzed by using an isocratic method at a flow rate of 1.5 mL min^{-1} during 4 min total run time. The column temperature was 30 °C and the injection volume 20 μL. The identification and quantification of TA in grape samples was based on the comparison of the retention times (t_R) and absorbance values of detected peaks in samples with those obtained by injection of pure standards of the analyte. Additionally, samples were spiked with known concentrations of compounds in order to verify the peak identification and the absence of interferences at the analyte t_R. Samples were quantified by using a matrix-matched calibration.

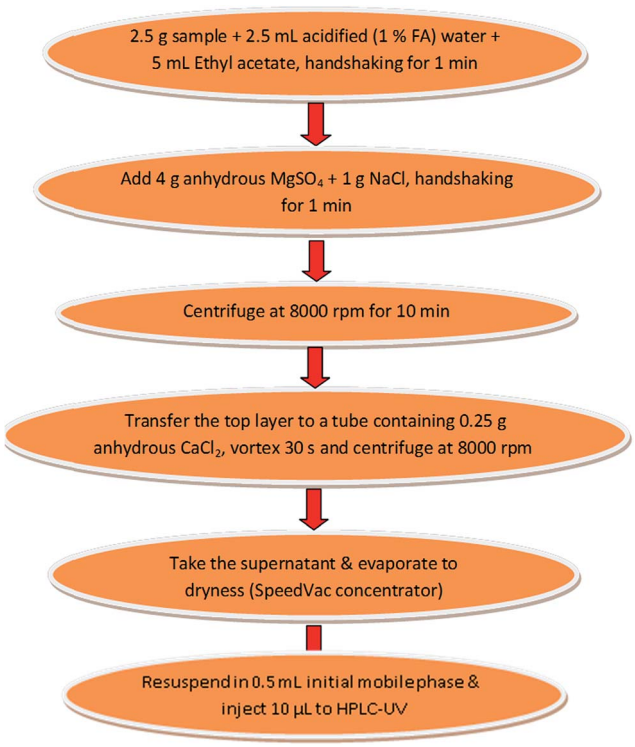

Fig. 1 Scheme of the proposed modified QuEChERS method for the sample preparation of TA in wine grapes.

2.5. Calibration curves, linearity, LOD and LOQ

To evaluate the linearity of the calibration curves, standard solutions were prepared at six concentration levels (0.25, 0.5, 1.25, 2.5, 5.0 and 12.5 μg mL^{-1}) in organic solvent and in grape matrix (both finally re-suspended in HPLC mobile phase). They were injected in triplicate into the HPLC-UV system and calculations were performed based on the average peak areas. Calibration curve regression equations with their determination coefficients (r^2) were calculated and the linear range for TA was determined.

The LOD of the target compound was set at signal to noise (S/N) of 3 being defined as the minimum concentration of target analyte that can be detected by the instrument with a response significantly higher than the background. The lowest concentration of analyte that has been validated with acceptable accuracy (recovery within the range 70–120% and RSDs ≤ 20%) by applying the complete analytical method is defined as method LOQ.[34] Its calculation is based on the accuracy and precision data obtained *via* the recovery study.

2.6. Interferences effect

Matrix effects (ME) are defined as positive or negative responses produced by compound/s (interferences) other than the target analyte that influence the measurement of its concentration or mass.[34] The interference is also referred to as "chemical noise" and ME are a subtle form of interference that could be minimized by a better detector selectivity. If interference cannot be eliminated or compensated, its effects may be acceptable if the impact on accuracy is not significant.[34] Potential ME (%) for TA caused by interferences occurring during HPLC-UV analyses

Table 1 Analytical performance, absolute recoveries (%, as an estimation of accuracy) and precision of the proposed method for grapes spiked at different concentration levels

Linear range (μg g^{-1})	Calibration curve	r^2	LOD (μg g^{-1})	LOQ (μg g^{-1})	Recovery (%) ± SD, $n = 3$ replicates			Precision (RSDs, %)	
					0.05 μg g^{-1}	0.5 μg g^{-1}	5 μg g^{-1}	Intra-day[a]	Inter-day[b]
TA LOQ-5	$y = 2.6606x + 0.0232$	0.9921	0.01	0.05	96 ± 6	82 ± 2	97 ± 7	5	13

[a] $n = 3$ replicates of the grape sample spiked at the 0.05 μg g^{-1} level processed in the same day. [b] $n = 9$ replicates of the grape sample spiked at the 0.05 μg g^{-1} level processed in 3 consecutive days.

were evaluated by comparing the responses from solvent and matrix-matched standards; and were calculated as follows:

$$\mathrm{ME} = \left[\frac{A_{ss} - A_s}{A_s}\right] \times 100$$

where A_{ss} is the response (peak areas) measured for spiked (addition was carried out over grape samples, before extraction), and A_s is the response obtained for a standard with the same concentration.

2.7. Recovery and precision

The accuracy and precision of the method were evaluated through recovery experiments by spiking TA to a portion of grape, at three different concentration levels, with three replicates at each level ($n = 3$). The spiked levels are shown in Table 1 and corresponded to LOQ, 10× LOQ and 100× LOQ. The spiking procedure was performed by adding an aliquot of the standard solution of TA to the mixture of grape and acidified water at the beginning of the procedure before applying the extraction method. Samples were mixed 30 s by vortex, kept 5 min for equilibration and subsequently submitted to the extraction process. The absolute recoveries (R, %) of the proposed method were calculated as the difference between the concentrations measured for extracts from spiked (C_s) and non-spiked aliquots (C_b) of grapes divided by the theoretical concentration (C_t) added to the sample, and multiplied by 100,

$$R\% = \left[\frac{C_s - C_b}{C_t}\right] \times 100$$

where C_s and C_b were established against a calibration curve obtained for matrix-matched standards.[35]

The precision in terms of repeatability and intermediate precision was determined at 0.05, 0.5 and 5 μg g^{-1}. Horwitz ratio (HorRat), a parameter for normalized performance that indicate the acceptability of a method related to laboratory precision (reproducibility),[36] was calculated as follows:

$$\mathrm{HorRat} = \frac{\mathrm{RSD_R}(\%)}{\mathrm{PRSD_R}(\%)}$$

where $\mathrm{RSD_R}$ (%) was the observed relative standard deviation calculated from the performance data, and $\mathrm{PRSD_R}$ (%) was the predicted relative standard deviation calculated from the Horwitz equation $\mathrm{PRSD_R}$ (%) = $2C^{-0.15}$, where C is the concentration found or added, expressed as a mass fraction (0.05 μg g^{-1} = 5×10^{-13}). Consistent deviations on the low side (values < 0.5), indicate an unreported averaging or excellent training and experience. Systematic deviations on the high side (values > 2) could be related to inhomogeneity of the tested samples, need further method optimization or training, operating below the limit of determination, or an unsatisfactory method.[36]

3. Results and discussion

3.1. Optimization of extraction conditions

The optimum extraction solvent was selected based on the solubility of TA, selectivity (lower co-extraction of matrix components) and R% of the analyte. Different extraction solvents including acetonitrile, hexane and ethyl acetate were evaluated. Other authors used acetonitrile as extraction solvent for extracting TA with QuEChERS in tomato and pepper products[30,32] achieving R% near to 100%, however other solvents were not evaluated. In this work, when acetonitrile was used, the chromatograms obtained did not have adequate resolution for TA which appeared partially overlapped with matrix interferences. In contrast, injections of the extracts obtained using ethyl acetate and hexane exhibited defined peaks and good selectivity. As can be observed from Fig. 2a, ethyl acetate showed the highest R% for TA, and hence it was chosen as the extraction solvent. These results could be explained since the medium polarity index of this solvent that reduces the extraction of high amount of interferences (such as phenolics) that are better recovered using acetonitrile at acidic pH. On the other hand, hexane has a very low polarity index, and thus its affinity for a polar compound such as TA is considerable lower than the observed for ethyl acetate.

The sample to solvent ratio was studied to achieve the highest recoveries with the minimum sample and solvent consumption, as well as to get good sensitivity for TA in grape samples. To determine the influence of extraction solvent volume, a series of separate sets of extractions was performed using 2.5 g sample diluted with 2.5 mL water (acidified with 1% FA), using 2.5, 5 and 7.5 mL ethyl acetate. Fig. 2b shows that the best results were achieved with 5 mL of ethyl acetate. A volume of 2.5 mL rendered a lower recovery, probably because the reduced volumetric recovery of extraction phase. The volumetric recovery for 2.5 mL was 72%, besides the obtained for 5 and 7.5 mL ethyl acetate were between 88–89% (data not shown). The sample/water/solvent ratio was studied maintaining the same spiking level for each assay and a constant volume of 5 mL for ethyl acetate as extraction solvent. The acidification level was

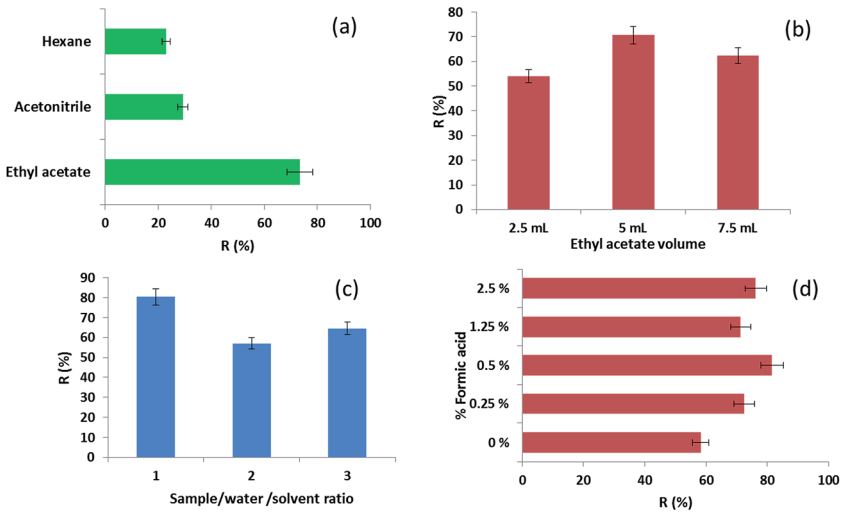

Fig. 2 (a) Effect of extraction solvent on the *R*% of TA; (b) effect of ethyl acetate volume on the *R*% of TA; (c) effect of sample/water/solvent ratio on the *R*% of the studied analyte, (1) 2.5 g grape : 2.5 mL water : 5 mL ethyl acetate; (2) 5 g grape : 2.5 mL water : 5 mL ethyl acetate and (3) 5 g grape : 0 mL water : 5 mL ethyl acetate; (d) effect of sample pH on the *R*% of TA. Extractions conditions: 2.5 g grape : 2.5 mL acidified water (FA according to each assay): 5 mL ethyl acetate; phase partitioning: 4 g anhydrous $MgSO_4$ + 1 g NaCl. No clean-up or drying step. The supernatant was collected in a glass khan tube and evaporated to dryness.

maintained at 0.5% FA for each separate assay. The results presented in Fig. 2c shows that the highest *R*% is obtained by using a combination of 2.5 g grape sample + 2.5 mL water + 5 mL ethyl acetate. By increasing the sample amount to 5 g, keeping the other factors constant, lower *R*% was obtained. It could be explained as the polar TA did not partition completely into the upper ethyl acetate phase due to the higher water content reached (higher sample amount). Anastassiades *et al.*[23] reported a similar result using different sample/solvent ratios with acetonitrile as extraction solvent. Avoiding the use of water (keeping the acidification to 0.5% FA by directly adding the acid), *R*% was 15% lower than the best condition. Both results were characterized by a reduced volumetric recovery. The dilution of samples at a compromising point appears critical to achieve the highest recoveries. In the light of the observed results, a sample/water/solvent ratio of 2.5 g grape : 2.5 mL water : 5 mL ethyl acetate was selected to perform further assays.

The sample pH adjustment prior to extraction is a commonly used strategy to increase the recovery of acidic or basic compounds. The strong acidity of TA (pK_a 3.5) makes the pH a critical parameter, where the addition of an acid solution to diminish sample pH increases recovery of the analyte from grapes. Working at acid pH, the equilibrium shifts towards the less polar form and the analyte is mostly as a neutral molecule, facilitating the extraction with medium polar organic solvents such as ethyl acetate. The pH not only has a strong influence on the recoveries, but also on the co-extraction of matrix components and in the selected clean-up strategy. The pH effect on the TA extraction was evaluated by adding different concentration of FA (0.25, 0.5, 1.25 and 2.5% v/v) and compared with a sample without pH modification. As can be observed from Fig. 2d, the acidification improved the *R*% of TA between 13 to 23% compared to results without FA addition. These results may be explained due to the pK_a of the studied analyte. At a sample pH lower than 3.5, the TA is in its neutral form favoring its partition to the ethyl acetate phase and increasing the *R*%. The best results were obtained with the addition of 0.5% FA, and thus it was the selected condition.

3.2. Optimization of clean-up

After QuEChERS extraction, the possibility and necessity of an additional clean-up of sample extract using d-SPE was evaluated. Various sorbents and salts for drying including PSA, C_{18}, GCB, $MgSO_4$ and $CaCl_2$ were evaluated alone and in different combinations. The best results, evaluated as recovery and minimization of matrix interferences, were obtained using no sorbent at all, but just applying a drying/clean-up step with anhydrous $CaCl_2$. The $CaCl_2$ gives a recovery of 83% for TA, having an adequate reduction in matrix components compared with other evaluated sorbents (see Fig. 3). The drying with $CaCl_2$ has a beneficial effect because the removal of water makes the final extracts less polar, which produces the precipitation of certain polar co-extractives; a visible precipitation of red grape pigments (purportedly anthocyanins) was observed to some extent during and after drying extracts with $CaCl_2$. This could also be attributed to the formation of Ca^{2+} complexes of the anthocyanins insoluble in ethyl acetate. This results agree with thus reported previously by Anastassiades *et al.* for strawberry extracts.[23] As well, anhydrous $CaCl_2$ increases the ionic strength of the medium. In fact, the lower amounts of water and the high ionic strength favor partition of neutral analytes (TA is well below its pK_a) to ethyl acetate phase. The use of $MgSO_4$ and a combination of $MgSO_4$ + $CaCl_2$, leads to *R*% of 72 and 74% respectively, with a background level similar to thus obtained with $CaCl_2$. The d-SPE step using different sorbents did not

show improvements, on the contrary, for all sorbents and combinations a reduction in *R*% was observed. It was interesting the achieved effect for PSA. A considerable reduction in the chromatograms background was observed by using this sorbent, reaching S/N comparable to $CaCl_2$. But the *R*% of the analyte was also lowered near to 24% probably due to the strong acidity of TA. PSA has primary and secondary amino groups that act removing acidity from extracts, thus increasing the risk of base-catalyzed degradation. PSA forms hydrogen bonds with compounds containing hydroxy or carboxy groups, and previous reports indicated that pesticides containing groups that have potential for hydrogen bond formation, such as basic nitrogen (–NH–, =N–) and hydroxy compounds (–OH), both included in the TA structure, are affected most deeply by these effects.[23] In this way, the analyte was able to interact with the sorbent, being strongly retained and reducing its recovery. As well, the basic nature of PSA also increase the pH of the medium, thus acids are in their ionic forms at pHs well above their pK_a's and the ionic strength from the $CaCl_2$ was not enough to force the ions into ethyl acetate. The same facts cause the lower background of the chromatograms (anthocyanins and phenolics are also retained in PSA). With the intention to improve the *R*% maintaining the beneficial clean-up facets of PSA, a buffering step using FA at different levels was tested (Fig. 3). The results obtained in these experiments showed an increase of *R*% (52 and 66% for 2 and 4% FA in the extract, respectively). On the contrary to *R*% results, the background of chromatograms also augmented (see Fig. 3), demonstrating the direct dependence of analyte *R*% and interferences retention on PSA sorbent with the pH of the medium. When C_{18} and GCB were evaluated, *R*% of 65 and 75% were obtained. Combining of C_{18} and GCB with $CaCl_2$ and PSA (buffering with 4% FA), rendered *R*% of 65 to 68%. In terms of chromatograms background, the use of GCB alone reduced the amounts of interfering peaks in the first segment of the chromatogram. This behavior is due to GCB has a strong affinity toward planar molecules, removing efficiently pigments and sterols. When GCB was combined with PSA an increase of interfering peaks was observed which is caused by the pH increase in the medium, as it was commented above. The same buffering strategy was also evaluated for clean-up with drying salts but no improvement in *R*% was observed. Grape extracts seem to have a low pH, which contributes to the effective extraction yields of TA. The dilution of grape samples with acidified water maintains the pH low enough for good recoveries, making the buffering step unnecessary. Taking in mind the discussed results, the usage of any sorbent material additional to anhydrous $CaCl_2$ was avoided, adding simplicity and saving costs regarding the application of this method in a routine work.

3.3. Performance of the analytical procedure

The analytical figures of merit of the optimized method are compiled in Table 1. The accuracy and precision of the method were assessed through recovery experiments by spiking TA to a portion of grape at three different concentration levels (0.05, 0.5 and 5 μg g^{-1}). In all cases, spiked and non-spiked aliquots were processed in triplicate and the concentrations of TA in the corresponding extracts determined by the calibration curve obtained for matrix-matched standards. According to established parameters, previously developed and accepted for pesticide analysis, the method performance criteria as to accuracy (trueness) and precision in the validation study are normally recoveries within the range of 70–120% and repeatability RSD ≤ 20%.[34] As can be seen in Table 1, the recoveries obtained for 2.5 g portions of grape were in the range of 82–97%, confirming the high accuracy of the method. The

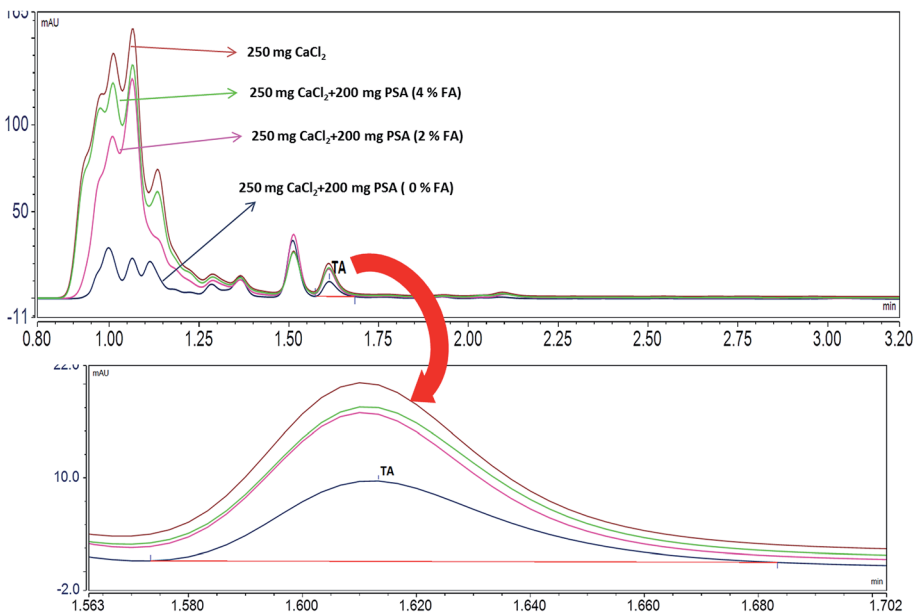

Fig. 3 Effect of PSA (and drying with $CaCl_2$) as d-SPE sorbent on the chromatograms background signal with and without using FA at different concentrations. Extraction conditions as described in Section 2.3.

associated standard deviations varied between 2 and 7%, showing very good repeatability (RSD%) results for all spike levels studied. HorRat values were also determined to confirm the acceptability of the observed RSD_R at the evaluated concentrations. HorRat of TA calculated at the three addition levels mentioned above were 0.02, 0.02 and 0.11, respectively. Observed repeatability was better than generally accepted guidance limits for repeatability based on blind duplicates, indicating satisfactory intra-laboratory precision. The inter-day accuracy and precision was assessed with 2.5 g portions of grape, spiked at the 0.05 μg g^{-1} (LOQ level) and processed in triplicate during 3 consecutive days. In this case, the recovery of the method was 112%, with a RSD of 13%. Consequently, the achieved results satisfied the validation criteria at all of the spike levels demonstrating the suitability of the proposed QuEChERS-HPLC-UV method for the accurate quantification of TA mycotoxin in grapes.

The comparison of the responses from solvent and matrix-matched standards showed a ME of −34% for TA caused by interferences occurring during HPLC-UV analyses. Thus, in order to compensate the errors associated with the observed interferences, matrix-matched standards were used as calibration technique to achieve accurate quantification of the target analyte.

The achieved LODs and LOQs of the analyte for extraction of 2.5 g grape sample, calculated as described in Section 2.5., showed that the proposed QuEChERS-HPLC-UV method shows a suitable sensitivity according to the levels expected in grapes. As well, the achieved LOQs are similar to those reported in previous works using SPE or LLE coupled with LC-MS/MS with and without previous derivatization step.[8,22]

The selectivity of the QuEChERS method for the determination of TA was evaluated by the comparison of t_R and spectral behavior achieved by analyzing a standard solution of TA and a QuEChERS extract of grape after applying the optimized method. Fig. 4 shows the chromatograms obtained for a standard, a spiked grape sample and a positive grape sample (sample M14). As can be observed, the t_R obtained after analyzing a grape sample did not show significant differences with the obtained for the standard as well as no interferences were detected at the analyte t_R.

3.4. Sample throughput, economics of analysis and comparison with other methodologies

The validated method, as compared with previous developed approaches, showed a substantial improvement in terms of total analysis time, simplicity and reduction of expenses of each analysis (Table 2). Considering the equipment and infrastructure of an average laboratory, a single chemist could prepare around 40 samples in 8 h working period with the proposed QuEChERS method. In terms of HPLC-UV analysis, we proposed a simple isocratic method with a total run time of 4 min, allowing the analysis of *ca.* 100 samples in 8 h cycle. Previously reported HPLC methods require between 10 to more than 20 min to achieving the determination of TA and most of them use different chromatographic gradients, requiring additional

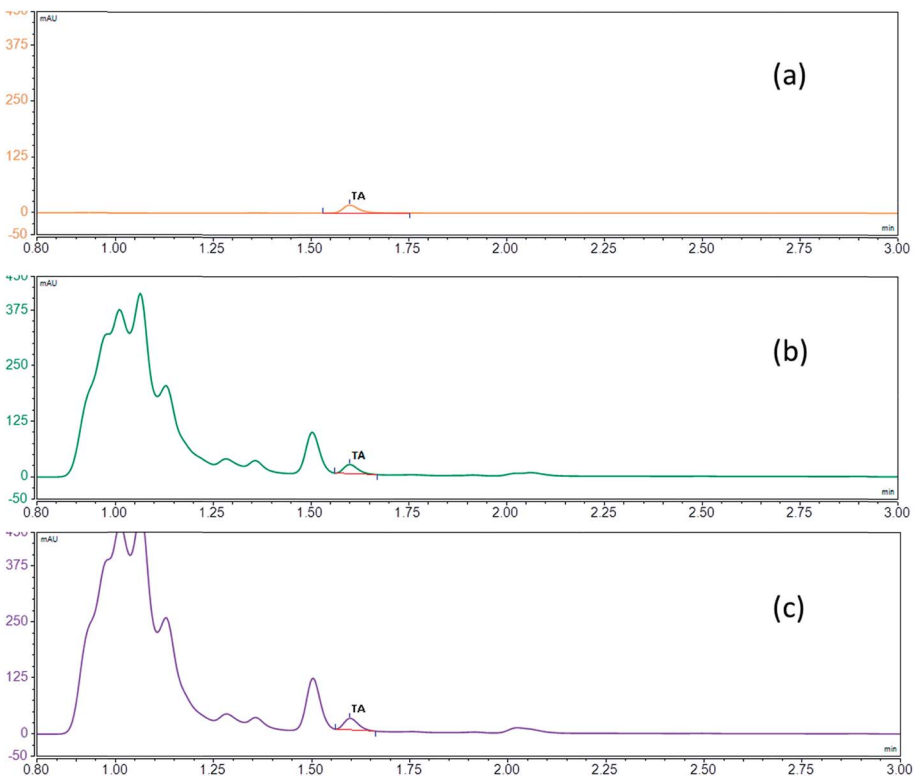

Fig. 4 Extracted chromatograms at 279 nm. (a) Solvent standard at 1.25 μg mL^{-1}; (b) QuEChERS extract of a grape sample spiked with 0.5 μg g^{-1} TA standard. (c) QuEChERS extract of a grape sample (code M14) without addition of TA.

Table 2 Determination of TA by using different analytical methodologies

Determination technique	Pretreatment method	Solvent volume (mL)	Extraction time (min)	Sample	LOD	Linear range	Recovery (%)	Ref.
HPLC-MS/MS	QuEChERS	10	25[b]	Tomato and pepper products	0.86 μg kg^{-1}	5–25 ng mL^{-1}	91–102	22
HPLC-MS/MS	QuEChERS	10	25[b]	Tomato products	0.29–0.38 μg kg^{-1}	Not informed	99–102	24
HPLC-MS/MS	SPE	100–200	≈40	Apple juice, tomato sauce, beer, olives and dried basil	1.2–7.1 μg mL^{-1}	20–1000 μg mL^{-1}	51–81	6
HPLC-UV	LLE + SPE	50	≈35	Carrots	20 μg kg^{-1}	Not informed	62–76	3
HPLC-MS/MS[a]	LLE	10	30	Cereals	10 μg kg^{-1}	50–5000 μg kg^{-1}	≈79	8
HPLC-UV	QuEChERS	5	≈12	Wine grapes	10 μg kg^{-1}	50–5000 μg kg^{-1}	82–97	This work

[a] With derivatization. [b] Only for the shaking step.

times for column equilibration.[3,6,8,30,32] The solvent exposure to the analyst was also considerably lower as well as the overall waste generation was minimized due to the fast chromatographic analysis. The input cost of sample processing for a single sample was below 1 USD, which is cheaper compared to the other available methods such as SPE or traditional LLE.[6,8,9,17,22] These previously reported methods require between 5 to 20 mL ethyl acetate or methanol/acetone mixtures for quantitative TA extraction as well as longer chromatographic methods are applied. In terms of sample throughput, our method has an absolute advantage, considering the time consuming SPE and traditional LLE techniques, much of them also using lengthy extra derivatization steps previous HPLC-MS. As can be seen from Table 2, the achieved LODs are similar to those reported in previous works using SPE or LLE coupled with HPLC-MS/MS or HPLC-UV with and without additional derivatization steps.[3,8,22] The previously QuEChERS reported methods for tomato and pepper products provided higher sensitivity and comparable recoveries for TA when are compared with the presented here. Nevertheless, these approaches involve more complex and lengthy enrichment procedures as well as determination techniques, with the additional increasing costs of sample analysis. Besides of that, the advantages in terms of superior resolutions, selectivity and sensitivity of these methods using HPLC-MS are undeniable being much times the choice when the complexity of the samples increases. However, from an economical point of view, these systems could be too costly or not accessible for most laboratories. Taking this in mind, the method proposed here with a core–shell column (with simple UV detection) and an efficient sample preparation based on a modified QuEChERS method is able to achieve cost-effective results on any standard laboratory, which is an advantage compared with the use of MS systems.

3.5. Testing the method for evaluating natural occurrence of TA in grapes

The developed and validated QuEChERS-HPLC-UV method was applied for the determination of TA to a total of 14 samples of red grapes from cv. Malbec grapevines of DOC San Rafael, Mendoza, Argentina. The levels of the toxin in the analyzed samples are summarized in Table 3 as well Fig. 5 show a map with the distribution and origin of samples in the DOC San Rafael, Mendoza, Argentina. TA was present in more than 57% of the analyzed samples, with a maximum concentration of 0.595 μg g^{-1} (sample M14, Fig. 4). It is convenient to mention that the sample collected from the visually infected bunch reach the highest level of TA, suggesting a positive correlation between the fungal development and the presence of TA, although its mycobiota was not determined. The results reported here represent an important evidence of the production of TA in natural conditions where grapes are cultured, being also relevant from the winemaking point of view. There is only one previous report of natural occurrence of TA in grapes from three Slovak winemaking regions, with concentrations ranging 0.7 to 31 μg g^{-1} but they were dried wine berries.[17] Additionally, we consider that our results are in agreement and complement those reported by Prendes *et al.*[16] Since *Alternaria alternata* has been reported the main component of the wine grape

Table 3 Occurrence of the target TA in non-spiked wine grape samples. Average concentrations (μg g^{-1}) with their standard deviations, $n = 3$ replicates[a]

Sample code	Level found
M1	n.d.
M2	n.d.
M3	n.d.
M4	n.d.
M5	n.d.
M6	n.d.
M7	<LOQ
M8	<LOQ
M9	0.096 ± 0.003
M10	0.057 ± 0.001
M11	0.093 ± 0.005
M12	<LOQ
M13	<LOQ
M14	0.595 ± 0.050
Occurrence frequency	57%

[a] n.d., not detected. <LOQ, below the limit of quantification of the method.

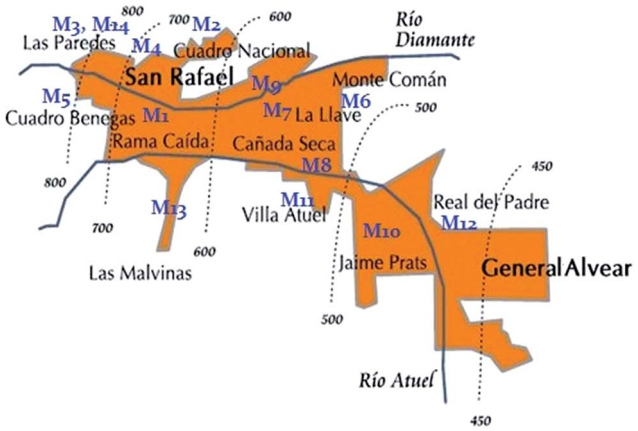

Fig. 5 Map showing the distribution of studied samples in the DOC San Rafael, Mendoza, Argentina.

mycobiota from DOC San Rafael, its well documented ability to produce high TA levels and pathogenic capacity,[16] the occurrence of TA reported here would be a consequence of the presence of this opportunistic pathogen. This emphasizes the importance of the analysis of micotoxigenic fungi in food commodities in order to predict and eventually prevent the risk of mycotoxin contamination.

4. Conclusions

A fast and simple method was developed and validated for the quantification of TA based on a modified QuEChERS method, drying/clean-up with anhydrous $CaCl_2$, and determination by HPLC-UV. No additional clean-up sorbents were necessary and just $CaCl_2$ was enough to kept matrix interferences at an acceptable level, saving time and costs. The developed method allows the determination of the studied mycotoxin in grape samples, showing good sensitivity, suitable precision and linear response ranges using matrix-matched standards. The results obtained in the validation procedure at three spike levels of TA showed adequate accuracy and precision. The proposed methodology has potentiality for the routine determination of the target mycotoxin with the aim of evaluating its content before winemaking or from a food safety point of view. The efficiency of the method was confirmed by the analysis of wine grapes, revealing the presence of TA at quantifiable levels. Data obtained in this study, for a limited number of samples, established the often occurrence of this compound in grapes used for wine elaboration. These results shows that the method has applicability for TA determination in an average laboratory, making it a successfully approach for high throughput routine quality control of grapes. Additionally, the present work contributes to evaluate the extent of contamination of grapes with the mycotoxin TA as well as to establish concentration limits for this toxin. Nevertheless, a large number of samples should be analyzed as well as the study of grape juices and wines should be carried out in the future to assess the extent of contamination and exposure of wine consumers.

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