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Assay of ochratoxin A in grape by high-pressure liquid chromatography coupled on line with an ESI–mass spectrometry

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Abstract

In this paper, we propose a method for detection of ochratoxin A (OTA) in grapes by using nano-reversed-phase high-performance liquid chromatography–electrospray ionization–mass spectrometry (nano-RP-HPLC–ESI–MS). The method is rapid, highly sensitive and reproducible. OTA is extracted preferably from the entire acinus, rather than must; using chloroform at long incubation time period, lyophilized, resolubilized in acetonitrile (AcCN) and injected onto a reversed phase capillary or analytical column. Capillary columns are the method of choice because it requires a reduced amount of injected sample and consequently the chloroform necessary for OTA extraction, which is a toxic agent. This method gives a detection limit of femtog/ml, without resorting to an immunoaffinity clean-up or concentration, which makes it by far superior to any other method reported. Moreover, by using MS as a detection method it is possible, in the case of a complex matrix, to measure its molecular mass and to confirm the presence of OTA by MS–MS, which cannot be done by fluorescent detection. The method has a high sample extraction throughput (24/h) and has adequate precision (between batch C.V. <8%) and sensitivity (limit of detection (LOD) = 1 pg/g; limits of quantification (LOQ) = 2 pg/g) for OTA measured.

Keywords: Ochratoxin A; Mycotoxin; Grape; *Penicillium* and *Aspergillus*; Nano-HPLC; Electrospray ionization mass spectrometry (ESI)

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by some species of *Penicillium* and *Aspergillus*. This molecule is widely present in cereal grains, vegetables and dried fruit as well as meat (pork and chicken) and fish, and so could contaminate human and animal food [1–6]. Due to the range of products in which OTA is found, avoidance of dietary intake by humans of OTA is almost impossible. The average human daily intake of OTA has been estimated at 85 ng/day or 1.2 ng/kg leading to plasma levels of 0.5 ng/ml though these levels can vary considerably depending on individual and regional dietary habits [7]. It has been demonstrated that OTA is nephrotoxic for a wide range of animals and particularly for pigs. It can cause liver diseases and it also has immunodepressive, tetrogenic, genotoxic and carcinogenic effects in humans [8–12]. For all of these reasons the International Agency for Cancer Research classified OTA in the B group as a possible cancerogenic substance for humans.

The presence of this toxin in must and wines was signalled for the first time in 1996 by Zimmerli and Dick [13] and later confirmed by others [14–17]. OTA was subsequently found in some Swiss wines as well as in some others produced in different regions; forcing the competent authorities to stop selling these drinks [9,12]. The presence of OTA in must and wine is due to the fact that fungi can contaminate grapes before they are harvested as well as later during the different steps involved in wine production but this will only occur if the grape skin is damaged [17]. Different authors have promoted different methodologies to detect OTA in foodstuffs where it could be present: immunoenzymatic tests were used to screen cereal grains [14,18], while immunoaffinity chromatography and high-performance liquid chromatography (HPLC) were used to investigate coffee [19–21]. In the particular case of wine, it has been demonstrated that good results can be obtained using IAC columns [22–23]. Although the procedures used to isolate and purify the OTA in these methodologies differ substantially [24], the protocols to

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detect OTA in wine consist of four different steps: isolation, purification, concentration, and OTA identification. However, when must is the starting material, OTA yields can be less than 50%, due to the fact that must has to be previously centrifuged, to avoid blocking the IAC columns, so a large part of the OTA in the sample is lost because it adheres to the solid portions of the grapes [22]. Moreover, problems arise during the solvent extraction of OTA from must, because this forms an emulsion that results in loss of analyte and hence results that cannot be reproduced [25]. To this regard when OTA is localized on the surface of the sample, such as green coffee or barley it is easy to extract exhaustively from solid matrix using liquid–liquid repartition and long incubation time [26,27]. On this line, considering that in the grape the toxin appears to be mainly present in the skin and is only found in the juice after the fruit has been crushed and macerated, it is highly desirable to develop new methods for extracting and detecting the presence of OTA in vineyards before grapes are harvested and used to produce wine. Regarding the OTA detection, it is well known that its determination using only spectroscopic techniques or fluorescence emission can generate artifacts which could compromise correct identification, especially when there are other chemicals present in the sample. Moreover, analytical problems such as coelution of interfering compounds or retention time shifts can lead to erroneous positive or negative results. This problem, that can be found in complex matrix when spectroscopic or fluorescent detection are used, was overcome in our case by the direct coupling of liquid chromatography (LC) and mass spectrometry (MS) using soft ionisation techniques. It is known that mass spectrometry is a powerful and sensitive technique with which traces of chemical compounds can be identified in the femtomole range. Measurement of intact molecular weight of an analyte by mass spectrometry is indisputable and has already been accepted by the scientific community as an established method for the unequivocal identification of an unknown compound which does not give false positives [28–34]. Nevertheless HPLC coupled with mass spectrometric detection is described in a very limited number of papers for a few sample matrices such as human blood [35,36], beer [22,37,38] and coffee [39] and wine [16,30,34,37,40].

So, our goal was to set up a method that could determine the presence of OTA in grapes by chloroform extraction from intact or slightly mashed grape with identification achieved by using nano-reversed phase columns coupled on-line with mass spectrometers. The method set up for OTA from must and grapes can be applied to any other food.

2. Experimental

2.1. Chemicals and reagents

The ochratoxin A standard used was obtained from Sigma–Aldrich, Oakville, Ont., Canada. The pure standard was in liquid form and was stored in the dark at 4 °C. a stock standard solution (50 μg/ml) was made in 100% acetonitrile (AcCN). The stock solution was calibrated spectrophotometrically at 333 nm, using the extinction coefficient 5550 M^{-1} cm^{-1} [41]. The stability of the stock solution was checked spectrophotometrically, OTA was stable in the refrigerator over a period of several months. Formic acid, ethanol, as well as HPLC-grade water and acetonitrile were obtained from Carlo Erba (Milan, Italy). Ammonia solution (33%) was from Riedel-de Haen (Seelze, Germany).

2.2. OTA extraction from grape using chloroform

When analytical columns were used, 100 g of grapes were dissolved in a cylinder with 100 ml of chloroform, while in the case of capillary columns 10 g of grapes were dissolved with 10 ml of chloroform. In both cases, extraction was obtained by turning the vial gently for 24 h, without any homogenization, on the assumption that OTA is localized on the surface of the grapes [26,27]. The mixture was centrifuged at 10,000 × *g* for 10 min. The pellet (about 30% of the total solution) was further treated with chloroform to check the presence of residual OTA, while 30 ml of the extract solution (or 3 ml when capillary columns were used) was evaporated under a gentle stream of nitrogen by rotavapor at 30 °C. The lyophilized was solubilized with 10 ml of H_2O or ethanol or AcCN (or 1 ml for capillary columns).

2.3. OTA extraction using immunoaffinity column

The method described by Visconti et al. [22] was used. Each sample of OTA extracted by chloroform from entire acinus and must (10 ml) were diluted with a solution (10 ml) containing 1% poly(ethylene glycol) and 5% sodium hydrogencarbonate, mixed and filtered through Whatman GF/A microfibre filter, and a 10 ml volume of diluted extract cleaned up through an OchraTest immunoaffinity column (Vicam Inc., Watertown, MA) at a flow-rate of about 1 drop per second. The column was washed with 5 ml solution containing sodium chloride (2.5%) and sodium hydrogenocarbonate (0.5%) followed by 5 ml distilled water at a flow-rate of 1–2 drops per second. OTA was eluted with 2 ml methanol and collected in a clean vial (Kimble Glass, USA). The eluted extract was evaporated under nitrogen stream at ca. 50 °C and reconstituted with 250 μl of the HPLC mobile phase.

2.4. High-performance liquid chromatographic (HPLC) method and calibration experiences for fluorescence detection and immunoaffinity column

The HPLC apparatus consisted of a Perkin-Elmer series 200 lc pump chromatographic system equipped with a Rheodyne Model 7125 injection valve (Rheodyne, Cotati CA, USA). The fluorescence detector used was a Perkin-Elmer Model LC 240 (λ_{ex} = 333 and 460 nm when using an acid mobile phase; λ_{ex} = 380 and 440 nm when using an alkaline mobile phase). For diluted samples, a loop of 250 μl was used as injection system; in the other cases the loop was 50 μl. The sample components were separated in a reversed-phase C18 column packed with 5-μm porous butyl silica particles (Vydac Protein C-18, 250 mm × 4.6 mm Group, Hesperia,

CA). The Vydac C-18 column was pre-equilibrated with 5% (v/v) aqueous acetonitrile solution containing 0.05% formic acid and samples were eluted using a gradient consisting of a first linear gradient from 5 to 60% (v/v) acetonitrile in 15 min, followed by a second gradient segment from 60 to 90% (v/v) acetonitrile in 5 min. At the end of the run, the column was flushed with 100% acetonitrile for 5 min. This post run gradient was used in order to ensure that hydrophobic contaminants were eluted from the column. Samples were eluted at a flow-rate of 1.0 ml/min.

The standard solutions needed to obtain the calibration lines were prepared in the same eluent as the one used in the mobile phase. Two sets of solutions were prepared. The first one was used to quantify the most concentrated samples and ranged from 0.1 to 1 mg/L the other set was used to determine the lowest contents of OTA and ranged from 10 to 50 μg/L.

2.5. Nano-HPLC–ESI–MS and calibration experiences

In order to reduce the amount of sample for analysis, and consequently the chloroform necessary for OTA extraction, capillary columns were used.

Liquid chromatography was carried out at 200 nl/min using an Ultimate nano-HPLC (LC-Packings, a Dionex Company, Italy). The column was integrated with an ion trap mass spectrometer Esquire 3000 plus (Bruker Daltonik, Germany). Samples were introduced onto the column by a sample injection valve with a 1 μl sample loop. The proteins were separated in a reversed-phase capillary column packed with 5-μm porous butyl silica particles (Vydac Protein C-18, 15 cm × 180 μm I.D., 5 μm 300 Å Group, Hesperia, CA). All solutions were filtered through a membrane filter (type FH 0.5-μm, Millipore, Milan, Italy) and degassed by sparging with helium during use. The Vydac C-18 capillary column was pre-equilibrated with 5% (v/v) aqueous acetonitrile solution containing 0.05% formic acid and samples were eluted using a gradient consisting of a first linear gradient from 5 to 60% (v/v) acetonitrile in 15 min, followed by a second gradient segment from 60 to 90% (v/v) acetonitrile in 5 min. At the end of the run, the column was flushed using 100% acetonitrile for 5 min. This post run gradient was used in order to ensure that hydrophobic contaminants were eluted from the column. ESI–MS was performed on an ion trap mass spectrometer (Esquire 3000 plus, Bruker Daltonik, Bremen, Germany). For analysis with pneumatically assisted ESI, an electrospray voltage of 3–4 kV and a Nebulizer 20 psi and Dry Gas 5 L/min were employed. The temperature of dry gas was set to 300 °C. Full-scan spectra were acquired over the range m/z 200–500 (scan duration 1 s). The product ion spectra were recorded using helium as collision gas.

The calibration curve was obtained by analysing OTA standard solutions at six concentration levels (range: 0.01–0.1 ng/ml; three determinations at each level have been performed). The significance of linear regression and intercept (not significantly different from 0) were calculated by SPSS 8.0 statistical software with the ANOVA model and the Student's t-test, respectively (regression: $Y = (42500 \pm 543)X$, $r^2 = 0.9881$).

3. Results and discussion

In order to develop a rapid and unequivocal reversed-phase high-performance liquid chromatography (RP-HPLC) method for the determination of ochratoxin A in foods such as grapes, a systematic study was carried out to establish the optimal chromatographic conditions compatible with using a mass spectrometer as detector, due to its high sensibility and accuracy.

Using grapes as starting material, our preliminary search led to the conclusion that chloroform extraction was the best method for OTA determination, being it exhaustive and reproducible (see later). Obviously, the chloroform must be eliminated before injecting a sample onto a C18 column, and we tested various solvents to find out which was most suitable to solubilize the OTA once the chloroform had been removed. So, a standard solution of commercial ochratoxin A was dissolved in three different solvents including H_2O and ethanol, which are commonly used for affinity columns [22], and the third was AcCN. Each sample was injected onto a C18 analytical column or capillary column. Capillary columns were used in preference to analytical ones because they require less analysis time and smaller samples. The latter aspect is of interest because, even though chloroform it is an exhaustive extracting agent of OTA using liquid–liquid repartition, it is an unpleasant solvent and therefore must be minimized as much as possible. In a pilot run OTA was detected by fluorescent detector, as it is well known that this chemical absorbs between 333 and 380 nm emitting a strong signal from 440 to 450 nm. Elution of OTA from reversed phase can be done either by an isocratic or gradient solution using AcCN as a basic eluent together with an appropriate buffer. We also investigated the effect of pH on spectroscopic emission of OTA during elution, as we knew it to be higher at alkaline pH [32]. Some studies reported that post-column fluorescence was enhanced by addition of ammonia solution enabling them to reach a detection limit of 0.003 ng/ml [38]. So we eluted OTA with either an acetonitril–ammonia buffer (NH_4Cl/NH_3, 20 mM, at pH 9.8) eluent was 15:85 (v/v) (Fig. 1A) or with acetonitrile–water (50:50 v/v), adding 1% acetic acid or formic acid to give a pH of 6.0 (Fig. 1B). When isocratic elution was used we found that in both cases chromatograms had a single peak (Fig. 1). As we moved from acid pH to alkaline solutions the elution time of OTA increased from 4 to 8 min, but there was a 10-fold enhancement in OTA fluorescence. There were no significant differences apparent in OTA detected by HPLC or fluorescence whether the solvent used was H_2O, ethanol or AcCN. However, elution at alkaline pH affords a distinct advantage, giving rise as it does to a stronger signal, which may be sufficient to detect OTA at concentrations as low as 0.1 ng/ml. This obviously represents a significant increase in OTA detection when HPLC is the only instrument available, but bearing in mind that the fluorescence signal of a chemical is strongly influenced by the solvent in which it has been dissolved, as well as other undesirable components which are inevitable when the sample comes from a food with a complex matrix, we decided to try to detect OTA using a mass spectrometer, which is more sensitive than fluorescence. Therefore, the outlet of capillary column was coupled on line with an ion trap interfaced by an electrospray

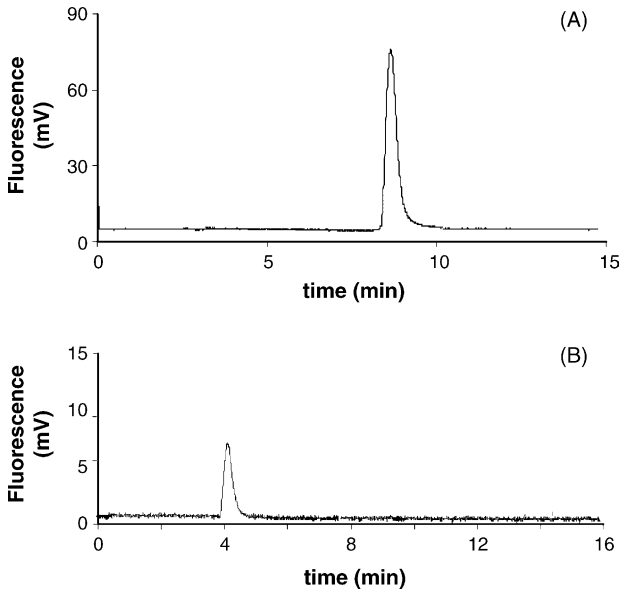

Fig. 1. Chromatograms of a OTA standard solution by using C18 column. (A) Elution by isocratic solution acetonitril-ammonia buffer (NH_4Cl/NH_3, 20 mM, at pH 9.8) eluent was 15:85 (v/v). (B) Elution by acetonitrile–water 50:50 (v/v) added of 1 % of acetic acid or formic acid, having a pH of 6.0.

source. This strategy allowed the injection of a small sample (1 μl), which resulted in a much reduced amount of chloroform for OTA extraction from solid matrix. Moreover, this strategy meant that we could not only measure the molecular mass of the chemical and consequently identify OTA unequivocally, but also take advantage of the greater sensitivity of the mass spectrometry, which is able to pick up chemicals in the nano-femtomole range, where spectroscopic signals are not normally detected. Obviously, we first had to calibrate the chromatographic separation to optimize mass spectrometer detection. Because of the alkaline buffer we had to use the negative ionization mode and the fragment $[M - H]^-$ with an $m/z = 402$ was the ion that could be monitored. However, in this modality AcCN adducts were found (data not shown) which could mask or interfere with the OTA signal. So, we decided to use positive mode and reverted to using an acid buffer containing formic acid as an ion-pair, even though this meant losing some of the fluorescence emission of OTA. In compensation however, it was possible to use gradient elution with this eluent, to obtain a better OTA resolution when trying to identify it from a complex chemical mixture. So, using this strategy the fluorescence detector could be bypassed and the reconstructed ion current (RIC) used for monitoring the HPLC run and detection of OTA. Fig. 2 shows the RIC recorded when different concentrations of OTA, previously solubilized

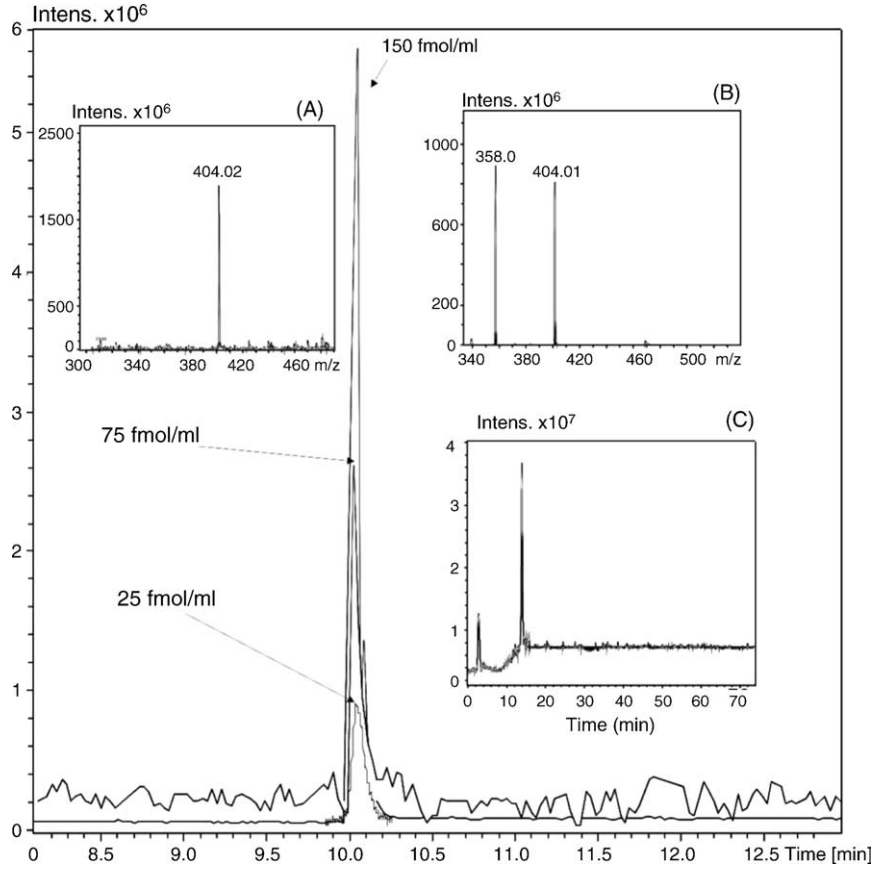

Fig. 2. Reconstructed ion current (RIC) recorded in positive modality upon loading onto the capillary different concentration of OTA (24.89 femtomole/ml; 74.67 femtomole/ml; 149.85 femtomole/ml) previously solubilized in AcCN. The mobile phase used was acetonitrile–water 50:50 (v/v) added of 0.05% of acetic acid or formic acid, having a pH of 6.0. (inset A) ESI spectra of OTA. (inset B) MS–MS–ESI spectra of OTA. (inset C) RIC recorded upon injection of OTA solubilized in ethanol. Electrospray parameters: electrospay voltage of 2–3 kV; temperature dry gas 300 °C; scan range 300–500.

in AcCN, were loaded onto the capillary. The inset A reports the *m*/*z* of the ESI spectra of standard OTA. The molecular mass found was 404 corresponding to the $[M+H]^+$ expected for Ochratoxin A. However, in order to be sure that the peak observed corresponds unequivocally to OTA a further MS–MS was performed using soft energy collision. The value *m*/*z* = 360 reported in inset B corresponds to the $[M+H\text{-}CO_2]^+$ ion which is formed at this low collision energy, where only the CO_2 group is removed. Fig. 2 clearly shows that with an ion trap OTA can be detected in femtomole concentrations. So, not only is the mass spectrometer method rapid and simple but unlike the fluorescence procedure it can identify trace amounts of the chemical. Interestingly, when OTA was solubilized in H_2O there was no appreciable RIC signal, whereas OTA solubilized in ethanol gave two peaks, one observed at 12 min, and a smaller peak near the front. Interestingly, MS analysis of both peaks gave a value of *m*/*z* = 404, MS–MS of which gave a *m*/*z* = 360, confirming that both peaks contained OTA (inset C Fig. 2). This indicates that OTA is partially fractionated in ethanol or two adducts are formed, therefore its concentration would be underestimated if the only peak observed was that at 12 min, as would have been the case with the fluorescent detector. Furthermore, since our parallel determination of OTA in meat gave successful results (data not shown), it can be concluded that OTA once extracted from any food can easily and rapidly detected with a simple HPLC run, without purification or prior clean up procedures, but by simply dissolving it in 5% AcCN and loading it onto a reversed phase capillary or analytical column. Moreover, by using a ion trap OTA can be detected in femtomole quantities, but sensitivity was also enhanced when the HPLC photodiode array and gas chromatography mass selective detection was used, revealing 0.1–2 mg/L [35], or immunoaffinity column [22] or fluorescence detection was used, which improved detection up to 0.1 ng/ml.

3.1. Application of method to grapes

Direct measurement of OTA in wine is important, but more so is the ability to detect its presence in grapes that are still in the vineyards, before they are harvested and used to produce wine, especially considering the fact that the toxin is mainly present on the grape skin [26,27] and is only found in the juice after crushing and maceration. Moreover, solvent extraction of OTA from must by stirring has the disadvantage of causing an emulsion, which leads to the loss of analyte and hence not reproducible results. Thus, taking into account that easy and rapid chloroform extraction of OTA from an entire acinus may be a way to achieve this, we compared the OTA extracted from entire acinus or must, starting from material clearly affected by *Aspergillus*. Interestingly, overnight chloroform extraction of OTA from grapes did not cause emulsion and resulted to be the most convenient and realistic way to determinate its concentration. The OTA peaks were well separated from interfering peaks in less than 10 min, as shown in Fig. 3A and B.

Table 1 compares the OTA in μg/kg determined on 10 g, when capillary column was used, or 100 g in the case of analytical column. The left column shows OTA extracted from must, whereas the right column shows OTA determined from

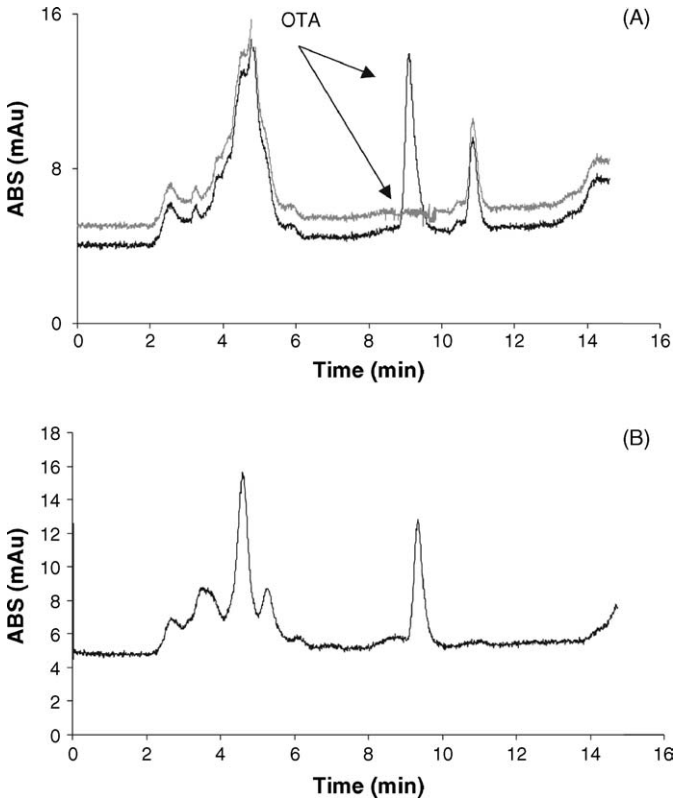

Fig. 3. (A) Chromatograms of a natural acinus sample (grey line) and of a spiked acinus sample (black line). (B) Chromatograms of a naturally contaminated commercial grape acinus. Conditions as described in the legend for Fig. 2.

entire acinus or slightly mashed acinus. It can be observed that the values reported in the right column are higher, indicating that extraction of OTA from entire acinus gives the greatest amount of mycotoxin. This corroborates the hypothesis that all OTA is localized on the grape skin and homogenization of acinus causes a reduction of liquid–liquid repartition. Moreover, when must is the starting material, a centrifugation step has to be included to avoid blocking the column, with the result that up to 30–40% of OTA is lost as it sticks to the pelleted solid material.

OTA extracted by chloroform from must or acinus was also determined by IAC column method. Interestingly, the OTA determined by IAC was 30–40% less than the value reported in Table 1 (data not shown). This loss is probably related to the numerous manipulations that the IAC method requires.

Table 1
Comparison of OTA amount detected in must or from entire acinus by using chloroform extraction two different extraction methods

Matrix	Extraction from must by chloroform (μg/kg)	Extraction from entire Acinus by chlorofprm (μg/kg)
White 1	0.21	0.26
White 2	0.17	0.33
White 3	0.09	0.19
Red 1	0.31	0.45
Red 2	2.20	2.27
Red 3	0.41	0.99

Finally, it is worth remarking that the sensitivity of the method used here allows the appearance of fungi to be seen shortly after they first infect when OTA spread is minimal. Thus, for the sake of consumer safety, an OTA analysis should be carried out on grapes directly before their harvesting, to ensure that OTA will not be present in end products like wine.

3.2. Analytical quality assurance

With our method nano-HPLC ESI–MS, within the spiking range of 0.01 0.05 and 0.1 μg/L, the mean recovery for OTA in spiked grape samples, was of 96 ± 1, 95 ± 5, and $93 \pm 6\%$, respectively, while the average R.S.D. was 3%. No matrix effect was observed. Recovery experiments were performed on OTA free samples (three determinations at each concentration level were performed). The limit of detection (LOD), defined as three times the chromatographic base line noise, was 0.01 μg/L (1 μL injected) and the limits of quantification (LOQ), defined as 10 times the signal-to-noise ratio, were 0.02 μg/L, whereas by HPLC–FLD the LOD was 0.05 and LOQ was 0.10 μg/L. A blank (the same grapes without OTA) was analyzed after every five samples. A reagent blank was analyzed at the beginning of each run.

Ochratoxin was considered as positively identified in grape samples when: (a) the ratio of the retention time of the analyte to that of the corresponding IS corresponded to that of the calibration solution within a ±2.5% tolerance and (b) the peak area ratios of the various transition reactions were within the tolerances set by the EU criteria [42].

Recoveries and precisions were calculated from the analysis of six blank grape samples performed by the same operator on three separate occasions over a two-week period. Within laboratory precision thresholds were obtained by following the same protocols but analyses were performed by two different operators on one occasion in a 1-month period. Repeatability at the 95% confidence level was deduced from the within-day precision using an expansion factor of 2.77. Similarly, the intermediate reproducibility was calculated from the within-laboratory precision using the same expansion factor.

4. Conclusion

The simplicity of sample preparation and high sensitivity make the nano-HPLC–ESI–MS method suitable for routine detection and assay of OTA in grapes or in any other solid matrix, by using liquid–liquid repartition and long incubation time and injection of small sample amount. This causes some concern because chloroform is an unpleasant agent, therefore new protocols must be based on micro-extraction for micro-analysis until new exhaustive solvents will be available. On the other hand a direct extraction of OTA by liquid–liquid repartition does not need pre-concentration techniques such as immunoaffinity. This is very useful not only for the rapid determination of OTA within the probable European Community (EC) legal limits, but also because it can be applied directly to entire acinus. This allows vineyards to be screened before grape harvesting revealing the possible presence of ochratoxinogenic strains in time to prevent wine contamination.

The most efficient way to protect consumers against OTA health hazards is therefore to implement good agricultural practice to minimize the presence of fungal strains on the grapes and therefore reduce the possibility of OTA production during the wine-making process. The main ways to achieve this would be appropriate antifungal treatment of vines and strict control of the wine-making process.

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