QUANTIFICATION OF OCHRATOXIN A AND FIVE ANALOGS IN NAVARRA RED WINES

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ABSTRACT

Ochratoxin A (OTA), B (OTB) and their methyl (MeOTA, MeOTB) and ethyl (OTC, EtOTB) esters were evaluated in 51 red wine samples from Navarra (Spain). Detectable levels of OTA and OTB were found in 100% of the samples, and 71% showed the presence of OTC. The six ochratoxins appeared simultaneously in 18% of the samples. Results indicated that OTC is hydrolyzed to OTA in red wine. Therefore, ochratoxin intake from wine can be underestimated when only assessed by OTA analysis. Analyzed Navarra wines are scarcely contaminated with ochratoxins and their contribution to human intake is low, with the worst case being 4.7% and 6.6% of the provisional tolerable weekly intake (PTWI) for OTA and for the sum of ochratoxins, respectively. No significant differences were generally found between vintages. With the exception of OTA, no significant differences were observed between organic and traditional farming. Levels of ochratoxins were positively correlated with temperature and inversely correlated with humidity and rainfall.

Keywords: Ochratoxin A, ochratoxin analogs, wine, occurrence, intake.

1. Introduction

Ochratoxins are a family of toxic compounds produced by several fungal species of the genera *Aspergillus* and *Penicillium.* Ochratoxin A (OTA) is the most important, due to its toxicity and occurrence. It may occur naturally in a variety of agricultural commodities such as cereals, coffee beans, spices and dried fruits. OTA can cause nephrotoxic, immunotoxic, teratogenic and carcinogenic effects (Pfohl-Leszkowicz & Manderville, 2007). The IARC classified this mycotoxin as a possible carcinogen for humans (Group 2B).

The OTA-producing fungi can also produce other related compounds, such as ochratoxin B (OTB), ochratoxin C (OTC), methyl ochratoxin A (MeOTA), and OTB methyl and ethyl esters (MeOTB and EtOTB, respectively) (Steyn, 1971) (Figure 1). The LD_{50} values for OTA, OTC and MeOTA were reported to be similar in ducklings (Steyn, 1971), whereas EtOTB presented a LD_{50} that was twice as high as the LD_{50} value for OTA in rainbow trout (Doster, Sinnhuber & Pawlowski, 1974). It has been demonstrated that OTC is more toxic than OTA in cell cultures (Müller, Burkert, Rosner & Köhler, 2003). Esterification facilitates the penetration of OTC into the cell and this compound is rapidly converted into OTA in the body, which in turn affects maximal tissue concentrations and overall toxicity, thereby showing synergistic toxic effects between OTA and OTC (Pfohl-Leszkowicz & Manderville, 2007). OTB was said to be tenfold less toxic than OTA (Marquardt & Frohlich, 1992), but some studies mention its cytotoxicity (Dietrich, O'Brien, Stack & Heussner, 2001), nephrotoxicity (Mally et al., 2005) and teratogenicity (O'Brien, Prietz & Dietrich, 2005). Knasmuller et al. (2004) examined OTA, OTB and citrinin (CIT) genotoxicity in human liver cells and they pointed out that the combined effects of these mycotoxins in food may have an

impact on the overall cancer hazard to humans. Heussner, Dietrich and O'Brien (2006) also showed the synergistic effects for OTA, OTB and CIT in renal cells.

The presence of ochratoxin A in wine has been reported worldwide (Soufleros, Bouloumpasi & Tricard, 2003, Rosa, Fraga, Santana, Magnoli & Dalcero, 2004, Leong, Hocking, Pitt, Kazi, Emmett & Scott, 2006, Brera et al., 2008), including in Spain (López de Cerain, González-Peñas, Jiménez, & Bello, 2002, Bellí, Marín, Duaigües, Ramos & Sanchis, 2004, Quintela, Villaran, Lopez de Armentia & Elejalde, 2011), which is one of the principal wine producers in the world (OIV, International Organisation of Vine and Wine, 2010). The SCOOP report (Miraglia & Brera, 2002) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have identified wine as the second largest source of OTA intake for humans in Europe (JECFA, 2007). The JECFA recommended a Provisional Tolerable Weekly Intake (PTWI) of 100 ng·kg b. $w.^{-1}$·$week^{-1}$. In order to minimize public health risk, the European Commission established a maximum level of 2 μg L^{-1} for OTA in wine (EC, 2006).

Due to the fact that ochratoxigenic fungi can produce OTA and its related compounds, it is more realistic to consider that under natural conditions, wine could be contaminated with a mixture of fungal metabolites. The co-occurrence of OTA and OTC in wine was described for the first time in 1996 (Zimmerli & Dick, 1996). However, the simultaneous presence of several ochratoxins in wine has yet to be investigated and knowledge of their combined toxicological effects is still limited. Both aspects are very important in order to avoid underestimating the total intake of ochratoxins and the possible adverse effects.

Several factors, such as climate, grape cultivation, and winemaking techniques, affect the presence of OTA in wines. Levels are influenced by climate, which obviously

depends on the latitude, but also on the particular circumstances of a given year, such as the temperature and relative humidity in the month before harvest (Stratakou & van der Fels-Klerx, 2010).

With regard to climate, the Navarra region (in northern Spain) is marked by the confluence of the Atlantic, Continental and Mediterranean climates. Its vineyard extension is limited, only spreading across 100 km and occupying just 15000 hectares. However, Navarra D. O. (Designation of Origin) wine is among the ten most marketed wines in the world and its production volume is one of the highest in Spain (Spanish Ministry of Environment and Rural and Marine Affairs, MARM 2010). Three-fourths of the Navarra wine production is dedicated to red wine and 30% of the total wine production is exported, mainly to Germany, Holland and the United Kingdom (Navarra Designation of Origin Regulatory Board, 2011).

In this study, the simultaneous presence of OTA and five analogs in 51 red wine samples from Navarra has been researched. Some factors that could have affected the levels that were found were also taken into consideration: the vintage year, organic or traditional farming practices and certain meteorological conditions, such as, temperatures, relative humidity and rainfall during the summer prior to harvesting. In addition, the possible transformation of OTC into OTA in red wine has been studied.

2. Material and methods

2.1. Samples

A total of 51 red wine samples corresponding to the Navarra Designation of Origin were analyzed. Thirty-five red wine bottles were purchased from different supermarkets throughout Navarra (Spain). They were classified on the basis of their vintage year as either “crianza” (2004) or young wines (2006 and 2007). Their different

alcoholic grades, shown on the labels, varied from 12 to 15.5% (v/v) and their measured pH was in the range of 3.3 to 3.9.

Furthermore, seven wineries in Navarra with ecological certification provided us with 16 ecological red wines, with vintages from 2007 and 2008.

2.2. Chemicals

Ochratoxin A (Ref: O1877) and B (Ref: O1382), acetonitrile and methanol CHROMASOLV® for HPLC were purchased from Sigma-Aldrich (St. Louis MO, USA). Sodium hydroxide pellets, ethanol absolute and formic acid were purchased from Panreac (Barcelona, Spain). Dichloromethane (DCM) and hydrochloric acid fuming 37% were obtained from Merck (Darmstadt, Germany), and sodium hydrogen carbonate was obtained from Riedel-de-Haën (Seelza, Germany). Instamed Phosphate Buffered Saline (Dulbecco) w/o Ca^{2+}, Mg^{2+} was purchased from Biochrom AG (Berlin, Germany). All of the reagents were of pro-analysis grade. Ochraprep® immunoaffinity columns (IAC) were obtained from R-Biopharm Rhône Ltd (Glasgow, UK). Millipore type I water was used to prepare all of the aqueous solutions and it was obtained daily from a Milli-Q water purifying system. Non-sterile Millex®-HV syringe filters 0.45 µm, PVDF, 13 mm, were purchased from Millipore Iberica S.A.U. (Madrid, Spain). Preassembled vial kit (amber screw top write-on, caps and septa) and deactivated glass inserts were acquired from Agilent Technologies (Madrid, Spain). MeOTA and OTC were synthesized from OTA after alcoholic esterification in acidic media.

2.3. Apparatus and chromatographic conditions

Analysis of ochratoxins was carried out using an Agilent Technologies 1100 liquid chromatographic system equipped with a fluorescence detector (model G1321A), controlled by Chemstation 3D software. The analytical conditions were: a Zorbax Eclipse XDB-C18 column (15 x 0.46 cm; 5µm) from Agilent Technologies with a ODS

precolumn from Teknokroma (Barcelona, Spain). The mobile phase consisted of A (formic acid 0.4%) and B (acetonitrile). The elution program was: 10 min isocratic at 42% B, followed by a gradient to 60% B at 15 min, maintained until 25 min. After the analysis, the column was re-equilibrated during 5 min at 42% B. The injection volume was 100 µL and the flow rate was 1.0 mL min^{-1}. Chromatography was performed at 40ºC and the fluorescence conditions were: excitation at 318 nm from 0 to 7.5 min and 333 nm from 7.5 to 25 min, emission at 461 nm for the entire analysis. In these chromatographic conditions, retention times were 5.6, 11.1, 18.5 and 21.3 min for OTB, OTA, MeOTA and OTC, respectively (Remiro, Ibáñez-Vea, Lizarraga & González-Peñas, 2010).

2.4. Analytical method

The extraction of ochratoxins and the method validation were described in Remiro et al. (2010). Briefly, 50 mL of red wine at pH 7.2 was passed through an immunoaffinity column and ochratoxins were eluted with 4 mL of methanol. Methanol was evaporated till dryness under a stream of nitrogen at 40º C. The residue was redissolved in 250 µL of mobile phase and injected into the HPLC system.

Recovery values for OTB, OTA, MeOTA and OTC (81.7, 93.5, 76.0, and 73.4%), limits of detection (LOD) (0.16, 0.32, 0.21 and 0.17 $ng \cdot L^{-1}$), and quantification (LOQ) (0.50 $ng \cdot L^{-1}$) values obtained during the validation process were used in this study.

2.5. OTB methyl and ethyl esters

During the chromatographic analysis of the samples, some showed two peaks at the retention times of 14.3 and 17.0 min. These peaks could possibly be due to the presence of the methyl and ethyl esters of OTB. In order to confirm this, these compounds were synthesized from OTB in the same way as MeOTA and OTC were synthesized from OTA (Remiro et al. 2010). They were then injected into the HPLC-

FLD system in order to compare retention times; their structures were confirmed by LC-MS.

2.6. Confirmation procedure

The presence of ochratoxins was confirmed in 10% of the red wine samples using an Agilent Technologies 1200 liquid chromatographic system coupled to a MSD Trap XCT Plus mass spectrometer (G2447A model) and equipped with an electrospray ionization source (ESI), in the analytical conditions described in Remiro et al. (2010).

2.7. Transformation of ochratoxin C in ochratoxin A

Ten 500 mL aliquots of red wine were spiked with OTC, five with 0.5 μg L^{-1} and five with 2 μg L^{-1}. Their initial spiked levels were measured and the aliquot containers were closed and maintained at room temperature. These ten samples were then analyzed at one week, two weeks, one month, two months and one year after being spiked.

2.8. Statistical methods

Ochratoxins levels obtained in different years were analyzed with the Kruskal-Wallis and the U de Mann-Whitney non-parametric tests for non-dependent samples. The U de Mann-Whitney test was applied to compare ecological and non-ecological samples. The nonparametric Spearman correlation test was used to associate ochratoxin concentrations between each other and with meteorological conditions. The study of OTC and OTA levels over time was analyzed with the Friedman test for dependent samples. The statistical significance was determined using the program UNStat+ v1.0 and a probability value of 0.05.

3. Results and discussion

3.1. OTB methyl and ethyl esters

The retention time of synthesized OTB methyl and ethyl esters coincided with those of the unidentified compounds found in some wine samples. Identification of the OTB ethyl and methyl esters was confirmed with the aid of their mass spectra obtained from both, standard solutions and real samples. Chromatograms and mass spectra obtained from a standard mixture as well as from a red wine sample naturally contaminated with ochratoxins are shown in figure 2.

Standards were not commercially available for MeOTB and EtOTB, and their molar absorption coefficients were not found in the reference literature. Therefore, an approximate quantification has been carried out assuming that they have analytical behavior similar to their respective analogs MeOTA and EtOTA (OTC).

3.2. Concentration of ochratoxins in traditional Navarra red wines

The co-occurrence of OTA and five analogs was evaluated in 35 traditional red wine samples. The results obtained are shown in table 1. Median values were calculated taking into account all the levels encountered, including those between LOD and LOQ. Values under LOD were assumed to be a half of LOD. Mean values were calculated for the > LOQ levels.

With regard to OTA, OTB, OTC and EtOTB, the statistical studies disclosed that there were no significant differences ($p > 0.05$) among the levels encountered in red wines of 2006, 2007, and 2004 vintages. This is in accordance with the hypothesis that aging of the wine does not affect the OTA concentration (Bellí et al., 2004, Quintela et al., 2011). With regard to MeOTA and MeOTB, there were significant differences ($p < 0.01$) due to the fact that the levels obtained for both micotoxins were below the limit of quantification in 2004 vintage.

3.3. Ecological red wines

In order to evaluate if organic farming has an influence on the ochratoxin levels in red wine, 16 wine bottles certified as ecological products by the CPAEN (*Consejo de la Producción Agraria Ecológica de Navarra*) were analyzed. The results are shown in table 1.

In this case, the U of Mann-Whitney non-parametric test for non-dependent samples indicated that there were no significant differences between 2007 and 2008 vintage wines.

Results obtained from ecological and non-ecological red wines samples have been compared. Significant differences between OTA median levels were observed ($p = 0.037$), being greater in traditional farming. This does not coincide with Chiodini, Scherpenisse and Bergwerff (2006), who after studying red, rosé and white sample wines, stated that conventional and organic viticulture produce equal amounts of OTA in wine. Significant differences do not exist in the case of other ochratoxins.

3.4. Ochratoxins concentration in relation to climatic conditions.

Comparing wine from two different years of harvest, López de Cerain et al. (2002) found different degrees of OTA contamination. Bellí et al. (2006) studied some meteorological variables in order to correlate them with the Aspergillus infection of grapes. Temperatures, relative humidity and rainfall in the month preceding each sampling date were analyzed by these authors.

The data regarding maximum, mean and minimum temperatures, relative humidity and rainfall of each municipality of each wine during the two months of summer (July and August) of their corresponding vintage year was acquired from the Meteorological National Agency. The Spearman correlation coefficients for nonparametric samples at $p < 0.05$ were studied.

Ochratoxin B and its ethyl ester do not correlate with these variables. However, OTA, MeOTA, OTC and MeOTB had similar behaviors. In general, they had a positive association with temperatures and they were inversely related with humidity and rainfall. This coincides with the fact that frequency and OTA concentration are higher in southern and warmer regions (Brera et al., 2008, Mateo, Medina, Mateo, Mateo & Jimenez, 2007, Otteneder & Majerus, 2000).

3.5. Total occurrence of ochratoxins and dietary intake

The OTA mean and median values found in this study were 0.016 and 0.005μg·L^{-1}, respectively (see table 1 for the values of the other ochratoxins). The highest OTA concentration was 0.142 μg·L^{-1}, fourteen times lower than the 2 μg·L^{-1} maximum permitted by the EU and it was found in an organic wine. The low limits of detection and quantification of this analytical method, much lower than others previously described, gave a great percentage of positive samples but the mean and median values obtained were lower than many of the LOD described in other works. In a recent research study, the mean OTA concentration (0.035 μg·L^{-1}) found in La Rioja (the nearest winemaking zone to Navarra) appears to be similar (Quintela et al., 2011). Red wines analyzed in southern regions of Spain (mean = 0.25 μg·L^{-1}) had higher concentrations of this mycotoxin (Blesa, Soriano, Moltó & Mañes, 2004).

This study describes the co-occurrence of 6 ochratoxins in wine. The fact that 100% of the samples had detectable levels for simultaneous occurrence of ochratoxin A and B confirms the assumption that OTA in food and feed is sometimes accompanied by its non-chlorinated analog (EFSA, 2006) and that their levels and incidence are comparable. The dispersion graphic of OTA concentration versus OTB concentration revealed a significant positive correlation, $r_s = 0.523$ ($CI_{95\%}$: 0.281; 0.703).

Zimmerli and Dick (1996) were the first authors to describe the simultaneous appearance of OTA and OTC in wine. Since then, no more studies have been carried out on this topic. In this study, 71% of the samples presented OTC. The median values of OTA and OTC concentrations obtained in this investigation verified that OTC was estimated to be approximately 10% of the corresponding OTA. A very large positive association (ρ = 0.837, $CI_{95\%}$: 0.725; 0.906) between OTA and OTC levels was evidenced by the Spearman correlation coefficient.

With regard to the simultaneous presence of ochratoxins, 20% of the samples presented levels for at least three of them. In 31% of the samples four ochratoxins were present and in 31% of the samples five ochratoxins were observed. A combination of the six mycotoxins appeared in 18% of the samples.

Consumer risk exposure has been evaluated taking into account the occurrence data obtained in this study (maximum and mean values of OTA and total ochratoxins). As recommended in the SCOOP Task (2002), a more accurate exposure should be assessed by studying data for consumers only as well as for the total population. With this purpose in mind, two different databases were researched. On one hand, the alimentary consumer data of the Spanish Ministry of Environment and Rural and Marine Affairs established a wine consumption in Spain of 24.2 mL day^{-1} per capita (MARM, 2009). On the other hand, recent researches regarding the healthy properties of moderate wine consumption, typical of the Mediterranean diet, recommended drinking 3 glasses of 120 mL of wine per day for men and two for women (FIVIN, 2008). Also, sex distinction was assumed for the average adult body weight. In accordance with the Statistical National Institute (2010), the different weights were as follows: 76.8 kg for men, 64.2 kg for women and 70.2 kg for the average body weight of the total Spanish adult population.

Basing our work on these assumptions, the weekly intake of ochratoxins is shown in table 2. Various organizations proposed different Provisional Tolerable Weekly Intake (PTWI) for OTA, ranging from 8.4 to 120 ng·kg b. w.$^{-1}$·week^{-1} (Miraglia & Brera, 2002). The most recent value of 100 ng·kg b. w.$^{-1}$·week^{-1} was adopted by the JECFA (2007). Therefore, in the worst case, using the maximum concentration found in this study for OTA or the total sum of ochratoxins and the maximum wine intake that corresponds to men and wine consumers, the intake value calculated in our study indicated that Navarra´s wine contribution represents 4.7% of the PTWI in the case of OTA and 6.6% of the PTWI in the case of the total sum of ochratoxins.

While wine consumption does not appear to be a serious risk factor for the Navarra population with respect to OTA, the simultaneous presence of several ochratoxins must be considered. Although the mycotoxin levels found were low, the ochratoxins ingested from wine can not be assessed by OTA analysis alone without underestimating their total intake. In addition, their presence in other matrices should be studied in order to determine the total ingestion of ochratoxins. It is also important to carry out more toxicological studies regarding the combinations of ochratoxins in order to evaluate the real and accurate risk of their simultaneous presence.

3.5. Transformation of OTC into OTA

The presence of ochratoxin A or OTC in wine is due to fungal activity, but the chemical transformation of OTC into OTA or vice versa should be taking into account. Zimmerli & Dick (1996) explained that the presence of OTC in wine could be due to the alcoholic and acidic nature of wine that would permit the transformation of the carboxylic acid into an ethanolic ester In order to observe the behavior of this ochratoxin, ten OTC spiked red wines were analyzed during a year.

Mean values of OTC and OTA concentrations against time are presented in figure 3. Quite clearly, OTC levels decreased with time, while the OTA levels increased, even to the point where both concentrations became similar (figure 3b). At just two months, there were significant differences ($p < 0.05$) with respect to both of the initial OTC concentrations, 0.5 and 2 $\mu g\ L^{-1}$. This suggests that OTC is hydrolyzed to OTA in wine. This fact can be due to the displacement (of the balance between the ester form and carboxylic form) towards the non-esterified form in the presence of water. If OTC was present in wines as a consequence of fungal metabolism, it would most likely transform into OTA. This fact makes it necessary to determine the concentrations of both ochratoxins in order to satisfy regulatory levels and to avoid underestimating the total intake of ochratoxins.

4. Conclusions

OTA, OTB and their methyl and ethyl esters were evaluated in 51 red wine samples from Navarra. Detectable levels for simultaneous occurrence of ochratoxin A and B were found in 100% of the samples. OTC was present in 71% of the samples. OTB methyl and ethyl esters were also found in some samples, and a combination of the six ochratoxins appeared in 18% of the samples. In addition, the results obtained when the transformation of OTC into OTA in red wine was examined suggested that OTC had hydrolyzed to OTA in wine. Therefore, there is a risk of underestimating ochratoxin intake from wine when only OTA analysis is performed.

Wines analyzed from Navarra are scarcely contaminated with ochratoxins; in fact, the levels of ochratoxins found in all of the samples were very low. Moreover, the worst case taken into account in this study indicated that Navarra´s wine contribution represents 4.7% of the PTWI for OTA and 6.6% of the PTWI in the case of the total sum of ochratoxins.

With regard to factors that could affect ochratoxin levels, no significant differences were generally found between the different vintages. With the exception of OTA, no significant differences were observed between organic and traditional farming. In general, ochratoxins levels had a positive association with temperature and were inversely related with humidity and rainfall.

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References

Bellí, N., Marín, S., Duaigües, A., Ramos, A. J. & Sanchis, V. (2004). Ochratoxin A in wines, musts and grape juices from Spain. *Journal of the science of food and agriculture*, 84 (6), 591-594.

Bellí, N., Bau, M., Marín, S., Abarca, M.L., Ramos, A.J. & Bragulat, M.R. (2006). Mycobiota and ochratoxin A producing fungi from Spanish wine grapes. *International journal of food microbiology*, 111, S40-S45.

Blesa, J., Soriano, J. M., Moltó, J. C., & Mañes, J. (2004). Concentration of ochratoxin A in wines from supermarkets and stores of Valencian Community (Spain). *Journal of Chromatography A*, 1054 (1-2), 397-401.

Brera, C., Debegnach, F., Minardi, V., Prantera, E., Pannunzi, E., Faleo, S., De Santis, B., & Miraglia, M. (2008). Ochratoxin A Contamination in Italian Wine Samples and

Evaluation of the Exposure in the Italian Population. *Journal of Agricultural and Food Chemistry*, 56 (22), 10611-8.

Chiodini, A. M., Scherpenisse, P., & Bergwerff, A. A. (2006). Ochratoxin A contents in wine: comparison of organically and conventionally produced products. *Journal of Agricultural and Food Chemistry*, 54 (19), 7399-7404.

Dietrich, D. R., O'Brien, E., Stack, M. E., & Heussner, A. H. (2001). Species- and sex-specific renal cytotoxicity of Ochratoxin A and B in vitro. *Experimental and Toxicologic Pathology*, 53 (2-3), 215-225.

Doster, R. C., Sinnhuber, R. O., & Pawlowski, N. E. (1974). Acute intraperitoneal toxicity of ochratoxin A and B derivatives in rainbow trout (Salmo gairdneri). *Food and cosmetics toxicology*, 12, 499-505.

EC (2006). Commission Regulation (EC) 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. (2006). *Official Journal of the European Union*, L364, 5-24.

EFSA (2006). Opinion of the Scientific Panel on Contaminants in the food chain on a request from the Commission related to ochratoxin A in food. Question Nº EFSA-Q-2005-154. *The EFSA Journal*, 365, 1-56.

FIVIN (2008). Fundación para la Investigación del Vino y Nutrición. Available from: http://www.lacienciadelvino.com/index.php?accion=documentacion.

Heussner, A. H., Dietrich, D. R., & O'Brien, E. (2006). In vitro investigation of individual and combined cytotoxic effects of ochratoxin A and other selected mycotoxins on renal cells. *Toxicology in vitro,* 20 (3), 332-341.

JECFA, Joint FAO/WHO Expert Committee on Food Additives (2007). Evaluation of certain food additives and contaminants: sixty-eight report of the Joint FAO/WHO Expert Committee on Food Additives. *WHO technical report series; nº 947.*

Knasmuller, S., Cavin, C., Chakraborty, A., Darroudi, F., Majer, B. J., Huber, W. W., & Ehrlich, V. A. (2004). Structurally related mycotoxins ochratoxin A, ochratoxin B, and citrinin differ in their genotoxic activities and in their mode, of action in human-derived liver (HepG2) cells: Implications for risk assessment. *Nutrition and Cancer*, 50 (2), 190-197.

Leong, S. L., Hocking, A. D., Pitt, J. I., Kazi, B. A., Emmett, R. W., & Scott, E. S. (2006). Australian research on ochratoxigenic fungi and ochratoxin A. *International journal of food microbiology*, 111 Suppl 1, S10-7.

López de Cerain, A., González-Peñas, E., Jiménez, A. M., & Bello, J. (2002). Contribution to the study of ochratoxin A in Spanish wines. *Food additives and contaminants*, 19 (1), 1058-1064.

Mally, A., Keim-Heusler, H., Amberg, A., Kurz, M., Zepnik, H., Mantle, P., Volkel, W., Hard, G. C., & Dekant, W. (2005). Biotransformation and nephrotoxicity of ochratoxin B in rats. *Toxicology and applied pharmacology*, 206, 43-53.

MARM, Spanish Ministry of Environment and Rural and Marine Affairs (2009). Food, Food consumption, marketing and distribution, Food consumption panel. http://www.marm.es/es/alimentacion/temas/consumo-y-comercializacion-y-distribucion-alimentaria/fichas_consumo_tcm7-7861.pdf

MARM, Spanish Ministry of Environment and Rural and Marine Affairs (2010). Food, Agro-food quality, Differentiated quality, Protected designations of origin (DOP).

http://www.marm.es/es/alimentacion/temas/calidad-agroalimentaria/Estadisticas_DOPs_2009-2010_tcm7-168422.pdf

Marquardt, R. R., & Frohlich, A. A. (1992). A review of recent advances in understanding ochratoxicosis. *Journal of animal science*, 70 (1), 3968-3988.

Mateo, R., Medina, A., Mateo, E. M., Mateo, F., & Jimenez, M. (2007). An overview of ochratoxin A in beer and wine. *International journal of food microbiology*, 119 (1-2), 79-83.

Miraglia, M., & Brera, C. (2002). Assessment of dietary intake of Ochratoxin A by the population of EU member states. *SCOOP 2002. Reports on Tasks for Scientific Cooperation*, task 3.2.7.

Müller, G., Burkert, B., Rosner, H., & Köhler, H. (2003). Effects of the mycotoxin ochratoxin A and some of its metabolites on human kidney cell lines. *Toxicology in Vitro*, 17, 441-448.

Navarra Designation of Origin Regulatory Board. Web revised in July, 2011. http://www.navarrawine.com/en/do_navarre/do-navarre.aspx

O'Brien, E., Prietz, A., & Dietrich, D. R. (2005). Investigation of the teratogenic potential of ochratoxin A and B using the FETAX system. *Birth defects research. Part B, Developmental and reproductive toxicology*, 74 (5), 417-423.

OIV, International Organisation of Vine and Wine, 2010. Web revised on November 2011. http://www.oiv.int/oiv/info/enstatistiquessecteurvitivinicole?lang=en.

Otteneder, H., & Majerus, P. (2000). Occurrence of ochratoxin A (OTA) in wines: Influence of the type of wine and its geographical origin. *Food additives and contaminants*, 17, 793-798.

Pfohl-Leszkowicz, A., & Manderville, R. A. (2007). Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Molecular nutrition & food research*, 51 (1), 61-99.

Quintela, S., Villaran, M. C., Lopez de Armentia, I., & Elejalde, E. (2011). Occurrence of ochratoxin A in Rioja Alavesa wines. *Food Chemistry*, 126 (1), 302-305.

Remiro, R., Ibañez-Vea, M., Gonzalez-Peñas, E., & Lizarraga, E. (2010). Validation of a liquid chromatography method for the simultaneous quantification of ochratoxin A and its analogs in red wines. *Journal of Chromatography A*, 1217 (52), 8249-8256.

Rosa, C. A. R., Fraga, M. E., Santana, D. M. N., Magnoli, C. E., & Dalcero, A. M. (2004). Occurrence of ochratoxin A in wine and grape juice marketed in Rio de Janeiro, Brazil. *Food additives and contaminants*, 21, 358-364.

Soufleros, E. H., Bouloumpasi, E. C., & Tricard, C. (2003). Occurrence of ochratoxin A in Greek wines. *Journal of the science of food and agriculture*, 83, 173-179.

Steyn, P. S. (1971). Ochratoxin and Other Dihydroisocoumarins. In A. Ciegler, S. Kadis, & S. J. Ajl, *Microbial Toxins*, vol. 6 (pp. 179-205). New York: Academic Press.

Zimmerli, B., & Dick, R. (1996). Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. *Food additives and contaminants*, 13, 655-668.

Table 1. Ochratoxin levels found in red wines

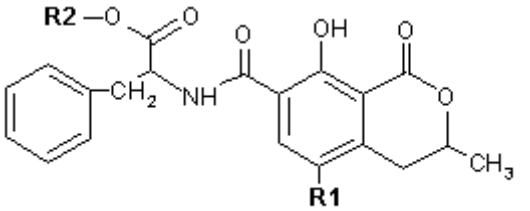
	Concentration ($ng \cdot L^{-1}$)						
	OTB	**OTA**	**MeOTA**	**OTC**	**MeOTB**	**EtOTB**	**Sum**
	Traditional wines						
% Positives[1]	100%	100%	40.0%	80.0%	88.6%	42.9%	
Range	3.21-70.0	0.49-94.9	<LOD-0.95	<LOD-4.47	<LOD-2.42	<LOD-2.42	6.71-174
Mean[2]	11.0	12.7	0.84	1.70	1.90	1.00	26.6
Median	7.96	6.10	< LOD	0.58	1.20	< LOD	17.8
	Ecological wines						
% Positives[1]	100%	100%	43.8%	50.0%	100%	43.8%	
Range	3.08-44.3	0.60-142	<LOD-4.27	<LOD-14.3	0.27-1.58	<LOD-1.24	5.11-200
Mean[2]	11.4	22.9	2.57	7.44	0.93	1.11	37.7
Median	5.54	2.84	< LOD	< LOD	0.53	< LOD	10.7
	Total levels (traditional + ecological wines)						
% Positives[1]	100%	100%	41.2%	70.6%	92.2%	43.1%	
Range	3.08-70.0	0.49-142	<LOD-4.27	<LOD-14.3	<LOD-8.98	<LOD-1.24	5.11-200
Mean[2]	11.1	15.9	1.34	2.63	1.70	1.03	30.1
Median	7.17	5.30	< LOD	0.42	1.00	< LOD	15.7

1: > LOD; 2: > LOQ:

Table 2. Weekly intake of OTA and total ochratoxins (OTs) ($ng \cdot kg$ b. $w.^{-1} \cdot week^{-1}$).

	Consumers				***Total population***	
	Men		**Women**			
	OTA	**Total OTs**	**OTA**	**Total OTs**	**OTA**	**Total OTs**
C max	4.70	6.61	3.70	5.22	0.34	0.48
C mean	0.53	1.00	0.42	0.79	0.04	0.07

Figure 1: Molecular structure of the six ochratoxins.



Ochratoxin	R1	R2
OTA	Cl	H
MeOTA	Cl	CH_3
OTC	Cl	CH_3CH_2
OTB	H	H
MeOTB	H	CH_3
EtOTB	H	CH_3CH_2

Figure 2. Chromatogram of a) standard mixture, b) a naturally contaminated wine sample. Mass spectra of MeOTB and EtOTB.

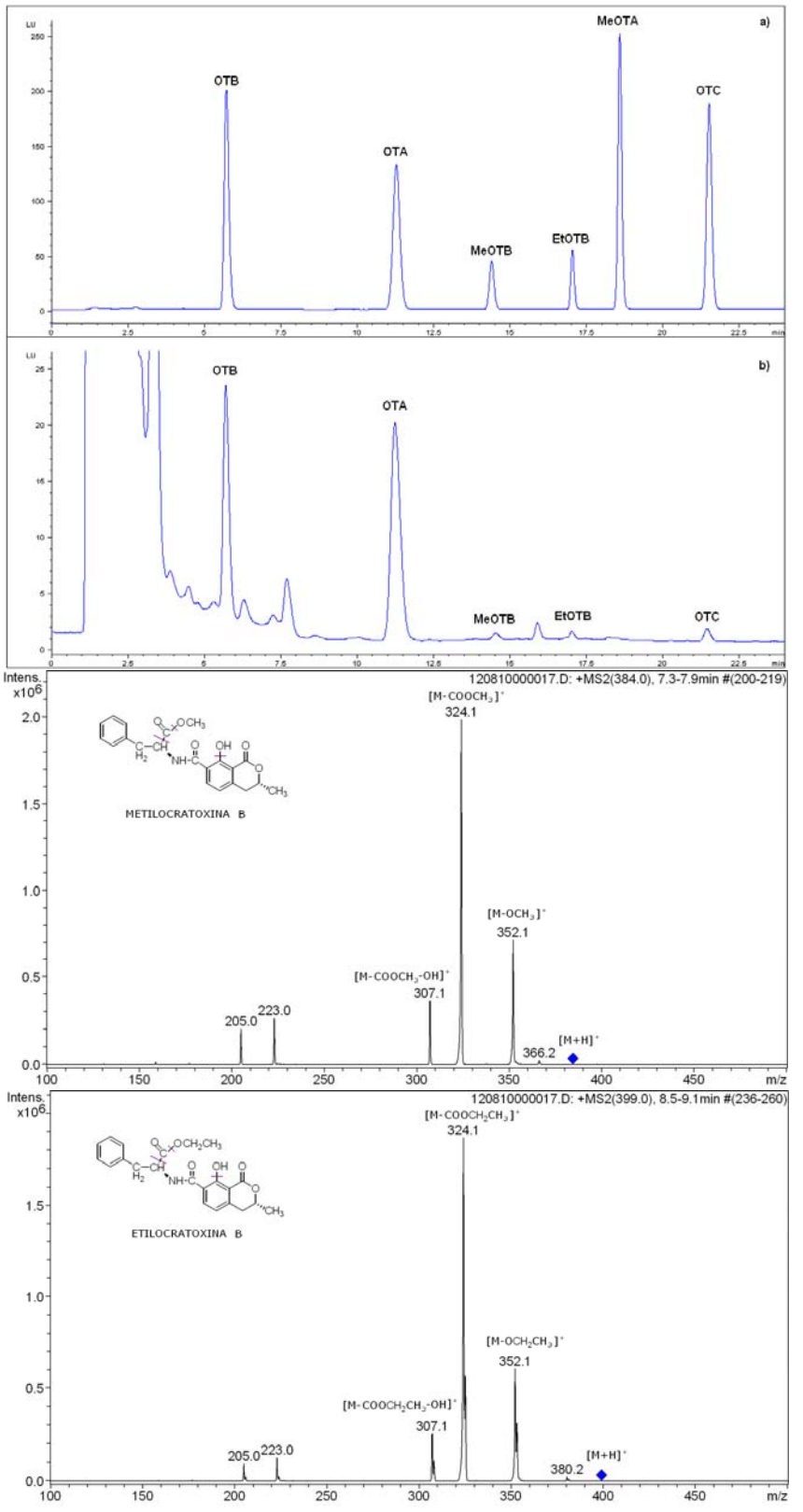


Figure 3. Evolution of OTC and OTA content in wine during a year, a) OTC initial spiked level 0.5 µg·L^{-1} and b) OTC initial spiked level 2 µg·L^{-1}.