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Ochratoxin A survey in Portuguese wine by LC–FD with direct injection

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ABSTRACT

Wine and grape juices were identified as one of the most important sources of ochratoxin A (OTA), a mycotoxin with diverse toxic effects that naturally appears in food and foodstuffs all over the world.

The aim of this study was to assess the OTA levels in Portuguese wines through the application of a simple and accurate method based on liquid chromatography (LC) with direct injection, followed by fluorescence detection (FD).

Randomly selected wine samples were used to evaluate the performance of direct injection as efficient, fast, inexpensive and safe sample preparation method. The proposed method was successfully validated. The limit of quantification (LOQ) was 1.0 μg/L and OTA recoveries from wine samples, spiked at the three fortification levels, were higher than 85.4%, with RSDs lower than 9.6% for both red and white wines. The presence of OTA was confirmed by methyl ester derivatization followed by LC analysis.

Data on OTA levels were obtained for 60 Portuguese red and white wine samples. OTA was found in 12 samples, nine (26%) red wine samples and three (12%) white wine samples. Only one red wine sample and one white wine sample presented a contamination level above the LOQ, with 1.23 and 2.4 μg/L, respectively. It should be pointed out that this white wine sample exceeded the EC maximum permitted level of 2.0 μg/L. The safe dose established as 120 ng/kg body weight/week was not exceeded by the weekly intake estimated for the samples contaminated above the LOQ.

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1. Introduction

Ochratoxin A (OTA) is produced by *Aspergillus ochraceus*, *Aspergillus carbonarius*, *Aspergillus niger*, and *Penicillium verrucosum* [1]. It has been related to Balkan Endemic Nephropathy (BEN), an endemic fatal disease in south-eastern Europe and the development of urinary tract tumours (ITT) in humans [1,2]. The International Agency for Research on Cancer (IARC) has classified this mycotoxin as a possible carcinogen to humans (group 2B) [3].

The natural occurrence of OTA is observed, all over the world, in raw cereal grains, coffee beans, cocoa beans, and dried fruits. It has been detected in various food products such as cereal products, milk, meat, coffee, wine, beer, spices, and grape fruit juice [4–8].

The presence of OTA in grape juices and wines has been reported by many authors [1]. Overall, wine is important to the European economy and populations and, therefore, is important to assure that it is free of harmful contaminants. In this sense, the Organisation Internationale de la Vigne et du Vin (OIV) and European Commission (EC) have proposed a limit of 2 μg/L for OTA in wine [2,9–12].

The main sources of daily OTA intake are cereals and cereal products, followed by wine, grape juice and coffee [1,8]. Wines, particularly red ones, were considered in a first approach to be the second most prominent source of OTA intake for humans, with 15% [13]. The first research group that found OTA in wine claimed that OTA exposure might be doubled by moderate red wine consumption [14].

Recent studies led to the identification of the predominant OTA producing moulds present on grapes, *A. carbonarius,* which is by far the species with the highest OTA production potential, and *A. niger*, whose activity is lower [15,16].

A survey from 2001, in four Portuguese winemaking regions, revealed the presence of these species [17]. In addition, Portugal shows a considerable climatic diversity between regions, dominated by Atlantic and Mediterranean influences being known that climatic and geographic differences influence mold growth and consequent OTA contamination of grapes. OTA levels increase in wines originating from southern areas of Europe with their warmer climates [1,17,18].

A revision of published reports evidences a clear relation between the type of fermentation and the presence of OTA in wines. Generally, white wines usually have lower OTA levels than rosé wines, which in turn have lower levels than red wines. This evidences a clear relationship between the process of maceration and OTA solubilisation in the grape must. On the other hand, sweet wines elaborated with a process that involves partial dehydra-

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tion in the sun tend to have higher OTA levels compared to dry wines [1].

Analytical methods for the analysis of OTA in wine were described using liquid-liquid extraction with toluene, chloroform, or hydrogen carbonate and PEG (polyethylene glycol) solution, and solid phase extraction analysis (SPE) with C_{18} and Oasis HLB cartridges, a water-wettable copolymer (from Waters). Immunoaffinity column (IAC) have been widely used as a clean-up tool and their use is highly recommended, allowing the isolation of the analyte from most matrix interferences, due to its specificity, and analyte pre-concentration, necessary when low limits of detection are required [19]. However, the results of an interlaboratory study comparing different analytical methodologies showed that the analysis and quantification of OTA is reproducible worldwide, for red, white, dessert wine samples included in this work [20].

Liquid chromatography (LC) with fluorescence or mass spectrometry detection, coupled with IAC clean-up, are the most employed analytical methodologies. Nonetheless, owing to its good native fluorescence properties OTA can be determined with less expensive equipment and with high sensitivity [5,7,21,22].

Inexpensive, fast methods based on OTA's direct determination in wine, without sample pre-concentration and clean-up, using LC–FD have been recently published, being the results comparable to those obtained with the IAC method [23,24].

Therefore, the analytical methodology proposed, based on LC–FD with direct injection, was in-house validated and proved to be accurate efficient, fast, and inexpensive for the determination of OTA in red and white wines, at the EU legal limit.

The analytical methodology was then applied for the rapid OTA determination in 60 Portuguese wine samples in order to contribute with new contamination data. On behalf of human health assurance the OTA daily intake in Portuguese population was also evaluated.

2. Experimental

2.1. Solvents and materials

LC grade acetonitrile and methanol were purchased from Carlo Erba (Milan, Italy). Acetic acid was obtained from Merck (Darmstadt, Germany). Ochratoxin A and boron trifluoride 14% methanolic solution were purchased from Sigma Chemicals Co. (St. Louis, USA).

Water was purified by distillation and passage through Milli Q system (Millipore, Bedford, MA). All chromatographic solvents and water were filtered through a 0.45 μm filter under vacuum and degassed for 15 min in ultrasonic bath.

Amber glassware was used to prevent light. The decontamination of the glassware was made by a sodium hypochlorite solution. Then, it was acid washed by immersion of the glassware in a solution of 4 mL/L H_2SO_4 followed by washing to neutral pH by rinsing with distilled water.

2.2. Standard solutions

The standard stock solution (250 μg/mL) and the intermediate standard solution (10 (g/mL) were prepared in 25 mL toluene–acetic acid (99:1), and stored at −20 °C. The working standard solutions, ranging between 0.5 and 10 ng/mL, were prepared in acetonitrile/water/acetic acid (49.5:49.5:1).

2.3. LC system and chromatographic conditions

The LC apparatus consisted of a pump model 307 Gilson (Gilson Medical Electronics, Villiers-le-Bel, France), an injector Rheodyne model 7125 (Cotati, CA, USA), and a PerkinElmer spectrofluorimeter, model LS45 (PerkinElmer, Beaconsfield, UK) operated at an excitation wavelength of 383 nm and an emission wavelength of 440 nm. The spectral bandwidth was 10 nm for both excitation and emission. The results were recorded on a 3390 A integrator (Hewllet-Packard, Philadelphia, PA).

Column oven for control LC column temperature (25 ± 0.5 °C), a guard column C_{18}-5 μm Nucleosil 120 KS (30 × 4 mm i.d.) and a column ACT Ace 5C_{18} (250 mm × 4.6 mm i.d.) were used given their ability to resist in alkaline pH range. The mobile phase, acetonitrile/NH_4Cl/OHNH4 buffer 20 mM (15:85), at pH 9.6, was maintained at a flow rate of 1.0 mL/min.

2.4. Sampling

Portuguese wine samples, randomly selected, included home-made wines and different brands collected from retail shops. A total of 60 samples of red, 35 samples, and white wines, 25 samples, from different regions of Portugal, were collected during 1 year and stored at 4 °C until analysis.

2.5. Sample preparation

Wine samples were filtrated twice through 0.45 μm membrane filters and directly injected into the chromatographic system, according Dall'Asta et al. [23] method.

2.6. Chemical confirmation of OTA by methyl ester formation

For confirmation, OTA was converted into its methyl ester and two different procedures were evaluated according to Zimmerli and Dick [25], using methanol and of 37% concentrated HCl, and Nesheim et al. [26] with boron trifluoride 14% methanolic solution. OTA methyl ester was then analyzed by LC–FD accordingly to the analytical procedure described above.

3. Results and discussion

3.1. Spectroscopic and LC conditions optimization

Excitation and emission wavelengths were optimized in order to maximize the optimum fluorescence emission.

Firstly, the excitation wavelength was established at 383 nm and the fluorescence intensity enhancement of the OTA emission spectra evaluated at 440, 450 and 495 nm. The better results were obtained at 440 nm (Fig. 1). Excitation spectra at 333, 380 and 383 nm for an emission wavelength of 440 nm were obtained (Fig. 2). The better results were achieved with 383 nm. Therefore, the wavelengths chosen were 383 nm for excitation and 440 nm for emission.

Moreover, since the pK_a of the OTA's carboxylic group is 4.4 and that of the phenolic moiety is 7.1, the working pH was also optimized to assure maximum fluorescence intensity and sensitivity. Therefore, spectroscopic measurements at different pH values, 3, 8.5, 9.6 and 10.5. For pH 3 the standard solution was redissolved in acetonitrile/water/acetic acid (49.5:49.5:1), for the other pH values the standard was dissolved in NH_4Cl:OHNH4 20 mM at the pH in study. The fluorescence intensity was higher at pH 9.6 when compared to pH 8.5, however, an increase of the pH to 10.5 resulted in a fluorescence decrease (Fig. 3). The pH 9.6 allows 4-fold fluorescence increase relatively to pH 3. These observations are according with the previous observations of [23]. According to these authors, the increased OTA conjugation of the phenate ring in alkaline solution causes a red shift of the adsorption spectra (λ_{abs} from 333 to 383 nm) and a blue shift of the emission spectra (λ_{em} from 450 to 440 nm).

These studies were fundamental to establish the chromatographic conditions, namely mobile phase composition. The

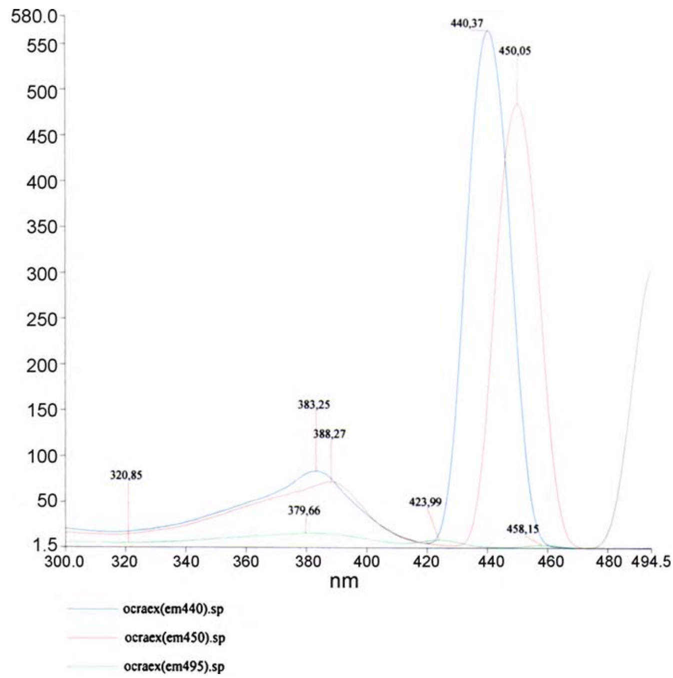

Fig. 1. OTA emission spectra at 440, 450 and 495 nm (λ_{exc} = 383 nm).

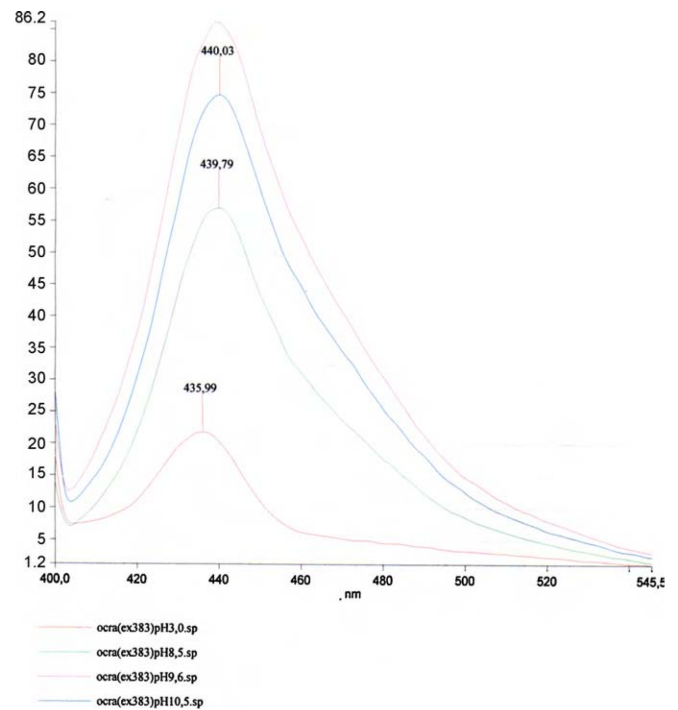

Fig. 3. OTA fluorescence intensity pH 3.0, 8.5, 9.6 and 10.5.

chromatographic column used was chosen due to its capacity to resist such alkaline pH.

The isocratic analysis under the conditions described allowed the elution of OTA with good resolution (Fig. 4).

The mean retention time for OTA was slight longer (rt = 13.25 min) than with the previous method used in our laboratory at lower pH [5]. On the basis of five parallel determinations, during 5 days, the precision standard deviation within-day and between-day (SD) of the OTA retention time, ranged from 0.016% to 0.041% and 0.033% to 0.074%, respectively.

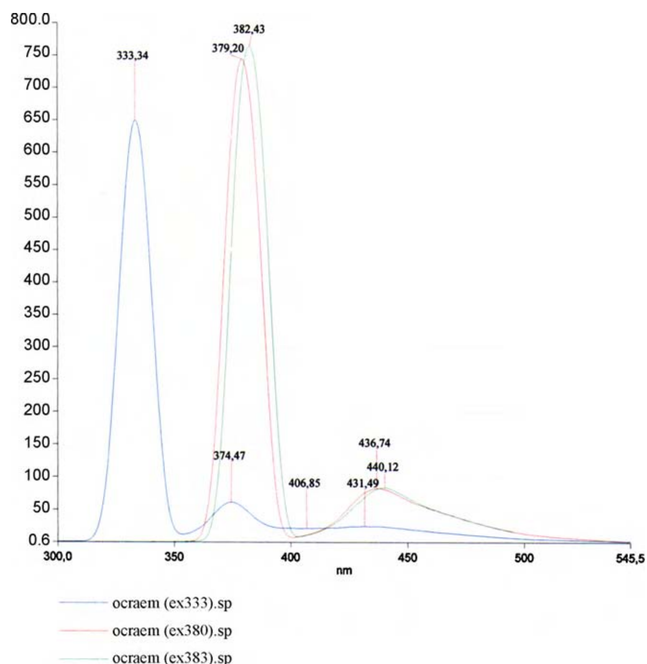

Fig. 2. OTA excitation spectra at 333, 380 and 383 nm (λ_{em} = 440 nm).

3.2. Extraction and clean-up optimization

Most of the current analytical methods for OTA determination in wine are based on IAC clean-up step that allows a highly selective isolation of the analyte from such a complex matrix, but is highly costly. Others SPE sorbents, such as octadecylsilica (RP-18) materials, leads to unsatisfactory results in the determination of OTA by LC–FD [27].

In the search of inexpensive, fast methods for OTA detection in wine, Dall'Asta et al. [23] and Tafuri et al. [24] successfully applied a method for its direct determination in wine without sample pre-concentration and clean-up, using LC–FD. Contrary to other authors statements the direct injection method compared favourably with published methods based on an immunoaffinity column clean-up [24].

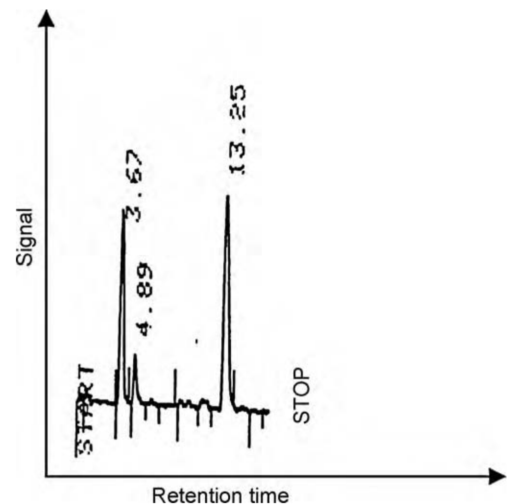

Fig. 4. Chromatogram of a standard solution of OTA at 10 ng/mL.

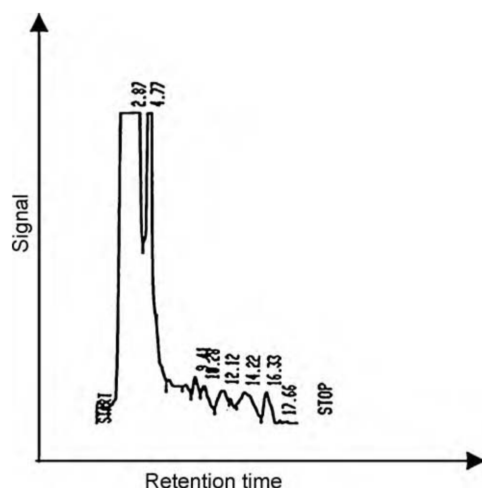

Fig. 5. Chromatogram of a blank sample.

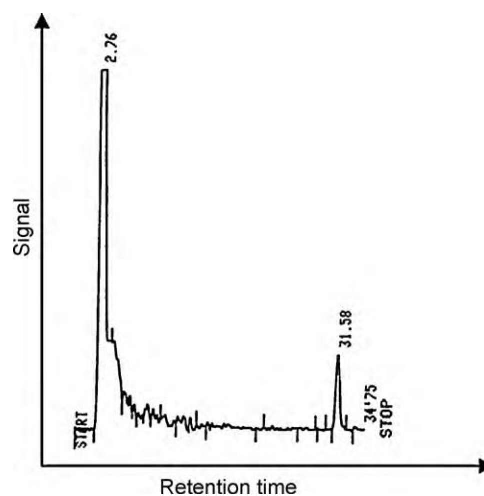

Fig. 6. Chromatogram of the OTA methyl ester.

Thus, this method was applied in the present study to analyze OTA in the red and white wine samples and clean blanks were obtained.

3.3. *Analytical method validation*

In order to verify the absence of potential interfering substances around the retention time of OTA, a number of representative blank red and white wine samples ($n = 10$) were analyzed in order to assess the specificity of the method. No interferences were observed in the region of interest where the OTA was eluted as is shown in the blank sample chromatogram (Fig. 5).

The calibration curves were obtained using the linear least squares regression procedure of the peak area versus the concentration, 0.5; 1.0; 2.5; 5.0 and 10.0 ng/mL. The linearity for OTA, in the working standard solutions at three determinations of five concentration levels, was good as shown by the fact that the determination of the correlation coefficients (r^2) were above 0.9990 for nine calibration curves, prepared in three different days. The equation of the curve was $y = 157427x - 32.599$.

The limit of quantification (LOQ) 1 μg/L for OTA was determined using lowering spiking assays, being the LOQ the smallest amount of the compound that can be quantified with a precision of 10%.

Within-day accuracy and the precision data were determined by analyzing, on the same day, three replicates of spiked samples at three levels (1.0, 2.0 and 4.0 μg/L) and one blank (to check interferences). The between-day accuracy and precision were also done by extracting batches of three fortification levels and analyzing them on three different days. Accuracy and intra-day and inter-day precision data are shown in Table 1. Under our conditions, for the three fortification levels, recoveries from spiked wine samples for OTA reached very high values, greater than 85% and the relative standard deviation was less than 9.6% demonstrating good method accuracy and precision.

Table 1
Accuracy and inter- and intra-assay repeatability results.

Fortification level (μg/L)	Recovery mean (%)	RSD within-day (%)	RSD between-day (%)
1.0	104.0	4.5	4.6
2.0	85.4	5.8	9.5
4.0	103.8	6.2	9.6

3.4. *Stability study*

OTA standard solutions in mobile phase were analyzed during 8 h under laboratorial conditions (temperature ≈ 25 °C). The results obtained showed that OTA was not stable under the pH 9.6. It was observed that the OTA stability was higher when the standards were prepared in the acid solution acetronitrile/water/acetic acid (49.5:49.5:1). These results are explained by the fact that the lactone ring of OTA is destroyed after just 2 h into contact with a solution of 0.5 M NaOH [28].

A stability study in naturally contaminated wine samples was performed weekly, during 1 month. Positive wine samples were prepared according the analytical procedure described above and injected into HPLC system. After 1 week we observed a decrease of 10% in the OTA concentration, and after 1 month control the OTA concentration still maintained this value (10% lower).

3.5. *Confirmation by methylation of OTA*

The presence of OTA in positive samples was confirmed by formation of the OTA methyl ester and identified by comparing with the retention time of standard methyl ester of OTA.

Positive confirmation was based in disappearance of the OTA peak and appearance of new one, corresponding to OTA methyl ester, at retention time 31.58 min (Fig. 6). The use of boron trifluoride [26] and concentrated hydrochloric acid [25] as catalysts to form the methyl ester of OTA were evaluated. A boron trifluoride in methanol solution (14%) is used for the preparation of the methyl ester in most methods and is also part of the official AOAC method for OTA determination on corn and barley by HPLC [29]. For boron trifluoride–methanol reagent, the recovery rate of the methyl ester was higher (93%) when compared with the method of Zimmerli and Dick [25] (79%) with good blanks (Fig. 7).

Following the Nesheim et al. [26] method the confirmation of OTA to methyl ester was achieved in all samples containing OTA levels upper than 1 μg/L wine, overcoming the problems reported by some authors regarding OTA confirmation via its methyl or ethyl ester due to the natural ethyl ester occurrence in wine and the difficulty to obtain an acceptable blank value [14,25].

3.6. *Occurrence of OTA in wine samples*

Sixty wine samples were analyzed under the conditions described before and nine (26%) red wine samples and three (12%) white wine samples were found to be contaminated with OTA.

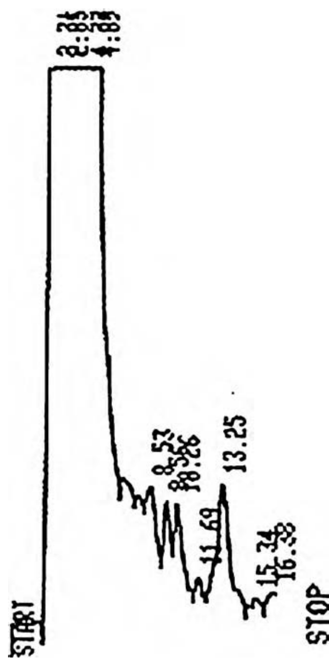

Fig. 7. White wine sample contaminated with OTA at 2.4 μg/L.

Only one red wine sample and one white wine sample presented a contamination level above the LOQ, with 1.23 and 2.4 μg/L, respectively. It should be pointed out that this white wine sample exceeded the EC maximum permitted level of 2.0 μg/L [9].

The worldwide occurrence of OTA contamination of wine has been amply documented. Most surveys carried out on wine showed a higher OTA concentration in red versus white and rosé wines [1,17]. In our study the highest level of contamination was found in a white wine sample. However, this might be explained by the low quality of this sample to be used in cooking procedures. Blesa et al. [5] also found the highest contamination in a white wine purchased in a food store (0.76 μg/L) comparing with other wines with designation of origin.

Generally, Europe southern regions, Morocco and South Africa are more susceptible due to climatic factors, grape cultivation, winemaking techniques and storage conditions. Therefore, surveys have been carried out in order to evaluate the levels of OTA in these regions [1].

In terms of the quantitative presence of OTA in different wines, the majority of the values reported are below 1 μg/L. However, some authors refer punctual OTA contamination up to 7 μg/L in red wines from southern Europe in Spain, France, Italy, Greece [1,30] and over 15 μg/L in the north and south of Africa [1,31]. Table 2 presents the OTA levels found worldwide.

Table 2
Worldwide occurrence of OTA in wine.

Country	Type of wine	Incidence	Contamination levels (μg/L)	Reference
Brazil	Red	3 (10)	0.028–0.042	[32]
	White	2 (10)	0.028	
	Rose	1 (5)	0.035	
Argentina	Red	2 (7)	0.028–0.042	[32]
Spain	Red	21 (61)	0.06–0.51	[5]
	White	4 (24)	0.09–0.76	
	Rose	12 (21)	0.11–0.46	
	Dessert	8 (18)	0.10–0.40	
	Red	13 (28)	0.056–0.316	[33]
	White	7 (12)	0.154–0.208	
	Red	24 (130)	0.06–4.24	[34]
	White	4 (50)	0.11–1.13	
Greece	Red	71 (104)	n.d.–2.69	[35]
	White	63 (118)	n.d.–1.72	
	Rose	13 (20)	n.d.–1.16	
	Dessert	15 (18)	n.d.–2.82	
	Red	9 (14)	n.d.–2.51	[36]
	White	7 (13)	n.d.–0.87	
	Rose	0 (1)	n.d.	
	Dessert	6 (7)	n.d.–3.20	
Italy	All types	175 (208)	0.01–4.00	[37]
	Red	16 (29)	0.31–2.92	[38]
	White	2 (4)	0.28–0.40	
	Rose	4 (10)	0.60–1.95	
Hungary	All types	0 (59)	n.d.	[37]
	All types	0 (57)	n.d.	[39]
Poland	Red	49 (53)	0.002–6.71	[40]
Portugal	All types	69 (340)	n.d.–2.1	[41]
Turkey	All types	82 (95)	0.006–0.815	[42]

3.7. *Daily intake*

The estimated weekly intake (EWI) was calculated for the samples contaminated above the LOQ. A red wine sample and a white wine sample were found to be contaminated with 1.23 and 2.4 μg/L, respectively. Assuming that wine consumption in 2008/2009 in Portugal was about 49.9 L/habitant/year [43] and that an adult body weights 60 kg, the EWI of OTA for each sample was 20 and 38 ng/kg bw/week, respectively.

These values represent about 17% and 32% of the tolerable weekly intake (TWI), 120 ng/kg bw/week, according to the Scientific Committee on Food of the European Commission [9]

OTA weekly intake values of 2 ng/kg bw/day in France, 25.9 ng/kg bw/day in Greece, 2.1 ng/kg bw/day in Spain, 1.4 ng/kg bw/day in Sweden, and 4.9 ng/kg bw/day in Switzerland have been reported [5].

4. Conclusions

The method was adequate for quantification of OTA in wine at levels half the EC limit and is simple, fast, efficient, inexpensive and safe. Therefore, it should be considered as an alternative analytical methodology to quantify OTA in wine without using any clean-up step, since is well-suited for high throughput of large amounts of samples in order to fulfill the tolerance levels established by EC Regulation.

This preliminary study has shown that OTA was found in 12 samples, nine (26%) red wine samples and three (12%) white wine samples. Only one red wine sample and one white wine sample presented a contamination level above the LOQ.

However, these studies are highly recommended in order to evaluate food safety.

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